

The Role of Hyaluronic Acid in the Prevention of Neoplastic Therapy-induced Oral Mucositis

By: Dr Ali Ibrahim Mohammed Mohammed

BDS, MSc

ORCID: 0000-0003-0017-5233

Doctor of Philosophy

April 2023

Melbourne Dental School

Faculty of Medicine, Dentistry & Health Sciences

The University of Melbourne

A thesis Submitted in total fulfilment for the degree of Doctor of Philosophy

*Supervisors: Prof. Nicola Cirillo, Prof. Michael John McCullough,
and Dr Antonio Celentano*

ABSTRACT

GENERAL SUMMARY

Mucositis is a common and most debilitating complication associated with cancer therapy. Approximately 30–40% of cancer patients treated with chemotherapy develop mucositis; this percentage rises to 60–85% for patients receiving conditioning regimens before hematopoietic stem cell transplantation (HSCT) and can increase to almost 100% for head and neck cancer (HNC) patients receiving radiotherapy concomitant with chemotherapy. The condition affects the entire alimentary canal from the mouth to the anus, causing ulceration and severe pain in both the oral cavity and intestinal tract. The consequences of mucositis are far reaching and can lead to a delay or even cessation of otherwise durable cancer treatment and negatively impacting the benefits of chemoradiation. Mucositis is not only an important driver of patients' symptoms and infection risk, but its onset is also associated with poor health outcomes, the use of health resources, and incremental costs. Given that mucositis is ultimately a predictable and potentially preventable condition, this project aimed at optimising a pre-clinical model of mucositis and testing the protective effect of a natural compound on chemotherapy-induced mucosal injury.

Chapter 1 discusses the generalities of mucositis, its causative agents and risk factors, its pathophysiology diagnosis, and management. After recalling basic concepts of cancer treatment (causative factor) and mucosal structure (target tissue), in this chapter I explain the clinical relevance and economic implications of mucositis. A large section is devoted to the pathophysiology of mucositis as this represents the starting point for the development of novel mechanism-based preventative and therapeutic strategies. The chapter then focuses on oral mucositis and, in this context, types of assessment and scoring scales are extensively discussed. Current management strategies of mucositis are scrutinised in the final part of Chapter 1. Despite its clinically devastating consequences, there is currently little to offer patients in the way of effective treatment to prevent or mitigate mucositis, and this provided the rationale for developing the aims of this project.

General materials and methods are detailed in **Chapter 2**. The experimental design included *in vitro* experiments using an oxidative stress-induced model of human oral mucosal injury and an *in vivo* dual murine model of 5-FU-induced oral/intestinal mucositis. Formulations of hyaluronic acid (HA) tested in these models included Mucosamin®, cross-linked (xl-), and non-crosslinked high molecular weight HA (H-MW-HA). Cell lines, culture conditions,

morphological, functional and molecular assays (e.g. cell viability, cytotoxicity, and proliferation; intracellular ROS production, superoxide dismutase enzyme activity) are described. For the animal study, methodology for clinical, histopathological and morphometric assessment of oral and intestinal mucositis is reported in detail. Specific materials and methods are additionally described in the relevant chapters.

In the experiments described in **Chapter 3**, we aimed to develop and characterise an *in vitro* model of oral mucosal injury that could mimic the initial events that trigger oral mucositis. Since the molecular mechanisms underlying chemotherapy and radiotherapy-induced oral mucositis involve the production of ROS at early stages and consequent activation of oxidative stress pathways, we established an oxidative stress model on human oral keratinocytes cultures whereby were able to identify optimum concentrations of hydrogen peroxide and incubation period for each of the cell line tested. Such a model allowed the screening of potentially xxx drugs reported later in this thesis.

In **Chapter 4**, the biocompatibility of several hyaluronic acid derivatives in our *in vitro* cell model system was assessed through an extensive series of experiments. We defined the optimal concentrations of the following compounds: Mucosamin[®] (1%, 5%, 7% and 10% v/v), native high molecular weight HA (H-MW-HA; 0.01%, 0.03%, 0.05%, 0.07% and 0.1% w/v), and cross-linked (xl-) HA (0.01% v/v of xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100). Oral keratinocytes were incubated for 24, 48, and 72 hours in the presence of these HA products and a dose-response curve was developed. All HA compounds tested significantly promoted oral epithelial cell proliferation compared to control, except for Mucosamin[®]; this commercial preparation did not affect cell growth at concentrations of up to 5% (v/v) and was cytotoxic at higher concentrations.

Drawing on the results of the pilot experiments described in the previous chapters, in **Chapter 5** we tested the protective effects of HA in a model of oxidative stress-induced oral mucosal injury. OKF6 cells were incubated with 400 μ M (IC₅₀ value) hydrogen peroxide (H₂O₂) and the effects of Mucosamin[®], H-MW-HA, Xl-HA 5/5, Xl-HA 30/30, and Xl-HA 100/100 were tested. While all HA compounds could attenuate to some extent the detrimental effects of ROS, the most marked effects were obtained with H-MW-HA. Specifically, pre- and then co-incubation of OKF6 cells with 0.01 % (w/v) H-MW-HA for 24 hours resulted in a sizeable increase in cell viability when compared to H₂O₂- treated cells. Remarkably, H-MW-HA also reduced the intracellular level of H₂O₂-induced ROS production, as measured by the ability of

cells to oxidize CM-H₂DCFDA. Hence, H-MW-HA at 0.01 % (w/v) was selected for the animal study.

Chapter 6 describes a detailed set of experiments to show that HA prevents oral and intestinal mucositis induced by chemotherapy *in vivo*. To overcome the limitations of current models, whereby oral and intestinal mucositis are studied separately using different, organ-specific models, we first characterized a pre-clinical dual (oral and intestinal) murine model of mucositis by using intravenous 5-FU injections (50 mg/kg) every 48 hours for 2 weeks. In the test group, the mice were pre-treated one day prior to the initial 5-FU treatment and then daily thereafter with high molecular weight HA (H-MW-HA) (0.01 % w/v) in drinking water. Mice were monitored clinically for weight loss, diarrhea (as a surrogate of intestinal injury), and incidence and extent of oral mucositis. Microscopically, histomorphometric analyses of the tongue and intestinal tissues were conducted. The results strongly indicated that H-MW-HA prevented 5-FU-induced damage to the intestinal mucosa and tongue epithelium. We also demonstrated that H-MW-HA enhanced the activity of the SOD enzyme in the blood serum of 5-FU treated mice.

This thesis comes to a conclusion with **Chapter 7**, where our results are discussed in light of current literature. These results reported in this thesis provide an experimental rationale to develop a novel HA-derived treatment as a therapeutic agent to protect against oral and intestinal mucositis associated with chemotherapy in cancer patients. We note that systemic administration of HA as a preventive or therapeutic tool in oral and intestinal mucositis may have profound clinical implications in patients, such as potential interference with cancer treatment, that require further elucidation. Future studies will address this important aspect of our research.

DECLARATION

This is to certify that:

- 1) The thesis comprises only my original work towards the PhD except where indicated
- 2) Due acknowledgement has been made in the text to all other material used
- 3) The thesis is less than 100, 000 words in length, exclusive of tables, maps, bibliographies and appendices.

Ali Ibrahim Mohammed Mohammed

Friday, 28th April 2023.

ACKNOWLEDGEMENTS

In submitting this thesis, I would like to express my deepest gratitude to all those who have supported me on this journey. The acknowledgments I include here are by no means exhaustive. There are too many to name individually, and I don't trust my memory after the late nights of correcting and trying to orchestrate this thesis. However, I now mention those who have been at the forefront of my mind as I finish compiling this work, which has been a large part of my life for the past few years.

I would like to express my most profound appreciation to my supervisors, my PhD Advisory Committee members, and other academic and support staff at the Melbourne Dental School.

Of these individuals, I extend a further heartfelt thanks to my supervisors, **Professor Nicola Cirillo, Dr. Antonio Celentano, and Professor Michael McCullough**, whose contributions and insights were what made the difference and elevated my work to produce what is now this body of research. I have been so fortunate for my research to have had the breadth to include the work being done in oral medicine and pathology under their supervision.

On a more personal note, to my principal supervisor, **Professor Nicola**; thank you for joining me on this journey from start to finish. Thank you for welcoming me onto the board and for being the best supervisor I can ever imagine with your strong wisdom, and endless enthusiasm. Your work in this area is what inspired this research, and I hope that our findings set the foundations for an important part of your legacy. Thank you for leading me through this journey, and for always having your time available to answer my questions, and for your endless generous support and guidance during this study. Many thanks for helping my family and me during our hard times. Your outstanding support was what made this thesis possible.

On that note, to my co-supervisor, **Dr. Antonio**; you have always been an ardent supporter of mine from the first time I met you during this long journey and especially during the most crucial times of the project. For this, and your wisdom, guidance, friendship, great sense of humor, and the laughs we have shared, I will forever be indebted.

Likewise, to my co-supervisor, **Professor Michael**; thank you for your unwavering support and enthusiasm towards my project, especially at the times when my own enthusiasm waned. I so much appreciate your guidance and supervision. After finishing this work, I am certain now that both you, and your green pen, deserve a well-earned rest.

I also extend a wider thanks to the chair of the Committee, **Professor Stuart Dashper**; I appreciated your guidance and support during my PhD study. In addition, I would like to thank **Rita Paolini** from Melbourne Dental School, who volunteered her time to participate in my research and offer all the support, expertise, and help I needed during my experiments.

I acknowledge Attila Gergely from Melbourne Dental School, Max Walker, the animal house manager at the Bio 21 Institute, and Paul McMillan and Ellie Cho from Biological Optical Microscopy Platform (BOMP) for their assistance with my experiments.

In particular, to my colleagues at Tikrit University, Dr. Farooq Abbas and Dr. Amir Abdullah; I left Iraq almost 7 years ago to the day to embark on this new journey. I will never forget the support you provided me in my career and for the best friendship you gave me. I am only here now because you helped encourage me to explore my interest in oral medicine. I hope I can find a way to support you in as valuable a way.

My final acknowledgment, which is certainly last, but not least, goes to my family: My parents Ibrahim and Amerah, my wife Huda, my son Alhasan, my daughters Jumana and Amenah, my brothers Mohammed, Ahmed, and Omar, and my sister Shaimaa who have been giving a lot of supports, prayers, and ‘doa’ throughout my study. I would also sincerely thank my extended family; those friends who are like brothers and sisters: Dr. Ibrahim Marsoomi, Lindsay Herbert and Barbara Herbert, and Margaret White. You have been there when I most needed you along this journey in ways which are too many to count and name. I am certain that I would not be here, writing these acknowledgments today, but for the part you played individually and collectively. Maybe, one day, when you can feel how heavy this thesis is when bound, you’ll not only be able to see exactly what I was working on for all those years, but also understand the load that you helped me carry. Thank you for always believing in me. I have only been able to do what I have done because of all of you.

I would like to thank all those who contributed to this work but were not mentioned in this acknowledgment.

Finally, I dedicate this thesis to my beloved wife, Huda, and my dear children, Alhasan, Jumana, and Amenah, for being very patient while their dad was so tired and moody. Thank you for everything that you have sacrificed. I hope that you understand and believe through my example that things that are hard work are worth the most.

PREFACE

PUBLICATIONS

Manuscript 1

Characterization of a Novel Dual Murine Model of Chemotherapy-Induced Oral and Intestinal Mucositis

Authors: Ali I. Mohammed¹, Antonio Celentano, Rita Paolini¹, Michael J. McCullough, Jun T. Low, Lorraine A. O' Reilly, and Nicola Cirillo

Article status: Published by Journal of Scientific Reports, Nature.

Mohammed, A. I., Celentano, A., Paolini, R., Low, J. T., McCullough, M. J., O' Reilly, L. A. & Cirillo, N. 2023. Characterization of a novel dual murine model of chemotherapy-induced oral and intestinal mucositis. Scientific Reports, 13(1), pp 1396.

Manuscript 2

High Molecular Weight Hyaluronic Acid Drastically Reduces Chemotherapy-Induced Mucositis and Apoptotic Cell Death

Authors: Ali Mohammed, Antonio Celentano, Rita Paolini, Jun Low, John Silke, Lorraine O'Reilly, Michael McCullough, and Nicola Cirillo

Article status: In revision following peer review by Cell Death and Disease Journal, Nature.

Abstract

The Role of Hyaluronic Acid Preparations in Preventing Antineoplastic Treatment-Induced Oral Mucositis.

Authors: A I Mohammed, A Celentano, M J McCullough, and N. Cirillo.

Abstract status: Published by Australian Dental Research Foundation Special Research Supplement 2020.

A I Mohammed, A Celentano, M J McCullough and N Cirillo. The Role of Hyaluronic Acid Preparations in Preventing Antineoplastic Treatment-Induced Oral Mucositis. 2020. Australian Dental Research Foundation Special Research Supplement 2020. Australian Dental Journal, 65(4), pp S7-S40. Available at: <https://onlinelibrary.wiley.com/doi/abs/10.1111/adj.12799>

CONFERENCES

e-poster

Protective effects of a hyaluronic acid-based compound in experimental models of oral mucositis

Authors: Mohammed Ali I, Celentano Antonio, McCullough Michael J, and Nicola Cirillo.

e-poster Status: Presented at 7th World Congress of the International Academy of Oral Oncology, Rome, 2019.

Mohammed Ali I., Celentano Antonio., McCullough Michael J., and Cirillo Nicola. 2019. Protective effects of a hyaluronic acid-based compound in experimental models of oral mucositis (e-poster). Presented at 7th World Congress of the International Academy of Oral Oncology, Rome, 2019. Available at: [25180--NEC_IAOO19 Progr165x230 02.indd \(iaoo2019.com\)](#).

FUNDING SOURCES

A PhD cannot be completed without funding and so in much appreciation I, Ali Mohammed, would like to acknowledge the financial support provided through:

- The Iraqi government, Iraqi ministry of higher education and scientific research (MOHESR) scholarship program;
- The University of Melbourne Peter Reade Oral Cancer Travel Award;
- The University of Melbourne Rowden White Scholarship;
- The Postgraduate Research Grant provided by the Australian Dental Research Foundation Inc (ADRF, Grant ID = 209-2017), The University of Melbourne;
- The Postgraduate Research Grant provided by the Iraqi ministry of higher education and scientific research (MOHESR); and
- Tuition fees offset scholarship by Melbourne Dental School, Faculty of Medicine, Dentistry, and Health Sciences, The University of Melbourne.

ABBREVIATIONS

AEC	Animal Ethics Committee
AEs	adverse events
AP-1	activator protein 1
ASCO	American society of clinical oncology
bFGF	basic fibroblast growth factor
BM	basement membrane
BMT	bone marrow transplantation
BW	body weight
C	crypts
CaCl ₂	calcium chloride
CCRT	concurrent chemo-radiotherapy
CIOM	chemotherapy-induced oral mucositis
CIM	chemotherapy induced mucositis
CM-H ₂ DCFDA	5-(and-6)-chloromethyl-2', 7'- dichlorodihydrofluorescein diacetate
CMMO	Celentano Murine Mouth opener
COX-2	cyclooxygenase-2
CRAMPs	chemoradiation-associated molecular pattern
CRT	chemotherapeutic or radiation regimens
CT	chemotherapy
CTCAE	common terminology criteria for adverse events
DAMPs	damage-associated molecular pattern molecules
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EB	ethidium bromide
ECM	extracellular matrix

EDC	1-ethyl-3-[3-(dimethylamino) propyl]-carbodiimide
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EORTC QLQ	European Organization for Research and Treatment of Cancer Quality of Life Questionnaire
ERK	extracellular-signal-regulated kinase
ESMO	European Society of Medical Oncology
FACT-HN	Functional Assessment of Cancer Therapy-Head and Neck
FBS	Foetal bovine serum
FCA	fluorometric cytotoxicity assay
FDA	fluorescein diacetate
FIP	filiform papillae
FFPE	formalin fixed paraffin embedded
GAG	glycosaminoglycan
GM-CSF/GCSF	granulocyte macrophage-colony-stimulating factor/granulocyte colony-stimulating factor
GSH	glutathione
GSH-Px	glutathione peroxidase,
GVHD	graft-versus-host-disease
HA	hyaluronic acid
hAECs	human amniotic epithelial cells
hAMSCs	human amniotic mesenchymal stem cells
HASs	hyaluronic acid synthases
HCE-2	human corneal epithelial cell line
H & E	hematoxylin and eosin
H-MW	high molecular weight
H-MW-HA	high molecular weight hyaluronic acid
HNC	head and neck cancer

HNSCC	head and neck squamous cell carcinoma
H ₂ O ₂	hydrogen peroxide
HOBt	1-hydroxybenzotriazole
HSCT	hematopoietic stem cell transplantation
HSV	herpes simplex virus
HUVECs	human umbilical vein endothelial cells
IARC	International Agency for Research on Cancer
IBS	irritable bowel syndrome
IGF-I	insulin-like growth factor I
IL-1 β	interleukins-1 β
IL-6	interleukins-6
IM	intestinal mucositis
i.p.	intraperitoneal
i.v.	intravenous
JNK	c-Jun N-terminal kinase
KGF	keratinocyte growth factor
K-SFM	keratinocytes serum-free medium
LLLT	low-level laser therapy
L-MW	low molecular weight
LP	lamina propria
LPS	lipopolysaccharide
MA	myeloablative
MAPK	mitogen activated protein kinase
MASCC/ISOO	multinational association of supportive care in cancer/International society of oral oncology
MF	muscle fibres
MMPs	matrix metalloproteinases

MPO	myeloperoxidase
NA	not applicable
NCCN	national comprehensive cancer network
NCI	National Cancer Institute
NCI-CTCAE	National Cancer Institute-Common Toxicity for Adverse Events
NCS	Newborn Calf Serum
NF- κ B	nuclear factor-kappa B
NHOF	normal human oral fibroblast
NK	natural killer
Nrf2	nuclear factor erythroid-2 related factor 2
NSAIDs	nonsteroidal anti-inflammatory drugs
OHCRC	Oral Health Cooperative Research Centre
OM	oral mucositis
OMAS	Oral Mucositis Assessment Scale
OMI	Oral Mucositis Index
ONS	oncology nursing society
OSCC	Oral squamous cell carcinoma
PAMPS	pathogen-associated molecular patterns
PBE	pituitary bovine extract
PBS	phosphate-buffered saline
PI	propidium iodide
PMB	photobiomodulation
PRRs	pathogen recognition receptors
QoL	quality of life
RAGEs	receptors for advanced glycation end products
RHAMM	receptor for hyaluronate-mediated motility
RIC	reduced-intensity conditioning

ROS	reactive oxygen species
RT	radiotherapy
RTOG	Radiation Therapy Oncology Group
SIPS	stress-induced premature senescence
SMase	sphingomyelinase
SOD	superoxide dismutase
TAOC	total antioxidant capacity
TB	toluidine blue
TBI	total body irradiation
TJs	tight junctions
TM	tunica muscularis
TNF- α	tumor necrosis factor-alpha
tp	time plateau
TRL	toll-like receptors
V	villi
VAS	visual analog scale
VEGF	vascular endothelial growth factor
v/v	volume/volume
WCCNR	Western Consortium of Cancer Nursing Research
WHO	World Health Organization
w/v	weight/volume
XOD	xanthine oxidase
2D	two-dimensional
3D	three-dimensional
5-FU	5-Fluorouracil

TABLE OF CONTENTS

ABSTRACT.....	ii
DECLARATION	v
ACKNOWLEDGEMENTS.....	vi
PREFACE	viii
ABBREVIATIONS	xi
TABLE OF CONTENTS.....	xvi
LIST OF FIGURES	xxiv
LIST OF TABLES	xxviii
CHAPTER 1 LITERATURE REVIEW	1
1.1 Introduction	2
1.2 Mucositis	4
1.2.1 Generalities of Cancer treatment-induced mucosal injury	4
1.2.2 Treatment of cancer and mucositis.....	5
1.2.3 The Economic implications of mucositis	6
1.3 Mucous membranes of oral and intestinal mucosa	8
1.3.1 Oral Mucosa	9
1.3.1.1 Oral mucosa layers.....	9
1.3.1.1.1 Epithelium	9
1.3.1.1.2 Lamina propria	9
1.3.1.1.3 Submucosa	10
1.3.1.2 Oral mucosa structure	10
1.3.2 Gastrointestinal tract.....	11
1.3.2.1 GI tract wall	12
1.3.2.1.1 GI Mucosa	12
1.3.2.1.1.1 <i>Epithelium</i>	13
1.3.2.1.1.2 <i>Lamina propria</i>	14
1.3.2.1.1.3 <i>Muscularis mucosa</i>	14
1.3.2.1.2 Submucosa	14
1.3.2.1.3 Muscularis	14
1.3.2.1.4 Serosa	15
1.4 Oral mucositis	16

1.4.1 Epidemiology of oral mucositis.....	17
1.4.1.1 Prevalence	17
1.4.1.2 Incidence	18
1.4.2 Risk factors of oral mucosal injury.....	19
1.4.2.1 Treatment–related factors	21
1.4.2.1.1 Chemotherapy-related factors	22
1.4.2.1.2 Radiation-related factors	23
1.4.2.1.3 The role of novel targeted anticancer treatments in mucositis development	25
1.4.2.2 Transplant-related factors	28
1.4.2.3 Patient-related factors	29
1.4.2.4 Role of Microbial Flora	30
1.4.2.5 Gene-related factors	31
1.4.2.6 Malignancy type.....	32
1.4.3 Pathogenesis of Mucositis	33
1.4.3.1 Historical model.....	33
1.4.3.2 Current model	34
1.4.3.2.1 Early Tissue Damage	34
1.4.3.2.2 Chemoradiation-induced oxidative stress	35
1.4.3.2.3 Inflammatory Infiltrate	36
1.4.3.2.4 Gene Expression Changes	36
1.4.3.2.5 Pro-Inflammatory Cytokines.....	37
1.4.3.2.6 Apoptosis as A Potential Initiator Of Mucositis	38
1.4.3.3 A Multiple Mechanism Model.....	39
1.4.3.3.1 Tissue structure and modified functional physiology	44
1.4.3.3.2 Microbiota and innate immune response	45
1.4.4 Differential diagnosis of oral mucositis.....	49
1.4.5 Assessment of Oral Mucositis	50
1.4.5.1 Oral Mucositis Scoring scales.....	50
1.4.5.1.1 Physician-reported outcome measures	51
1.4.5.1.2 Nurse-reported outcome measures	52
1.4.5.1.3 Detailed objective scoring scales	54
1.4.5.1.4 in vitro measurement scale	54
1.4.5.1.5 Patient-reported outcome measures of Mucositis	54

1.4.5.2 World Health Organization Oral Toxicity Scale (WHO)	56
1.4.5.3 National Cancer Institute- Common Toxicity Criteria	56
1.4.5.4 The Radiation Therapy Oncology Group Scale.....	56
1.4.5.5 Western Consortium for Cancer Nursing Research Scale	57
1.4.5.6 Oral Mucositis Assessment Scale	58
1.4.5.7 The Oral Mucositis Index	59
1.4.5.8 Oral mucositis measurement in children.....	59
1.4.6 Management of Oral Mucositis	61
1.4.6.1 Preventive Strategies:.....	66
1.4.6.1.1 Basic oral Care/Maintaining Oral Hygiene:.....	66
1.4.6.1.2 Oral care protocols and patient education	67
1.4.6.2 Palliative care (including Pain management)	68
1.4.6.2.1 Pain Management	68
1.4.6.2.2 Topical preparations and other approaches	69
1.4.6.3 Chemoradiotherapy-induced mucositis management	70
1.4.6.3.1 Nutritional Support.....	70
1.4.6.3.2 Oral decontamination	70
1.4.6.3.3 Cryotherapy	71
1.4.6.3.4 Photobiomodulation (laser and other light therapy).....	71
1.4.6.3.5 Growth factor	72
1.4.6.3.6 Management of bleeding.....	72
1.4.6.3.7 Anti-inflammatory agents	72
1.4.6.3.8 Antioxidants	73
1.4.6.4 Novel Approaches for Oral Mucositis Management	74
1.4.7 OM prevention is an unmet health need.....	76
1.4.8 Hyaluronic acid for management of oral mucositis.....	76
1.5 Hypothesis and Aims of the study.....	78
1.5.1 Overall study Hypothesis:	78
1.5.2 Overall study Aim	79
CHAPTER 2 MATERIALS AND METHODS.....	80
2.1 Abstract	81
2.1 Introduction	83
2.2 <i>In Vitro</i> Experiments	85

2.2.1 Reagents.....	85
2.2.2 Equipment.....	85
2.2.3 HA Preparations	86
2.2.4 Cell Lines.....	86
2.2.5 Culture Conditions.....	87
2.2.6 Cell culture assessment and observation by Phase Contrast Inverted Microscope .	88
2.2.7 Preparation of HA Solutions.....	88
2.2.7.1 Mucosamin [®]	89
2.2.7.2 Native HA (H-MW-HA).....	89
2.2.7.3 Cross-linked (xl-)HA EDC/HOBT 5/5 mmol/mL (xl-HA 5/5)	89
2.2.7.4 xl-HA EDC/HOBT 30/30 mmol/mL (xl-HA 30/30) and xl-HA EDC/HOBT 100/100 mmol/mL (xl-HA 100/100)	90
2.2.8 Cell Number Titration Assay.....	91
2.2.8.1 Cell preparation and incubation	91
2.2.8.2 Preparation of the FDA solution	91
2.2.8.3 Cell viability measurement	92
2.2.9 Cell Viability and Growth Assay.....	94
2.2.9.1 Induction of oxidative stress	94
2.2.9.2 Morphological analysis of cells by phase contrast inverted microscope	94
2.2.9.3 Determination of cell viability	94
2.2.10 Fluorescence-Based Live-Dead Cytotoxicity Assay	97
2.2.10.1 Cell preparation and treatments	97
2.2.10.2 Preparation of the FDA/PI staining solution.....	97
2.2.10.3 FDA/PI staining assay.....	97
2.2.11 Fluorometric Cytotoxicity Assay (FCA)	100
2.2.11.1 Cell preparation and culture treatments	100
2.2.11.2 Preparation of the FDA solution	101
2.2.11.3 Fluorometric cytotoxicity assay (FCA) assay.....	101
2.2.11.4 Determination of cell viability	102
2.2.12 Proliferation Assay	105
2.2.12.1 Cell preparation and culture treatments	105
2.2.12.2 Fluorescein diacetate (FDA) assay	105
2.2.12.3 Determination of cell viability	106
2.2.13 Evaluation of Intracellular ROS Production and Kinetics in Oral Keratinocytes	108

2.2.13.1 Reagents preparation.....	108
2.2.13.1.1 CM-H ₂ DCFDA Solution.....	108
2.2.13.1.2 Hydrogen Peroxide Solution (Positive Control)	108
2.2.13.1.3 Compounds/Diluents of Interest.....	109
2.2.13.2 Cell preparation and culture treatments	109
2.2.13.3 CM-H ₂ DCFDA stain loading.....	109
2.2.13.4 Determination of intracellular ROS generation	110
2.2.14 Real-Time Quantification of Oxidative Stress Using Fluorescent Dye-Based Redox Probes in Oral Keratinocytes.	112
2.2.14.1 Reagents preparation.....	112
2.2.14.2 Cell preparation and culture treatments	112
2.2.14.3 CM-H ₂ DCFDA stain loading.....	113
2.3 Animal Experiments.....	116
2.3.1 Chemicals and Reagents.....	116
2.3.2 Preparation of 5-FU	116
2.3.3 Preparation of Hyaluronic Acid.....	116
2.3.4 Animals.....	117
2.3.5 5-FU Administration.....	117
2.3.6 Intravenous Injection of mice.	118
2.3.7 Monitoring of Animals.	119
2.3.8 Diarrhea Assessment	119
2.3.9 Oral Mucositis Scoring.....	119
2.3.10 Euthanasia and Blood Sample Collection	122
2.3.11 Tissue Sample Preparation	122
2.3.12 Macroscopic Examination of the Tongue.....	123
2.3.13 Superoxide Dismutase (SOD) Enzyme Activity Assay	125
2.3.13.1 Preparations of Assay Solutions (for one 96-well plate)	126
2.3.13.2 General Protocol	127
2.3.13.3 Calculating SOD Activity Unit (U)	127
2.3.14 Tissue Processing and Embedding for Histology.....	129
2.3.15 Tissues Sectioning	131
2.3.16 Hematoxylin and Eosin Staining and Mounting	131
CHAPTER 3 DEVELOPMENT OF AN <i>IN VITRO</i> MODEL FOR OXIDATIVE STRESS-INDUCED CYTOTOXICITY IN ORAL KERATINOCYTES.....	133

3.1 Abstract	134
3.2 Introduction:	135
3.3 Material and Methods.....	137
3.3.1 Cell lines and culture conditions	137
3.3.2 Optimizing seeding density of cells.....	137
3.3.3 H ₂ O ₂ treatment of cells (induction of oxidative stress)	138
3.3.4 Cell viability and growth assay	138
3.3.4.1 Evaluation of epithelial cell viability rate with fluorescence-based live-dead assay	139
3.3.4.2 Evaluation of epithelial cell viability rate with fluorometric cytotoxicity assay (FCA)	139
3.3.5 Statistical Analyses.....	139
3.4 Results	141
3.4.1 Optimization of cell number for cell viability/proliferation assay	141
3.4.2 H ₂ O ₂ inhibits epithelial cell proliferation in a time- and dose-dependent manner	144
3.4.3 Cytotoxic effect of H ₂ O ₂ on oral keratinocytes	147
3.4.4 Establishment of an oxidative stress-induced mucosal injury model with H ₂ O ₂ -treated oral keratinocyte cells	150
3.4.5 Effect of H ₂ O ₂ -induced oxidative stress on morphological changes in oral keratinocyte cells	154
3.5 Discussion	158
3.6 Conclusion.....	163
CHAPTER 4 DETERMINING THE EFFECTS OF HYALURONIC ACID DERIVATIVES IN THE REGULATION OF EPITHELIAL CELL PROLIFERATION IN VITRO	164
4.1 Abstract	165
4.2 Introduction	166
4.3 Materials and Methods.....	173
4.3.1 Cell line and culture conditions	173
4.3.2 HA preparations.....	173
4.3.3 Preparation of HA solutions	174
4.3.4 Viable cell proliferation assay	174
4.3.5 Statistical analysis.....	176
4.4 Results	177

4.4.1 Treatment of human oral keratinocytes with Mucosamin [®] at concentrations greater than 5% v/v reduces cell viability.....	177
4.4.2 H-MW-HA promotes human oral keratinocytes proliferation	181
4.4.3 Differential effect of cross-linked HA derivatives on keratinocyte proliferation .	184
4.5 Discussion	186
4.6 Conclusion.....	191
CHAPTER 5 HYALURONIC ACID-BASED COMPOUNDS AMELIORATE OXIDATIVE STRESS IN AN <i>IN VITRO</i> MODEL OF ORAL MUCOSAL INJURY.	192
5.1 Abstract	193
5.2 Introduction	194
5.3 Materials and Methods	201
5.3.1 Cell line and culture conditions	201
5.3.2 HA preparations.....	201
5.3.3 Preparation of HA solutions	202
5.3.4 OKF6 cells treatment with HA constituents	202
5.3.5 Cell Viability Measurement.....	203
5.3.6 Determination of Intracellular ROS Production and Kinetics in OKF6 cells	204
5.3.7 Real-Time Quantification of Oxidative Stress in Oral Keratinocytes Using Fluorescent Dye-Based Redox Probes.	204
5.3.8 Statistical analysis.....	206
5.4 Results	207
5.4.1 HA Constituents Effectively Protect Oral Keratinocytes Against H ₂ O ₂ -Induced Cytotoxicity and Oxidative Stress <i>In Vitro</i>	207
5.4.2 H-MW-HA Protects Human Oral Keratinocytes Against H ₂ O ₂ -Induced ROS Production.....	217
5.4.3 Dynamic Real-Time Detection of the effect of H-MW-HA on the H ₂ O ₂ -induced Redox Changes in Oral Keratinocytes Using ROS Sensitive Fluorescent Sensors.	221
5.5 Discussion	225
5.6 Conclusion.....	234
CHAPTER 6 HYALURONIC ACID PREVENTS ORAL AND INTESTINAL MUCOSITIS INDUCED BY CHEMOTHERAPY <i>IN VIVO</i>	235
6.1 Abstract	236
6.2 Introduction:	237
6.3 Materials and Methods	243
6.3.1 Preparation of 5-Fluorouracil (5-FU) and Hyaluronic Acid (HA)	243

6.3.2 Animals.....	243
6.3.3 Chemotherapy induced oral mucositis (CIOM) model	243
6.3.4 Oral Mucositis Index	247
6.3.5 Diarrhea Assessment	247
6.3.6 Macroscopic Examination of the tongue	247
6.3.7 Tissue sample preparation	249
6.3.8 Histological processing.....	249
6.3.9 Morphometric analysis	251
6.3.9.1 Small intestine.....	251
6.3.9.2 Tongue	252
6.3.10 Superoxide dismutase (SOD) enzyme activity assay	253
6.3.11 Statistical Analyses.....	253
6.4 Results:	254
6.4.1 Overall response to 5-FU and H-MW-HA treatment	254
6.4.2 H-MW-HA administration does not reverse 5-FU associated weight loss but increase overall survival rate of mice	254
6.4.3 H-MW-HA ablates Chemotherapy-induced diarrhea.....	258
6.4.4 H-MW-HA decreases the severity and duration of Chemotherapy-induced oral mucositis in mice	262
6.4.5 H-MW-HA reduces chemotherapy-induced oral ulcerations	264
6.4.6 Histological Evaluation of the Mice Tongue and Intestine in the control group ..	268
6.4.7 Effects of H-MW-HA on Oral Histology in 5-FU-Treated Mice.....	268
6.4.8 H-MW-HA treatment decreases intestinal injury elicited by 5-FU.....	272
6.4.9 Serum superoxide dismutase (SOD) activity is induced by H-MW-HA in 5-FU treated mice.....	277
6.5 Discussion:	279
6.6 Conclusion.....	287
CHAPTER 7 DISCUSSION AND CONCLUSION.....	288
7.1 Discussion	289
7.2 Conclusion and future studies	302
REFERENCES	306

LIST OF FIGURES

Figure 1.1 Relationships among oral complications of cancer and cancer therapy.....	7
Figure 1.2 Cross section showing the principal components of oral mucosa.	10
Figure 1.3 Cross-section of a typical segment of the GI tract wall showing the four principal layers and associated structures: mucosa, submucosa, muscularis, and serosa..	12
Figure 1.4 Schematic representation of the anatomical and histological organization of the digestive tract..	15
Figure 1.5 The effect of dynamic changes of the microbiota on all stages of mucositis, including pre-treatment risks..	47
Figure 1.6 The schematic diagram of the five stages represents the biological sequence of OM pathogenesis..	48
Figure 1.7 The Children's International Mucositis Evaluation Scale (ChIMES).....	60
Figure 2.1 Schematic diagrams summarising the experiments undertaken in the project.	84
Figure 2.2 Standard plate layout of a 96-well plate for cell titration assay to be analysed in the Synergy HTX microplate reader (Bio-Tek, USA).....	93
Figure 2.3 Plate layout of two 6-well plates for cell proliferation assay analysed by TC10™ Automated cell counter.....	96
Figure 2.4 Plate layout of a 4-well plate for the cell proliferation assay to be analysed by Fluorescein diacetate (FDA)/ Propidium iodide (PI) staining assay.....	99
Figure 2.5 Standard plate layout of a 96-well plate for dose-response characterization of hydrogen peroxide (H₂O₂) to be analysed in a Synergy HTX microplate reader (Bio-Tek, USA) using fluorometric cytotoxicity assay (FCA)..	103

Figure 2.6 Standard plate layout of 96-well plate for the fluorometric cytotoxicity assay (FCA) to be analysed in a Synergy HTX microplate reader (Bio-Tek, USA)..	103
Figure 2.7 Schematic diagram of the fluorometric cytotoxicity assay (FCA) experimental procedure. OKF6 cells were seeded in 96-well plates overnight under standard conditions..	104
Figure 2.8 Standard plate layouts of a 96-well plate for fluorescein diacetate (FDA) assay to be analysed in the Synergy HTX microplate reader (Bio-Tek, USA)..	107
Figure 2.9 Experimental design of intracellular ROS production evaluation assay....	111
Figure 2.10 Experimental design of intracellular ROS level evaluation..	115
Figure 2.11 Oral cavity assessments..	120
Figure 2.12 Mouse tongue morphology..	124
Figure 2.13 Superoxide dismutase (SOD) inhibition assay mechanism	126
Figure 2.14 Samples and blank arrangement on 96-well plate	128
Figure 3.1 The proliferation of H400, H357, and OKF6 cells over 72 hours at varying cell seeding densities..	143
Figure 3.2 Effect of H₂O₂ treatment on the cell growth of H400 cell..	146
Figure 3.3 H₂O₂-induced cell death in oral keratinocytes..	148
Figure 3.4 H₂O₂-induced loss of cell viability in oral cancer and oral normal epithelial cells.....	152
Figure 3.5 Effect of H₂O₂ on the morphology of the cells.....	155
Figure 3.6 H₂O₂ induced morphological alteration consistent with apoptotic features in oral epithelial cells.....	157
Figure 4.1 Molecular configuration of the HA's repeating branch	166

Figure 4.2 The effects of Mucosamin® on OKF6 cell proliferation at 24/48/72 hours' time points..	179
Figure 4.3 Effect of Mucosamin® on epithelial cells morphology..	180
Figure 4.4 The effect of H-MW-HA on OKF6 cells' proliferation after 24 hours..	181
Figure 4.5 The effects of H-MW-HA on OKF6 cell proliferation after 24, 48, and 72 hours..	183
Figure 4.6 The effects of H-MW-HA on the percentage of viable OKF6 Cells relative to control (100%) after 24, 48, and 72 hours..	184
Figure 4.7 Differential effect of xl-HA products on OKF6 cells proliferation after 24 and 48 hours..	185
Figure 5.1 The protective effect of hyaluronic acid (HA) constituents on hydrogen peroxide (H₂O₂)-induced cytotoxicity in oral epithelial cells.....	209
Figure 5.2 Protective effect of Mucosamin® on H₂O₂-induced cytotoxicity in oral epithelial cells.....	210
Figure 5.3 Protective effect of High molecular weight hyaluronic acid (H-MW-HA) on H₂O₂-induced cytotoxicity in oral epithelial cells..	211
Figure 5.4 The protective effect of HA constituents on hydrogen peroxide (H₂O₂)-induced cell death in oral epithelial cells..	212
Figure 5.5 Protective effect of H-MW-HA (0.01%, and 0.05% w/v) against H₂O₂-induced ROS production..	219
Figure 5.6 Protective effect of H-MW-HA (0.05% w/v) against H₂O₂-induced ROS production..	222
Figure 5.7 Effect of H-MW-HA on H₂O₂-induced ROS production in oral keratinocytes.....	223
Figure 6.1 Schematic diagram of the experimental procedure timeline..	245

Figure 6.2 Oral cavity assessment..	248
Figure 6.3 Histological sections of the various sections used for mucositis assessment.	250
Figure 6.4 Schematic diagram of the wall of the small intestine..	251
Figure 6.5 Mucosa of the tongue.	252
Figure 6.6 The effects of high molecular weight Hyaluronic acid (H-MW-HA) treatment on body weight and survival of mice..	257
Figure 6.7 Effect of High molecular weight hyaluronic acid (H-MW-HA) on diarrhea induced by 5-Flurouracil (5-FU) in mice..	259
Figure 6.8 Incidence and severity of diarrhea overtime in group C and group D mice..	261
Figure 6.9 Effect of high molecular weight hyaluronic acid (H-MW-HA) co-treatment on oral mucositis severity in 5-FU treated mice..	263
Figure 6.10 Chemotherapy-induced oral mucositis (CIOM) ulcers in (5-FU)-treated mice..	266
Figure 6.11 Chemotherapy-induced oral mucositis (CIOM) ulcer size percentage after HA treatment in the 5-fluorouracil (5-FU)-treated mice..	267
Figure 6.12 Effect of H-MW-HA on the 5-FU-induced tongue mucosa damage..	270
Figure 6.13 Effect of H-MW-HA on the 5-FU-induced intestinal mucosa damage.	274
Figure 6.14 Effect of H-MW-HA on the 5-FU-induced intestinal mucosa damage.. ...	275
Figure 6.15 High molecular weight hyaluronic acid (H-MW-HA) increased activity superoxide dismutase (SOD) enzyme in 5-FU treated mice..	278

LIST OF TABLES

Table 1.1 Risk factors related to developing oral mucositis.....	21
Table 1.2 Five-Stage Model of Oral Mucositis	40
Table 1.3 Canonical pathways that were prioritized based on gene frequency and expected relevance to regimen-related toxicity..	49
Table 1.4 Measurement of Oral mucositis.	52
Table 1.5 Measurement of Oral mucositis.	53
Table 1.6 Measurement of Oral mucositis..	55
Table 1.7 Clinical Oral Mucositis Assessment Scales.	58
Table 1.8 Oral Mucositis Assessment Scale	59
Table 1.9 Oral Mucositis Management.....	63
Table 2.1 Visual oral ulcerative mucositis score.	121
Table 2.2 Solution amount in each well	128
Table 2.3 Steps involved in tissue processing..	130
Table 2.4 Steps involved in hematoxylin and eosin (H&E) staining technique.	132
Table 5.1 Difference in cell viability (% of control) of OKF6 cells treated with HA constituents compared to untreated control group (100%)..	213
Table 5.2 Difference in cell viability (% of control) of OKF6 cells treated with HA constituents compared to H₂O₂-treated group..	214
Table 5.3 The Protective effect of Mucosamin[®] on H₂O₂-induced cytotoxicity in oral epithelial cells.....	215
Table 5.4 The Protective effect of H-MW-HA on H₂O₂-induced cytotoxicity in oral epithelial cells.....	216

Table 5.5 protective effect (in ratio) of H-MW-HA 0.01% w/v and 0.05% w/v against H₂O₂-induced ROS production..	220
Table 5.6 protective effect (in ratio) of H-MW-HA (0.05% w/v) against H₂O₂-induced ROS production..	224
Table 6.1 Visual oral ulcerative mucositis score.	246
Table 6.2 Treatment allocations and body weights from 5-Flurouracil (5-FU) and hyaluronic acid (HA) treated mice..	256
Table 6.3 Description of diarrheal status stratified by treatment received.....	259
Table 6.4 The effect of H-MW-HA on the 5-FU-induced intestinal injury..	276

CHAPTER 1 LITERATURE REVIEW

1.1 Introduction

The term “mucositis” has been coined to indicate cytotoxic neoplastic therapy-induced mucosal barrier injury (Sonis, 2012a). Mucositis has been defined as a common and most debilitating complication associated with cytotoxic cancer therapy. It remains a significant burden for those who are undergoing anti-neoplastic drug or radiation therapy (Villa and Sonis, 2015; Lalla et al., 2014; Al-Dasooqi et al., 2013; Sonis et al., 2004a). There is a sizeable incremental cost for patients with mucositis, and these additional healthcare resources are due to increased rates of hospitalization, the use of opioids, and a greater need for nutritional and fluid support. The greater the severity of mucositis, the higher the cost of treatment (Murphy, 2007), with an incremental cost of supportive care in the patients population estimated to be more than US \$17,000 per patient, for those who had head and neck cancer (HNC) (Nonzee et al., 2008). A recent meta-analysis by Elting and Chang,. (2019) has estimated the additional cost of oral mucositis to be between US\$5,000 and US\$30,000 per patient receiving radiation therapy and US\$3,700 per cycle in patients undergoing chemotherapy (CT). In addition, hospitalization costs associated with mucositis in stem cell transplant patients have been estimated to overreach US\$70,000 per patient (Elting and Chang, 2019).

Oral cavity mucositis, or oral mucositis (OM), is one of the cancer regimen-related toxicities and is typically associated with radiation therapy, CT or the combination of the two, such as in patients receiving hematopoietic stem cell transplantation (HSCT) (Georgiou et al., 2012; Sonis, 2011). OM has been the most extensively studied among alimentary tract mucositis (Al-Dasooqi et al., 2013; Villa and Sonis, 2015). Virtually all patients receiving radiotherapy (RT) alone or concomitant with chemotherapy develop OM (Trotti et al., 2003a). Its onset depends on the type of treatment, the schedule of drug therapy and patient-based variables (Bentzen et al., 2001; Georgiou et al., 2012; Trotti et al., 2003a; Vera-Llonch et al., 2006).

Established research indicates that the incidence of mucositis ranges from 30-40% to nearly 100% when considering all grades of mucositis (Keefe et al., 2007; Mortensen et al., 2012). About 30–40% of cancer patients treated with CT develop mucositis; this percentage rises to 60–85% for patients receiving conditioning regimens before HSCT and can increase to almost 100% for patients with HNC undergoing radiation therapy concomitant with CT (Sonis et al., 2004a; Blijlevens et al., 2008; Villa and Sonis, 2016b). However, according to Sonis (2007), the incidence of OM is underreported. Reasons for this could be the lack of standardized

clinical scoring criteria, the site of the tumour, and the variety of treatment regimens (Sonis, 2007).

Three of the most commonly used OM grading systems, recently appraised by Villa et al., (2021) include the World Health Organization (WHO), the common terminology criteria for adverse events (CTCAE), and the score of Radiation Therapy Oncology Group (RTOG).

OM is characterized clinically by erythema of the OM that develops to ulcerative lesions that are associated with severe pain and may need the use of analgesics, alterations in nutritional status; parenteral or gastrostomy feeding reliance, hospitalization, and can predispose to bacteraemia or sepsis in case of neutropenic patients. This adversely affects patients' quality of life (QoL), increasing healthcare resources with accompanying costs (Elting et al., 2008b; Sonis et al., 2004a; Elting et al., 2003; Scully et al., 2003). OM is reported as the considerable undesirable and bothersome side effect of cancer treatment experienced by patients (Sonis, 2011). Further, in some cases, it can turn into a dose limiting toxicity that leads to delay or even cessation of cancer treatment, thereby negatively affecting patient's cancer treatment outcome (Sonis et al., 2004a).

The pathobiology of mucositis encompasses a series of related and interacting biological and cellular events that initially occur in the submucosal and culminate in targeting the epithelium. The pathogenesis of OM has been proposed to follow sequentially and mechanically linked and overlapped five phases, including initiation, primary damage response, signalling and amplification, ulceration, and healing, with microbiota's role in mucositis pathogenesis has been considered in all phases (Sonis, 2007; Bowen et al., 2019).

Several therapeutic and preventive modalities are being used in the management of OM, including growth factors, essential oral care, anti-microbial and anti-inflammatory agents, low-level laser therapy (LLLT), natural agents, and cryotherapy, among others (Al-Rudayni et al., 2021; Miranda-Silva et al., 2021; Elad et al., 2020; Saunders et al., 2020; Yarom et al., 2020; Ariyawardana et al., 2019; Yarom et al., 2019; Lalla et al., 2014; Migliorati et al., 2013; Saunders et al., 2013). So far, palifermin is the singular compound authorized as a medicine for the treatment of OM by the European Medicines Agency and the US Food and Drug Administration (Elad et al., 2020). Nevertheless, Various agencies have developed many guidelines on OM management, including the American society of clinical oncology (ASCO), the oncology nursing society (ONS), the national comprehensive cancer network (NCCN), and

the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO), based on different evidence of effectiveness (Moslemi et al., 2016).

Though the negative impact of OM on the patient's cancer treatment outcome is well established, the accessible therapeutic agents for mucositis are, to a great extent, palliative (Sonis, 2011). Currently, there is no effective treatment that mechanistically targets the pathogenesis of the disease and surprisingly, up to date, there is no drug that has been approved to prevent the disease (Cirillo et al., 2015). New effective therapeutic approaches based on mechanistic insights are required. Understanding the pathobiology and the pharmacogenomics of toxicities is the main objective of mucositis researchers to develop effective targeted mucositis therapy.

HA preparations are widely used in treating several diseases and have proven recently to be potentially effective in treating OM (David-Raoudi et al., 2008; Favia et al., 2008; Cirillo et al., 2015; Ruggiero et al., 2016). However, the mechanisms underlying its effect remain unclarified yet. The present study aimed to assess the potential protective effects of hyaluronic acid (HA) preparations in an *in vitro* oxidative stress-induced model of oral mucosal injury. Further, we tested a selected HA preparation in an *in vivo* mouse model of chemotherapy-induced oral and intestinal mucositis, by focusing on oxidative stress as a readout.

Below, an insight into cancer, head and neck cancer, OM, hyaluronic acid, and evidence of hyaluronic acid therapy for OM is given in this chapter.

1.2 Mucositis

1.2.1 Generalities of Cancer treatment-induced mucosal injury

Cancer treatment is associated with many adverse events (AEs) that may be of acute or chronic onset. Mucositis is a common and most debilitating complication associated with cytotoxic RT and/or CT (Scully et al., 2006; Krishna et al., 2011). It is an inflammatory condition that affects the entire alimentary canal from the mouth to the anus, precipitating ulceration and pain in both the oral cavity (oral mucositis) and intestinal tract (intestinal mucositis), and for this reason, often referred to collectively as alimentary tract mucositis (Krishna et al., 2011; Sonis et al., 2004a). Ancillary associated complications include pain, ulceration, abdominal bloating, anorexia, nausea, vomiting, and diarrhea (Keefe et al., 2000; Keefe, 2004; Gibson and Keefe,

2006; Pico et al., 1998) that remain a significant burden for 40-80% (Lalla and Peterson, 2006; Campos et al., 2014) of patients undergoing chemotherapeutic or radiation regimens (CRT) (Sonis et al., 2004a; Al-Dasooqi et al., 2013; Villa and Sonis, 2015; Lalla et al., 2014). Assessment of mucositis often focuses on OM, partly due to the practical difficulties in directly studying gastrointestinal tissues from cancer patients (Kuiken et al., 2015; Al-Dasooqi et al., 2013; Sonis et al., 2004a). In addition, intestinal mucositis (IM) is primarily assessed by clinical symptoms or biomarkers given it cannot be visually inspected (Herbers et al., 2010; Kuiken et al., 2017).

1.2.2 Treatment of cancer and mucositis

The efficacy of various antitumor treatment modalities on cancer response and survival of the patients with tumour has been the main focus of studies in oncology for long decades. With the advancement of time, and to reduce the comorbidities associated with the earlier radical ways, treatment modalities have undergone several progressions from the radical strategies of treatment such as, radical surgeries and radical RT. to the current organ saving procedures which particularly targeted the tumour involving tissues only. Furthermore, parallel advancements have occurred for the locoregional control of cancer, like concurrent chemoradiotherapy (CCRT), postoperative RT /CCRT, simulated CCRT, and Intensity-modulated radiation therapy. In this evolution of modern medicine, the concentration is not only increasing survival among cancer patients by adding quantity to the life, but also enhancing the QoL of cancer patients (Debela et al., 2021).

Surgery, RT, and / or CT remain the gold standard treatment modalities for HNCs (Argiris et al., 2008). The proper surgery, radiation therapy targeted area, dose-quantity and fractionation, and signs for CT rely upon the particular site and the spread (stage) of cancer and pathologic discoveries (Pfister et al., 2020). for patients having early-stage cancer (I or II), around 30% - 40% of the recommended treatment is single-methodology therapy, typically with surgical procedures or RT (Funk et al., 2002; Pfister et al., 2020). Commixed style treatment is advised for about 60% of patients with locally or widespread advanced cancer at finding (Funk et al., 2002; Adelstein et al., 2017). The pillar of treatment for HNC squamous cell carcinoma (HNSCC) that progressed locoregionally is a surgical procedure followed by adjuvant radiation therapy or standard concurrent chemoradiotherapy keeping surgical intervention as salvage treatment, alluded to as the organ-preservation approach (Anderson et al., 2021). In addition, there are new therapies that are designed to target specific molecular components, which could

help comprehension of the molecular genetics for HNSCC (Kumar et al., 2008). Biologic therapies, that aim at targeting specific mechanisms important for tumour growth and metastasis, have also provided an opportunity to personalize therapy depending on patient or tumour special characteristics (Shin and Khuri, 2013; Urba et al., 2012).

Overall, a multidisciplinary team should supervise the case to deal with the symptoms and problems associated, such as pain, xerostomia, speech difficulties, and depression, to name a few. Comorbidities should be treated alongside with ensuring the best achievable QoL during and after the treatment (Farwell et al., 2002).

All types of HNC treatments can lead to complications that may impair the patient's life and cause certain organ mutilation (Galbiatti et al., 2013). At least 50% of patients who have locally advanced HNCs are at a high risk of developing local or distant deteriorations, recurrence, secondary tumours and metastasis (Agra et al., 2010; Li et al., 2010; Lim et al., 2010; Argiris et al., 2008). An increased risk of acute and long-term side effects is associated with more aggressive cytotoxic anticancer therapy (Langer, 2008). For example, RT damages the salivary glands, resulting in xerostomia, pain, and difficulty with speech, swallowing, and eating, with an expanded risk of oral infections (Dirix et al., 2006; Rosenthal et al., 2006). Mucositis is another significant acute cancer therapy-related toxic side effect. An average incidence of mucositis in HNC patients undergoing RT, CT, or radio-chemotherapy is as high as 80-100% (Trotti et al., 2003a). These conditions cause substantial morbidity and significantly impair cancer patients' QoL.

1.2.3 The Economic implications of mucositis

Oral complications of cancer and cancer treatment are interrelated, although they are often described as separate clinical entities in the literature. For this reason, they share not only clinical aspects but also functional, psychosocial, and economic consequences. Figure 1.1 shows the correlation between oral complications of cancer and cancer treatments. OM is one of the most well-studied toxicities associated with cancer therapy. Mucositis is not just a significant cause of patient symptoms and risk of infection; Yet, its existence is likewise associated with worsening health conditions, increased utilization of healthcare resources, and significantly increased expenses (Carlotto et al., 2013). For instance, OM patients experience more infection and pain, are intolerant of standard diets, and frequently depend on enteral nutrition for nourishing support. They also have high rates of unscheduled doctor visits,

emergency room visits, and hospitalizations and spend a more extended stay in the hospital than those who do not develop the disease (Carlotto et al., 2013; Elting et al., 2003; Murphy, 2007). These can add a substantial burden to the total cost of care. Various studies have reported the incremental costs of OM (Elting and Shih, 2004; Mittmann et al., 2010; Elting et al., 2003; Sonis et al., 2001; Elting et al., 2007; Murphy et al., 2009; Nonzee et al., 2008; Peterman et al., 2001).

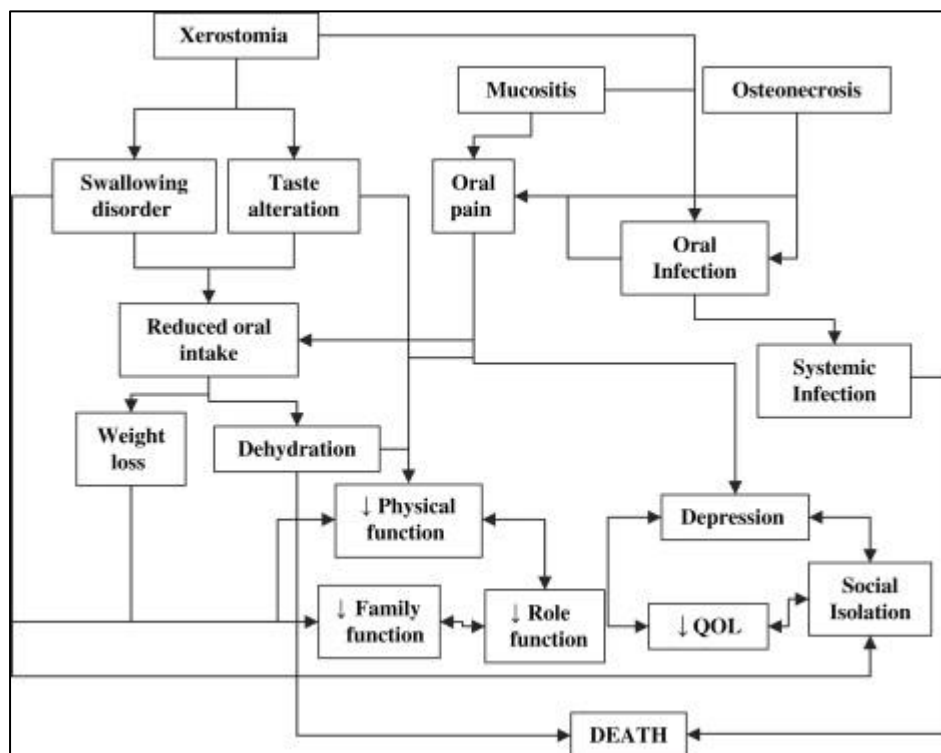


Figure 1.1 Relationships among oral complications of cancer and cancer therapy. Abbreviation: QoL; quality of life. Adapted from Elting et al.,(2008a).

The overall mucositis incremental costs follow the mucositis severity grade (Carlotto et al., 2013). For example, when severe grades were considered, the additional cost associated with severe mucositis was reported at \$3,600, more than double the additional cost of mild/moderate mucositis, which was reported at US\$1,700 (Elting et al., 2003). Other studies have reported similar results when comparing incremental costs for mucositis grades: US\$2,949 for mild/moderate mucositis versus US\$4,037 for severe mucositis (Peterman et al., 2001) and US\$2,725 for mild/moderate grades versus US\$5,565 for severe grades (Elting and Shih, 2004). The estimated hospitalization costs for mucositis related to any regimen or cancer was US\$7,985 per patient, based on identifying International Classification of Diseases-9 codes from a Texas state-wide registry of claims (Elting and Shih, 2004). For example, when patients with lymphoma or solid tumours receiving CT develop mucositis, it is associated with

increased risks of hospitalization and an incremental cost of US\$3000 per cycle with OM and US\$6000 per cycle with both oral and gastrointestinal mucositis (Elting et al., 2003). In patients receiving RT for HNC, mucositis is associated with increased requirements for gastrostomy tube insertions, hospitalizations, and incremental costs ranging from US\$1700 to US\$6000 per patient, based on mucositis grade (Elting et al., 2007; Peterman et al., 2001; Vera-Llonch et al., 2006). The economic burdens of radiation are increased by the concomitant administration of CT and accelerated schedules (Elting et al., 2007; Sonis et al., 2004a; Garden, 2001). A review study (Nonzee et al., 2008) outlined an additional cost of US\$18,515 among patients with mucositis being treated for HNC or lung cancer, greater than what had been observed in other studies. A study conducted by Sonis et al., (2001) reported that the increase in the maximum OM score by one-point in the (HSCT) patients was correlated with an extra 1 day of fever, up to 2.7 extra days of complete parenteral feeding, increased risk of infection by 2.1 times, and a further 2.6 days of analgesics, another 2.6 days of hospital stay, increased risk of 100-day death by 3.9 times, contributing to overall hospital costs of approximately \$25,000 per patient. Finally, given the high economic burdens associated with OM, an effective mucositis intervention product has a substantial potential financial return estimated by experts to be in the range of US\$1 billion annually (Villa and Sonis, 2020).

1.3 Mucous membranes of oral and intestinal mucosa

Mucous membrane (also called mucosa) is a moist epithelial membrane that lines internal organs and body cavities exposed to the external environment, mainly the respiratory, digestive, and urogenital tracts. The mucous membrane contains mucosal cells that secrete mucus, a gelatinous fluid that comprises mainly of water (~ 95%), mucins (0.5-5%), proteins (0.5-1%), inorganic salts (~ 1%), lipids, and mucopolysaccharides (Bansil and Turner, 2006; Lai et al., 2009; Dwivedy and Aich, 2011). Examples of the mucous membranes that line various tracts and structures of the body include the lips, mouth, nose, trachea, eyelids, conjunctival membranes, middle ears, and eustachian tube. Other mucosal examples include lungs, stomach and intestines, ureters, urethra, vagina, and urinary bladder (Dwivedy and Aich, 2011). Mucous membranes vary in structure but are mainly composed of one or more layers of epithelial cells overlying a deeper layer of loose connective tissue (Hall and Guyton, 2011). The function of mucosal lining is to protect the underlying tissue from mechanical damage and the invasion of microorganisms and toxic substances. Mucous membranes also help keep underlying tissues moist and play a role in the absorption and transformation of nutrients

(Squier and Kremer, 2001; Hall and Guyton, 2011). The mucous membrane that lines the oral cavity is called the oral mucosa (Squier and Kremer, 2001).

1.3.1 Oral Mucosa

The oral mucosa is a moist membrane composed of soft tissue. It extends from the junction between the border of labial mucosa and the lips anteriorly to the folds of the palatopharyngeal posteriorly (Brizuela and Winters, 2021).

1.3.1.1 Oral mucosa layers

Oral mucosa configures of three different layers (figure 1.2). The surface epithelial layer followed by basement membrane, and lies on the underlining fibrous connective tissue layer, called lamina propria. The oral mucosa is connected to underlying structures in many mouth regions by loose and a dense submucosal connective tissue (Johnston, 2015; Groeger and Meyle, 2019). Together, these three layers are comparable to the dermal, epidermal, and hypodermal skin layers (Moharamzadeh et al., 2007).

1.3.1.1.1 Epithelium

The surface epithelium of the oral mucosa is stratified squamous epithelium, which may be keratinized or non-keratinized in accordance with the area of the mouth. It can modify in thickness in different oral regions. The epithelium displays four layers of cells: the spinous layer, the basal layer, the granular layer, and the keratinized superficial layer (Johnston, 2015). The keratinization process is the transformation of viable keratinocytes in the granular layer into dead surface cells free of organelles and full of masses of cytokeratin fibres. The granular layer is replaced by the surface layer in non-keratinized oral epithelium, which lack keratohyalin granules (Squier and Hill, 1989). The oral epithelium also involves non-keratinocyte cells, including Langerhans, melanocytes, and Merkel cells. The epithelial cells are attached to each other by desmosomes, while the basal layers are connected to the underlying lamina propria via hemidesmosomes and the basement membrane (Moharamzadeh et al., 2007).

1.3.1.1.2 Lamina propria

Lamina propria is a connective tissue layer consisting of fibroblasts, blood vessels, nerves, mast cells, macrophages, mast cells, and inflammatory cells, all immersed in an amorphous

substance made up of proteoglycans and glycoproteins. Lamina propria is subdivided into the superficial papillary and reticular deep layers. Fibroblast is the primary cell found in the lamina propria. It takes part in synthesizing and reintegrating connective tissue fibers and amorphous substance and participates in wound healing, where fibroblasts' number increases (Kydd and Daly, 1982).

1.3.1.1.3 Submucosa

Submucosa is a fibro collagenous and elastic tissue layer that contains blood vessels and nerves. Depending on its location, the submucosal layer may contain adipose tissue, lymphoid tissue, minor salivary glands, and muscle fibres. The submucosa is found in all areas of the oral cavity except for the hard palate and attached gingiva, which are covered by the masticatory mucosa. Where the submucosal layer is absent, the lamina propria are attached directly to the underlying bone and form a mucoperiosteum (Johnston, 2015).

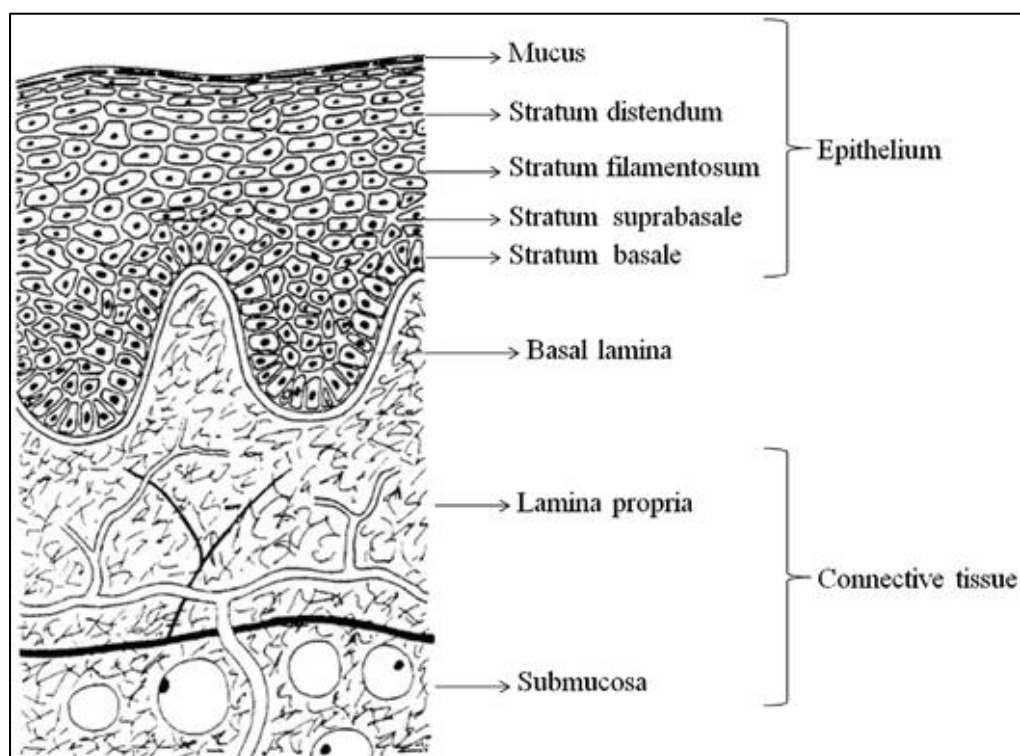


Figure 1.2 Cross section showing the principal components of oral mucosa. *Adapted from (Johnston, 2015)*

1.3.1.2 Oral mucosa structure

The mucous membrane lining the oral cavity (oral mucosa) is covered everywhere by stratified squamous epithelium (Squier and Hill, 1989). The oral mucosa may appear to be slightly

different from the rest of the moist lining of the digestive tract, to which it is attached. According to the functional requirements put upon different regions of the oral cavity, oral mucosa is classified into three different types that are histologically, clinically, and functionally different (Squier and Hill, 1989; Squier and Kremer, 2001). **1) Masticatory mucosa** covering the gingiva and hard palate regions, which are subject to mechanical forces of mastication, causing erosion and shearing, is made up of a keratinized stratified squamous epithelium. This tissue resembles the skin's epidermis in its maturation pattern and is tightly attached to the underlying structures by a collagenous connective tissue, or lamina propria. **2) Lining mucosa** covers the remaining areas of the oral cavity (floor of the mouth, buccal regions, and ventral surface of the tongue), except for the dorsal surface of the tongue. It provides an elastic and deformable surface that can stretch to accommodate movements such as mastication, speech, or bolus swallowing. Lining mucosa is covered with a non-keratinized stratified squamous epithelium and can vary in its thickness considerably in different areas of the mouth. It is attached to the underlying structures by a loose, elastic connective tissue, which is more flexible and elastic than masticatory mucosa's connective tissue. **3) A specialized mucosa**, which has the characteristics of both masticatory and lining mucosa, covers the dorsal surface of the tongue. Its surface consists of areas of the keratinized and non-keratinized epithelium. This epithelium is tightly attached to the underlying muscle of the tongue (Squier and Hill, 1989; Sloan et al., 1991; Squier and Kremer, 2001).

The different types of oral mucosa have different relative extent in the oral cavity. According to the measurements made by Collins and Dawes (1987), it has been calculated that the masticatory mucosa represents approximately 25% of the total surface area of the oral cavity lining while lining mucosa and the specialized mucosa (dorsum of the tongue) represents approximately 60% and 15% of the total surface area of the oral lining, respectively.

1.3.2 Gastrointestinal tract

The gastrointestinal (GI) tract is the largest mucosal surface in the human body, which is in continuous contact with dietary antigens and diverse commensal microorganisms that reside within the intestinal lumen. Thus, GI mucosal surfaces, particularly in the intestines, are crucial sites of innate and adaptive immune regulation (Turner, 2009; Helander and Fändriks, 2014). The tubular parts of this system, the oesophagus, stomach, small intestine, cecum, large intestine, colon, and rectum, are potential spaces that expand to adapt ingested substances (Gelberg, 2014).

1.3.2.1 GI tract wall

Through its extend from the oesophagus to the anal canal, the GI tract's wall has the same four layers of tissue; the details of their structural organization vary to fit their specific functions. Starting from the lumen and moving outwards, these layers are **1)** the Mucosa (tunica mucosa), **2)** Submucosa (tunica submucosa), **3)** Muscular coat (tunica muscularis), and **4)** Serosa (tunica serosa) or adventitia (tunica adventitia) (figure 1.3) (Patton and Thibodeau, 2019).

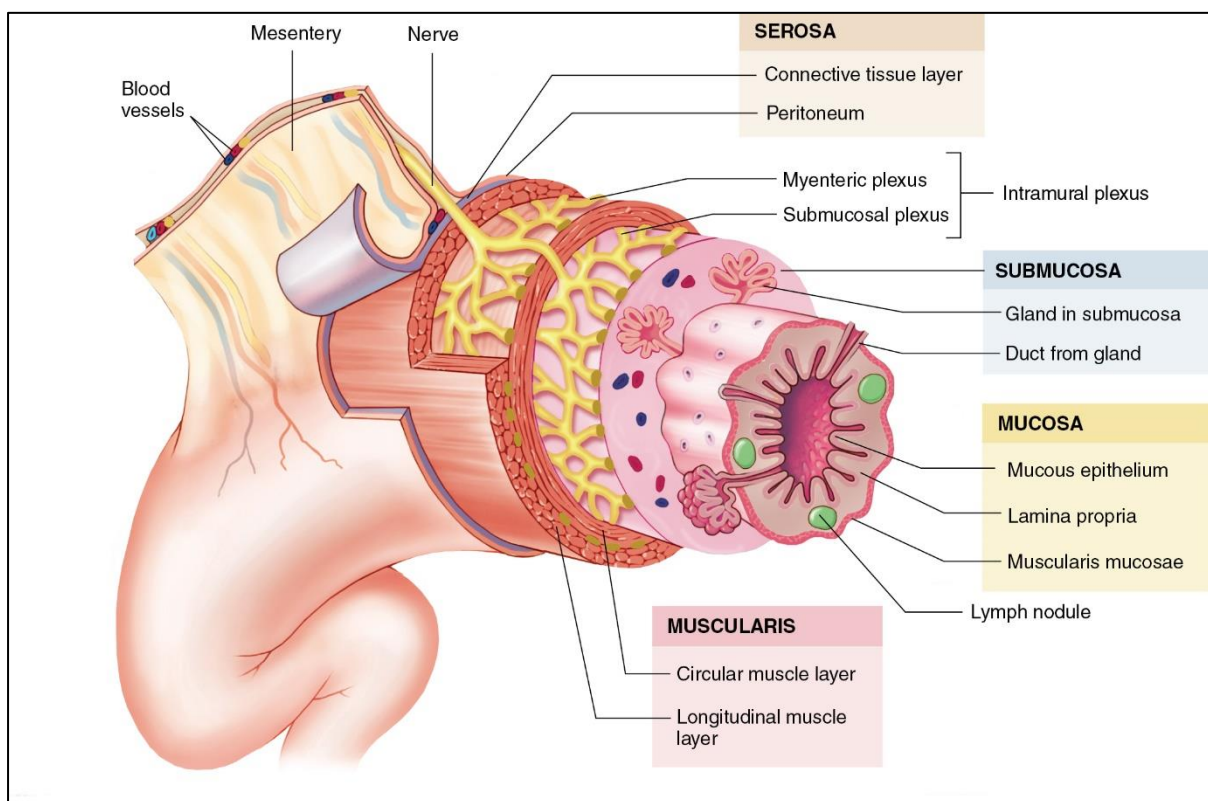


Figure 1.3 Cross-section of a typical segment of the GI tract wall showing the four principal layers and associated structures: mucosa, submucosa, muscularis, and serosa. Although different areas of the GI tract specialize in function, the anatomy of the wall is similar in structure. Adapted from (Patton, 2019).

1.3.2.1.1 GI Mucosa

Tunica mucosa is the inner-most layer of the GI wall found throughout most of the intestinal tract and is followed by the submucosa, the muscularis externa, and the serosa (Luissint et al., 2016). Three principal functions of intestinal mucosa include, protection of the inner

environment, secretion of antimicrobial peptides and absorption of immune sensing and nutrients (Salvo Romero et al., 2015). The intestinal mucosa itself is made up of 3 layers. A unicellular epithelium layer that lines the inner lumen of the GI tract. Immediately adjacent to the epithelial layer is a layer of loose fibrous connective tissue namely the lamina propria, an interstitial tissue with a rich vascular and lymphatic network and abundant leukocytes. The third is a thin layer of smooth muscle fibres called muscularis mucosae (Luissint et al., 2016).

1.3.2.1.1.1 Epithelium

Intestinal mucosal surface is covered by epithelial layer, which is in direct contact with alimentary tract contents. Within the epithelium, there are many individual cell types, each with its own specialized functions, including absorption of nutrients and barrier function, mucus production, antimicrobial molecules production, growth factors production, and cell regeneration (Luissint et al., 2016). For example, In the esophagus and anal canal the epithelium is protective and it is composed of nonkeratinized stratified squamous epithelium. The remainder is a simple columnar epithelium. This simple columnar epithelium also has absorption function in the intestine, and secretory function, especially in the stomach (Peterson and Artis, 2014; Salvo Romero et al., 2015).

Structurally, the epithelium surface of the small intestine forms tiny finger-like projections called villi, inside of which is supplied by the rich vascular and lymphatic network of the lamina propria. The mature villous epithelial enterocytes are fed by epithelial stem and progenitor cells found in adjacent invaginations on the mucosal surface, called crypts of Lieberkühn. Intestinal epithelial cells (IECs) are interconnected by tight junctions that create a physical barrier between the luminal contents and the intestinal lamina propria (Jenq et al., 2015). IECs and the tight junctions between them are essential components of the intestinal barrier and are primarily responsible for the absorption of nutrients (Peterson and Artis, 2014). Intestinal stem cells (ISCs) are located at the base of the crypt and are able to generate all types of cells in the intestinal epithelium. Thus, they are essential for both intestinal homeostasis and post-injury regeneration (Sato et al., 2009). Close to the ISCs, Paneth cells are located at the bases of the crypt and produce ISC growth factors (Sato et al., 2011). In addition, Paneth cells contain a variety of potent antimicrobial molecules with efficacy against intestinal bacteria (Peterson and Artis, 2014).

1.3.2.1.1.2 Lamina propria

The lamina propria is a loose connective tissue lying below the IEC layer that contains nerve fibres, blood vessels, lymphatic vascular systems, smooth muscles that regulate blood flow and villus movement, and immune cells such as neutrophils, regulatory T cells, macrophages, and mast cells (Mowat and Agace, 2014). It also contains the innate lymphoid cells, most recently identified cells of the innate immune system, which are involved in tissue homeostasis and coordination, during, for example, infection, inflammation, and cancer by promoting rebuilding, healing, and repair (Artis and Spits, 2015).

1.3.2.1.1.3 Muscularis mucosa

It is a thin layer of smooth muscle, in a state of constant tension, pulls the stomach and small intestine mucosa into wavy folds. These folds greatly increase the intestinal surface area available for digestion and absorption (Luissint et al., 2016).

1.3.2.1.2 Submucosa

The submucosa layer of the digestive tube lies immediately beneath the mucosa. It is a broad layer of dense connective tissue, thicker than the mucosal layer, that connects the overlying mucosa to the underlying muscularis. It contains blood and lymphatic vessels, numerous small glands that release digestive secretions. Additionally, it includes a dense branching network of parasympathetic nerves that form the submucosal plexus (Bernier-Latmani and Petrova, 2017).

1.3.2.1.3 Muscularis

The muscularis layer (also called the muscularis externa) is the third layer of the alimentary canal. It is a thick layer of muscle tissue that surrounds the submucosa. This wall portion comprises a double layer of smooth muscle: an inner circular layer and an outer longitudinal smooth muscle layer. Like the submucosa, the muscularis contains nerves organized into a plexus that lies between the two muscle layers, called the myenteric plexus. Both submucosal plexus and myenteric plexus are termed intramural plexus (figure 1.3). They both comprise the major part of the enteric nervous system and thus play an essential role in regulating digestive system movement and secretion (Pangtey et al., 2017; Geesman et al., 2021).

1.3.2.1.4 Serosa

The outermost layer of the GI wall is termed serosa (or serous layer). It is present only in the region of the alimentary canal within the abdominal cavity and consisting of serous membrane. The serosa is actually made up of a visceral peritoneum layer overlying a layer of loose connective tissue; the serous membrane that connects the abdominopelvic cavity and covers its organs. The coating attached and covers the walls of the abdominopelvic cavity is called the parietal layer of the peritoneum. The fold of serous membrane that connects the parietal and visceral portions is called a mesentery (figure 1.3). All together, these tissues serve to keep the alimentary canal in place near the ventral surface of the vertebral column (Patton and Thibodeau, 2019). A general gastrointestinal morphology rendering is represented in figure (1.4).

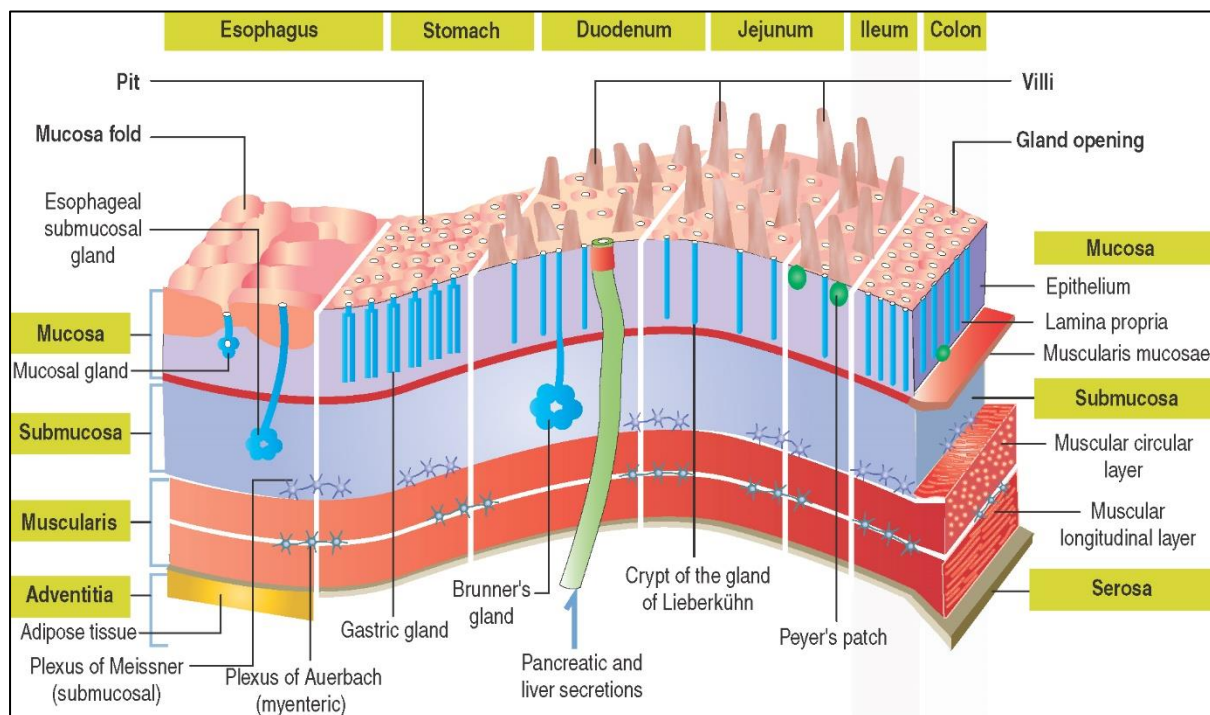


Figure 1.4 Schematic representation of the anatomical and histological organization of the digestive tract. Adapted from (Kierszenbaum, 2020).

Oral cavity has been described as a mirror reflecting of an individual's health. The GI tract is also a highly specialized chemosensory organ. Anticancer therapy always poses a significant challenge to oral mucosa integrity. CT and RT limit the ability of the epithelium to proliferate so that it becomes thin or ulcerated. This occurs first in the most rapidly proliferating tissues, such as oral and gastrointestinal lining mucosa (Squier and Kremer, 2001).

1.4 Oral mucositis

When mucositis inflammation occurs in the oral mucosa and oropharyngeal segment, it is called “oral mucositis” (Sonis, 2013; Lalla et al., 2014; Rosenthal and Trotti, 2009; Sonis, 2004b). Historically, the term mucositis has not been clearly defined by the literature. Injuries of the oral mucosa that were related to malignant tumour therapy were alluded to as “stomatitis” (Sonis, 2012a; Rosenthal and Trotti, 2009). Both terms, stomatitis, and mucositis, have been utilized reciprocally in the literature (Rosenthal and Trotti, 2009; Maiya et al., 2006; Freitas et al., 2014). However, a more specific term is needed, particularly, because “stomatitis” also refers to other conditions in the oral mucosa with damage not relating to cancer treatment etiological factors (e.g., infection). The term “Mucositis” has been recognized to be used for cytotoxic neoplastic therapy-induced mucosal barrier injury, and was adopted and assigned in 2007 (Sonis, 2012a; Shankar et al., 2017).

OM represents a serious clinical problem in oncology and remains the most common and disturbing complication in patients undergoing non-surgical cancer treatment. It happens in almost 100% of patients under conditioning therapies of HSCT, or RT, with/without concurrent CT, for HNCs (Sonis, 2007; Silverman, 2007; Trotti et al., 2003a).

Similarly, as with numerous different toxicities, It is logically evident that the reported OM prevalence is under-announced (Al-Ansari et al., 2015). Although some medical services experts may underestimate the clinical effect of OM, this consideration is not the same among patients who refer to OM as one of the most challenging and bothersome inconveniences of their tumour therapy (Scully et al., 2003; Sonis, 2011). Pain related to mucositis frequently debilitates QoL and the functional status of the patients (Elting et al., 2008b).

OM varies in severity from mild erythema, causing mucosal discomfort, to extensive ulcerative areas requiring high dosages of narcotics to reduce efficacy (Sonis, 2007). Severe OM may result in a reduction of cancer therapy dose or even abrupt cessation of cancer treatment. Moreover, severe OM with ulcers is portrayed by disturbance of the epithelial boundary integrities, leading to breaks in the mouth’s mucosa tissue, which implies an important risk factor for systemic infection (Sonis et al., 2001). The role of mucositis in bacteraemia and sepsis is also well proven (Elting et al., 1992; Beighton et al., 1994; Ruescher et al., 1998).

1.4.1 Epidemiology of oral mucositis

OM remains the most common and disturbing complication of anticancer therapy and a common cause of oral pain (Scully et al., 2006; Dale et al., 2004). It occurs in virtually all patients receiving radiation therapy for oral and oropharyngeal cancers and in over 50% of those treated for hypopharyngeal and laryngeal cancer (Trotti et al., 2000; Sonis and Fey, 2002; Trotti et al., 2003a; Elting et al., 2007; Villa and Sonis, 2020). The OM incidence, prevalence, and severity can vary significantly depending both on the treatment modalities and the patient's setting (Colella et al., 2010; Di Liberto et al., 2007; Sonis, 2007; Scully et al., 2006; Rubenstein et al., 2004; Sonis and Fey, 2002). Like with many complications of cancer treatment, studies have reported that OM is underreported and its incidence is possibly underestimated (Sonis et al., 2004a). The data that exist concerning the prevalence and incidence of mucositis are inconsistent (Murphy, 2007; Sonis, 2007), and the reason behind this conflict is due to patients age variability, tumour location, different treatment regimens, and the lack of standardized definitions/criteria for scoring OM (Chapko et al., 1991; Schubert et al., 1992; Wardley et al., 2000; Rubenstein et al., 2004; Sonis, 2004b; Sonis et al., 2004a; Blijlevens et al., 2008).

1.4.1.1 Prevalence

Over the last few years, the entrance of new CT and RT protocols has led to increase in the prevalence of OM among cancer patients (Rosenthal and Trotti, 2009). The occurrence of oral lesions varies according to the utilized treatment, and the mouth status before the appearance of the condition, and pathogenesis. Although it is known to be underestimated, as do many other toxicities, the overall frequency of OM varies between 15% and 50% (Keefe et al., 2007; Murphy, 2007; Sonis, 2007; Mortensen et al., 2012; Villa and Sonis, 2016b). Studies have shown that 20-40% of patients undergoing conventional CT cite mucositis as the most debilitating side effect of cancer treatment, even worse than vomiting, nausea, hair loss, diarrhea, or other common toxicities (Rose-Ped et al., 2002; Villa and Sonis, 2020). Approximately 9-19% of delays and interruptions in oncological treatment are attributed to OM (Trotti et al., 2003a; Rubenstein et al., 2004). Around 80% of HNC patients who develop RT -induced OM experience the ill effects of ulcers or pseudomembranes (Mercadante et al., 2015). Other studies (McGuire et al., 1992; Woo et al., 1993; Bellm et al., 2000) reported that OM prevalence might reach up to 80% in patients receiving high-dose CT protocols, in particular, when CT used as a conditioning regimen. However, another study reported, and depending on the assessment method used, that the prevalence of OM varied between 30% and

69% in patients receiving conditioning CT (Nottage et al., 2003). This discrepancy in OM prevalence reflects the underreporting of OM. Additionally, a higher prevalence of OM has been reported in paediatric patients compared to adult patients with the same malignancy and receiving the same antineoplastic regimens. (Sonis et al., 1978; Miller et al., 2012).

1.4.1.2 Incidence

The reported incidences of OM were extensive and varied according to the type of cancer and the treatment method (Carlotto et al., 2013). Radiation therapy has consistently resulted in high levels of mucositis. OM develops in virtually all patients receiving radiation therapy for HNC, especially if combined with CT (Trotti et al., 2003a; Elting et al., 2007). Current radio- and CT protocol studies show that OM has an 85-100% incidence rate, depending on three factors: the type of CT drug, the radiation dose received, and the treatment administration plan (whether fractionated or not) (Rodríguez-Caballero et al., 2012). Trotti et al (2003a) indicated that the overall incidence of OM is 80% in patients receiving treatment for HNC, with the incidence rate of severe grade 3-4 mucositis being 39%. The rate varies between as high as 100% for altered fractionation radiation treated patients, and 22% in those treated with CT alone. In patients undergoing conventional RT and concurrent chemoradiotherapy, the incidence is 97% and 89%, respectively (Trotti et al., 2003a). For radiation with altered fraction schedules, the incidence of severe grade 3-4 OM is highest. In contrast, in CT alone treated patients, the incidence of higher grade 3-4 OM is almost negligible (0%) (Trotti et al., 2003a). Another review by Sonis et al, showed that the mucositis incidence rate in HNC patients was 42%; the total number of patients was 2206 (Sonis et al., 2004a).

Most studies of the severity and incidence of OM have been on patients with cancer obtaining chemotherapeutic agents on the basis of an inpatient, which notified an incidence of OM ranging from 22.3% to 71.7% (Mercadante et al., 2015; Maree et al., 2012; Nishimura et al., 2012; Cheng et al., 2010). In another study of outpatients. OM incidence was higher (75.4%) (Chen, 2008). In patients receiving CT for solid tumours OM can develop in approximately 40% of patients (Elting et al., 2003). Furthermore, the frequency of ulcerative mucositis among HSCT recipients varies from 83% in autograft-receiving patients to 88% in allograft-receiving patients, depending on the stomatotoxicity of the regimens (Sonis et al., 2001). However, for the majority of conditioning regimens, eliminating whole-body irradiation reduced the incidence of severe OM in autologous-receiving patients to approximately 40% (Filicko et al., 2003; Spielberger et al., 2004; Fanning et al., 2006; Grazziutti et al., 2006).

Literature has shown a wide variation in OM rates (Wilberg et al., 2014; Kim et al., 2012; Elting et al., 2003). For example, a study by Çakmak and Nural, (2019), which included 147 patients undergoing outpatient cancer CT, reported that half of the patients (51.7%) developed OM. Furthermore, most of the patients with OM (81.9%) had grade 1 OM, while 17.1% and 1.3% of patients with OM developed grades II and III, respectively.

Research shows that the incidence of severe grade OM is positively correlated with the introduction of alternative fractionations of RT in HNC patients (Peracchia and Salti, 1981; Horiot et al., 1990; Geara et al., 1996). Wilkes (1998), reports that the overall incidence of OM in patients with locally advanced HNC undergoing concurrent radio-chemotherapy is 100%, with the occurrence of higher grade OM varied from 20%-47%. Additionally, data about the incidence rate of OM in young patients compared to older patients are conflicting. While some research reported that paediatric patients, who receive similar anticancer treatment have a higher incidence of OM than older patients with the same malignancy (Sonis et al., 1978; Miller et al., 2012), other research indicates a significant correlation between OM and advanced age (Mattsson et al., 1992; McCarthy et al., 1998; Zalcberg et al., 1998).

1.4.2 Risk factors of oral mucosal injury

Prioritized by funding agencies, the major goals of current research in cancer therapy-induced OM are the prediction of risk factors and severity of OM and the application of personalized treatment or preventive targeted molecular therapy (Sonis, 2015). Recognizing risk factors for OM can be an essential aid that can be used for early interventions and to facilitate personal support for affected patients (Wardill et al., 2020). Not all cancer patients, who are diagnosed with the same malignancies, and receive the same treatment, are at an equivalent hazard of creating mucositis or different toxicities. Moreover, It has been widely observed that different anatomical sites of the oral mucosa vary in the risk of developing clinically significant OM. For example, CT with high doses often results in at least moderate mucositis of the buccal mucosa, soft palate, floor of the mouth, and lateral and ventral surface of the tongue, while adherent gingiva and hard palate are rarely affected (Lalla et al., 2008).

Although a plurality of research studies on OM have been inducted during the past few decades, a clear and comprehensive description of risk aspects related to lesion development and progression remains unclear and needs more investigation (Barasch and Peterson, 2003). For example, a study by Sonis (2009) reported that approximately 8% of new cancer patients would

be at high risk of being affected with ulcerative mucositis. These include patients receiving RT with/without concurrent CT to treat HNC and patients receiving antagonistic types of conditioning CT, particularly combined with total-body irradiation (TBI), before HSCT.. On the contrary, about 40% of new cancer patients treated with surgical intervention, peripheral radiation, or low-dose CT would be at small or never be at risk of developing mucositis (Sonis, 2009). Furthermore, approximately 20% to 50% of new patients of cancer are at moderate risk of developing mucositis, and at some point in their therapy, they would have ulcerative mucositis (Sonis, 2009).

Given the difficulties of performing cellular and molecular studies in human cancer patients and the inherent limitation of animal models, understanding the full range of pathophysiology of OM and therapeutic approaches requires additional evidence on epidemiological factors and risk factors. This, in turn, enhance researchers to set new clinical trials incorporating mucositis risk factors in patient stratification (Barasch and Peterson, 2003). Additionally, recently emergent data, including a study by Logan et al. (2009) that provides insights into differences and similarities of pathobiology caused by different chemotherapeutic agents, has changed conceptions about the classical modelling of commonalities among injury mechanisms across the alimentary tract mucosa (Peterson and Lalla, 2010). This modelling provides the basis for potentially identifying the relative risk degree for patients who develop oral and/or gastrointestinal mucositis. Risk factors related to developing OM have been grouped into two main classifications; treatment-related and patient-related factors (Pico et al., 1998; Barasch and Peterson, 2003; Cawley and Benson, 2005; Eilers and Million, 2007). However, additional factors also seem to be contributed to the mucositis risk, including genetic-related factors (Filippi et al., 2006; Peterson et al., 2012) and tumour-related factors (Sonis, 2012b), and oral microbiota (de Farias Gabriel et al., 2022) (Table 1.1).

Table 1.1 Risk factors related to developing oral mucositis.

Risk factors categories
Treatment-related variables
Therapy type (chemotherapy, radiotherapy, or combined chemo-radiotherapy)
- Chemotherapy related variables
- Radiation-related variables
- The role of novel targeted anticancer treatments in mucositis development
Transplant-related variables
Patient-related variables
Oral microbiota
Gen-related variables
Malignancy type

1.4.2.1 Treatment-related factors

Treatment-related factors are of great significance in OM. Therefore, they need to be evaluated prior to initiating cancer treatment (Barasch and Peterson, 2003). Treatment-related risk factors attributed to mucositis development are linked to the type of anti-neoplastic modalities administrated. These include the type of therapy (radiation, CT, combined chemo-radiotherapy, or targeted therapy), dose, schedule, and route of administration (Sonis, 2010b; Villa and Sonis, 2015; Villa and Sonis, 2016a; Villa and Sonis, 2016b).

Treatment type and dose can be among the considerable overwhelming risk factors shown to influence the subsequent incidence and progression (severity and duration) of mucositis (Sonis, 2004a; Cawley and Benson, 2005). OM has been reported to be correlated with a large number of cytotoxic agents (Wilkes, 1998). However, few studies have analysed the specific relation of these regimens to the incidence and severity of the toxicity (Barasch and Peterson, 2003). Virtually, 40% of cancer patients undergoing CT regimens as a treatment for most common types of tumour, such as breast, lung, and colorectal cancer are at risk of developing OM. The relative risk increases to become 100% in patients receiving high-dose radiation therapy for HNC. Furthermore, nearly all patients (> 90%) receiving high conditioning doses of CT in HSCT are at risk of affecting with OM (Barasch and Peterson, 2003; Scully et al., 2003; Trotti et al., 2003a; Sonis et al., 2004a; Cheng et al., 2010; Sonis, 2012b; Peterson, 2006; Shouval et al., 2019).

Previous exposure to RT was reported as a risk factor for developing OM in autologous HSCT recipients (Bolwell et al., 2002), adding strength to reports of residual DNA damage as an independent predictor of OM risk (Goutham et al., 2012). Similarly, HNC patients undergoing adjunctive RT who underwent previous CT treatment were reported to have a high risk of developing OM (Porock et al., 2004).

A number of mucositis risk factors in the treatment-related category have been investigated epidemiologically or microbiologically, while others have been hypothesized according to the outcomes of prevention and/or treatment studies (Muller et al., 1999; Barasch et al., 1995; Woo et al., 1993; Zerbe et al., 1992; Mattsson et al., 1991; Wingard et al., 1991). Therefore, specific mechanistic relationships for many of these factors must be identified (Barasch and Peterson, 2003).

1.4.2.1.1 Chemotherapy-related factors

Mucositis onset and severity rates depend on the used type of CT (Shi et al., 2016). The chemotherapeutic agents differ in their mucosal toxicity (Peterson, 2006). For instance, the anti-metabolites like methotrexate and 5-fluorouracil (5-FU) (Abdel-Rahman et al., 2016; Schwab et al., 2008), plant alkaloids like, irinotecan (Mayo et al., 2017; Stein et al., 2010), taxanes and anthracyclines (Kwon, 2016), and alkylating agents such as cisplatin and cyclophosphamide (Villa and Sonis, 2015), are tend to correlate more consistently with mucotoxicity compared to etoposide, hydroxyurea, or bleomycin (Cinausero et al., 2017). Furthermore, bolus infusion of CT tends to be more toxic, and the combination of different chemotherapeutic agents in a cancer treatment further increases the likelihood of mucositis (Peterson et al., 2011; Dodd, 2004; Balis et al., 2002; Pico et al., 1998; Wilkes, 1998; Barasch et al., 1995).

Among anti-metabolites, capecitabine and S-1 treatments hold a minimal mucositis risk compared to 5-FU. A recent meta-analysis demonstrated that S-1 fluoropyrimidines provoked a less mucositis risk than 5-FU, while capecitabine-treated patients had a similar S-1 toxicity profile. On the contrary, combining irinotecan and fluoropyrimidine is related to a high hazard of gastrointestinal mucositis (Abdel-Rahman et al., 2016), mainly in combination with capecitabine. Skof et al. (2009) reported that patients who underwent irinotecan and capecitabine (XELIRI regimen) showed more elevated gastrointestinal mucositis rates compared to those exposed to irinotecan and 5-Fu (FOLFIRI regimen).

Furthermore, Some preclinical studies have shown that cisplatin tends to be more toxic to the ileal mucosa than the other parts of the gastrointestinal tract (Yamamoto et al., 2013). However, lung and gastrointestinal cancer patients who received 5-FU and platinum showed a lower risk of severe mucositis related to platinum (Al-Ansari et al., 2015). Notably, patients developing carboplatin and oxaliplatin-induced intestinal mucositis showed a lower degree of mucotoxicity than that induced by cisplatin (Hartmann and Lipp, 2003).

The mucositis risk in patients receiving large-dose CT and allogeneic HSCT is significantly related to the conditioning regimen. In comparison with other CT regimens, high doses of melphalan as conditioning regimens in HSCT were considered a risk factor for OM, promoting mucositis in 60–80% of the cases (Vokurka et al., 2009; Blijlevens et al., 2008; Grazziutti et al., 2006). The Prospective Oral Mucositis Audit trail (Blijlevens et al., 2008) reported an incidence level of OM for up to 87% after Carmustine, Etoposide, Cytarabine and Melphalan (BEAM) and high-doses of melphalan followed by autologous HSCT. In this study incidence of higher grade OM was 87%. In addition, studies show that severe OM is seen in patients undergoing myeloablative (MA) drugs or high-dose of melphalan as a conditioning regimen in allogeneic stem cell transplantation (Svanberg et al., 2010; Svanberg et al., 2007; Aisa et al., 2005; Sonis et al., 2001).

The doses of CT also affect the risk for the development of mucositis. Research shows that an increase in the intensity of CT will, in turn, increase the risk for OM development (Bensinger et al., 2008). OM risk also depends on the CT cycles and duration, and each cycle of chemotherapeutic agents elevates OM risk (Çakmak and Nural, 2019; Kim et al., 2012; Naidu et al., 2004). Additionally, the risk of having mucositis significantly increases by adding RT to CT or vice versa (Zerbe et al., 1992; Trotti et al., 2000).

Given that the chemotherapy-induced mucosal damage mechanism has not been elucidated (Bowen et al., 2019), it is not yet clear why and how particular agents with identical toxic prospects could apply varying adverse impacts on the oral mucosa throughout patients (Barasch and Peterson, 2003). Further studies are needed to evaluate risk factors for OM induced by CT.

1.4.2.1.2 Radiation-related factors

Various studies demonstrate that the development of OM, with a progressive worsening, represents the most common and clinically significant side effect during RT involving the head

and neck region (mainly oropharyngeal tissues) (Sonis, 2013; Elting et al., 2007; Trotti et al., 2003a; Spijkervet et al., 1991; Spijkervet et al., 1989; Al-Tikriti et al., 1984). Nearly all patients undergoing RT for HNCs develop OM (Baker, 1982; Trotti et al., 2003a). The severity of OM varies widely depending on the volume of tissues exposed to radiation, ionizing radiation type, the daily radiation dose, and the cumulative radiation dose (Vera-Llonch et al., 2006; Scully et al., 2004; Scully et al., 2003; Spijkervet et al., 1989). In a conventional fractionation of RT setting; when an irradiation dose of 2 Gy/day, and 5 fractions per week are used, the early developed patchy mucositis becomes more evident during the third week of RT and progresses to more confluent ulcerative pseudomembranous mucositis if radiation therapy continues (Kaanders and Ang, 1994; Parsons, 1994).

Patients receiving altered fractionation of RT for HNC are expected to develop more severe OM (Vera-Llonch et al., 2006; Merlano et al., 1991). Horiot et al (1992) report that the incidence of OM in patients receiving hyper-fractionation RT (with no acceleration) for the treatment of oropharyngeal carcinoma is 66.5%, as opposed to 49% in patents receiving conventional fractionation. Furthermore, Geara et al show a high incidence of high-grade OM (grade 3-4) in patents with head and neck neoplasm treated with hyper-fractionation RT with no acceleration (Geara et al., 1996).

Adding concomitant CT, often cisplatin, to RT is correlated with an increased risk of mucositis (Trotti et al., 2003a). In addition, Sanguineti et al. (2012) reported that HNC patients undergoing CT plus RT showed an increase in mucositis development risk when using intensity-modulated radiation therapy and conventional RT fractions by 4.1-fold and 5.1-fold, respectively. However, Walsh et al. (2011) showed that adding cetuximab (an epidermal growth factor (EGF) receptor inhibitor) to a standard RT regimen caused an increase in both the incidence and onset of OM in HNC patients when compared to RT plus cisplatin ($p = 0.014$). Other studies reported a grade 3-4 OM rate ranging between 45% to 74% by adding cetuximab to RT in HNC patients (Levy et al., 2014; Rampino et al., 2012; Alongi et al., 2012; Pryor et al., 2011).

Patients receiving regimens that include large chemotherapeutic doses with TBI accompanied by HSCT are at high risk of developing OM (Mattsson et al., 1991; Zerbe et al., 1992; Sonis et al., 2004a). The incidence rate of OM in HSCT patients undergoing conditioning regimens that combined TBI and CT ranges from 75%-85% and it is reported as the most debilitating effects (Bellm et al., 2000; Sonis et al., 2004a). However, the data published on the cytotoxicity

of various regimens of cancer treatment are inconsistent sometimes. For instance, Woo and colleagues (1993) demonstrated that OM severity and incidence provoked by busulfan (Bu)-based medication or TBI were comparable in bone marrow transplantation (BMT) patients. While Barasch et al (1995) reported a 100% incidence of OM in patients receiving conditioning regimens that include high-dose CT without TBI. Moreover, a study by Wingard and colleagues (1991) showed that OM was 19 times more likely to develop in patients receiving Bu-containing regimens compared to patients treated with cyclophosphamide plus TBI.

Of note, radiation therapy is constantly associated with oesophagitis incidence. Simultaneous RT and CT at high doses significantly increase the risk of developing severe oesophagitis (Adebahr et al., 2016). RT to the abdominal or pelvic region causes enteritis with a prevalence ranging from 0.5% - 50% (Webb et al., 2013; Theis et al., 2010). Complications associated with the small bowel are proportional to the small intestine volume in the radiation site. With a low hospitalization rate, this side effect was often delayed, grade 1 or 2. Notably, mucotoxicity was raised by adding CT to the treatment (Hernández-Moreno et al., 2015).

Finally, an increase in the incidence and severity of mucositis is associated with protocols utilizing accelerated dose plans where the accumulative everyday dose surpasses 2 Gy (Cinausero et al., 2017).

1.4.2.1.3 The role of novel targeted anticancer treatments in mucositis development

Besides standard CT and radiation therapy, an increasing number of targeted agents are currently used in clinical practice to treat various types of cancer. The target-specificity allows these new agents to have high potency and at the same time lower toxicity than standard CT. Nonetheless, clinicians are faced with new toxicities whose properties vary according to the target drugs administered (Pulito et al., 2020). Among them, the mammalian target of rapamycin (mTOR)-inhibitor promotes severe mucosal toxicity different from conventional OM. The mTOR-inhibitor-associated stomatitis (mIAS) is smaller, relatively shallow, and extremely painful. Macroscopically it shows a central necrotic area and a surrounding erythematous halo (Bossi et al., 2015; Elting et al., 2013; Sonis, 2010b). It usually develops within five days of the first course of treatment and either improves or resolves spontaneously, even during mTOR inhibitor therapy (Vigarios et al., 2017); however, it is often the cause of remodulation of therapy dose or discontinuation of therapy in the presence of high-grade mIAS. In the BOLERO-2 trial study, the combination treatment of everolimus, an mTOR inhibitor,

with exemestane was limited by a high incidence of all grade stomatitis in women with metastatic breast cancer (67% all grade stomatitis, 33% grade 2, and 8% grade 3. Class) (Shameem et al., 2015). In particular, patients treated with mTOR inhibitors such as everolimus, tengerolimus or ridaforolimus are at increased risk of developing oral stomatitis and enteritis (Abdel-Rahman and Fouad, 2015). The incidence of mIAS toxicity changes according to cancer types. In fact, patients with renal cell carcinoma (RCC) have a lower risk of developing mIAS than patients with astrocytoma, gastric and breast cancer (Shameem et al., 2015). This is also due to the properties of the drug associated with the mTOR inhibitor, which vary between different types of cancer depending on clinical guidelines.

The mucosal lesions associated with EGFR inhibitors occur in 15% of treated patients (Basile et al., 2019). Clinically they appear as limited lesions characterized by moderate erythema, sometimes not unlike aphthous lesions (Lacouture et al., 2011). As with mIAS, onset occurs concurrently with the first treatment cycle, may affect any non-keratinized area, and may resolve on its own during treatment (Lacouture et al., 2011). Less than 1% of patients treated in monotherapy with anti-EGFR antibodies, cetuximab or panitumumab or EGFR tyrosine kinase inhibitor (TKi), erlotinib or gefitinib develop a high-grade mucosal lesion, which in very few cases requires treatment remodulation or suspension (Cunningham et al., 2004; Price et al., 2014; Shepherd et al., 2005). Conversely, the lesion's incidence and severity are higher in patients treated with a multi-targeted TKi such as afatinib, lapatinib, or dacomitinib (Geyer et al., 2006; Park et al., 2016; Ramalingam et al., 2014). In fact, these cancer treatments are associated with a higher incidence of all grade mucositis than other EGFR-TKi (40% vs. 15% at all grades, 8.7% vs. 1% at grade ≥ 3) (Soria et al., 2015) or anti-EGFR monoclonal antibody therapy (Vigarios et al., 2017). Although monotherapy with anti-EGFR antibodies or TKis causes few mucosal lesions, these drugs are often associated with cytotoxic agents that cause an increase in mucositis incidence and severity. In fact, the relative risk of high-grade mucosal lesions (≥ 3) increases significantly when cetuximab or panitumumab are co-administered with cisplatin, 5-FU, FOLFIRI, or FOLFOX (Vigarios et al., 2017). Furthermore, Although the addition of cetuximab to RT does not alter the risk of developing mucositis in HNC patients compared to RT alone (about 60% of high-grade ≥ 3) (Bonner et al., 2010; Bonner et al., 2006), it does increase the risk of generating high-grade mucositis when combined with RT plus CT, compared to RT plus CT treatment alone (Maddalo et al., 2020; Magrini et al., 2016).

Mucositis associated with ado-trastuzumab emtansine (T-DM-1) treatment is attributable to mucosal telangiectasia (Sibaud et al., 2014). The T-DM-1 regimen is currently approved for HER2+, a member of the EGF receptors aberrantly expressed in some tumors, for the treatment of metastatic breast cancer. This treatment promotes vascular malformation of the mucosa, leading to epigastaxy and gastrointestinal or gynecologic bleeding in 30% of patients (Sibaud et al., 2014; Sibaud et al., 2015).

OM, particularly stomatitis characterized by an aphthous ulcer, occurs in a very small number of patients treated with cyclin-dependent kinase 4/6 (CDK4/6) inhibitors (Sledge et al., 2017; Lasheen et al., 2017), which are used as first- or second-line treatment for hormone-positive/HER2-negative metastatic breast cancer. However, this regime encourages GIM rather than OM. Abemaciclib induces early onset GIM in approximately 80% of patients in the first treatment cycles (Sledge et al., 2017; Dickler et al., 2017). In particular, CDK4/6 inhibitors bind cyclin D3 in GI epithelial cells, inhibit their proliferation and consequently induce mucosal damage (Xu et al., 2017).

Dafrafenib, vemurafenib, and the BRAF and v-raf murine sarcoma viral oncogene homolog B1 inhibitors, were recently approved by the Food and Drug Administration for the treatment of BRAFV600-mutant metastatic melanoma. Patients treated with any of these inhibitors develop asymptomatic hyperkeratotic lesions that ascend in both keratinized and non-keratinized mucosa, including mucosal lesions characterized by a verrucous or papillomatous appearance present in the tongue, lip mucosa, and the linea alba (Lacouture et al., 2013; Gençler and Gönül, 2016; Boussemart et al., 2013).

Nonspecific stomatitis, characterized by hypersensitivity of the oral mucosa and associated with moderate erythema or inflammatory pain, is an adverse event of antiangiogenic drug treatment (Yuan et al., 2015). The probability of developing a stomatitis event changes according to the administered targeted drug. In fact, bevacizumab or ramucirumab, two monoclonal antibodies directed against the vascular endothelial growth factor receptor (VEGFR), rarely induce stomatitis. Conversely, 25% of patients treated with multi-kinase inhibitors (MKIs) such as sunitinib, sorafenib or cabozantinib complain of stomatitis within the first two months of treatment (Yuan et al., 2015). However, less than 10% of them require a drug dose adjustment, while only 1% discontinue treatment (Yuan et al., 2015).

Low-grade stomatitis has also been reported, albeit in rare cases, as an immunotherapy-associated adverse event (irAE) in patients treated with anti-programmed death 1 (PD1), pembrolizumab and nivolumab, or anti-programmed death ligand 1 (PDL1). were) drug, atezolizumab and durvalumab (Vigarios et al., 2017). Patients treated with an immune checkpoint drug experience varying degrees of diarrhea, often associated with abdominal pain, dehydration, and constipation (Collins et al., 2017; Hofmann et al., 2016). Endoscopic analysis revealed a colicky mucosa with mild inflammation and/or ulceration after PD1 or PDL1 treatment, while the same appeared ulcerated and brittle in patients treated with a cytotoxic T lymphocyte-associated antigen 4 (CTLA4) inhibitor (Basile et al., 2019; Marthey et al., 2016). Between 27-54% of patients treated with a CTLA4 inhibitor complain of GI toxicities, often requiring either remodulation or treatment discontinuation (Gupta et al., 2015).

1.4.2.2 Transplant-related factors

About 75% - 100% of patients receive allogeneic (using stem cells from donor origin) or MA autologous (using the same patient's stem cells) HCST develop OM of varying grades (Sonis et al., 2004a). Various studies report that the severity of mucositis may be related to the type of transplant. For instance, an increase in OM severity was noted in autologous vs. allogeneic BMT patients (1999; 1991). On the contrary, other studies showed no observed OM severity discrepancies between the two groups (Zerbe et al., 1992; Woo et al., 1993). Although particular HSCT regimens, such as those containing total body irradiation or high-dose melphalan (Wardley et al., 2000), have been clearly implicated in inducing severe OM. Relatively scant data has been published correlating the relationship between different intensities of condition regimens and the incidence and severity of OM among patients with allogeneic HSCT (Chaudhry et al., 2016; Al-Ansari et al., 2015). A systematic review by Chaudhry et al. (2016) analysed the relationship between the intensity of prophylaxis graft-versus-host-disease (GVHD) treatment and conditioning treatment protocol on OM prevalence and consequences in patients with allogeneic HSCT. Groups were studied according to the power of the regimen, such as MA and reduced-intensity conditioning (RIC) (including non-myeloablative, NMA). This study reported that MA treatment had a higher OM incidence, likewise RIC treatment. On the contrary, a study by Shouval et al. (2019) reported lowest OM risk with RIC compared to MA. Other studies (Takahashi et al., 2010; Ringden et al., 2013) have reported that either RIC or NMA treatments are related to decreased OM occurrence and

grade. However, patients receiving allogeneic transplant treatment need prophylaxis GVHD, which might expand the OM danger.

1.4.2.3 Patient-related factors

Patient-linked risk aspects are better complex and, in the majority of the cases, not well characterized. Although resemblances in identification and therapy, not all cancer patients fall under the same risk-degree of OM development. Age, gender, body mass index, malnutrition, pre-existing medical conditions, poor oral health, smoking, mucosal injury, and variations in salivary composition and production, are among the accounted patient-related risk factors that might significantly impact OM development (Shouval et al., 2019; Chaveli-Lopez and Bagan-Sebastian, 2016; Villa and Sonis, 2016b; Vasconcelos et al., 2016; Krishna et al., 2011; Raber-Durlacher et al., 2010; Sonis, 2010b; Schwab et al., 2008; Sonis et al., 2004a). Nonetheless, none of these factors have been consistent or accurate (Cinausero et al., 2017). Revealing fundamental patient-linked risk factors would improve the designing of better observations and intervention pathways.

The influence of gender on gastrointestinal mucositis risk is highly determined in patients receiving 5-FU, and the risk is more elevated for females than males (Schwab et al., 2008; Chansky et al., 2005; Sloan et al., 2002). In addition, several studies associated females with more severe chemotherapy-induced OM and gastrointestinal mucositis (Valeh et al., 2018; Yang et al., 2013; Vokurka et al., 2009; Vokurka et al., 2006). However, contradicting evidence has supported male gender as a predictor of OM in HNC patients receiving CT (Nishii et al., 2020). OM seems to be more likely to develop in the outermost age groups of cancer patients, which is evidenced by the higher rate of OM incidence among children and elderly patients (Zalcberg et al., 1998; McCarthy et al., 1998; Sonis et al., 1978). Body composition also could play a role in the metabolism of the drug. Drug exposure can be higher in patients with lower body mass index, females and those with slim bodies. Thus, they can be susceptible to more intense toxic effects (Prado et al., 2007). Smoking was also determined as a risk factor in predicting OM in HNC patients and transplant recipients (Shouval et al., 2019; Chen et al., 2017; Tao et al., 2017).

An increase or decrease prevalence of mucositis has been reported to be associated with quantitative salivary function (Barasch and Peterson, 2003). For example, xerostomia has been identified as one of the predictor factors for developing mucositis in patients treated with 5-FU

(McCarthy et al., 1998). In contrast, Oblon et al. (1997) and Ahmed et al. (1993) demonstrated that propantheline can protect patients from ulcerative OM induced by etoposide. The reason was attributed to the ability of propantheline to protect the oral mucosa from etoposide in the saliva and thereby reduce the incidence of OM. Nonetheless, propantheline may have other unexplained mucoprotective properties (Barasch and Peterson, 2003), which warrants further research in this direction.

Several biological/laboratory parameters have been associated with mucositis risk, including low expression of colonic thymidylate synthase (Santini et al., 2004), , Neutropenia (Reyna-Figueroa et al., 2015), low urea (Tebbutt et al., 2003), low platelet count, decreased haemoglobin levels, lymphopenia (Nishii et al., 2020), impaired renal function (Mizuno et al., 2019), and serum creatinine (Grazziutti et al., 2006); however, the evidence was sparse and limited to a few stand-alone studies (Wardill et al., 2020).

1.4.2.4 Role of Microbial Flora

For many years, OM severity has been conceptually linked to oral microbiota (Barasch and Peterson, 2003). Studies indicate that shifts in oral microbiome composition during the CT influence the severity of OM (Ye et al., 2013). Bad oral hygiene, specifically gum disease, has been recognized as an ecological factor that might be related to OM severity (Coracin et al., 2013; Wuketich et al., 2012). In addition, periodontal pathogens such as *porphyromonas gingivalis* have been identified as variables to explain oral ulceration in HSCT patients (Laheij et al., 2012). A reduction in gum infection and oral bacterial burden was related to a diminished incidence of OM in HSCT-treated patients (Kashiwazaki et al., 2012; da Silva Santos et al., 2011; Soga et al., 2009). In paediatric oncology patients, positive herpes simplex virus (HSV) type 1 was described as a risk factor for OM, and oral candida was also identified as a predictor of OM (de Farias Gabriel et al., 2022). Furthermore, the potential role of fungi and viruses, typically associated with mucosal injury, in developing OM ulceration has been investigated (Mendonça et al., 2015; Laheij et al., 2012; van der Beek et al., 2012; Nicolatou-Galitis et al., 2001; Woo et al., 1990). However, no conclusive evidence can be drawn.

To date, no convincing evidence for role of the oral and intestinal microbiota in the initiation of mucositis (Basile et al., 2019; Barasch and Peterson, 2003). However, there has been recently increasing attention on oral microbiota's role in the risk of OM (Napeñas et al., 2007; Laheij and de Soet, 2014; Vanhoecke et al., 2015). Microflora's ability in proinflammatory

cytokines stimulation is well known (Stringer and Logan, 2015). This ability could attributed to the vital role of the microbiome in the activation of the innate immune cells, such as dendritic cells, macrophages, mast cells, and natural killer (NK) cells, by identifying Pathogen-Associated-Molecular-Patterns—PAMPs (Menezes-Garcia et al., 2018). These molecules are recognized by Toll-like receptors (TRL) that activate NF- κ B and Promote proinflammatory cytokines generation that induces leukocytes resulting in secretion of more proinflammatory cytokines (Menezes-Garcia et al., 2018). Microflora composition may become imbalanced during anti-cancer treatment and consequently, the physiological microflora may become under-represented (dysbiosis) (Touchefeu et al., 2014). The existence and varieties in the composition of the microbiota differ throughout the gastrointestinal tract based on the type, timing, and doses of CT. In particular, these differences are likely to contribute to the mucosal injury's initiation and aggravation (Basile et al., 2019).

1.4.2.5 Gene-related factors

By assessing the mutations of the germline related to signalling pathways that affect the development of mucositis or the metabolism of the drug, it is now possible to identify pharmacodynamic factors and pharmacokinetic genomic drivers that influence the risk of mucositis (Cinausero et al., 2017). The first genomic toxicity tests were linked to identifying mutations affecting enzymes engaged in drug metabolism. Polymorphism affecting a variety of gene clusters, such as genes encoding enzymes involved in the metabolism of drugs., was evaluated for their ability to predict the risk of mucositis (Basile et al., 2019).

For instance, patients deleted thymidylate synthase polymorphism gene (Cho et al., 2007) or a reduction of the dihydropyrimidine dehydrogenase gene (Meulendijks et al., 2015; Amstutz et al., 2018) tend to have more severe 5-FU-induced toxicity. Since these genes do not affect most patients receiving CT who develop toxicity and none of those treated with RT, a more mechanistic-based approach characterizes many more recent studies (Bachour and Sonis, 2018). Patients carrying the 677TT genotype for methylenetetrahydrofolate reductase, and receiving methotrexate for HCT, had more severe mucositis (Robien et al., 2004). Furthermore, a study by Sakamoto et al. (2006) on the Japanese who underwent 5-FU and cisplatin reported that the increased severity of mucositis correlated with the cytokine genes' polymorphisms. Furthermore, patients with systematic diseases distinguished by decreased (such as psoriasis) or increased (such as Addison's disease) rates of apoptosis appear to have a lower or higher risk of developing mucositis, respectively (Sonis, 2010b). Most recently, a genome-wide

association study by Schack et al. (2022) of RT -induced toxicity in HNC patients identified an association between a locus on chromosome 5 and the development of moderate/severe mucositis.

Genomically derived analyses by Bachour and Sonis et al. (2018) have implied six common pathways as being associated with mucositis. These pathways include protein-tyrosine kinase 6 (PTK6) signalling and the Wnt signalling pathway, two transforming growth factor (TGF)- β associated pathways, extracellular-signal-regulated kinase (ERK) signalling, and the inflammatory response pathway. While the top two pathways appear to be highly mucositis specific, the remaining four are also implicated in developing other pathologies such as irritable bowel syndrome (IBS) (Bachour and Sonis, 2018). TK6 is expressed normally in intestinal cells (Gierut et al., 2012) and is thought to be associated with an epithelial barrier function (Haines et al., 2016). On the contrary, Wnt signalling pathways can be implicated in antigen exhibition and immune reaction by interacting with the NK-kB signalling (Gierut et al., 2012). On the other hand, the Wnt pathways, and through crosstalk with NF-kB pathway, could be involved in innate immune response (Ma and Hottiger, 2016) and antigen presentation (Swafford and Manicassamy, 2015). TGF- β pathway stimulation is correlated to regimen-induced toxicity, and its arranging via β 1 integrin suppressions or Smad7 alters further mucositis development (Bian et al., 2015). ERK pathways function in tandem with intra- and extracellular signalling changes, whereas the inflammatory reaction process may be connected with the immune response activation (Russi et al., 2014).

Interestingly, the earlier pathways participate with diseases that have the same clinical manifestations (like IBS), thus supporting the theory that the risk of developing a particular clinical condition (such as diarrhea) is associated with patient-particular and individual genetic profiling (Bachour and Sonis, 2018). Consequently, genomics impact on the risk of toxicity must be more pervasive and related to genes influencing these pathogenesis-related pathways.

1.4.2.6 Malignancy type

The tumour itself has recently been considered as a risk factor for OM (Sonis, 2012a; Russi et al., 2014). Tumour location (Nishii et al., 2020), size (Xu et al., 2015), and stage (Chen et al., 2017) have been reported as predictors of oral and gastrointestinal mucositis. Tumor-induced inflammatory response, along with inflammation caused by regimen-induced cytotoxicity, may contribute to side effects, including OM. Non-Hodgkin's lymphoma (compared to other

haematological malignancies) has also been reported as a predictor of OM (Bolwell et al., 2002). In children, germinal (compared to non-germinal) and haematological malignancies versus central nervous system cancers (particularly Hodgkin's lymphoma) have been reported to increase HSCT-induced OM risk (Fadda et al., 2006; Allen et al., 2018).

1.4.3 Pathogenesis of Mucositis

1.4.3.1 Historical model

Over the last few decades, researchers are trying to understand the mechanism underlying mucositis pathogenicity in an attempt to develop an effective targeted therapy. In fact, until in the late 1990s, and for many years, the conventional understanding of mucositis pathobiology was based on the hypothesis of direct and non-specific epithelial cell death mediated by either CT or RT (Lockhart and Sonis, 1981; Lockhart and Sonis, 1979; Guggenheimer et al., 1977; Bloomer and Hellman, 1975; Sonis, 1998). Historically, mucositis induced by RT was presented as a cellular response to the radiation-reaction process, which incorporates basal epithelial DNA damage of the oral cells (Bloomer and Hellman, 1975). On the other hand, CT-induced mucositis was attributed to the damage of the epithelial basal cells as a result of permeation of toxic drugs from the sub-mucosal blood supply in these cells (Lockhart and Sonis, 1981).

The paradigm was simple and was based on the assumption that CT and RT would non-specifically kill rapidly dividing normal cells of the basal epithelium as well as rapidly dividing cancer cells. Thereby, direct DNA injury would provoke programmed cell death in the GIT mucosa, ending in epithelium renewal capacity loss, and as a result the mucosa would become atrophic because imbalance in the equilibrium of cell depletion and cell proliferation. Atrophic damage would cause thinning of the epithelium to the point of breaks and cause ulceration. Ulcers would become secondarily infected and colonized with bacteria which together with the functional trauma were viewed as additional factors to modulate mucositis development, duration, and healing. These ulcers were then ultimately healed when the treatment was reduced or stopped (Lockhart and Sonis, 1981; Lockhart and Sonis, 1979; Guggenheimer et al., 1977; Bloomer and Hellman, 1975; Sonis, 1998). However, this straightforward process of injury, ulcer development, and curing was no longer deemed entire, and it could not explain some subsequent findings about other molecules and cells' roles in the submucosal region (Sonis et al., 2000).

1.4.3.2 Current model

It has been almost two decades since the conventional pathogenic paradigm that defined the pathogenesis of mucositis was revised (Sonis, 1998). In (1998) Sonis suggests that mucositis is not based only on the consequences of basal cell death. But on the contrary, the process is more biologically complex. It is now thought, however, that Mucositis is actually the result of a complicated series of cellular and biological circumstances that occur primarily in the cells and tissues of the submucosa and culminates in targeting the epithelium tissue (Sonis, 2009).

Although epithelial injury is the main contributor to the clinical symptoms of mucositis, a substantial amount of data show a more complicated picture of the condition and portray mucositis as resulting from a sequence of interactive and dynamic cellular and molecular circumstances throughout the different compartments of mucosa (Sonis, 2004b). Subsequent studies reveal more complex picture of what is happening around OM and implicated microvascular injury (Paris et al., 2001), proinflammatory cytokines (Logan et al., 2009; Sonis et al., 2000; Yeoh et al., 2005; Manzano et al., 2007), host–microbiome interactions (Stringer et al., 2009a; Stringer et al., 2009b; Stringer et al., 2007) and extracellular matrix (ECM) alterations (Al-Dasooqi et al., 2010; Al-Dasooqi et al., 2011). This concept has been supported by a number of biological and clinical compliances from extensive research in the biology of mucositis, primarily as a means to develop therapeutic targets. These observations include a number of facts that are explained in the following paragraphs.

1.4.3.2.1 Early Tissue Damage

Understanding the pathobiology of OM has been expanded during the last 2 decades to show that connective tissue and submucosal endothelial changes come before the epithelial tissue damage and begin almost immediately after RT or CT. Consistently, histologic and electron microscopic evaluation, within one week after radiation of oral mucosa in animals, demonstrates early damage to both the endothelium and connective tissue and at least 5 days before the manifestations of radiation-induced mucositis were observed (Bowen et al., 2019). Furthermore, Paris et al (2001) importantly suggests that endothelial injury might play a major role in the initiating of radiation-induced mucositis, as they find that preventing apoptosis in endothelial cells, by basic fibroblast growth factor (bFGF) addition, could attenuate radiation-induced IM in a murine model. A previous study of radiation-induced renal injury is also consistent with Paris study (Jaenke et al., 1993). Similarly, several chemotherapeutic agents

are also associated with endothelial toxicity, for example, 5-fluorouracil (Kinhult et al., 2003), cisplatin (Kuenen et al., 2003) and melphalan (Watts et al., 1997).

Further evidence suggests the endothelial damage role as an early event in the development of mucositis and platelet aggregation as a part of mucosal damage induced by RT. This role is denoted by the observation of a significant increase in salivary platelet-activating factor expressions in patients with OM developed as a complication to RT for oral cancer (McManus et al., 1993). Moreover, the platelet-activating factor role is approved by the finding that the use of clopidogrel, an adenosine diphosphate suppressor, prompted platelet accumulation and effectively ameliorates RT -induced mucositis in rat intestine (Wang et al., 2002).

1.4.3.2.2 Chemoradiation-induced oxidative stress

Although the main contributor to the clinical manifestations of mucositis is the tissue injury and break of the epithelium continuity (Elad et al., 2022), a substantial amount of data demonstrates a more complicated picture of the condition and indicates that, at the molecular level, mucosal injury induced by CT and RT is triggered by excessive reactive oxygen species (ROS) generation during the initial preclinical phase, followed by consequential activation of free radical damage pathways (Yoshino et al., 2013; Criswell et al., 2003).

Cancer management therapies, such as 5-FU, cisplatin, or RT, generate ROS that target the DNA of cancer cells, causing cell damage and death. However, ROS from these therapies also randomly target the DNA of healthy non-tumour cells (Nguyen et al., 2022). Increased ROS production leads to oxidative stress (Pisoschi and Pop, 2015). Biologically, oxidative stress can increase cancer risk and susceptibility to inflammatory diseases and conditions such as mucositis, among other things (Perillo et al., 2020).

The genes that regulate ROS metabolism have been the subject of several investigations. Two clinical studies provided direct evidence about the association of OM with genetic polymorphisms (Venkatesh et al., 2014; Pratesi et al., 2011). Both studies found that variations in the genes involved in the DNA damage and repair, in particular genes related to protection against oxidative stress, are related with high risk of OM development in HNC patients. Furthermore, other studies reported that rises in pro-inflammatory cytokine production resulting from the nuclear factor-kappa B (NF- κ B) activation during chemoradiotherapy culminate in mucosal ulceration leading to the clinical presentation of regimen-induced OM (Sonis, 2002; Maria et al., 2017). As such, the oxidative stress pathway has been assessed as a

potential target for therapeutic effect, including OM prevention. Nonetheless, whether a certain oxidative damage pathway mechanism modulation could prevent OM has not been clarified as yet (Nguyen et al., 2022).

Various compounds and enzymes are involved in the oxidative stress pathway, such as hydrogen peroxide (H₂O₂), Superoxide dismutase (SOD), glutathione (GSH), catalase, and myeloperoxidase (MPO). Furthermore, upregulation of the pro-apoptotic pathways, such as NFκB, are also signalled by this pathway (Pisoschi and Pop, 2015), which makes it not only further contributing to complications but also a potential medicinal target for the prophylaxis of OM. However, it is still unclear the exact mechanism underlying oxidative stress contribution in chemoradiotherapy-induced OM. Therefore, more research is required on ROS production and reduction mechanism, in addition to ROS level detection.

1.4.3.2.3 Inflammatory Infiltrate

Noticing T-lymphocytes to be involved in inflammatory processes of some oral diseases, such as recurrent aphthous ulceration (Pedersen et al., 1992), oral lichen planus, and chronic graft-versus-host disease (Mattsson et al., 1992) calls attention to the role of inflammatory cells during radiation-induced OM (Handschel et al., 2001). Various studies label mucositis incorrectly as an acute inflammatory disorder and demonstrate that the predominant histological changes in irradiated mucosal and submucosal tissues are oedema and inflammatory cells infiltration (Lockhart and Sonis, 1981; Handschel et al., 1999). However, this concept does not correspond with the results of histological studies of radiation-induced mucositis both in animal model and human. In fact, Sonis et al, (2000) indicate that inflammatory infiltrates usually appear at advanced levels of the toxic procedure and are highly related to the ulcerative mucositis stage. Similarly, Handschel et al, (2001) indicate that radiation-induced OM seems to represent intermediate stage of an inflammatory response, and it is not an acute inflammatory response or an aggressive form of a chronic inflammation.

1.4.3.2.4 Gene Expression Changes

Among evidence reflecting the intricacy of the mechanism underlying pathobiology of mucositis is the finding of 14 canonical pathways as being mostly related to the development of anti-neoplastic therapy-induced toxicities, including mucositis (Sonis et al., 2007). These pathways were specified by the analysis of genomes of venous blood cells for patients being treated for HNC (Sonis et al., 2007). Among these pathways is the NF-κB signalling. NF-κB

is one of the transcription factors that is triggered by ROS, CT, and RT and proposed as an essential factor in terms of both tumour cell reluctance to treatment and normal cell cytotoxicity (Sonis, 2002). This factor is not only involved in the modulation of a broad spectrum of physical reactions but also activates numerous cell-specific genes, including proinflammatory cytokines (Pahl, 1999). A study conducted by Logan and colleagues (2008) affirms that the peaking in the elevated levels of NF- κ B expression precedes the peaking in the proinflammatory cytokines levels in both oral and intestinal tissues following the administration of the chemotherapeutic agent. Furthermore, upregulated pro-inflammatory cytokines are among activating agents of NF- κ B (Beg et al., 1993; Pugazhenthii et al., 2013). Once upregulated, TNF- α is speculated to provide a positive-feedback loop to amplify injurious pathways by activation of NF- κ B (Beg et al., 1993; Pugazhenthii et al., 2013; Sonis, 2004b).

Cyclooxygenase-2 (COX-2), a catalytic enzyme implicated in the inflammatory process via its prostaglandin production role (Dubois et al., 1998; Vane et al., 1998), is another gene shown to be upregulated by NF- κ B (Roshak et al., 1996; Yamamoto et al., 1995). Sonis et al (2004b) indicate that the level of COX-2 expression markedly increased 10 and 16 days following RT, mainly in submucosal fibroblast and endothelium of animals' oral mucosa. In addition, the parallel of the kinetics of COX-2 expression with the mucositis progression suggests the role of this enzyme in amplifying OM (Sonis et al., 2004a). These results are consistent with the clinical finding, which indicates a dramatic increase in the expression of NF- κ B and COX-2 in the chemotherapy-treated patients' oral mucosa (Logan et al., 2007a). However, inhibition of COX-2 is found ineffective for OM treatment in preclinical model (Haagen et al., 2009).

1.4.3.2.5 Pro-Inflammatory Cytokines

Further evidence confirming the complexity of mucositis pathogenesis comes from investigating the correlation between mucositis and pro-inflammatory cytokines in *in vivo* studies. Cytokines high levels are noted in peripheral blood in patients that develop toxicities, including mucositis, and are also prevalent within the affected mucoasal tissues. Earlier research points out that elevated levels of interleukins-1 β (IL-1 β), interleukins-6 (IL-6), and tumour-necrosis factor-alpha (TNF- α) in the peripheral blood are consistently seen in patients who experience non-hematologic toxicities following CT compare with those who do not experience toxicity (Sleijfer et al., 1998; Hall et al., 1995). A similar finding has been reported in patients developing acute GVHD as a post-transplantation complication during conditioning transplantation of bone marrow (Remberger et al., 1995). Furthermore, Sonis et al (2000),

found that TNF- α expression and IL-1 β levels in hamsters' submucosal cells rise precipitously after sharply targeted irradiation and that the increase in both cytokines correlated with mucositis development in those model. The use of those cytokines attenuating agents has approved efficacy in preventing both practical (Sonis et al., 1999a; Sonis et al., 2000) and clinical (Epstein et al., 2001) mucositis. Importantly, IL-1 β and IL-6 are also described as early markers of circulation for different complications induced by RT, such as pneumonia (Chen et al., 2002).

Recently, various studies have shown the patterns of pro-inflammatory cytokines in the development of both GI and OM. IL-1 β and TNF- α tissue levels and gene expression are noted to be associated with RT -induced gut and oral mucosal injury (Ong et al., 2010; Sonis et al., 2002). Likewise, the development of chemotherapy- induced GI mucositis is also associated with the proinflammatory cytokines' upregulation, in particular IL-6, IL-1 β , and TNF- α (Logan et al., 2009; Logan et al., 2008; Logan et al., 2007b). Furthermore, according to Logan et al (2009), elevated levels of TNF- α , IL-6, and IL-1 β are considered as a good markers of chemotherapy-induced toxicities. Inhibition of pro-inflammatory cytokines through targeting them by anti-inflammatory agents, though results are not consistently positive, shows promise for reducing the course of experimental mucositis (Haagen et al., 2009; Melo et al., 2008; Lima et al., 2005).

1.4.3.2.6 Apoptosis as A Potential Initiator Of Mucositis

Tissue damage mainly observed in mucositis is an ultimate apoptosis result (Sonis, 2007; Hannun, 1997). Apoptosis not only occurs within the oral epithelium, but is also seen within the cells of the submucosa as an indicator of the intricacy of RT - or chemotherapy-induced mucositis (Paris et al., 2001; Chrzanowski et al., 2001; Stefanelli et al., 2002; Maňáková et al., 2003; Li et al., 2011). In the case of radiation therapy, it has been noted that both fibroblasts and endothelial cells undergo apoptotic changes (Bowen et al., 2019). Paris et al (2001) suggest that apoptosis, in particular, endothelial apoptosis is the primary lesion and precedes epithelial injury in the evolution of the GI mucositis, and in certain instances, interfering with apoptosis can attenuate the onset of mucositis in animal models. In addition, several antineoplastic drugs, such as etoposide (Stefanelli et al., 2002), ara-C (Maňáková et al., 2003) and melphalan (Chrzanowski et al., 2001), resulting in acute apoptosis fibroblasts.

According to the prevailing theories, in addition to NF- κ B pathway, apoptosis processes could be modified by different pathways; i.e. beside the role of NF- κ B pathway in inducing apoptosis (Lin et al., 1999), The ceramide pathway may function in tandem or successively to prompt elementary apoptosis in submucosal and basal epithelial cells (Maddens et al., 2002). Billis et al. (1998) demonstrated that the ceramide pathway, mediated by the enzymes sphingomyelinase and ceramide synthase, can participate in RT -induced endothelial apoptosis and may be a likely contributor to initiating mucositis. In favour of this, a study by Paris et al., (2001) demonstrated increased antagonism to radiation-induced mucositis of the gastrointestinal tract of mice as a result of the inhibition of endothelial apoptosis, pharmacologically by intravenous (i.v.) bFGF or genetically by deletion of the acid sphingomyelinase gene. Later on, Hwang et al (2005) extended this study and found that ceramide pathway inhibition at various parts significantly reduced radiation-induced OM severity in animal models.

Another factor that modifies apoptosis is activator protein 1 (AP-1). It is nuclear transcription factor composed of dimers of Fos and Jun proteins family and ATF and JDP protein subfamilies (Hess et al., 2004) . Radiation- and chemotherapy-induced tissue damage has been linked with two AP1 factor family members; c-JUN and c-FOS (Sherman et al., 1990; Bamba et al., 2003). Both proteins are found to be localized in epithelial cells (De Sousa et al., 2002) and in other cell types in the submucosa, including fibroblasts (Angel et al., 2001), endothelial cells (Maselli et al., 2002) and macrophages (Guha and Mackman, 2001). Fibroblast damage is also mediated by AP-1 activation, leading to caspase-3 activation and apoptosis (Kolbus et al., 2000; Davis, 2000).

1.4.3.3 A Multiple Mechanism Model

According to one theory (Sonis, 2004b), mucositis develops and heals through five overlapping processes (phases); initiation; the primary damage response; signalling and amplification; ulceration; and finally healing. Table 1.2 outlines this five-stage model of OM. Each phase comprises a series of biological events representing possible therapeutic agents' actionable targets (Russi et al., 2014; Reyes-Gibby et al., 2017).

Table 1.2 Five-Stage Model of Oral Mucositis

Initiation	Primary damage Response	Signal Amplification	Ulceration	Healing
X-rays (Adler and Hardigree, 1965; Lliakis, 1991) or CT (Jamieson and Lippard, 1999; Kopjar et al., 2002; Cheung-Ong et al., 2013) cause direct DNA damage which culminate in clonogenic cell death. In addition, cancer treatment cause epithelial barrier dysfunction because of tight junction defects, as important factor in exacerbating injury (Al-Dasooqi et al., 2013)	CRAMPs (Sonis, 2010a) or DAMPS (Seong and Matzinger, 2004) molecules bind to (PRRs) (Palm and Medzhitov, 2009) including (RAGEs) and (TLRs) (Sims et al., 2010).	Intracellular and intercellular feedback loops intensify the damage response pathways (Sonis, 2004b).	Submucosal cell apoptosis eliminates epithelial nutrient factors such as KGF (Sonis, 2007).	New angiogenesis enhanced by COX-2 activation (Sonis et al., 2004b) and help in the regeneration of the submucosa (Sonis, 2007).
X-rays (Leach et al., 2001) or CT (Zhu et al., 2014; Kasiappan and Safe, 2016) generate ROS. Host microbial dysbiosis (oral and gut) following anticancer therapies, including bacterial and endotoxin translocation through penetrable TJs, with succeeding innate immune stimulation (Bowen et al., 2019).	At least, 14 canonical pathways are triggered (Sonis et al., 2007) (Table 1.3).	TNF- α activates NF- κ B (Pahl, 1999), sphingomyelinase (Andrieu-Abadie and Levade, 2002; Dimanche-Boitrel and Rebillard, 2013), and MAP kinase signalling pathway (Moriguchi et al., 1997).	halt of epithelial proliferation results in mucosal thinning (Sonis, 2011).	RM3/1 macrophages downregulate inflammatory responses (Hamann et al., 1995).

Table 1.2 continued

ROs induce oxidative DNA damage (Cirillo et al., 2015), protein oxidation, membrane lipid peroxidation (Gate et al., 1999), connective tissue damage (Cirillo et al., 2015), and activate a cascade of biomolecules such as, NF-κB, AP-1 (Sen and Packer, 1996), ceramide pathway (Jiang et al., 2011) and Nrf2 transcription factor (McDonald et al., 2010).	NF-κB elevates over 150 target genes expression (Pahl, 1999), which linked with production of pro-inflammatory cytokines (such as IL-6, TNF-α, and IL-1β), cell adhesion molecules, stress responders (such as SOD, inducible NO-synthase, and COX-2), and cytokine modulators (Jankovic et al., 2012; Logan et al., 2008; Logan et al., 2007a).	NF-κB-activated COX-2 produces prostaglandins (Sonis et al., 2004b).	Robust inflammatory infiltrate comprised of macrophages, neutrophils, and mast cells (Sonis et al., 2000; Sonis, 2004b).	ECM (Meredith et al., 1993) promotes multiplication, migration, and differentiation of epithelial cells to close the ulcerative wound (Sonis, 2007; Al-Dasooqi et al., 2013; Bowen et al., 2019).
Damaged cells release CRAMPs (Scaffidi et al., 2002) ,which play an integral role in initiating toxicity by activating the host's innate immune response (Sonis, 2010a).	NF-κB induces pro-apoptotic effects by modulating of apoptosis genes in the BCL family (Huang, 2000; Sonis, 2002).	MAPK signalling activates JNK signalling (Moriguchi et al., 1997), which activate AP-1 pathway (Yang et al., 1997).	MMPs degrade the ECM , thereby abolishing the scaffold on which the epithelium lies (MacDonald et al., 1999; Pallone and Monteleone, 2001).	Submucosal cells regenerate (Sonis, 2007).

Table 1.2 continued

X-rays (Brach et al., 1991) and CT (Gurujeyalakshmi et al., 2000) stimulate NF- κ B.	Damaged cell membranes stimulate SMase (Fuks et al., 1995).	Ceramide pathway activated by TNF- α activate apoptosis (Kolesnick and Fuks, 2003, Dimanche-Boitrel and Rebillard, 2013).	The oedematous and infiltrated ECM (Russi et al., 2014) weakening the attachment between submucosa and epithelium (Sonis, 2007).	New tissues generated during healing that are not similar to the old tissue (Denham and Hauer-Jensen, 2002).
CT stimulates <i>de novo</i> ceramide synthesis (Bose et al., 1995).	The ceramide pathway signals submucosal cells to enter apoptosis (Paris et al., 2001; Hwang et al., 2005).	Activation of cytokines attracts and activate immune cells causing local infiltration and oedema (Antin et al., 2002; Logan et al., 2009; Ong et al., 2010).	Programmed cell death, reduced epithelial regeneration, and apoptosis thin the epithelium (Sonis, 2007).	
Cells die in epithelia (Sonis et al., 2000), endothelia (Paris et al., 2001), and submucosal tissue (Maňáková et al., 2003).	AP-1 modulates fibroblast damage by inducing caspase-3 (Davis, 2000), and promotes the secretion of MMPs (Al-Dasooqi et al., 2013).	Upregulation in ECM components activates immune cells which produce ROS and amplify the oxidative damage (Russi et al., 2014).	Painful, deep, and broad ulcers, transcend the full epithelial thickness and covered by a pseudomembrane, are formed (Sonis, 2011).	

Table 1.2 continued

	Nrf2 transcription factor activates antioxidant-related genes (McDonald et al., 2010).	Damage responses are amplified in space and intensity (Sonis, 2007).	Microorganisms colonize the ulcers (Napeñas et al., 2010) and release cell wall products (Engels-Deutsch et al., 2003; Jaresova et al., 2007) which stimulate inflammatory response through the innate immune system pathway (Janeway and Medzhitov, 2002; Bahri et al., 2010), thereby amplify and accelerate local tissue damage (Tao et al., 2008; Zunt et al., 2009).	

Abbreviation: AP-1, activator protein 1; COX-2, cyclooxygenase-2; CRAMPs, chemoradiation-associated molecular pattern; CT, chemotherapy; DAMPs, damage-associated molecular pattern molecules; ECM, extracellular matrix; IL-6, interleukin-6; IL-1 β , interleukins-1 β ; JNK, c-Jun N-terminal kinase; KGF, keratinocyte growth factor; MAPK, mitogen activated protein kinase; MMPs, matrix metalloproteinases; NF- κ B, nuclear factor kappa-B; Nrf2, nuclear factor erythroid-2 related factor 2; PRRs, pathogen recognition receptors; RAGEs, receptors for advanced glycation end products; ROS, reactive oxygen species; RT, radiotherapy; SMase, sphingomyelinase; TLRs, Toll-like receptors; TNF- α , tumour necrosis factor-alpha.

Pathophysiology of OM can also be altered by a number of factors:

1.4.3.3.1 Tissue structure and modified functional physiology

It is now thought that the sequential biological and cellular events related to mucositis pathogenicity predominantly arise in the tissues and cells of the submucosa (endothelium and connective tissue) and culminate in targeting the epithelium cells, ending in degeneration and breakdown of the epithelial barrier resulting in the development of mucositis ulcers (Sonis, 2004b; Logan et al., 2007b; Sonis et al., 2007; Bowen et al., 2019). Recent studies highlight tight junctions (TJs) and their role in regulating the epithelial barrier function (Al-Dasooqi et al., 2013). These junctions regulate the cellular permeability through epithelium, both *in vitro* and *in vivo* (Madara, 1998; McCarthy et al., 1996; Wong and Gumbiner, 1997; Mitic et al., 2000; Bowen et al., 2019). The effect of chemoradiotherapy on altering TJs function and increasing mucosal permeability has been well known (Melichar et al., 2005). The importance of TJs in the pathobiology of mucositis is becoming increasingly clear (Zuo et al., 2015), linking mucositis severity to endotoxin and bacterial translocation through permeable TJs (Bowen et al., 2019). Furthermore, the emerging role of toll-like receptors (TLRs) in mediating mucosal injury has been shown recently in genetic knock-out studies. However, condition progression appears to be receptor- and drug-class specific (Bowen et al., 2019).

More recently, extensive research has been conducted on the roles played by specific TJs and how intestinal permeation directs microbiota translocation and consequent activation of innate immune reactions, mediating the pathogenesis of mucositis. Furthermore, TJs may also be necessary for systematic anti-cancer reactions (Alexander et al., 2017). However, information on the direct effect of TJs alteration on the clinical symptoms of mucositis is poorly understood and not yet elucidated. One study (Chen et al., 2011) showed a link between prevention from OM and TJ retention characteristics after RT. In addition, Wardle and co-workers (2016) demonstrated a correlation between diarrhea and the levels of endotoxin in mice, which was associated with TJs' alterations and translocation of FITC-dextran (Wardill et al., 2014). Using surrogate markers of endotoxin in a RT -induced mucositis murine model, Biju et al. reported a specific association between mucositis and altered mucosal permeability through a combination of epithelial TJs disruption and insufficient villus epithelium replacement (Biju et al., 2012). Given that increased mucosal permeability is somewhat correlated to TJ loss, further pre-clinical studies are required to assess mucositis interventions ability to stabilize these proteins as a measure of a potential outcome.

1.4.3.3.2 Microbiota and innate immune response

Changes in the composition of oral microorganisms during OM development have been determined (Sixou et al., 1996) and targeted (Donnelly et al., 2003) for decades. Nevertheless, gastrointestinal flora's role in intestinal damage has only been recognized recently. Technical progress in using the genetic sequence to identify diverse microbial species has been the bases of much new knowledge. An intricate interaction between the dynamic changes resulting from cancer therapy and the baseline arrangement of different species is evolving and considered influential in mucositis development (Bowen et al., 2019).

Recent clinical studies have examined the general variety of the oral microbiota and transformations during CT (Ye et al., 2013) to identify an association with OM. Other studies have demonstrated how microbes affect healing *in vitro* (De Ryck et al., 2015; De Ryck et al., 2014), and functioning changes in the microbiome itself when exposed to radiation (Vanlancker et al., 2016; Vanhoecke et al., 2016). Furthermore, microbial dysbiosis measurement in faecal samples of cancer patients with IM (Wang et al., 2015) has led to presume that the composition of the gut microflora could be utilized as a surrogate marker for transformations that lead to diarrhea. In addition, a mechanistic correlation between microbial signature alteration and the ability to metabolize nutrition and xenobiotics has been recognized in patients receiving high-dose CT (Montassier et al., 2015).

Pre-clinical studies utilizing mucositis animal models also identified overlapping between mucositis and changes in the microflora, which in turn encourages further investigation of the association between mucositis and microbiome changes *in vivo*. For instance, after cancer therapy there is a general reduction in microbial diversity (Pontoppidan et al., 2015; Nam et al., 2013; Lin et al., 2012) and increasing proteobacteria relative proportions that involve facultative anaerobes, like *Salmonella spp* and *E. coli* (Montassier et al., 2014; Stringer et al.).

The advent of genetic knock-out mucositis models has increased the opportunities for targeting the microbial interaction with the mucosa, with the main focus being on TLRs as a direct mediator between signalling cascades and bacterial ligands across immune, neural, and epithelial cells (Cario, 2016). For example, in IM model induced by irinotecan, deletion of MYD88 (Wong et al., 2015), adapter protein for TLR signalling pathway, or TLR4 germ-line (Wardill et al., 2016), was noticed to be protective. In addition, knockdown of TLR2 protected against irinotecan-induced mucositis (Frank et al., 2015), but not methotrexate-induced IM

(Wong et al., 2015), which give perception that the protective effect of TLR knock out could be receptor and drug class-specific. It is also noteworthy to mention that TLRs are also capable of signalling cellular danger signals that are not associated with microbes (damage-associated molecular patterns) (Piccinini and Midwood, 2010). As such, further studies are required to explain the role of the pathogenic than the non-pathogenic associated stimulation of TLR signalling in mucositis development.

In germ-free mice that received irinotecan, the mice showed protection against chemotherapy-induced IM. However, those mice lost their protection once colonized with the various microbiome (Pedroso et al., 2015). This finding reinforced the theory of the direct contribution of the gut microbiomes in IM pathogenesis. On the other hand, there have been other studies where antibiotic treatment was used to break down the oral microbial load but did not show clear protection against OM (Donnelly et al., 2003).

Furthermore, in some settings, TLR agonism has been shown to play a part in protection against oral and intestinal mucositis. For instance, Burdelya et al. (2012) reported that CBLB502, a TLR5 agonist, protected mice, and reduced RT -induced OM. Another study by Jones et al. (2011) reported that flagellin, a natural ligand, protected mice against radiation-induced IM. Finally, it has previously been shown that adding lipopolysaccharide (LPS), the cogent TLR4 agonist, before abdominal radiation reduced mucosal barrier damage induced by irradiation through a cyclooxygenase-mediated pathway (Riehl et al., 2000). It is not yet known whether TLR agonists protect the epithelium directly or through resident bacteria. However, one possible mechanisms may be correlated to the type of TLR agonist by gram-positive species such as *Lactobacillus* (Bowen et al., 2019).

In contrast to the gut microbiome, studies investigating the associations between the composition of oral microbiota and OM development are lacking. There is general agreement about the role played by the oral microbiota in the vulnerability to ulcerative mucositis and its infectious consequences, and it's altered by cancer therapy. However, mechanistic understanding is lacking (Vanhoecke et al., 2015). Two studies with different cohorts and detection methods examined oral microbiota composition changes related to cancer therapy and determined species involved in the pathogenesis of mucositis (Laheij et al., 2012; Ye et al., 2013). Extra research is needed to understand the intricacies of oral microflora and mucositis pathogenesis. Figure 1.5 shows the role of the dynamic microbial changes in relation to the mucositis development in all phases of mucositis pathogenesis.

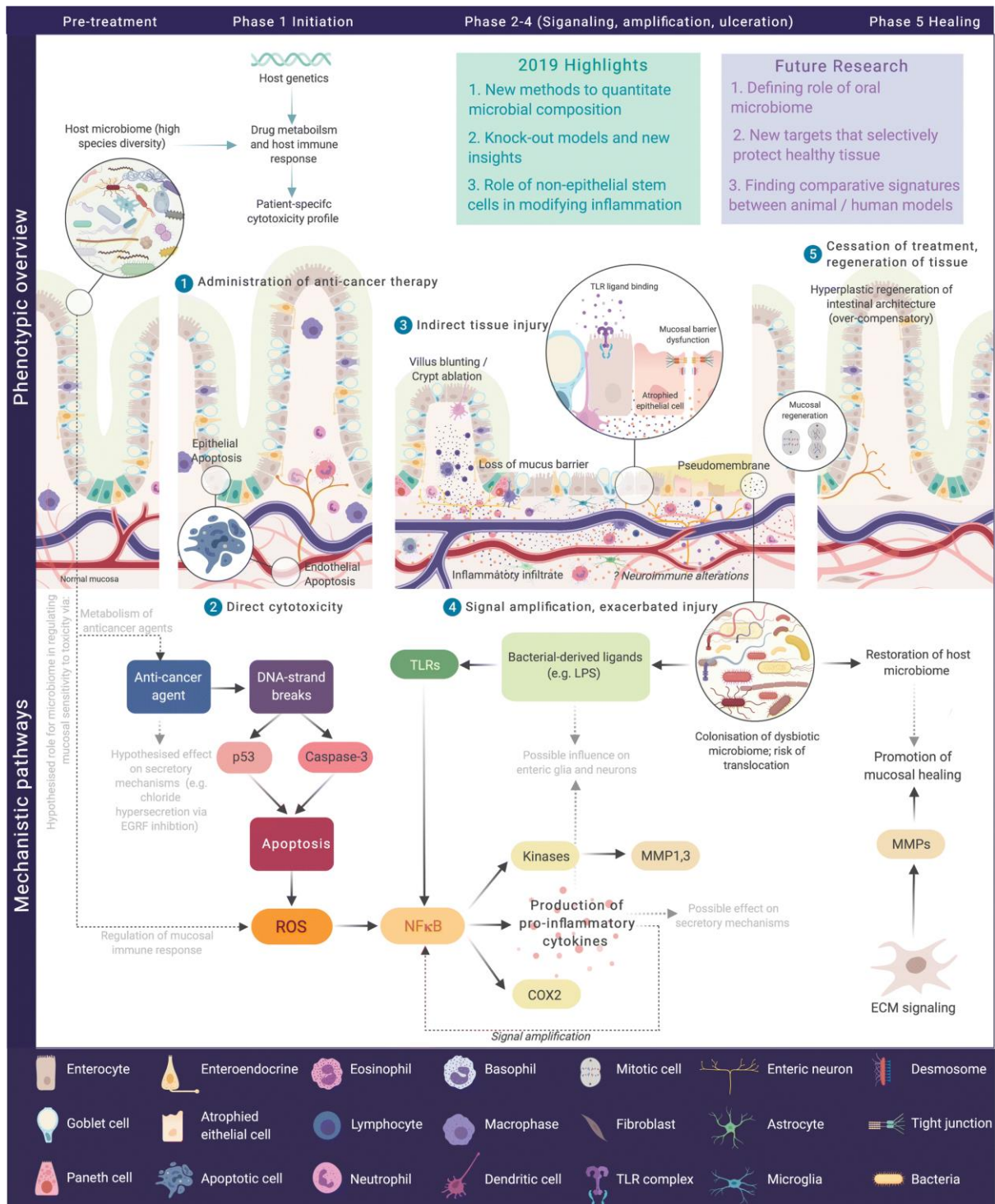


Figure 1.5 The effect of dynamic changes of the microbiota on all stages of mucositis, including pre-treatment risks. An example showing the interaction of gut microbes in causing mucositis. However, the oral microbes will also play a similar role in the development of oral mucositis. Adapted from Bowen et al. (2019).

The model of the development of mucositis is better explained in the oral mucosa; however, it can also occur in other parts of the gastrointestinal tract. Although these events happen in sequence following an acute mucotoxic challenge, clinically many appear at the same time, especially when fractionated radiation dosing protocols are used in which the mucosal tissues are challenged frequently. The description of the model seems linear, but in fact, all events occur very quickly and simultaneously in all mucosal elements (Sonis, 2007; Elad et al., 2022) (Figure 1.6), especially when fractionated radiation dosing protocols are used in which the mucosal tissues are challenged frequently (Denham and Hauer-Jensen, 2002). In the case of CT regimens, such as conditioning protocols for HSCT, the damage is associated with a brief but intense duration (Sonis, 2004b).

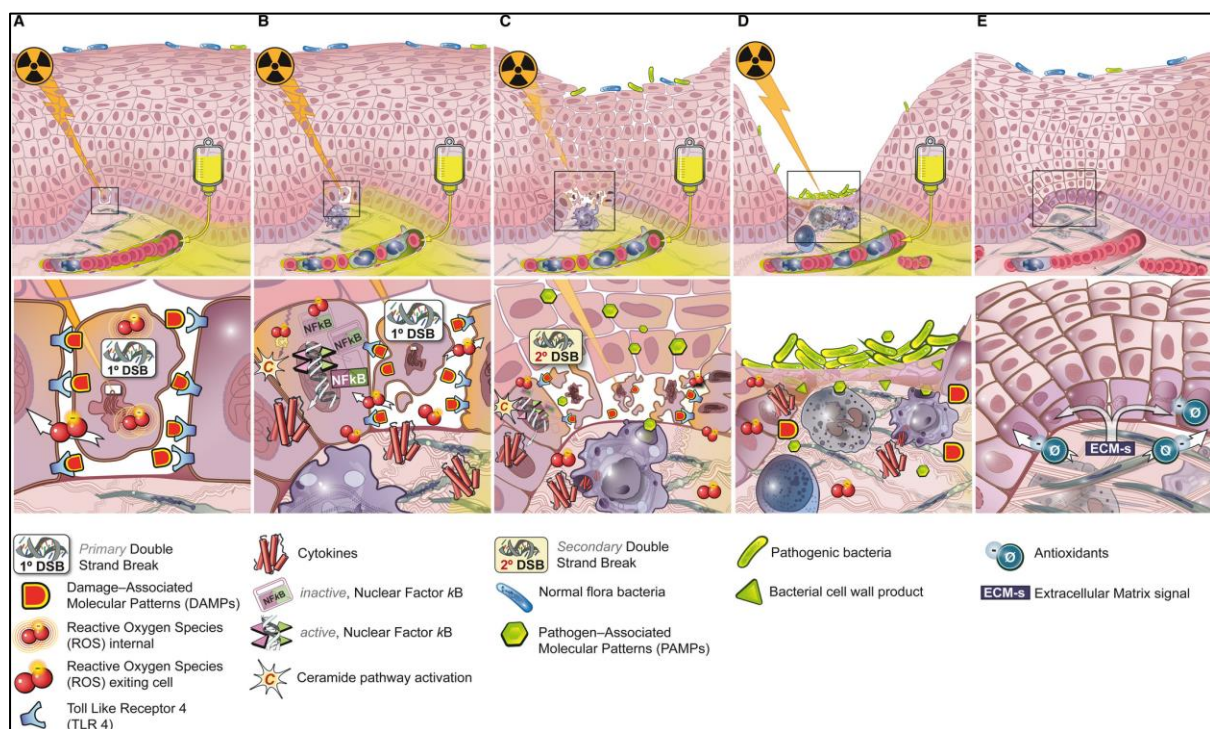


Table 1.3 Canonical pathways that were prioritized based on gene frequency and expected relevance to regimen-related toxicity. Adapted from (Sonis et al., 2007).

Pathways	Reference
Nitrogen metabolism	(Robbins and Zhao, 2004)
Toll-like receptor signalling	(Bowie and O'Neill, 2000)
NF-κB signalling	(Bowen et al., 2005; Sonis, 2002)
B Cell receptor signalling	(Kishimoto, 2005)
P13K/AKT signalling	(Vara et al., 2004)
Cell Cycle: G2/M DNA Damage checkpoint receptor	(Weng et al., 1998)
P38 MAPK signalling	(Roux and Blenis, 2004)
Wnt/B-catenin signalling	(Widelitz, 2005)
Glutamate receptor signalling	(Hinoi et al., 2004)
Integrin signalling	(Ginsberg et al., 2005)
VEGF signalling	(Giles, 2001)
IL-6 signalling	(Jones et al., 2005)
Death receptor signalling	(de Thonel and Eriksson, 2005)
SAPK/JNK signalling	(Nishina et al., 2004)

Abbreviations: IL-6, interleukin-6; MAPK, mitogen-activated protein kinase; NF-κB; nuclear factor kappa-B; VEGF, vascular endothelial growth factor;

1.4.4 Differential diagnosis of oral mucositis

OM diagnosis can be completed based on the disease history, onset context, and clinical observations. However, OM can be confounded with other oral diseases. Secondary fungal infection (such as candidiasis) or viral infection (such as HSV) can complicate the OM clinical course, and when lesions appear unusual or last longer than expected, these infections must be considered. HSV reactivation-induced ulcers are clinically different from OM ulcers in that they are usually localized to the keratinized mucosa of the dorsal surface of the tongue, hard palate, and gingivae. Furthermore, in myelosuppressive patients, deep fungal infections, non-HSV viral infections, and bacterial infections, of the oral cavity should be considered. They

can be distinguished from OM by clinical symptoms, swab tests, or biopsies for deep fungal infections. Since multiple medications are often used to treat cancer patients, the differential diagnosis of oral ulcers may go further and comprises drug eruptions lesions and Stevens-Johnson syndrome/erythema multiforme (Elad et al., 2022).

1.4.5 Assessment of Oral Mucositis

Measurement is a principal factor of scientific research. Thus, it is necessary to establish measurement scales for OM to use in research studying therapeutic and prophylactic agents in clinical practice to improve patient outcomes. Mucositis must be assessed accurately for the development of effective new therapies. Mucositis measurement tools must accurately depict mucosal injury, classify changes objectively, measure severity reproducibly, and identify underlying molecular mechanisms associated with the relevant clinical results, such as degree of improvement and pain relief (Kuten-Shorrer et al., 2021). Several scoring systems for assessing OM severity and providing direction for health care interventions and modifications of cancer treatments have been developed. Nevertheless, none have proved to be of ideal standard having feasibility, reproducibility, reliability, validity, sensitivity, and specificity for measuring mucositis in various clinical and research settings. Furthermore, none of the scales developed so far has been accepted universally for assessing mucositis. Most of the available measurement scales measure OM; however, a few measure gastrointestinal mucositis as well (Sonis et al., 2004a).

Systematic-periodic objective evaluation of the oral cavity should be conducted in all patients at risk of OM development, regardless of the utilized scale. Besides the objective physical symptoms of mucositis (redness, ulceration, and bleeding) subjective and functional symptoms, like pain and swallowing difficulty (dysphagia) should be carefully evaluated. Patients undergoing radiation therapy to the head and neck should have a thorough oral examination before starting treatment and periodically during treatment (Sonis et al., 2004a). Various scoring systems used to assess the OM objectively and subjectively are shown in Tables 1.4 -1.6 below.

1.4.5.1 Oral Mucositis Scoring scales

Various terminologies have been used to describe mucosal injury; for example, stomatitis or mucositis has been used interchangeably while describing oral inflammatory conditions. For decades, the most commonly used mucositis scale was developed to define oral toxicity

resulting from various cancer treatments. Several types of scales were developed to assess OM (Sonis et al., 2004a; Elad et al., 2022).

1.4.5.1.1 Physician-reported outcome measures

The first group of scales is mainly based on the WHO scale which was developed to assess the patients receiving cancer treatment clinically (World Health Organization, 1979). These are simple, combined, and variable toxicity scales comprising 4-5 point rating scales that assess the overall condition of the mouth in relation to mucosal appearance, severity, pain, and in some cases, the patient's functional limitations related to the patient's oral condition (such as food tolerance, and swallowing). In addition, the National Cancer Institute-Common Toxicity for Adverse Events (NCI-CTCAE), version 5.0 , and the Radiation Therapy Oncology Group's (RTOG) scales come under this group (Sonis et al., 2004a) (Table 1-4).

Scales used for clinical research combine both subjective and objective measures of OM severity. For example, the OM Rating Scale measures erythema and ulceration, allowing for sub-analysis of mucosal damage at anatomical sites. The measurements of this scale have been validated in adults and children and were closely associated with patients' symptoms (Sung et al., 2007; Elad et al., 2022).

Table 1.4 Measurement of Oral mucositis. Simple, combined-variable mucositis scoring scales

Scale	Use	Reference	Measured elements	Advantages	Disadvantages
NCI-CTCAE	Clinical and research	(Trotti et al., 2000; National Cancer Institute, 2017)	Combined elements: signs (redness, ulceration), symptom (burn, pain); function: type of daily nutritional intake	Widely used in setting of clinical care and research; Specific measures of mucositis in patients receiving radiation therapy, CT, or HSCT to the head and neck	Research evaluation potentially confusing by the combination of symptoms, signs and functional changes
WHO	Clinical and research	(World Health Organization, 1979)	Combined elements: signs (redness, ulceration), symptom (burn, pain); function: patient daily nutritional intake	Widely used in setting of clinical care and research; Specific measures of mucositis in patients receiving radiation therapy, CT, or HSCT to the head and neck	Research evaluation potentially confusing by the combination of symptoms, signs and functional changes
RTOG	Clinical and research	(Cox et al., 1995)	Combined elements: signs (unspecified), symptom (pain); function: unspecified	Widely used in setting of clinical care and research	Research evaluation potentially confusing by the combination of symptoms, signs and functional changes

Abbreviations: HSCT: hematopoietic stem cell transplantation; RTOG: Radiation Therapy Oncology Group; NCI-CTC: National Cancer Institute Common Toxicity Criteria; WHO: World Health Organization.

1.4.5.1.2 Nurse-reported outcome measures

The second group of scales has evolved from the above is mainly developed as nursing management and research tools. These scales combine objective, functional and symptomatic

variables by applying them to specific anatomic sites, adding greater specificity to various aspects of oral function and subjective patient responses. Western Consortium of Cancer Nursing Research (WCCNR) scale comes under this category of scales (Sonis et al., 2004a) (Table 1.5).

Table 1.5 Measurement of Oral mucositis. Combined objective/functional/symptom scales

Scale	Use	Reference	Measured elements	advantages	Disadvantages
Oral Assessment Guide	Clinical	(Eilers et al., 1988)	Signs (redness), symptoms (pain, salivary changes), functional disorders (swallowing, voice)	Universal scale that may reflect clinical outcomes/conditions; Suitable for making nursing care decisions	Not all variables are necessarily associated with clinical conditions; Some variables are not constant
Western Consortium for Cancer Nursing Scale	Clinical	(WCCNR, 1991)	subjective variables injuries, bleeding, colour	Universal scale that may reflect clinical outcomes/conditions; the scale were refined in 1998 based on elimination of five measures other than colour, lesions, or bleeding.	Mixed functional, subjective, and objective variables; Difficult to record accurately
Tardieu Quantitative Scale of Oral Mucositis for HSCT	Research	(Tardieu et al., 1996)	Mucosal alterations, salivary functional changes, functional disorders (voice, swallowing), pain	Includes four anatomical sites, severity range	Not verified (used in pilot study only); tested only on HSCT patients; detailed, requires moderate to heavy training
Walsh Quantitative Scoring System for Oral Mucositis	clinical and research	(Walsh et al., 1990)	Mucosal alterations, functional alterations, salivary function, pain	Conceptual elements of the NCI or WHO scale applied to specific anatomical regions; require moderate training	Not verified yet; tested only on HSCT patients

1.4.5.1.3 Detailed objective scoring scales

The third group of scales was designed mainly for clinical research trials. These detailed objective scoring scales focus on direct, separately scored, objective, and subjective endpoints. Oral Mucositis Index (OMI) and Oral Mucositis Assessment Scale (OMAS) come under this category of scales (Sonis et al., 2004a). (Table 1.6)

1.4.5.1.4 *in vitro* measurement scale

The fourth group of scales has been developed for the *in vitro* measurement of mucositis. These scales are purely for research, for example, Epithelial Viability Scale (Sonis et al., 2004a). (Table 1.6)

1.4.5.1.5 Patient-reported outcome measures of Mucositis

Patient-reported outcome data make essential contributions to the overall assessment of OM severity. The 0 to 10 Numerical Rating Scale is a widely used one-dimensional measure due to its ease of implementation (Elad et al., 2022). Patients measure the severity of their oral pain on an 11-point horizontal bar, with no pain recorded as 0 and 10 being the worst possible pain. The Daily Oral Mucositis Questionnaire and its weekly version (Weekly Oral Mucositis Questionnaire) measure symptoms of OM, including mouth and sore throat, and their impact on the patient's well-being and function (Stiff et al., 2006; Epstein et al., 2007).

One of the scales related to patient-reported outcomes is the Functional Assessment of Cancer Therapy-Head and Neck (FACT-HN) scale, which includes the FACT-General. It is a multidimensional QoL tool explicitly designed for use in cancer patients and a specific subscale of H&N cancer (List et al., 1996). FACT-HN is used to assess OM's impact on the QoL of H&N cancer patients in specific scopes, including physical, functional, emotional, and social domains. Likewise, a Quality of Life Questionnaire (QLQ) has been designed by the European Organization for Research and Treatment of Cancer, namely (EORTC QLQ), to evaluate the QoL of cancer patients and has been complemented by disease-specific modules. In its most recent version, the EORTC QLQ-HN43 module captures all HNC treatment modalities' side effects and QoL effects across 19 scales (Singer et al., 2019). Being one of the standard tools for measuring QoL in H&N cancer patients, the EORTC QLQ-HN43 has been validated internationally and is available in 17 languages (Elad et al., 2022).

The first group of scales which are based on WHO design, are the most relevant scales for clinical management of OM. A systematic review of approximately 400 trials revealed that

most of the studies utilized the NCI (43%) or WHO (38%) scales, followed by a study-specific scale (10%) and a cooperative group (RTOG/ECOG) scale (5%) (Sonis et al., 2004a).

Table 1.6 Measurement of Oral mucositis. Detailed, objective mucositis scoring scales and *in vitro* measurements.

Scale	Use	Reference	Measured elements	advantages	Disadvantages
OMI for HSCT	Research	(Schubert et al., 1992)	Thirty-four mucosal alterations: signs (erythema, atrophy, ulceration/pseudo-membrane, edema, and selected sites); separate pain scores (VAS)	specific to 11 anatomical sites of the mouth, allowing substitution analyses of changes across the oral mucosa; eliminates symptoms confusion and functional disorders; Scores are consistent with NCI and WHO scores.	Requires more experience and examiner time than NCI-CTC and WHO scales; tested only on patients receiving HSCT
Twenty-item OMI for HSCT	Research	(McGuire et al., 2002)	Twenty mucosal alterations: signs (erythema, atrophy, ulceration/pseudo-membrane, edema, and selected sites)	specific to nine anatomical sites of the mouth, Objective clinical changes were recorded as in full OMI	Requires less experience than OMI
OMAS for chemotherapy, radiation, and HSCT	Research	(Tardieu et al., 1996)	Signs (redness, ulceration)	Same advantages as OMI with fewer anatomical oral sites scored	Requires more experience and examiner time than NCI-CTC and WHO scales but less time than OMI
Epithelial Viability Scale (<i>in vitro</i> measurement)	Research	(Wymenga et al., 1997)	Trypan blue based exclusion assay, based on oral epithelial smears	Easily administered; objective <i>in vitro</i> measurement; studied with both chemo/and radiotherapy-induced mucositis	At an early stage of development; Requires additional verification

Aberrations: VAS: visual analog scale; OMI: Oral Mucositis Scale; OMAS: Oral Mucositis Assessment Scale.

1.4.5.2 World Health Organization Oral Toxicity Scale (WHO)

These were the first established guidelines proposed by the WHO in 1979, which were developed to clinically assess the oral toxicity in patients receiving cancer therapy. The WHO Oral Toxicity Scale measures OM's anatomical, symptomatic, and functional components. The condition severity is graded from 0 (no OM) to 4 (alimentation is not possible, and the patient needs total parenteral nutrition) (World Health Organization, 1979) (Table 1.7). This is one of the most widely used scales in various clinical and research settings. Easy to assess and less time-consuming, reliability and validity are not established.

1.4.5.3 National Cancer Institute- Common Toxicity Criteria

In 1982, the National Cancer Institute (NCI) created the Common Toxicity Criteria (NCI-CTC) to evaluate chemotherapy-related effects. In 1997, the NCI revised the original CTC as NCI CTC version 2.0 (Trotti et al., 2000). Later another revision of the NCI-CTC version 2.0 was done as NCI-CTC version 3.0 on April 30, 1999. This updated NCI-CTC version 3.0 was released in December 2003 for widespread implementation (Trotti et al., 2003b). In 2009, NCI again revised the toxicity criteria, and a new NCI-CTC version 4.0 was formulated (National Cancer Institute, 2011). In 2017, NCI again revised the toxicity criteria, and a new NCI-CTCAE, version 5.0, has been formulated, and it is now being used widely in clinical trials to document AEs of antineoplastic therapies in cancer research (National Cancer Institute, 2017) (Table 1.7).

1.4.5.4 The Radiation Therapy Oncology Group Scale

The Radiation Therapy Oncology Group (RTOG) created the Acute Radiation Morbidity Scoring Criteria for evaluating RT effects (different criteria were also developed for the late effects of RT). In 1997, the RTOG appointed a committee to review the RTOG evaluation criteria, and the revised RTOG was integrated into the NCI-CTC to produce version 2.0; since March 1998, all NCI-sponsored clinical trials have used the modified version. This RTOG criterion is an essential part of NCI-CTC Version 2.0. The RTOG assessment scale has effectively measured the mucosal toxicity of anticancer therapeutic regimens. The RTOG Acute Radiation Morbidity Scoring Criteria for mucous membranes measure only the anatomical changes associated with OM. The severity of OM is graded from 0 (no OM) to 4 (necrosis or deep ulceration present, with or without bleeding) (Cox et al., 1995).

1.4.5.5 Western Consortium for Cancer Nursing Research Scale

Western Consortium for Cancer Nursing Research (WCCNR) has been developed from the earlier scales for nursing management and research. The revised OM staging system of the WCCNR is a 4-grade assessment tool that only measures the anatomical alterations associated with OM (WCCNR, 1991).

Table 1.7 Clinical Oral Mucositis Assessment Scales.

Scale	Grade 0	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Sever)	Grade 4 (Life- threatening)	Grade 5 (Death)
WHO	No finding	Soreness ± erythema	Redness and, ulcers, but patient still can tolerate solid diet	Extensive erythema with ulcers and solid food can not be swallowed. Liquid diet only	Mucositis to the extent that nourishment is impossible (mucositis malnutrition)	NA
NCI-CTCAE (version-5)	None	Asymptomatic or mild symptoms; no intervention specified	Moderate pain or ulcer; not interfering with oral intake; modified diet indicated	Intense pain; Interfere with oral intake	Life-threatening consequences; indicate for urgent intervention.	Death related to toxicity
RTOG	No change on baseline	Irritation, mild pain may occur that does not require analgesics	Patchy mucositis which may produce serous inflammatory exudate; may experience moderate pain that requires analgesia	Confluent inflammatory, fibrinous mucositis; may include severe pain that require narcotic	Ulceration, bleeding, or necrosis	NA
WCCNR	Lesions: none Colour: pink Bleeding: none	Lesions: 1-4 Colour: slight red Bleeding: N/A	Lesions: > 4 Color: median red Bleeding: spontaneous	Lesions: combined Colour: very red Bleeding: spontaneous	NA	NA

Abbreviations: NA, not applicable; NCI-CTCAE, National Cancer Institute-Common Terminology Criteria for Adverse Events; WCCNR, Western Consortium for Cancer Nursing Research; WHO, World Health Organization; RTOG, Radiation Therapy Oncology Group.

1.4.5.6 Oral Mucositis Assessment Scale

The oral mucositis assessment scale (OMAS) was first developed by Sonis et al. (1999b); This scale separates subjective and objective consequences. For example, redness and ulceration levels scored at specific areas in the mouth were considered primary indicators of OM, whereas

oral soreness, swallowing tribulation, and eating ability were secondary indicators. The OMAS provides an objective OM assessment based on scoring of ulcerations presence and size or pseudo-membranes (score 0 to 3: 0 = no lesion; 1 = lesion < 1cm²; 2 = lesion of 1cm² to 3cm²; 3 = lesion > 3cm²) and erythema (score 0 to 2: 0 = none; 1 = not severe; 2 = severe) on the lower and upper lips, left and right cheeks, left and right lateral and ventral tongue, soft palate, floor of the mouth, and hard palate (**Table 1.8**). OMAS has been demonstrated to be quite reproducible between observers, more sensitive over time, and accurate in recording mucositis-related features (Sonis et al., 1999b). This scale is somewhat suitable for clinical research, but can be difficult to applied in regular clinical care.

Table 1.8 Oral Mucositis Assessment Scale

Location	Ulceration†	Erythema‡
Lip		
Upper	0, 1, 2, or 3	0, 1, or 2
Lower	0, 1, 2, or 3	0, 1, or 2
Buccal mucosa		
Right	0, 1, 2, or 3	0, 1, or 2
Left	0, 1, 2, or 3	0, 1, or 2
Ventro-lateral tongue		
Right	0, 1, 2, or 3	0, 1, or 2
Left	0, 1, 2, or 3	0, 1, or 2
Floor of mouth	0, 1, 2, or 3	0, 1, or 2
	0, 1, 2, or 3	0, 1, or 2
Palate		
Soft	0, 1, 2, or 3	0, 1, or 2
Hard	0, 1, 2, or 3	0, 1, or 2

†0 = none; 1 = <1 cm²; 2 = 1–3 cm²; 3 = >3 cm², ‡0 = none; 1 = not severe; 2 = severe.

1.4.5.7 The Oral Mucositis Index

The Oral Mucositis Index (OMI) evaluates OM severity based on erythema, atrophy, ulceration, and edema (each is graded on a scale from 0 to 3, where 0 = none and 3 = severe). The OMI internally corresponds to high test-retest and reliability among ratters and shows strong evidence of the validity of construct (McGuire et al., 2002).

1.4.5.8 Oral mucositis measurement in children

OM severity validation can be challenging in children. Depending on the child's age, patients' ability to understand instructions, bear oral examination, and expression of symptoms may require dependence on parental or proxy reports (Elad et al., 2022). Several of the most

common OM scales evaluated in adults (such as the Oral Mucositis Daily Questionnaire and the Oral Mucositis Rating Scale) have also been assessed in children (Sung et al., 2007; Tomlinson et al., 2011). In addition, the Children's International Mucositis Evaluation Scale, a specific scale to measure OM in paediatrics, has been verified for use in children and adolescents undergoing HSCT who are eight years of age or older (Jacobs et al., 2013)(Figure 1.7).

CHILDREN'S INTERNATIONAL MUCOSITIS EVALUATION SCALE (ChIMES)

PAIN

1. Which of these faces best describes how much pain your child feels in their mouth or throat today? Circle one.

					
0 No hurt	1 Hurts a little bit	2 Hurts a little more	3 Hurts even more	4 Hurts a whole lot	5 Hurts worst

FUNCTION

2. Which of these faces shows how hard it is for your child to SWALLOW their saliva/spit today because of mouth or throat pain? Circle one.

						☐ Can't tell
0 Not hard	1 Little bit hard	2 Little more hard	3 Even harder	4 Very hard	5 Can't swallow	

3. Which of these faces shows how hard it is for your child to EAT today because of mouth or throat pain? Circle one.

						☐ Can't tell
0 Not hard	1 Little bit hard	2 Little more hard	3 Even harder	4 Very hard	5 Can't eat	

4. Which of these faces shows how hard it is for your child to DRINK today because of mouth or throat pain? Circle one.

						☐ Can't tell
0 Not hard	1 Little bit hard	2 Little more hard	3 Even harder	4 Very hard	5 Can't drink	

PAIN MEDICATION

5. Has your child taken medicine for any kind of pain today?

☐ Yes ☐ No

If yes, did your child need the medicine because they had mouth or throat pain?

☐ Yes ☐ No

APPEARANCE

6. Please look in your child's mouth. Can you see any mouth sores (ulcers)?

☐ Yes ☐ No ☐ Can't tell

Figure 1.7 The Children's International Mucositis Evaluation Scale (ChIMES). Adapted from (Jacobs et al., 2013).

1.4.6 Management of Oral Mucositis

In general, several principles are adopted for oral care of cancer patients; these include preventing infection, controlling pain, preserving oral function, improving QoL, and managing concomitant oral complications (Elad et al., 2015). These guidelines are not limited to OM but rather apply to any oral complication.

Given that mucositis has become increasingly dose-limiting toxicity, efforts to treat and prevent OM gained significance in scientific and clinical studies (Raber-Durlacher et al., 2013). Several treatment modalities have been evaluated for the prevention and management of mucositis, but all have a limited success rate. None of the modalities has proved to be successful in preventing mucositis, though few interventions have been shown to be effective in reducing the progression towards higher grades. Research is ongoing to find a definitive preventive and treatment modality for mucositis. Currently, no gold standard of clinical practice has been set for preventing and treating OM. Recently, the FDA has approved the use of Palifermin (recombinant growth factor) for the prevention of mucositis in HSCT settings. At present, the standard of care for mucositis is symptomatic management. Modalities targeting salivary production like Amifostine have been tried. Various oral hygiene protocols have been formulated to maintain oral hygiene, and different types of mouthwashes (alcohol and non-alcohol based) have been attempted to reduce the incidence of mucositis. Various anti-inflammatory agents have been tried to reduce inflammation in the mucosa. To reduce the pain severity of OM, multiple types of local as well as systemic analgesics and anaesthetics have been tested. Various antimicrobial agents like antibacterial, antifungal, and antiviral agents have been tried to reduce the opportunistic pathogens infection in the mucosa. Even modifications in standard treatment modalities of cancer (i.e., Radiation/ CT) have been attempted to decrease the occurrence of this severe adverse event (Elad et al., 2020).

Mucositis management has become an important area in head and neck symptom research and formulating management guidelines. For the last two decades, many treatment techniques for radio-chemotherapy-induced OM in cancer patients have been established. Oncologists have employed a variety of techniques to reduce the side impacts of cancer treatment, including dosage adjustments and other therapeutic and preventative measures (Elad et al., 2022). After intensive reviews of various clinical trials involving different modalities for the management of mucositis, various international organizations have published their recommendations for managing OM. These include the Mucositis Study Group of the MASCC/ISOO (Rubenstein et

al., 2004; Keefe et al., 2007; Lalla et al., 2014; Elad et al., 2020), NCCN (Bensinger et al., 2008), European Society of Medical Oncology (ESMO) (Peterson et al., 2011), European Oncology Nursing Society (Quinn et al., 2008), and a Cochrane review (Worthington et al., 2011; Clarkson et al., 2010; Worthington et al., 2007; Worthington et al., 2004). In addition, several strategies have been tried to prevent and treat the OM induced by cancer therapies.

Treatment of OM has mainly been palliative until now, although a number of preventive and therapeutic strategies have been developed (Sonis, 2011; Colella et al., 2010; Lalla et al., 2014; Cohen et al., 2013). Specific measures for managing OM include either prevention or symptom management. Currently, there is no intervention for therapeutic intent, and little to offer patients in the way of effective treatment to prevent or mitigate mucositis (Elad et al., 2022). The key factor in preventing OM is tissue preservation through planning treatment with RT and using RT techniques capable of preserving the uninvolved oral mucosal surface (Elad et al., 2022). Further measures are addressed in the evidence-based guidelines for OM management published by the MASCC/ISOO (Table 1.9) (Elad et al., 2020).

The categories of the guideline include a recommendation, a suggestion, or no possible guidelines. Interventions with the most evident evidence, supported by a large number of randomized controlled clinical trials, with one of them at least with no significant drawbacks, were categorized as recommended. In comparison, a suggestion was reserved for the interventions where the results in multiple randomized controlled clinical trials were consistent. As such, many of the interventions that did not reach a recommendation or suggestion level of guidelines were assigned as no possible guidelines (Elad et al., 2022). The full set of guidelines articles contains the evaluation of the evidence related to these interventions (Ariyawardana et al., 2019; Yarom et al., 2019; Yarom et al., 2020; Elad et al., 2020; Elad, 2019; Hong et al., 2019; Zadik et al., 2019; Correa et al., 2020; Elad, 2020; Logan et al., 2020; Saunders et al., 2020).

Table 1.9 Oral Mucositis Management. Based on the Guidelines published in 2020 Multinational Association for Supportive Care in Cancer/International Society of Oral Oncology article (Elad et al., 2020).

Intervention	Objective	MASCC/ISOO guideline category	Reasoning	Specifications
Basic oral health care (Hong et al., 2019)	Prevention	Suggestion (expert examiner opinion)	Educate patients and improve their compliance with routine oral hygiene	Includes dental evaluation and treatment, as indicated, before cancer therapy; educating patients about the importance of primary oral care to improve self-control during cancer therapy; the use of sodium bicarbonate and saline rinses to raise oral hygiene; In addition, the implementation of multi-agent combined oral hygiene protocols.
Cryotherapy (Correa et al., 2020)	Prevention	Recommendation	The local cold-induced vasoconstriction reduces chemotherapeutic agent delivery to the oral mucosa.	Used in patients undergoing 5-Fluorouracil bolus doses and in patients receiving autologous HSCT concomitant with a high dose of melphalan.
Photo-biomodulation therapy (Zadik et al., 2019)	Prevention	Recommendation	Wound healing stimulation and promotion	Specific protocols for patients undergoing HSCT as well as patients receiving RT with or without concomitant CT for HNC; the use of this protocol should be cautiously considered in areas with known tumours.
Benzydamine (Ariyawardana et al., 2019)	Prevention	Recommendation or Suggestion ((expert opinion)	Anti-inflammatory	Used in patients receiving moderate-dose RT for HNC (up to 50 grays) and those undergoing chemoradiotherapy for HNC.
KGF-1 (Logan et al., 2020)	Prevention	Recommendation	KGF-1 Induces epithelial cell differentiation and proliferation; KGF-1 has anti-inflammatory, antioxidant, and antiapoptotic effects, stimulating tissue protection.	In patients receiving high-dose of CT conditioned with TBI prior to autologous HSCT.

Table 1.9 continued

Glutamine (Yarom et al., 2019)	Prevention	Suggestion	Preserve cellular homeostasis and enhance cell protection against metabolic stress	In patients undergoing chemoradiation therapy for HNC; Caution should be considered when using glutamine in patients receiving HSCT due to higher mortality rates noticed in HSCT patients who received parenteral glutamine.
Honey (Yarom et al., 2020)	Prevention	Suggestion	Promotes wound healing and have an anti-microbial effect	In patients receiving RT with or without concomitant CT for HNC; given systemically and topically; strict oral hygiene is recommended.
Analgesia (Patient-controlled) (Saunders et al., 2020)	Treatment	Recommendation	Pain relief	In patients receiving HSCT
Topical morphine 0.2% (Saunders et al., 2020)	Treatment	suggestion	Management of pain	In patients with HNC undergoing chemoradiotherapy

Abbreviations: HNC, head and neck cancer; HSCT, hematopoietic stem cell transplantation; KGF-1, keratinocyte growth factor-1; MASCC/ISOO, Multinational Association for Supportive Care in Cancer/ International Society of Oral Oncology; PMB, photobiomodulation; TBI, total body irradiation.

The recommended or suggested interventions specific to certain cancer patient populations are listed in table 1.9. According to the 2020 MASCC/ISOO guidelines (Elad et al., 2020), cryotherapy is recommended concurrently with a high-dose bolus injection of melphalan in patients receiving autologous HSCT (Correa et al., 2020). Literature describes several protocols of cryotherapy, in which ice water must be kept in the mouth for varying lengths of time, and all protocols have been reported to be effective (Correa et al., 2020); Nonetheless, more extended periods may be more difficult for patients. By applying Photobiomodulation (PBM) therapy protocols, PBM has demonstrated a beneficial effect in preventing OM (Zadik et al., 2019). Based on the present literature, 0.15% benzydamine mouthwash efficaciously prevents OM in patients receiving up to 50 Gy RT or chemoradiotherapy. For example, those patients who undergoing RT to head and neck area for lymphoma (Ariyawardana et al., 2019). In addition, Keratinocyte Growth Factor-1 (Palifermin) at a dose of 60 mcg/kg once daily for 3 days before and after myelotoxic HSCT has also shown a beneficial effect in OM prevention (Logan et al., 2020). Oral administration of 10 grams of glutamine one to three times daily over a 6-week chemoradiotherapy course has proven efficacy in reducing OM severity and concomitant pain in HNC patients (Yarom et al., 2019). Combined topical and systemic application of various honey products has proven efficacy consistently (Yarom et al., 2020). Patient-controlled analgesia involves the i.v. infusion of morphine with a patient-controlled pump. Finally, A 0.2% topical morphine rinse has been recommended to manage OM-related pain associated with RT (Saunders et al., 2020).

Although the guidelines are evidence-based, they may not be universally applicable. For example, benzydamine has been approved for use in Europe and is available without a prescription in some European countries but not in other countries such as the United States (Ariyawardana et al., 2019). Additionally, PBM treatment encompasses numerous types of laser devices and protocols, and each type is only registered in some countries, limiting access to this treatment method. Therefore, widespread collaboration is required to translate protocols used on one unit to other units successfully (Zadik et al., 2019). Furthermore, palifermin reduced the incidence of severe OM in multiple randomized controlled clinical trials, but not completely; 63% of the patients receiving palifermin still developed severe mucositis (Raber-Durlacher et al., 2013). Therefore, guidelines should be tailored based on clinical practice considerations and patient preferences.

Maintenance of optimal nutritional support is necessary throughout cancer treatment duration to mitigate the risk of nutritional deterioration associated with OM and subsequent discontinuation of anticancer regimen. Possible irritation sources, such as acidic, spicy, or hard (crispy) foods, should be avoided because they can exacerbate OM. In cases where dietary measures are insufficient, liquid nutritional supplements should be considered. In addition, the need for using gastric feeding or complete parenteral nutrition may be warranted in severe cases of OM (Elad et al., 2022)

Although various interventions have been tried in paediatric populations to prevent or treat OM, there are currently no specific guidelines available for children receiving cancer treatment due to inadequate and/or incompatible evidence (Miranda-Silva et al., 2021). Although the implementation of basic oral care protocols is proposed to cover all cancer treatment modalities and age groups, including paediatric patients, other interventional protocols in this patient population are estimated from the evidence currently available for adult cancer patients. Modifications are recommended to increase patient acceptance and tolerance for the intervention. For example, oral cryotherapy using flavoured popsicles in place of ice or ice water may improve compliance in younger patients (Sung et al., 2017).

Based on the latest clinical practice guidelines for mucositis management published by the MASCC/ISOO (Elad et al., 2020), several strategies have been tried to prevent and treat the OM induced by cancer therapies. We will go through them below:

1.4.6.1 Preventive Strategies:

1.4.6.1.1 Basic oral Care/Maintaining Oral Hygiene:

Basic oral care aims to reduce the impact of the oral microbial flora, reduce cancer therapy-related symptoms of pain and bleeding, and prevent soft tissue infections that may have systemic effects. In addition, maintaining good oral hygiene is an essential step in preventing severe mucositis, as poor oral hygiene has been considered a significant risk factor for developing and progressing OM. Despite limited evidence of the benefits of oral care protocols for preventing OM, almost all reviews and clinical practice guidelines have recommended basic oral care as a best clinical practice. The rationale behind this is clear that good oral hygiene gives fewer portals for opportunistic pathogens to grow, preventing the development of severe grades (Hong et al., 2019). Few authors have also suggested that patients should abstain from smoking and avoid hot, spicy, and coarse foods, fruits and beverages with a high acid content,

and alcohol (including alcohol-containing elixirs) as these cause mucosal irritation (Eilers, 2004; Eilers and Million, 2007; McGuire et al., 2006; Hong et al., 2019; Elad et al., 2020).

Basic oral care protocol is a multidisciplinary approach that involves the patient, family/caregivers, nurse-physician, dentists, dental hygienist, dietician, and pharmacist. It has been recommended that prior to initiating the cancer treatment, patients at risk of developing mucositis must require a thorough evaluation of the oropharyngeal region by a dentist. In addition, any condition hampering mucosal health, such as dental caries, periodontal disease, or mucosal damage and/or sites of the irritation, must be appropriately dealt with before starting the cancer treatment (Hong et al., 2019; Yu et al., 2020).

Good oral hygiene maintaining before, during, and after radiation therapy will reduce the risk for oral complications like infections, caries, gingivitis, and osteoradionecrosis. Basic oral care during radiation therapy involves gentle brushing with a soft bristle toothbrush, dental flossing as tolerated, frequent oral rinsing with a bland solution like normal saline with sodium bicarbonate and the use of moisturizing agents, regular dental evaluations and cleanings, and the use of lifelong daily dental fluoride prophylaxis. Mouthwashes containing alcohol may not be favourable to be tolerated by patients during clinical oral ulceration, so, alcohol-free preparations are recommended (Lalla et al., 2008; Elad et al., 2020). A study by Bonnaire et al. (1998) demonstrated that tooth brushing reduced the number of oral lesions in patients receiving cancer CT. They also reported that using foam toothbrushes is not equivalent to tooth brushing and cannot be recommended for plaque control or caries prevention (Bonnaire-Mallet et al., 1998).

1.4.6.1.2 Oral care protocols and patient education

For the successful use of oral care protocol, education of the patient, caregivers, and professional staff and increasing their awareness regarding the use of such protocols is also recommended. With the specific objective of reducing mucositis, Graham et al. initiated a unit-based oral care protocol and teaching program and documented a reduction in mucositis (Graham et al., 1993). Similarly, Larson et al. (1998) used the PRO-SELF Mouth Aware Program in a study of outpatients receiving CT and demonstrated the program's feasibility in maintaining oral hygiene (Larson et al., 1998). To improve the consistency of oral care, Yeager et al. (2000) implemented an oral care standard in two inpatient hematology/ oncology units and demonstrated feasibility, tolerability, and adherence in patients with leukemia and those

undergoing transplantation (Yeager et al., 2000). Improved oral status (i.e., reduced mucositis or increased oral comfort) was reported in three randomized clinical studies, and only one study reported no change in mucositis at the conclusion of the study (Kenny, 1990; Levy-Polack et al., 1998; Cheng et al., 2001). One randomized clinical trial did not show a statistically significant difference between controls and those undergoing the intervention (McGuire et al., 1998); however, participants who were taught the protocols performed oral hygiene routines more frequently compared with the control group and reported feeling more prepared to manage their symptoms.

1.4.6.2 Palliative care (including Pain management)

Pain associated with mucositis depends on tissue damage rate, sensitization of pain receptors, and elaboration of inflammatory and pain mediators. In addition, OM-induced pain has a negative effect on dietary intake, oral hygiene, and QoL (Sadrzadeh-Afshar et al., 2016). Therefore, the palliative care of mucositis and its associated pain is most important during chemoradiation therapy. Systemic analgesics and other modalities, a palliative mixture of agents (Magic or Miracle Mouthwashes), mucosal coating agents, and topical anesthetics/analgesics have been used to decrease mucositis-associated pain (Yarom et al., 2020).

1.4.6.2.1 Pain Management

Reducing pain associated with mucositis is an essential aspect of symptoms management in patients receiving radiation treatment for HNC. Most patients require both systemic as well as local analgesics. A recent study by Chen et al. (2004) reported that only a few HNC patients are given adequate narcotic analgesia. Dosage, frequency, and duration of analgesics should be adjusted periodically to meet the pain intensity levels of the patient (Chen et al., 2004). Mouthwashes containing an anaesthetic, like 2% viscous lidocaine, may provide short-term relief even though no experimental studies have assessed the efficacy of these agents (Saadeh, 2005). The latest published MASCC-ISOO guidelines (Elad et al., 2020), has strongly recommended patient-controlled analgesia with intravascular morphine sulfate for mucositis-associated pain management in HSCT settings (even for the paediatric population), but its use in CT and radiation settings is inconclusive. Mouth washing of 0.2% morphine and 0.5% doxepin approved to be effective for OM-induced pain relief (Saunders et al., 2020). Few studies have reported using transdermal fentanyl in patients with swallowing difficulty

(Saunders et al., 2020). Other topical or systemic factors such as sucralfate are also available as an oral antiulcer agent that promotes gastric and duodenal ulcer healing by forming a protective barrier on the ulcer site. Sucralfate is the most widely studied coating agent, and it has also been used as an oral rinse for the prevention and treatment of mucositis (Lalla et al., 2008; Saadeh, 2005). However, the 2020 MASCC/ISOO guidelines have recommended avoiding the use of sucralfate to prevent or treat OM in patients undergoing CT and RT because of the lack of effectiveness (Elad et al., 2020).

Clinical practice guidelines given by WHO (World Health Organization, 1996), and the Agency for Healthcare Research and Quality for managing acute pain (Jacox et al., 1994) should be followed while dealing with mucositis-associated pain.

The FDA approved Gelclair in 2002 as a drug for managing and alleviating oral lesions-associated pain, including CT or RT -induced OM. Gelclair was found to be effective and safe in improving pain scores, nutritional and swallowing endpoints (Buchsel, 2003; Buchsel, 2008).

Most patients suffering from intense mucositis also need systemic analgesics involving opioids to manage pain satisfactorily (Lalla et al., 2008). The MASCC/ISOO guidelines suggest using morphine with patient-controlled analgesics in patients receiving HSCT and recommend using trans-dermal fentanyl in patients undergoing traditional or high-dose CT. The conventional opioids are often less effective than expected for the management of mucositis pain, and there is contradictory evidence for gabapentin (Elad, 2020).

1.4.6.2.2 Topical preparations and other approaches

Various types of topical agents have been used alone or in combination for the management of mucositis and its associated pain. The most common ingredients used in combination include viscous lidocaine, milk of magnesia, benzocaine, pectin, kaolin, chlorhexidine, and diphenhydramine, while benzydamine and morphine have been used as single topical agents (Saunders et al., 2020). Nevertheless, their effectiveness is inconclusive, even though they may be slightly better than normal saline. Regarding the use of topical anaesthetics (like lidocaine), their absorption through damaged mucosal surfaces is of great concern because of their cardiac effects (Elad et al., 2020).

1.4.6.3 Chemoradiotherapy-induced mucositis management

1.4.6.3.1 Nutritional Support

In patients with severe mucositis, it is so essential for weight and dietary intake to be monitored by a dietician or other professionals. Insufficient intake of food and fluids because of the pain related to severe OM may end in malnutrition and dehydration. Furthermore, changes in taste may also happen after CT and/or RT. A gentle diet and fluid supplements are easier to tolerate than normal feeding in the presence of OM. For prophylactic purposes, some centres often use gastrostomy tubes for patients at high risk of developing severe mucositis. However, this varies widely from medical place to another. Total parenteral nutrition using a fixed catheter such as the Hickman line is usually given to patients undergoing hematopoietic cell transplantation. (Yarom et al., 2019; Elad et al., 2020).

1.4.6.3.2 Oral decontamination

Oral decontamination can help to decrease mucositis. It was also found that patients who develop OM after undergoing HSCT are three times more likely to be infected with bacteraemia, leading to more hospital stay days than those without mucositis (Lalla et al., 2008). In addition, oral disinfection can also decrease oral cavity infection by opportunistic pathogens, and this function can minimize the systemic sepsis risk from resident oral microbes (Yoneda et al., 2007).

The MASCC/ISOO guidelines suggest using a patient-control oral care protocol including the use of soft and new toothbrush for brushing with each CT cycle, flossing every day, and utilizing rinses (saline or sodium bicarbonate) (Elad et al., 2020). Patients have to be educated about the important role of oral hygiene. Chlorhexidine mouthwashes containing alcohol may not be favourable to be tolerated by patients during clinical oral ulceration, so, alcohol-free preparations are utilized at some centres. Furthermore, many studies have not shown the major efficacy of chlorhexidine mouth rinse in decreasing the severity of mucositis (Elad et al., 2020; Roopashri et al., 2011; Diaz-Sanchez et al., 2015). For this, the MASCC/ISOO guidelines have suggested not applying chlorhexidine mouth rinse to prevent or treat OM (Elad et al., 2020).

Nystatin-containing mouthwash has been demonstrated not to be successful in decreasing chemotherapy-induced mucositis severity (Köstler et al., 2001; Lalla et al., 2008; Elad et al., 2020). Nevertheless, a novel study showed that systemic fluconazole versus no treatment

significantly decreased candidal carriage and incidence of severe mucositis resulting from RT (15% versus 45%) in patients suffering from HNC (Nicolatou-Galitis et al., 2006).

The MASCC/ISOO guidelines are against antimicrobial lozenges or acyclovir routine use to prevent OM. However, medications like acyclovir and valacyclovir have an important role in the prevention and management of damage resulting from HSV (Köstler et al., 2001; Elad et al., 2020).

1.4.6.3.3 Cryotherapy

Cryotherapy may decrease the intensity of OM in patients receiving short bolus infusions of CT (Correa et al., 2020). Setting ice chips in the mouth during the CT bolus administration reduces the drug delivery to the oral mucosa by causing local vasoconstriction (Correa et al., 2020). The MASCC/ISOO guidelines advise the cryotherapy utilization to decrease OM in patients undergoing bolus infusions of 5-fluorouracil and propose its use in patients undergoing high-dose melphalan for HSCT (Elad et al., 2020). Ice chunks are put in the mouth, starting 5 minutes before CT, adding when needed, down to 30 minutes. However, cryotherapy has only been shown to be effective for a short-dose infusion of CT, cannot be tolerated by some patients, and has no impact on OM resulting from RT (Elad et al., 2020).

1.4.6.3.4 Photobiomodulation (laser and other light therapy)

Photobiomodulation therapy (PBM), such as laser and other light therapies, has been proven effective in preventing OM in specific patient populations. In addition, LLLT may decrease the severity, need for opioid analgesia, dysphagia, and inflammation of OM induced by chemo/radiotherapy and can support wound healing in patients undergoing high-dose CT or chemoradiotherapy before undergoing HSCT (Zadik et al., 2019).

LLLT been assumed to decrease ROS and pro-inflammatory cytokines concentrations that play a part in OM pathogenicity (Lalla et al., 2019). The MASCC/ISOO guidelines advise using LLLT to prevent OM in patients undergoing high-dose of CT for HSCT and propose its use in patients undergoing radiation therapy without concomitant CT (Elad et al., 2020). However, the long-term safety of LLLT and its potential effect on tumour response is open to question yet (Villa and Sonis, 2016b).

1.4.6.3.5 Growth factor

Intravenous recombinant human keratinocyte growth factor-1 (palifermin) significantly decreases the incidence, intensity, and period of OM in patients with hematologic malignancies receiving high-dose of CT and TBI for autologous HSCT (Logan et al., 2020). In addition, it can promote the differentiation and reproduction of monocyte and neutrophils of the oral mucosa and increase the phagocytosis process, chemotaxis, and metabolism of intracellular oxygen of neutrophils (Spielberger et al., 2004; Barasch et al., 2009). Therefore, the MASCC/ISOO guidelines suggest utilizing these growth factors in this specific population (Elad et al., 2020).

Palifermin is the first and only pharmacological drug approved via the US FDA for patients suffering from haematological cancers undergoing HSCT (Barasch et al., 2009; Villa and Sonis, 2020). The epithelial growth factors' safety has not been proven for patients with other cancers other than hematologic malignancies (Lalla et al., 2019).

1.4.6.3.6 Management of bleeding

Bleeding may appear from the OM ulcerations in thrombocytopenic patients, resulting from high-dose CT. Topical intraoral bleeding may usually be controlled by utilizing topical haemostatic drugs such as gelatine sponge or fibrin glue (Lalla et al., 2008).

When platelet counts fall below 20,000/ml, patients should receive platelet transfusion because of the high risk of developing internal bleeding, which may have adverse outcomes and affect the central nervous system (Raber-Durlacher et al., 2004; Lalla et al., 2008).

Haemorrhage does not seem directly related to OM, but ulcerative lesions increase the bleeding when submucosal blood vessels are exposed to shock in patients suffering from thrombocytopenia. Nevertheless, bleeding in the mouth floor region may be serious in very ill patients because of the risk of blood aspiration. Particularly in children, oral haemorrhage may be a frightening activity. On top of that, coagulated blood precipitate can develop infection (Raber-Durlacher et al., 2004).

1.4.6.3.7 Anti-inflammatory agents

Benzydamine hydrochloride, a topical nonsteroidal anti-inflammatory agent with antimicrobial properties, inhibits the production of pro-inflammatory cytokines involving TNF- α

(Ariyawardana et al., 2019). One Phase III trial study reported that the use of benzydamine hydrochloride mouthwash in patients with HNC receiving RT was able to decrease mucositis severity (Ariyawardana et al., 2019). As a result, the MASCC/ISOO guidelines proposed using this drug to prevent and treat RT -induced mucositis. Nevertheless, this drug has not yet been approved by the FDA (Elad et al., 2020)

Safaris, a commercial L-glutamine suspension, increases glutamine take-up into the epithelial cell. Mucosal damage can be reduced by glutamine through declining pro-inflammatory cytokines expression and apoptosis, and glutamine may stimulate healing by promoting the synthesis of collagen and fibroblast (Yarom et al., 2019). Phase III study demonstrated that the topical use of glutamine significantly reduced chemotherapy-induced OM incidence (Yarom et al., 2019). However, systemic administration of glutamine is not recommended by the MASCC / ISOO guidelines for gastrointestinal mucositis management due to efficacy lack (Elad et al., 2020).

1.4.6.3.8 Antioxidants

Vitamin A apply significant inhibitory impacts on epithelial reproduction and inflammation, improves the resistance of the mucosa to cycle-specific cytotoxic therapy and process a temporary arrest of the oral epithelium cell cycle. In addition, Tretinoin preventive topical use has decreased oral complications related toBMT. (Yarom et al., 2019).

Vitamin E topical use of 100 mg showed a potential efficacy in reducing OM (Rodríguez-Caballero et al., 2012; Yarom et al., 2019).

Allopurinol mouthwash topical application has been demonstrated to directly affect oxidative stress by scavenging free radicals and inhibiting proteases and superoxide dismutase. Furthermore, oxypurinol, a metabolite of allopurinol, suppresses the pyrimidine synthesis enzyme, resulting in intracellular accumulation of orotic acid, which in turn leads to reduced 5-FU- induced toxicity and could provide prevention OM. (Al-Taie et al., 2020). Whereas initial studies of topically administered allopurinol reported a reduction of mucosal toxicity in patients receiving 5-FU-based CT, consecutive trials failed to confirm these findings. In contrast, one double-blind, randomized clinical trial found a higher incidence of OM in patients treated prophylactically with allopurinol. No guideline was possible due to insufficient evidence (Jensen et al., 2013; Köstler et al., 2001).

Pilocarpine is another significant factor of the salivary function modifiers. The topical application of pilocarpine proved its ability to preserve the function of the salivary gland and palliate radiation-induced OM symptoms (Al-Taie et al., 2020). A MASCC/ISOO guideline references that pilocarpine administered orally cannot be utilized to prevent OM for HNC patients undergoing radiation therapy or CT (Elad et al., 2020).

Amifostine is thought to act as a ROS scavenger known to increase mucositis severity (Lalla et al., 2008). However, due to limited evidence of the advantage, MASCC/ISOO guidelines about administering Amifostine in OM in patients undergoing chemo-radiotherapy cannot be established (Elad et al., 2020). Nevertheless, this drug was recommended to prevent chemotherapy- or radiation-induced esophagitis (Nicolatou-Galitis et al., 2013; Lalla et al., 2019).

1.4.6.4 Novel Approaches for Oral Mucositis Management

Although mouth rinses are commonly used in managing OM, they are indicated to have a limited effect on OM due to the short time the preparations are in contact with the oral mucosal tissues. Additionally, gargling is very difficult for patients with OM because they usually suffer periods of severe pain, which will decrease pharmaceutical formulation efficacy. Local application, on the other hand, is a simple way with a comfortable application that prolongs the contact time with the oral mucosa. Nevertheless, this pathway faces many difficulties because of the enzymatic and environmental conditions, salivary washout effect, mechanical forces caused by the functions of the oral cavity, and the oral mucosa's relative *impermeableness* (Al-Taie et al., 2020).

Between these new approaches, rebamipide has a set of pharmacological effects comprising PGE2 synthesis promotion, mucus secretion inducement, upregulation of EGF, antioxidant properties, and suppression impact on the production of proinflammatory cytokines (Yasuda et al., 2012; Elad et al., 2020). Many studies have demonstrated the efficacy and safety of rebamipide mouth rinse in the prophylaxis and management of OM associated with cancer therapy. However, it is not possible to locally maintain the concentrations of rebamipide on the oral mucosal surface, and the prophylaxis effect has a short residency time inside the mouth (Al-Taie et al., 2020). Poly (d,l-lactide-co-glycolide) nanoparticles have been demonstrated to reduce oral ulcer site and period in a significant way; thus, the rebamipide-nanoparticles, covered with chitosan hydroxypropyltrimonium chloride, has been suggested as a potentially useful intervention for OM treatment (Takeuchi et al., 2018; Al-Taie et al., 2020).

The topical application of nonsteroidal anti-inflammatory drugs (NSAIDs) is beneficial in oral ulcer management. Therefore, many new formulations have been designed to enhance the distribution of NSAIDs to the oral mucosa, such as proniosomal gels, mucoadhesive films, and electrospun nanofibers (NFs), buccal tablets, and liquid crystalline films (Haley and von Recum, 2019). A good example of models is the electrospun NFs and mucosal-adhesive ketoprofen (KET) that have been experimentally designed and tested in animal models for the management of OM-related inflammation and pain as an alternate delivery method to orally taken KET.

Another study tested ginsenoside Rb1 derived from ginseng and loaded in mucosal adhesive films composed of chitosan–sodium alginate (Watanabe et al., 2009). This study reported that ginsenoside mucoadhesive film topical use has anti-inflammatory and curing efficacy on severe chemotherapy-induced OM (Watanabe et al., 2009).

Topical film dosage forms are a useful choice for OM because they can regulate drug release and preserve oral mucosa (Aksungur et al., 2004). Film dosage models containing allopurinol have been designed with the expectation to be effective as a delivery method, especially for patients with dysphagia associated with OM due to chemoradiation therapy. This form is designed in a way that ensures instant degradation in saliva. In addition, The allopurinol homogeneity after degradation leads to its diffusion in the mouth (Murata et al., 2012).

Propolis is a natural material that is significant in managing OM; this resinous substance contains compounds like amino acids, phenolic aldehydes, flavonoids, steroids, and polyphenols, and. Propolis has broad spectrum immunomodulatory, anti-inflammatory, antioxidant, and antimicrobial efficacy, making it beneficial for managing many oral conditions (Yarom et al., 2020; Oryan et al., 2018). Green propolis 5% in a mucosal adhesive gel was tested clinically to assess its efficacy in radiation-induced OM (Noronha et al., 2014). The outcomes showed that most of the patients who received propolis-based medication did not have mucositis. Furthermore, debilitating symptoms such as stopping food intake, oral infection, or oral pain were not reported by any patient of the propolis medication group (Noronha et al., 2014). These outcomes proposed the propolis-based gel as a potential and promising topical choice for the prevention of radiation-induced OM.

Moreover, a clinical study was conducted using topical mucosal adhesive films composed of high concentrations of licorice and triamcinolone acetonide to manage OM during HNC radiotherapy (Ghalayani et al., 2017), and the results were promising for pain relief and OM

treatment related to radiation therapy. Another clinical trial showed a sticky mucosal adhesive hydrogel, which acts as a barrier over damaged mucosa, as a promising agent for delaying progression and mitigating symptoms of OM (Allison et al., 2014).

It has been demonstrated that melatonin may have a role in radioprotection related to many functions, including lipid peroxidation, DNA damage reduction, and antioxidant properties (Mihandoost et al., 2014). In addition, melatonin gel plays an important role in preventing mucosal injury related to HNC (Abdel Moneim et al., 2017).

Despite a major clinical and economic impact of OM, the accessible therapeutic agents are to a great extent palliative (Elad et al., 2020). Many supportive and palliative treatments are available for managing OM induced by cancer therapy, but prophylaxis of this exhausting complication has to be emphasized. The treatment approach to manage OM has a palliative and supportive character. It aims to mitigate symptoms and prevent secondary complications (Elad et al., 2020). Hence, effective treatments for OM would improve dramatically patient's QoL and also, would decrease the use of health resources.

1.4.7 OM prevention is an unmet health need

None of the drugs used for treating OM are specific to the mechanism of action of the disease (Colella et al., 2010), and surprisingly, no single preventive measure has been approved to date. A certain level of efficacy has been shown in preventing the development or severity of OM with local application of PTA (polymyxin E, tobramycin, and amphotericin B), granulocyte macrophage-colony-stimulating factor/granulocyte colony-stimulating factor (GM-CSF/G-CSF), oral cooling, LLLT and the systemic administration of amifostine together with GM-CSF/G-CSF (Stokman et al., 2006), however, the most recent MASCC/ISOO guidelines did not confirm any of these treatments (Elad et al., 2020). Therefore, up to our knowledge, there is no single intervention that can completely prevent or reverses OM. Hence, OM prevention or treatment must be considered ***an unmet health need***. The inadequacy of current routine prevention modalities strongly calls for novel, more specific, and mechanism-based approaches.

1.4.8 Hyaluronic acid for management of oral mucositis.

HA is a naturally occurring, linear, non-sulfated glycosaminoglycan (GAG) that consists of repeating disaccharide unit of D-glucuronic acid and N-acetyl-D-glucosamine, connected by

β -1,3- and β -1,4-glycosidic bonds (Lapcák et al., 1998; Milas and Rinaudo, 2004). This relatively simple structure is preserved throughout all mammals, implying that HA is a biomolecule of considerable importance (Chen and Abatangelo, 1999). In physiological conditions, HA is in the salt form and negatively charged and referred to as sodium hyaluronate (Hargittai and Hargittai, 2008; Schanté et al., 2011). HA is secreted by many cell types and is a major component of the ECM (Fraser et al., 1997). It is synthesized by HA synthase (HASs) at the plasma membrane's inner wall and then extruded to the ECM space without any further modifications (Prehm, 1984; Karamanos et al., 1995; Prehm and Schumacher, 2004). Depending on enzymes that catalyse its synthesis, HA can be found in a wide range of molecular weights ranging from 20,000 up to several million Daltons. The number of repeated disaccharide units in HA molecules can extend up to 2×10^5 which generates a large HA chain of up to several million Daltons in weight and up to 25 μm in length. In mammals, three HAS isoforms (HAS-1, HAS-2, and HAS-3) synthesize HA molecules of different chain lengths and are subject to differential regulation (Itano et al., 2002; Itano et al., 2004). HA has been widely used in clinical practice to accelerate skin wound repair (David-Raoudi et al., 2008). HA preparations are widely used in treating several diseases and have proven recently to be potentially effective in treating OM (David-Raoudi et al., 2008; Favia et al., 2008; Cirillo et al., 2015; Ruggiero et al., 2016). However, the mechanisms underlying its effect have not been elucidated yet. The effect of HA on keratinocytes and mesenchymal cells is apparent and has been demonstrated recently; however, the underlying mechanisms have not been elucidated yet (Colella et al., 2010). Recently, several reports have shown the HA's potential efficacy in treating OM both *in vitro* and *in vivo* (Favia et al., 2008). In 2001, a gel based on sodium hyaluronate (SH) was approved by the US FDA as a Class 1 treatment device for use in the management of pain relief and which also proved to be useful for pain management in patients with OM (Colella et al., 2010). Preliminary data suggest that HA may be effective in treating the cytotoxic effect of chemo- and radiotherapy (Ruggiero et al., 2016). Additionally, Cirillo et al. (2015) have demonstrated that HA may have a preventive role to play in OM. However, the mechanisms underlying HA activity are still to be elucidated.

At a molecular level, OM induced by radiation and/or CT is suggested to be related to producing ROS at an early pre-clinical point and subsequent activation of oxidative stress routes (Criswell et al., 2003). The changes occurring in the mesenchyme would, in the end, lead to cell death of the keratinocytes in the basal epithelium, but it remains unclear how oxidative stress can stimulate alteration of the oral mucosa; these mechanisms need to be

elucidated. Furthermore, to develop effective therapeutic and/or preventive strategies, it is very important to understand and target the pathogenic procedures that cause mucositis. Recently, it has been shown that fibroblasts control significant points of keratinocyte function, including invasion and adhesion through paracrine mechanisms (Hassona et al., 2014).

A recent study demonstrated that a commercial combination based on HA lessened the paracrine effects of fibroblast senescence on keratinocyte behaviour. Further, the same study indicated that it could have important clinical use. The study used a combined molecular-clinical approach to prove that the *in vitro* results of preventing senescence in oral fibroblasts undergoing oxidative stress may be used clinically in patients with OM induced by chemoradiation. In addition, it provided evidence for the efficacy of the hyaluronic acid-based combination as a preventive procedure for this condition (Cirillo et al., 2015).

Given the high incidence and prevalence of OM in cancer patients undergoing chemoradiation and given the potential efficacy of the HA in the treatment of OM, in addition to the easy accessibility and relatively low cost of HA compound, a study on the development of HA-based medications for preventing mucositis is warranted ethically, scientifically, and financially.

The present study aimed to assess the potential protective effects of HA preparations in an *in vitro* oxidative stress-induced model of oral mucosal injury. Further, we tested a selected HA preparation in an *in vivo* mouse model of chemotherapy-induced oral and intestinal mucositis, by focusing on oxidative stress as a readout.

1.5 Hypothesis and Aims of the study

1.5.1 Overall study Hypothesis:

This study hypothesizes that hyaluronic acid can effectively prevent or mitigate chemotherapy-induced mucosal damage *in vitro* and prevent oral mucositis *in vivo*.

Specifically

- 1- That hydrogen peroxide (H₂O₂) treatment can exert a cytotoxic effect on oral epithelial cells *in vitro*
- 2- That hyaluronic acid directly affects normal oral epithelial cell proliferation *in vitro*

- 3- That hyaluronic acid can effectively prevent and/or mitigate oxidative stress-induced damage *in vitro* by excreting antioxidant effects.
- 4- That High molecular weight hyaluronic acid (H-MW-HA) can completely prevent or effectively attenuate the chemotherapy-induced mucositis *in vivo* in a mice model of 5-flurouracil (5-FU)-induced oral and intestinal mucositis.

1.5.2 Overall study Aim

This study aimed to assess the suitable hyaluronic acid that can effectively prevent oral mucositis.

Specifically:

- 1- To establish an *in vitro* model of oxidative stress-induced oral mucosal toxicity, using hydrogen peroxide and three monolayer cultures of oral mucosa keratinocytes to simulate radiation/or chemotherapy-induced oral mucositis.
- 2- To determine whether hyaluronic acid is involved in regulating oral epithelial proliferation during normal growth.
- 3- To assess the protective characteristics of hyaluronic acid in an oxidative stress-induced model of oral mucosal injury *in vitro* with the main focus on the antioxidant effects of HA.
- 4- To assess *in vivo* the ability of hyaluronic acid to prevent or attenuate chemotherapy-induced oral and intestinal mucositis in a mice model.

CHAPTER 2 MATERIALS AND METHODS

2.1 Abstract

Oral and intestinal mucositis is a debilitating inflammatory disease seen in cancer patients undergoing chemo-radiotherapy and is initiated through various mechanisms, including excessive production of ROS. 5-Fluorouracil (5-FU), a widely used anticancer agent, is known to frequently induce mucositis, which often leads to CT disruptions and, for which, there remains a need for a safe and effective prevention strategy and/or treatment. HA preparations are widely used in treating several diseases and have recently been proven to be potentially effective in treating OM. However, the mechanisms underlying its effect have not been elucidated yet. This study aimed to assess the potential protective effects of HA preparations in an oxidative stress-induced model of oral mucosal injury *in vitro*. Further, we tested a selected HA preparation *in vivo* in a mouse model of chemotherapy-induced oral and intestinal mucositis, especially in relation to oxidative stress. We first established an *in vitro* model of oral mucosal toxicity using three oral keratinocyte cell lines (OSCC cell lines H357 and H400 and immortalized normal human oral keratinocytes, OKF6). The cells were incubated with increasing concentrations of H₂O₂ (100μM -1200μM) for up to 24 hours. A commercial formulation of HA enriched with amino acids (Mucosamin[®]), cross-linked (xl-), and non-crosslinked high molecular weight HA (H-MW-HA) were tested at concentrations of 0.01% and 0.05% w/v to assess the protective effect of HA compounds in counteracting the oxidative stress-induced damage in an *in vitro* model of oral mucosal toxicity using immortalized normal human oral keratinocytes, namely OKF6 cells. Cell growth was evaluated using the FDA cell viability fluorescent assay, while ROS production was measured using the redox-sensitive dye, CM-H₂DCFDA. For the animal study, we characterized a mouse model of alimentary tract mucositis using 5-FU. 6-week-old C57BL/6 female mice received i.v. 5-FU injections (50 mg/kg) or saline every 48 hours for 2 weeks. H-MW-HA (0.01 % w/v) was administered orally in drinking water one day prior to the initial 5-FU injection and then daily thereafter until the end of the experiment. Post initial injection, mice were monitored every 48 hours until day 10, and then every 24 hours until the end of the experiment, for signs of morbidity, and clinically for weight loss and diarrhea, as surrogates for intestinal injury. In addition, the incidence, development, and extent of OM were assessed clinically by systematic oral examination using a visual oral ulcerative mucositis score, every 48 hours until day 10, and then every 24 hours until the end of the experiment. Microscopical and histomorphometric analyses of the tongue and intestinal tissue were also conducted at 3 interim time points during the experimental

period, coupled with the assessment of superoxide dismutase (SOD) enzyme activity in blood serum taken at the endpoint, as a surrogate of HA's antioxidant activity.

2.1 Introduction

Chapter 2 contains the details of general materials and methods used in this work. The experimental design included in vitro experiments using an oxidative stress-induced human oral keratinocyte injury model and an in vivo dual murine model of 5-FU-induced oral and intestinal mucositis (Figure 2.1). Formulations of HA tested in these models included Mucosamin®, cross-linked (xl-), and non-crosslinked H-MW-HA. In addition, cell lines, culture conditions, morphological, functional, and molecular assays (e.g., cell viability, cytotoxicity, and proliferation; intracellular ROS production, and superoxide dismutase enzyme activity) were described. For the animal study, the methodology for clinical, histopathological, and morphometric assessment of oral and intestinal mucositis was reported in detail, including the procedure to evaluate the protective effect of systematically administered H-MW-HA against 5-FU-induced mucositis. Specific materials and methods were additionally described in the relevant chapters. In this chapter, we discussed in details general materials and methods used in this study.

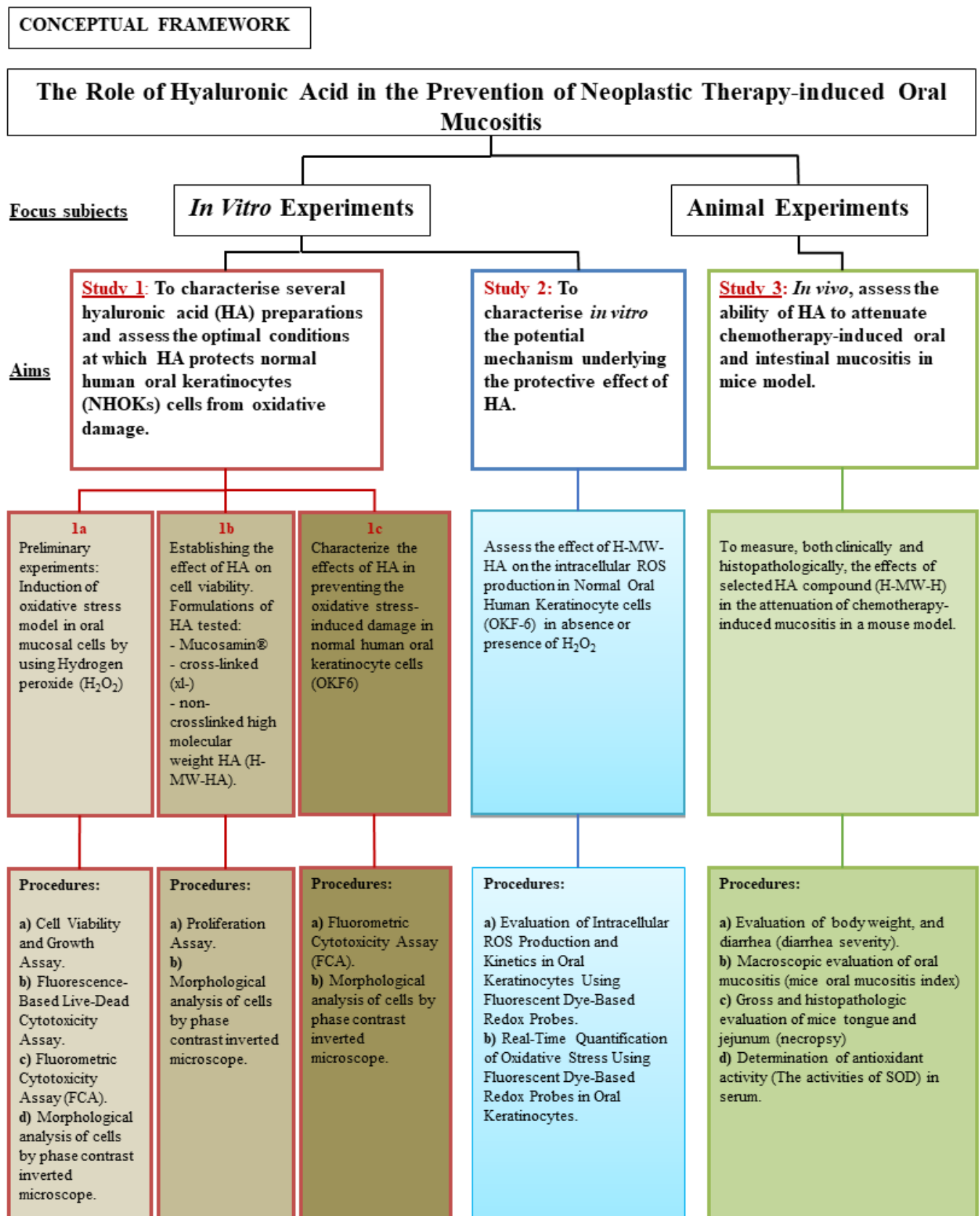


Figure 2.1 Schematic diagrams summarising the experiments undertaken in the project.

2.2 In Vitro Experiments

2.2.1 Reagents

Dulbecco's Modified Eagle's Medium (DMEM) (Cat#: D5796), F-12 HAMS nutrient mixture (Cat#: N6658), Newborn Calf Serum (NCS) (Cat#: #N4637), Hydrocortisone (Cat#: H0135), Penicillin-Streptomycin (Cat#: P4333), 0.25% Trypsin-EDTA solution (Cat#: T4049), Dulbecco's Phosphate Buffered Saline (Cat#: D8662), Trypsin-EDTA solution (Cat#: T4049), Hanks' Balanced Salt solution (HBSS) (Modified, with sodium bicarbonate, without phenol red, Cat# H8264), Propidium iodide (PI) (Cat#: P4170), Fluorescein diacetate (FDA) (Cat#: F7378), and Hyaluronic acid sodium salt, from *Streptococcus equi* (Cat#: 53747) were all purchased from Sigma-Aldrich, Castle Hill, NSW, Australia. Foetal Bovine Serum (FBS)–Sterile (Cat#: SFBS-F) was purchased from Bovogen Biologicals, VIC, Australia. Keratinocyte-SFM (serum free medium) (#17005-042), with complementary keratinocytes supplements (human recombinant epidermal growth factor (rEGF), and bovine pituitary extract (BPE), were all purchased from ThermoFisher Scientific, Life Technologies, Australia. 5-(and-6)-chloromethyl-2', 7'- dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (Cat# C6827) was purchased from Molecular Probes, Life Technologies, Australia. Trypan blue dye (Cat#: 1450021) was purchased from Bio-Rad, NSW, Australia. Hydrogen peroxide (H₂O₂) 30% w/w AR (100 volumes) (Cat#: HA154) was purchased from Chem-Supply, Gillman, SA, Australia. Mucosamin[®] spray, a commercial adjuvant gel, was purchased from Errekappa Euroterapici Spa, Milan, Italy. All other reagents including, ethanol, ddH₂O (double distilled water), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), and Calcium chloride (CaCl₂) were of analytical grade and were commercially available.

2.2.2 Equipment

100-mm tissue culture plate (Cat#: CLS430167, Corning[®] tissue-culture treated culture dishes), 6-well cell culture plates (Cat#: CLS3516, Corning[®] Costar, TC-Treated Multiple Well Plates), dark, clear bottom 96-well tissue culture microplates (Cat#: CLS3603, Corning[®] 96 Well Black Polystyrene Microplate), 50 mL centrifuge tubes (Cat#: CLS430829, Corning[®] 50 mL centrifuge tubes), 15 mL centrifuge tubes (Cat#: CLS430791, Corning[®] 15 mL centrifuge tubes), 25 mL serological pipettes (Cat#: CLS4489, Corning[®] Costar[®] Stripette[®] serological pipettes), 10 mL serological pipettes (Cat#: CLS4488, Corning[®] Costar[®] Stripette[®] serological pipettes), 5 mL serological pipettes (Cat#: CLS4487, Corning[®] Costar[®] Stripette[®] serological

pipettes), Corning® bottle-top vacuum filter system (CAT #; CLS430767) were all purchased from Sigma–Aldrich, Castle Hill, NSW, Australia. 4-well cell culture plates (Cat#176740 Nunc™ Cell-Culture Treated Multidishes) were purchased from ThermoFisher Scientific, Life Technologies Australia. Cell counting slides for TC10™/TC20™ Cell Counter (Cat#: 1450015) were purchased from Bio-Rad, NSW, Australia.

2.2.3 HA Preparations

Mucosamin® spray, a commercially available adjuvant gel composed of 1.33% (w/v) HA with a molecular mass of 600–800 kDa, not crosslinked, in combination with a pool of collagen precursor synthetic amino acids, namely L-proline (0.75% w/v), L-leucine (0.15% w/v), L-lysine (0.1% w/v), and glycine (1% w/v), was purchased from Errekappa Euroterapici Spa, Milan, Italy. A natural non-sulphated H-MW-HA powder (Hyaluronic acid sodium salt derived from *Streptococcus equi*), non-crosslinked, with a molecular weight of $\sim 1.5\text{--}1.8 \times 10^6$ Da, (Cat#53747) was purchased from Sigma–Aldrich, Castle Hill, NSW, Australia. Three cross-linked (xl-) HA-derivatives powder (xl-HA (5/5); EDC/HOBT 5/5 mmol/mL, xl-HA (30/30); EDC/HOBT 30/30 mmol/mL, and xl-HA (100/100); EDC/HOBT, 100/100 mmol/mL) were used in our study. The numbers refer to the concentration of carbodiimides (EDC) and 1-hydroxybenzotriazole (HOBt) for amidation of carboxylic acid group. The concentrations are used in terms of (mmol/mL). Crosslinked HA derivatives were kindly provided by Dr Annalisa La Gatta (Department of Experimental Medicine, Section of Biotechnology, Medical Histology and Molecular Biology, School of Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy).

2.2.4 Cell Lines

Three monolayer cultures of oral mucosal keratinocyte were used in this study as *in-vitro* models of OM. These were the oral squamous cell carcinoma (OSCC) cell lines, H400 and H357, and the immortalized normal human oral keratinocytes OKF6. The H400 cell line was established by Prime et al. (1990) from an OSCC originating from the alveolar process of a female patient aged 55 year old. The H357 cell line was established by Prime et al. (1990) from an OSCC originating from the tongue of a male patient aged a 74 year old. OKF6 Immortalized normal human oral keratinocytes were established by Dickson et al. (2000) from normal oral keratinocytes originating from the floor of the mouth of a 57 year old male patient. H400 cell line (Cat#06092006), and H357 cell line (Cat#06092004) were purchased from Sigma-Aldrich,

Castle Hill, NSW, Australia. OKF6 cells were provided by Oral Health Cooperative Research Centre (OHCRC), The University of Melbourne, Australia. OSCC cell lines were derived in the UK and OKF6 cell line were derived in the USA, before 2001 and thus those cells were not subject to approval of the ethical committee (Hassona et al., 2013).

2.2.5 Culture Conditions

Details of the keratinocyte cell lines have been documented previously (Hassona et al., 2013; Dalley et al., 2014; Celentano et al., 2019). Experiments of cell culture were undertaken in a laminar flow hood using an aseptic technique. Cells were grown as adherent cultures in 100-mm tissue culture-treated culture plates. H400 and H357 cell lines were grown in complete growth medium consisting of DMEM and nutrient mixture F-12 Ham in a 1:1 ratio, supplemented with 10% (v/v) FBS, 0.5 µg/ml hydrocortisone, 100 IU/ml penicillin, and 1% penicillin-streptomycin. OKF-6 cells were grown in keratinocytes serum-free medium (K-SFM) supplemented with 25 µg/mL pituitary bovine extract (PBE), 0.2 ng/mL EGF, 1% (v/v) NCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin, and containing 0.4 mM CaCl₂. OKF6 cells were grown under the same standard conditions (humidified atmosphere, 5% CO₂ at 37°C). All cell lines were incubated in a humidified atmosphere with 5% CO₂ at 37°C for five to seven days to reach 70-80% confluency. The growth medium was replaced every 48-72 hours. Cells were assessed using the FLoid™ Cell imaging Station digital inverted microscope (ThermoFisher Scientific, USA), with the filter set on the bright field and 20x magnification power. The cells were subcultured/passaged when reached 70-80% confluency.

For cells passaging, the old growth medium was firstly removed by using 10 mL serological pipette. Then, the confluent cultures were adequately washed once with 3-4 mL phosphate-buffered saline (PBS) warmed to 37°C to remove all traces of serum in the aim to avoid trypsin enzyme inhibition. After removing the PBS, the washed monolayer was pre-treated with (3 mL) 10 mM EDTA for 10 minutes in the CO₂ incubator. To detach the cells from the plate, the EDTA was discarded and the cells were then treated with 2-3 mL of the pre-mixed 0.25% trypsin-1mM EDTA solution for up to 10 mins in the CO₂ incubator. The dishes were observed under the inverted microscope using the bright field with 20x magnification to confirm the detachment of the cell monolayer. Serum containing fresh culture medium (5-6 mL) was then added to the cells to inhibit the trypsin. Cell suspensions were subsequently collected by using 10 mL serological pipette, placed in a 15 mL falcon tube and centrifuged at 4°C at 800 x g using the ThermoFisher Scientific ST16R refrigerated centrifuge (ThermoFisher Scientific,

USA). The supernatant was discarded using 5 mL serological pipette without disturbing the pellet and the cells pellet was gently resuspended in at least 5mL of fresh complete growth medium using 10 mL serological pipette. The cells were then seeded to new culture dishes in desired aliquots of the new cell suspension for ideal sub-cultivation requirements with a seeding density of approximately 2.2×10^6 cells per 100 mm culture dish and placed in the incubator.

When not being actively cultured, cell lines were cryopreserved to maintain adequate stock for future experiments. Cells were trypsinized as described above, resuspended in a freezing medium comprised of a complete growth medium supplemented with 10% DMSO at a density of approximately 2×10^6 cells/mL. The suspension was aliquoted to be stored in a 1.2 mL Corning® cryogenic vial at -70°C (1 mL of cell suspension in a 1.2-mL cryovial). For recovery, the cryovials were taken out of -70°C storage and the cell suspension was thawed immediately in a 37°C water bath. Once completely thawed, the cells were seeded onto the culture dish and 6mL of complete growth medium added and placed in the incubator for approximately 6 hours to allow for cells attachment. After the attachment of the cells to the plate was established, the medium was changed in order to remove the DMSO containing supernatant. For this purpose, before adding fresh medium the plates were washed with PBS as described above.

2.2.6 Cell culture assessment and observation by Phase Contrast Inverted Microscope

Cell confluency was assessed using the FLoid™ Cell imaging Station digital inverted microscope (ThermoFisher Scientific, USA) with the filter set on the bright field and 20x magnification power. The cells' confluency was assessed in 20 different, random representative microscopic fields, and the mean confluence (%) of the cells was calculated accordingly.

2.2.7 Preparation of HA Solutions

The tested HA compounds solution was prepared in complete culture medium composed of K-SFM supplemented with 25 µg/mL PBE, 0.2 ng/mL EGF, 0.4 mM CaCl_2 , 1% (v/v) NCS, containing 100 IU/ml penicillin, and 100 µg/ml streptomycin (Yiannis et al., 2020).

2.2.7.1 Mucosamin[®]

A stock solution of Mucosamin[®] at 10% v/v concentration (with HA final concentration equal to ~0.1% v/v) was prepared. We diluted 1 mL of Mucosamin[®] in 9 mL of complete culture medium (10:100 ratio) using 15 mL falcon tube. The sample was then vortexed for 1 minute and then stored in the dark at 4°C. The Mucosamin[®] solution was prepared fresh on the day of the experiment. The 15 mL falcon tubes used to prepare the Mucosamin[®] stock solution were UV protected using aluminium foil. This solution was used to prepare the working solution by directly adding it to the culture medium to reach the desired final concentrations.

2.2.7.2 Native HA (H-MW-HA)

Natural non-sulphated H-MW-HA powder was dissolved in complete culture medium in 15 mL falcon tube at a ratio of 5mg/1mL to prepare 2mL of 0.5% weight/volume (w/v) H-MW-HA stock solution as per manufacturer instructions. The 15 mL falcon tube used to prepare the H-MW-HA stock solution was covered with aluminium foil to keep the solution UV protected. The stock solution was shaken at room temperature on the orbital shaker at 200 rpm for 2 hours until all particles were dissolved and then immediately saved UV protected at 4°C. The H-MW-HA stock solution was always prepared fresh on the day of the experiment. Working solutions were prepared by directly adding the stock solution to the complete culture medium to reach the wanted final concentrations.

2.2.7.3 Cross-linked (xl-)HA EDC/HOBT 5/5 mmol/mL (xl-HA 5/5)

A stock solution of xl-HA 5/5 at a concentration of 0.1% (w/v) was prepared as per the supplier's instructions by dissolving 10mg of xl-HA 5/5 powder in 10 mL of complete culture medium in a 15 mL falcon tube and vortexed for 1 minute. The 15 mL falcon tube used to prepare the xl-HA 5/5 stock solution was covered with aluminium foil to keep the solution protected from light. The falcon tubes were put in a dry incubator (Heraeus B 15 Compact Incubator, with opaque cover; ThermoFisher Scientific, USA, #: 50044698), and the temperature was set at 50 °C, and the incubator was put on an orbital shaker. The solution was then shaken on the orbital shaker at 100 rpm for 96 hours until all particles were visually dissolved. Subsequently, the stock was filtered using a 0.22 Millipore filter and preserved in a 15 mL falcon tube covered with aluminium foil and saved at 4°C protected from light until the day of the experiment. The stock solution was used to make the working solution by adding it directly to the culture medium to reach the wanted final concentrations.

2.2.7.4 xl-HA EDC/HOBT 30/30 mmol/mL (xl-HA 30/30) and xl-HA EDC/HOBT 100/100 mmol/mL (xl-HA 100/100)

Stock solutions of xl-HA 30/30 and xl-HA 100/100 at a concentration of 0.1% (w/v) were prepared separately as per the supplier's instructions by dissolving 10 mg of xl-HA 30/30 and xl-HA 100/100 powder in 10 mL complete culture medium in a 15 mL falcon tube and vortexed for 1 minute. The 15 mL falcon tubes used to prepare both xl-HA stock solutions were uv protected. The falcon tubes were put in a dry incubator (Heraeus B 15 Compact Incubator, with opaque cover; ThermoFisher Scientific, USA, #: 50044698), and the temperature was set at 50 °C, and the incubator was put on an orbital shaker. The solutions were then shaken on the orbital shaker at 100 rpm for ten days until approximately all particles were visually dissolved. Subsequently, the stocks were filtered using a 0.22 Millipore filter (Corning® bottle-top vacuum filter system, Sigma-Aldrich) and kept in 15 mL falcon tubes covered with aluminium foil and saved at 4°C protected from light until the day of the experiment. The stock solution was used to make the working solution by adding it directly to the culture medium to reach the wanted final concentrations.

2.2.8 Cell Number Titration Assay

2.2.8.1 Cell preparation and incubation

Human oral keratinocyte cells (OKF6, H400, and OKF6) were cultured in complete growth medium in 100-mm Petri plastic dishes as described in sections (2.3.4 and 2.3.5) and grown to 70-80% confluence, so that $3\text{--}4 \times 10^6$ cells were obtained on the day of the experiment. Cells were grown in a humid incubator with 5% CO₂ at 37 °C. When oral epithelial cells grew to 80% confluence, they were subsequently detached, as described in the section (2.3.5). The viability of the oral keratinocytes used in the experiment was confirmed to be > 90% when determined with trypan blue exclusion (trypan blue dye, 0.4% solution, Bio-Rad).

For the cell titration assay, four cell suspensions (5–10 ml/selected density) for each cell line were prepared in culture medium at four different densities (2×10^5 cells/mL, 1.5×10^5 cells/mL, 1×10^5 cells/mL, and 5×10^4 cells/mL). Each cell line was seeded into four dark, clear bottom, black side 96-well tissue culture microplates (Sigma-Aldrich, Australia) at four different densities (2×10^4 cells/well, 1.5×10^4 cells/well, 1×10^4 cells/well, and 5×10^3 cells/well) with a total volume of 100 µL of cell suspension per well using a multichannel pipette. Normally, five technical replicates were run per each seeding density. Furthermore, five empty wells were filled with 100 µL culture medium and were used as blanks. The plates were incubated overnight to allow for cells to attach. Incubation was performed in a humidified atmosphere (5% CO₂, 37°C). After cell attachment, the plates were washed with 100 µL of 37°C PBS, and the growth medium was replenished. The cells were subsequently placed in incubator for 0, 24, 48, and 72 hours under standard conditions with the replenishment of the growth medium every 24 hours. Figure 2.1 shows an example of a standard 96-well plate setup we used in our experimentation. 2.1. Cell confluence was assessed at the end of each time point using the FLoid™ Cell imaging Station digital inverted microscope (settings: white light filter, 20x magnification)

2.2.8.2 Preparation of the FDA solution

Stock solution of FDA was made by dissolving 5 mg of FDA (Sigma-Aldrich) in 1 ml of acetone in a 15 ml falcon tube (FDA stock solution can be stored in dark place for up to 3 months at ~20 °C). FDA solution (12 µM) was prepared in a 15 ml falcon tube by adding 1 µl FDA-stock per 1 ml fresh growth medium without serum and supplements, followed with vortexing for 5 seconds. The FDA solution was prepared immediately before addition to the

plates to avoid precipitation. The 15 ml falcon tubes used to prepare the FDA stock and solution were covered with aluminium foil because the FDA dye is sensitive to light.

2.2.8.3 Cell viability measurement

FDA fluorescent assay was used to measure cell viability. The assay was carried out according to the procedure reported Lindhagen et al. (2008) with some modifications. Briefly, at each time point, the growth medium was removed, and the plates were rinsed once with 100 µl PBS/well using a multichannel pipette. The FBS was removed from the well, and 100 µl of FDA solution/well were added using a multichannel pipette. Subsequently, the plates were incubated in standard conditions for 40 minutes, and the FDA fluorescence was measured at the respective excitation and emission wavelength of 485/20 nm and 528/20 nm using the Synergy HTX Multi-Mode Reader (Bio-Tek, USA). Data are expressed as mean cell proliferation attributed to the FDA fluorescent intensity, with blank value subtracted.

	1	2	3	4	5	6	7	8	9	10	11	12
A		M	M	M	M	M	M	M				
B		M	20K	20K	20K	20K	20K	M				
C		M	15K	15K	15K	15K	15K	M				
D		M	10K	10K	10K	10K	10K	M				
E		M	5K	5K	5K	5K	5K	M				
F		M	B	B	B	B	B	M				
G		M	M	M	M	M	M	M				
H												

Figure 2.2 Standard plate layout of a 96-well plate for cell titration assay to be analysed in the Synergy HTX microplate reader (Bio-Tek, USA). Each row represents the seeding density in five replicates for each cell line. Each labelled well was filled with 100 μ l of cell suspension prepared with growth medium at seeding densities 2×10^4 cells/well (“20k”), 1.5×10^4 cells/well (“15k”), 1×10^4 cells/well (“10k”), and 5×10^3 cells/well (“5k”). “B”, blank (medium only); Blank values were deducted from the signal values of the wells containing cells; “M”, medium only (placed peripherally to avoid evaporation); Due to medium evaporation from outer wells during incubation, medium is added to wells in the outermost rows.

2.2.9 Cell Viability and Growth Assay

2.2.9.1 Induction of oxidative stress

Induction of concentration- and time- dependent oxidative stress in oral keratinocyte cells was based on the procedure reported by Datta et al. (2002). Briefly, after passaging, OSCC (H400) cells were seeded into two 6-well plates (Sigma-Aldrich, Australia). Wells were plated with 2 mL of cell suspension prepared in complete growth medium at a density of 1×10^5 cells/well and cultured in standard conditions, with the growth medium replenished every 48-72 hours. The viability of the seeded cells was confirmed to be >90% when determined with trypan blue exclusion (trypan blue dye, 0.4% solution, #1450021, Bio-Rad). At 60-80% confluence, cells were washed once with 1 mL of PBS, and then exposed to increasing concentrations of H₂O₂ (200, 400, and 800 μ M) and incubated in standard conditions for three different time intervals (2, 6, and 24 hours). H₂O₂ test solutions were prepared from a 1 M stock solution prepared in 1.5 mL of complete cell culture medium containing DMEM-F12 (1:1) supplemented with 10% (v/v) FBS, 0.5 mg/ml hydrocortisone and 1% penicillin/streptomycin. Each time point was performed in three technical replicates for each tested H₂O₂ concentration. Three wells containing untreated cells were used as a control group (Figure 2.2).

2.2.9.2 Morphological analysis of cells by phase contrast inverted microscope

After the incubation period ended, the media was removed, the cells were washed once with 1 mL of PBS, and the morphological changes were assessed and photographed using a FLoid™ Cell Imaging Station digital inverted microscope (ThermoFisher Scientific, USA) with the filter set on the bright field at 20x magnification..

2.2.9.3 Determination of cell viability

To determine the H₂O₂ mediated cell death, the cell numbers and cell viability in wells of each group were counted progressively at 2, 6 and 24 hours after culture, by using the TC10™ Automated cell counter (Bio-Rad) according to the supplier's protocol. At each time point, the supernatants were collected in 1.5 ml microcentrifuge tubes and the dead cells in the supernatant were counted by using cell counting slides as described below. A 10 μ L aliquot was pipetted at a 45° angle into each chamber of the cell counting slide (Bio-Rad) and inserted into the TC10™ Automated cell counter. Six readings were recorded per counting slide. Attached cells were subsequently rinsed once with 1 mL PBS and then trypsinized by pre-

treatment of 10 mM EDTA for 5 min, followed by incubation with a 0.25% trypsin in 1 mM EDTA solution (Sigma-Aldrich) for up to 5 min until approximately detachment of all the cells. Cells in the suspension were then collected in a 15 mL falcon tube and centrifuged at 4°C, at 800 x g. The cell pellet was gently resuspended in 1 mL of fresh complete growth medium and transferred in a 1.5 ml microcentrifuge tube. Thereafter, cell viability was determined using a trypan blue exclusion assay using the TC10™ Automated cell counter. By crossing the cell membrane selectively of the dead cells only, the trypan blue dye does not penetrate live cells that possess intact cell membranes. A 10 µL volume of well-mixed cell suspension was added to 10 µL of 0.4% Trypan Blue Dye (Bio-Rad) and mixed by pipetting the solution 10 times. A 10 µL aliquot from this solution was then loaded at a 45° angle into the cell counting slide chamber. The same procedure was repeated with the same cell suspension, and 10 µL aliquot from this solution was loaded at a 45° angle into the other chamber of cell counting slide. Subsequently, the counting slide was inserted into the automated cell counter. Six readings were recorded per counting slide.

Control cells' viability was considered 100 %, and mediated cell death was evaluated by comparing treated cells' viability with the viability of the control cells using the following formula:

$$\text{Cell viability\%} = \frac{\text{The total cell viability of treated cells}}{\text{Mean of total cell viability of control cells}} \times 100$$

The IC₅₀ value was defined as the concentrations of H₂O₂ that inhibited cell viability of treated cells to 50% compared to unexposed control and was estimated from the results.

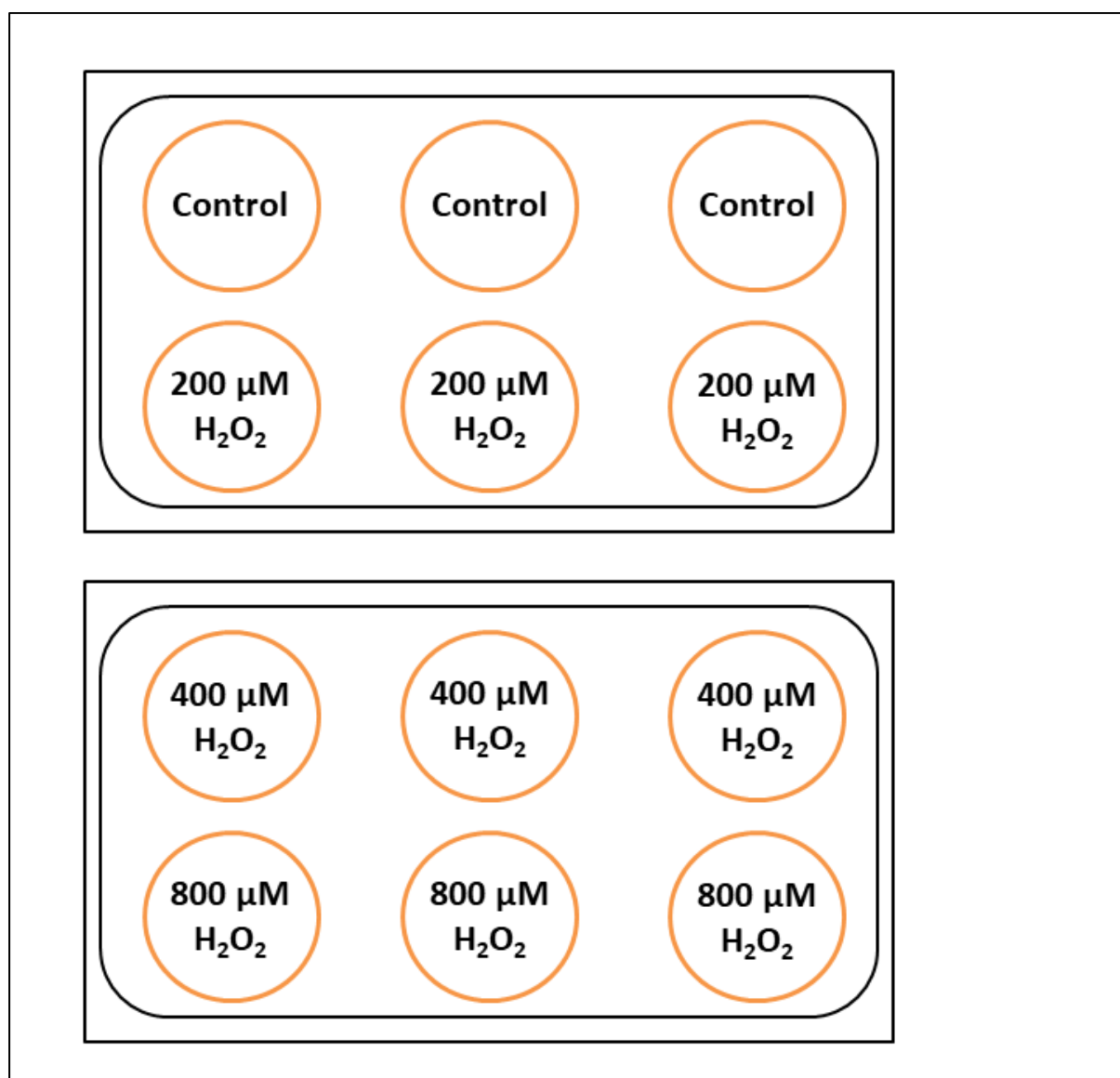


Figure 2.3 Plate layout of two 6-well plates for cell proliferation assay analysed by TC10™ Automated cell counter. H400 cells were seeded in two 6-well plates at a density of 1×10^5 cells/well and exposed to increasing concentrations of H₂O₂ (200, 400, and 800 μM) for three different time points (2, 6, and 24h). At each time point, the cell numbers and cell viability were counted by using trypan blue exclusion assay using the TC10™ Automated Cell Counter (Bio-Rad). Each time point was performed in triplicate per each tested H₂O₂ concentration.

2.2.10 Fluorescence-Based Live-Dead Cytotoxicity Assay

2.2.10.1 Cell preparation and treatments

The induction of dose-dependent oxidative stress in oral keratinocytes was based on the procedure reported by Datta et al. (2002), with some modifications. Briefly, after passaging, OSCC cells (H400) were seeded into 4-well plates (ThermoFisher Scientific, Australia). Wells were plated with 500 μ L of cell suspension prepared in complete growth medium at a density of 5×10^4 cells/well and cultured in standard conditions, with the growth medium replenished every 48-72 hours. The viability of the seeded cells was confirmed to be > 90% when determined with trypan blue exclusion (trypan blue dye, 0.4% solution, 1450021, Bio-Rad). At 60-80% confluence, cells were washed once with 500 μ L PBS, and then exposed to different concentrations of H_2O_2 (400, 800, and 1200 μ M) and incubated for 24 hours in standard conditions. H_2O_2 test solutions were prepared in 500 μ L of complete cell culture medium containing DMEM-F12 (1:1) supplemented with 10% (v/v) FBS, 0.5 mg/ml hydrocortisone and 1% penicillin/streptomycin. The experiment was performed in biological triplicate. One well (in triplicate) containing untreated cells was used as a control group (Figure 2.3).

2.2.10.2 Preparation of the FDA/PI staining solution

Stock solution of FDA was made by dissolving 5 mg of FDA (Sigma-Aldrich) in 1 ml acetone in 1.5 ml microcentrifuge tubes and stored in dark place at -20 °C. Propidium iodide (PI) stock solution was prepared by dissolving 2 mg of PI (Sigma-Aldrich) in 1 ml PBS in 1.5 ml microcentrifuge tubes and stored in a dark place at 4°C. FDA/PI solution was prepared by adding 20 μ l FDA-stock solution and 50 μ l PI-stock solution to 5 ml fresh culture media without FBS or supplements (only DMEM) in 15 ml falcon tubes. Thereafter, the solution was vortexed for 5 seconds. The FDA/PI solution was prepared immediately before addition to the plates to avoid precipitation. The tubes used to prepare the stocks and solutions were covered with aluminium foil because the FDA dye is a light-sensitive compound.

2.2.10.3 FDA/PI staining assay

The assay was carried out according to the procedure reported by Jones and Senft (1985) with some modifications. Briefly, when incubation time ended, we removed the growth medium, and the cells were rinsed once with 500 μ l PBS, followed by adding 500 μ l of FDA/PI solution to each well. The plate was then incubated at 37 °C with 5% CO_2 for 5 minutes. After that, the

staining solution was removed, and the samples were washed twice with 500µl PBS, followed by adding 500 µl of fresh medium without FBS and supplements (only DMEM) to the wells. Cells were then examined under an inverted fluorescence microscope (FLoid™ Cell Imaging Station, Life Technologies) with filters set for FITC and Texas Red for the FDA dye and PI dye detection, respectively. Fluorescence of vital cells was measured in six random fields per well per condition (treatment) with three biological replicates. Photomicrographs obtained were transferred, as TIFF files, to a computer where the vital stained cells were counted manually using Fiji (ImageJ) software (Schneider et al., 2012; Schindelin et al., 2012).

Control cells' (without treatment) viability was considered 100 %, and mediated cell death was evaluated by comparing treated cells' viability with the viability of the control cells using the following formula:

$$\text{Cell viability\%} = \frac{\text{The average of cell viability of treated cells in six different fields}}{\text{Mean of total cell viability of control cells in six different fields}} \times 100$$

Viability data are expressed as mean percentage viability relative to the untreated control (100%). IC₅₀ values were estimated from the results, and were defined as the concentrations of H₂O₂ inducing 50% loss of cell viability.

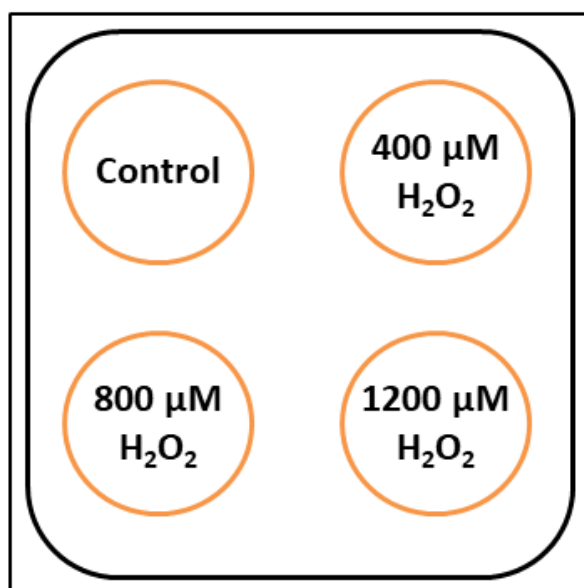


Figure 2.4 Plate layout of a 4-well plate for the cell proliferation assay to be analysed by Fluorescein diacetate (FDA)/ Propidium iodide (PI) staining assay. H400 cells were seeded in 4-well plates at a density of 5×10^4 cells/well and exposed to increasing concentrations of H_2O_2 (400, 800, and 1200 μM) for 24 hrs. Cell viability was evaluated by using fluorescein FDA/PI stains in a simultaneous staining procedure.

2.2.11 Fluorometric Cytotoxicity Assay (FCA)

2.2.11.1 Cell preparation and culture treatments

Induction of dose-dependent oxidative stress in oral keratinocyte cells was based on the procedure reported by Datta et al. (2002), with some modifications. Briefly, after passaging, OSCC cell lines (H400 and H357), and benign human oral keratinocyte cell line (OKF6) were seeded in dark, clear bottom 96-well tissue culture microplates (Sigma-Aldrich). 96-well black microplates with clear bottoms were used for the fluorescent assays to lower the background fluorescence and prevent well-to-well crosstalk. For the fluorometric cytotoxicity assay (FCA), wells were plated with 100 μ L of cell suspension prepared in a complete growth medium at a density of 1.5×10^4 cells/well. The viability of the seeded cells was confirmed to be > 90% when determined with trypan blue exclusion (trypan blue dye, 0.4% solution). After overnight incubation in standard conditions to allow for cells attachment, plates were washed once with 100 μ L/well PBS, and in the dose-response experiments, cells were treated with 100 μ L/well increasing concentrations of H₂O₂ (H₂O₂ concentrations used for the cytotoxicity assays ranged between 100 - 1200 μ M) prepared in complete growth medium. One row of the wells (five technical replicates) was used as a control group. Control wells contained untreated cells (no H₂O₂) cultured in 100 μ L of complete growth medium only. In one row, a 100 μ L/well growth medium was placed in empty wells (five technical replicates) and was used as blank. The plates were then incubated in standard conditions for 24 hours. 3-5 wells were used for each control, blank, and tested H₂O₂ concentrations per microplate (three to five technical replicates, figure 2.4). At the end of the incubation period, the media was removed, and the cells were washed once with PBS and the morphological changes were assessed as described earlier (section 2.3.9.2).

In further experiments, HA constituents potential protective effect on H₂O₂-induced damage in oral keratinocytes was determined *in vitro* using the normal human oral keratinocyte cell line OKF6. For FCA, wells were plated with 100 μ L of cell suspension prepared in a complete growth medium at a density of 1.5×10^4 cells/well in dark, clear-bottom 96-well microplates (Sigma-Aldrich). The viability of seeded cells was confirmed to be > 90% when determined with trypan blue. After overnight incubation in standard conditions to allow for cell attachment, plates were washed once with 100 μ L/well PBS, and the cells were pre-treated with 100 μ L/well of HA constituents prepared in a complete culture medium. The plates were moved to a humidified 37 °C/5% CO₂ incubator and incubated for 24 hours. After incubation, the plates

were washed once with 100 µl/well PBS, and the cells were then co-treated with 100 µl/well of HA constituents with or without 400 µM H₂O₂ prepared in fresh complete culture medium. The concentrations of HA constituents used for the FCA were 1% and 5%, v/v for the Mucosamin[®], 0.01% and 0.05%, w/v for H-MW-HA, and 0.01% v/v for the xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100. Subsequently, the plates were moved to a humidified (5% CO₂ at 37 °C) incubator and incubated for further 24 hours. One row of the wells (five technical replicates) was used as a control group. Control wells contained cells and were filled with 100 µl complete culture medium. One row of empty wells (five technical replicates) was filled with 100 µl of culture medium and used as blank. Normally, three to five wells were used for each blank, control, and tested HA concentrations per microplate (three to five technical replicates, figure 2.5). Figure 2.6 represents the schematic diagram of the experimental procedure timeline.

2.2.11.2 Preparation of the FDA solution

Stock solution of FDA was made by dissolving 5 mg of FDA (Sigma-Aldrich) in 1 ml of acetone in a 15 mL falcon tube (FDA stock solution can be stored in dark place for up to 3 months at ~20 °C). FDA solution (12 µM) was prepared in a 15 mL falcon tube by adding 1 µL FDA-stock per 1 mL fresh growth medium without serum and supplements, followed with vortex for 5 seconds. 10 -15 mL of the FDA solution was prepared each time. The FDA solution was prepared immediately before its addition to the plates to avoid precipitation. The 15 mL falcon tubes used to prepare the FDA stock and solution were covered with aluminium foil because the FDA dye is sensitive to light.

2.2.11.3 Fluorometric cytotoxicity assay (FCA) assay

At the end of each incubation time, cell viability was assessed using FDA. The FCA was carried out according to the procedure reported by Lindhagen et al. (2008) with some modifications. Briefly, the growth medium was removed at each time point, and the plates were rinsed once with 100 µl PBS/well using a multichannel pipette. The FBS was removed from the well, followed by adding 100 µl of FDA solution per well using a multichannel pipette. After that, the plates were incubated at 37 °C with 5% CO₂ for 40 minutes. After incubation, the plates were removed from the incubator, and the FDA fluorescence was counted at the respective excitation and emission wavelengths of 485/20 nm and 528/20 nm using Synergy HTX Multi-Mode Reader (Bio-Tek, USA).

2.2.11.4 Determination of cell viability

After incubation, the non-fluorescence probe, FDA, is hydrolysed by esterases in viable cells with intact plasma membranes and converted into the green fluorescent metabolite fluorescein (Rubinstein et al., 1990). The conversion is proportional to the amount of metabolically active cells.

Control cells (without treatment) viability was considered to be 100 %, and H₂O₂ mediated cell death was measured by comparing the viability of treated cells with control cells viability using the following formula:

$$\text{Cell viability \%} = \frac{(\text{Fluorescence of treated cells} - \text{Mean fluorescence of the blank wells})}{(\text{Mean fluorescence of control cells} - \text{Mean fluorescence of the blank wells})} \times 100$$

Viability data are showed as mean percentage viability relative to the untreated control (100%), with the blank values subtracted. IC₅₀ values were estimated from the results, and it is defined as the concentrations of H₂O₂ inducing 50% loss of cell viability.

	1	2	3	4	5	6	7	8	9	10	11	12
A	M						M					
B	M						M					
C	M						M					
D	M						M					
E	M						M					
F	M						M					
G	M	CTL	CTL	CTL	CTL	CTL	M					
H	M	BLK	BLK	BLK	BLK	BLK	M					

Figure 2.5 Standard plate layout of a 96-well plate for dose-response characterization of hydrogen peroxide (H₂O₂) to be analysed in a Synergy HTX microplate reader (Bio-Tek, USA) using fluorometric cytotoxicity assay (FCA). Each one of the gray rows represents the tested concentration in five replicates for hydrogen peroxide (H₂O₂). “BLK”, blank (medium only); Blank values were subtracted from the signal values of the wells containing cells. “CTL”, control (cell suspension only). “M”, medium (to avoid evaporation); Normally, due to evaporation from outer wells during incubation, medium was added to the wells in the outermost rows.

	1	2	3	4	5	6	7	8	9	10	11	12
A	M	M	M	M	M	M	M					
B	M						M					
C	M	HA+ H ₂ O ₂	HA+ H ₂ O ₂	HA+ H ₂ O ₂	HA+ H ₂ O ₂	HA+ H ₂ O ₂	M					
D	M	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	M					
E	M	HA	HA	HA	HA	HA	M					
F	M	CTL	CTL	CTL	CTL	CTL	M					
G	M	BLK	BLK	BLK	BLK	BLK	M					
H	M	M	M	M	M	M	M					

Figure 2.6 Standard plate layout of 96-well plate for the fluorometric cytotoxicity assay (FCA) to be analysed in a Synergy HTX microplate reader (Bio-Tek, USA). Each one of the grey rows represents the tested concentrations in five replicates for hydrogen peroxide (H₂O₂) and hyaluronic acid (HA) constituent. “BLK”, blank (medium only); Blank values were deducted from the signal values of the wells containing cells. “CTL”, control (cell suspension only). “M”, medium (to avoid evaporation); Normally, due to evaporation from outer wells during incubation, medium was added to wells in the outermost rows.

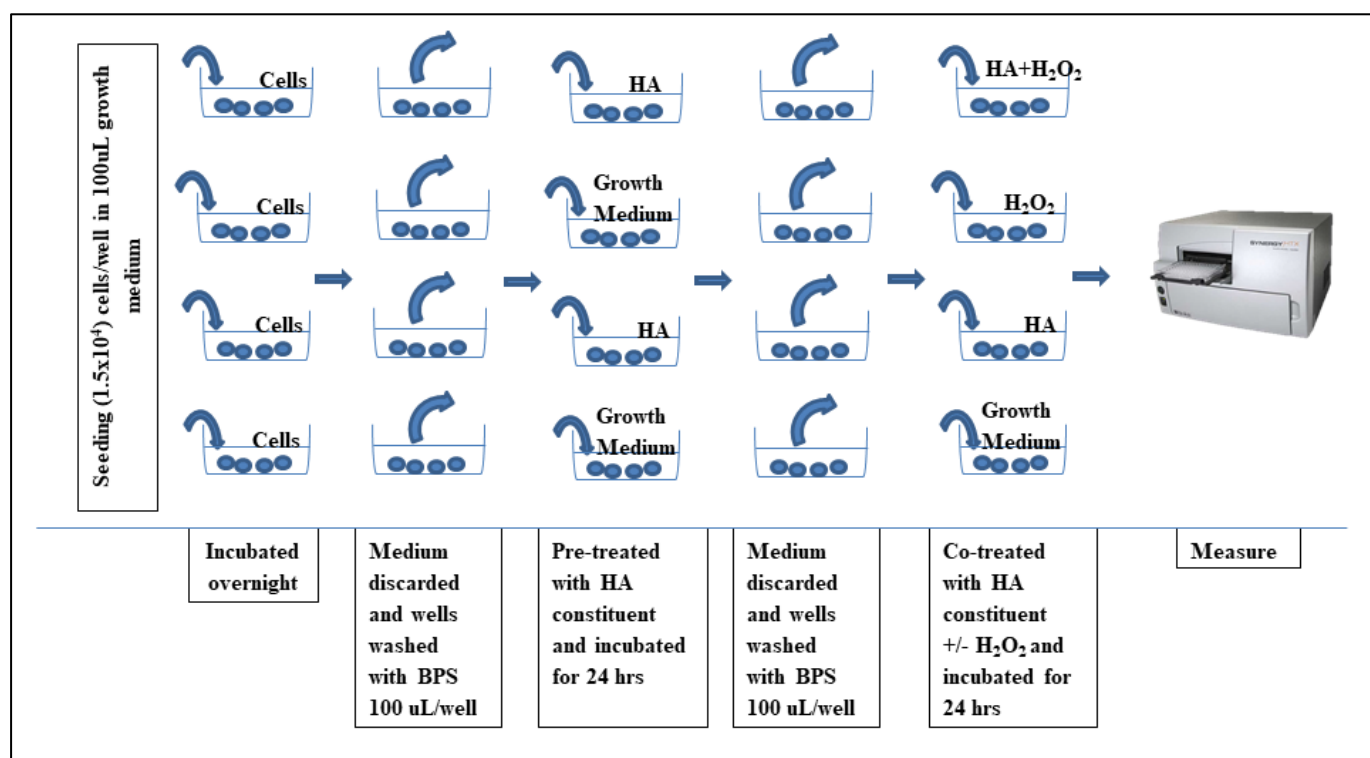


Figure 2.7 Schematic diagram of the fluorometric cytotoxicity assay (FCA) experimental procedure. OKF6 cells were seeded in 96-well plates overnight under standard conditions. The cells were then pre-treated with a HA constituent for 24 hrs, and then co-treated with 400 μ M H₂O₂ and the same HA constituent and incubated for 24 hrs. Cell viability was measured by fluorescein diacetate (FDA) fluorescence in a Synergy HTX Multi-Mode Reader (Bio-Tek, USA) at the respective excitation and emission wavelengths of 485/20 nm and 528/20 nm.

2.2.12 Proliferation Assay

2.2.12.1 Cell preparation and culture treatments

Viable cell proliferation assay was based on the procedure reported by Gonzalez-Reyes et al. (2013), with some modifications. Briefly, after passaging, benign human oral keratinocyte cells (OKF6) were seeded in a dark, clear-bottom 96-well tissue culture microplate (Sigma-Aldrich). For the proliferation assay, wells were plated with 100 μ L of cell suspension prepared in complete growth medium at a density of 1.5×10^4 cells/well. The viability of seeded cells was confirmed to be > 90% when determined with trypan blue exclusion (trypan blue dye, 0.4% solution). After overnight incubation in standard conditions to allow for cell attachment, plates were washed once with 100 μ L/well PBS. The cells were then treated with 100 μ L/well of HA constituents prepared fresh in complete growth medium from the stock solutions on the experiment's day. Concentrations of HA constituents used for the cell proliferation assay were 1%, 5%, 7% and 10% v/v for the Mucosamin[®], 0.01%, 0.03%, 0.05%, 0.07% and 0.1% w/v for H-MW-HA, and 0.01% v/v for xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100. One row of the wells (three or six technical replicates) was used as a control group. Control wells contained untreated cells cultured 100 μ L complete growth medium only. In one row, 100 μ L growth medium was placed in an empty wells (three or six technical replicate) and was used as blank. The plates were then incubated in standard conditions for 24, 48, and 72 hours. 3-6 wells were used for each control, blank, and tested HA constituents per microplate (three or six technical replicates, figure 2.7). At the end of the incubation period, the media was removed, and the cells were washed once with PBS, and the morphological changes were assessed as described in section (2.3.9.2).

2.2.12.2 Fluorescein diacetate (FDA) assay

At the end of each incubation time, cell viability was assessed using fluorometric FDA assay, as described previously (Lindhagen et al., 2008). After incubation, the supernatants were removed and the cells were rinsed once with 100 μ L PBS using a multichannel pipette. Then, 100 μ L of 12 μ M FDA solution, prepared in K-SFM serum as described in section (2.3.8.2), was added to each well, and the plates were incubated for 40 minutes in standard conditions. The plate was then removed from the incubator, and the FDA fluorescence intensity was evaluated at the respective excitation and emission wavelengths of 485/20 nm and 528/20 nm using Synergy HTX Multi-Mode Reader (Bio-Tek, USA).

2.2.12.3 Determination of cell viability

After incubation, the non-fluorescence probe, FDA, was hydrolysed by esterases in viable cells with intact plasma membranes and converted into the green fluorescent metabolite fluorescein (Rubinstein et al., 1990). The conversion is proportional to the amount of metabolically active cells. Data were expressed as mean cell proliferation attributed to the FDA fluorescent intensity, and the proliferation graph was determined by plotting the FDA fluorescent intensity values versus time.

In addition, control cells (without treatment) viability was considered to be 100%, and HA mediated cell proliferation was assessed by comparing treated cells viability with the viability of control cells using the following formula:

$$\text{Cell viability \%} = \frac{(\text{Fluorescence of treated cells} - \text{Mean fluorescence of the Blank wells})}{(\text{Mean fluorescence of control cells} - \text{Mean fluorescence of the Blank wells})} \times 100$$

Viability data are expressed as mean viability percentage relative to the untreated control (were control is 100%).

	1	2	3	4	5	6	7	8	9	10	11	12
A	M	M	M	M	M	M	M	M				
B	M							M				
C	M							M				
D	M							M				
E	M							M				
F	M							M				
G	M	CTL	CTL	CTL	CTL	CTL	CTL	M				
H	M	BLK	BLK	BLK	BLK	BLK	BLK	M				

Figure 2.8 Standard plate layouts of a 96-well plate for fluorescein diacetate (FDA) assay to be analysed in the Synergy HTX microplate reader (Bio-Tek, USA). Each of the grey rows represents the tested concentration in six replicates for hyaluronic acid (HA) constituent. “BLK”, blank (medium only); “CTL”, control (cell suspension only); “M”, medium (to avoid evaporation).

2.2.13 Evaluation of Intracellular ROS Production and Kinetics in Oral Keratinocytes

Intracellular ROS accumulation was monitored in OKF6 cells by using CM-H₂DCFDA, a chloromethyl derivative of H₂DCFDA (Invitrogen, C6827), as per manufacturers' instruction. In brief, CM-H₂DCFDA is a fluorogenic dye and a useful indicator of hydroxyl, peroxy, and other ROS activity within the cell. It is a non-fluorescent cell-permeable probe that deacetylated, after permeating into the cell, by cellular esterases into a non-fluorescent compound (2',7'-dichlorodihydrofluorescein; CM-H₂DCF), which ROS later oxidizes into (5-and-6-chloromethyl-2', 7'-dichlorofluorescein; CM-DCF), which is a highly fluorescent compound. (Keston and Brandt, 1965; Ro et al., 2014). The oxidation of these probes was detected by monitoring the increase in fluorescence by a fluorescence microplate reader with maximum excitation and emission spectra of 495 nm and 529 nm, respectively.

2.2.13.1 Reagents preparation

2.2.13.1.1 CM-H₂DCFDA Solution

First, the CM-H₂DCFDA vial was centrifuged at a low speed prior to opening. As per manufacturers' instruction, a 2mM stock solution of the fluorescent substrate reagent, CM-H₂DCFDA, was prepared by adding 43.3 μ L water-free DMSO to the 50 μ g vial and gently vortex until solid particles were dissolved. This substrate solution was then diluted to a 5 μ M working solution by adding 25 μ L of the 2mM CM-H₂DCFDA solution to 10mL of warm Hanks' balanced salt solution (HBSS)/Ca/Mg without phenol red (Sigma-Aldrich). CM-H₂DCFDA solution is naturally unstable; therefore, it was prepared immediately before addition into the plates.

2.2.13.1.2 Hydrogen Peroxide Solution (Positive Control)

A fresh working solution of 400 μ M H₂O₂ was prepared by diluting 50 mM H₂O₂ stock solution in the complete growth medium. H₂O₂ concentration was chosen on the basis of concentration that reduced OKF6 cell viability by about 50%, as determined by the FCA.

2.2.13.1.3 Compounds/Diluents of Interest

Compounds of interest (native H-MW-HA) were prepared in complete growth medium to final desired concentrations (0.01% and 0.05% w/v). Concentrations of H-MW-HA used for the assay were chosen based on concentrations that reduced H₂O₂- induced cells cytotoxicity as determined by the FCA.

2.2.13.2 Cell preparation and culture treatments

Intracellular ROS accumulation was monitored in OKF6 cells by adding the CM-H₂DCFDA, as per kit instruction (Molecular Probes, Life Technologies, Victoria, Australia). Intracellular ROS measurement was based on the procedure reported by Ferrer et al (2009). After the passaging of the benign human oral keratinocyte cell line (OKF6) (section 2.3.5), these cells were seeded in a dark, clear-bottom 96-well tissue culture microplate (Sigma-Aldrich). Wells were plated with 100 µL of cell suspension prepared in complete growth medium at a density of 1.5×10^4 cells/well and incubated overnight in a humidified atmosphere in standard conditions to allow for cell attachment before the assay. The viability of seeded cells was confirmed to be > 90% when determined with trypan blue exclusion (trypan blue dye, 0.4% solution). After overnight incubation, plates were washed once with 100 µL/well PBS. The cells were then pre-treated with 100 µL/well of native H-MW-HA prepared from the stock solutions in fresh complete growth medium on the day of the experiment. Concentrations of H-MW-HA used for the assay were 0.01%, 0.05% w/v. One row was used as a control vehicle (-ve CTR). Control wells contained unexposed cells cultured in 100 µL complete growth medium only. Another row held untreated cells cultured in 100 µL of complete growth medium only to be used later as a non-stained control vehicle (background blank). Besides, wells in another empty row contained untreated cells cultured in 100 µL of complete growth medium only, which were used later as positive control vehicle (+ve CTR). The plates were then incubated in standard conditions for 24 hours.

2.2.13.3 CM-H₂DCFDA stain loading

At the end of the incubation period, the media was removed, and OKF-6 cells were washed twice with 100µL/well of HBSS. Then, HBSS was removed, and the cells were stained with 100 µL/well of 5 µM CM-H₂DCFDA solution and incubated in the dark in a humidified incubator for 30 minutes under standard conditions. After that, the HBSS with CM-H₂DCFDA was removed, and the plate was washed twice with 100 µL/well of HBSS before the addition

to the wells of 100µL of the previously prepared growth medium with the compounds of interest (HA +/- H₂O₂). One row was left unstained to be used as a background blank. Besides, cells in the positive control wells were treated with 400µM H₂O₂ prepared in 100 µL of complete growth medium. Three biological replicates were run per experiment, and 4 wells were used for each -ve CTR, +ve CTR, background blank, and the tested compounds of interest (H-MW-HA +/- H₂O₂) concentrations per microplate (four technical replicates). Figure 2.8 represents the experimental design of the standard 96-well plate layouts for the ROS generation determination assay.

2.2.13.4 Determination of intracellular ROS generation

After co-treatment of the OKF6 cells, the cells were incubated in a humid atmosphere at standard conditions, while the kinetic increases in fluorescence of CM-DCF were recorded at the respective excitation and emission wavelength of 485/20 nm and 528/20 nm using the Synergy HTX Multi-Mode Reader (Bio-Tek, USA) for 6 hours (1 read at time points 0, 15, 30, and 45 minutes, and at 30 minutes intervals thereafter) to determine a time course for intracellular ROS generation.

Backgrounds (non-stained cells) were subtracted from all measurements and fold change was determined from assay control (control vehicle). ROS generation of control vehicle was considered as (1 unit), and ROS formation was measured as the change in CM-DCF fluorescence (-fold increase) relative to control vehicle using the following formula:

$$\text{ROS generation (fold changes)} = \frac{(\text{Fluorescence of treated cells} - \text{Mean fluorescence of the background blank})}{(\text{Mean fluorescence of control cells} - \text{Mean fluorescence of the background blank})}$$

ROS levels are reported as the mean CM-DCF fluorescence change (-fold increase) ± SD relative to control as Control=1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Growth medium only											
B	400µM H ₂ O ₂ + H-MW-HA (0.05% v/v)											
C	400µM H ₂ O ₂ + H-MW-HA (0.01% v/v)											
D	400µM H ₂ O ₂											
E	H-MW-HA (0.05% w/v)											
F	H-MW-HA (0.01% w/v)											
G	Control vehicle (–ve CTR)											
H	(Non-stained OKF6 cells) (Background Blank)											

Figure 2.9 Experimental design of intracellular ROS production evaluation assay. Standard plate layouts of 96-well plate for CM-H₂DCFDA staining assay to be analysed in the Synergy HTX microplate reader (Bio-Tek, USA). Gray rows represent the wells loaded with 100 µL/well of 5 µM CM-H₂DCFDA solution in four replicates.

2.2.14 Real-Time Quantification of Oxidative Stress Using Fluorescent Dye-Based Redox Probes in Oral Keratinocytes.

Intracellular ROS production levels were measured in OKF6 cells by using CM-H₂DCFDA, a chloromethyl derivative of H₂DCFDA (Invitrogen, C6827), as per manufacturers' instruction adherent cells protocol for fluorescence microscopy. Briefly, CM-H₂DCFDA is a chemically reduced and acetylated form of 2',7'-dichlorofluorescein (DCF), and calcein. It is a non-fluorescent probe until hydrolysed by intracellular esterases to release 2',7'-dichlorodihydrofluorescein (CM-H₂DCF), which is readily oxidized to the highly fluorescent CM-DCF in the presence of ROS (Keston and Brandt, 1965; Ro et al., 2014). Fluorescence from the CM-DCF is detected under a fluorescence microscope equipped with filters appropriate for fluorescein (FITC), 488 nm excitation, and 530 nm emission.

2.2.14.1 Reagents preparation

CM-H₂DCFDA solution, H₂O₂ solution (positive control), and compounds of interest (0.05% w/v H-MW-HA and 0.05% w/v H-MW-HA +/- 400µM H₂O₂) were prepared as described in section (2.3.13.1). H₂O₂ concentration was chosen based on the concentration that reduced OKF6 cell viability by about 50% as determined by the FCA. Concentration of H-MW-HA used for the assay were chosen based on concentrations that reduced H₂O₂- induced cells cytotoxicity as determined by the FCA.

2.2.14.2 Cell preparation and culture treatments

The intracellular ROS production level was measured in OKF6 cells by adding the CM-H₂DCFDA as per the manufacturers' instruction. Intracellular ROS measurement was based on the procedure reported by Ro et al (2014) with some modifications. Briefly, OKF6 cells were passaged (section 2.3.5), and then seeded in a cell culture treated 6-well plate (Sigma-Aldrich). Wells were plated with 2 mL of cell suspension prepared in complete growth medium at a density of 0.3×10^6 cells/well. The viability of seeded cells was confirmed to be > 90% when determined by trypan blue exclusion (trypan blue dye, 0.4% solution). Cells were incubated in a humidified atmosphere in standard conditions and allowed to adhere for 24 hours before being washed once with 100 µL/well PBS and having the medium replenished. The cells were then incubated in 2mL/well growth medium in standard conditions, with the growth medium replenished every 48-72 hours, until they reached 70-80% confluence. At 60-80% confluence, cells were washed once with 1 mL of PBS and then pre-treated with 2 mL/well of 0.05% w/v

H-MW-HA prepared in the fresh complete growth medium. A fresh complete growth medium was also added to the wells containing non-treated cells. The plates were then incubated in standard conditions for 24 hours.

At the end of the incubation period, the media was removed, and OKF-6 cells were washed twice with 1 mL/well of DPBS. The cells were then co-treated with 2 mL/well of the compounds of interest (0.05% w/v H-MW-HA with or without 400 μ M H₂O₂), which were previously prepared in the fresh complete culture medium. One well (in triplicate) was used as a control vehicle (-ve CTR) and contained untreated cells cultured in 2 mL complete growth medium only. Another well was used as a positive control vehicle (+ve CTR). Cells in the positive control well were treated with 400 μ M H₂O₂ prepared in 2 mL of complete growth medium. Figure 2.9 represents the experimental design of the standard 6-well plate layouts for the ROS generation level determination assay. The plate was then incubated in standard conditions for 3 hours. The later incubation time (3 hours) was chosen based on the time when ROS production in cells treated with the oxidative stimulus (400 μ M H₂O₂) reached the maximum level as determined by the CM-H₂DCFDA staining kinetic assay.

2.2.14.3 CM-H₂DCFDA stain loading

At the end of the incubation period, the media was removed, and OKF-6 cells were washed twice with 2 mL/well of HBSS using a single channel pipette. Then, HBSS was removed, and the cells were stained with 2 mL/well of 5 μ M CM-H₂DCFDA solution and incubated in the dark in a humidified incubator for 30 minutes under standard conditions. After that, the HBSS with CM-H₂DCFDA was removed, and the plate was washed gently twice with 2 mL/well of HBSS. After washing, cells were covered with 2mL/well of HBSS/Ca/Mg and were viewed under FLoid™ cell imaging station digital inverted microscope (ThermoFisher Scientific, USA) using filter was set for fluorescein (FITC) cell imaging (488 nm excitation and 530 nm emission). The fluorescence intensity excited at 488/530 nm was used as a surrogate indicator of the cells' redox (ROS production) status, with an anticipated increase in fluorescence intensity in oxidative stress. The intensity of CM-DCF fluorescence was captured in seven different fields per each well per condition in three independent experiments. The obtained images were transferred as tiff files to a computer and were analysed using the ImageJ software (Schneider et al., 2012; Schindelin et al., 2012).

ROS level of control vehicle was normalized to 1 unit, and the changes in CM-DCF fluorescence are expressed as -fold increase relative to control vehicle using the following formula:

$$\text{ROS level (fold changes)} = \frac{(\text{Fluorescence of treated cells})}{(\text{Mean fluorescence of control cells})}$$

ROS levels are reported as the mean CM-DCF fluorescence change (-fold increase) $\pm$ SD relative to control.

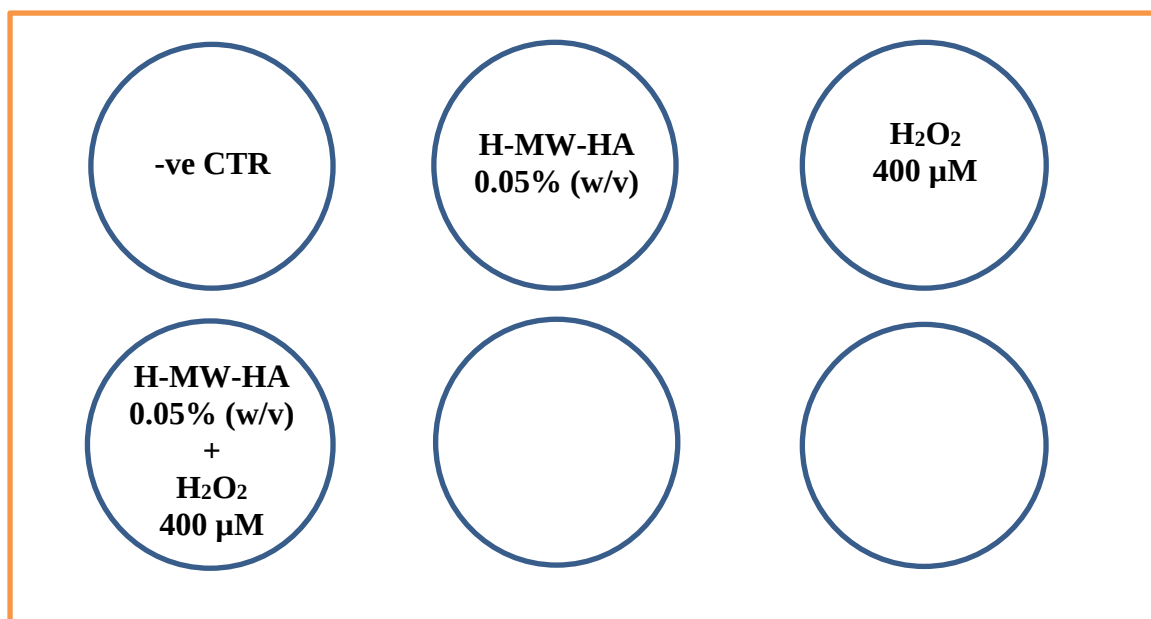


Figure 2.10 Experimental design of intracellular ROS level evaluation. Standard plate layouts of 6-well plate for CM-H₂DCFDA staining assay to be viewed under FLoid™ Cell Imaging Station digital inverted microscope (ThermoFisher Scientific, USA).

2.3 Animal Experiments

2.3.1 Chemicals and Reagents

5-Flurouracil (5-FU) powder (Cat#F6627), a natural non-sulphated H-MW-HA powder (non-crosslinked, mol wt $\sim 1.5\text{-}1.8 \times 10^6$ Da, Hyaluronic acid sodium salt, from *Streptococcus equi* (Cat#53747), SOD assay kit-WST (Cat# 19160), 10% Neutral buffered formalin (NBF) solution (Cat# HT50112), Toluidine Blue O (TBO) powder (Cat# 198161) were all purchased from Sigma–Aldrich, Castle Hill, NSW, Australia. All other reagents were of analytical grade and were commercially available.

2.3.2 Preparation of 5-FU

The 5-FU powder was dissolved in saline to final volume of 10 mg/mL according to the following protocol:

- 1) 10 mg of 5-FU was weighed with a precision analytical balance (XT 120A, Precisa Inst. AG, Swiss) in a 1 ml Eppendorf tube. Then the powder was dissolved in 1ml of saline.
- 2) The solution was vortexed for 5 minutes and then sonicated for 5 minutes.
- 3) After that, the solution was centrifuged for 5 minutes at 1000 rpm using an Eppendorf centrifuge 5418R (ThermoFisher Scientific, Australia).
- 4) Last, the solution was visually checked for any precipitate at the bottom of the tube.
- 5) If powder not completely dissolved, the solution was then warmed up to 60 °C for 5 minutes.

Vortex, sonication, and centrifugation processes were repeated until the powder was completely dissolved.

2.3.3 Preparation of Hyaluronic Acid

A Natural non-sulphated H-MW-HA powder was dissolved in drinking water at a concentration of 0.01% (w/v), and 1000 ml of the solution was prepared according to the following procedure:

- 1) 100 mg of powder was weighed with a precision analytical balance (XT 120A, Precisa Inst. AG, Swiss), and was dissolved in 1000 ml autoclaved sterile water in

a sterile glass bottle (Fisher Bottle lab 10000mL graduated with PP screwcap, Cat # FSBFB33181, ThermoFisher Scientific, Australia).

- 2) The solution was shaken on the orbital shaker at 100 rpm and left overnight until all particles were dissolved.

2.3.4 Animals

C57BL/6 female mice aged 6-12 weeks and weighing between 15 and 20 g at baseline were used in this study. Animals were purchased from Bio21 animal facility (Bio21 institute, The University of Melbourne, Parkville, Victoria, Australia). Mice were housed in groups of three or four in clear cages with wood chips under specific pathogen-free conditions and maintained on a 12-hour light/dark cycle in a temperature-controlled and humidity-controlled room ($21 \pm 1^\circ\text{C}$ and 40%-60%, respectively) with free access to an autoclaved food pellets and water provided ad libitum. Mice were monitored daily for signs of morbidity, and body weights were recorded using digital weighing balance starting from day 2 (one day post initial injection) every 48 hours until day 10, then every 24 hours until the end of the experiment. This study was approved by the Animal Ethics Committee (AEC) of The University of Melbourne (ID 1814636.3). All in vivo experiments were conducted in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes determined by the National Health and Medical Research Council 8th edition (Hubrecht, 2013), and reported in compliance with the ARRIVE guidelines. All efforts were made to minimize animal suffering.

2.3.5 5-FU Administration

For the Chemotherapy induced mucositis (CIM) mouse model, representative CT drug 5-FU (Sigma-Aldrich, Castle Hill, NSW, Australia) was used because it causes severe muco-toxic effects in patients with cancer (Longley et al., 2003; Köstler et al., 2001). The model was established according to a method previously described (Bertolini et al., 2017) with a slight modification. Briefly, C57BL/6 female mice aged 6-12 weeks were given intravenous (i.v.) injections, via tail vein, of 5-FU (50 mg/kg in saline) every 48 hours for 13 days (starting at day 1), considering that the first 5-FU injection day is day 1. As a control, saline alone was administered in the same manner in the control group mice. 5-FU dose and frequency were selected based on a similar case when the same dose and frequency of 5-FU effectively reduced tumour volume in a xenograft murine model (Brown et al., 2015). Furthermore, the dose is within the range of intermittent therapeutic doses in humans (Reagan-Shaw et al., 2008;

Kanetaka et al., 2012), and comparable to what has been recently used in infusion doses for the treatment of HNC patients, then approximately 800 mg/m² compared to human (Winqvist et al., 2014).

2.3.6 Intravenous Injection of mice.

Intravenous injection procedure and post-procedure monitoring were conducted following the Bio 21 institute's safe work procedure, namely "intravenous injection of a mouse" issued on the 10th of October 2017 and Authorised by the Bio21 EHS Coordinator. The procedure was according to the following protocol:

- 1) The work area was sprayed with F10 veterinary disinfectant (1:125).
- 2) 80% alcohol was used to spray the operator hands.
- 3) The mouse was placed in a clean cage with a secured wire lid, and these were positioned under the heating lamp (no closer than 20cm) for 8-10 minutes to cause vasodilation of the tail vein.
- 4) Once lateral veins became physically more visible, the mouse was placed in a restraining device with the tail exposed.
- 5) The tail was wiped with alcohol-moistened tissue or gauze to increase vein/s identification, and one of the two lateral tail veins was identified at the middle third of the tail.
- 6) The non-dominant hand was used to restrain the tail, and the injection was given in the lateral tail vein of the mouse using 1mL syringe and 25-27 gauge needles with the needle facing upwards and almost parallel to the vein.
- 7) To confirm that the needle was correctly placed in the vein, negative pressure was applied until some blood could be seen in the hub of the syringe. If not, the needle was promptly slightly pulled back without removing it from the tail and redirected until blood was seen.
- 8) The plunger was slowly pressed to release the substance to the vein.
- 9) Once the required amount of substance was injected, the needle was removed from the vein, and the syringe and needle were placed in a sharps container, and a slight pressure was applied with tissue/gauze to the injection site to stop the bleeding.
- 10) Injection sites were rotated during the experiment to reduce the cumulative welfare burden.

- 11) The animal was removed from the restrainer and placed back in its cage, visually monitored for the next 5-10 minutes to ensure bleeding was ceased.
- 12) A visual check on the mice was completed upon removal from the change station, ensuring all mice behaved normally, and no abnormal signs were visible around the injection site.

2.3.7 Monitoring of Animals.

Over 19 days observation period, mice were daily monitored for morbidity signs, and the animal's body weight (BW) was recorded during the study beginning and then every 48 hours, from day 2 (one-day post initial injection), until day 10, and then every 24 hours until the end of the experiment. Monitoring sheets with the clinical records for the presence of ruffled/dull coats, temperament change (including stress marks on face and paws and squealing when handled), weight loss, and reluctant to move were maintained. Where an animal's ability to consume food and water affected (grade 2 OMI or weight loss of >5% of starting weight), a semi-solid diet, where the food moistened, was placed in the cage floor to improve nutrition.

2.3.8 Diarrhea Assessment

In the present study, we evaluated the severity of 5-FU-induced gastrointestinal mucositis by assessing diarrhea severity in animals. Stool passages/diarrhea severity of all mice were recorded, starting from day 2 (one day post initial injection), every 48 hours until day 10, and then every 24 hours until the end of the experiment. Bowen's score system was used to determine diarrhea severity as an IM surrogate (Gibson et al., 2003) Based on stool consistency, the scoring system is classified into four grades:

- Grade 0, Normal; the stool is normal.
- Grade 1, mild diarrhea; the stool is slightly wet and soft.
- Grade 2, moderate diarrhea; the stool is wet and unformed.
- Grade 3, severe diarrhea; the stool is watery.

2.3.9 Oral Mucositis Scoring

For OM index scoring, mice were anesthetized with isoflurane every 24-48 hrs. Mice induction was briefly done in a chamber at 5% isoflurane in oxygen until the loss of the righting reflex

occurred. Animals were then left in the chamber with isoflurane 2% in oxygen for 2-3 minutes before being removed to perform the OM scoring. The time required for mice examination and image collection for each mouse was approximately 2-3 minutes. The oral cavity was examined for mucositis/ulcer formation and general appearance by macroscopic means by using diagnostic tools (figure 2.10 A). The incidence, development, and extent of OM were assessed clinically by using the visual oral ulcerative mucositis score, which was modified by Nakajima et al. (2015), based on the method of Sonis et al. (2000), (Table 2.1). Two independent trained observers (AM, and AC) scored the mice, who graded the animals independently using the above-described scale. Image acquisition (figure 2.10 B) was made with a digital camera (Canon EOS 60D; Canon Inc., Tokyo, Japan). Mice were returned to their original cages after image acquisition and recovery from anaesthesia. The general anaesthesia procedure and post-procedure monitoring were conducted following the University's "General anaesthesia for mice and rats" Animal Care and Use Standard.

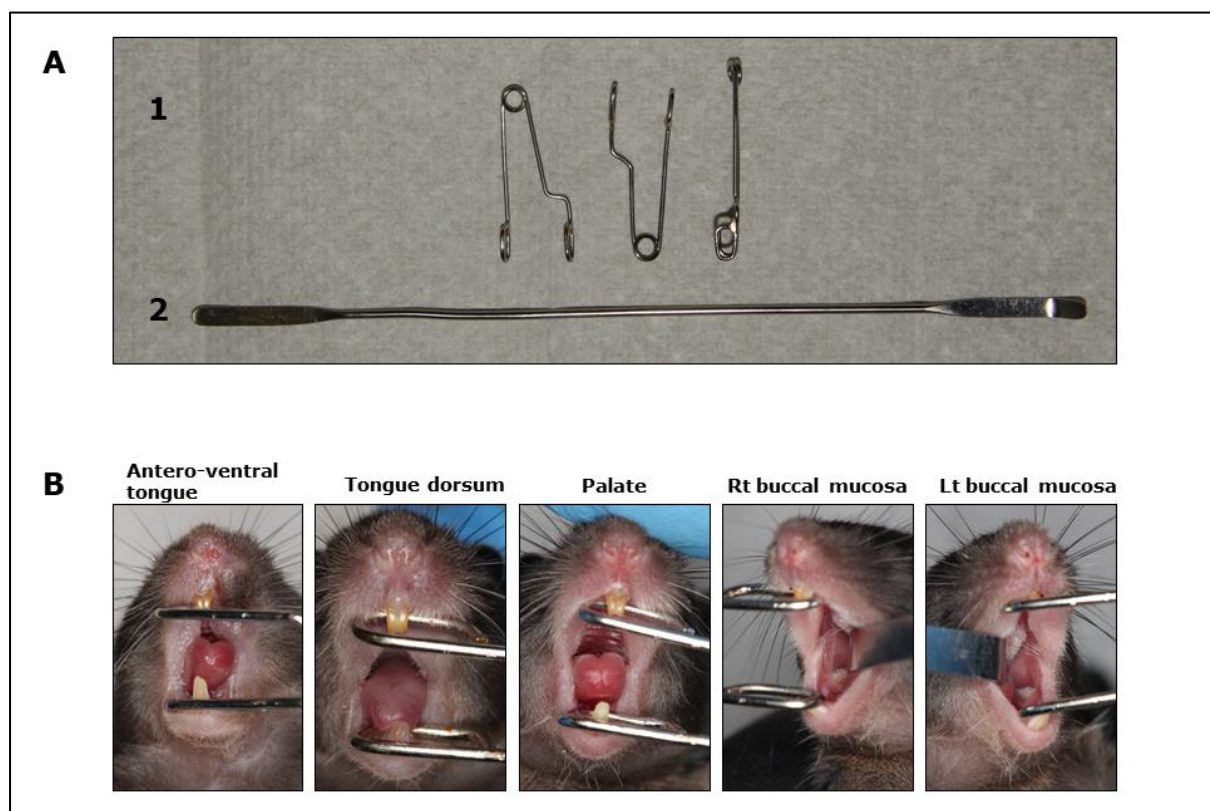


Figure 2.11 Oral cavity assessments. A: Oral cavity examination tools; 1: Stainless steel laboratory micro spatula; 2: three views of modified Celentano Murine Mouth opener (CMMO). B: Oral cavity examination views. Right (Rt); Left (Lt).

Table 2.1 Visual oral ulcerative mucositis score, (Nakajima et al., 2015): based on a modification of Sonis' method (2000).

Grade	Severity	Description
0	Normal	Normal (no abnormalities)
1	Mild	Partial hyperaemia, erythema and swelling, but no evidence of mucosal erosion (intact mucosa).
2	Mild	Overall hyperaemia, erythema and swelling and superficial erosion, such that there may be mild sloughing.
3	Moderate	Epidermolysis, hyperaemia and erythema; moderate mucositis characterized by frank ulcer formation; Ulcers typically have areas of necrosis (erosion) with associated yellowish/grey coloration with pseudomembrane formation. Cumulative area of ulceration $\leq 25\%$ of tongue surface area.
4	Severe	Extensive epidermolysis and bleeding; severe mucositis with the formation of ulcers that affect 25% to 50% of the tongue or buccal mucosal area; Significant erythema with the formation of the pseudomembrane. Pliability loss.
5	Severe	Bleeding and abscesses; virtually complete ulceration of the mucosa, all areas of mucosa are at risk ; Pliability and mobility loss.

2.3.10 Euthanasia and Blood Sample Collection

At the end of each experimental time point (specifically days, 14, 16, and 19 of the experiment), and after weighing the animals, mice were euthanized by exsanguination via cardiac puncture under general anaesthesia. Briefly, general anaesthesia was induced in a chamber at 5% isoflurane in oxygen until loss of the righting reflex occurred. Animals were then maintained on 2-3% isoflurane via a nose cone and laid on the back, and a 25-26 gauge needle was inserted into the heart at a 15-30° angle from the point just beyond the sternum. Negative pressure was applied slowly to withdraw the maximum amount of blood (~ 1ml) from the heart. To ensure the mice were dead, cervical dislocation was also performed. Blood samples were obtained and left at room temperature for 30 minutes to allow for clotting and then centrifuged at 2500g for 20 minutes in an Eppendorf centrifuge 5418R (ThermoFisher Scientific, Australia). Then the blood serum was collected in 1 ml Eppendorf tubes and frozen at -80°C until analysis.

2.3.11 Tissue Sample Preparation

For mucositis' histological evaluation, tongue, and jejunum were harvested at necropsy. The procedure was carried out as follows: animals were placed on a dissecting board. The angle of the lips and buccal mucosa was retrieved at necropsy using dissection forceps and scissors. An incision was cut at the upper side of the corner of the mouth extended from the labial skin deep to the oral mucosa. With forceps, the skin was held at the incision and pulled gently, and another cut was done on the lower side of the mouth angle. The angle of the mouth was then held with one hand using a forceps, and scissors were used to cut a longitudinal sample of 3-5 mm length from the cheek. The tongue was excised at the level of the trachea.

For tongue removal, a cut was made through the buccal surfaces on each side of the oral cavity to the temporomandibular joint. Then, the lower jaw was dislocated, and the upper jaw was reversed to expose the epiglottis and the base of the tongue. Next, the tongue was gently extended using forceps, and the attachments underneath were carefully cut to the tongue base. Finally, while maintaining reflection, the base of the tongue was horizontally cut to release it from the oral cavity.

For jejunum retrieval, the abdominal cavity was accessed via laparotomy. First, an incision was made in the abdominal side skin using dissecting forceps and scissors. Then the skin was held with forceps and gently removed from the tissue of the abdominal muscle. The abdominal skin was then cut, and the abdominal muscles were exposed using a pair of scissors. Next, an

incision was carefully made in the peritoneal tissue and abdominal muscles to expose the intestine, and the cutting was continued until the intestine was exposed. Next, the stomach was identified and delineated to where it connects to the small intestine. The intestine was cut about 1 cm distal to the stomach with a pair of scissors. Since there was no definite anatomical boundary between the duodenum, jejunum, and ileum for jejunal specimen collection, the middle of the small intestine was taken into account during specimen collection (Bialkowska et al., 2016; Williams et al., 2016). The small intestine was held with a pair of forceps or one hand, and approximately 6-10 cm section of the proximal jejunum (located nearer to the stomach) was cut using a pair of scissors.

The samples were collected and promptly fixed in 10% buffered formalin, pH 7.4 for 48 hours, and then transferred to 70% ethanol and stored at 4°C for histological processing and scoring. Animal experiments were replicated thrice unless otherwise indicated.

2.3.12 Macroscopic Examination of the Tongue

The tongue was excised at the level of the trachea. To detect erosive or ulcerative lesions on the tongue surface, tongues were stained with a solution composed of 1% toluidine blue prepared in 10% acetic acid for 1 min, followed by repeated washes with acetic acid until there was no further recovery of dye (Muanza et al., 2005). Tongues were placed on a blue dissection board. Tongues images were acquired with a digital camera (Canon EOS 60D; Canon Inc., Tokyo, Japan) using a 100mm lens (Canon 100mm f/2.8 Macro USM lens) connected with a ring flash (Sigma EM-140 DG Ring Flash). Four images were captured for each tongue, representing four different aspects of the tongue (dorsal, ventral, and two lateral surfaces) and scored according to the following criteria; Negative, identified as no uptake of dye or light-coloured, spready flecked distribution of dye, positive, identified as deep, dark blue colouring of the epithelium and loss of epithelium (specified as an ulcer). The ratio of surface area with positive toluidine blue staining (excision stained area excluded) was calculated using Fiji (ImageJ) software.(Schneider et al., 2012; Schindelin et al., 2012). The calculation was carried out according to the following equation (Figure 2.11):

"The ulcer size percentage of chemotherapy

– induced oral mucositis (CIOM) relative to the total epithelialized surface of the tongue
 = toluidine blue positive surface area of the tongue
 /total epithelialized surface area of the tongue."

The measurements were carried out as follows:

- 1.) ImageJ software was opened.
- 2.) The picture was dragged and dropped into the software interface.
- 3.) A series of line segments were drawn along the selected surface area at the tongue image using the “polygon selection” tool provided by the software.
- 4.) From the main bar, analyse menu > measure was chosen. The software automatically calculated the area of the selected segment in pixels.



Figure 2.12 Mouse tongue morphology. Representative photomicrograph of a toluidine blue (TB) –stained tongue surface displaying the ulcer of the epithelium (in blue) at the upper surface of the tongue. The Fiji software (ImageJ) was utilised to measure the percentage of ulcer size of chemotherapy-induced oral mucositis (CIOM) relative to the total epithelial tongue surface; ratio = tongue surface area positively stained with TB (excluding excision trauma)/total epithelial tongue surface area. Numbers define the selected measured surface area.

2.3.13 Superoxide Dismutase (SOD) Enzyme Activity Assay

The initial line of the antioxidant defence system against ROS-induced oxidative stress *in vivo* are antioxidant enzymes, including Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). SOD catalyses the superoxide anion dismutation, forming H_2O_2 , which in turn is decomposed by GSH-Px and CAT to water and oxygen, thereby preventing hydroxyl radicals formation (Cui et al., 2010). Accordingly, this enzyme works synergistically with other antioxidant enzymes at different sites in the free radical metabolic pathway. In this study we observed the influence of H-MW-HA on the SOD antioxidant enzyme activity with the main focusing was on the antioxidant effects of HA.

Total SOD activity was measured in blood serum by using SOD determination kit (Cat# 19160, Sigma–Aldrich, Castle Hill, NSW, Australia). Using SOD ELISA kit to measure the SOD in each sample was based on WST-1 method. The method is based on the colorimetric reaction of Dojindo's highly water-soluble tetrazolium salt, WST-1(2-(4-Iodophenyl) - 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2Htetrazolium, monosodium salt). In the reaction, the use of Xanthine oxidase (XOD) catalyses the oxidation conversion of Xanthine to generate superoxide anions (O_2^-), which cause a reduction of WST-1 to produce water-soluble formazan dye. However, these superoxide anion concentrations are reduced in the presence of SOD, yielding less colorimetric signal (Figure 2.12). Therefore, the inhibition levels were used to indicate the SOD activities in serum.

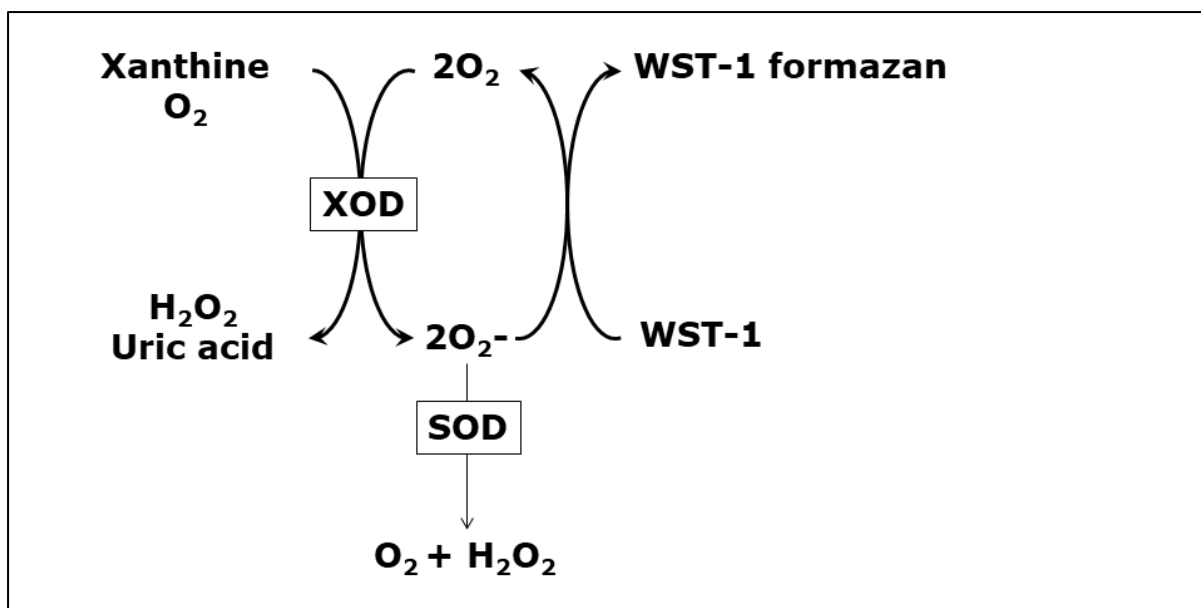


Figure 2.13 Superoxide dismutase (SOD) inhibition assay mechanism

The assay was carried out according to the manufacturer's instructions and according to the following protocol:

2.3.13.1 Preparations of Assay Solutions (for one 96-well plate)

- 1) To prepare working solution of WST, 1 ml of WST Solution was diluted in 19 ml of the buffer solution supplied from the original stock.
- 2) To prepare working solution of the enzyme, the tube of the enzyme solution was centrifuged for 5 seconds and then mixed by pipetting and diluted by adding 15 μ l of the mixed enzyme solution into 2.5 ml of dilution buffer supplied from the original stock.
- 3) Each serum sample was divided into 3-4 aliquots after the step of serum preparation. Use up one aliquot was used in each assay to ensure replicate and to avoid repetitive freezing-thawing of the samples.
- 4) An original serum sample was entirely thawed on ice and mixed well. Then, it diluted at a dilution rate of 1:5 with the dilution buff sample from the original stock during the sample preparation. The diluted sample was used as a working serum sample solution (X5).
- 5) To ensure the readings are within the standard value range, a serial dilution process of the X5 sample solution was performed. X5 sample solution was diluted with dilution buffer to prepper samples solution with the dilution ratios of : X5(no dilution), X5/5, X5/5², X5/5³, X5/5⁴, X5/5⁵, and X5/5⁶.

2.3.13.2 General Protocol

All materials, samples, and prepared reagents were equilibrated to room temperature and gently agitated before use. There were three blanks in each assay; blank 1, staining without inhibitor, blank 2, blank of the sample and blank 3, blank of the reagent. The test was conducted in Corning 96 well microplates (Cat# CLS3595, Sigma–Aldrich, Castle Hill, NSW, Australia). For the amount of solutions in each well, refer to Table 2.2. Briefly, 20µl of sample solution was put to each well of the sample and blank 2, and 20µl of ddH₂O (double distilled water) was put to each well of blank 1 and blank 3. Then, 200µl of the WST working solution was added to each well and mixed by using a pipette. Then, 20µl of dilution buffer was added to each blank 2 and blank 3 well. After that, a multi-channel pipette was used to add 20µl of enzyme working solution to each well of the sample and blank 1, followed by thoroughly mixing. Refer to figure (2.13) for sample and blank arrangement on a 96-well microplate. The plate was then incubated at 37 °C for 20 min. At the end of incubation time, the absorbance was read at 450 nm using a microplate reader (Perkin Wallac Victor 2V Multilabel Microplate Reader 1420, Shelton, USA).

2.3.13.3 Calculating SOD Activity Unit (U)

For statistical reasons, each sample and blank were assayed with three replicates (triplicates). The SOD activity (inhibition rate %) was calculated using the following equation (A refers to absorbance).

$$\text{SOD activity (inhibition rate \%)} = \frac{(\text{Ablank 1} - \text{Ablank 3}) - (\text{Asample} - \text{Ablank 2})}{(\text{Ablank 1} - \text{Ablank 3})} \times 100$$

The point where a 20µl of sample solution inhibited reduction of WST-1 to 50% of maximum (IC₅₀) was represented as 1 unit (U) of SOD activity. Fit spline/LOWESS test was used for Inhibition curve fitting and IC₅₀ calculation. Then the dilution rate at 50% inhibition (IC₅₀) was read from inhibition curve. The SOD U in the original sample was calculated by multiplying the dilution rate at IC₅₀ and at the sample preparation. The assay was run in biological triplicate and all the activities were expressed as U/ml of blood serum.

Table 2.2 Solution amount in each well

	Sample	Blank 1	Blank 2	Blank 3
Sample Solution	20μl	-	20μl	-
ddH ₂ O	-	20μl	-	20μl
WST Working Solution	200μl	200μl	200μl	200μl
Dilution Buffer	-	-	20μl	20μl
Enzyme Working Solution	20μl	20μl	-	-

1	2	3	4	5	6	7	8	9	10	11	12
Sample 1,X5											
Sample 1,X5/5											
Sample 1,X5/5 ²											
Sample 1,X5/5 ³	Sample 2			Sample 3			Sample 4				
Sample 1,X5/5 ⁴											
Sample 1,X5/5 ⁵											
Sample 1,X5/5 ⁶											
Blank 1	Blank 2			Blank3							

Figure 2.14 Samples and blank arrangement on 96-well plate

2.3.14 Tissue Processing and Embedding for Histology

Tissue samples were processed by Peter MacCallum's Centre for Advanced Histology and Microscopy (CAHM, Melbourne, Victoria, Australia). Initially, the fixed tissues were transversely trimmed under a fume hood into slices of 2-4 mm in length and were put in labelled cassettes and then refixed in 70% ethanol until further processing. To be processed, the tissues underwent multiple steps for several hours, which involve Dehydration, Clearing, and Infiltration. The process was completely automatic by using Tissue-Tek® VIP® 5 vacuum infiltration processor (Sakura Finetek USA, Torrance, CA, USA) and was conducted according to the manufacturer's protocol. First, tissue was dehydrated from an aqueous environment to an alcohol environment by using a series of increasing concentrations of alcohol. Then alcohol was replaced with a clearing agent (xylene) to make the tissue receptive to paraffin. Next, xylene was replaced, and the specimens were infiltrated with paraffin. Table 2.3 shows the steps, chemicals, and timings used for processing techniques.

The next step was embedding. After the final infiltration, tissue cassettes were transferred to the embedding station, Sakura – Tissue Tek TEC embedding centre (Sakura Finetek USA, Torrance, CA, USA). This machine contains tanks of melted wax, heating plates and a cold plate for setting the blocks. The entire cassette was left for 15 minutes in a paraffin bath at 58°C. Then, the infiltrated tissue was dragged from the cassette using warm forceps and orientated inside a metal mould of suitable size containing a small amount of molten paraffin, with the cut side facing down. The mould was transferred into a cold plate to allow the paraffin to solidify in a thin layer that held the tissue in position. After confirming that the tissue was in the desired orientation, the mould was filled with molten wax, and the main part of the labelled cassette was placed on top, and this was also filled with wax. The entire mould was then transferred to the cold plate for final solidification. When the wax was completely cooled and hardened (in about 30 minutes), the paraffin block was easily popped out of the mould in readiness for cutting sections on a microtome.

Table 2.3 Steps involved in tissue processing. Processor: Tissue-Tek® VIP® 5, with each step representing the next station on the processor. All steps were carried out with a vacuum. Heat was only applied on the paraffin stations.

Process	Solution name	Time (minutes)	Temperature (°C)
Dehydration	70% Ethanol	30	--
Dehydration	70% Ethanol	30	--
Dehydration	100% Ethanol	60	--
Dehydration	100% Ethanol	60	--
Dehydration	100% Ethanol	60	--
Dehydration	100% Ethanol	60	--
Clearing	Xylene	60	--
Clearing	Xylene	60	--
Clearing	Xylene	60	--
Clearing	Xylene	90	--
Infiltration	Paraffin	60	60
Infiltration	Paraffin	60	60
Infiltration	Paraffin	60	60
Infiltration	Paraffin	60	60

2.3.15 Tissues Sectioning

4µm thick sections were made of each sample tissue of the intestine and tongue. This was achieved using a rotary microtome (Leica RM2255 Rotary Microtome). The process was carried out according to the routine histological processing steps as mentioned elsewhere (Slaoui and Fiette, 2011). First, to expose the tissue, the wax was removed from the block surface. The blocks to be sectioned were placed face down on a cold surface (such as ice or refrigerated cold plate) for 10 minutes to harden the cutting surface before sectioning. Then, the paraffin block was mounted on the microtome holder, so the wax block faced the blade and was aligned in the vertical plane. The blade was angled at 5°. The dial was set to a 10 µm cut-off in order to flatten the block; Once cut smoothly, the dial was set to 5 µm sections. A series of paraffin sections were cut as a ribbon and picked up with forceps or a fine paintbrush and flowed on a 45°C water bath to stretch the paraffin section and eliminate wrinkles and distortion in the tissue. The selected tissue section was then removed out of the water path by putting a standard microscope glass slide, labelled with a pencil or histo-pen. Tissue sections were then dried at 37°C in a thermostatic laboratory oven. To melt the wax, the slides containing the paraffin sections were put on the heating block for 20 minutes in an oven set at 60°C, which helped the sections adhesion to the slide. Then, the slides were transferred (upright positioned) to a laboratory oven and incubated overnight at 37 °C in preparation for the next stage of staining.

2.3.16 Hematoxylin and Eosin Staining and Mounting

The hematoxylin and eosin (H&E) staining technique is used to stain the nuclei blue to purple (hematoxylin) and counterstain the cytoplasm pink to red (eosin). A fully automated process (Jung Auto Stainer XL, Leica Microsystems, Victoria, Australia) was used to achieve H & E staining and to put the cover-slipping. The process was carried out according to the routine histological processing steps as mentioned elsewhere (Slaoui and Fiette, 2011). To achieve staining, tissue sections underwent a series of steps: rehydration to remove paraffin (utilizing xylene and alcohol solutions ending in water), staining, and dehydration again (using alcohol and xylene). Table 2.4 shows the steps, chemicals, and timings used for processing techniques.

After staining, a fragile glass (coverslip) was placed on top of the tissue section to protect it and to enhance visual assessment of the tissue. The process of cover-sliding involves mounting a coverslip glass over a tissue section prepared on a microscope slide. The mounting process

was achieved by applying two or three drops of the Eukitt® mounting medium (Cat# 03989, Sigma–Aldrich, Castle Hill, NSW, Australia), while keeping the clearing agent covering the tissue section. Then the coverslip was placed on the slide over the tissue section. Finally, The slides were left overnight to dry on a flat surface within the fume hood at room temperature.

Table 2.4 Steps involved in hematoxylin and eosin (H&E) staining technique.

Process	Solution name	Time (minutes)
Rehydration	Xylene	3:00
Rehydration	Xylene	3:00
Rehydration	Xylene	3:00
Rehydration	100% Ethanol	1:00
Rehydration	100% Ethanol	1:00
Rehydration	100% Ethanol	1:00
Rehydration	70% Ethanol	0:30
Washing	Tap water	1:00
Staining	Hematoxylin (Harris)	4:00
Washing	Tap water	1:00
Washing	Tap water	1:00
Decolorization	Scott's water	0:45
Washing	Tap water	2:00
Staining	Eosin	4:00
Washing	Tap water	0:15
Dehydration	100% Ethanol	0:45
Dehydration	100% Ethanol	0:45
Dehydration	100% Ethanol	0:45
Dehydration	100% Ethanol	1:00
Clearing	Xylene	2:00
Clearing	Xylene	2:00
Clearing	Xylene	2:00

**CHAPTER 3 DEVELOPMENT OF
AN *IN VITRO* MODEL FOR
OXIDATIVE STRESS-INDUCED
CYTOTOXICITY IN ORAL
KERATINOCYTES**

3.1 Abstract

Background: With its significant adverse impact on anti-neoplastic treatment delivery, OM remains one of cancer treatments' most common side effects. The exact mechanisms underlying cancer therapy-induced OM have not been totally understood, mainly because of the lack of suitable experimental models. We aimed at establishing an *in vitro* oxidative stress model that could mimic the initial events that trigger OM using human oral epithelial cells with hydrogen peroxide (H_2O_2) as the causative factor. Such a model will allow the screening of potential anti-oxidant drugs.

Materials and Methods: We used one immortalized normal human oral keratinocytes cell line, OKF6, as well as two types of OSCC cell lines derived from human, H357 and H400. The cells were incubated with increasing concentrations of H_2O_2 (100 μM -1200 μM) for up to 24 hours. Cell viability was evaluated using trypan blue exclusion assay, fluorescence-based live-dead assay, and FCA, while morphological changes were analysed by phase-contrast inverted microscope.

Results: H_2O_2 induced cytotoxic effects in a concentration and time-dependent manner. FCA assay showed that the survival rates of oral epithelial cells were significantly lower than the control groups 24 hours after exposure to 800 μM , 400 μM , and 100 μM of H_2O_2 and higher for H400, H357 and OKF6 cells, respectively. Incubation with H_2O_2 for 24 hours at concentrations of 800 μM , 800 μM , and 400 μM was able to decrease cell viability ratio to levels close to the IC_{50} value for H400 cells (54.21 ± 9.04 , $p < 0.01$), H357 cells (53.48 ± 4.01 , $p < 0.01$), and OKF6 cells (48.64 ± 3.09 , $p < 0.01$) respectively. In addition, morphological studies showed that H_2O_2 was able to induce apoptosis in the tested cell lines in a concentration-dependent manner.

Conclusions: We successfully established an oxidative stress model on human oral keratinocyte cultures via H_2O_2 administration. We were able to identify optimum concentrations of H_2O_2 and incubation period for each of the cell line tested.

3.2 Introduction:

Chemotherapy and radiotherapy are highly effective treatments for cancer. However, these treatments can also damage healthy tissues. In particular, the rapidly dividing epithelial progenitor cells of the alimentary tract can be severely affected (Sonis, 1998; Duncan and Grant, 2003; Sonis, 2009; Campos et al., 2014; Bertolini et al., 2017). OM is a pathological condition clinically characterized ulceration in the oral mucosa resulting in severe functional-altering soreness and distress that can affect the patient's nutritional intake and oral hygiene. (Murphy and Gilbert, 2009; Rosenthal, 2007; Yeoh et al., 2006). Despite its severity, frequency, and economic impact, to date there is no single intervention that effectively prevents or treats OM, with most current standard therapies for mucositis are predominantly palliative (Sonis, 2011; Nicolatou-Galitis et al., 2013).

Identifying innovative strategies to treat OM cannot disregard the pathogenic mechanisms underlying the development of this condition (Biswal, 2008; Cinausero et al., 2017). It is now thought that mucositis is represented by a sequence of several biological and cellular events, including the interaction of oral mucosal cells and tissues, ROS, proinflammatory cytokines, apoptosis mediators, and local factors such as oral microbiota and saliva. These events predominantly arise in the submucosal tissues and cells (endothelium and connective tissue) and culminate in targeting the epithelial cells, and ending in degeneration and breakdown of the epithelial barrier resulting in mucositis ulcers development (Sonis, 2004b). Therefore, epithelial cells targeting to manage OM appear to be an encouraging strategy.

It has been shown that excessive ROS production, such as H_2O_2 , superoxide, and hydroxyl radicals, plays an essential role in the damage of cells and functional loss in many tissues and organs (Afonso et al., 2007). ROS generation is a primary mode of action of many chemotherapeutic agents and ionizing radiation (Sonis, 2009; Zhao and Robbins, 2009). At the molecular level, mucosal injury induced by cancer treatment is triggered at early pre-clinical stages by ROS production and consequent oxidative stress pathways activation (Yoshino et al., 2013; Criswell et al., 2003).

H_2O_2 is a highly reactive component that leads to cell injury. The H_2O_2 -induced oxidation model has been widely used to study the cellular response to oxidative damage (Gille and Joenje, 1992). Redox is one of the main etiological factors for cell apoptosis induction (Holbrook and Ikeyama, 2002). Incubating cells with potentially destructive molecules, such

as H₂O₂, renders sub-cytotoxic stress and puts the cells into a senescence-like state called “*stress-induced premature senescence*” (Fripiat et al., 2002; Kiyoshima et al., 2012).

Several *in vitro* models of oral mucosal barriers have recently been developed where the effects of either CT agents or ionizing radiation on oral keratinocyte viability, responses of inflammatory cytokine, and proliferation were examined to optimize *in vivo* noticed epithelial changes associated with mucositis (Bierbaumer et al., 2018; Sobue et al., 2018; Sonis et al., 2017; Tschachojan et al., 2014; Lambros et al., 2011; Tobita et al., 2010). Although they are two-dimensional (2D) and do not represent exactly what is happening *in situ* where cells live in 3D, monolayer cultures from oral keratinocytes have been widely used to mimic a specific part of the oral mucosal epithelial barrier (Abbott, 2003; Bierbaumer et al., 2018). Artificially regenerated skin or mucosa substitutes (*ex vivo* organ cultures) have been used for basic or clinical science research to assess the cytotoxic effect of radiation on the oral mucosa (Xu et al., 2009). Developing a highly reproducible 3D oral mucosa organotypic model has provided an organizational complexity between *ex vivo* organ cultures and single-cell cultures. In such a model, epithelial cells form multilayers that resemble the mucosa of the upper alimentary tract histomorphologically and functionally (Dongari-Bagtzoglou and Kashleva, 2006).

It has been shown that ROS has distinctive effects in oral keratinocytes and fibroblasts. Specifically, oxidative stress in fibroblasts induces cellular senescence and this, in turn, leads to the production of ROS. Keratinocytes are more sensitive to oxidative stress and undergo apoptosis in the presence of potent pro-oxidant stimuli, which clinically translates into mucosal ulceration seen in OM (Cirillo et al., 2015). Taking into account the pro-oxidant mechanisms involved in the pathophysiology of OM, the need for new research models of oxidative stress-induced OM in which the new therapeutic interventions can be characterized appears evident. In particular, it is essential to establish a dose-response curve of the impact of ROS in oral keratinocytes. Therefore, the goal of this study was to establish an *in vitro* model of oxidative stress-induced oral mucosal toxicity, using H₂O₂ and three monolayer cultures of oral mucosa keratinocytes to emulate what is thought to occur in chemoradiotherapy-induced OM.

In this study, we demonstrated that H₂O₂ treatment exerted cytotoxic effects on all the tested epithelial cell lines. This effect was found to be both time and dose-dependent. A novel *in vitro* model of oxidative stress-induced OM was developed on the basis of human oral epithelial cell lines.

3.3 Material and Methods

3.3.1 Cell lines and culture conditions

To establish oxidative stress-induced oral mucosal toxicity *in vitro* model with H₂O₂, we used three monolayer cultures of oral mucosa keratinocytes (OSCC cell lines, H400 and H357, and immortalized normal human oral keratinocytes, OKF6). H400 (Prime et al., 1990), a human alveolar OSCC cell line (Cat#06092006), and H357 (Prime et al., 1990), a human tongue OSCC line (Cat#06092004), were purchased from Sigma-Aldrich, Castle Hill, NSW, Australia. We used the human oral-derived squamous cell carcinoma cell line in our model because they are commercially available, easy to culture, and the results have high reproducibility, in analogy with the existing oral mucosa models (Bierbaumer et al., 2018). OKF6 (Dickson et al., 2000), immortalized normal human floor of the mouth keratinocyte, was provided by OHCRC, The University of Melbourne, Australia. This cell line was chosen based on previous research work, which showed that the OKF6/TERT cells are a reproducible and valuable model of normal oral epithelial cells because they compare primary oral keratinocytes in cytotoxicity studies or beta-defensin and inducible cytokine expression studies (Dongari-Bagtzoglou and Kashleva, 2003; Dongari-Bagtzoglou and Kashleva, 2003; Feucht et al., 2003; Dongari-Bagtzoglou et al., 2004). Cell lines and culture conditions details have been explained in sections (2.3.4 and 2.3.5).

3.3.2 Optimizing seeding density of cells

In preliminary experiments, the optimization of human oral keratinocyte cell number for FDA fluorescent assay in 96-well plates was experimented in a cell proliferation assay using monolayer cultures of oral keratinocytes (H400, H357, and OKF6) as described in section 2.3.8. Briefly, H400, H357, and OKF6 cells were sowed in 96-well plates at four different seeding densities (5×10^3 , 1×10^4 , 1.5×10^4 , and 2×10^4 cells/well). Cells were then incubated in a humid incubator overnight to allow cell adhesion to the plate, and subsequently studied over 72 hours. The replenishment of growth medium was performed every 24 hours. Fluorescent reading was performed and cell proliferation as a function of increasing fluorescence was measured every 24 hours at four different time points (24, 48, and 72 hours, respectively). Data were expressed as mean cell proliferation attributed to the FDA fluorescent intensity. Seeding density that still fall in the exponential phase from day-0 till day-3 was chosen as a standardized seeding density for each cell line. Cells confluence overtime was also

measured by inverted microscopy imaging (FLoid™ Cell Imaging Station, Cat#: 4471136, Life Technologies Australia Pty Ltd.) (section 2.3.6).

3.3.3 H₂O₂ treatment of cells (induction of oxidative stress)

A series of preliminary experiments to establish an *in vitro* oxidative stress-induced OM model by using H₂O₂, a well-known oxidative stress inducer (Gille and Joenje, 1992; Cross et al., 2015), was performed. The effect of H₂O₂ dose and duration inducing cellular injuries without completely compromising cell culture viability were evaluated in cell proliferation assays using monolayer cultures of oral mucosa keratinocytes (H400, H357, and OKF6 cell lines). Briefly, after the overnight incubation period following seeding, the cells were exposed to oxidative stress by incubating them with H₂O₂ for up to 24 hours. Various concentrations of H₂O₂ ranging between 100 and 1200 µM diluted in culture medium were tested over the given time points. The initial range of concentrations was determined based on existing literature (Lin et al., 2019; Hseu et al., 2019; Cirillo et al., 2015; Man et al., 2014; Royack et al., 2000). At each incubation period ends, morphological changes were assessed, and the cells were photographed with a FLoid cell imaging system (FLoid™ Cell Imaging Station, Life Technologies Australia) using white light and 20× magnification (section 2.3.9.2). Subsequent to treatment with H₂O₂, the cells viability/proliferation were measured by using trypan blue exclusion assay, fluorescence-based live-dead assay, and FCA to determine the best time and the most optimal concentration of H₂O₂ to induce cellular injuries.

3.3.4 Cell viability and growth assay

In a preliminary experimental phase, the effect of different H₂O₂ doses and duration on cell proliferation was assessed using monolayer H400 cultures, as described in section 2.3.9. Briefly, keratinocytes cells were seeded into 6-well plates (1×10⁵ cells/well) and grown in a humid atmosphere under standard conditions (5% CO₂, 37°C). At 60% confluence, cells were then incubated with increasing concentrations of H₂O₂ (0, 200, 400, and 800 µM) diluted in 1.5 mL of experimental medium and incubated for up to 24 hours at 37°C. To determine the H₂O₂ mediated cell death, the cell number and cell viability were counted chronologically in three-wells of each group at 2, 6 and 24 hours timepoints, by using trypan blue exclusion assay (TC10™ Automated Cell Counter, Bio-Rad, UK) (section 2.3.9.3). Control cells viability (without treatment) was deemed to be 100%, and H₂O₂-mediated cell death was evaluated by comparing treated cells' viability with the viability of the control cells. The IC₅₀ value was

defined as the concentrations of H₂O₂ that inhibited cell viability of treated cells to 50% compared to untreated control. This preliminary study aimed to characterize the best time and the optimal concentration of H₂O₂ to induce cellular injury.

3.3.4.1 Evaluation of epithelial cell viability rate with fluorescence-based live-dead assay

Selected H₂O₂ dosage range and duration, as determined by preliminary experiments described above, were further confirmed by assessing the effect of H₂O₂ on cell viability and proliferation in monolayer H400 cultures. Briefly, cells were incubated for 24 hours with increasing H₂O₂ concentrations (400, 800, and 1200 µM) by using the fluorescence-based live-dead assay. 24 hours after H₂O₂ treatment H400 cells were labelled with FDA and propidium iodide (PI) stains (two fluorescent dyes allow two-colour discrimination of the of living cells population from the dead-cell population) as described in section 2.3.10. All experiments in this phase were performed in technical triplicates.

3.3.4.2 Evaluation of epithelial cell viability rate with fluorometric cytotoxicity assay (FCA)

Subsequently, keratinocytes cells (H400, H357, and OKF6) were treated with increasing concentrations of H₂O₂ (100µM -1200 µM) for up to 24 hours, as described in section (2.3.11). Cell viability/proliferation was evaluated using FCA (Section 2.3.11.3) to determine the H₂O₂ mediated cell death. Control cells viability (without treatment) was deemed to be 100%, and H₂O₂ mediated cell death was evaluated by comparing treated cells' viability with the viability of the control cells. The IC₅₀ value was defined as the concentrations of H₂O₂ that inhibited cell viability of treated cells to 50% compared to untreated control. 3-5 technical replicates were used for each group/cell line.

3.3.5 Statistical Analyses

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 42 (IBM Corp., Armonk, N.Y., USA) and GraphPad Prism version 8.0.1 for Windows (GraphPad Software Inc, San Diego, CA). Cell viability data were expressed as mean percentage viability relative to the untreated control (100%) ± standard deviation (SD). Cell number data were expressed as mean ± SD. Data were presented as means ± SD, unless otherwise mentioned. Statistical analysis was conducted using a one-tailed paired Student's t-test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to detect inter-group differences. Multiple linear regressions were evaluated using the regression tool in Microsoft

Excel 2010 to identify collinearity among the independent variables. Differences were considered statistically significant if $p < 0.05$. “n” indicates the number of independent experiments.

3.4 Results

3.4.1 Optimization of cell number for cell viability/proliferation assay

To identify the optimal seeding density of oral keratinocytes cells using FDA fluorescent assay, cell number titration assay was conducted in 96-well plates using three cell lines (H400, H357, and OKF6). Fluorescent intensity over time and experimental replicates for each seeding density per each cell line were plotted using GraphPad Prism software to visualize exponential growth. On average, a cell culture density of 2×10^4 cells/well showed the highest fluorescence signal over time in all the investigated cell lines. In contrast, a seeding density of 5×10^3 cells/well showed the lowest fluorescence signal during all investigated time points (time 0, 24, 48, and 72 hours; Figure 3.1a). We observed a steadier proliferation of cells as the seeding density of primary cells increased. Furthermore, the amount of time that cells can grow exponentially also increased. (Figure 3.1a). We also noticed that the increase in the initial cell seeding density ensured that the confluence of the cells stayed below ~90% throughout the experiment, or that this confluence level was reached later in the test, and this trend was specific to the cell line. We found out that when cells were seeded at a concentration of 2×10^4 cells/well, full confluence was reached at 72 hours timepoint (Figure 3.1c). However, within the time point range 48-72 hours, the exact time when the cells became fully confluent was not assessed. Interestingly, despite the presence of mitotic activity, when cells were seeded at 5×10^3 cells/well, the confluence of the cell stayed below ~40% during the experiment (figure 3.1c). In case of the H400 cell line, cells showed exponential growth between 0-72 hours when seeded at densities of 1×10^4 , 1.5×10^4 , and 2×10^4 cells/well. Nevertheless, H400 cells seeded at 5×10^3 cells/well showed exponential growth until 48 hours, with a growth curve reduced thereafter (Figure 3.1a). In regard to H357 and OKF6, both cell lines grew exponentially between 0-72 hours when seeded at 1.5×10^4 and 2×10^4 cells/well. However, when seeded at 5×10^3 and 1×10^4 cells/well, both H357 and OKF6 cells showed exponential growth until they plateaued at 48 hours, after which they showed a growth decrease (Figure 3.1 a).

Subsequently, we investigated the correlation between viable oral keratinocyte cell number and fluorescence intensity over time (Figure 3.1b). This correlation proved to be linear for cells seeded at a concentration of to 2×10^4 cells/well with a correlation coefficient of $R = 0.9570$, 0.9471 , and 0.9685 for H400, H357, and OKF6 cell lines, respectively. A slight decrease of linearity was observed at cell concentrations below 2×10^4 cells/well; at an initial seeding

density of 1.5×10^4 , $R = 0.9433$, 0.9393 , and 0.9384 for H400, H357, and OKF6 cell lines, respectively; at initial seeding density of 1×10^4 , $R = 0.9507$, 0.845 , and 0.8127 for H400, H357, and OKF6 cell lines, respectively. The strength of linearity reached the lowest value at cell concentration of 5×10^3 ($R = 0.7396$, 0.2297 , and 0.4800 for H400, H357, and OKF6 cell lines, respectively (Figure 3.1b).

Based on the current findings, we concluded that, to ensure that:

- (1) the growth of the cells following an exponential pattern after seeding,
- (2) confluence is attained later in the experiment, and
- (3) a reliable linear response is proved even if the cells are doubling during the incubation time, the seeding density should be chosen based on the growth kinetics of the individual cell lines. In our subject, we found that plating densities of $1 - 2 \times 10^4$ cells/well for H400, $1.5 - 2 \times 10^4$ cells/well for H357, and $1.5 - 2 \times 10^4$ cells/well for OKF6 fulfil these criteria.

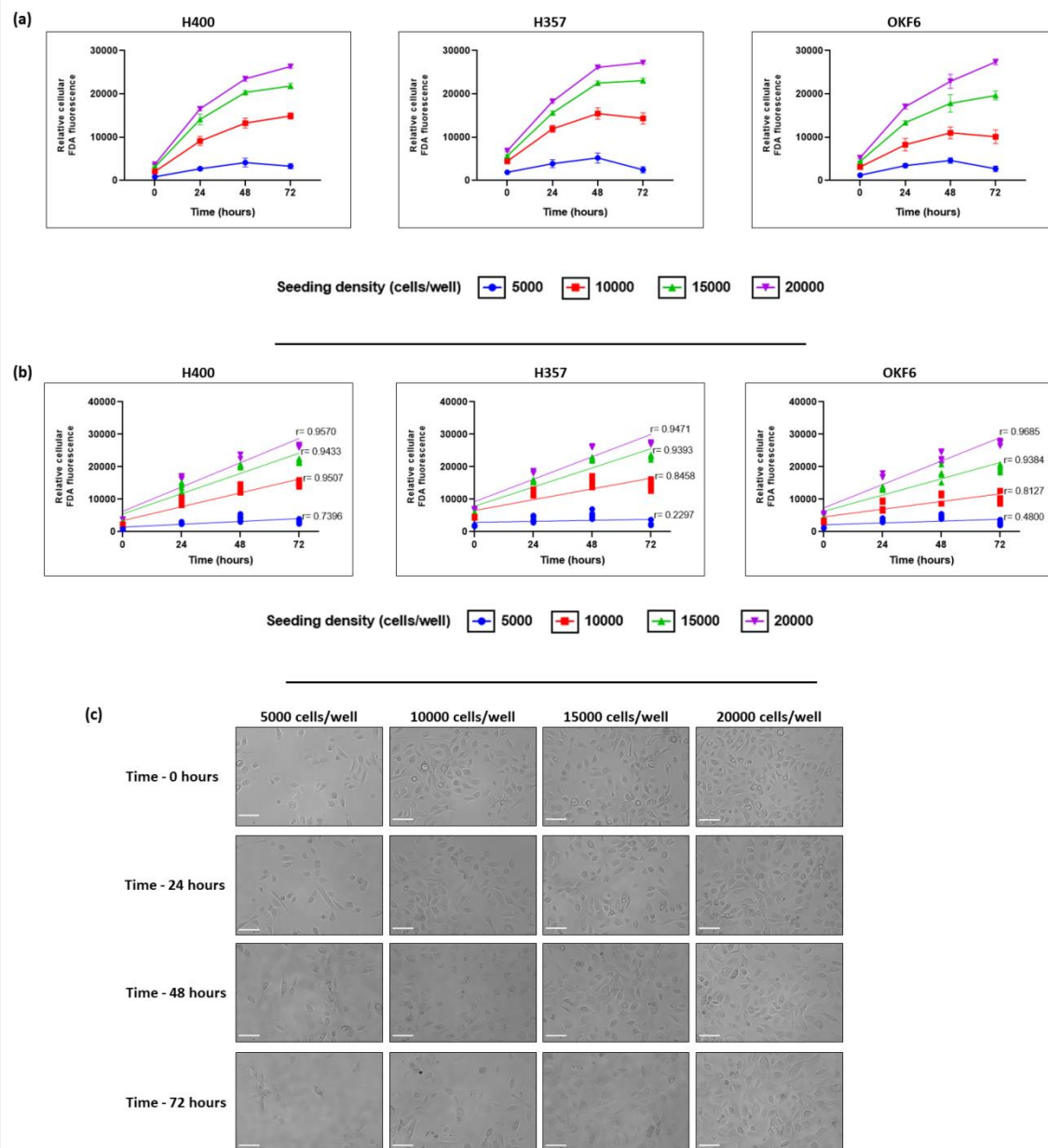


Figure 3.1 The proliferation of H400, H357, and OKF6 cells over 72 hours at varying cell seeding densities. Three monolayer cultures of oral mucosa keratinocyte (OSCC cell lines, H400 and H357, immortalized normal human oral keratinocytes, OKF6) seeded in 96-well plates at 5×10^3 , 1×10^4 , 1.5×10^4 , and 2×10^4 cells/well were grown under standard conditions (37°C , in a 5% CO_2 incubator). Cells were observed over 72 hours, replenishing culture medium every 24 hours. **(a)** Cell proliferation and viability at four different time points (0, 24, 48, and 72 hours) were assessed by fluorescein diacetate (FDA) fluorescence in Synergy HTX Multi-Mode Reader (Bio-Tek, USA) at the respective excitation and emission wavelengths of 485/20nm and 528/20nm. **(b)** Pearson correlation index between viable oral keratinocyte cell number and fluorescence intensity over time. **(c)** Representative micrographs of OKF6 cells showing confluence over time. Images (FLoid cell imaging system, Life Technologies) with magnification at 20x. A scale bar of 100 μm applies to all panels. All

measurements were performed in triplicate with mean $\pm$ SD for 5 technical replicates per cell line.

3.4.2 H₂O₂ inhibits epithelial cell proliferation in a time- and dose-dependent manner

In a preliminary experiments series that was designed to establish an *in vitro* model of OM induced by oxidative stress by using H₂O₂, we initially utilized monolayer cultures of epithelial cells (H400 cells) to characterize a duration and concentration range of H₂O₂ treatment that can inhibit the proliferation of the cells and induce oxidative cell injury without ultimately compromising the viability of cell culture. (Figure 3.2a). H-400 cells were incubated for 2 hours, 6 hours, and 24 hours with three different concentrations of H₂O₂ (200 μ M, 400 μ M, and 800 μ M), and then each of the important elements, such as time of incubation and dose H₂O₂, were validated by determining the best time and the optimal concentration of H₂O₂ to induce cellular injury. As reflected by the trypan blue exclusion test, the results displayed that cell viability decreased after incubation with H₂O₂, and this was correlated with treatment duration and concentration. Comparing with the untreated control group, H400 cells treated with 200 μ M, 400 μ M, and 800 μ M H₂O₂ showed a decreasing trend in cell viability after 6 hours, and 24 hours, but not after 2 hours. In contrast, after 2 hours exposure to increasing H₂O₂ concentrations (200, 400 and 800 μ M), H400 cell viability was even slightly increased. Nonetheless, the increase was not significant as compared to control ($p > 0.05$; figure. 3.1a). A marked decrease in cells survival observed after 24 hours of incubation with 400 μ M and 800 μ M H₂O₂, as compared to control ($62.99\% \pm 8.32$, $p < 0.005$, and $47.13\% \pm 4.88$, $p < 0.001$, respectively; Figure 3.2a).

The effect of H₂O₂-mediated oxidative stress on H400 proliferation was also investigated (Figure 3.2b). A gradual increase in cell numbers was observed in the controls and samples treated with 200 μ M H₂O₂ overtime. Conversely, treatment with 400 and 800 μ M H₂O₂ induced a time-dependent biphasic effect during cell proliferation. In fact, at these concentrations, H400 cell treatment showed an initial increase in proliferation until 6 hours after treatment, whereas at 24 hours this trend was reversed with a marked decrease in cell numbers compared with the control ($p < 0.05$) (Figure. 3.2b). Furthermore, assessment of the correlation between total cells number and H₂O₂ dose over time, a strong negative correlation between total cells number and H₂O₂ dose at time 24 hours timepoint was observed. This correlation proved to be a statistically

significant with a linear relationship and a correlation coefficient of $R = -0.9277$ ($p < 0.001$) (Figure 3.2c).

Based on our results, we conclude that exposure of H400 cells to H_2O_2 induces cellular damage through oxidative stress and reduces the viability and proliferation of cells in a manner depending on time and concentration. We identified $800\ \mu M$ as the concentration of H_2O_2 that at 24 hours was able to inhibit H400 cell viability to a proximate level of the IC_{50} . Hence, a range of concentrations close to $800\ \mu M$ and the 24 hours timepoint were furtherly explored in subsequent experiments establishing the oxidative stress-induced injury model in H400 cells.

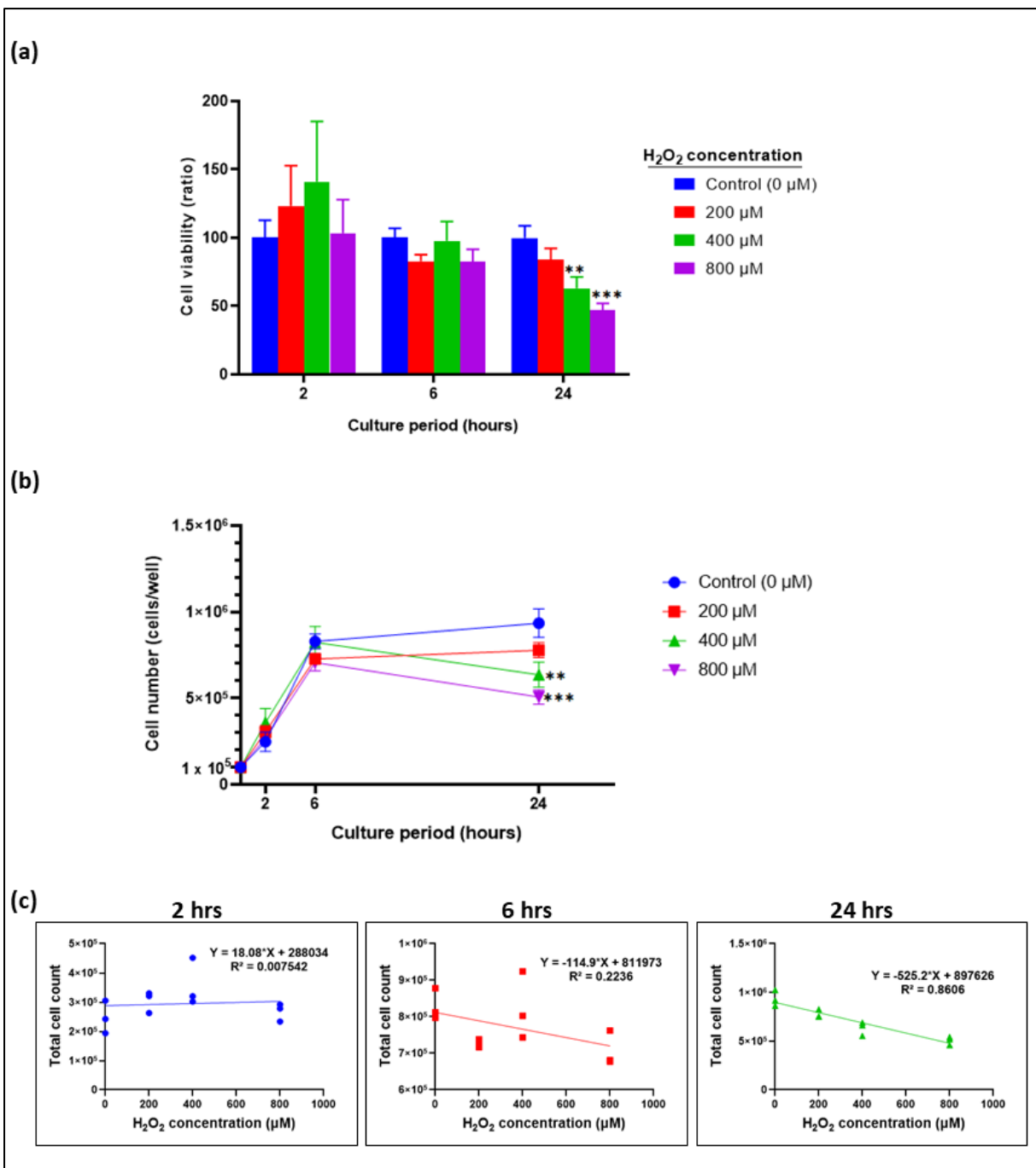


Figure 3.2 Effect of H₂O₂ treatment on the cell growth of H400 cell. (a) : H400 cells were a subject to increasing concentrations of H₂O₂ for indicated times. The viability of cells was measured at time points by using a trypan blue exclusion test using a TC10™ automated cytometer (Bio-Rad). Bars indicate mean cell viability ratios in H₂O₂-treated over untreated cells. Error bars depict one standard deviation from the mean of three technical replicates. Statistical significance is given as follows: **p<0.005 and ***p<0.001 as compared to control. (b): H400 cultures were incubated with increasing concentrations of H₂O₂ for the times indicated and cell count was measured by utilizing a TC10™ automated cytometer. Data are represented as mean cell number ± SD of three technical replicates. Statistical significance is accustomed as follows: **p < 0.005 and ***p < 0.001 as compared to control. (c): Pearson correlation index between total H400 cells number and H₂O₂ dose over time.

3.4.3 Cytotoxic effect of H₂O₂ on oral keratinocytes

Some limitations of this study involved the use of a viability test that excludes certain dyes by means of a live cell membrane. Although trypan blue exclusion is one of the commonly used tests for cell viability, it is a time consuming and expensive test. Additionally, given that the number of stained cells tends to vastly increase overtime after the addition of the dye, we were forced to count cells unceasingly within 3-5 minutes after the dye was administered to the experimental plates. Particularly while counting a large number of samples, it was extremely inappropriate to perform all tests on the same day when counting each cell suspension before staining the following sample. Therefore, there was a need for a more reliable and rapid method of distinguishing live and dead cells. The FDA staining test is a better viability test given that identifies live cells rather than dead cells, as it stains only living cells. While the FDA selectively stained live cells, impaired and dead cells were stained with propidium iodide (PI), an ethidium bromide (EB) analog. Therefore, in a subsequent experiment, we tried to validate the results obtained from previous experiments using a simultaneous double-staining procedure with FDA-PI stains.

We found that FDA-stained cell identification was clear, with live cells stained glowing green and non-viable cells stained glowing red when investigated under an inverted fluorescence microscope with the filter set on the FITC and Texas Red fields for the FDA and PI detection, respectively (Figure 3.3a). Subsequently, the oxidative effect of H₂O₂ was evaluated (Figure 3.3b, and 3.3c). Using FDA fluorescence, we demonstrated that exposure of H400 cells to 800 and 1200 μ M H₂O₂ for 24 hours induced a dose-depending decrease in the viability of cells (Figure 3.3b). In particular, incubation with 800 μ M H₂O₂ for 24 hours decreased cell viability by about 50% when compared to control cells ($43.1\% \pm 8.45$, $p < 0.05$). Furthermore, a strong negative correlation between cell viability ratio and H₂O₂ dose after 24 hours of H₂O₂ incubation was noticed ($R = -0.9033$, $p < 0.001$; Figure 3.3c). These findings were consistent with our previous findings while using trypan blue assay.

To conclude, we found that the FDA-PI staining is a more reliable, rapid, and cost-efficient, method to distinguish live cells when compared to trypan blue exclusion. FDA-PI assay was therefore chosen for subsequent experiments investigating the H₂O₂-mediated toxicity in other cell lines.

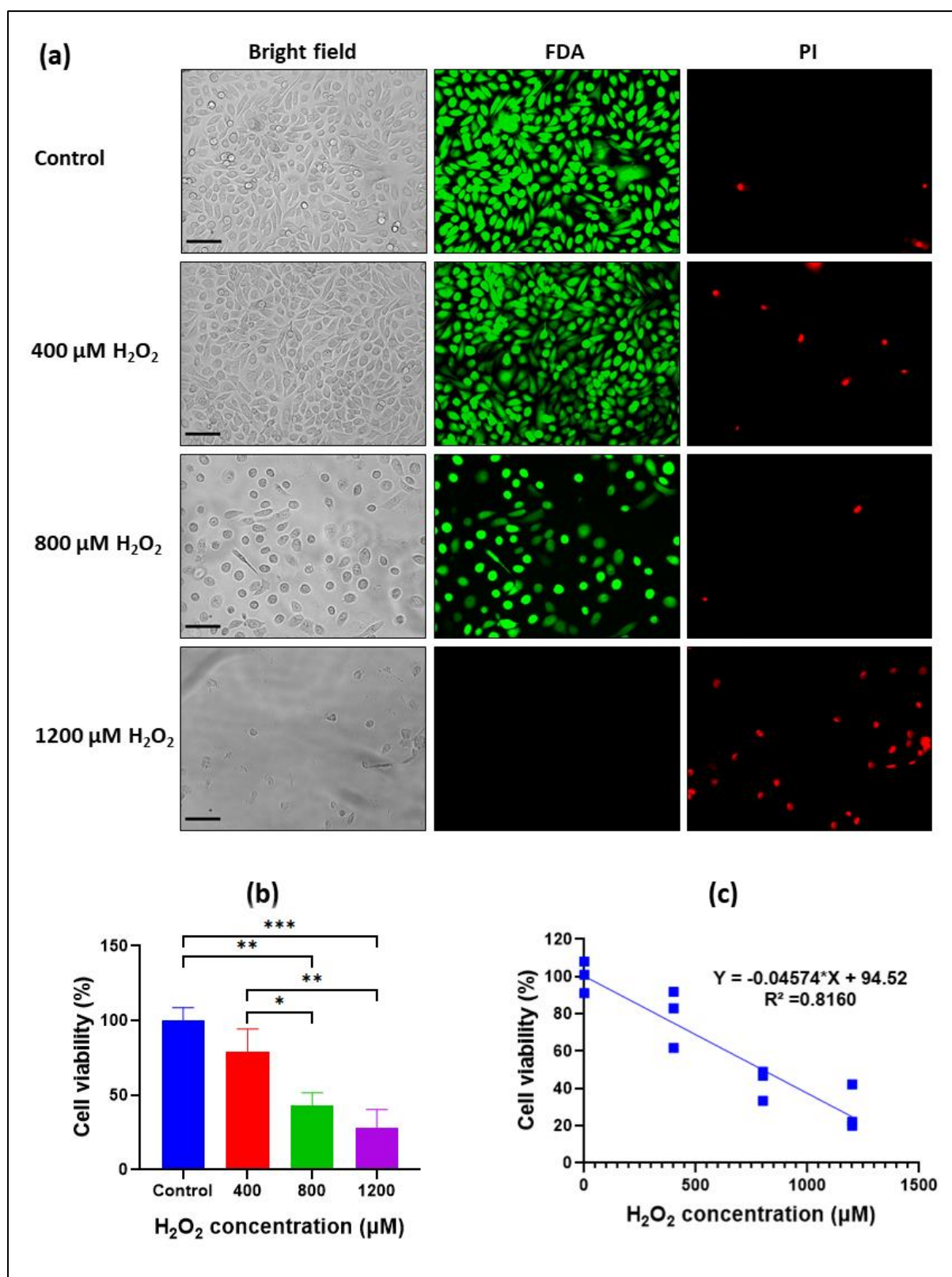


Figure 3.3 H_2O_2 -induced cell death in oral keratinocytes. Fluorescein diacetate (FDA) and propidium iodide (PI) staining of H400 cells that exposed to increasing concentrations of H_2O_2 over 24 hours stained. Cell viability was quantified after 24 h by inverted fluorescence microscopy (FLoid™ Cell Imaging Station, Life Technologies Australia). Fluorescence of vital cells was measured in six different random fields/well per treatment in three technical

replicates. Captured images were transferred to a computer and investigated to count stained vital cells utilizing Image J software (ImageJ v. 1.50i, National Institutes of Health). **(a)**: Representative micrographs (20x) of H400 cells treated with H₂O₂ showed vitality staining of the cells (from left to right: Bright-field, FDA signal, and PI signal). The same field is displayed in each image filter. The scale bar denotes 100 μ m and used to all panels. **(b)**: Mean cell viability ratios in H₂O₂-treated over untreated H400 cells. Error bars depicts one standard deviation of the mean from three technical replicates. **(c)**: Pearson correlation index between cell viability ratio and H₂O₂ dose after 24 hours of H₂O₂ incubation. Data are displayed as mean $\pm$ SD of three technical replicates. *p < 0.05, **p < 0.005 and ***p < 0.001.

3.4.4 Establishment of an oxidative stress-induced mucosal injury model with H₂O₂-treated oral keratinocyte cells

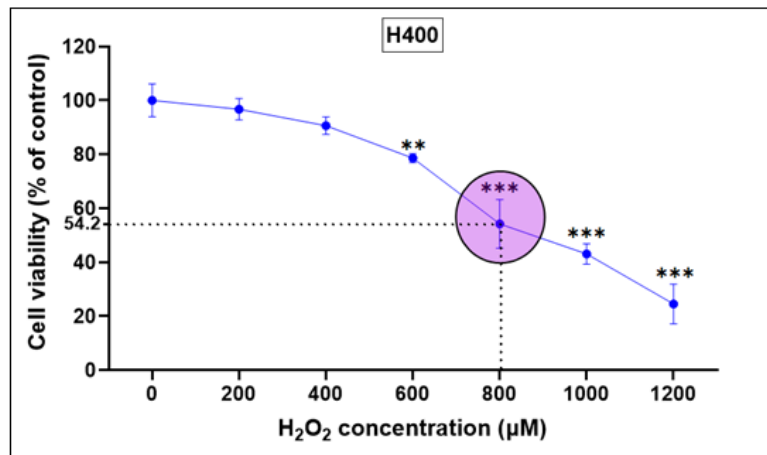
In a previous series of preliminary experiments we determined the optimal experimental conditions, including cell seeding densities, a range of concentrations for H₂O₂, and incubation time to establish an free radical damage-induced model in monolayer cultures of oral keratinocytes. In a subsequent series of experiments, we established an H₂O₂-induced oxidative stress model using three monolayer cultures of oral mucosal keratinocyte derived from cancer (H400, and H357), and normal (OKF6) oral epithelial cells to emulate what is thought to occur OM induced by chemoradiation therapy. Based on the findings from the preliminary experiments, oral epithelial cells were incubated with a broad range of increasing H₂O₂ concentration (100-1200 μ M) for 24 hours. The *in vitro* effects of H₂O₂ on H400, H357, and OKF6 cells growth and viability were examined after 24 hours using FCA by using FDA stain. Cells incubated with only a growth medium were used as control and the fluorescence density was in proportion to the number of viable cells. Results showed that administration of H₂O₂ clearly decreased cell viability in H400 cells after 24 hours in a concentration-dependent manner over the examined ranges (200-1200 μ M H₂O₂). Loss of cell viability after 24 hours of exposure to H₂O₂ was remarkably lower in H400 cells than in untreated control cells for H₂O₂ concentrations of 600 μ M and higher (Figure 3.4a). Similarly, the administration of increasing concentrations of H₂O₂ (ranging from 100-1300 μ M H₂O₂) was able to markedly reduce cell viability after 24 hours in both H357 and OKF6 cell lines. This effect was significantly different compared to control untreated cells for concentration at 400 μ M and higher, and 100 μ M and higher for H357 and OKF6 cells, respectively (Figure 3.4 b, and 3.4c).

Similarly, administration of increasing concentrations of H₂O₂ (ranging from 100-1300 μ M H₂O₂) was able to markedly reduce cell viability after 24 hours in both H357 and OKF6 cell lines. This effect was significantly different compared to control untreated cells for concentration higher than 400, and 100 μ M for H357 and OKF6 cells, respectively (Figure 3.4 b, and 3.4c).

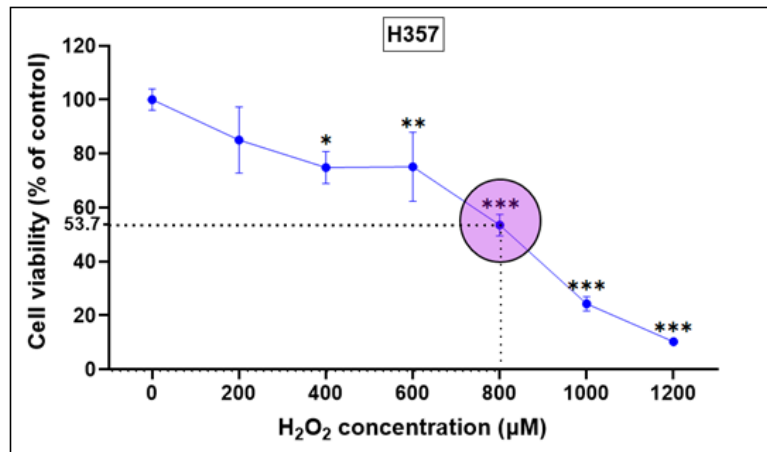
The calculated IC₅₀ values following 24 hours exposure to H₂O₂ were approximately 800, 800 and 400 μ M for H400, H357, and OKF6 cells, respectively (Figure 3.4 b, and 3.4c). A concentration close to the IC₅₀ values for the three cell types (800 μ M for H400 cells; 800 μ M for H357 cells; 800 μ M for OKF6 cells) were considered to be the most appropriate

concentrations of H₂O₂ to induce cellular injuries in oral epithelial cells and were chosen for successive experiments to investigate H₂O₂-mediated toxicity.

(a)



(b)



(c)

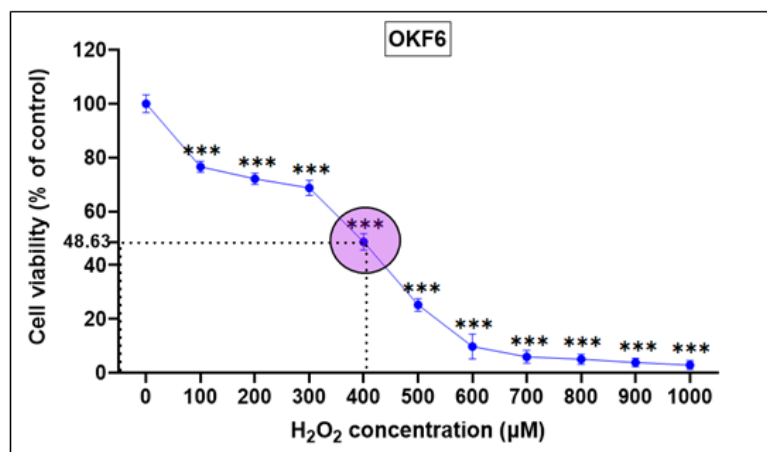


Figure 3.4 H₂O₂-induced loss of cell viability in oral cancer and oral normal epithelial cells. H400 (a), H357 (b), and OKF6 (c) cells, were seeded at densities of 1.5×10^4 in 96-well plates and incubated for 24 hours with increasing concentrations of H₂O₂. Cell viability (a, b,

and c) after 24 hours of incubation with H₂O₂ was measured by (a) fluorescein diacetate (FDA) fluorescence in a Synergy HTX Multi-Mode Reader (Bio-Tek, USA) as described in the Materials and Methods section. The data are displayed as the relative response of treated cells as compared to untreated controls (100%) and represent means $\pm$ SD from at least three technical replicates of 1-3 biological replicate experiments. Statistical significance is accustomed as follows: * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.005$ as compared to untreated controls.

3.4.5 Effect of H₂O₂-induced oxidative stress on morphological changes in oral keratinocyte cells

To evaluate the H₂O₂ impact on cell morphology, H400, H357, and OKF6 cells were treated with a wide range of H₂O₂ concentrations (100-1200 μ M) for up to 24 hours. Cell morphology was assessed in bright field micrographs (Figure 3.5). Morphological observation of untreated cells showed that the cells retained their original polygonal morphology and were stably adherent to the culture plates. In contrast, cells treated for 24 hours with H₂O₂ at concentrations $\leq$ 600, 400, and 100 μ M, for H400, H357, and OKF6 cells, respectively displayed morphological alterations compatible with apoptotic cell death. These features included cell shrinkage, blebbing, condensation and fragmentation of nuclear chromatin, and formation of apoptotic bodies. In addition, affected cells also displayed other types of morphological alterations, such as loss of polygonal shape at the expense of a round appearance, loss of adhesion with neighboring cells, tendency to remain as single cells rather than forming cell colonies, and loss of adhesion from the culture plates (Figure 3.5, and 3.6a, and 3.6b).

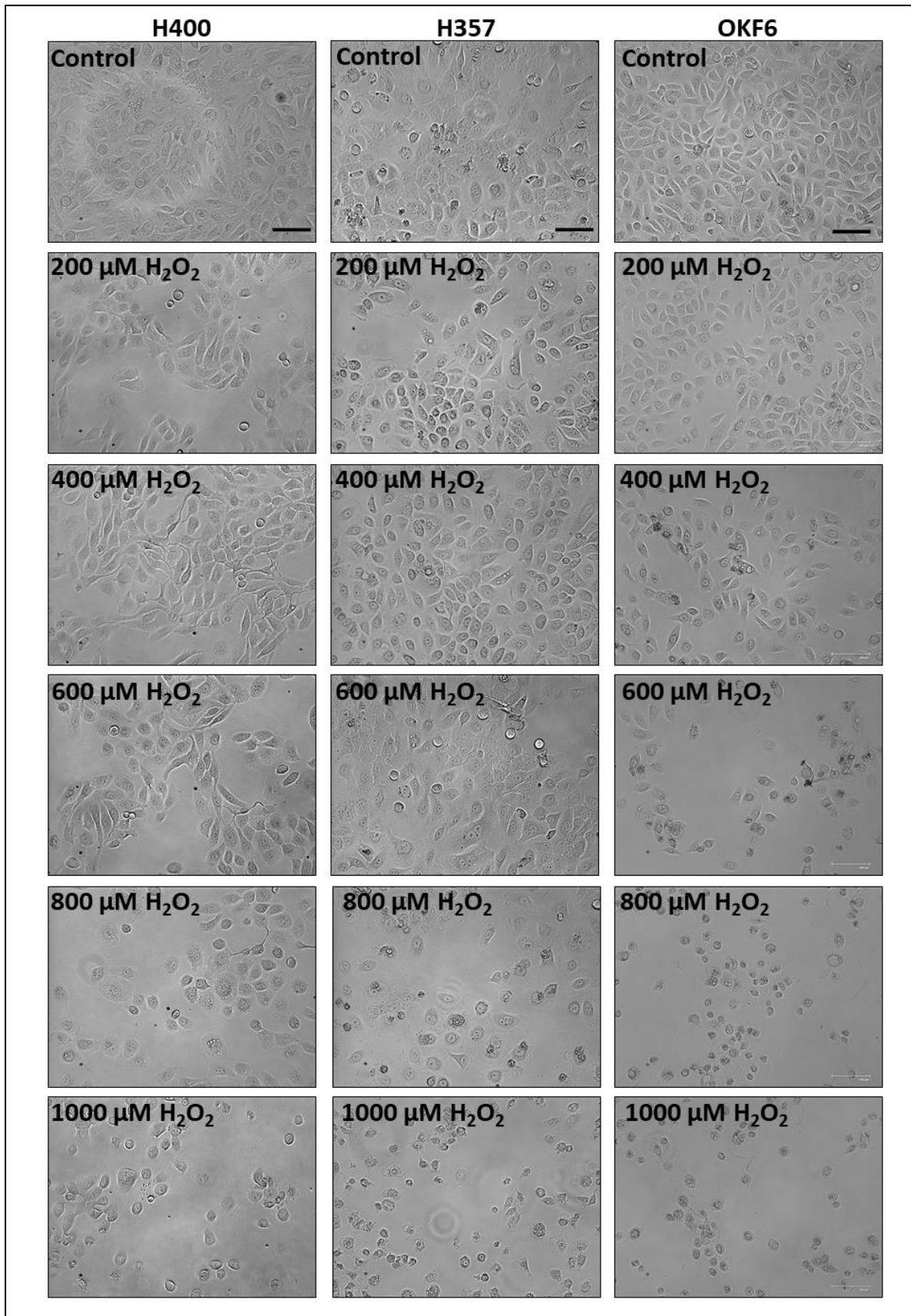


Figure 3.5 Effect of H_2O_2 on the morphology of the cells. Representative micrographs of oral keratinocytes treated with H_2O_2 . H400, H357, and OKF6 cells were exposed to H_2O_2 with various increasing concentrations (100-1200 μM) over 24 hours. By the end of the incubation

period, morphological changes were assessed, and images were acquired utilizing fluid cell imaging (FLoid™ Cell Imaging Station, Life Technologies Australia) with filter sets on the bright field at 20× magnification. Scale bar represents 100 μm and applies to all panels.

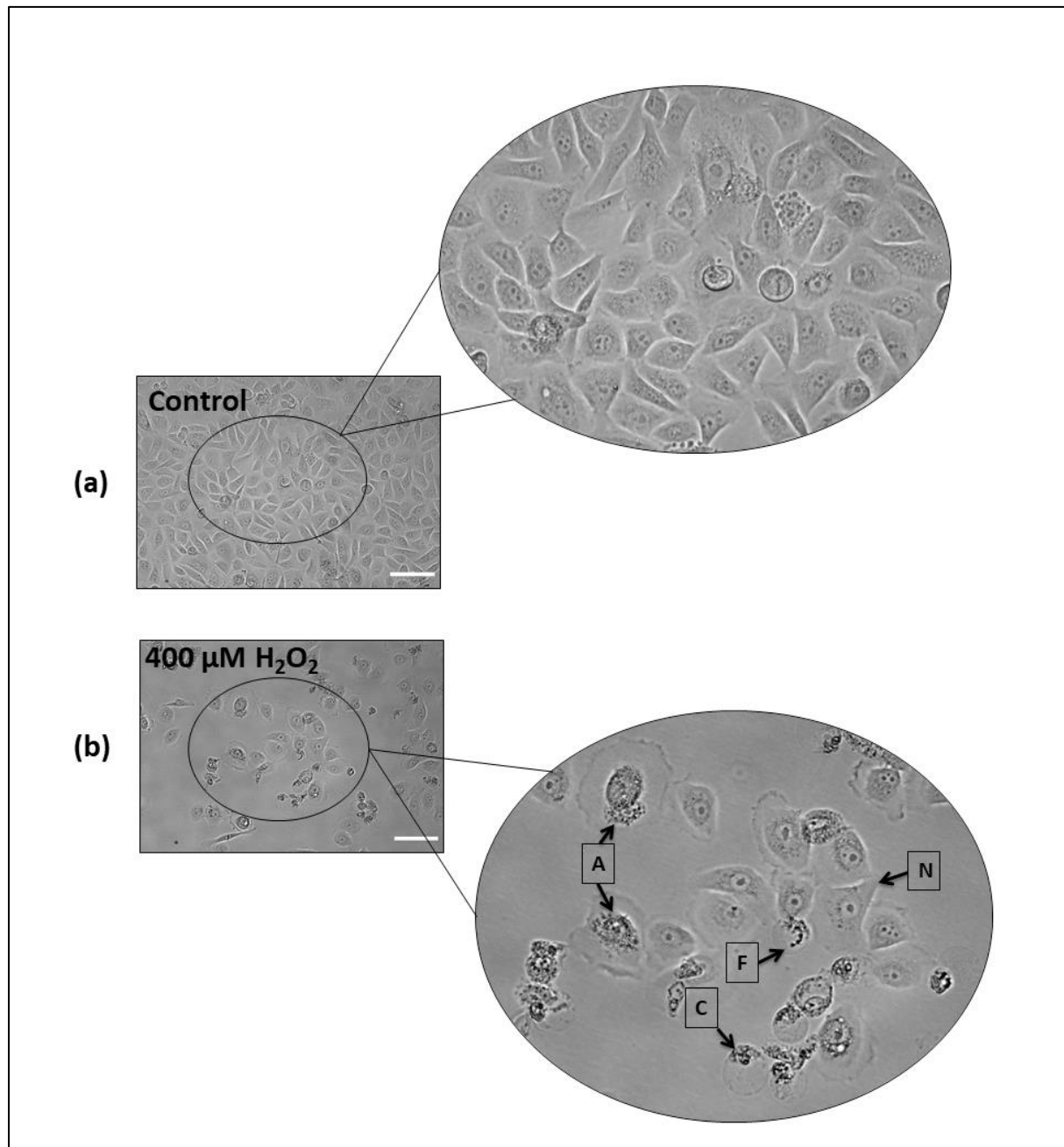


Figure 3.6 H₂O₂ induced morphological alteration consistent with apoptotic features in oral epithelial cells. Bright field representative micrographs (20x) of OKF6 cells. **(a):** Untreated (control) OKF6 cells retained their original morphology and were stably adherent to the culture plates. **(b):** OKF6 cells after 24 hours treatment with an IC₅₀ value of H₂O₂ (400 μM) displayed obvious apoptotic features, labelled with black arrows: A denotes apoptotic cells; F denotes fragmented nucleus; C denotes condensed nucleus; N denotes normal cell). Scale bar denotes 100 μm and display to all panels.

3.5 Discussion

The molecular mechanisms underlying CT and RT -induced OM involves ROS production at early stages and consequent redox imbalance pathways activation (Cirillo et al., 2015; Yoshino et al., 2013; Sonis, 2010a; Criswell et al., 2003), we, therefore, wanted to establish a new experimental model of oxidative damage-induced OM *in vitro* in which cellular activities and new therapeutic interventions could be characterized. Given that the H₂O₂-induced oxidant model has been widely utilized to study the response of cells to oxidative damage (Gille and Joenje, 1992), here we successfully developed a novel *in vitro* free radical damage-induced OM model using H₂O₂ to treat human oral epithelial cell lines. Specifically, in the current study, we studied the cytotoxic impact of H₂O₂ on the proliferation, viability and apoptosis of oral keratinocytes and our results showed that H₂O₂ led to an anti-growth effect on H400, H357, and OKF6 cells by both cell proliferation suppression and cell apoptosis induction. This *in vitro* model of H₂O₂-induced OM allowed for the detection of the alterations in cellular activities in a culture of oral keratinocytes, excluding other submucosal cellular components.

OM is a complex disease process involving the effects of CT and radiation on keratinocytes and other types of cells in the submucosa, like endothelial cells and fibroblasts (Sonis, 2004b; Sonis, 2007; Yeoh et al., 2006). To date, the exact underlying mechanisms of the development of OM have not been entirely clarified (Bertolini et al., 2017; Al-Dasooqi et al., 2013; Lalla et al., 2008). The absence of an appropriate *in vitro* laboratory model for OM could be a part of the possible difficulties in understanding the exact mechanism underlying the development of this condition. Monolayer cultures from oral keratinocytes have been widely utilized to mimic a specific part of the oral mucosal epithelial barrier (Abbott, 2003; Bierbaumer et al., 2018). Recently, the HaCaT, an untransformed human immortalized keratinocyte cell line, (Boukamp et al., 1988) has been utilized more widely to model oral mucosa and to compare with head and neck squamous cell carcinoma (Chen et al., 2011; Wu et al., 2012; Bell et al., 2012; Maggioni et al., 2013; Seto et al., 2013; Uchida et al., 2014; Chen et al., 2016), whereas immortalized oral OKF6 keratinocytes (Dickson et al., 2000) have been sparingly used to model OM (Shrivastava and Deshmukh, 2013). In our model, we also used the human OSCC cell lines (H400 and H357), in analogy with the existing oral mucosa models (Bierbaumer et al., 2018). Moreover, these cell lines are currently one of the best-characterized oral epithelial cell lines available, easy to culture, and generate highly reproducible results (Prime et al., 1990). However, our results show that the model can also be applied successfully using immortalized

oral keratinocytes (OKF6) cell lines. Hence, this cell line was chosen for successive experiments to generate epithelial models of the oral mucosa.

In addition to ethical and cost relations, *in vitro* models' advantages over animal models include the ability to monitor the mucosal epithelium's rapid and dynamic biological responses to stress injury induced by oxidation. Recently, several models have been invented where CT and RT impact on the proliferation of oral keratinocytes have been tested to reiterate the observed *in vivo* epithelial changes associated with mucositis (Sonis et al., 2017; Tschachojan et al., 2014; Tobita et al., 2010; Lambros et al., 2011). By comparison, in our model we used H₂O₂ to induce the oxidative stress-induced cellular injury. Although this model can be utilized to ask focused mechanistic questions about the aetiology of OM and the role of interventions in disease prevention, it should be acknowledged that one of the limitations of this study is the use of the oral keratinocytes monolayer cultures which represent 2D cultures and do not accurately represent what happens *in situ* when cells live in 3D (Abbott, 2003). In fact, using a 3D model of OM would add more to the study and provide a better organotypic model. Compared to 2D monolayer cultures, the 3D oral mucosal tissue models offer many advantages. Most importantly, the 3D models more accurately reproduce the structure, function, and underlying gene and protein expression of native mucosal tissues. However, despite these advantages of 3D tissue models, 2D systems are much less expensive and more amenable to high-throughput screening and hence are useful for initial studies related to drug screening or studying isolated reactions or pathways (Klausner et al., 2021). Furthermore, Thus far, organotypic models of OM have relied on cost-prohibitive commercially available constructs (Sonis et al., 2017) or primary epithelial cultures, which vary in life span and other phenotypic characteristics and are associated with ethical considerations for harvest (Tobita et al., 2010). Other limitation of this study is the lack of different cell populations oral mucosa, such as the lack of connective tissues and the lack of endothelial cells. Literature tested the effect of compounds In OM models *in vitro* using normal human oral fibroblasts (Cirillo et al., 2015; Picciolo et al., 2022). However, the main rationale for the *in vitro* choice of this study was the selection of normal human oral keratinocytes, as they are the main actors in the mucositis pathological processes, where epithelial injury is the main contributor to the clinical symptoms of mucositis. Furthermore, OM is a condition where complex multi factors are included in its pathogenesis, which cannot be entirely optimized *in vitro*, including local and systemic factors (e.g., oral microbiota, xerostomia, neutropenia).

Although the use of H₂O₂-induced- oxidative stress model on oral keratinocytes is a simplification of the pathogenesis of mucositis, in vitro cell culture and an oxidative stress model is a well-established methodology to mimic oxidative stress-induced cellular injury models in vitro and has been widely used in the literature (Giri et al., 2022; Lee et al., 2022; Ying et al., 2022; Velayutham et al., 2021; Zoico et al., 2021; Yiannis et al., 2020; Lin et al., 2019; Man et al., 2014).

In the current study, the cytotoxic potential of H₂O₂ on oral keratinocyte cell lines was assessed by a trypan blue exclusion test, fluorescence-based live-dead assay, and FCA. Trypan blue exclusion test is the commonly utilized test of cell viability. However, blue-stained cells numbers increases over time after dye addition, so cells should be counted within 3-5 minutes after dye staining (Jones and Senft, 1985). Additionally, when many samples had to be stained and calculated, counting the cell suspension in triplicate required much time and a large number of counting slides to perform all the experiments on the same day. Therefore, this method was considered time-consuming and expensive. A more reliable and fast contrasting live and dead cells method was used in the following experiments. Since the late 1980s, the FCA test has been used in semi-automated 96-well setups for a long time, and several publications have demonstrated its advantages in drug development using cancer cell lines (Dhar et al., 1996; Lindhagen et al., 2007; Wickström et al., 2008).

The FCA belongs to a non-clonogenic microplate-based tests group that measures the total density of living cells after a short period of incubation (Larsson et al., 1990; Larsson et al., 1992). FCA applications are analogous to the widely used MTT colorimetric assay with slightly different endpoints (Blumenthal, 2005); In healthy cells where the plasma membrane is intact, FCA measures the cells esterase activity by measuring the fluorescence produced when the non-fluorescent FDA probe is hydrolysed (Rubinstein et al., 1990; Larsson et al., 1992). FCA becomes a more sensitive test when compared to the MTT assay (Larsson et al., 1990).

Optimization of the influence of experimental parameters on cell viability is crucial for data reliability regardless of the model type (e.g., 2D monolayers, 3D models, or in vivo models) used to evaluate drug sensitivity (Eilenberger et al., 2018; Larsson et al., 2020). Here, we pinpointed sources of experimental variability in conventional 2D cell-based oxidative stress screens to determine the effect of confounders on cell viability for the oral epithelial cell line treated with H₂O₂ (a well-known oxidative stress inducer).

In the current study, we describe several biological (cell type and seeding density) and technical (drug type, dose, and treatment time) factors that should be taken into consideration to achieve more reliable and reproducible drug-dose sensitivity screens in 2D models. Researchers conventionally use the 50% inhibitory concentration (IC₅₀) to determine drug potency with cell-based cytotoxicity tests. However, drug treatment time differs frequently between studies, ranging from minutes to days (Larsson et al., 2020). Here, we conducted drug sensitivity screening for 2, 6, and 24 hours and observed that IC₅₀ for H400 cells was reached after 24 hours of exposure to 800 μM of H₂O₂. As expected, the variation in cell viability was associated with the choice of dose and treatment time (Hafner et al., 2016). Therefore, 24 hours incubation time was chosen for successive experiments to investigate H₂O₂-mediated toxicity. In agreement with previous studies, we demonstrated that cell line, drug dose, and treatment time substantially affected cell viability (Hatzis et al., 2014; Hafner et al., 2016; Niepel et al., 2019).

In our study, a broad range of concentrations of H₂O₂ (100-1200 μM) were tested to assess the toxic effects of persistent exposure to H₂O₂ at the viability and growth of oral keratinocyte cells. Our data show that H₂O₂ was able to exert a induction and concentration-dependent toxic effect on the tested epithelial cell lines. In fact, a significant change in cell survival was shown after 24 hours incubation of H400 with 400 μM H₂O₂ or higher. H400, H357, and OKF6 cells exposed to various H₂O₂ concentrations for 24 hours resulted in a marked decrease in cell survival which was dose-dependent. The IC₅₀ value in the H400 cells, H357 cells, and OKF6 cells were approximately 800, 800, and 400 μM, respectively, based on FCA assays. These findings are consistent with other previous observations suggesting that H₂O₂ concentration-dependently and time-dependently promoted damage in different normal and malignant cells lines (Datta et al., 2002; Liu and Zhao, 2014; Cirillo et al., 2015; Zhou et al., 2012; Moll et al., 2007). However, the approximate IC₅₀ value of H₂O₂ in our study after 24 hours exposure was higher than the IC₅₀ (150 μM) appraised by Zhou et al. utilizing HTR-8/SVneo cells (Zhou et al., 2012). In a separate study, Moll et al. (2007) used H₂O₂ concentrations of up to 1000 μM to assess human term placentas apoptosis and proliferation. Furthermore, Murata et al. (2013) used a 100 μM H₂O₂ for 24 hours to measure invasion rates and apoptosis in a mature extravillous trophoblast cell line. The variability in the results of these previous studies could be postulated to variability in the studied cells' sensitivity to H₂O₂.

H₂O₂ dose-dependently increased dead cell numbers based on trypan blue exclusion test, and propidium iodide (PI)-positive cells in the H400 cells, suggesting that H₂O₂ induced epithelial cell death occurred via apoptosis. H₂O₂ induced apoptotic cell death in tested epithelial cell lines, was revealed from morphological studies (PI staining and phase contrast). Apoptosis is one of the most important objectives for cancer therapy (Kang et al., 2007). Apoptosis of dying cells is characterized by particular morphological and biochemical alteration (Kerr et al., 1972). Changes include cell shrinkage, nuclear condensation and fragmentation, blebbing of the cell membrane, apoptotic bodies formation, in addition to the loss of cell attachment to neighbours are common features of apoptotic cells (Acebedo et al., 2014). In this study, three different types of assays were achieved to prove the cell death mode induced by H₂O₂ against H400, H357, and OKF6 cells: trypan blue exclusion, phase contrast and fluorescence-based live-dead assay. Exclusion of trypan blue is the most usual test that can exclude live cells from non-viable cells (Jones and Senft, 1985). Trypan blue exclusion study showed that H₂O₂ induced apoptosis in H400 OSCC cells through a shift in live cell population proportional to increasing H₂O₂ concentrations from 2 to 24 hours. One of the best methods to determine cell apoptosis is the morphological analysis through phase contrast and fluorescence inverted microscopy (Doonan and Cotter, 2008). Following our observation, noticeable morphological changes of apoptosis in treated cells included loss of cellular adhesion to their neighbours, membrane blebbing, and chromatin condensation and fragmentation along with the creation of apoptotic bodies. FDA stain was used in conjugation with propidium iodide to determine apoptotic cells after the cell membrane integrity loss, and outcomes showed nonviable cells stained bright red.

In summary, we have successfully created an *in vitro* model of mucositis induced by free radical oxidative damage using H₂O₂ based on human oral epithelial cell lines (H400, 357, and OKF6) and by evaluating cytotoxicity, and morphological changes induced by H₂O₂. The study demonstrated inhibition of H400, H357, and OKF6 cells proliferation, and induction of marked apoptotic morphological changes. The oxidative stress-induced model in OKF6 cell line was chosen for the subsequent experiments. Further research to understand the cellular apoptosis signalling pathways will help to understand better the toxic effect of exogenous H₂O₂ on epithelial cells. This might include evaluating the caspase pathway and other protein signalling pathways such as p43 protein via proteomic assays and western blot test.

3.6 Conclusion

In conclusion, we found that exposure of the oral epithelial cell lines to H₂O₂ for up to 24 hours exerted a cytotoxic effect on the tested cell lines. This toxic effect was found to be time- and dose-dependent. H400, H357, and OKF6 cells exhibited cell proliferation inhibition and marked apoptotic morphological changes. Our oxidative stress model based on human oral epithelial cell lines was successfully established, with the optimal concentration of H₂O₂ being 800, 800, and 400 µM for H400, H357, and OKF6 cells, respectively, and the treatment period being 24 hours. OM established in the OKF6 cell line was chosen for the subsequent experiments.

**CHAPTER 4 DETERMINING THE
EFFECTS OF HYALURONIC ACID
DERIVATIVES IN THE
REGULATION OF EPITHELIAL
CELL PROLIFERATION *IN VITRO***

4.1 Abstract

Background: Hyaluronic acid (HA) is a naturally occurring non-immunogen, biodegradable, and biocompatible linear polysaccharide with unique viscoelastic and hygroscopic properties. HA preparations are widely used in treating several diseases and have proven to be beneficial in clinical practice to accelerate wound healing. Several reports have recently shown the potential efficacy of HA's topical application in the prevention and treatment of OM. However, the underlying mechanisms of beneficial efficacy remain unclear, specifically, if HA can directly affect oral keratinocytes. This study aimed to determine whether HA directly affects the proliferation of oral epithelial cells.

Materials and Methods: Immortalized human normal oral keratinocytes cells, namely OKF6, were grown with increasing concentrations of five different products containing HA for 24, 48, and 72 hours. HA derivatives used were Mucosamin® (1%, 5%, 7% and 10% v/v), native H-MW-HA(0.01%, 0.03%, 0.05%, 0.07% and 0.1% w/v), and cross-linked (xl-) HA (0.01% v/v of xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100). At each timepoint, cells were morphologically assessed by inverted phase-contrast microscopy and the viability of the cells was assessed using a proliferation test.

Results: All HA products used (Mucosamin®, native H-MW-HA, xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100) were demonstrated to have a direct effect on oral keratinocyte cell proliferation. Specifically, the compounds tested significantly promoted oral epithelial cell proliferation compared to control, except for Mucosamin®; this commercial preparation did not affect cell growth at concentrations of up to 5% (v/v) and was cytotoxic at higher concentrations. In addition, cells incubated for 72 hours in the existence of the tested HA products showed typical morphology comparable to the control, except for cells cultured with Mucosamin® at concentrations higher than 5%, which displayed apoptotic-like features.

Conclusion: We demonstrated the HA direct effect on the regulation of normal human oral keratinocyte proliferation. Overall, our outcomes showed that all tested HA preparations were safe in that these were not cytotoxic even at relatively high concentrations, with the exception of Mucosamin® (7% and 10% v/v).

4.2 Introduction

HA is a linear, non-sulfur glycosaminoglycan (GAG) chain consisting naturally of repeating polysaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, connected through β -1,3- and β -1,4-glycosidic linkages (Figure 4.1) (Lapcík et al., 1998; Milas and Rinaudo, 2004). This element is preserved in all mammalian species, implying that HA is a biomolecule of great significance (Chen and Abatangelo, 1999). Under physiological conditions, HA is in the form of salt, negatively charged, and is known as sodium hyaluronate (Hargittai and Hargittai, 2008; Schanté et al., 2011). HA is secreted by many cell types and is a significant element of the ECM (Fraser et al., 1997). On the inner wall of the plasma membrane, HA synthase (HAS) synthesizes HA, which is then turned out into the ECM space without further modification (Prehm, 1984; Karamanos et al., 1995; Prehm and Schumacher, 2004). Depending on enzymes that catalyse its synthesis, HA can be seen in a broad molecular weight (MW) span ranging from a few million down to 20,000 Daltons. Repeated unit numbers of disaccharides in HA molecules can span up to 2×10^5 generating a big HA chain with an MW of up to several million Daltons and a length of up to 25 μm . HAS-1, HAS-2, and HAS-3 are three differentially regulated isoforms responsible for making HA of different chain extents (Itano et al., 2002; Itano et al., 2004). Importantly, HA's biological properties are in part related to its MW (Tavianatou et al., 2019).

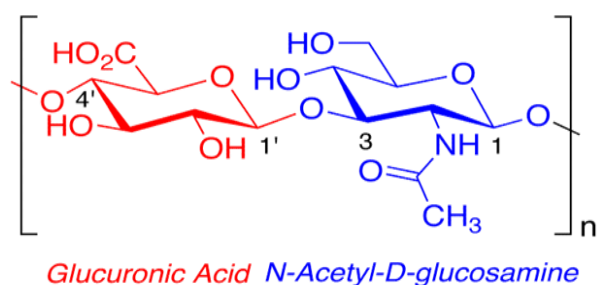


Figure 4.1 Molecular configuration of the HA's repeating branch

Manufacturing processes for HA are leaned on extraction from animal resources or production by microbial fermentation. HA was initially extracted from the umbilical cords, rooster comb, or bovine vitreous. However, these procedures are expensive and produce limited HA quantities, along with the presence of protein impurities in the resulting HA extracts, which requires extensive purification of the crude product (Shiedlin et al., 2004). Advancements in biotechnology have directed to developing less costly ways to produce high yields of HA. The

new methods are based on microorganism fermentation, which avoids contamination risk by animal pathogens. Most HA derivatives today are obtained from bacteria (*Streptococcus equi* and *Streptococcus zooepidemicus*). HA obtained from bacteria has a higher degree of purity than HA produced by animal sources (Shiedlin et al., 2004; Rangaswamy and Jain, 2008).

HA's physicochemical properties have been widely reviewed (Day and Sheehan, 2001; Lapcák et al., 1998; Laurent and Fraser, 1992). HA is biocompatible, biodegradable, non-toxic, and a non-immunogenic polysaccharide with unique viscoelastic and hygroscopic properties. Such features enable it to serve many critical roles in the human body (Laurent, 1998; Almond, 2007; Ambrosio et al., 1999; Termeer et al., 2003; Jansen et al., 2004; Amarnath et al., 2006; Oprenyeszk et al., 2013; Milas and Rinaudo, 2004). In physiological solution, the HA molecular structure is reinforced by combining disaccharide chemistry, interactions with solvents, and interior hydrogen bonds. As a result, the axial atoms of hydrogen form relatively hydrophobic nonpolar faces, whereas side chains form more polar hydrophilic faces, giving rise to a twisted band structure (Cowman and Matsuoka, 2005; Garg and Hales, 2004; Zhu et al., 1994). In the solution form, the HA string takes the form of a rigid, randomly extended coil, which varies in size with the variation of physicochemical properties, such as the basicity or acidity, salt concentration, and shear rate. For example, these chains are intertwined at low concentrations, contributing to distinctive rheological properties. In comparison, Hydrated HA solutions are supremely thick and dilute shear at higher concentrations. (Scott et al., 1991; Lapcák et al., 1998; Cowman and Matsuoka, 2005). Likewise, a more flexible molecular structure is formed in an alkaline pH (Maleki et al., 2008).

In addition to its viscoelastic properties, HA also exhibits strong water retention properties. Because of its strong anionic nature, the HA chain structure acts to trap water between the coiled chains, giving it a strong water-retaining ability (Lapcák et al., 1998; Hargittai and Hargittai, 2008; Rah, 2011; Cowman and Matsuoka, 2005). HA's strong water-holding property may also be due to the joining of the hydroxyl groups throughout the length of the chain, resulting in a helical structure formation that, in water, is estimated to be able to trap approximately 1000 times its weighing (Hargittai and Hargittai, 2008).

HA is ubiquitous in nature and has been found in both bacteria and vertebrates. In the human body, HA is most abundantly formed in the ECM of the submucosal tissues. Along with different molecules, High MW (H-MW) HA plays an essential role in the network integrity of most tissues' ECM (Laurent and Fraser, 1992; Lapcák et al., 1998). The HA network acts like

a screen, limiting the migration of plasma proteins, pathogens, and proteases and managing water transport (Laurent and Fraser, 1992). The largest amount of HA resides in the skin (epidermis and dermis), lung, intestine, and brain. HA is also abundant in eyes vitreous body, disc nucleus, synovial joint fluids, hyaline cartilage, and umbilical cord (Fraser et al., 1997; Robert et al., 2010; Monheit and Coleman, 2006; Barbucci et al., 2002; Laurent and Fraser, 1992; Kuo, 2005; Toole, 2004).

HA has a significant biological and physiological part in the body of human. Given its H-MW and ability to retain a large amount of water, it participates in maintaining homeostasis, the mechanical integrity of tissues, lubrication, viscoelasticity, and shock absorption in most tissues' ECM, particularly joints (Dicker et al., 2014). Furthermore, it is likewise recognized that HA play a position in intracellular functions, including migration, cell adhesion, differentiation, and proliferation, and thus processes such as wound healing, vascularity, morphogenesis, tissue development, inflammation, metastasis, and tumor progression (Lee and Spicer, 2000; D'Agostino et al., 2015; Dicker et al., 2014; Toole, 2004; Alho and Underhill, 1989; Slevin et al., 2002). Proteins, namely hyaladherins, carry out these processes (Vigetti et al., 2014). Examples of hyaladherins are receptors for hyaluronate-mediated motility (RHAMM) (Entwistle et al., 1996), cluster determinant 44 (CD44) (Aruffo et al., 1990), , toll-like receptors (TLRs) 2 and 4 (Jiang et al., 2005), HA receptor for endocytosis (HARE) (Takei et al., 2004; Asayama et al., 1998), and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (Schledzewski et al., 2006). Among these receptors, RHAMM and CD44, which are thought to be involved in metastasis, have enticed awareness (Toole et al., 2002). RHAMM is defined on the surface of the cell and is also found in the nucleus and cytosol (Turley et al., 2002). It has been shown to be involved in regulating cellular responses to growth factors and during cell migration, especially for smooth cells and fibroblasts (Cheung et al., 1999). CD44 is the most distinctive HA receptor recognized on several cells' plasma membranes, including epithelial cells, keratinocytes, fibroblasts, endothelial cells, and leukocytes (Isacke and Yarwood, 2002). This receptor is implicated in various cellular processes such as proliferation, migration, cell adhesion, and differentiation (Turley et al., 2002). In addition, HA's ability to react with ROS confers an antioxidant effect of HA on ROS (Hiramoto et al., 2012). Finally, due to its potential efficacy in macrophage aggregation and migration inhibition and in preventing immune complexes' adhesion to polymorphonuclear leukocytes, HA is considered a potent mediator of inflammation (Laurent and Fraser, 1992).

HA half-life *in vivo* ranges from minutes into hours or days, depending on tissue type (Lee and Spicer, 2000; Lepperdinger et al., 2004). HA is hydrolyzed naturally in the body through a complicated enzymatic mechanism implicating the enzyme hyaluronidase (Hyal) (Jedrzejewski and Stern, 2005; Girish and Kemparaju, 2007). The degraded HA is either immediately reabsorbed by CD44 cell surface receptors, hydrolyzed in lysosomes, or emptied into circulation, where they are evacuated by the drainage system such as kidneys, liver, or lymph nodes (Aruffo et al., 1990; Lepperdinger et al., 2004). In addition, HA is correspondingly naturally hydrolyzed in the body by ROS (Stern et al., 2007; Lurie et al., 2003). Degradation of HA is a highly organized process that generates a precisely defined size of HA fragments for the desired biological function. The most widely expressed hyaluronidases are Hyal-1 and Hyal-2. Molecular modeling has indicated that HA's carboxylic groups are the distinction sites of the CD44 receptors and Hyal-2 enzymes (Banerji et al., 2007).

HA's biological activity is a function of its molecular weight and biophysical characteristics (Ferguson et al., 2011; Stern et al., 2006). For instance, native HA (H-MW-HA, 2000 - 10000 kDa) displays anti-inflammatory and anti-angiogenic activities, while low and medium MW-HA (100 - 250 kDa) show contrasting biophysical properties; they have immunostimulatory, angiogenic, and inflammatory properties (Stern et al., 2006; Liu et al., 2008; Ke et al., 2013). Furthermore, chemotaxis has been induced with the small HA fragments (< 4 saccharides) (Frenkel, 2014). The correlation between the MW of HA and physiological properties has been studied recently (Cyphert et al., 2015; Cowman et al., 2015; David-Raoudi et al., 2008; Ferguson et al., 2011; Ke et al., 2013). Under physiologic circumstances, HA is present in a H-MW form (> 106 Da) and functions to support tissue hydration and volume expansion, such as that found in synovial fluids (Stern et al., 2006). In reaction to inflammation and tissue damage, HA experiences dynamic regulation, resulting in raised HASs-induced synthesis coupled with hyaluronidases (HYALs)-mediated degradation into fragments of smaller WM. In addition, HA with a shorter chain length (250 kDa) prompts the expression of inflammatory genes in macrophages (Fraser et al., 1997; McKee et al., 1996; McKee et al., 1997). Therefore, when testing the biological conditions of HA-based derivatives, it is crucial to specify their biophysical features.

When injected *in vivo*, HA does not trigger adverse reactions (Bergman et al., 2007; Prestwich and Kuo, 2008; Burdick and Prestwich, 2011). However, native HAs does not have long-lasting mechanical integrity when injected *in vivo*. The short half-life of injected HA may be due to its rapid hyaluronidase-mediated degradation and insufficient mechanical properties (Stern,

2004). To produce more stable HA with customized degradation rates and mechanical properties while maintaining the material's fundamental properties, controlled chemical modifications such as conjugation or crosslinking have recently been developed (Allison and Grande-Allen, 2006; Kuo and Prestwich, 2010; Price et al., 2007; Ambrosio et al., 1999; Schanté et al., 2011). In conjugation, the HA chain is joined to the compounds by only single bonds, while in crosslinking, different chains of HA are joined by two or more bonds (Šoltés and Mendichi, 2003; Schanté et al., 2011). The crosslinked HA, also known as Hylan, has a long residence time *in vivo* (1.5 - 9 days) and can arrive very H-MW (up to 23×10^6 Da) (Reichenbach et al., 2007). HA's chemical transformation can be conducted on the three general functional groups of HA: glucuronic acid carboxylic group, the primary and secondary hydroxyl groups, and the amino group, which can also be retrieved by reduction of the N-acetyl group (Schanté et al., 2011). Hydroxyls are usually modified through crosslinking with divinyl sulfone (Oh et al., 2008), etherification (Nobuhiko et al., 1992), esterification (Eenschooten et al., 2010), and bis-epoxide crosslinking (Malson and Lindqvist, 1987). While carboxylates are typically modulated by carbodiimide-mediated interactions (Kuo et al., 1991), amidation (Oh et al., 2010), and esterification (Campoccia et al., 1998).

Amidation with carbodiimides is the most commonly used HA carboxylic group modification reaction (Oh et al., 2010; Bulpitt and Aeschlimann, 1999). Among carbodiimide-based activators, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) is one of the most commonly used crosslinking agents, given its ability to dissolve in water (Schanté et al., 2011; Follain et al., 2008; Danishefsky and Siskovic, 1971). HA's carboxylic acids react with EDC to form O-acylisourea-HA mediators, which interact with amines of choice to create amide bonds. However, the O-acylisourea mediator is highly reactive and can be degraded and rearranged to the steady N-acylurea byproduct. In this respect, Bulpitt (1999) used EDC together with 1-hydroxybenzotriazole (HOBt) and N-hydroxysuccinimide (NHS) to form non-reconstitutable and less degradable mediators, thereby un-enabling the N-acylureas formation.

HA plays an essential role in tissue repair, including wound healing. What underscores the relevance of HA in wound healing is the HA presence in the injured tissues ECM (Chen and Abatangelo, 1999; Goa and Benfield, 1994; Buchanan et al., 2009). During wound healing and inflammation, HA participates in active cellular processes like cell recognition and cell migration. Due to the high capability of HA to absorb water and its capability of inflammatory pathway stimulation for wound healing, HA expedites solutes and nutrient delivery to the injured area. It can also promote early inflammation by enhancing cellular infiltration and

proinflammatory cytokines production, such as IL-8, TNF- α , IL-1b (Wisniewski et al., 1996; Kobayashi and Terao, 1997). The wound matrix rich with HA may also expedite cell proliferation and motility, which is crucial for wound repair (West et al., 1997). In addition, HA ability to reduce ROS deleterious effects on wound healing by its ROS scavenging activity has been reported (Hawkins and Davies, 1998; Trabucchi et al., 2002). Intact HA keeps tissues hydrated, whereas released degraded HA following tissue injury reaches the wound area and stimulates motility of the cells, proliferation, and angiogenesis, thereby promoting a scar-free wound recovery process (Zhu et al., 2006; Etscheid et al., 2005), and angiogenesis (West and Kumar, 1989), an essential step of the wound-healing process. Several studies have demonstrated the prospect that the products resulting from HA degradation may play an important part in the sequence of stages of wound curing, but the underlying molecular mechanisms of their action role remain unknown (Chen and Abatangelo, 1999). These degraded fragments of HA have the potential to react with RHAMM and CD44. Nevertheless, ECM composition and cell type are likely to have a role in determining the effects resulting from this interaction (Toole, 2004).

Given its superior biocompatibility, biodegradability, non-immunogenicity, chemical functionalization simplicity, and unique biological and physicochemical properties, HA and HA-derivatives have generated growing interest among researchers, and they are already extensively investigated and used in several biomedical applications (Kogan et al., 2007) such as visco-supplementation (Moreland, 2003), ophthalmic surgery (Rah, 2011), regenerative medication (Mironov et al., 2005), wound healing (Jiang et al., 2007), therapeutics (Platt and Szoka Jr, 2008) and drug delivery (Peer and Margalit, 2004), and tissue engineering (Collins and Birkinshaw, 2013).

HA is widely employed in wound healing because of its essential physiological part in regeneration and tissue repair (Lin et al., 2009; King et al., 1991). In addition, Aminogam[®], a mercantile formulation based on sodium salt of HA(sodium hyaluronate, SH), has recently been shown to directly act on epithelial tissue and promote healing of the wound in stratified epithelia such as mucous membranes and skin (Colella et al., 2010; Cirillo et al., 2015; Neuman et al., 2015; Colella et al., 2012). Although, HA's ability to target keratinocytes as well as mesenchymal is highly recognized (Colella et al., 2012), the underlying potential mechanisms remain unknown (La Gatta et al., 2019).

Having found that HA-based preparations can accelerate wound healing and promote epithelial migration and repair in the epithelium of the mucous membranes and skin (La Gatta et al., 2019; Neuman et al., 2015; Cirillo et al., 2015; Colella et al., 2012), in the present study we investigated the functional properties of HA and HA derivatives. We aimed in this preliminary study to determine whether HA is implicated in regulating the proliferation of oral epithelial cells during normal growth. The comparative effects of native HA, non-crosslinked HA-based preparations, and crosslinked HA derivatives on cell proliferation were investigated in monolayer cultures of normal human oral keratinocytes. We hypothesized that HA and HA derivatives have a direct effect on normal oral epithelial cell proliferation.

4.3 Materials and Methods

4.3.1 Cell line and culture conditions

To evaluate the biological properties of HA derivatives immortalized normal human oral keratinocytes (OKF6) cultures were used in our experiments as an OM *in vitro* model. OKF cells, a benign cell line that express hTERT and evade a p16^{INK4a} cell cycle control mechanism, is derived from the floor of the mouth and established by Dickson et al. (2000). OKF6 cells were provided by OHCRC, The University of Melbourne, Australia, and subjected karyotype analysis prior to testing (VCGS Cytogenetics Laboratory, Royal Children's Hospital, Parkville, VIC). Details of cell line and the conditions of cell cultures have been described in sections (2.3.3 and 2.3.4). Briefly, OKF-6 cells were cultured as adherent cultures in 100-mm tissue culture-treated culture plates and grown in keratinocytes serum-free medium (K-SFM) (#17005-042, ThermoFisher Scientific) supplemented with 25 µg/mL PBE, 0.2 ng/mL EGF, 1% (v/v) NCS (#N4637, Sigma-Aldrich), 100 IU/ml penicillin, and 100 µg/ml streptomycin, and containing 0.4 mM CaCl₂ (P4333, Sigma-Aldrich). Cells were grown in a humidified atmosphere at standard conditions (5% CO₂, 37°C). When reached 70-80% confluence, the cells were subcultured/passaged as explained in section (2.3.4).

4.3.2 HA preparations

The tested compounds were Mucosamin[®] spray (Errekappa Euroterapici Spa, Milan, Italy), a commercially available adjuvant gel comprised of 1.33% (w/v) HA with 600–800 kDa molecular mass, non-crosslinked, in mixture with a collection of collagen prototype synthetic amino acids, namely L-lysine (0.1% w/v), L-leucine (0.15% w/v), L-proline (0.75% w/v), glycine (1% w/v), and L-leucine (0.15% w/v); A natural non-sulphated H-MW-HA powder (Hyaluronic acid sodium salt derived from *Streptococcus equi*), non-crosslinked with a molecular weight of ~1.5-1.8 X 10E6 Da, (Cat#53747, Sigma-Aldrich); and three cross-linked (xl-) HA-derivative powders (xl-HA (5/5); EDC/HOBT 5/5 mmol/mL, xl-HA (30/30); EDC/HOBT 30/30 mmol/mL, and xl-HA (100/100); EDC/HOBT, 100/100 mmol/mL). Crosslinked HA derivatives were kindly provided by Dr Annalisa La Gatta (Department of Experimental Medicine, Section of Biotechnology, Medical Histology and Molecular Biology, School of Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy).

4.3.3 Preparation of HA solutions

HA test solutions were prepared in a complete cell culture media composed of K-SFM topped up with 25 µg/mL PBE, 0.2 ng/mL EGF, 0.4 mM CaCl₂, and 1% (v/v) NCS, containing 100 IU/ml penicillin, and 100 µg/ml streptomycin (section 2.3.7). A stock solution of HA-based compound (Mucosamin[®]) at a 10% concentration v/v (which equal to 0.1% v/v HA) was prepared by diluting Mucosamin[®] in complete culture medium at a ratio of 10:100 as described in Section 2.3.7.1. Concentrations of HA-based compound (Mucosamin[®]) used for the cell proliferation assay ranged from 1%-10% v/v. H-MW-HA Stock solution (0.5% w/v) was prepared by dissolving a natural non-sulphated H-MW-HA (~1.5-1.8 X 10⁶ Da), in complete culture medium at a ratio of 5mg/1ml, according to the manufacturer's instructions (Section 2.3.7.2). Concentrations of H-MW-HA used for the cell proliferation assay ranged from 0.01% to 0.1% w/v. xl-HA stock solutions (0.1% w/v) were made by dissolving 10 mg of xl-HA powder (xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100) in 10mL complete culture medium at a 0.1:100 ratio, according to the supplier's instructions (Section 2.3.7.3 and 2.3.7.4).

One of the limitations of this study was that the final concentration of the stock solutions for two of the xl-HA compounds (xl-HA 30/30, and xl-HA 100/100) was not quantifiable. In fact, xl-HA 30/30, and xl-HA 100/100 did not completely dissolve in the growth medium during preparation, requiring prompt filtration of the compounds using 0.22 µm Millipore filters (Cat # CLS430767, Sigma-Aldrich). For this reason, only one dilution (1:10 v/v) of the three xl-HA stocks was used for the cell proliferation assay. Resulting diluted xl-HA samples concentrations were referred to as 0.01% v/v depending on the original dissolved powder ratio.

4.3.4 Viable cell proliferation assay

A series of preliminary experiments tested the effects of five HA derivatives on epithelial cell proliferation in cell proliferation assays using the OKF6 cell line, as outlined in section (2.3.12). Briefly, OKF6 cells were cultured in a dark, clear-bottom 96-well tissue culture microplate (Cat#: CLS3603, Sigma-Aldrich). Wells were plated with 100 µL of cell suspension prepared in complete growth medium at a density of 1.5×10⁴ cells/well. Incubation was performed in a humidified atmosphere with 5% CO₂ at 37°C. After overnight incubation to allow for cell attachment, plates were washed once with PBS (100 µL/well), and then cells were treated by incubating them with various concentrations of HA derivatives prepared in complete medium. Concentrations of HA-derivatives used for the cell proliferation assay were:

1%, 5%, 7% and 10% v/v for the Mucosamin[®]; 0.01%, 0.03%, 0.05%, 0.07% and 0.1% w/v for H-MW-HA; 0.01% w/v for xl-HA 5/5; and 1:10 v/v dilutions of 0.1% w/v stock solutions of xl-HA 30/30, and xl-HA 100/100. The concentrations of all the HA derivatives were chosen based on the potential therapeutic efficacy of HA-based compound (Mucosamin[®]), as reported by previous literature (Cirillo et al., 2015; La Gatta et al., 2019). The cells were incubated for 24, 48, and 72 hours for the Mucosamin[®] and H-MW-HA, or for 24 and 48 hours for xl-HA under standard conditions. HA treatment incubation was replenished every 24 hours. Before replenishment of HA incubation, the plates were washed once with PBS (100 µL/well). At each timepoint, cells were observed and photographed using a fluid cell imaging system (FLoid™ Cell Imaging Station, Life Technologies Australia) using white light and 20× magnifications. At each timepoint end, the viability of the cells was assessed using fluorometric FDA assay, as outlined in (section 2.3.12.2). Briefly, after incubation, the tested solutions were taken away, cells were rinsed once with 100 µl PBS. 100 µl of FDA solution, prepared in K-SFM serum, was then added to each well, and the plates were incubated for 40 minutes at 37 °C with 5% CO₂ (Lindhagen et al., 2008). After incubation, the non-fluorescence probe, FDA, is hydrolysed by esterases in viable cells with intact plasma membranes and converted into the green fluorescent metabolite fluorescein (Rubinstein et al., 1990). The conversion is proportional to the number of metabolically active cells. The FDA fluorescence intensity was measured at the respective excitation and emission wavelengths of 485/20 nm and 528/20 nm using a Synergy HTX Multi-Mode Reader (Bio-Tek, USA). Data were expressed as mean cell proliferation attributed to the FDA fluorescent intensity, and the proliferation graph was determined by plotting the FDA fluorescent intensity values versus time. Cell viability was expressed as the mean percentage viability relative to the unexposed control (100%).

4.3.5 Statistical analysis

The assay for each concentration of HA compounds was performed in 3-6 replicates. Statistical analysis was performed using GraphPad Prism version 8.0.1 for Windows (GraphPad Software Inc, San Diego, CA). All data are proposed as the mean $\pm$ standard deviation (SD). Differences between groups were analysed by using the one-way analysis of variance (ANOVA) test, followed by Tukey's post hoc multiple comparison test to detect inter-group differences. Whenever two independent variables were studied against one dependent variable, we used two-way ANOVA test to determine differences between groups, followed by Tukey's post hoc multiple comparison test to detect inter-group differences. Multiple linear regressions were evaluated using the regression tool to identify collinearity among the independent variables. Differences were considered statistically significant if $p < 0.05$.

4.4 Results

4.4.1 Treatment of human oral keratinocytes with Mucosamin[®] at concentrations greater than 5% v/v reduces cell viability.

Mucosamin[®] effect on OKF6 cells proliferation was examined *in vitro* using fluorometric FDA proliferation assay. This assay was performed in four different concentrations of Mucosamin[®] (1%, 5%, 7%, and 10% v/v) for 24, 48, and 72 hours, while in control groups, we used untreated OKF6 cells. In this assay, the fluorescence intensity was proportional to viable cell number. As shown in Figure (4.2a), untreated OKF6 cells exhibited an increase in fluorescence intensity over time. Treatment of OKF6 cells with Mucosamin[®] at concentrations of 1% and 5% (v/v), for 72 hours, had no direct effect on cell viability compared to control. Furthermore, OKF6 cells incubated with Mucosamin[®] at concentrations of 7% and 10% at 24 hours showed an increase in cell viability compared to untreated control cells. This increase was significant at the concentration of 10% (v/v) when compared to the untreated control. However, Mucosamin[®] treatment at concentrations of 7% and 10% (v/v) after 48 and 72 hours induced a transient cytotoxic effect on OKF6 cells, as demonstrated by a reduction in fluorescence intensity when compared to untreated controls. In fact, the cytotoxic effect of Mucosamin[®] (7% and 10%, v/v) was transient relative to internal control; compared to cells treated with 7% and 10% of Mucosamin[®] at 24 hours, there was a decrease in cell viability which was only apparent in cells treated with 7% and 10% of Mucosamin[®] at 48 hours, then the cells resume their growth at 72 hours (Figure 4.2a). Figure 4.2b shows the percentage of viable cells relative to the unexposed control after 24, 48 and 72 hours treatment with 1%, 5%, 7%, and 10% (v/v) of Mucosamin[®]. The percentage of viable cells decreased in cells treated with 7% and 10% of Mucosamin[®] after 48 and 72 hours (Figure 4.2b).

Morphological observation of untreated cells showed that the cells were adherent to the plates and preserved their original polygonal morphology. On the other hand, OKF6 cells treated with Mucosamin[®] at concentrations higher than 5% for 24 hours displayed some morphological changes like an average increase in cell size and tendency to remain as single cells rather than as larger aggregated cell colonies. In addition, continuous treatment of the cells with Mucosamin[®] at concentration 10% for 72 hours caused induction of cellular apoptotic modifications such as cells body shrinkage, blebbing, condensation and fragmentation of nuclear chromatin, and formation of apoptotic bodies (Figure 4.3, black arrows). This effect culminated with some cells detaching from the plates (Figure 4.3). Thus, based on the current

findings, we concluded that Mucosamin[®] at concentrations higher than 5% (v/v) decreased proliferation of oral keratinocytes and induced cytotoxic effects, at least transiently. Hence these concentrations were deemed unsuitable for further use in oral keratinocytes.

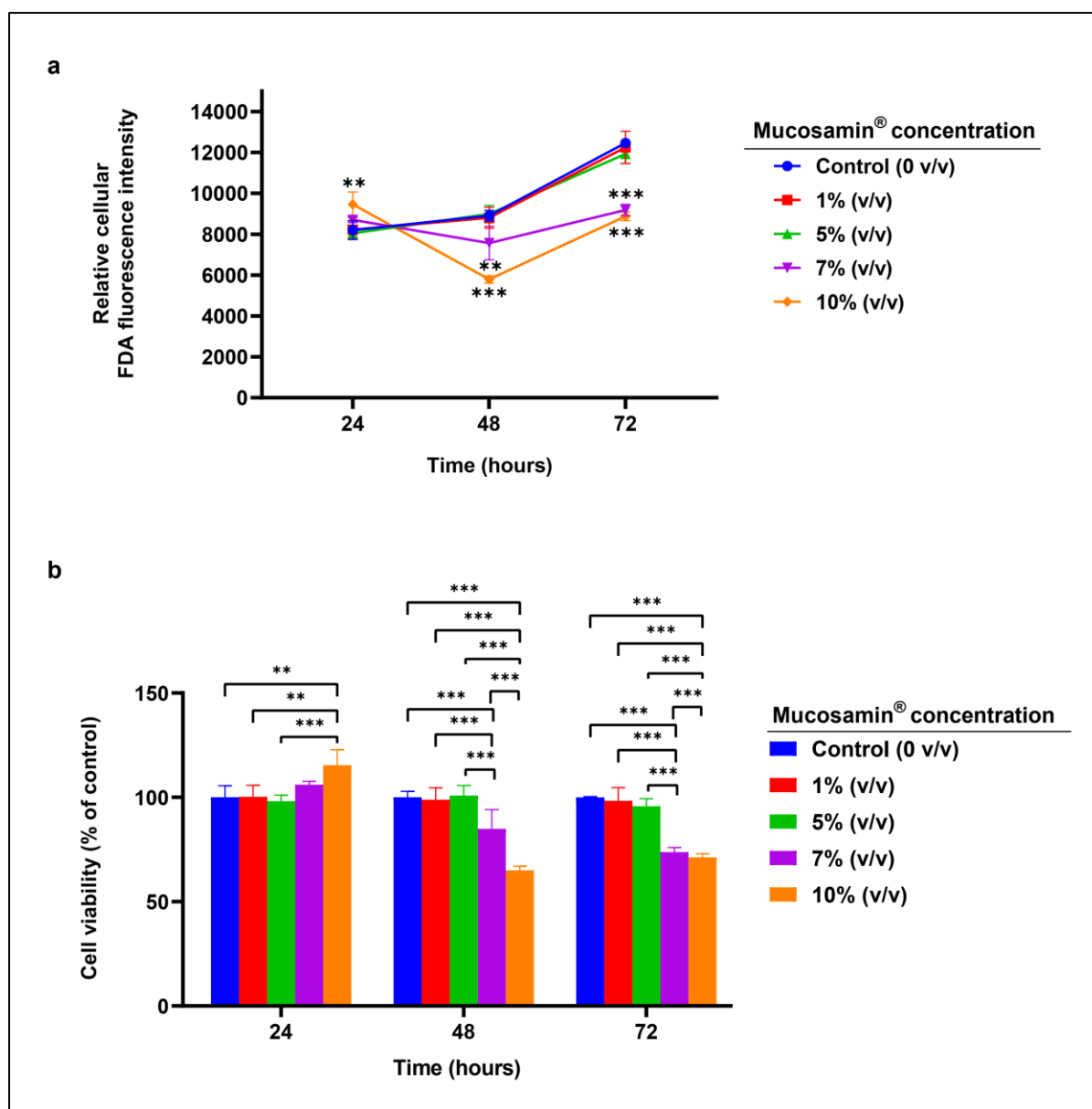


Figure 4.2 The effects of Mucosamin® on OKF6 cell proliferation at 24/48/72 hours' time points. (a): Cell proliferation relative to the FDA fluorescent intensity. **(b):** The percentage of viable OKF6 cells relative to control (100%). OKF6 cells were cultured in 96-well plates at a density of 1.5×10^4 cells/well and grown under standard conditions as a monolayer. Cells were incubated with Mucosamin® (1%, 5%, 7%, and 10% v/v) for 24, 48 and 72 hours, with the replenishment of culture medium performed every 24 hours. At each time point, cell viability was assessed by using fluorometric FDA Cell Proliferation Assay. The assay for each concentration of the HA-based compound was performed in triplicate, and control samples include cells without treatment. Error bars indicate mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared to control group (figure a), or as indicated by connectors in (figure b).

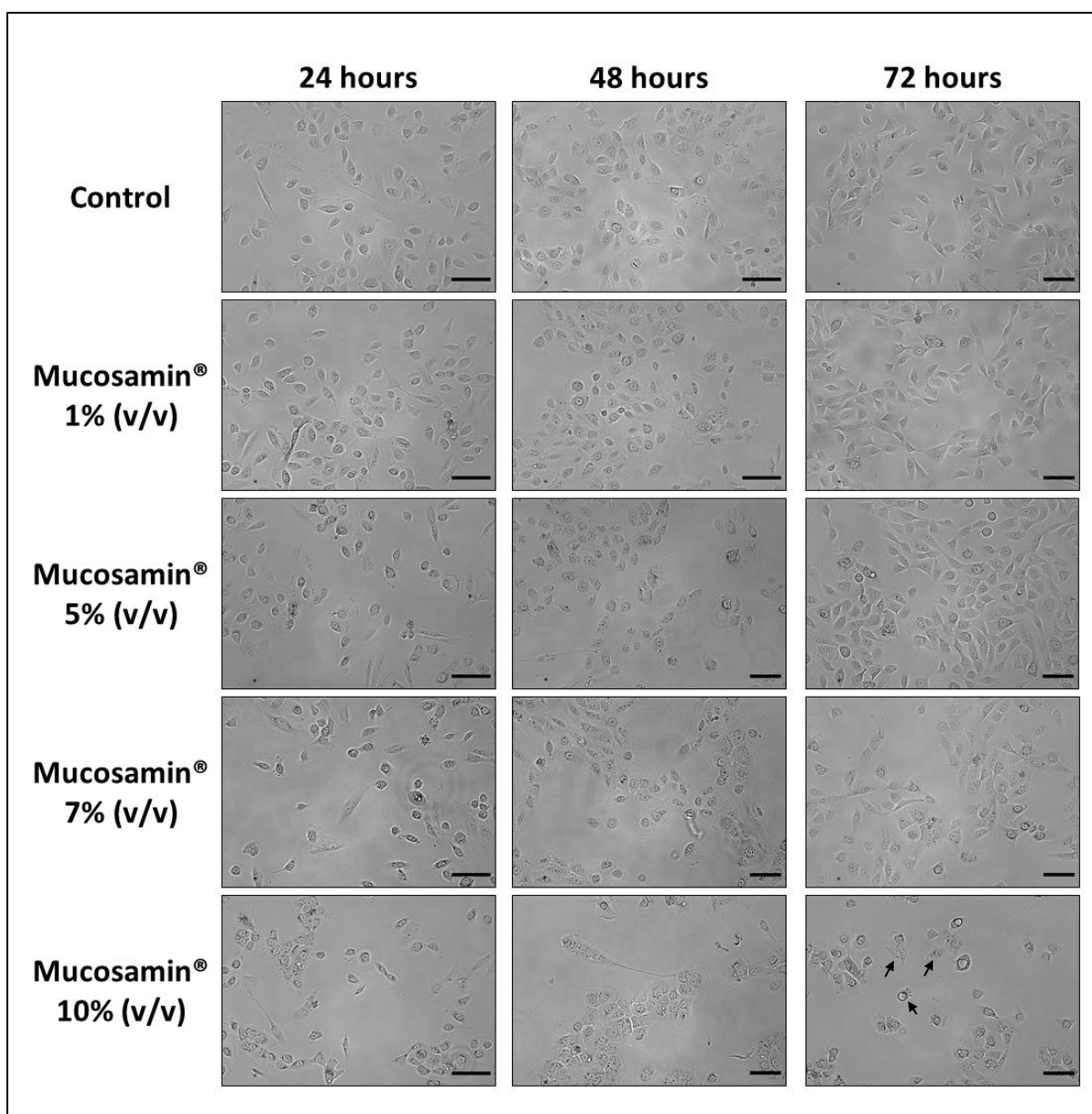


Figure 4.3 Effect of Mucosamin® on epithelial cells morphology. Representative micrographs of oral keratinocytes treated with Mucosamin®. OKF6 cells were incubated with increasing concentrations of Mucosamin® (HA-based compound) (1%, 5%, 7%, and 10% v/v) for 24, 48, and 72 hours. Morphological changes were assessed at each timepoint, and images were acquired using white light inverted microscopy (FLoid™ Cell Imaging Station, Life Technologies Australia) at 20× magnifications. The scale bar in the right bottom corner denotes 100 µm used for all panels. Black arrows labelled noticeable apoptotic features.

4.4.2 H-MW-HA promotes human oral keratinocytes proliferation

In a subsequent series of experiments, we determined if H-MW-HA directly affects oral epithelial cell proliferation. To do so, we set up the experimental conditions by running pilot experiments of incubation OKF6 cells with rising concentrations of H-MW-HA (0.01%, 0.03%, 0.05%, 0.07%, and 0.1% w/v). As reflected by the FDA fluorescence intensity, cell viability ratio relative to the untreated control increased with an increasing H-MW-HA dose (Figure 4.4). A significant increase in FDA fluorescence was noted after 24 hours treatment with all the tested concentrations (0.01% - 0.1% w/v).

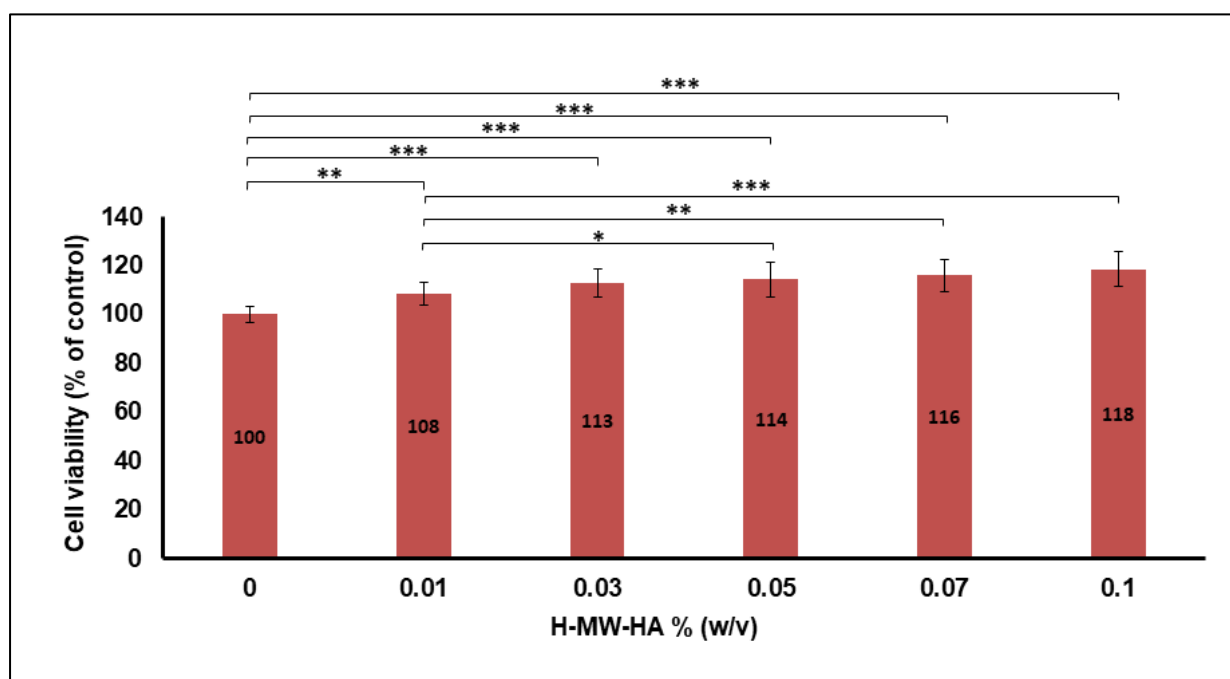


Figure 4.4 The effect of H-MW-HA on OKF6 cells' proliferation after 24 hours. The Percentage of viable OKF6 Cells relative to control (100%). OKF6 cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well and grown under standard conditions as a monolayer. The cells were treated with various concentrations of H-MW-HA (0.01%, 0.03%, 0.05%, 0.07%, and 0.1% w/v) and incubated for 24 hours. Cell Proliferation Assay was used to measure cell viability by using fluorometric FDA. Data shown is from 3 experiments, n=6 per group. Error bars show mean $\pm$ SD; *p < 0.05, ** p < 0.01, ***p < 0.001.

Subsequently, in a separate experiment, we tested the H-MW-HA effect on cell proliferation treating the OKF6 cells with increasing concentrations of H-MW-HA (0.01%, 0.03%, 0.05%, 0.07%, and 0.1% w/v), and increasing the observation time to 24, 48, and 72 hours. As shown in figure 4.5a, treating the cells with concentrations higher than 0.03% w/v of H-MW-HA enhanced cell proliferation remarkably ($p < 0.001$) in a dose- and duration-dependent manner over 72 hours of incubation, compared to control. We also investigated the correlation between OKF6 cells proliferation, as reflected by the FDA's fluorescence intensity, and H-MW-HA dose over time (Figure 4.5b). This correlation proved to be linear relationship with a correlation coefficient of $r = 0.9198$ ($P < 0.001$), $r = 0.9617$ ($P < 0.001$), and $r = 0.9291$ ($P < 0.001$) for the time points 24, 48, and 72 hours, respectively. Further, figure 4.6 shows the percentage of viable cells relative to untreated control after 24, 48 and 72 hours treatment with 0.01%, 0.03%, 0.05%, 0.07%, and 0.1% (w/v) of H-MW-HA. The percentage of viable cells relative to the untreated control significantly increased after treating the cells with H-MW-HA at concentrations higher than 0.01% after 24, 48, and 72 hours. According to these results (Figure 4.6), the highest increase in epithelial cell viability ratio was observed when OKF6 cells were treated with 0.1% (w/v) H-MW-HA for 48 hours. We also noted that at concentrations 0-0.5% (w/v), the number of viable cells remained relatively stable or proliferated slowly for the first 48 hours to then undergo a sharp increase at 72 hours (Figure 4.5a). Conversely, oral keratinocytes incubated with 0.07% and 0.1% (w/v) H-MW-HA displayed a steady, sustained growth over the 72-hr observation points. Furthermore, after being treated for 72 hours with H-MW-HA, the cells showed their typical morphology, retaining their original polygonal shape.

By combining our results, we concluded that the treatment of OKF6 cells with native HA (H-MW-HA) promoted a significant epithelial cell proliferation at a concentration as low as 0.01% (w/v). This effect was also present with the higher concentrations of H-MW-HA and proved to be time and dose-dependent.

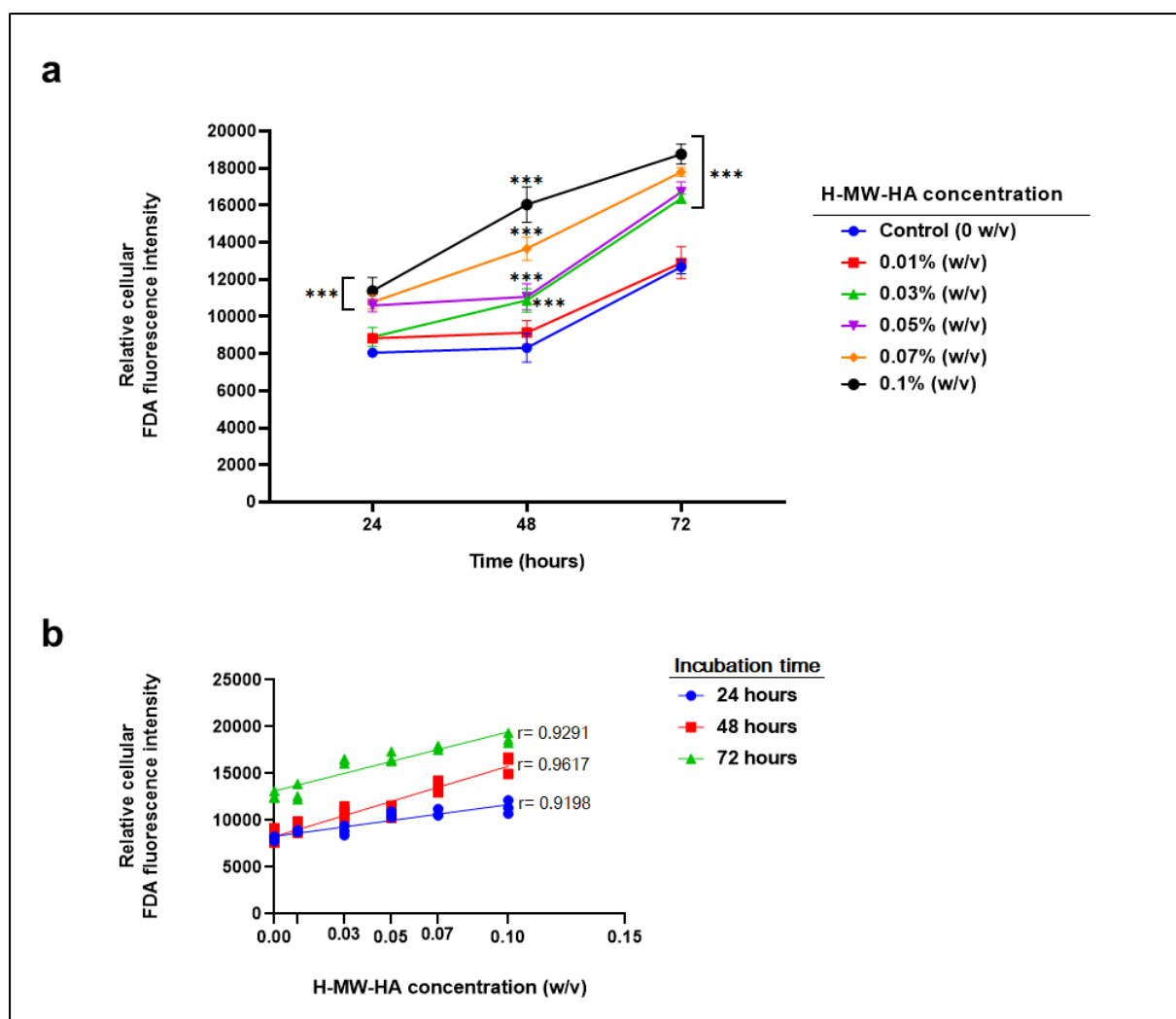


Figure 4.5 The effects of H-MW-HA on OKF6 cell proliferation after 24, 48, and 72 hours. (a): Cell proliferation relative to the FDA fluorescent intensity. **(b):** Pearson correlation index between cell proliferation as reflected by the FDA fluorescence intensity and H-MW-HA dose over time. OKF6 were seeded as a monolayer in 96-wll plates at a density of 1.5×10^4 cells/well and grown under standard conditions. The cells were treated with various concentrations of H-MW-HA (0.01%, 0.03%, 0.05%, 0.07%, and 0.1% w/v) and incubated for 24, 48 and 72 hours, with the replenishment of culture medium performed every 24 hours. At each time point, assessment of cell viability was achieved using fluorometric FDA Cell Proliferation Assay. The assay for each H-MW-HA concentration was performed in triplicate, and control samples include cells without treatment. Error bars indicate mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared to the control group.

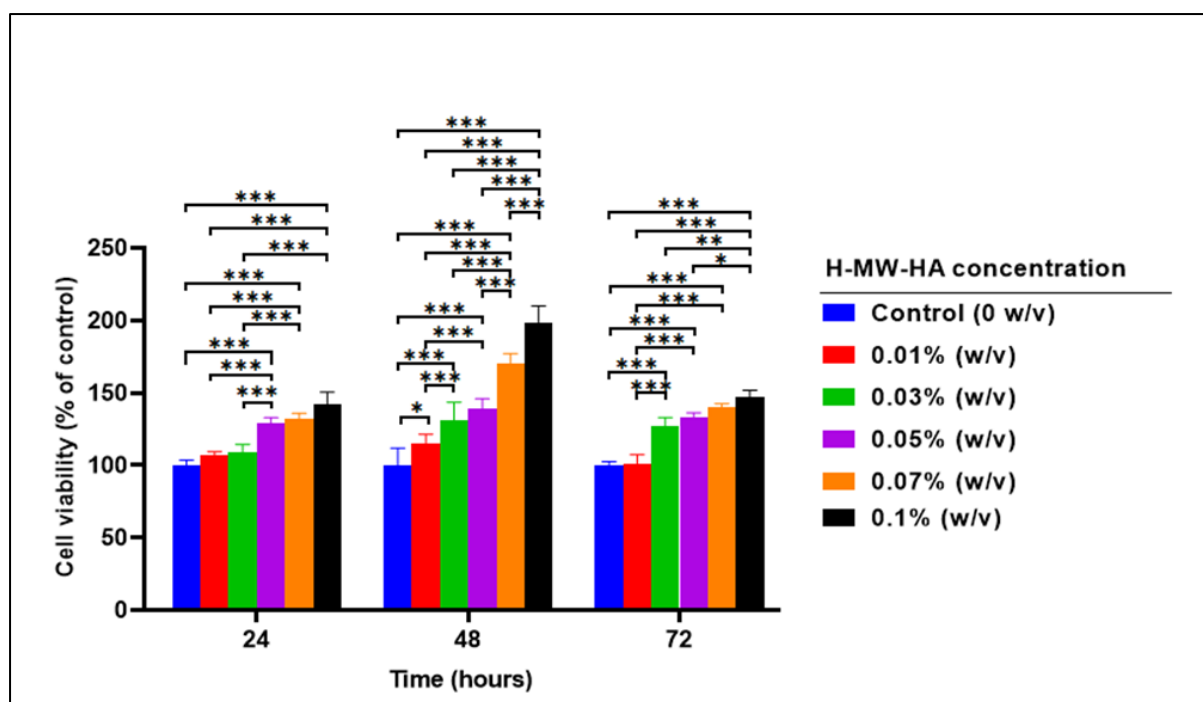


Figure 4.6 The effects of H-MW-HA on the percentage of viable OKF6 Cells relative to control (100%) after 24, 48, and 72 hours. In a 96-well plates, OKF6 cells were seeded as a monolayer at a concentration of 1.5×10^4 cells/well and grown under standard conditions. Various H-MW-HA concentrations (0.01%, 0.03%, 0.05%, 0.07%, and 0.1% w/v) were utilized to treat the cells and cells incubated for 24, 48 and 72 hours, with the replenishment of culture medium performed every 24 hours. At each time point, cell proliferation test was used to assess cell viability by using fluorometric FDA. The assay for each H-MW-HA concentration was performed in triplicate, and control samples include cells without treatment. Error bars denotes mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.00$.

4.4.3 Differential effect of cross-linked HA derivatives on keratinocyte proliferation

We next compared the effect of xl-HA derivatives (xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100) on the proliferation of the oral epithelial cells. To identify whether xl-HA derivatives have a direct effect on cell vitality/number, OKF6 cells were incubated with xl-HA products (0.01% v/v) for 24, and 48 hours. As shown in Figure 4.7 0.01% xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100 stimulated cell proliferation from 24 hours to 48 hours after incubation without exerting any cytotoxic effect. This effect was significant at 0.01% (v/v) of xl-HA 100/100 for 24 and 48 hours compared to control cells. Furthermore, cells treated with xl-HA products for 48 hours, showed typical morphology and retained their original polygonal shape. Based on our findings, we concluded that the treatment of OKF6 cells for up to 48 hours with xl-HA

products was non-toxic to the oral epithelial cells and induced an increase in OKF6 cell proliferation at a concentration as low as 0.01% (v/v).

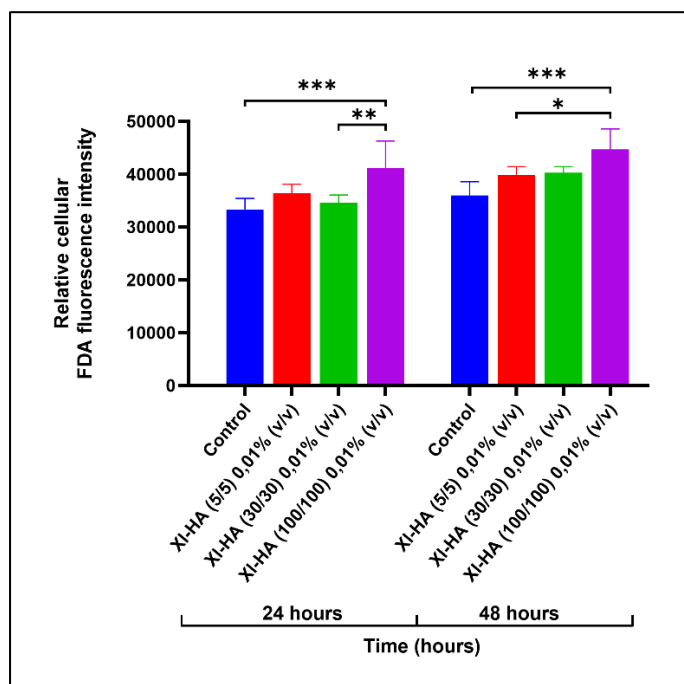


Figure 4.7 Differential effect of xl-HA products on OKF6 cells proliferation after 24 and 48 hours. 96-well plates were used to grow OKF6 cells as a monolayer at seeding density of 1.5×10^4 cells/well and grown under standard conditions. The cells were incubated with xl-HA derivatives (xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100) at concentration of 0.01% (v/v), for 24 and 48 hours, with the replenishment of culture medium performed every 24 hours. Cell proliferation relative to the FDA fluorescent intensity was assessed At each timepoint by using fluorometric FDA Cell Proliferation test. Data is from 3 experiments and the assay for each xl-HA product was performed in 5 replicates, and control samples include cells without treatment. Error bars indicate mean $\pm$ SD and data were; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.5 Discussion

Given the biocompatibility, biodegradability, and non-immunogenicity of HA (Laurent and Fraser, 1992), in addition to its wide clinical use (David-Raoudi et al., 2008) and efficacy for its potential results in the treatment and prevention of OM (Cirillo et al., 2015), the overarching aim of the project was to test the efficacy of varying derivatives of HA in the prevention of OM. However, we first wanted to test how different HA preparations are tolerated by normal oral epithelial cells and hence designed preliminary experiments, using monolayer cultures of normal human oral keratinocytes (OKF6) to ascertain that the tested HA products did not impair oral epithelial cell viability. We adopted an extended observation time of up to 72 hours to account for the possible effects of degradation products. Indeed, the half-life of H-MW-HA is relatively short, and all the resulting HA fragments often have critical physiological functions in ECM biogenesis, tissue homeostasis, and healing processes. For example, following tissue injury, natural HA in the ECM has been described to be chipped into smaller metabolites to trigger the response of the innate immunity by promoting macrophages with other characteristic cells to create chemokines needed for initiating tissue integrity rehabilitation or repair (Campo et al., 2010). Multiple lines of proof advocate HA as an important component of the ECM implicated in various biological functions, including migration and proliferation (Zhu et al., 2006; Etscheid et al., 2005). Therefore, to answer the research question of whether HA is involved in oral mucosa regenerative procedures, we used OKF6 cells to examine HA effects on proliferation *in vitro*. The aim of this work was to establish if, and to what extent, the varying preparations of HA may impact normal epithelial cells' health and to exclude those preparation with possible detrimental effects.

In the present study, we limited our investigation to only immortalized normal human oral keratinocytes (OKF6) cells for an *in vitro* model of normal oral keratinocytes and refrained from testing other oral keratinocytes, such as normal human keratinocytes NHK. The rationale for selecting only OKF-6 was based on the results from Chapter 3, where all preliminary experiments were undertaken using different monolayer cultures of oral mucosal keratinocyte as *in-vitro* models, including oral squamous cell carcinoma (OSCC) cell lines, H400 and H357, and the immortalized normal human oral keratinocytes OKF6. These cell lines were chosen in analogy with the existing oral mucosa models (Bierbaumer et al., 2018; Celentano et al., 2020). our results from Chapter 3 showed that the model can be applied successfully using immortalized oral keratinocytes (OKF6) cell lines. In addition, This cell line was chosen based

on previous research work, which showed that the OKF6/TERT cells are a reproducible and valuable model of normal oral epithelial cells because they compare primary oral keratinocytes in cytotoxicity studies or beta-defensin and inducible cytokine expression studies (Dongari-Bagtzoglou and Kashleva, 2003; Dongari-Bagtzoglou and Kashleva, 2003; Feucht et al., 2003; Dongari-Bagtzoglou et al., 2004). Hence, this cell line was chosen for all successive experiments to generate epithelial models of the oral mucosa. Furthermore, using primary keratinocytes to generate epithelial models of the oral mucosa is restricted by several disadvantages, including limited commercial supply of normal oral keratinocyte (NOK), poor longevity of NOK, and inherent donor-to-donor variation from one batch of keratinocytes to another (Jennings et al., 2016).

Our study analysed the functional activity of five different HA derivatives *in vitro*. Recent literature investigated several *in vitro* (La Gatta et al., 2020; La Gatta et al., 2019; Nyman et al., 2019; Stellavato et al., 2016; D'Agostino et al., 2015) and *in vivo* models (Riehl et al., 2012) to assess HA functional activity. However, currently, only a little information is available about HA's effect on the oral epithelial cells (Cirillo et al., 2015). Surprisingly, to date, no studies have explored HA constituent's direct effects on normal oral keratinocyte cell proliferation. OKF6 was used as a model of normal oral mucosal stratified squamous epithelial in our experiment. Immortalized normal oral epithelial (OKF6) monolayer culture has been used widely in modelling oral mucosa *in vitro* (Shrivastava and Deshmukh, 2013; Yiannis et al., 2020). Further, OKF6 was the same cell line we used in our study in Chapter 3 to create our *in vitro* oxidative damage-induced cytotoxic model mimicking OM. OKF6 cells treated with increasing concentrations of varying HA products (Mucosamin[®]; HA-based compound, native HA; H-MW-HA, and xl-HAs; xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100 (EDC/HOBT, mmol/mL)), and the proliferation of cells was assessed throughout 24, 48, and 72 hours. Successively, the correlation between different concentrations of different-sized HA products and relative cellular proliferation was investigated. All HA products have proved to promote cell proliferation except for Mucosamin[®] which was cytotoxic at concentrations higher than 5% v/v.

In particular, the data showed that Mucosamin[®] at concentrations up to 5% v/v had no direct influence on the proliferation of oral epithelial cell for up to 72 hours. However, after incubation for 48 hours, Mucosamin[®] at concentrations of 7% and 10% v/v showed a cytotoxic effect and significantly reduced the epithelial cell viability ratio compared to controls,

consistent with results reported in the literature (La Gatta et al., 2019). In a recent study where a scratch (wound healing) test *in vitro* was used (La Gatta et al., 2019), results showed that the migration of keratinocytes was enhanced in the presence of 1% (v/v) Aminogam® (HA-based compound which has the identical composition of Mucosamin®) but attenuated at 10% (v/v). However, it is not clear whether amino acids, but not HA, play any role in the cytotoxic effects of Mucosamin®. D-alanine, D-lysine, D-amino acid, and D-proline were not included in the tested compounds used in this study, and they were cytotoxic when tested in several epithelial cells lines (Bardaweel et al., 2013). Besides, H-MW-HA fragments have not shown a cytotoxic effect on epithelial cells *in vitro* in the relevant literature (D'Agostino et al., 2017; La Gatta et al., 2019; Riehl et al., 2012). Nonetheless, the mechanisms underlying Mucosamin® cytotoxicity against normal oral human epithelial cells requires further investigation. The results from our study, in combination with others that proved Aminogam® (La Gatta et al., 2019) was able to impair epithelial cells migration at 10% v/v, have demonstrated that Mucosamin® at concentration of 5% v/v and less is a suitable candidate as preventive agent for OM. Further studies for Mucosamin® are warranted.

Mucosamin® induced apoptotic cell death in tested epithelial cell lines, was evident from morphological studies (phase contrast inverted microscope). One of the best methods to determine cell apoptosis is the morphological analysis through phase contrast and fluorescence inverted microscopy (Doonan and Cotter, 2008). Following our observation, we demonstrated noticeable morphological changes of apoptosis in treated cells included loss of intercellular adhesion, membrane blebbing, and chromatin condensation and fragmentation along with the creation of apoptotic bodies. The observed morphological changes were consistent with the peculiar apoptotic morphological alteration (Kerr et al., 1972; Acebedo et al., 2014). Based on the present findings, we concluded that Mucosamin® exerted cytotoxic effects via inducing apoptosis in oral keratinocyte.

This study also indicated that the treating oral epithelial cell with native HA (H-MW-HA) at concentration up to 0.1% w/v can promote a significant dose-dependent increase in proliferation with a remarkable effect on cell viability. This finding is consistent with previous reports indicating H-MW-HA's ability to increase epithelial cell proliferation in *in vitro* model of colonic epithelial (Caco-2) cells (Sammarco et al., 2019). H-MW-HA was also reported to increase prostate epithelial cell proliferation *in vitro* (Damodarasamy et al., 2015). Furthermore, Riehl et al., (2012) demonstrated that HA promoted normal intestinal and colonic

epithelial cell proliferation *in vivo* but not *in vitro* in monolayer cell cultures. Overall, by combining our results with those suggested by other authors, we are confident that the H-MW-HA compounds selected in this study are not cytotoxic and can also promote oral epithelial proliferation.

Subsequently, when we examined xl-HA effects on the proliferation of oral keratinocytes, we demonstrated that xl-HA did not show any cytotoxicity. At a concentration of 0.01% v/v, xl-HA100/100 promoted significant epithelial cell proliferation, whereas, at the same concentration, xl-HA5/5 and xl-HA30/30 did not show significant proliferative inducing properties. A potential limitation of xl-HA solutions is that the typical cell response can be dose-dependent in particular. The concentration of carbodiimides (EDC) and 1-hydroxybenzotriazole (HOBt) for amidation of the carboxylic acid group might play a role in the biophysical properties of xl-HA, which should be fully taken into account in future research. Consistent with our findings, La Gatta et al., (2020) reported that regardless of the HA molecular weight, xl-HA did not show any cytotoxic effect on human dermal fibroblast cells. Overall, these results indicate a safe profile for xl-HA in the treatment of oral keratinocytes.

The mechanism underlying exogenous HA's ability to induce oral epithelial cell proliferation remains incomplete. The increased epithelial growth correlated with exogenous HA treatment might be attributed to the direct influence of HA on the cells and mediated through HA interacting with receptors such as RHAMM, TLRs, and CD44 (Vigetti et al., 2014; Viola et al., 2015; Tolg et al., 2006). Studies have indicated that HA itself might influence CD44 protein expression in skin cells (Gruber et al., 2022; Mazzucco, 2019). Gruber et al., (2022) have noted that form of HA fractions with a molecular weight distribution of 5–1,500 kDa increased CD44 protein expression, and the influence appears to be molecular weight dependent. However, the exact mechanism underlying the HA effect was unknown (Gruber et al., 2022). Likewise, Mazzucco found that a form of HA with a molecular weight distribution of 50 - 2,500 kDa was able to increase CD44 protein expression (Mazzucco, 2019). However, from the test data in Mazzucco's studies, the actual molecular weight fraction of HA that caused the upregulation of the CD44 protein was unclear. As mentioned before, in physiological conditions, hyaluronan is an essential component of ECM. In addition to its significant role in the hydration of tissue, HA also organizes several functions of the cells. It is highly distributed in many smooth connective tissues (for example, the skin) and has a primary role in the wound healing process

(encompassing granulation and inflammation phases), enhancing and reducing inflammation, and facilitating cell proliferation, motility, and angiogenesis (Lee and Spicer, 2000; D'Agostino et al., 2015; Dicker et al., 2014; Toole, 2004; Alho and Underhill, 1989; Slevin et al., 2002). In this regard, HA is found to exert biological activity through reacting with its cell surface receptors (Vigetti et al., 2014). Specifically, interaction of HA with CD44 activates various biophysical reactions varying from angiogenesis and cell proliferation to inflammation and ECM degradation (Viola et al., 2015). Literature reported that H-MW-HA (ranging from 1800-50 kDa) mainly triggers CD44 (D'Agostino et al., 2017; Misra et al., 2015; Yang et al., 2012). The Molecular weight of HA-products used in our experiments was in line with what found in the literature. HA-CD44 interaction triggers different signalling cascades, such as PKC and ERK1/2: both are participated in the process of cellular proliferation and migration (Viola et al., 2015). Furthermore, RHAMM is another important HA's cell surface receptor that shares the ERK1/2 activation pathway with CD44, and affecting the migration of the cells (Tolg et al., 2006; Vistejnova et al., 2014). In this respect, cell proliferation assay was used in our research to correlate various products of H-MW-HA and function.

From what we know, this study is the first to demonstrate that five different HA products (Mucosamin[®], native H-MW-HA, xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100) have a direct effect on oral keratinocyte cell proliferation. By using the same model, all tested HA products have proved to promote OKF6 proliferation except for Mucosamin[®]. Further, our experimental results indicate that incubation of OKF6 cells with all tested HA products has proven to be safe for up to 72 hours, except for Mucosamin[®] at a concentration higher than 5% v/v that appeared to be cytotoxic after incubation for 48 hours. Based on our findings, Mucosamin[®] (1% and 5% v/v), native H-MW-HA (0.01% and 0.05%), and xl-HA (0.01% v/v) were chosen for further study as preventive agents for OM. One limitation of this study was investigating the impact of HA on other biological processes beyond proliferation, such as differentiation and migration in normal oral keratinocytes. However, the functional role of HA in some biological processes of epithelial cells, such as accelerating keratinocyte proliferation, migration, and differentiation, and the regulation of normal epithelial turnover was informed by evidence in the literature (Endo et al., 2021; La Gatta et al., 2019; Cirillo et al., 2015; Colella et al., 2012; D'Agostino et al., 2015; Neuman et al., 2015; Slevin et al., 2002; Oksala et al., 1995a; Alho and Underhill, 1989). Further experiments to study cellular biological reactions to particular sizes of HA in a conception that likens HA binding to specific receptors and signals would

help more understand the mechanism underlying HA's direct effect on oral epithelial cell proliferation.

4.6 Conclusion

Unprecedented, an *in vitro* comparative parallel study was conducted utilizing a monolayer oral epithelial model and testing various pharmaceutical grade HA products at different concentrations to ultimately evaluate their specific characteristics. All the tested compounds have been shown to promote oral epithelial cell proliferation except for Mucosamin[®] (HA-based compound). Unlike all other compounds tested, Mucosamin[®] treatments at up to 5% v/v (~0.05% w/v HA) could not prompt cell proliferation and was cytotoxic at a concentration higher than 5% (v/v).

In conclusion, here, we selected HA preparations and concentrations that are bioactive, safe, and can support the stimulation of epithelial cell proliferation in our cell model system. Based on the present experimental results, all HA derivatives used except Mucosamin[®] at high concentrations may be tested in further experiments as preventive agents for OM.

**CHAPTER 5 HYALURONIC ACID-
BASED COMPOUNDS
AMELIORATE OXIDATIVE STRESS
IN AN *IN VITRO* MODEL OF ORAL
MUCOSAL INJURY.**

5.1 Abstract

Background: OM is the most debilitating complication associated with the cytotoxicity of cancer therapy. The initial injury to the mucosa is triggered by reactive oxygen species (ROS). Despite its severity and prevalence, no single preventative measure has been approved to date, and the accessible therapeutic agents are largely palliative. Hyaluronic acid (HA) is a biocompatible, biodegradable, and non-immunogen polysaccharide that is potentially effective in preventing and treating OM. However, the mechanisms underlying its protective effects have not been elucidated yet. This study aims to assess the protective effects of hyaluronic acid (HA) in an oxidative stress-induced *in vitro* model of oral mucosal injury.

Materials and Methods: An *in vitro* model of oral keratinocytes cytotoxicity was established by treating for up to 24 hours immortalized normal human oral keratinocytes, namely OKF6 cells, with 400 μM (IC_{50} value) hydrogen peroxide (H_2O_2), a well-known oxidative stress inducer. Five HA-based compounds, namely Mucosamin[®], H-MW-HA, X1-HA 5/5, X1-HA 30/30, and X1-HA 100/100 were tested at concentrations ranging from 0.01 % to 0.05 % w/v to assess their protective effect in counteracting the H_2O_2 -induced oxidative damage in OKF6 cells. Cell growth was evaluated using the FDA cell viability fluorescent assay, while ROS production was measured using the redox-sensitive dye CM- H_2DCFDA .

Results: Treatment of OKF6 cells with H_2O_2 for 24 hours showed a 50% reduction in cell proliferation. when compared to control, all tested HA preparations protected OKF6 cells against H_2O_2 -induced damage without exerting any cytotoxic effect, with the most marked effects obtained with H-MW-HA. Specifically, pre- and then co-incubation of OKF6 cells with 0.01 % and 0.05 % (w/v) H-MW-HA for 24 hours resulted in increased cell viability (39.03 % and 42.7 %, respectively) when compared to H_2O_2 - treated cells. Remarkably, H-MW-HA also reduced the intracellular level of H_2O_2 -induced ROS production, as measured by the ability of cells to oxidize CM- H_2DCFDA . H-MW-HA at concentrations of 0.01 % w/v and 0.05 % w/v decreased the ROS production induced by H_2O_2 by 11.5 % and 23.6 %, respectively.

Conclusion: We have provided the first evidence for the protective effect of HA-based compounds against oxidative stress-induced cellular damage in oral keratinocytes *in vitro* and shown that H-MW-HA exerts significant ROS scavenging activity. The demonstrated cytoprotective effect of HA preparations against oxidative stress-induced cell death was associated with their antioxidant properties, highlighting their potential use as preventative agents for OM.

5.2 Introduction

Radiotherapy (RT) and chemotherapy (CT) are two of the most common and widely used treatment methods for both hematologic and solid tumours (Jia et al., 2015; Kusiak et al., 2020; Slade, 2020). Continuous progress is being made to improve CT and RT specificity towards cancer cells in order to reduce cytotoxic effects on surrounding tissues. However, both therapies often cause numerous toxic side effects, including nausea, diarrhea, vomiting, and mucositis (Al-Dasooqi et al., 2013; Alterio et al., 2007; Keefe, 2004). Epidemiological studies on cancers of the head and neck region have reported that OM disorder concerns 75% of high-risk patients undergoing RT or high-dose CT (Al-Dasooqi et al., 2013; Scully et al., 2006). According to Sonis, almost all patients who receive RT alone or concomitant with CT for head and neck malignancies are likely to develop OM (Sonis, 2011).

OM significantly impairs patients' quality of life (Bockel et al., 2018; Sonis et al., 2004a; Trotti et al., 2003a), as it causes oral pain, dysphagia, and inability to independently consume fluid and food, with the consequent need for hospitalization and establishment of parenteral nutrition and opioid-level analgesics regimen (Elting et al., 2003; Vera-Llonch et al., 2006). In severe cases, anticancer regimens have to be either reduced or discontinued, with a significant negative impact on patient's overall prognosis (Elting et al., 2008b; Sonis, 2009; Aprile et al., 2015; Nonzee et al., 2008). OM has constantly been reported as the most bothersome side effect of the total cancer treatment that patients experience (Scully et al., 2003; Sonis, 2011).

OM remains a significant burden for those who are undergoing antineoplastic drug treatment or radiation therapy (Al-Dasooqi et al., 2013; Lalla et al., 2014; Sonis et al., 2004a; Villa and Sonis, 2015). This is translated in higher healthcare costs spent for HNC patients with OM due to increased hospitalization rates, the use of opioids, and a greater need for nutritional and fluid support. The greater the severity of mucositis, the higher the cost of treatment, with an incremental cost in the patients' population estimated to be more than \$17,000 (Murphy, 2007; Nonzee et al., 2008).

Management of OM is currently directed at prevention, palliation, infection prevention, and treatment. The accessible therapeutic agents for OM are predominantly palliative while focusing on pain control and maintaining nutrition. The cornerstone of therapy remains the use of topical anaesthetics (lidocaine, capsaicin) and systemic analgesics. (Sonis, 2011; Colella et al., 2010; Lalla et al., 2014; Cohen et al., 2013). The only approved treatments for mucositis to date are palifermin (Kepivance¹) and benzydamine (Cirillo et al., 2015). Palifermin application

is limited to mucositis in patients with hematological malignancies undergoing conditioning regimens prior to stem cell transplant (Raber-Durlacher et al., 2013), while benzydamine use has been approved in Europe only, and its efficacy is limited to patients receiving RT (Sonis, 2011; Nicolatou-Galitis et al., 2013). None of the drugs used for OM treatment are explicitly targeting the key pathways involved in mucositis disorder, and surprisingly, not a single preventive measure has been approved to date (Colella et al., 2010; Cirillo et al., 2015).

Alternative treatments are being studied, with some level of effectiveness that has been shown in preventing the development or reducing the severity of OM (Bensadoun and Ciais, 2002; Migliorati et al., 2006; Lalla et al., 2014). These include the local application of PTA (polymyxin E, tobramycin, and amphotericin B), GM-CSF/G-CSF, natural agents, cryotherapy, low-energy laser (LEL) irradiation, antimicrobial agents, anti-inflammatory medications, and the systemic administration of amifostine together with GM-CSF/G-CSF (Gori et al., 2007; Svanberg et al., 2007; Barasch et al., 2006; Lalla et al., 2006; Stokman et al., 2006; Lalla et al., 2008; Bensinger et al., 2008; Migliorati et al., 2013). However, none of these has been confirmed to be effective according to systematic reviews (Lalla et al., 2014; Raber-Durlacher et al., 2013). The latest published guidelines from MASCC/ISOO suggest that specific prophylactic measures, including LLLT, benzydamine, palifermin, oral cryotherapy, oral care protocols, and mouthwash may be influential in preventing OM, but only can be used for specific populations and therapy strategies (Elad et al., 2020). To date, therefore, there is no single treatment that completely prevents or reverts OM. Effective treatment for OM would dramatically improve patients' quality of life and decrease the use of health resources. Hence, new mechanism-based treatment or prevention strategies are urgently needed.

Understanding the pathogenic mechanisms underlying OM development is crucial in identifying innovative preventive and/or therapeutic strategies. The aetiology and the pathogenesis of OM have not been fully elucidated yet, and to date, they are still the subject of intense debate. Several studies have reported that slowly appearing but long-lasting changes take place in the oral mucosa during the development of OM, in addition to keratinocyte apoptosis-induced by acute toxicity (Talwar et al., 2014; Pauloski et al., 2011). Moreover, the mucosal changes that cause impaired oral intake often persist for a prolonged period until the first year after cancer treatment, when the effect of OM is expected to have dissipated (Pauloski et al., 2011).

The complexity of chemoradiation-induced mucosal injury as a biological process has only recently been appreciated (Sonis, 2004b; Logan et al., 2007b; Sonis et al., 2007; Bowen et al., 2019). The condition is now thought to be caused by a complex series of biological and cellular events and represents a sequential interaction of tissues and oral mucosal cells, ROS, proinflammatory cytokines, apoptosis mediators, and local factors such as saliva and oral microflora (Bowen et al., 2019; Sonis, 2009). The changes associated with chemoradiation-induced mucosal toxicity predominantly arise in the cells and tissues of the submucosa (endothelium and connective tissue) and culminate in targeting the underlying epithelium cells, the most important layer of the mucosal barrier. This ultimately results in the epithelial barrier's degeneration and breakdown, the most critical step in mucositis development (Sonis, 2004b; Bowen et al., 2019). Today, mucositis is known as an epithelial and sub-epithelial injury, and its pathogenesis is reported to develop in a five-stage model: (1) initiation, (2) primary damage response, (3) signalling and amplification, (4) ulceration, and (5) healing, with the main contributor to the clinical manifestations being the epithelium injury (Sonis, 2007).

At a molecular level, several studies have reported that DNA damage, ROS production at early preclinical phases, and consequent activation of oxidative stress pathways play an important role in the pathogenesis of oral and intestinal mucositis (Criswell et al., 2003; Moss, 2007; Yoshino et al., 2013; Russi et al., 2014; Rtibi et al., 2018). The changes taking place in the mesenchyme would ultimately culminate in keratinocyte cell death in the basal epithelium (Sonis, 2004b). However, the mechanisms underlying oxidative stress ability to induce alteration of the oral mucosa have not been elucidated yet. Hence, given the vital role of ROS in OM pathogenesis, either limiting the generation of ROS or increasing their detoxification during CT/RT stands as a rational and promising strategy to prevent OM onset and/or reduce its severity (Block et al., 2007; Urbain et al., 2012; de Freitas Cuba et al., 2015; Shen et al., 2018).

Production of free radicals such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($\cdot OH$), H_2O_2 , and hypochlorous acid (commonly designated as reactive oxygen species, ROS) has been observed to take part in tissue damage and loss of function in several tissues and organs (Afonso et al., 2007). Almost all living organisms possess antioxidant and repair systems that have evolved to protect them against the deleterious effect of oxidative stress. Nevertheless, these systems are not sufficient to completely prevent damage. Therefore, there has been growing attention paid to antioxidants and their role in preventing ROS-induced damage (Vertuani et al., 2004). The antioxidants could attenuate oxidative damage of tissue either directly by

scavenging the free radical species and/or indirectly by enhancing natural enzymes' defences of cells (Conner and Grisham, 1996; Cui et al., 2010). Characterized as natural antioxidant products, Polysaccharides, which are widely distributed in animals, plants, and microorganisms, have received increasing attention (Chen et al., 2012). Polysaccharides have been reported to possess potential and potent capabilities in preventing ROS-induced damage in living organisms and tissues by scavenging free radicals or enhancing the antioxidant enzyme activities (Cui et al., 2010; Xin et al., 2011).

HA is a naturally occurring, linear, non-sulfated polysaccharide chain composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, connected by β -1,3- and β -1,4-glycosidic bonds (Lapčík et al., 1998; Milas and Rinaudo, 2004; Rah, 2011). HA can be extracted from bovine vitreous, umbilical cords, and rooster combs, and it can also be produced by bacteria (*Streptococcus equi* and *Streptococcus zooepidemicus*) (Rah, 2011; Milas and Rinaudo, 2004; Lapčík et al., 1998). HA's physicochemical properties have been widely reviewed (Day and Sheehan, 2001; Lapčík et al., 1998; Laurent and Fraser, 1992). It is a biocompatible, biodegradable, non-toxic, and non-immunogenic polysaccharide with unique viscoelastic and hygroscopic properties. Such features enable it to serve many critical roles in the human body (Laurent, 1998; Almond, 2007; Ambrosio et al., 1999; Termeer et al., 2003; Jansen et al., 2004; Amarnath et al., 2006; Oprenyeszk et al., 2013; Milas and Rinaudo, 2004). For example, HA contributes to maintaining tissue's mechanical integrity, homeostasis, viscoelasticity, lubrication, and shock absorption in the ECM of most tissues, especially in joints (Dicker et al., 2014). Furthermore, HA is also known to play a role in intracellular functions, including cell adhesion, migration, proliferation, and differentiation and, consequently, processes like inflammation, wound healing, angiogenesis, tissue development, morphogenesis, tumor progression, and metastasis (Lee and Spicer, 2000; D'Agostino et al., 2015; Dicker et al., 2014; Toole, 2004; Alho and Underhill, 1989; Slevin et al., 2002).

HA can be found with a wide range of molecular weights ranging from greater than 1×10^6 Da, H-MW-HA, to 0.8×10^5 Da, low molecular weight (L-MW-HA), and can reach up to $<6 \times 10^3$ Da, oligo-HA (Maharjan et al., 2011). In fact, HA's biological activity is a function of its molecular weight and biophysical characteristics (Ferguson et al., 2011; Stern et al., 2006). For example, H-MW-HA (ranging from 2000 to 10000 kDa) exhibits anti-angiogenic and anti-inflammatory properties. In contrast, low and medium molecular weight HA (100–250 kDa) have the opposite biological activity; they are angiogenic, inflammatory, and immunostimulatory (Stern et al., 2006; Liu et al., 2008; Ke et al., 2013; Maharjan et al., 2011).

In the human body, HA's highest occurrence is in the ECM of connective tissues. Along with other structural macromolecules, HA contributes to the network's mechanical integrity in the ECM of most tissues (Laurent and Fraser, 1992; Lapcík et al., 1998; Luo et al., 2000). The largest amount of HA resides in the skin (dermis and epidermis), lung, intestine, brain, and central nervous system. HA is also abundant in synovial joint fluids, a vitreous body of the eyes, hyaline cartilage, disc nucleus, and umbilical cord (Fraser et al., 1997; Robert et al., 2010; Monheit and Coleman, 2006; Barbucci et al., 2002; Laurent and Fraser, 1992; Kuo, 2005; Toole, 2004). The *in vivo* half-life of HA ranges from minutes to hours, or days, depending on the tissue types (Lee and Spicer, 2000; Lepperdinger et al., 2004). HA is naturally degraded in the body by a complex enzymatic mechanism involving hyaluronidase (Hyal) enzymes (Jedrzejewski and Stern, 2005; Girish and Kemparaju, 2007), and can also be naturally degraded in the body by ROS (Lurie et al., 2003; Stern et al., 2007). Under physiologic conditions, HA exists in a high molecular weight form ($> 10^6$ Da) (Stern et al., 2006). In response to tissue injury and inflammation, HA undergoes dynamic regulation, resulting in both increased synthesis through induction of HASs, and degradation by hyaluronidases (HYALs) and ROS to smaller molecular weight fragments (Banerji et al., 2007).

Although it is clear that HA acts directly on keratinocytes and mesenchymal cells, the underlying mechanisms have not yet been elucidated (Colella et al., 2012). Current literature has demonstrated that HA has antioxidant effects on various human cell types, both *in vitro* and *in vivo* (Campo et al., 2004b; Balogh et al., 2003; Halicka et al., 2009; Trabucchi et al., 2002). In murine model of carbon tetrachloride-induced liver injury, treatment with HA has been shown to successfully reduce lipid peroxidation and increased antioxidant enzymes (SOD and GSH-Px) activities in the liver (Campo et al., 2004b). Furthermore, substantial evidence has shown that, in inflammatory diseases, HA undergoes degradation by HYALs and ROS to smaller molecular weight fragments (Lurie et al., 2003; Stern et al., 2007; Balogh et al., 2003; Soltés et al., 2006). HA's ability to react with oxygen-derived free radicals imparts HA with antioxidant scavenging effects towards ROS (Hiramoto et al., 2012). The enzymatically digested HA revealed a better ability to scavenge free radicals than intact HA (El-Safory and Lee, 2010). The participation of HA in activation and modulation of the inflammatory response includes its antioxidant scavenging effect towards ROS, such as hydroxyl radical ($\bullet\text{OH}$) (Šoltés et al., 2006). It was also reported that HA was able to permeate the skin and protect granulation tissue against ROS-induced damage, and stimulate wound healing (Trabucchi et al., 2002). Although the most fundamental component in OM pathogenesis is oxidative stress, no studies

have currently explored HA's antioxidant activity and its effect on the ROS-induced damage of oral keratinocyte cells. A little information was available about HA's efficacy in preventing or reducing the ROS-induced damage of the oral mucosal tissues. This, came from a study using hyaluronic acid-based compounds (Mucosamin[®]) to inhibit fibroblast senescence and epithelial cytotoxicity induced by oxidative stress (Cirillo et al., 2015). However, to date the underlying mechanisms are poorly understood and need to be better elucidated.

Owing to its superior biocompatibility, biodegradability, non-immunogenicity, and unique physicochemical and biological properties, HA and HA-derivatives have generated growing interest among researchers, and they are already extensively investigated and used in several biomedical and pharmaceutical applications (Kogan et al., 2007; Mero and Campisi, 2014; Chun et al., 2016) such as regenerative medicine (Mironov et al., 2005), visco-supplementation (Moreland, 2003), ophthalmic surgery (Rah, 2011), wound healing (Jiang et al., 2007), therapeutics (Platt and Szoka Jr, 2008), drug delivery (Peer and Margalit, 2004), and tissue engineering (Collins and Birkinshaw, 2013). However, injected HA is highly soluble and often exhibits very poor mechanical properties with short half-life *in vivo* (Stern, 2004). Therefore recently HA has been chemically modified by crosslinking or coniugation to improve its properties, including mechanical properties, solubility, viscosity, degradation, and biophysical properties (Allison and Grande-Allen, 2006; Kuo and Prestwich, 2010; Price et al., 2007; Ambrosio et al., 1999; Schanté et al., 2011).

HA's direct effect on keratinocytes and mesenchymal cells, including oral mucosa, is clear and has been recently demonstrated (Colella et al., 2012). Several reports show the potential efficacy of HA in treating OM both *in vitro* and *in vivo* (Favia et al., 2008). In 2001, the US Food and Drug Administration approved a gel based on sodium hyaluronate (SH) as a Class 1 medical device for pain relief management in patients with OM (Colella et al., 2010). Besides, a spray compound (Aminogam[®]) containing a pool of synthetic amino acids (L-proline, L-leucine, L-lysine, and glycine) combined with SH accelerated healing (Favia et al., 2008) and helped manag pain in patients with OM (Colella et al., 2010). Furthermore, a preliminary data study suggested that HA combined with amino acid precursors of collagen (AAs) (Mucosamin[®]) might be effective in the treatment of the cytotoxic effect of chemo-and radiotherapy (Ruggiero et al., 2016). However, the mechanisms underlying HA ability to promote the healing of OM are poorly understood and have not been elucidated yet. Studies on a bioadherent gel (Gelclair[®]) composed of poly-vinylpyrrolidone and HS suggest that it work merely as a physical barrier between the oral environment and oral mucosa, thus reducing pain

and possibly, promoting healing (Buchsel, 2008). On the other hand, other studies suggest that the mechanisms underlying amino acid-enriched HA compound (Aminogam®) activity in the healing of OM are more likely to involve biomolecular and physiological changes in keratinocytes and mesenchymal cells (Mariggiò et al., 2009; Colella et al., 2012; Ruggiero et al., 2016; La Gatta et al., 2019).

Surprisingly, despite the relatively low cost of commercial HA preparations, HA-based compounds' effectiveness in preventing, rather than treating, OM has been poorly investigated. Among several mechanistically based approaches, only one study has provided some evidence of the beneficial role of HA in the prevention of the detrimental effects of ROS in mucosal fibroblasts *in vitro*, and the HA-based compound has shown promising results in preventing OM *in vivo* (Cirillo et al., 2015). By using an *in vitro* model of mucositis, Cirillo et al showed that oxidative stress-induced senescence in normal oral fibroblast triggers cytotoxicity in keratinocytes, a process that can be prevented by HA-based compounds. Yet, the exact mechanism by which HA prevents or mitigates OM is not clearly understood and needs to be more elucidated.

In this context, a study on the development of HA-based medications to prevent or mitigate OM has become crucial. In the previous chapter, HA constituents' functional properties were assessed to determine whether HA has been involved in regulating oral epithelial proliferation during normal growth. In particular, the effect of a commercial formulation of HA enriched with amino acids (Mucosamin®), native H-MW-HA, and Cross-linked (xl-) HA (xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100) on cell proliferation were investigated in monolayer cultures of normal human oral keratinocytes. Our results showed that except for Mucosamin® higher than 5% v/v, all HA preparations tested were safe, biologically active, and can support cell activation prompting proliferation. Accordingly, this study aimed to assess the protective characteristics of HA in an oxidative stress-induced model of oral keratinocytes injury *in vitro* with a main focus on the antioxidant effects of HA. Our results have clearly demonstrated the protective effect of HA preparations against oxidative stress-induced damage in oral keratinocytes and shown that H-MW-HA exerts significant ROS scavenging activity.

5.3 Materials and Methods

5.3.1 Cell line and culture conditions

To evaluate the protective effects of HA derivatives in an oxidative stress-induced model of oral mucosal injury *in vitro*, immortalized normal human oral keratinocytes (OKF6) cultures, were used in our experiment. OKF6, a benign human oral keratinocyte cell line that express hTERT and also bypass a p16^{INK4a} cell cycle control mechanism, is derived from the floor of the mouth and established by Dickson et al. (2000). OKF6 cells were provided by OHCRC, The University of Melbourne, Australia, and underwent karyotype analysis prior to experimentation (VCGS Cytogenetics Laboratory, Royal Children's Hospital, Parkville, VIC). Details of the cell line and culture conditions have been described in sections (2.3.3 and 2.3.4). Briefly, OKF-6 cells were cultured as adherent cultures in 100-mm tissue culture-treated culture plates (Cat#: CLS430167, Sigma-Aldrich) and grown in keratinocytes serum-free medium (K-SFM) (#17005-042, ThermoFisher Scientific) supplemented with 25 µg/mL pituitary bovine extract (PBE), 0.2 ng/mL EGF, 1% (v/v) NCS (#N4637, Sigma-Aldrich), 100 IU/ml penicillin, and 100 µg/ml streptomycin (P4333, Sigma-Aldrich), and containing 0.4 mM CaCl₂. Cells were grown in a humidified atmosphere at standard conditions (5% CO₂, 37°C). When reached 70-80% confluency, the cells were subcultured as described in section (2.3.4).

5.3.2 HA preparations

The tested compounds were Mucosamin[®] spray (Errekappa Euroterapici Spa, Milan, Italy), a commercially available adjuvant gel comprised of 1.33% (w/v) HA with 600–800 kDa molecular mass, non-crosslinked, in mixture with a collection of collagen prototype synthetic amino acids, namely L-lysine (0.1% w/v), L-leucine (0.15% w/v), L-proline (0.75% w/v), glycine (1% w/v), and L-leucine (0.15% w/v); A Natural non-sulphated H-MW-HA powder (Hyaluronic acid sodium salt derived from *Streptococcus equi*), non-crosslinked with a molecular weight of ~1.5-1.8 x 10E6 Da, (Cat#53747, Sigma-Aldrich); and three cross-linked (xl-) HA-derivatives powder (xl-HA (5/5); EDC/HOBT 5/5 mmol/mL, xl-HA (30/30); EDC/HOBT 30/30 mmol/mL, and xl-HA (100/100); EDC/HOBT, 100/100 mmol/mL). Crosslinked HA derivatives were kindly provided by Dr Annalisa La Gatta (Department of Experimental Medicine, Section of Biotechnology, Medical Histology and Molecular Biology, School of Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy).

5.3.3 Preparation of HA solutions

HA test solutions were prepared in complete cell culture media composed of K-SFM supplemented with 25 µg/mL pituitary bovine extract (PBE), 0.2 ng/mL EGF, 0.4 mM CaCl₂, 1% (v/v) NCS, containing 100 IU/ml penicillin, and 100 µg/ml streptomycin (section 2.3.3). A stock solution of HA-based compound (Mucosamin[®]) at a 10% concentration v/v (which equal to 0.1% v/v HA) was prepared by diluting Mucosamin[®] in complete culture medium at a ratio of 10:100 as described in section 2.3.3. Tested concentrations of HA-based compound (Mucosamin[®]) used for the FCA were 1% and 5% v/v. H-MW-HA Stock solution (0.5% w/v) was prepared by dissolving a natural non-sulphated H-MW-HA (~1.5-1.8 x 10E6 Da), in complete culture medium at a ratio of 5mg/1ml, according to the manufacturer's instructions (Section 2.3.3). Concentrations of H-MW-HA used for the FCA were 0.01% and 0.05% w/v. Xl-HA stock solutions (0.1% w/v) were prepared by dissolving 10 mg of xl-HA powder (xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100) in 10 mL complete culture medium at a ratio of 0.1:100 according to the supplier's instructions (Section 2.3.3). The concentration of xl-HAs used for the FCA was 0.01% v/v.

This study limitation was that two of the xl-HA powder (xl-HA 30/30, and xl-HA 100/100) were not completely solubilized in the tested growth medium, which required the stock solutions of the three xl-HAs to be immediately filtered using 0.22 Millipore filter (Cat # CLS430767, Sigma-Aldrich). This led that determination of the final concentration of the stock solutions of xl-HA 30/30, and xl-HA 100/100 was not possible. For this reason, only one dilution (1:10 v/v) of the three xl-HA stocks was used for the FCA. Resulting diluted xl-HA samples concentrations were referred to as 0.01% v/v depending on the original dissolved powder ratio.

5.3.4 OKF6 cells treatment with HA constituents

Oxidative stress model of oral keratinocytes cytotoxicity was established *in vitro* using monolayer (OKF6 cells) cultures as described in chapter three. Briefly, oxidative stress was induced by treating OKF6 cells for 24 hours with 400 µM H₂O₂ prepared in complete keratinocyte serum free media (K-SFM, #17005-042, ThermoFisher Scientific). H₂O₂ concentration to induce oxidative stress in OKF6 was chosen based on a previously identified concentration that reduced cell viability by about 50% (IC₅₀) as determined by FCA. The potential protective effect of HA constituents on oxidative stress-induced damage in oral

keratinocytes was determined *in vitro* using OKF6 cells as described in section (2.3.11). Briefly, OKF6 cells were seeded at 1.5×10^4 cells/well in a dark, clear-bottom 96-well microplate (Sigma-Aldrich) in complete keratinocyte serum free media (K-SFM, #17005-042, ThermoFisher Scientific) supplemented with 25 µg/mL pituitary bovine extract (PBE), 0.2 ng/mL EGF, 1% (v/v) NCS (#N4637, Sigma-Aldrich), 100 IU/ml penicillin, and 100 µg/ml streptomycin, and containing 0.4 mM CaCl₂ (P4333, Sigma-Aldrich). Cells were incubated overnight in a humidified atmosphere at 37°C, in a 5% CO₂ incubator. The cells were then pre-treated for 24 hours with 100 µl/well of HA constituents prepared in complete culture medium. Pre-treatment of the cells with HA constituents for 24 hours is to allow for HA metabolism and chemical transformation in cells (Lee and Spicer, 2000; Lepperdinger et al., 2004). Pre-treated cells were then co-treated with 100 µl/well of HA constituents with or without 400 µM H₂O₂ and incubated for 24 hours under standard conditions. At the end of this time, the viability of cells was measured. The concentrations of HA constituents used were 1% and 5%, v/v for the Mucosamin[®], 0.01% and 0.05%, w/v for H-MW-HA, and 0.01% v/v for the xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100. The concentrations of all HA constituents were chosen for their potential safe efficacy, as supported by results in Chapter four. In addition, these concentrations were chosen based on the potential therapeutic efficacy of HA-based compound (Mucosamin[®]), as supported by current literature (Cirillo et al., 2015; La Gatta et al., 2019). Control wells contained cells and were filled with 100 µl complete culture medium. Some well were filled with 100 µl of culture medium and used as blanks. Normally, three to six wells were used for each blank, control, and tested HA concentrations per microplate (three to five technical replicates).

5.3.5 Cell Viability Measurement

Cell viability was assessed using FDA fluorescent assay as outlined in section (2.3.11.3). Briefly, after incubation, the tested solutions were removed, and the cells were rinsed once with 100 µl PBS and treated with 100 µl/well of 12 µM FDA for 40 minutes at 37 °C with 5% CO₂ (Lindhagen et al., 2008). FDA is a non-fluorescence cell permeable probe that hydrolysed by esterases in viable cells with intact plasma membranes and converted into the green fluorescent metabolite fluorescein (Rubinstein et al., 1990). The conversion is proportional to the number of metabolically active cells. The FDA fluorescence intensity was quantified at the respective excitation and emission wavelength of 485/20 nm and 528/20 nm using Synergy HTX Multi-Mode Reader (Bio-Tek, USA). Cell viability was expressed as a percentage of fluorescence

intensity. Viability of control cells (without treatment) was considered as 100%. The results obtained from the treated cells incubated were compared with those of control cells. Data are expressed as mean percentage viability relative to the unexposed control (100%).

5.3.6 Determination of Intracellular ROS Production and Kinetics in OKF6 cells

Intracellular ROS production was measured in OKF6 cells by using the cell-permeant CM-H₂DCFDA, Invitrogen, ThermoFisher Scientific, C6827), as per manufacturers' instruction and as outlined in section (2.3.13). In brief, 1.5×10^4 cells/well were seeded in a dark, clear-bottom 96-well microplate (Sigma-Aldrich) in complete culture media and incubated overnight under standard conditions. The cells were then pre-treated with H-MW-HA (0.01% and 0.05% w/v) for 24 h. The culture medium was then replaced and OKF6 cells were exposed to 5 μ M of CM-H₂DCFDA solution prepared in Hanks' balanced salt solution (HBSS) without phenol red (Sigma-Aldrich) and incubated for 30 minutes in the dark in a humidified incubator under standard conditions. The medium with CM-H₂DCFDA was then removed, and the plate was washed with HBSS before the addition of growth medium with the compounds of interest (H-MW-HA +/- H₂O₂) to the wells. CM-H₂DCFDA is a chloromethyl derivative of H₂DCFDA, and it is a fluorogenic dye and a useful indicator of hydroxyl, peroxy, and other ROS activity within the cell. It is a cell-permeable non-fluorescent probe, and after diffusion into the cells, CM-H₂DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, 2',7'-dichlorodihydrofluorescein (CM-H₂DCF), which is later oxidized by ROS into 5-(and-6)-chloromethyl-2', 7'-dichlorofluorescein (CM-DCF), a highly fluorescent compound, staining the cell cytoplasm with bright green fluorescence (Keston and Brandt, 1965; Ro et al., 2014). To determine a time course for intracellular ROS generation, the kinetic increases in fluorescence of CM-DCF were measured at time points 0, 15, 30, and 45 minutes, and at 30 minutes intervals thereafter for 6 hours at the respective excitation and emission wavelength of 485/20 nm and 528/20 nm using the Synergy HTX Multi-Mode Reader (Bio-Tek, USA). ROS generation of control vehicle was considered (1 unit), and ROS formation was measured as the change in CM-DCF fluorescence (-fold increase) relative to the control vehicle.

5.3.7 Real-Time Quantification of Oxidative Stress in Oral Keratinocytes Using Fluorescent Dye-Based Redox Probes.

In further experiments, The real-time measurement of intracellular ROS production at a time point of 3 hours was performed using the fluorescent dye-based ROS probes CM-H₂DCFDA

as outlined in section (2,3.14). In brief, OKF6 cells were seeded in a cell culture treated 6-well plate (Sigma-Aldrich) at a density of 0.3×10^6 cells/well in complete culture media and incubated in a humidified atmosphere at standard conditions (5% CO₂, 37 °C). The cells were allowed to grow until they reached 70-80% confluence, with the growth medium replenished every 48-72 hours. At 80% confluence, the cells were pre-treated with 0.05% H-MW-HA and incubated at standard conditions for 24 hours. Pre-treated cells were then co-treated with 0.05% w/v H-MW-HA with or without 400 μ M of H₂O₂ and incubated at standard conditions for 3 hours. After cell culture treatments, OKF6 cells were treated with 5 μ M of CM-H₂DCFDA solution prepared in Hanks' balanced salt solution (HBSS) without phenol red (Sigma-Aldrich) and incubated for 30 minutes in the dark in a humidified incubator under standard conditions. After that, the plate was washed twice with 2 mL/well of HBSS, and the cells were covered with 2mL/well of HBSS/Ca/Mg and were examined under FLoid™ cell imaging station digital inverted microscope (ThermoFisher Scientific, USA) using a filter set for fluorescein (FITC) cell imaging (488 nm excitation and 530 nm emission). The fluorescence intensity excited at 488/530 nm was used as a surrogate indicator of the cell redox (ROS production) status, with an expected increase of the intensity in the presence of oxidative stress. The intensity of fluorescence was measured in seven random and different fields per well per condition in three independent experiments. The images were transferred as “.tiff” files into a computer and were analyzed using the ImageJ software (Schneider et al., 2012; Schindelin et al., 2012). The ROS level of the control vehicle was normalized to 1 unit, and the changes in CM-DCF fluorescence were expressed as a fold increase relative to control vehicle.

5.3.8 Statistical analysis

The assay for each concentration of HA compounds was performed in 3-6 replicates. Statistical analysis was performed using GraphPad Prism version 8.0.1 for Windows (GraphPad Software Inc, San Diego, CA). Differences between groups were analysed by using the one-way analysis of variance (ANOVA) test, followed by Tukey's post hoc multiple comparison test to detect inter-group differences. Whenever two independent variables were studied against one dependent variable, a two-way ANOVA test was used to determine differences between groups followed by Holm-Sidak multiple comparison test to detect inter-group differences. The paired samples t-test was also used to determine the mean difference between the paired two groups on a given outcome and whether this difference was significant. All data are presented as the mean $\pm$ standard deviation (SD). Viability data are expressed as mean percentage viability $\pm$ standard deviation (SD) relative to the unexposed control (100%). ROS levels are reported as the mean CM-DCF fluorescence change (-fold increase) $\pm$ SD relative to control as Control=1. Differences were considered statistically significant if $p < 0.05$.

5.4 Results

5.4.1 HA Constituents Effectively Protect Oral Keratinocytes Against H₂O₂-Induced Cytotoxicity and Oxidative Stress *In Vitro*.

Our results showed that exogenous treatment with H₂O₂ inhibited OKF6 cell proliferation. Treatment of OKF6 cells with IC₅₀ value of H₂O₂ (400 μ M) induced a decrease in the cell viability by about 50% ($p < 0.05$) after 24 hours of incubation compared to untreated control cells (Figures 5.1, 5.2, and 5.3). Furthermore, microscopical cell observation showed that H₂O₂ induced apoptotic-like morphological changes in OKF6 cells. Cell morphology was verified in bright field micrographs using a Fluid Cell Imaging System (Life Technologies, Australia). The results showed morphological changes compatible with apoptotic cell death in OKF6 cells treated with 400 μ M of H₂O₂ (figure 5.4). These alterations included shrunken and round appearance of the cells and intracellularly the presence of condensed and fragmented nuclear chromatin. Affected cells also displayed a loss of adhesion with neighbouring cells, a tendency to remain as single cells rather than forming cell colonies, and a detachment from the culture plate for the most damaged cells. Pre-treatment and then co-treatment of the OKF6 cells with 1 % v/v Mucosamin[®] or 0.01% w/v of the other HA constituents (Mucosamin[®], H-MW-HA, XI-HA 5/5, XI-HA 30/30, and XI-HA 100/100) did not induce any morphological changes in the OKF6 cells. Regular and normal cells with polygonal shape and a network of normal processes were prominent throughout the field (Figure 5.4). Cell viability of OKF6 cells remained unchanged at 1 % Mucosamin[®] when compared to untreated control. However, all other HA constituents at concentration 0.01% w/v were able to induce oral keratinocyte cell proliferation (Figure 5.1) and (table 5.1).

The protective effect of HA against H₂O₂-induced damage was then assessed by using FCA. HA constituents significantly decreased oxidative stress-induced cytotoxicity in OKF6 cells at all tested concentrations ($F(11, 24) = 31.922, p < 0.05$), as shown in figure (5.1). In particular, treatment of OKF6 cells with 1% v/v of Mucosamin[®], and 0.01% w/v of H-MW-HA, and 0.01% v/v of XI-HA 5/5, XI-HA 30/30, and XI-HA 100/100 prevented the H₂O₂-induced cell death, and the cell viability increased from 58.91 ± 4.93 in H₂O₂ treated cells to 76.66 ± 8.65 , 81.62 ± 2.49 , 73.32 ± 2.85 , 80.77 ± 6.14 , and 78.63 ± 10.49 for Mucosamin[®], H-MW-HA, XI-HA 5/5, XI-HA 30/30, and XI-HA 100/100, respectively (table 5.2). Furthermore, the mean difference in cell viability ratio compared to the H₂O₂-treated cells was 17.7 ± 8.25 , 22.7 ± 6.26 , 14.4 ± 4.16 , 21.8 ± 10.6 , and 19.7 ± 7.01 for Mucosamin[®], H-MW-HA, XI-HA 5/5, XI-HA 30/30,

and XI-HA 100/100, respectively (table 5.2). Morphologically, pre-treatment and then co-treatment of the OKF6 cells with 1 % v/v Mucosamin[®] or 0.01% w/v of the other HA constituents (Mucosamin[®], H-MW-HA, XI-HA 5/5, XI-HA 30/30, and XI-HA 100/100) attenuated the H₂O₂-induced cytotoxicity. Regular and normal cells with polygonal shape and a network of normal processes were prominent throughout the field (Figure 5.4).

In further experiments, Mucosamin[®] and H-MW-HA's protective effect at higher concentrations up to 5% v/v of Mucosamin[®], and 0.05% w/v of H-MW-HA were assessed *in vitro*. OKF6 cells were pre-treated with 1% and 5% v/v of Mucosamin[®], or with 0.01% and 0.05% w/v of H-MW-HA for 24 hours, and then co-treated with 1% and 5% v/v of Mucosamin[®] with or without H₂O₂, or 0.01% and 0.05% w/v of H-MW-HA with or without H₂O₂ for 24 hours and the cell viability was assessed using fluorescein diacetate (FDA) fluorescence. Results showed that both Mucosamin[®] (1% and 5% v/v) and H-MW-HA (0.01% and 0.05% w/v) were able to significantly decrease H₂O₂-induced cell death in OKF6 cells at all concentrations tested ($p < 0.05$) (Figures 5.2, and 5.3). More precisely, the percentage of cell death prevention was 27.89% and 34.19% with 1% and 5% v/v Mucosamin[®] (tables 5.3). Furthermore, the most marked effects were obtained with H-MW-HA. Specifically, pre-and then co-incubation of the OKF6 cells with 0.01% and 0.05% (w/v) H-MW-HA for 24 hours resulted in an increase in cell viability, and the percentage of prevention of cell death was 39.03% and 42.7%, respectively when compared to H₂O₂-treated cells (table 5.4). Based on our results, we found that exogenous treatment with H₂O₂ inhibited oral keratinocyte cell proliferation and induced apoptosis, a process that was prevented by using HA constituents (Mucosamin[®], H-MW-HA, XI-HA 5/5, XI-HA 30/30, and XI-HA 100/100). H-MW-HA treatment protocol was considered the most reliable protocol in terms of cost, availability, producibility, and protective effect against H₂O₂-induced oxidative stress. Hence, H-MW-HA constituent (0.01% w/v, and 0.05% w/v) was chosen for the subsequent experiments investigating the H₂O₂-mediated ROS production in human oral keratinocytes.

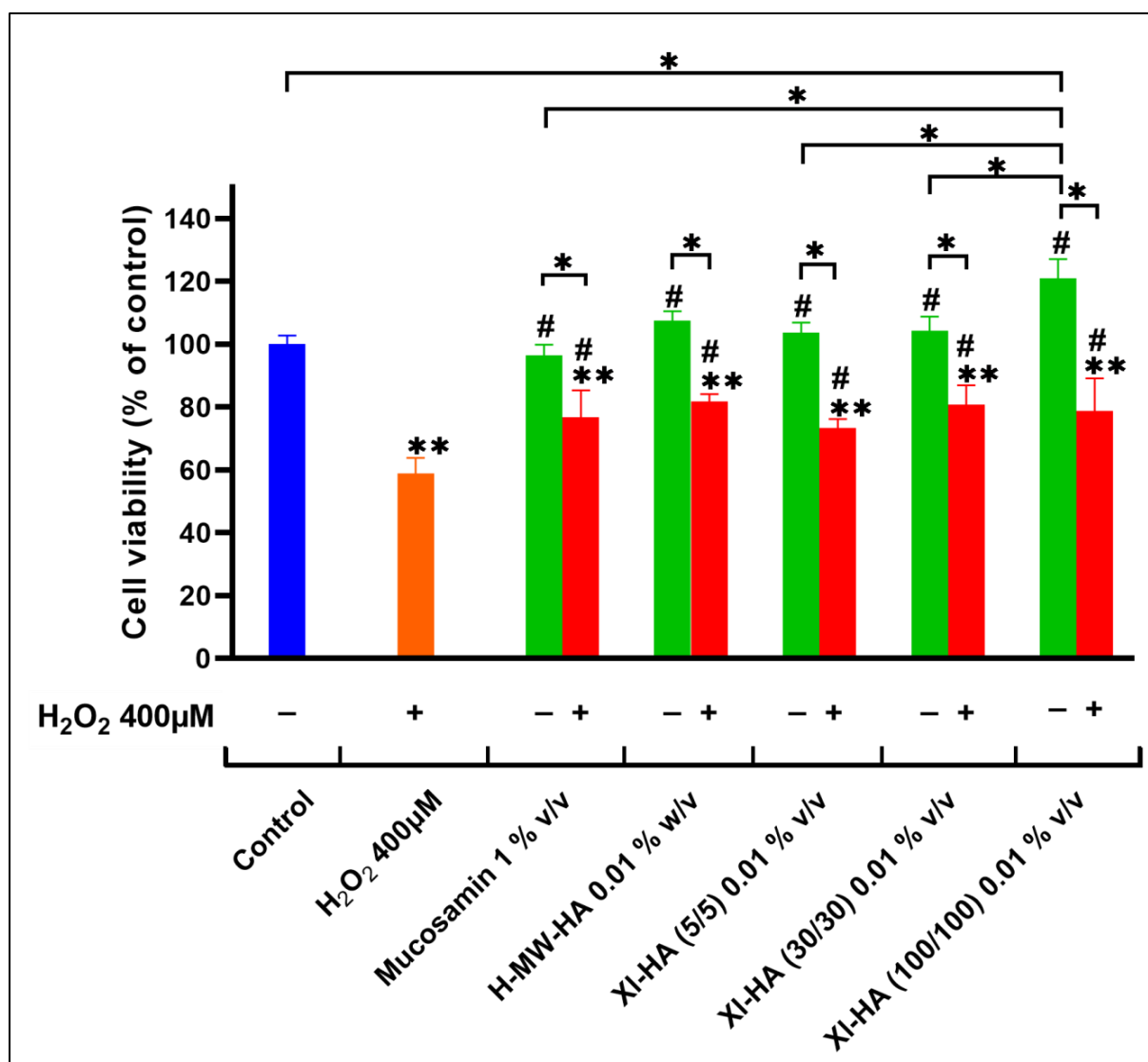


Figure 5.1 The protective effect of hyaluronic acid (HA) constituents on hydrogen peroxide (H₂O₂)-induced cytotoxicity in oral epithelial cells. OKF6 cells were pre-treated with HA constituents (HA) for 24 hours (Mucosamin[®] 1% v/v, H-MW-HA 0.01% w/v, XI-HA 5/5 0.01% v/v, XI-HA 30/30 0.01% v/v, and XI-HA 100/100 0.01% v/v), and then were co-incubated with the same HA constituents with or without H₂O₂ (400 µM) for further 24 hours. At the end of experiment, cell viability was quantified by fluorescein diacetate (FDA) fluorescent assay and expressed as the relative response (%) of treated cells as compared to untreated controls (as control cell viability = 100%). Data are expressed as means ± SD of readings from three wells per data point (technical triplicate). * p< 0.05; ** p< 0.05 as compared to control group; # p< 0.05 as compared to H₂O₂ group.

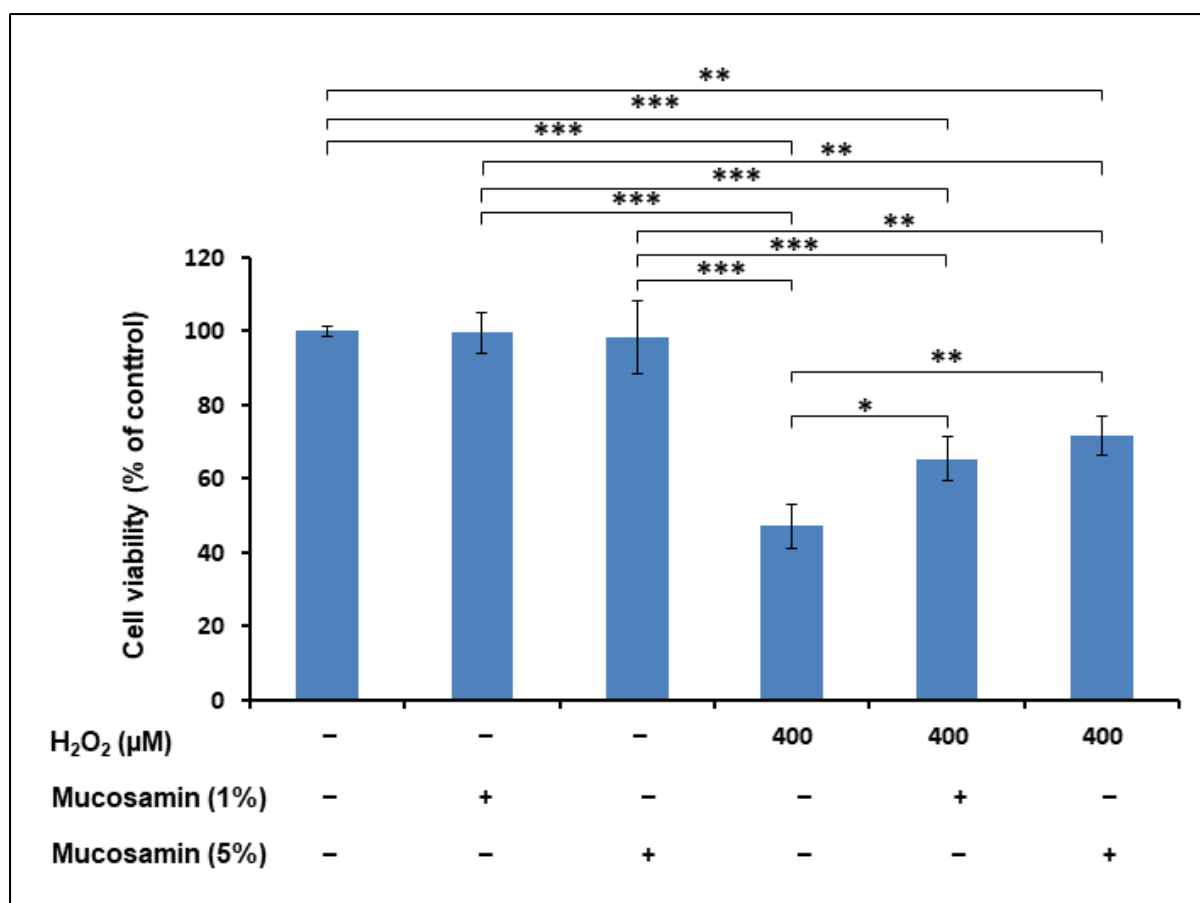


Figure 5.2 Protective effect of Mucosamin® on H₂O₂-induced cytotoxicity in oral epithelial cells. OKF6 cells were pre-treated with Mucosamin® (1% and 5% v/v) for 24 hours, and then were co-incubated with Mucosamin® (1% and 5% v/v) with or without H₂O₂ (400 μM) for further 24 hours. At the end of experiment, cell viability was quantified by fluorescein diacetate (FDA) fluorescent assay and expressed as the relative response (%) of treated cells as compared to untreated controls (as control cell viability = 100%). Data are expressed as means ± SD of readings from three wells per data point (technical triplicate). * p < 0.05; ** p < 0.01, *** p < 0.001.

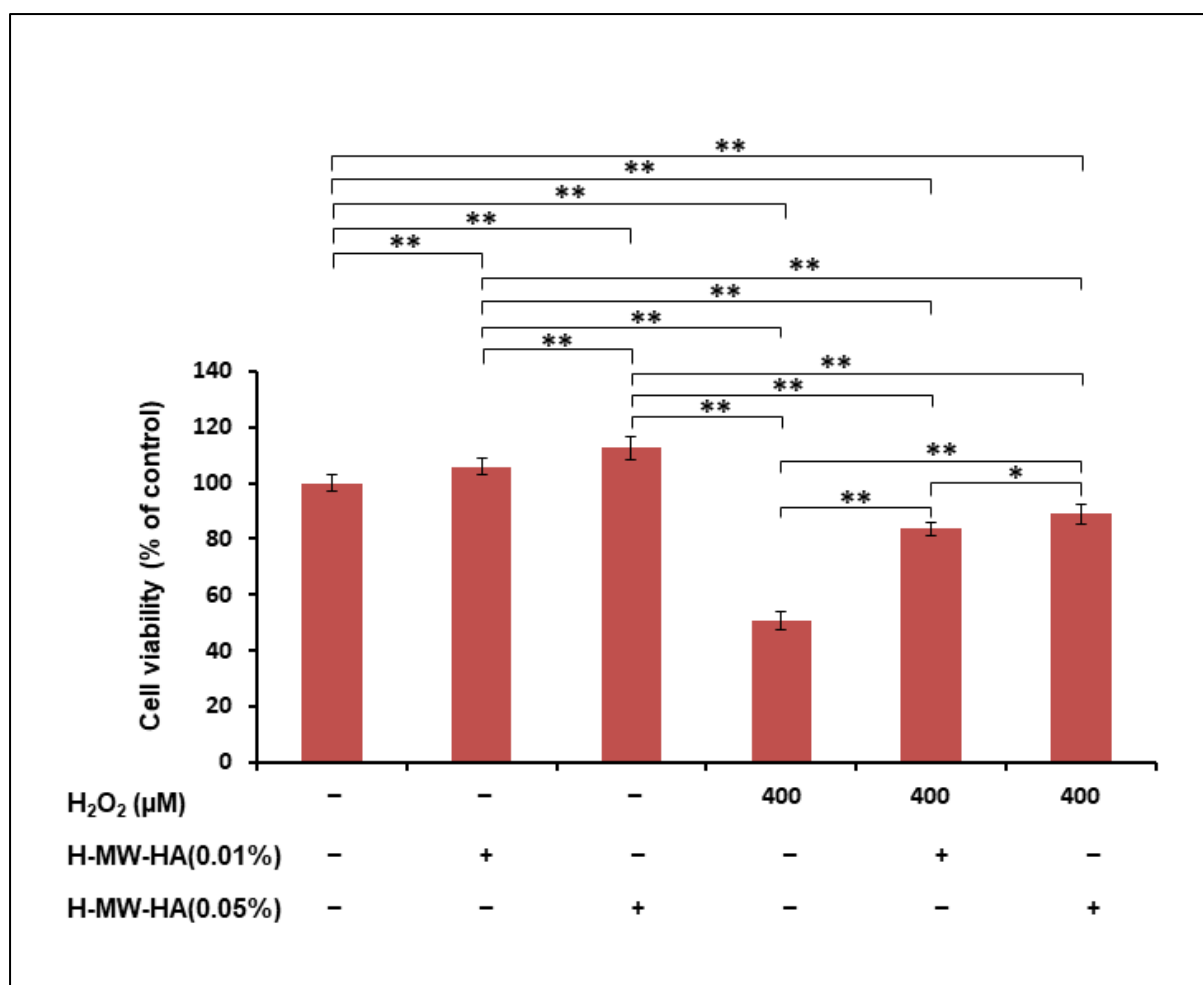


Figure 5.3 Protective effect of High molecular weight hyaluronic acid (H-MW-HA) on H₂O₂-induced cytotoxicity in oral epithelial cells. OKF6 cells were pre-treated with H-MW-HA (0.01% and 0.05% w/v) for 24 hours, and then were co-incubated with H-MW-HA (0.01% and 0.05% w/v) with or without H₂O₂ (400 μM) for further 24 hours. At the end of experiment, cell viability was quantified by fluorescein diacetate (FDA) fluorescent assay and expressed as the relative response (%) of treated cells as compared to untreated controls (as control cell viability = 100%). Data are expressed as means ± SD of readings from three wells per data point (technical triplicate) from three experiments (biological triplicate). * p < 0.005; ** p < 0.001.

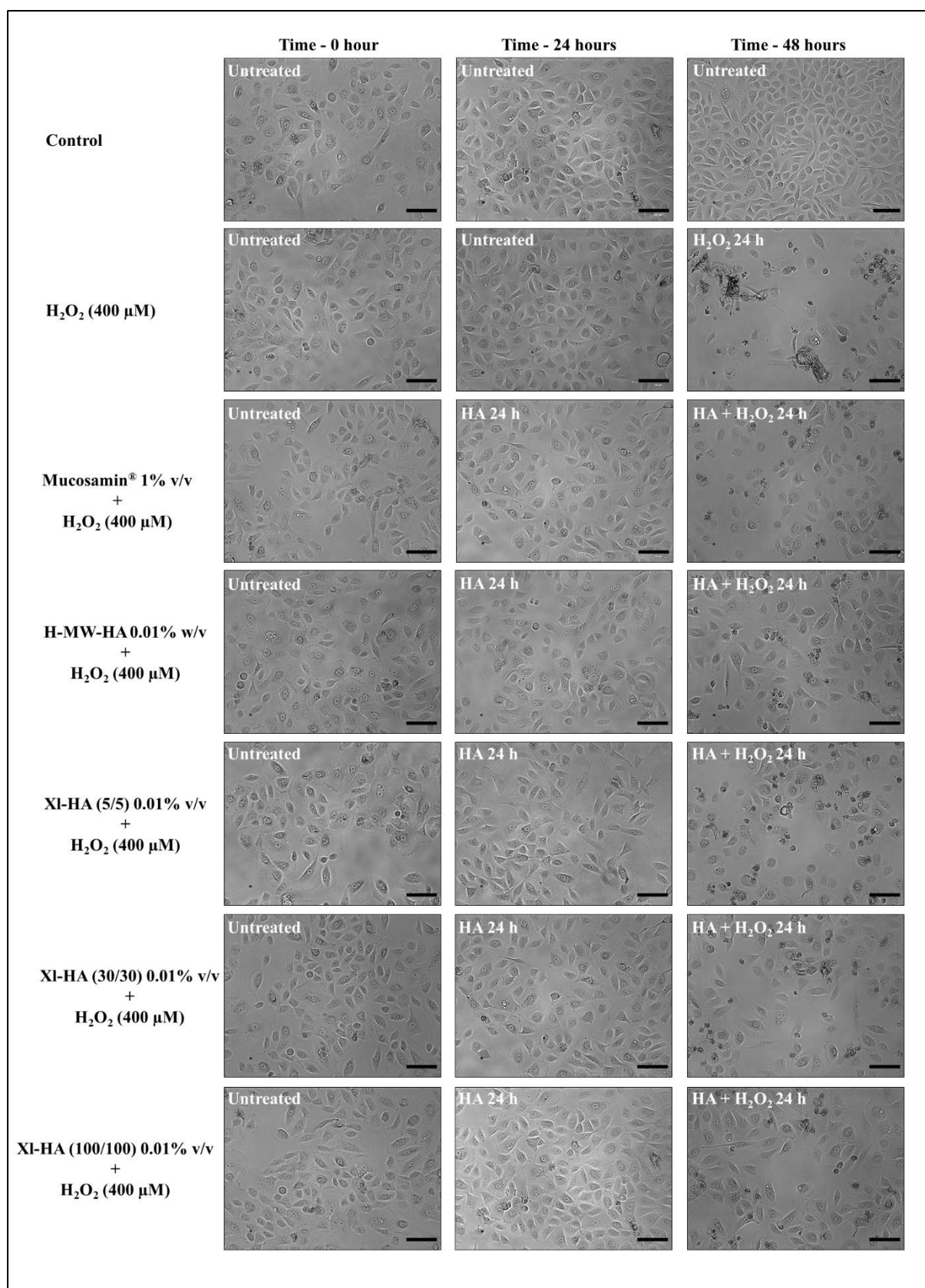


Figure 5.4 The protective effect of HA constituents on hydrogen peroxide (H₂O₂)-induced cell death in oral epithelial cells. OKF6 cells (representative images shown) were pre-treated with HA constituents (HA) for 24 hours (Mucosamin® 1% v/v, H-MW-HA 0.01% w/v, XI-HA 5/5 0.01% v/v, XI-HA 30/30 0.01% v/v, and XI-HA 100/100 0.01% v/v), and then were co-

incubated with the same HA constituents with or without H₂O₂ (400 µM) for further 24 hours. At each time point (0 hour, 24 hours, and 48 hours), morphological changes were assessed, and images were acquired using the Fluid Cell Imaging System (FLoid™ Cell Imaging Station, Life Technologies, Australia) with the filter sets on the bright field at 20× magnification. The scale bar represents 100 µm and applies to all panels.

Table 5.1 Difference in cell viability (% of control) of OKF6 cells treated with HA constituents compared to untreated control group (100%). OKF6 cells were pre-treated with HA constituents (HA) for 24 hours (Mucosamin® 1% v/v, H-MW-HA 0.01% w/v, XI-HA 5/5 0.01% v/v, XI-HA 30/30 0.01% v/v, and XI-HA 100/100 0.01% v/v), and then were co-incubated with the same HA constituents with or without H₂O₂ (400 µM) for further 24 hours. At the end of experiment, cell viability was quantified by fluorescein diacetate (FDA) fluorescent assay and expressed as the relative response (%) of treated cells as compared to untreated controls (as control cell viability = 100%). Data are expressed as means ± SD of readings from three wells per data point (technical triplicate). * p< 0.05 compared to control group.

HA constituents	Cell viability (HA treated group; % of control)	Difference in cell viability (% of control) compared to Control group (100%)
XI-HA 100/100	120.955 ± 6.142	20.9 ± 3.58*
H-MW-HA	107.483 ± 3.018	7.4 ± 0.98
XI-HA 30/30	104.261 ± 4.502	4.2 ± 2.18
XI-HA 5/5	103.696 ± 3.189	3.6 ± 5.61
Mucosamin®	96.418 ± 3.427	-3.5 ± 2.51

Table 5.2 Difference in cell viability (% of control) of OKF6 cells treated with HA constituents compared to H₂O₂-treated group. OKF6 cells were pre-treated with HA constituents (HA) for 24 hours (Mucosamin[®] 1% v/v, H-MW-HA 0.01% w/v, Xl-HA 5/5 0.01% v/v, Xl-HA 30/30 0.01% v/v, and Xl-HA 100/100 0.01% v/v), and then were co-incubated with the same HA constituents with or without H₂O₂ (400 μ M) for further 24 hours. At the end of experiment, cell viability was quantified by fluorescein diacetate (FDA) fluorescent assay and expressed as the relative response (%) of treated cells as compared to untreated controls (as control cell viability = 100%). Data are expressed as means $\pm$ SD of readings from three wells per data point (technical triplicate). * $p < 0.05$ compared to H₂O₂-treated group.

HA constituents	Cell viability (HA+H ₂ O ₂ 400 μ M treated group; % of control)	Difference in cell viability (% of control) compared to H ₂ O ₂ (400 μ M) treated group
H-MW-HA	81.627 $\pm$ 2.491	22.7 $\pm$ 6.26*
Xl-HA 30/30	80.775 $\pm$ 6.142	21.8 $\pm$ 10.6*
Xl-HA 100/100	78.632 $\pm$ 10.499	19.7 $\pm$ 7.01*
Mucosamin [®]	76.661 $\pm$ 8.656	17.7 $\pm$ 8.25*
Xl-HA 5/5	73.324 $\pm$ 2.853	14.4 $\pm$ 4.16*

Table 5.3 The Protective effect of Mucosamin[®] on H₂O₂-induced cytotoxicity in oral epithelial cells. OKF6 cells were pre-treated with Mucosamin[®] (1% and 5% v/v) for 24 hours, and then were co-incubated with Mucosamin[®] (1% and 5% v/v) with or without H₂O₂ (400 µM) for further 24 hours. At the end of experiment, cell viability was quantified by fluorescein diacetate (FDA) fluorescent assay and expressed as the relative response (%) of treated cells as compared to untreated controls (as control cell viability = 100%). Difference between groups was quantified by using pair sample t-test. Data are expressed as means ± SD of readings from three wells per data point (technical triplicate).

Pairs	Groups	Mean	SD	P-value	Protection (%)
Group 1	H ₂ O ₂ (400 µM)	47.091	6.023		
Group 2	Mucosamin [®] 1% (v/v) + H ₂ O ₂ (400 µM)	65.305	6.031		
Group 3	Mucosamin [®] 5% (v/v) + H ₂ O ₂ (400 µM)	71.561	5.404		
Mean difference (group 2- group 1)	[Mucosamin [®] 1% (v/v) + H ₂ O ₂ (400 µM)] - H ₂ O ₂ (400µM)	18.214	11.88	p<0.05	27.89
Mean difference (group 3- group 1)	[Mucosamin [®] 5% (v/v) + H ₂ O ₂ (400 µM)] - H ₂ O ₂ (400 µM)	24.47	4.05	p<0.01	34.19

Table 5.4 The Protective effect of H-MW-HA on H₂O₂-induced cytotoxicity in oral epithelial cells. OKF6 cells were pre-treated with H-MW-HA (0.01% and 0.05% v/v) for 24 hours, and then were co-incubated with H-MW-HA (0.01% and 0.05% v/v) with or without H₂O₂ (400 μ M) for further 24 hours. At the end of experiment, cell viability was quantified by fluorescein diacetate (FDA) fluorescent assay and expressed as the relative response (%) of treated cells as compared to untreated controls (as control cell viability = 100%). Difference between groups was quantified by using pair sample t-test. Data are expressed as means $\pm$ SD of readings from three wells per data point (technical triplicate) from three experiments (biological triplicate).

Pairs	Groups	Mean	SD	P-value	Protection (%)
Group 1	H ₂ O ₂ (400 μ M)	50.89	3.15		
Group 2	H-MW-HA 0.01% (w/v) + H ₂ O ₂ (400 μ M)	83.49	2.53		
Group 3	H-MW-HA 0.05% (w/v) + H ₂ O ₂ (400 μ M)	88.90	3.65		
Mean difference (group 2- group 1)	[H-MW-HA 0.01% (w/v) + H ₂ O ₂ (400 μ M)] - H ₂ O ₂ (400 μ M)	32.59	2.91	<0.001	39.03
Mean difference (group 3- group 1)	[H-MW-HA 0.05% (w/v) + H ₂ O ₂ (400 μ M)] - H ₂ O ₂ (400 μ M)	38.004	4.45	<0.001	42.74

5.4.2 H-MW-HA Protects Human Oral Keratinocytes Against H₂O₂-Induced ROS Production.

Based on the results obtained using the different HA constituents, particularly H-MW-HA, in protecting human oral keratinocytes against the H₂O₂-induced oxidative stress, we wanted to ascertain that H-MW-HA was capable of reducing ROS production in keratinocytes treated with H₂O₂. To monitor ROS production changes in H₂O₂-treated keratinocytes upon H-MW-HA incubation, we employed a fluorescent dye-based probe based on the redox-active green fluorescent (CM-H₂DCFDA), a highly sensitive thiol peroxidase that is oxidized by H₂O₂, yielding a fluorescent adduct that is trapped intracellularly (Ro et al., 2014). By introducing the CM-H₂DCFDA probe in keratinocytes, we could monitor any change in the intracellular redox state upon exposure to H-MW-HA either in the absence or the presence of H₂O₂-induced oxidative stress. This time we compared the effect of the two concentrations (0.01% w/v and 0.05% w/v) of H-MW-HA that have been so far considered for treating H₂O₂-induced oxidative stress *in vitro*. Three independent experiments were run, where OKF6 cells were first pre-treated with H-MW-HA (0.01% w/v and 0.05% w/v) for 24 hours and then co-treated with H-MW-HA (0.01% w/v and 0.05% w/v) either in the absence or the presence of H₂O₂ (400 μ M). ROS production was monitored in OKF6 cells over 6 hours time period.

First, we determined whether H-MW-HA induces any change in the redox state of OKF6 cells at baseline. The results obtained from the three independent experiments demonstrated that the two tested H-MW-HA did not induce any change in the redox state of OKF6 cells at baseline (Figure 5.5). As expected, the addition of H₂O₂ determined a rapid increase in ROS levels in all experimental groups. The increase in ROS levels was seen from the first few minutes after adding H₂O₂ and continued throughout all measured time points over the 6-hour monitoring time (Figure 5.5). Strikingly, a significant reduction in H₂O₂-induced ROS production levels was observed upon pre-treatment and the co-treatment with 0.05% w/v H-MW-HA ($F(70, 990) = 14.29$; $p < 0.01$; Figure 5.5). More precisely, a significant reduction in the ROS levels in H-MW-HA (0.05% w/v) + H₂O₂ (400 μ M) group compared with that in H₂O₂ (400 μ M) treated group started 30 minutes after the addition of H₂O₂ and continued during the all tested time points over 6 hours monitoring period. Additionally, results showed that pre-treatment of the OKF6 cells with H-MW-HA (0.01% w/v) for 24 hours and then co-treatment with H-MW-HA (0.01% w/v) was also effective in reducing the oxidative status upon exposure to H₂O₂

(Figure 5.5). The reduction in H₂O₂-induced ROS production level was significant ($p < 0.05$) only at 4 hours and 4.5 hours' tested time points (Figure 5.5).

Interestingly, ROS production levels in the H₂O₂-treated group had biphasic behaviour. Specifically, ROS levels first plateaued (Time plateau-1) between 0.5–1 hour and the ROS levels in the H₂O₂ treatment group plateaued at about 5.32 ± 1.13 (folds increase) compared to the control group. After that, the ROS production levels started to increase again until they plateaued (Time plateau-2) between 3–4.5 hours at about 9.23 ± 1.75 (folds increase) compared to the control group. Time plateau-2 was when H₂O₂-induced ROS production reached the highest level. More precisely, 4 hours was the time point when ROS production in cells treated with 400 μ M H₂O₂ reached the maximum level as determined by the CM-H₂DCFDA staining kinetic assay (Figure 5.5).

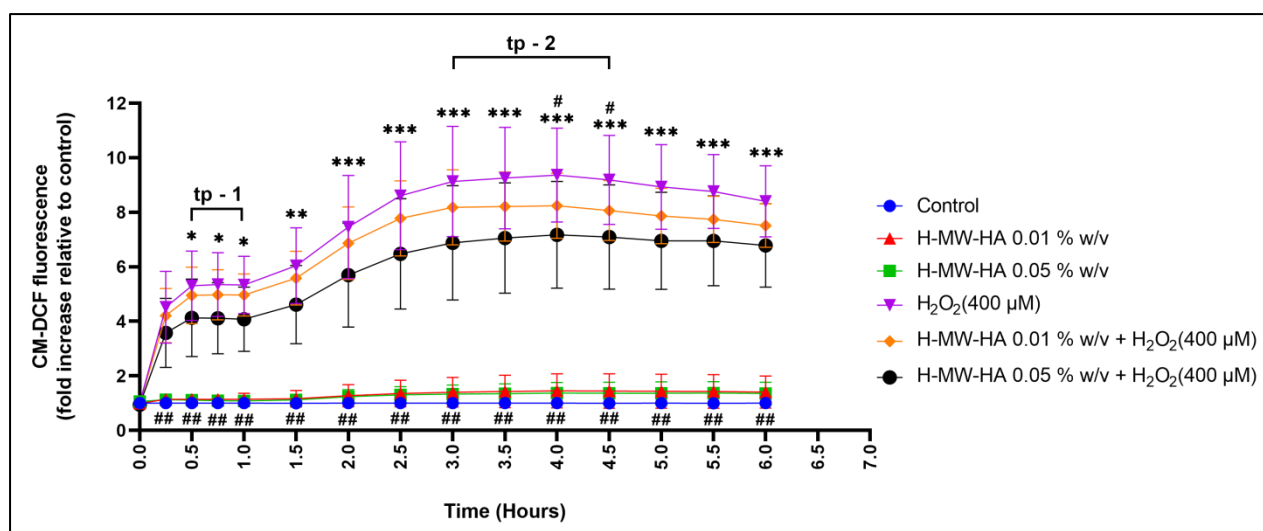


Figure 5.5 Protective effect of H-MW-HA (0.01%, and 0.05% w/v) against H₂O₂-induced ROS production. OKF-6 cells were cultured with H-MW-HA (0.01% w/v, and 0.05% w/v) in complete keratinocyte serum free media (K-SFM, #17005-042, ThermoFisher Scientific) for 24 hours in 96-well culture plates (Cat#: CLS3603; Sigma-Aldrich) under standard conditions (5% CO₂ at 37 ° C). Culture medium was then removed and cells were incubated with the fluorescent probe, 5-(and-6)-chloromethyl - 2', 7'- dichlorodihydrofluorescein diacetate (CM-H₂DCFDA), for 30 minutes. Fluorescent probe was then replaced with complete culture medium supplemented with H-MW-HA (0.01% w/v, and 0.05% w/v) either in absence or presence of H₂O₂ (400 μM). ROS production was measured as the change in CM-DCF fluorescence (-fold increase) relative to control as control=1 at timepoints 0, 15 min, 30 min, 45 min, and every 30 min for up to 6 hrs. CM-DCF fluorescence intensity was measured using Synergy HTX Multi-Mode Reader (Bio-Tek, USA) with maximum excitation and emission spectra of 495 nm and 529 nm, respectively. Data are from 3 independent experiments (three biological replicates), n= 4 per group (four technical replicates). Values represent mean ± SD. Where, # p< 0.05; compared to H-MW-HA (0.01% w/v) + H₂O₂ (400 μM) treated group, * p< 0.05, ** p< 0.01, *** p< 0.001; compared to H-MW-HA (0.05% w/v) + H₂O₂ (400 μM) treated group, and ## p < 0.05 compared to H₂O₂ (400 μM) treated group, H-MW-HA (0.01% w/v) + H₂O₂ (400 μM) treated group, or H-MW-HA (0.05% w/v) + H₂O₂ (400 μM) treated group. Abbreviations: tp-1, time plateau-1; tp-2, time plateau-2.

In plateaus 1 and 2, both tested H-MW-HA concentrations (0.01% w/v and 0.05% w/v) were able to reduce significantly ($p < 0.001$) the levels of H_2O_2 induced ROS production (table 5.5). In time plateau-1, the protective effect (%) of H-MW-HA (0.01% w/v and 0.05% w/v) against ROS production induced by H_2O_2 was 6.83% and 22.9%, respectively. While in time plateau-2, H-MW-HA's protective effect (%) against H_2O_2 -induced ROS production was 11.5 % and 23.67 % for H-MW-HA 0.01% w/v and 0.05% w/v, respectively (table 5.5). Thus, the reduction in the H_2O_2 - induced ROS production levels in OKF6 cells treated with 0.01% w/v and 0.05% w/v H-MW-HA can be attributed to the protective effect of the HA. On the basis of the data presented above, time plateau-2, and more specifically, 4 hours incubation with H_2O_2 (400 μ M) was chosen as the time point when the H_2O_2 -treated OKF6 cells produced the highest level of ROS and was furtherly explored in the subsequent experiment by measuring the internal ROS production by using CM- H_2 DCFDA and fluorescent microscope.

Table 5.5 protective effect (in ratio) of H-MW-HA 0.01% w/v and 0.05% w/v against H_2O_2 -induced ROS production. Data are from 3 independent experiments (three biological replicates), $n = 4$ per group (four technical replicates). Values represent mean (folds increase) $\pm$ SD.

Time plateau - 1 (0.5 hr - 1 hr)					
Pairs	Groups	Mean	SD	P-value	Protection (%)
	H_2O_2 (400 μ M)	5.3292	1.1347		
	HA 0.01% (w/v) + H_2O_2 (400 μ M)	4.9654	0.8895		
	HA 0.05% (w/v) + H_2O_2 (400 μ M)	4.1092	1.2685		
Pair 1	[H_2O_2 (400 μ M)] - [HA 0.01% (w/v) + H_2O_2 (400 μ M)]	0.3638	0.5148	$p < 0.001$	6.83
Pair 2	[H_2O_2 (400 μ M)] - [HA 0.05% (w/v) + H_2O_2 (400 μ M)]	1.2202	0.6334	$p < 0.001$	22.9
Time plateau - 2 (3 hr - 4.5 hr)					
Pairs	Groups	Mean	SD	P-value	Protection (%)
	H_2O_2 (400 μ M)	9.2355	1.7589		
	HA 0.01% (w/v) + H_2O_2 (400 μ M)	8.1737	1.1996		
	HA 0.05% (w/v) + H_2O_2 (400 μ M)	7.0498	1.9351		
Pair 1	[H_2O_2 (400 μ M)] - [HA 0.01% (w/v) + H_2O_2 (400 μ M)]	1.0618	0.9263	$p < 0.001$	11.5
Pair 2	[H_2O_2 (400 μ M)] - [HA 0.05% (w/v) + H_2O_2 (400 μ M)]	2.1856	1.1394	$p < 0.001$	23.67

5.4.3 Dynamic Real-Time Detection of the effect of H-MW-HA on the H₂O₂-induced Redox Changes in Oral Keratinocytes Using ROS Sensitive Fluorescent Sensors.

Given the vital role that ROS production and oxidative stress play in tissue injury and activation of inflammatory conditions, including OM (Afonso et al., 2007), we further investigated H-MW-HA's potential to modulate ROS production in H₂O₂-treated OKF6 cells *in vitro*. By introducing the CM-H₂DCFDA probe in keratinocytes, we could monitor any change in the ROS production state upon exposure to H-MW-HA either in the absence or the presence of H₂O₂- induced oxidative stress by measuring the changes in CM-DCF fluorescence intensity under the fluorescent microscope. OKF6 cells were treated with H₂O₂ (400 μ M) for 4 hours, the time point that, as shown in our previous experiments, coincides with the highest levels of ROS production for OKF6 when treated with H₂O₂. This time, we investigated H-MW-HA's effect at a concentration that was approved to have the highest protection ratio from the previous experiment (0.05% w/v).

First, we found that the used H-MW-HA protocol (pre-treatment of the OKF6 cells with H-MW-HA (0.05% w/v) for 24 hours and then treatment of the cells with H-MW-HA (0.05% w/v) for further 4 hours) did not induce any significant change in the redox state of cells at baseline compared to the control group (Figure 5.6). As expected, and in agreement with the results obtained in measuring the ROS production and kinetics in OKF6, treatment of OKF6 cells with 400 μ M H₂O₂ for 4 hours resulted in increased ROS production ($P < 0.001$) in 5.54 (± 1.37) folds increase compared to the control group value (Figure 5.6). In all examined fields, the increase in the fluorescent intensity of cells in the H₂O₂-treated group was apparent compared to all other experimental groups when the cells were examined under the fluorescent microscope (Figure 5.7). Of note, pre-treatment of the OKF6 cells with H-MW-HA (0.05% w/v) for 24 hours and then co-treatment of the cells with H-MW-HA (0.05% w/v) for another 4 hours was very effective in reducing the oxidative status upon exposure to 400 μ M of H₂O₂ ($p < 0.01$; Figure 5.6). Furthermore, 0.05% (w/v) of H-MW-HA protected the oral keratinocyte cells ($p < 0.01$) by 79.06 % against ROS production induced by H₂O₂ (table 5.6). Thus, we suggest that at the time point 4 h, the reduction in ROS production level can be attributed to the protective effects of HA. Our results demonstrated that the increased ROS production induced by H₂O₂ was reduced to the levels of the controls following treatment with 0.05% w/v H-MW-HA (Figure 5.6), indicating that H-MW-HA has a scavenging effect on ROS produced in response to H₂O₂ in OKF6 cells.

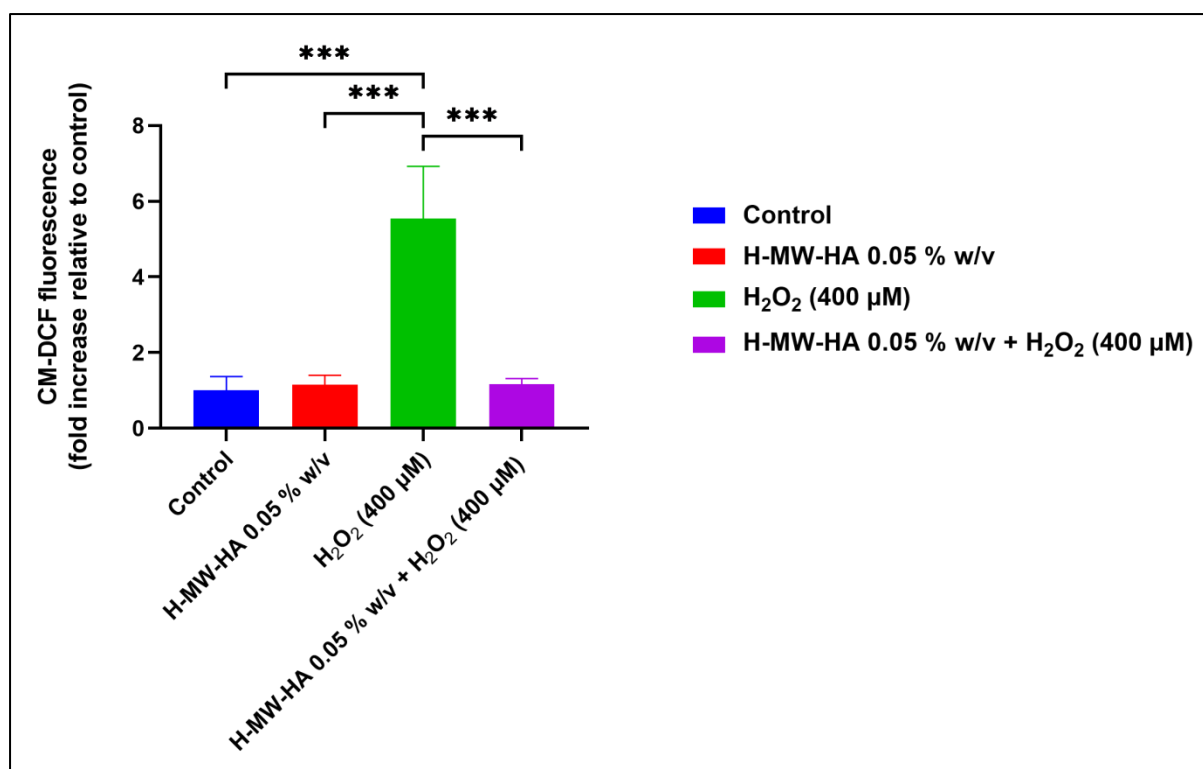


Figure 5.6 Protective effect of H-MW-HA (0.05% w/v) against H₂O₂-induced ROS production. OKF-6 cells were cultured with H-MW-HA (0.05% w/v) in complete keratinocyte serum free media (K-SFM, #17005-042, ThermoFisher Scientific) for 24 hours in 6-well culture plates (Cat#: CLS3516; Sigma-Aldrich) under standard conditions (5% CO₂ at 37 ° C). Culture medium was then replaced with complete culture medium supplemented with H-MW-HA (0.05% w/v) either in the absence or the presence of H₂O₂ (400 μM) and the cells were incubated for 4 hours. Culture medium was then removed and the cells were incubated with the fluorescent probe, 5-(and-6)-chloromethyl - 2', 7'- dichlorodihydrofluorescein diacetate (CM-H₂DCFDA), for 30 minutes. Then, fluorescent probe was replaced with Hank's buffer saline solution (HBSS), and the plates were viewed under the FLoid™ cell imaging station digital inverted microscope (ThermoFisher Scientific, USA). The microscope settings selected included the fluorescein (FITC) filter (488 nm excitation and 530 nm emission). The intensity of CM-DCF fluorescence was measured in seven different fields per each well per condition (seven technical replicates) in three independent experiments (three biological replicates). ROS production was measured as the change in CM-DCF fluorescence (-fold increase) relative to control as control=1. Values represent mean ± SD. Where, *** p < 0.001.

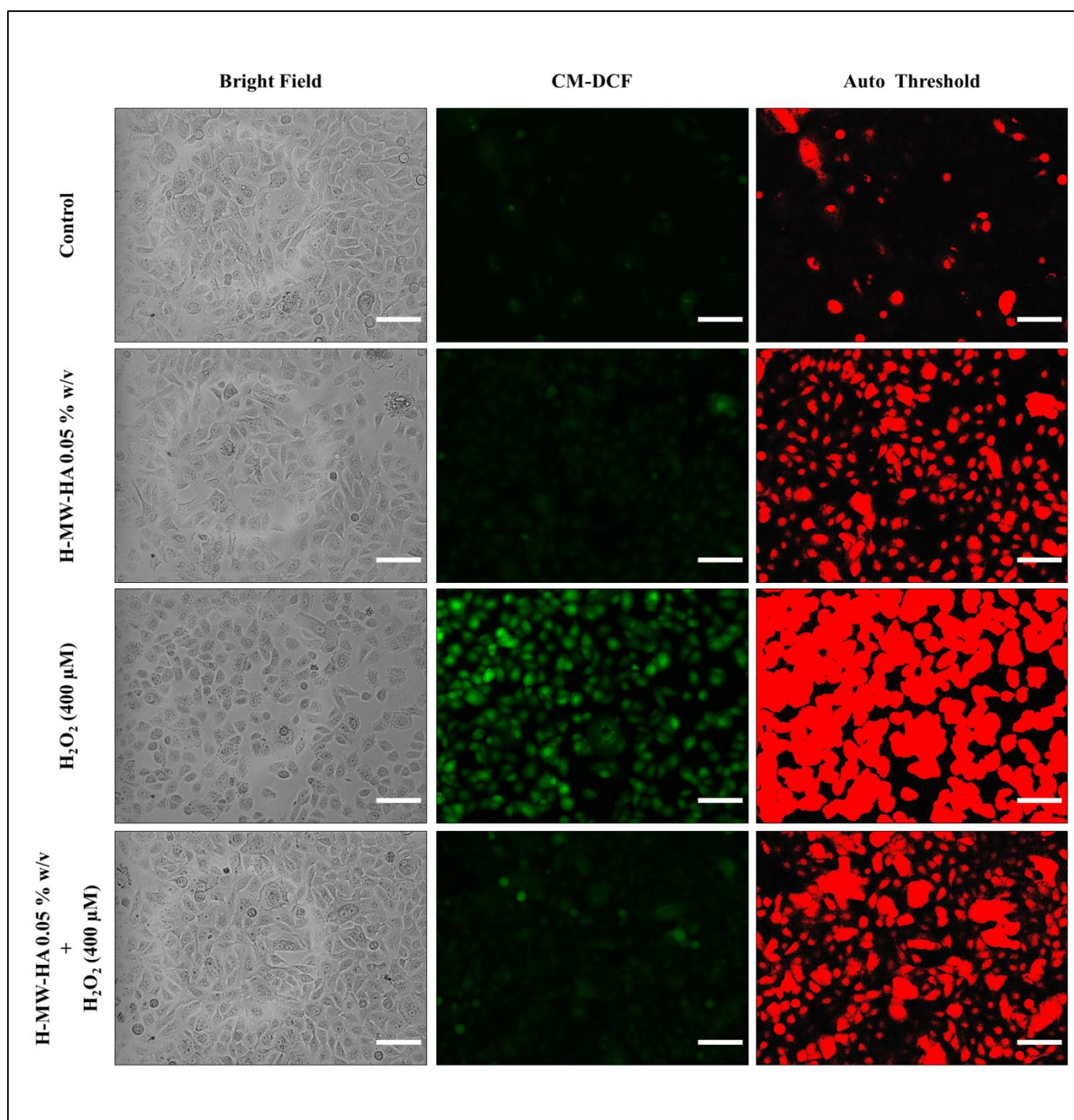


Figure 5.7 Effect of H-MW-HA on H₂O₂-induced ROS production in oral keratinocytes. Representative micrographs, at 20× magnifications, of oral keratinocytes treated with H-MW-HA (0.05% w/v) either in the absence or presence of H₂O₂ (400 μM). OKF-6 cells were cultured with H-MW-HA (0.05% w/v) in complete keratinocyte serum free media (K-SFM, #17005-042, ThermoFisher Scientific) for 24 hours in 6-well culture plates (Cat#: CLS3516; Sigma-Aldrich) under standard conditions (5% CO₂ at 37 ° C). Culture medium was then removed and replaced with complete culture medium supplemented with H-MW-HA (0.05% w/v) either in the absence or the presence of 400 μM H₂O₂ and the cells were incubated for 4 hours. Culture medium was then removed and cells were incubated with the fluorescent probe, 5-(and-6)-chloromethyl - 2', 7'- dichlorodihydrofluorescein diacetate (CM-H₂DCFDA), for 30 minutes. Then, the fluorescent probe was replaced with Hank's buffer saline solution (HBSS), and the

plates were viewed under FLoid™ cell imaging station digital inverted microscope (ThermoFisher Scientific, USA). Filter was set for fluorescein (FITC) cell imaging (488 nm excitation and 530 nm emission). Fluorescence intensities of oxidized probes (CM-DCF from CM-H2DCF) were quantified using the ImageJ software. Image threshold was set using Auto-threshold method (Huang) and the mean intensity of the threshold area was measured. The representative micrographs showed the bright field (left panel), fluorescent intensity of 5-(and-6)-chloromethyl-2',7'- dichlorofluorescein (CM-DCF) (middle panel), and Auto threshold (right panel). The same field is shown in each image filter. Scale bar represents 100 μ m and applies to all panels.

Table 5.6 protective effect (in ratio) of H-MW-HA (0.05% w/v) against H₂O₂-induced ROS production. Data are from 3 independent experiments (three biological replicates), n= 7 per group (seven technical replicates). Values represent mean (folds increase) $\pm$ SD.

Paired sample t-test					
Pairs	Groups	Mean	SD	P-value	Protection (%)
	H ₂ O ₂ (400 μ M)	5.54	1.379		
	HA 0.05% (w/v) + H ₂ O ₂ (400 μ M)	1.162	0.157		
Pair	[H ₂ O ₂ (400 μ M)] - [HA 0.05% (w/v) + H ₂ O ₂ (400 μ M)]	4.38	1.41	p < 0.01	79.06

5.5 Discussion

Despite the biocompatibility, biodegradability, and a non-immunogenicity of HA (Laurent and Fraser, 1992), and increasing evidence of HA and its derivatives effectiveness in the treatment and prevention of OM and other inflammatory conditions, HA still does not stand as a universally recognized therapy for OM, essentially because of the variety of HA constituents and treatment protocols employed in various trials in terms of molecular weight, concentration, mixing with other compounds, and cross-linking. Current literature has demonstrated anti-oxidative properties of HA and its constituents, such as High molecular weight (H-MW) HA, L-MW-HA, cross-linked HA, and HA-based compounds both *in vitro* and *in vivo* (Moseley et al., 2003; Krasinski et al., 2009; Ke et al., 2011; Mendoza et al., 2009; Trabucchi et al., 2002; Halicka et al., 2009). However, there is limited research on the anti-oxidative effects of HA on oral keratinocytes (Cirillo et al., 2015). Therefore, a significant effort is needed to understand whether the different HA constituents exert specific biological effects and to establish the optimal parameters to be used to achieve the best therapeutic activity.

The present study is the first to demonstrate the cell-protective properties of five different HA constituents (Mucosamin[®], H-MW-HA, XI-HA 5/5, XI-HA 30/30, and XI-HA 100/100) towards H₂O₂-induced damage using oxidative stress *in vitro* model that mimics OM. Besides, it is the first study to demonstrate H-MW-HA's antioxidant properties towards H₂O₂-induced oxidative stress in an *in vitro* model of oral keratinocyte injury. All biomaterials studied were determined to possess a cell-protective effect against H₂O₂-induced damage, with H-MW-HA having the greatest protective potential towards oxidative stress-induced damage, followed by XI-HA 30/30, XI-HA 100/100, Mucosamin[®], and XI-HA 5/5. In addition, H-MW-HA and Mucosamin[®] exhibited varying dose-dependent protective effects against H₂O₂-induced cellular damage in oral keratinocytes. Consistent with previous studies in Chapter 4, all HA treatments, except Mucosamin[®], effectively improved OKF6 proliferation, with XI-HA 100/100 having the greatest effect on cell proliferation, followed by H-MW-HA, XI-HA 30/30, and XI-HA 5/5. We then moved to assess ROS levels in oral keratinocyte cells and observed a marked antioxidant activity exerted by H-MW-HA. Most importantly, H-MW-HA showed significant antioxidant capacity against H₂O₂-induced ROS production, with the most substantial reduction in the ROS levels detected in OKF6 cells exposed to 0.05% w/v H-MW-HA. Taken together, these results demonstrate that the protective effects of HA against H₂O₂-induced damage in normal human oral keratinocytes occur through the regulation of ROS

production. Oxidative stress and inflammation are two fundamental components in OM's pathogenesis (Sonis, 2010a; Bowen et al., 2019). With this in mind, HA constituents (H-MW-HA) that demonstrate antioxidant properties in the absence of cytotoxic side effects are likely to be used as a preventive therapy in the treatment of OM.

The latest data on the pathophysiology of chemoradiotherapy-induced mucosal injury, including OM, suggest that cytotoxic agents initiate a complex sequence of biologic events in both mucosal and submucosal compartments in which oxidative stress plays a pivotal initiating role (Iglesias-Bartolome et al., 2012; Yoshino et al., 2013; Chen et al., 2020). According to multiple mechanistic models proposed by Sonis (Sonis, 2004b; 2009), the complex pathophysiological processes of chemoradiotherapy-induced OM are comprised of 5 overlapping stages, including initiation/primary damage response, upregulation and activation, signaling and amplification, ulceration, and healing (Sonis, 2010a; Al-Dasooqi et al., 2013; Bowen et al., 2019). The initiation phase, which occurs immediately after the cytotoxic agents' administration, is characterized by clonogenic cell death and ROS production, which ultimately results in DNA damage, activation of various signalling pathways, and also activation of the innate immune response. The innate immune system responds to chemoradiotherapy-induced damage-associated molecular patterns (DAMPs) and exogenous microbial components, which help to initiate the inflammatory cascade. These activated inflammatory responses impair cell proliferation, induce cell senescence/apoptosis, and inhibit cell regeneration in both mucosal and submucosal compartments, which ultimately cause ulceration (Elting et al., 2007; Cinausero et al., 2017; Maria et al., 2017; Lotze et al., 2007; Subramaniam and Muthukrishnan, 2019; Bowen et al., 2019). Severe OM can have a major impact on cancer treatment delivery and can often result in a reduction and even cessation of cancer treatment delivery, high health care cost, and significant adverse effects on patient's quality of life (QOL) due to extended patient's hospitalization, increased demand for parenteral nutrition, antibiotics, and narcotic analgesia. Additionally, ulcerative OM can become secondarily infected, especially causing bacteraemia or septicaemia, which was found to be mainly caused by *staphylococci* and *streptococci species* of the viridans group (Girmenia et al., 2003; Kersun et al., 2005; Han et al., 2006).

In recent years, much attention has been devoted to ROS's putative role as mediators of tissue damage, and ROS is attributed to participating in the pathogenesis of a large number of diseases, ranging from cancer to inflammation (Roberts, 1988; Senel et al., 1997; Valko et al., 2007; Genestra, 2007). Among these inflammatory diseases is OM, in which there is

convincing evidence implicating ROS (Zhao and Robbins, 2009; Moss, 2007). Oxidative stress plays a key role in developing chemoradiotherapy-induced OM (Yoshino et al., 2013; Rtibi et al., 2018; Russi et al., 2014). If the ROS level is higher than that of cellular antioxidant potential, tissue-damaging oxidative stress will occur. Several key ROS are available, which include H₂O₂, [•]OH, hypochlorous acid, and O₂^{•-}. Among ROS, and because of its solubility in both aqueous environments and lipid and its comparatively low reactivity, H₂O₂ can spread freely through cellular membranes to a distance of numerous cell diameters before it reacts with specific molecular targets (Rhee et al., 2005; Vilhardt and van Deurs, 2004). H₂O₂ is not highly reactive itself, but it is an intermediate to both hypochlorite radical and [•]OH production (Schieber and Chandel, 2014), and there is much evidence that H₂O₂ is a mediator of oxidative damage in cells (Rice-Evans et al., 1995). Excessive production of these ROS affects several cellular structures and leads to DNA damage, protein modification, lipid peroxidation, disruption of cell signalling, and cellular death (Cross et al., 2015; Cross et al., 1987), causing destruction to ECM components, such as collagen, hyaluronan, and proteoglycans (Halliwell et al., 1992; Waddington et al., 2000), and further alter the metabolism of cells responsible for the synthesis of dermal ECM (Vessey et al., 1992; Kawaguchi et al., 1997; O'toole et al., 1996).

H₂O₂ has been widely used to mimic the oxidative stress model *in vitro* (Xu et al., 2018; Wang et al., 2019). In normal human plasma, there is an average H₂O₂ of about 3 μM (Lacy et al., 2000). However, activated macrophages or neutrophils have been shown to release H₂O₂ with a local concentration that might reach up to 1000 μM (Hyslop et al., 1995; Burgoyne et al., 2012). First, we have adopted a free radical H₂O₂-induced oxidative stress model that we have established *in vitro* in our previous experiments (Chapter 3), which reasonably represents the major ROS sources of ROS in OM (Pelle et al., 2005). Five HA constituents, namely Mucosamin[®], H-MW-HA, XI-HA 5/5, XI-HA 30/30, and XI-HA 100/100 were considered in the present study. Cell viability following exposure to the HA constituents in a benign human oral keratinocyte cell line (OKF6) allowed the effect of each constituent to be assessed. When exposed to the pathological range (400 μM) of H₂O₂, OKF6 cells showed significantly decreased viabilities. Strikingly, all tested HA derivatives protected oral keratinocyte cells against H₂O₂-induced damage without exerting any cytotoxic effect against the treated cells, and a decrease in cell viability was massively reduced by pre-incubating the oral keratinocyte cells with an HA-containing product diluted in culture medium. To better understand the cellular mechanism and the dynamics underlying this effect, with the ultimate goal of optimizing our *in vivo* protocol, we wanted to assess the effect of H-MW-HA on redox status

in oral keratinocytes cells. CM-DCF-based fluorometric assay revealed that H-MW-HA acts as a scavenger of ROS during H₂O₂-induced oxidative stress. More importantly, 0.01% w/v H-MW-HA exerted moderate antioxidant activity, whereas a striking reduction in the levels of ROS was detected in cells exposed to 0.05% H-MW-HA. Taken together, these results demonstrate the ability of our HA constituents to be used as a radical scavenging protective system in the form of locally delivered antioxidant therapy.

Our results indicated that H₂O₂ could induce ROS in human normal oral keratinocyte cells even after 15 min exposure and the ROS levels increased after H₂O₂ treatment in a time- and dose-dependent manner. Similar results were reported in previous studies conducted in HaCaT human keratinocytes cells (Bae et al., 2014) or in human umbilical vein endothelial cells (HUVECs) (Park, 2013), but the exposure time of H₂O₂ in these studies was usually longer than 1 h. Furthermore, Cirillo et al. (2015) demonstrated that a significant increase in ROS level was observed in normal human oral fibroblast (NHOF) cells transiently exposed to 600 μ M of H₂O₂ for more than 1 h for 5 days, and it was in accordance with our results. More recently, literature demonstrated that a significant increase in ROS level was observed in HaCaT cells exposed to 1 mM (Zheng et al., 2013), or 1.6 mM (Lee et al., 2019) of H₂O₂ for less than 1 h. Also this was in accordance with our results. Overproduced ROS can cause damage to important biomolecules such as DNA, proteins, and lipids (Beneš et al., 1999). For instance, Cirillo et al. (2015) found that H₂O₂ could cause oxidative DNA damage in NHOF. Therefore, we speculated that the balance between ROS generation and an antioxidant system was destroyed by the surplus ROS produced by H₂O₂, thus leading to DNA damage. Further experiments, however, are needed to substantiate the oxidative DNA damage in normal human oral keratinocytes in a model of oxidative stress.

Unique HA's molecular structure and physicochemical properties, including biocompatibility, nontoxicity, and biodegradability, make it a fascinating macromolecule suitable for various biomedical applications (Sudha and Rose, 2014; Turley et al., 2002). HA plays important physiological functions *in vivo*, such as lubrication of joints, regulating vascular wall permeability, regulating the diffusion and transport of proteins and electrolytes diffusion and transport regulation, promoting wound healing, and it is involved in important cell functions such as cell motility, cell-matrix adhesion and cell organization (Šoltés and Mendichi, 2003; Chen and Abatangelo, 1999; Mori et al., 2004; Fusco et al., 2007; Borzacchiello et al., 2010). Among HA physicochemical characteristics, antioxidant properties are the most relevant and support most of its physiological and therapeutic functions (Toole, 2004; Borzacchiello et al.,

2010; Dovedytis et al., 2020). Several studies indicate the antioxidant properties of HA both *in vitro* and *in vivo* (Suzuki and Yamaguchi, 1993; Cortivo et al., 1996; Fukuda et al., 1997; Mytar et al., 2001; Campo et al., 2004a; Lym et al., 2004; Campo et al., 2008a; Campo et al., 2008b; Zhao et al., 2008; Moseley et al., 2003; Halicka et al., 2009; Ke et al., 2011). H-MW HA has been demonstrated to protect corneal epithelial cells against oxidative damage induced by benzalkonium chloride (Pauloin et al., 2008; Wu et al., 2011a), ultraviolet B radiation (UVB) (Pauloin et al., 2009a; Li et al., 2013), EDTA disodium salt (Ye et al., 2012), and sodium lauryl sulfate (Pauloin et al., 2009b). HA-containing product (Mucosamin[®]) protected NHOV cells against senescence induced by oxidative stress, and protected normal human oral keratinocyte cells against the secondary detrimental effect of senescent fibroblasts (Cirillo et al., 2015). Other studies have pointed to the antioxidant activity of cross-linked HA derivatives such as HYALAN[®] used in wound healing and ophthalmology, which has been shown to be more resistant to $\cdot\text{OH}$ degradation than uncross-linked HA (Al-Assaf et al., 1995; Larsen et al., 1992). In the present study, we confirmed the protective role of five tested HA biomaterials (Mucosamin[®], H-MW-HA, Xl-HA 5/5, Xl-HA 30/30, and Xl-HA 100/100) against oxidative-induced cytotoxicity of H_2O_2 on normal human oral keratinocyte cells. HA pre-incubation effectively enhanced the relative cell survival rates and reduced the cytotoxicity induced by H_2O_2 in OKF6 cells. This study has also highlighted the antioxidant properties of H-MW-HA towards H_2O_2 -induced ROS production in OKF6 cells. We confirmed that H-MW-HA pre-incubation effectively reduced the ROS levels induced by H_2O_2 in OKF6 cells.

The mechanisms underlying the protective role and antioxidant properties of HA against oxidative stress-induced damage in OKF6 cells have yet to be fully elucidated. One possible scenario is that HA may exert protective effects by acting either as a direct or indirect antioxidant. ROS scavenging capacity of HA (direct antioxidant effect) is mainly attributed to its structure as a linear polysaccharide made of repeating disaccharide units of D-glucuronic acid and N-acetyl glucosamine linked together by $\beta(1,4)$ and $\beta(1,3)$ glycosidic linkages, which its long chain is very sensitive to cleavage by ROS (Balazs et al., 1967; Matsumura et al., 1966; Stern et al., 2007). HA is susceptible to ROS attack, which leads to its degradation and changes in its structure and its degradation, thus modulating oxidative damage (Saari et al., 1993). Recent literature reveals a relation between the depolarization of HA by ROS and its ability to scavenge these species, and therefore, exert its antioxidant efficacy (Balogh et al., 2003; Moseley et al., 2003; Trommer et al., 2003; Parsons et al., 2002; Pigman et al., 1961). The ability of HA to achieve this function is more accepted to be both molecular weight- and dose-

dependent, as H-MW-HA forms a long chain than a lower molecular weight HA (Presti and Scott, 1994; Kvam et al., 1995; Moseley et al., 2002; Cortivo et al., 1996). HA provides protection against cellular damage induced by $\cdot\text{OH}$ in a MW- and dose-dependent manner in avian embryonic fibroblasts (Presti and Scott, 1994) and in articular chondrocytes (Kvam et al., 1995). This study also supports the dependency of the antioxidant properties of HA upon molecular weight and concentration. Such conclusions were proven by the greater protective effects of H-MW-HA towards oxidative stress-induced cellular damage compared to the other tested HA derivatives. Furthermore, the concentration-dependent antioxidant characteristic was approved when higher protective efficacy against H_2O_2 -induced cellular damage was obtained with 0.05% w/v H-MW-HA and 5% w/v Mucosamin[®] compared to 0.01% w/v H-MW-HA and 1% w/v Mucosamin[®]. Besides, higher ROS scavenging activity was obtained with 0.05% w/v H-MW-HA, compared to 0.01% w/v H-MW-HA.

The molecular weight of HA is also a significant factor in the ability of HA to directly compete with other cellular and molecular targets for the attack of ROS, with the later formed radicals being confined within the structure of the HA, thus preventing the attack of the alternative sites. During the such antioxidant activity, HA is known to undergo modification and chain depolymerization, yielding HA molecules with lower molecular weights and less viscosity, the mechanisms of which are well-documented (Waddington et al., 2000).

Besides the direct HA antioxidant effect, other authors have highlighted the importance of the HA antioxidant role based on its indirect antioxidant effect. Onodera et al. (2015) demonstrated that HA could also exert an indirect antioxidant effect by modifying Nuclear factor-erythroid-2-related factor 2 (Nrf2) regulation through interaction with CD44/RHAMM and using Akt as an intracellular messenger. Under physiological conditions, the free radicals are detoxified by numerous antioxidants and phase II detoxifying enzymes such as superoxide dismutase (SOD), catalase, glutathione S-transferase, heme oxygenase-1 (HO-1), and GSH-Px. These enzymes are coordinated at the transcription level by Nrf2 (Nguyen et al., 2009; Kansanen et al., 2012). In cyclophosphamide (CY) induced immunosuppressed mice model *in vivo*, HA administration was able to overcome CY-induced immunosuppression and significantly raised the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and total antioxidant capacity (TAOC) in immunosuppressed mice (Ke et al., 2011). Moreover, HA was considered to specifically bind to CD44 receptors, expressed on human epithelial cells, and protected cell membrane against oxidative stress damage induced by benzalkonium chloride by interacting with the CD44 receptor (Pauloin et al., 2008). We hypothesized that the CD44

receptor and Nrf2 pathway activation could be a key to understanding how H-MW-HA protects NHOK cells from cytotoxicity of H₂O₂. However, further research is needed to confirm our hypothesis.

Another key consideration about the antioxidant properties of HA towards H₂O₂-derived ROS is that it may be attributed to the ability of HA to form a viscous, pericellular meshwork around cells that restricts the movement of ROS in close proximity with cells or other biomolecules (Presti and Scott, 1994; Kvam et al., 1995; Moseley et al., 2002; Cortivo et al., 1996). Although, the mechanism by which HA forms this meshwork structure has not been clearly explained, these "meshworks", formed by self-aggregation of the HA molecules (Scott et al., 1991; Scott, 1992) keep the ROS-producing cells further from the target cells or tissues. Such halos of HA around cells reduce tissue injury caused by ROS by creating a mechanical barrier that limits ROS access to the cell surface, thus reducing DNA damage and NF- κ B and caspase activation (Heldin and Pertoft, 1993). It has been shown that the ability of HA to achieve this function is MW- and dose-dependent, as H-MW-HA forms a more effective exclusion zone than L- MW-HA (Campo et al., 2004a; Campo et al., 2008a; Campo et al., 2008b; Zhao et al., 2008). This could explain the higher protective effect obtained with H-MW-HA compared to other tested HA derivatives in our experiment.

In the present study, we also explored the effects of HA constituents on oral epithelial cell proliferation. We demonstrated that the 5 tested HA biomaterials did not show any cytotoxic effect on the OKF6 cells. Quite the opposite, at a concentration of 0.01% v/v, xl-HA100/100 promoted significant epithelial cell proliferation. The possible limitation of this study is the lack of use of various concentrations of the xl-HA solutions and the absence of the biophysical properties of the tested xl-HA constituents. It is possible that the optimal response of oral keratinocytes towards xl-HA may be particularly dose-sensitive. This will need to be fully considered in future experiments. Indeed, the concentration of carbodiimides (EDC) and 1-hydroxybenzotriazole (HOBt) for amidation of the carboxylic acid group might play a role in the biophysical properties of xl-HA (Schanté et al., 2011). A study by LA Gatta et al. (2020) demonstrated that regardless of the HA molecular weight, xl-HA did not show any cytotoxic effect on epithelial cells, which was consistent with our finding. Taken together, these results demonstrate a safe application of xl-HA to treat oral epithelial cells.

The role of HA-based biomaterials containing sodium hyaluronate combined with a pool of amino acids of precursor collagen (Mucosamin[®]) in OM treatment has been elucidated from

clinical trials of OM healing studies (Buchsel, 2003; Buchsel, 2008; Favia et al., 2008; Colella et al., 2010; Ruggiero et al., 2016). It is, however, becoming clear that OM pathogenesis is quite a complex process (Bowen et al., 2019), and the mechanisms underlying the ability of HA-based compounds to mitigate or treat OM are poorly understood and have not been elucidated yet (Colella et al., 2012; Cirillo et al., 2015). As suggested above, the ROS production at early pre-clinical stages and consequent activation of oxidative stress pathways would appear to be working in concert leading to rapid tissue damage, further activation of innate immune response, and continually promoted inflammation (Sonis, 2004b; Al-Dasooqi et al., 2013). Whether HA-based biomaterials are beneficial in mitigating, preventing, or treatment of OM is yet to be proven, although recent studies have suggested that externally applied Mucosamin[®] (HA-based compound) may be beneficial in preventing (Cirillo et al., 2015), or treating (Ruggiero et al., 2016) chemoradiotherapy-induced OM. Although these results are from small studies, they indicate that HA-based biomaterials may play a role in modulating OM pathology.

As shown in this study, H-MW-HA appears to be capable of scavenging H₂O₂-induced ROS levels. Such observations suggest possible mechanisms for preventing an H₂O₂ source for the secondary formation of [•]OH via the Haber–Weiss reaction (Aust et al., 1985). H₂O₂ is also necessary for [•]OH generation by the Fenton reaction (Aust et al., 1985), and the reduction in H₂O₂ concentrations would also reduce the [•]OH generation. H₂O₂ and/or its derivatives are suggested to be responsible for activating the NF-κB pathway (Logan et al., 2007b). The NF-κB is likely to be responsible for inducing inflammation and apoptosis (Logan et al., 2007b). By combining results from our study and proposed antioxidative mechanisms suggested by other literature (Cirillo et al., 2015), H-MW-HA presents a promising therapeutic candidate for reducing oxidative stress in OM. Indeed, attempts at enhancing the antioxidative properties of HA and other polysaccharide-based biomaterials have already been reported (Sakurai et al., 1997; Wenk et al., 2001). Notwithstanding the limitations of the study, in our opinion, the results presented here are important and suited to the purpose of the study. Specifically, the experimental question was triggered by a popular clinical scenario, and the hypothesis was formulated and assessed *in vitro*.

In summary, in the current study, we provided the evidence for the protective effect of HA biomaterials against H₂O₂-induced oxidative stress and explored its mechanism of action in OKF6 human normal oral keratinocytes. According to our findings, five HA constituents, namely Mucosamin[®], H-MW-HA, XI-HA 5/5, XI-HA 30/30, and XI-HA 100/100 protected

OKF6 cells against H₂O₂-induced damage in an oxidative stress model mimicking oral keratinocytes cytotoxicity. This was demonstrated in a MW- and dose-dependent manner. We also showed that H-MW-HA reversed the increased intracellular ROS production and oxidative cellular damage caused by H₂O₂ in oral keratinocytes. In this process, H-MW-HA likely contributed to alleviating oxidative stress and improving oxidative resistance. These results provide the first evidence that HA, particularly H-MW-HA, may have high applicability as a therapeutic for maintaining oral keratinocyte function during oxidative stress.

However, treatment with H-MW-HA would be unjustified if it caused the growth of tumour cells or inhibited the antineoplastic effects of CT. This concern was diminished in a mouse model of ovarian carcinogenesis. Instead, SN-38, the active metabolite of irinotecan (CPT 11), conjugation to HA significantly improved the profile of *in vivo* tolerability and enhanced therapeutic efficacy for ovarian cancer treatment, demonstrating tumour suppressor property as was observed by Montagner et al. (2015). Further studies have also reported that HA derivatives and different types of HA-paclitaxel conjugates have potential synergistic antitumor effects (Banzato et al., 2008; Kim et al., 2013; Lee et al., 2013; Mero and Campisi, 2014; Chen et al., 2018). Furthermore, combinations of antioxidants have been shown to have greater potential synergistic anticancer effects *in vivo* (Lamson and Brignall, 1999).

The use of HA alone or in combination with anticancer agents (5-fluorouracil, doxorubicin, methotrexate, imatinib, gemcitabine, cisplatin, paclitaxel etc.) seems to be a promising new path for antitumor therapeutics, and may have the potential for overcoming multidrug resistance in cancer. (Toole et al., 2008; Seton-Rogers, 2012; Lee et al., 2013; Lokeshwar et al., 2014; Negi et al., 2015; Han et al., 2016; Diao et al., 2019). These observations allowed us to suspect that H-MW-HA does not influence the antitumor effect of 5-FU. However, whether this mechanism is occurring in the combination treatment of 5-FU and H-MW-HA needs further investigation. In addition, further studies are required to identify the effect of H-MW-HA on bioavailability and pharmacokinetics *in vivo* and 3D-OM organotypic models. Therefore, we decided to test the protective effect of H-MW-HA *in vivo*. We considered that these further studies might suggest additional information, including the route of administration and safety of H-MW-HA for clinical application. To that end, doses of 0.01% H-MW-HA were chosen for further studies to confirm whether the prophylactic use of H-MW-HA prevents or ameliorate OM *in vivo* in a mouse model of chemotherapy-induced mucositis.

5.6 Conclusion

The present research work is the first to suggest a potential role of five HA constituents, namely Mucosamin[®], H-MW-HA, Xl-HA 5/5, Xl-HA 30/30, and Xl-HA 100/100 on OM prevention, in relation to their activity against oxidative stress in an *in vitro* model of oral keratinocytes injury. This activity was demonstrated to be MW- and dose-dependent manner in correlation with oral keratinocytes viability. Our results also demonstrate that H-MW-HA can protect in a dose-dependent manner H₂O₂-treated normal human oral keratinocyte (OKF6) cells against oxidative stress by decreasing intracellular ROS generation. Overall, the findings of this study suggest that HA can reduce oxidative stress as a robust antioxidant candidate and alleviate or prevent OM disease associated with chemoradiotherapy-induced oxidative stress. Nonetheless, *in vitro* studies may not reflect the whole real *in vivo* environment, so further studies are needed *in vivo* to translate these findings to pre-clinical applications.

**CHAPTER 6 HYALURONIC ACID
PREVENTS ORAL AND
INTESTINAL MUCOSITIS
INDUCED BY CHEMOTHERAPY *IN*
*VIVO***

6.1 Abstract

Background: Chemotherapy-induced oral and intestinal mucositis are debilitating inflammatory conditions observed in patients undergoing cancer treatment. 5-Fluorouracil (5-FU), one of the most commonly used chemotherapeutic agents used to treat head and neck cancer patients, has been associated with a high incidence of mucositis that often leads to disruptions of the CT course. We have characterized the role of H-MW-HA in promoting the growth of cultured immortalized normal human oral keratinocytes and mitigating the oxidative stress-induced damage in an *in vitro* model of OM. This study aimed to assess the potential protective effects of HA against chemotherapy-induced oral and intestinal mucositis in a murine model with a main focus on the antioxidant effects of HA.

Methods: Oral and intestinal mucositis were induced in female C57BL/6 mice by administering intravenously 5-FU (50 mg/kg) every 48 hours, for two weeks. In addition, mice were pre-treated one day prior to the initial 5-FU treatment and then daily thereafter with either i) H-MW-HA (0.01 % w/v) in drinking water; or ii) drinking water alone. Mice were monitored throughout the experimental period to record the incidence of weight loss and diarrhea. In addition, the incidence, development, and extent of OM were assessed clinically every 24-48 h using the visual oral ulcerative mucositis score. Epithelial histo-morphometric analyses of the tongue and intestinal tissue were conducted 14, 16 and 19 days after the first 5-FU injection. Superoxide dismutase (SOD) enzyme activity was evaluated in blood serum taken at endpoint.

Results: The clinical parameters collected suggested that prophylactic use of the H-MW-HA is effective in preventing the onset or significantly reducing the severity of OM and diarrhea. Histological analysis indicated that H-MW-HA prevented 5-FU-induced damage to the intestinal mucosa and tongue epithelium. We also demonstrated that H-MW-HA enhanced the activity of the SOD enzyme in the blood serum of 5-FU treated mice.

Conclusion: H-MW-HA significantly counteracted the 5-FU-induced damage to the oral and intestinal tissues *in vivo*. The therapeutic effect exerted by H-MW-HA was linked to the downregulation of oxidative stress. Our results support the translation of H-MW-HA to clinical use as an adjunct treatment for cancer patients undergoing CT.

Keywords: hyaluronic acid, oral, intestinal, mucositis, chemotherapy, mouth neoplasm

6.2 Introduction:

Mucositis is a common and most debilitating complication associated with the cytotoxicity of RT and CT (Scully et al., 2006). The condition affects the entire alimentary canal from the mouth to the anus and causes ulceration and pain in both the mouth and intestine. It is also associated with anorexia, vomiting, and diarrhea (Pico et al., 1998; Keefe et al., 2000; Keefe, 2004) and remains a significant burden for 40-80% (Lalla and Peterson, 2006; Campos et al., 2014) of those who are undergoing CRT (Sonis et al., 2004a; Al-Dasooqi et al., 2013; Villa and Sonis, 2015; Lalla et al., 2014). Specifically, nearly all patients, who receive CRT for the treatment of HNC, experience OM during their therapy course (Trotti et al., 2003a; Trucci et al., 2009). Mucositis severity and duration depend on the type of treatment, drug therapy schedule, and patient-based variables (Bentzen et al., 2001; Georgiou et al., 2012; Trotti et al., 2003a; Vera-Llonch et al., 2006).

Oral mucositis (OM, characterized by erythema and ulcerative lesions) and Intestinal mucositis (IM, characterized by \ shortening of villi and disruption in the homeostasis of the crypt cells), collectively referred to as alimentary tract mucositis, are considered to be due to excessive inflammation and epithelial atrophy and apoptosis, together with cellular hypo-proliferation, resulting in a decrease in mucosal thickness (Duncan and Grant, 2003; Campos et al., 2014). Ulcerative lesions in OM are associated with severe pain that requires the use of painkillers, sometimes up to the use of opioids. The consequences of OM are far-reaching and include changes in nutritional status, gastrostomy or parenteral feeding dependence, hospitalization, and the predisposition to bacteraemia or sepsis in severe cases. These sequelae can adversely affect patient's quality of life and are compounded by the use of additional health resources with its accompanying costs (Elting et al., 2003; Elting et al., 2008b; Scully et al., 2003; Sonis et al., 2004a). OM has been reported as the most undesirable and bothersome side effect of cancer treatment experienced by patients (Sonis, 2011). Dose-limiting toxicity may be a consequence in some cases, which leads to delay or even cessation of cancer treatment, negatively impacting the benefit of CT (Sonis et al., 2004a).

Treatment for the highly debilitating condition of mucositis would dramatically improve patients' quality of life and enable patients to receive a higher dosages of radiation or CT for durable remission or cure. Effective prevention or treatment for mucositis also has a predicted potential economic return, estimated to be in the range of USD 1 billion annually (Yuan and Sonis, 2014). This estimated economic return refers to the USA only, and the estimation was

based on a review of the literature and analysis of the economic burden of toxicities associated with cancer treatment, published by Carlotto et al. (2013). Minimal estimates for the incremental costs for mild/moderate mucositis (USD 1,936.06 in 2012 values) and severe (USD 4,099.89 in 2012 values) are considerable, and given that approximately 400,000 patients per year suffer from OM during cancer therapy (Brown and Wingard, 2004), OM costs exceed US\$1 Billion/year. This calculation does not include the cost of drugs to fight the pain, nor the societal costs, including the loss of productivity for patients and their families.

5-fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents for treating a variety of cancers, including breast, gastrointestinal tract, and head & neck cancers (Grem, 2000; Curra et al., 2018), and is also commonly associated with mucositis (Curra et al., 2018). It acts primarily by inhibiting thymidylate synthase and incorporating its metabolites into DNA and RNA to block replication and transcription, resulting in apoptotic cell death and hence, reduced cellular proliferation (Di Gennaro et al., 2009). However, 5-FU does not act selectively, exerting its cytotoxic effects on both malignant and normal cells, mainly targeting rapidly-proliferating cells, such as the alimentary canal, capillary, and immune cells resulting in inflammation and ulceration of the mucosa (Sonis et al., 2001; Cool et al., 2005; Vera-Llonch et al., 2007; Kwon, 2016). Despite its efficacy, the serious side effects of 5-FU such as mucositis and diarrhea frequently cause a reduction of CT dose or even treatment cessation, thereby compromising otherwise durable remission (Sonis et al., 2001; Benson et al., 2004; Manzi et al., 2016).

Despite the significant clinical and economic impact of OM, the accessible therapeutic agents are generally palliative (Sonis, 2011; Colella et al., 2010; Lalla et al., 2014; Cohen et al., 2013), with none specifically targeting the key pathways involved, such as oxidative stress (Colella et al., 2010). Many guidelines for the management of OM, based on contradictory evidence of effectiveness, have been defined by various organizations, such as the MASCC/ISOO, Oncology Nursing Society (ONS), ASCO, and NCCN (Moslemi et al., 2016). A large number of therapeutic modalities, including anti-inflammatories, biologic response modifiers, cytoprotectants antimicrobials, antifungals, coating agents, and anaesthetics are used in managing OM (Lalla et al., 2014; Saunders et al., 2013). Surprisingly, only a few agents, such as palifermin and benzydamine, have been approved to date. Palifermin is an expensive agent, and its use is limited to patients with haematological malignancies receiving stem cell transplants (Raber-Durlacher et al., 2013). Benzydamine has been approved for use in Europe,

but it has an overall limited activity, and its efficacy seems to be limited for patients receiving radiation therapy (Sonis, 2011; Nicolatou-Galitis et al., 2013). Some levels of effectiveness in the prevention of the development, or the reduction in the severity of OM have been reported with a local application of cryotherapy, LLLT, PTA (polymyxin E, tobramycin, and amphotericin B), and - GM-CSF/G-CSF (Stokman et al., 2006; Lalla et al., 2008; Bensinger et al., 2008; Lalla et al., 2014; Migliorati et al., 2013). However, none of these have been confirmed in a systematic review (Raber-Durlacher et al., 2013). Currently, no single intervention that universally prevents or reverts oral or intestinal mucositis exists to improve quality of life and decrease the use of health resources for cancer patients.

Identifying innovative ways to treat OM cannot be separated from the pathogenic mechanisms underlying the development of this condition. The complexity of mucositis's biological process has only been recognized recently (Sonis, 2004b; Logan et al., 2007b; Sonis et al., 2007; Bowen et al., 2019). It is now thought that mucositis represents a sequential series of biological and cellular events, including the interaction of oral mucosal cells and tissues, ROS, proinflammatory cytokines, apoptosis mediators, and local factors such as saliva and the oral microbiota. These events predominantly arise in the cells and tissues of the submucosa (endothelium and connective tissue) and culminate in targeting the epithelial cells, the most important layer of the mucosal barrier, and ending in degeneration and breakdown of the epithelial barrier resulting in the development of ulcers (Sonis, 2004b). Recent studies highlighted the tight junctions (TJs) and their role in regulating the epithelial barrier function (Al-Dasooqi et al., 2013). These junctions regulate the paracellular permeability across the epithelium in both physiological and pathological states in monolayer cell culture and *in vivo* (Madara, 1998; McCarthy et al., 1996; Wong and Gumbiner, 1997; Mitic et al., 2000; Bowen et al., 2019). The effect of cancer therapy on TJ function and the consequent increase in mucosal permeability has been reported (Melichar et al., 2005). The importance of TJs in the pathobiology of mucositis is becoming increasingly clear (Zuo et al., 2015), linking mucositis severity to endotoxin and bacterial translocation through permeable TJs (Bowen et al., 2019). Furthermore, the emerging role of toll-like receptors (TLRs) in mediating mucosal injury has been shown recently in genetic knock-out studies (Bowen et al., 2019). Regardless of the drug-specific downstream signalling, at a molecular level, CT and RT -induced mucosal injury triggered by the excessive production of ROS at early preclinical stages result in consequent activation of oxidative stress pathways (Criswell et al., 2003; Yoshino et al., 2013). Hence,

given the vital role played by ROS in OM pathogenesis, the use of a compound that inhibits oxidative stress-induced damage could represent a valid therapeutic strategy.

HA is a linear, unbranched polymer of disaccharides composed of alternating β -1, 4-glucuronic acid (GlcA) and β -1,3-N acetylglucosamine (GlcNAc) moieties. It is an essential constituent of connective tissues, and the most important glycosaminoglycan produced by fibroblasts during wound and ulcer healing (Rah, 2011). HA is naturally biocompatible, biodegradable, and a non-immunogen, and when in the salt form (sodium hyaluronate, SH), it is widely used in clinical practice to accelerate skin wound repair (David-Raoudi et al., 2008). The effect of HA on keratinocytes and mesenchymal cells is apparent and has been demonstrated recently (Papakonstantinou et al., 2012). Several reports showed the potential efficacy of HA in the treatment of OM, both *in vitro* and *in vivo* (Favia et al., 2008). US Food and Drug Administration, in 2001, approved a gel-based on sodium hyaluronate (SH) as a Class 1 medical device for use in the management of pain relief and which also proved to be useful for pain management in patients with OM (Buchsel, 2008). Also, a spray compound (Aminogam®) containing a pool of synthetic amino acids (L-proline, L-leucine, L-lysine, and glycine) combined with SH, accelerated healing (Favia et al., 2008) and helped manage pain in patients with OM (Colella et al., 2010). Furthermore, preliminary data from a clinical study suggested that HA combined with amino acid precursors of collagen (AAs) (Mucosamin®) may be effective in the treatment of the cytotoxic effect of chemo-and radiotherapy (Ruggiero et al., 2016).

Recently, the same HA-based compound (Mucosamin®) has shown promising results in preventing OM in a pilot clinical study (Cirillo et al., 2015). In addition, using an *in vitro* model of mucositis, Cirillo et al. (2015) showed that oxidative stress-induced senescence in normal oral fibroblast triggered cytotoxicity in keratinocytes, a process that was prevented by HA-based compound (Mucosamin®). However, the mechanisms underlying HA's ability to prevent, mitigate, or treat OM are poorly understood and have not been elucidated yet. Studies on an adherent gel (Gelclair®) composed of poly-vinylpyrrolidone and HS suggest that it works merely as a physical barrier between the oral environment and oral mucosa, thus reducing pain and possibly, promoting healing (Buchsel, 2008). On the other hand, other researchers suggest that the mechanisms underlying amino acid-enriched HA compound (Aminogam®) activity in the healing of OM are more likely to involve biomolecular and physiological changes in

keratinocytes and mesenchymal cells (Favia et al., 2008; Colella et al., 2012; Cirillo et al., 2015).

Animal models of mucositis have been widely used recently as a suitable alternative to human pre-clinical studies, providing novel insights for understanding mucositis pathobiology, as well as assessing innovative interventions (Wardill et al., 2019). The development of an animal model of 5-FU-induced OM by Sonis et al. (1990) has marked an essential step in mucositis research and has since been extensively used in both rats and mice for preclinical testing of new mucositis curative and preventive agents, as it may offer endpoints of therapy close to human clinical pathology. However, despite the evidence that mucositis involves the entire gastrointestinal tract, oral and intestinal mucositis are often studied independently utilizing distinct organ-specific pre-clinical models, and this approach has hindered the development of potentially effective whole-patient treatment strategies. For example, while intraperitoneal (i.p.) protocols that cause IM are easy to conduct, unfortunately, the i.p. 5-FU injection protocol is insufficient to induce OM in mice unless local mechanical or chemical injuries are additionally applied. As such, research has predominantly focused on either oral or intestinal mucosal damage as single entities, using diverse experimental designs and clinical or histopathological assessments (Takeuchi et al., 2018; Shimamura et al., 2018; Tancharoen et al., 2018; de Koning et al., 2007; Zhang et al., 2018; Huang et al., 2019). There is an urgent need for a more holistic experimental animal model that is suitable for the assessment of both oral and gastrointestinal damage induced by CT.

In the previous chapters, we have identified the protective effect of HA preparations against oxidative stress-induced damage in oral keratinocytes *in vitro*, and shown that H-MW-HA exerts significant ROS scavenging activity. In this *in vitro* model of mucosal toxicity induced by H₂O₂, a protective effect of a commercial formulation of HA enriched with amino acids (Mucosamin[®]), cross-linked (xl-), and non-crosslinked H-MW-HA were tested at concentrations of 0.01%, and 0.05% w/v. All HA formulations reduced oxidative stress-induced cell death, but the most marked effects were obtained with the use of H-MW-HA. Specifically, pre- and then co-incubation of H₂O₂-treated OKF6 cells with 0.01% and 0.05% (w/v) H-MW-HA for 24 hours increased cell viability when compared to H₂O₂-treated cells and promoted cell proliferation compared to untreated control. Furthermore, 0.05% (w/v) of H-MW-HA decreased ROS production induced by H₂O₂. This study aimed to examine the

effects of orally administered H-MW-HA in the prevention of chemotherapy-induced mucositis in mice.

6.3 Materials and Methods

6.3.1 Preparation of 5-Fluorouracil (5-FU) and Hyaluronic Acid (HA)

5-FU powder (Cat#F6627, Sigma-Aldrich, Castle Hill, NSW, Australia) was dissolved in saline to a final concentration of 10 mg/ml as described (Section 2.4.2). A Natural non-sulphated H-MW-HA powder (non-crosslinked, m.w. $\sim 1.5\text{--}1.8 \times 10^6$ Da, Hyaluronic acid sodium salt, from *Streptococcus equi*, Cat#53747, Sigma-Aldrich, Castle Hill, NSW, Australia) was dissolved in drinking water at a concentration of 0.01% (w/v) (Section 2.4.3). The water used in the drinking bottles was tap water, filtered (1-micrometer pore diameter), and then autoclaved, and was replenished every three days.

6.3.2 Animals

C57BL/6 female mice aged 6-12 weeks and weighing between 15 and 20 g at baseline were used in this study. Animals were purchased from the Bio21 animal facility (Bio21 Institute, The University of Melbourne, Parkville, Victoria, Australia). Mice were housed in groups of three or four in clear cages with wood chips under specific pathogen-free conditions and maintained on a 12-hour light/dark cycle in a temperature-controlled and humidity-controlled room ($21 \pm 1^\circ\text{C}$ and 40%-60%, respectively) with free access to an autoclaved food pellets and water provided ad libitum. Mice were monitored daily for signs of morbidity, and BWs were recorded using a digital weighing balance starting from day 2 (one day post initial injection) every 48 hours until day 10, then every 24 hours until the end of the experiment. This study was approved by the AEC of The University of Melbourne (ID 1814636.3). All work was conducted in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes determined by the National Health and Medical Research Council 8th edition (Hubrecht, 2013). All efforts were made to minimize animal suffering.

6.3.3 Chemotherapy induced oral mucositis (CIOM) model

C57BL/6 mice were used to study potential protective effects of HA against chemotherapy-induced oral mucositis (CIOM) according to a previously described method (Bertolini et al., 2017). A total of sixty mice were used in this study, randomly divided into 4 groups:

- Group A (Control group, n=12). These mice received only i.v. saline injections every 48 hours, from day 1 to day 13.

- Group B (HA treatment only group, n= 12). These mice received HA compound (0.01% w/v) orally in drinking water from day 0 until the endpoint day
- Group C (5-FU group, n=18). These mice received a single i.v. 5-FU injection (50 mg/kg/day) every 48 hours, from day 1 to day 13.
- Group D (HA treatment + 5 FU group, n= 18). These mice received a single i.v. 5-FU injection (50 mg/kg/day), every 48 hours, from day 1 to day 13, plus HA compound (0.01% w/v) orally in drinking water from day 0 until the endpoint day (Figure 6.1).

In all cases i.v. (intravenous) administration of 5-FU and saline was performed via tail vein injections (Section 2.4.5 and 2.4.6).

Starting from day 2 (one day post initial injection), mice were assessed clinically for the incidence, development and extent of OM using the Oral Mucositis Index (Table 6.1), every 48 hours until day 10, then every 24 hours until the end of the experiment (Section 2.4.9). Animals were sacrificed on days 14, 16, and 19. Blood samples were obtained by cardiac puncture and allowed to clot at room temperature for 30 minutes, and then centrifuged at 2500 g for 20 minutes, and blood serum was frozen at -80°C until analysis (Section 2.4.10). A 6-cm section of the proximal jejunum, and tongue specimens were collected at necropsy (Section 2.4.11). For histological analysis and scoring, the jejunum and tongue samples were fixed in 10% neutral buffered formalin (4% formaldehyde) with pH 7.4 for 48 hours at room temperature and then transferred to 70% ethanol and stored at 4°C for long term storage. About half of the posterior third of the tongue and small part of the jejunum weight between 0.5 – 1.0 gram were put in labelled cryovials tube (Cat# CLS430487, Sigma Aldrich, Castle Hill, NSW, Australia) and frozen in liquid nitrogen <30 min after sample harvesting. Then, they were stored at -80°C for further analysis.

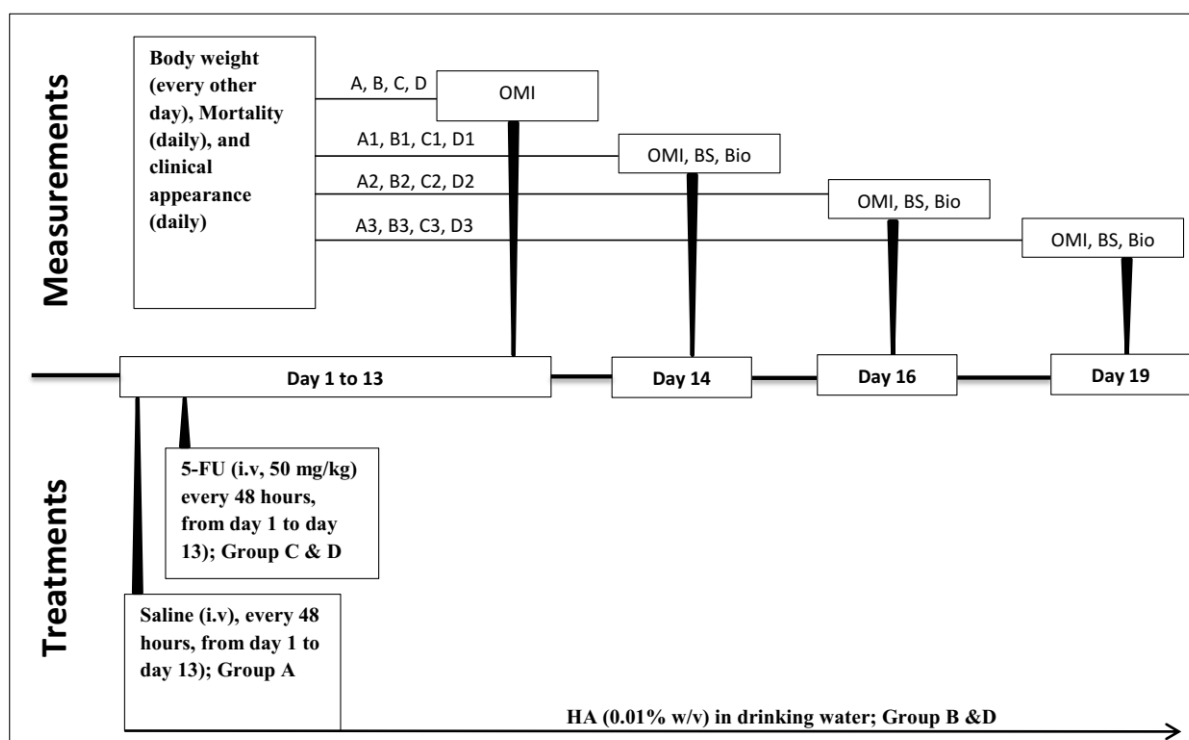


Figure 6.1 Schematic diagram of the experimental procedure timeline. 6-12 weeks-old C57BL/6 female mice were divided into 4 groups and treated as follows: (1) Group A; Saline as double control (control); (2) Group B; HA treatment only (H-MW-HA); (3) Group C, 5-FU; (4) Group D, 5-FU+HA (5-FU+H-MW-HA). The HA treatment in drinking water was started at day 0. 5-FU was injected 7 times at 2-day intervals, from day 1 to day 13. At days 14, 16, and 19 mice were sacrificed, and the blood serum samples were collected. Tongue and jejunum were retrieved at necropsy. Abbreviations: BS, post-mortem blood sampling; Bio, post-mortem biopsy; OMI, oral mucositis index scoring.

Table 6.1 Visual oral ulcerative mucositis score (Nakajima et al., 2015). adapted from Sonis et al. (2000).

Grade	Severity	Description
0	Normal	Normal (no abnormalities)
1	Mild	Partial hyperaemia, erythema and swelling, but no evidence of mucosal erosion (intact mucosa)
2	Mild	Diffuse hyperaemia, erythema and swelling and superficial erosion, such that there may be mild sloughing
3	Moderate	Epidermolysis, hyperaemia and erythema; moderate mucositis characterized by frank ulcer formation. Ulcers typically have areas of necrosis (erosion) with associated yellowish/grey coloration with pseudomembrane formation. Cumulative area of ulceration $\leq 25\%$ of tongue surface area
4	Severe	Extensive epidermolysis and bleeding; severe mucositis with ulcer formation affecting 25% to 50% of tongue or buccal mucosal area. Marked erythema and pseudomembrane formation. Loss of pliability
5	Severe	Bleeding and abscesses; virtually complete ulceration of the mucosa, all mucosa areas at risk. Loss of mobility and pliability

6.3.4 Oral Mucositis Index

Mice were anesthetized with isoflurane every 48 hours, starting from day 2 until day 10, then every 24 hours until the end of the experiment and the incidence, development, and extent of OM assessed clinically by using the visual oral ulcerative mucositis score, adapted from Nakajima et al. (2015), (Table 6.1) (Figure 6.2) (Section 2.4.9). Image acquisition (Figure 6.2B) was recorded digitally (Canon EOS 60D; Canon Inc., Tokyo, Japan).

6.3.5 Diarrhea Assessment

In the present study, we assessed the severity of 5-FU-induced gastrointestinal mucositis by assessing diarrhea severity in animals as a surrogate marker of gastrointestinal mucositis. Stool passages/diarrhea severity of all mice were recorded, starting from day 2 (one day post initial injection), every 48 hours until day 10, and then every 24 hours until the end of the experiment (Section 2.4.8). Diarrhea severity was assessed by using Bowen's score system (Gibson et al., 2003) and classified into four grades according to the stool consistency: 0, normal stool; 1, slightly wet and soft stool indicating mild diarrhea; 2, wet and unformed stool indicating moderate diarrhea; 3, watery stool indicating severe diarrhea (Section 2.4.8).

6.3.6 Macroscopic Examination of the tongue

The tongue was excised at the level of the trachea. To reveal erosive or ulcerative lesions of the surface, tongues were stained with 1% toluidine blue in 10% acetic acid for 1 minute, followed by repeated washes with 1% acetic acid until there was no further recovery of dye (Muanza et al., 2005). Tongues images were acquired with a digital camera (Canon EOS 60D; Canon Inc., Tokyo, Japan) using a 100 mm lens (Canon 100mm f/2.8 Macro USM lens) connected with a ring flash (Sigma EM-140 DG Ring Flash) and scored accordingly to the following criteria: Negative – lack of dye uptake or light, diffusely stippled uptake of dye; positive – deep, royal blue staining of the epithelium and lack of epithelium (identified as an ulcer). The percentage of toluidine blue positive surface area (excluding excision trauma) was calculated using Fiji (ImageJ) software (Schneider et al., 2012; Schindelin et al., 2012) (Section 2.4.12).

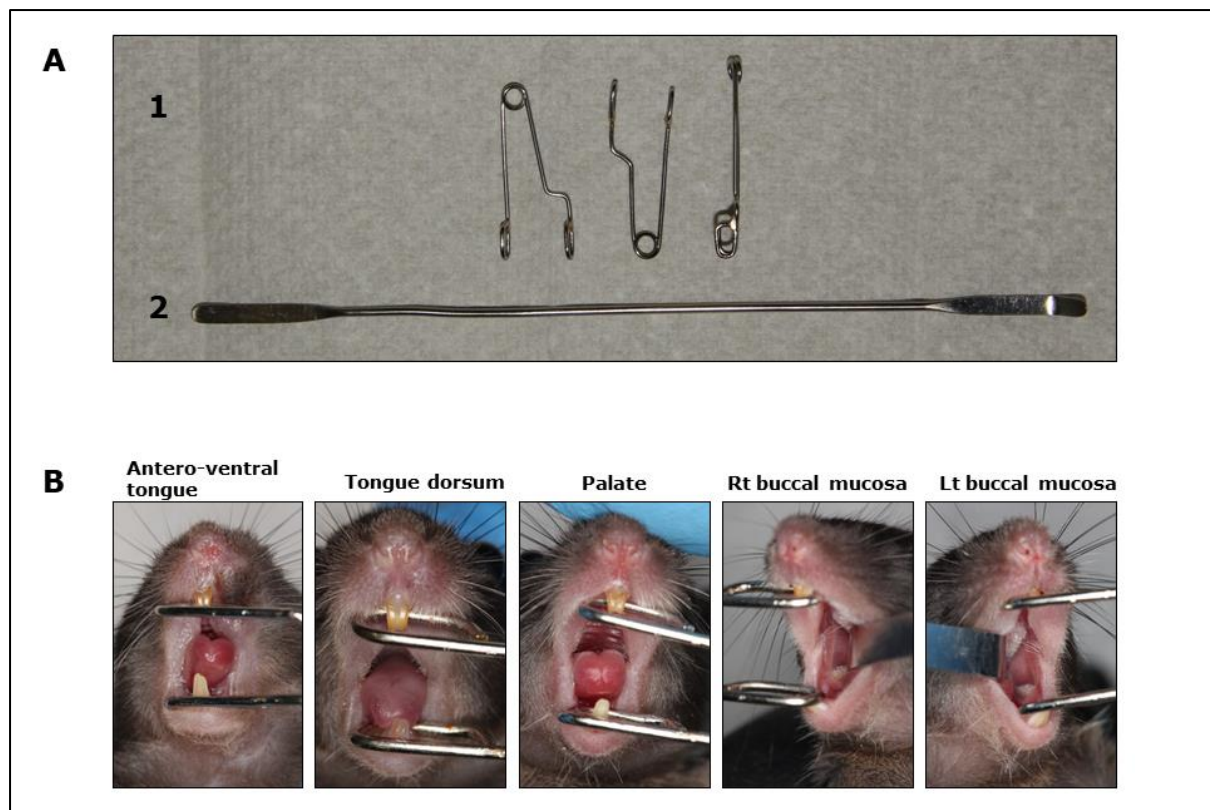


Figure 6.2 Oral cavity assessment. **A:** Oral cavity examination tools; **1:** Stainless steel laboratory micro spatula; **2:** three views of Celentano Murine Mouth opener (CMMO). **B:** Oral cavity examination views. Right (Rt); Left (Lt).

6.3.7 Tissue sample preparation

At necropsy, a 6-cm section of the proximal jejunum, and tongue specimens were collected. For analysis of the intestinal jejunum, 3-4 transversely cut samples of 1 mm in length and one longitudinally cut sample of 4 mm in length were taken at random positions, considering the complete length of the sample. All transverse samples were taken at similar positions throughout the gastrointestinal tract. For tongue analysis, each tongue segment, depending on length, was divided into 3-4 transversely cut samples of 2-3 mm each.

6.3.8 Histological processing

Samples were processed by the Peter MacCallum's Centre for Advanced Histology and Microscopy (CAHM, Melbourne, Victoria, Australia). Briefly, samples were dehydrated in a Tek VIP 6 tissue processor (Sakura Finetek USA, Torrance, CA, USA) and paraffin embedded with Tissue-Tek TEC (Sakura Finetek USA, Torrance, CA, USA) according to standard protocols (Section 2.4.14). 4 µm thick transverse section (Leica RM2255 Rotary Microtome) was made of each transverse sample of intestinal ring and tongue (Figure 6.3a) and of each longitudinal section of intestinal lumen (Figure 6.3b) (Section 2.2.15). All sections were mounted on microscope slides, stained with H & E using a fully automated process (Jung Auto Stainer XL, Leica Microsystems, Victoria, Australia) and cover-slipped (Section 2.2.16). All slides were scanned as digital images using Olympus VS120 automated slide scanner equipped with a BX61VS microscope with a 20× objective (Olympus VS120-S6-W, Olympus VS-ASW software). Scanned digital images were examined using QuPath open source digital software v. 0.2.0 (Bankhead et al., 2017).

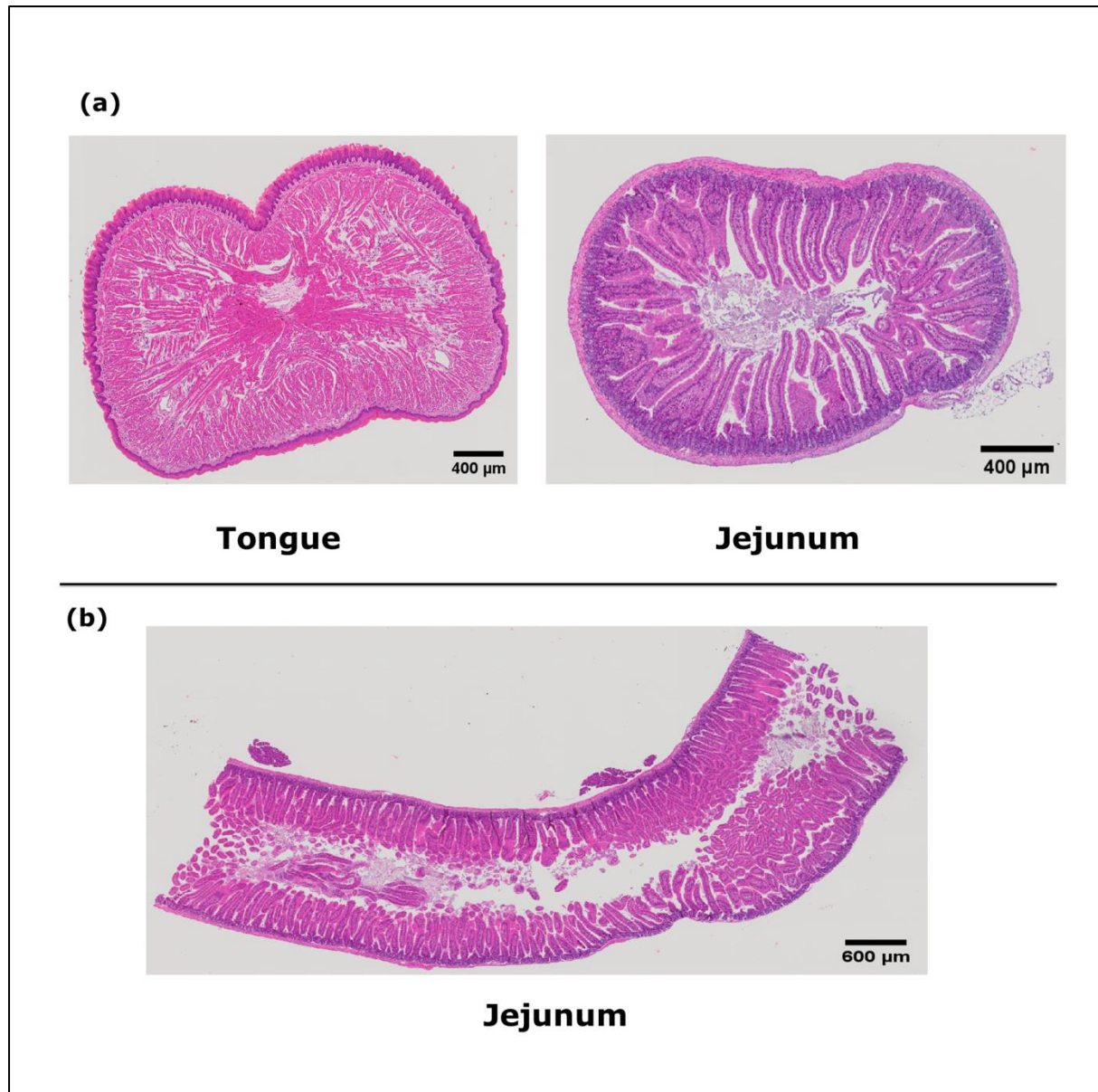


Figure 6.3 Histological sections of the various sections used for mucositis assessment. H&E-stained tissue sections (20x). **(a)**. transversely cut segments of tongue and jejunum. **(b)**. longitudinal cut segment of jejunum.

6.3.9 Morphometric analysis

6.3.9.1 Small intestine

For the morphometric analysis of the intestinal wall of the jejunum, tissue samples were oriented with longitudinally cut villi to assess tunica mucosa thickness, intestinal villi length and crypt depth (Figure 6.4). Analysis was conducted as described by Navarrete et al (2015) using QuPath open source digital software v. 0.2.0 (Bankhead et al., 2017). Briefly, three mice per group were analyzed, with the analysis consisting of 3 intestinal ring sections and one longitudinal intestinal section per mouse. Each analysis incorporated 3 fields per section and 1 measurement per field, with twelve intact villi and crypts measured and averaged (Navarrete et al., 2015). All scores were allocated in a blinded manner.

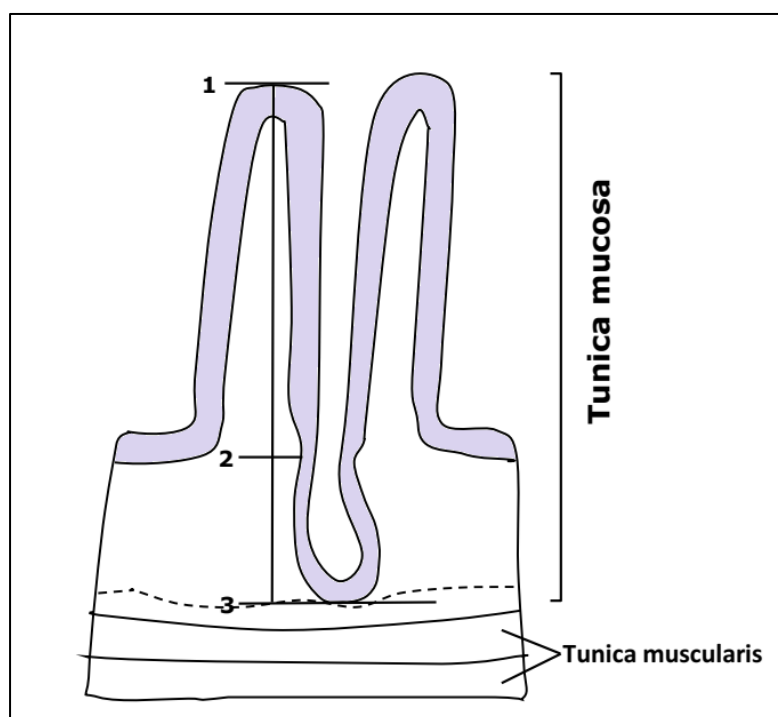


Figure 6.4 Schematic diagram of the wall of the small intestine. Morphometric measurements of the tunica mucosa, intestinal villi and crypts of the ileum. **1-3:** Thickness of the tunica mucosa; **1-2:** Length of the intestinal villi; **2-3:** Depth of the intestinal crypt.

6.3.9.2 Tongue

Epithelial thickness of the tongue was measured using QuPath open source digital software v. 0.2.0 (Bankhead et al., 2017) as described by Carrard et al (2008). Briefly, epithelial thickness of the tongue was measured from the basal membrane to the granular layer. Three mice per group, and three transverse sections per tongue, consisting of 3 fields per section and 3 measurements per field, were analysed, in a blinded manner (Figure 6.5).

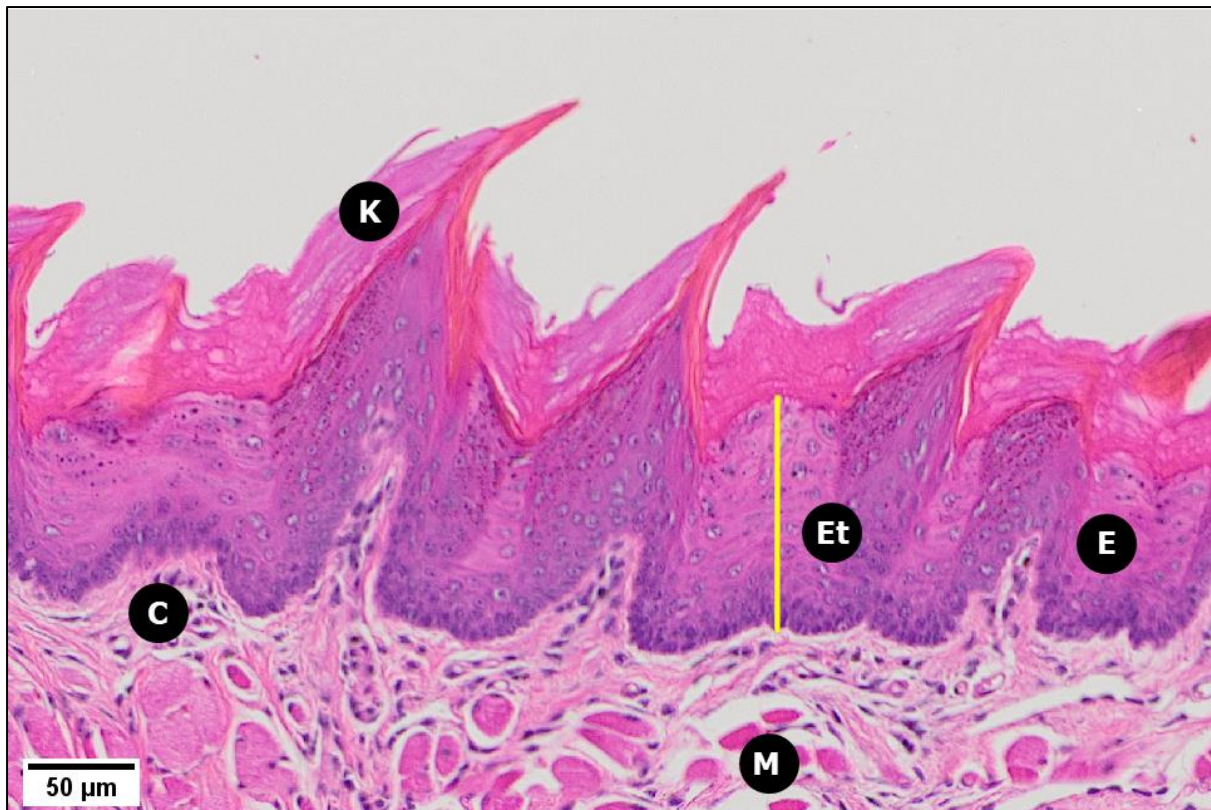


Figure 6.5 Mucosa of the tongue, showing keratin layer (K), epithelial tissue (E), connective tissue (C) and muscle tissue (M). Epithelial thickness (Et) was measured from basal membrane to epithelial granular layer. H&E stain of dorsal tongue section (20x).

6.3.10 Superoxide dismutase (SOD) enzyme activity assay

Total superoxide dismutase activity was measured in blood serum using an indirect enzyme assay (SOD Assay Kit, 19160, Sigma Aldrich, Castle Hill, NSW, Australia) and carried out according to the manufacturer's instructions (Section 2.2.13). Briefly, total SOD activity was assayed spectrophotometrically (Perkin Wallac Victor 2V Multilabel Microplate Reader 1420, Shelton, USA) by measuring absorbance at 450 nm by a method using xanthine and xanthine oxidase for generation of superoxide anion and highly water-soluble tetrazolium salt, WST-1, as the indicator reagent. The assay was run in triplicate with a 20 µl of sample solution placed in 96 well plates in a total volume of 240 µl/well. The rate of WST-1 reduction by superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, the inhibition activity of SOD was quantified by measuring the decrease in colour development. The point where a 20 µl of sample solution inhibited the reduction of WST-1 to 50% of maximum (IC₅₀) was defined as 1 U of SOD activity. A dilution ratio, where the inhibition curve gives 50% inhibition, was calculated, and the SOD unit in the original sample was calculated by multiplying the dilution rate. The assay was run in biological triplicate and all the activities were expressed as U/ml of blood.

6.3.11 Statistical Analyses

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 42 (IBM Corp., Armonk, N.Y., USA) and GraphPad Prism version 8.0.1 for Windows (GraphPad Software Inc, San Diego, CA). Data are presented as means ± SD, unless otherwise mentioned, and evaluated by One-way analysis of variance ANOVA followed by Tukey's post hoc test to detect inter-group differences. Pearson chi-square tests for independence (categorical variables) were used to examine differences in ulcer status and diarrheal status between different treatment groups. Fit spline/LOWESS test was used to calculate IC₅₀. The Logrank test was used for comparative analysis of survival rates. Differences were considered to be statistically significant if $p < 0.05$. "n" indicates the number of independent experiments performed.

6.4 Results:

6.4.1 Overall response to 5-FU and H-MW-HA treatment

Sixty mice were used in the study and divided into four groups as described above; A (control), B (H-MW-HA), C (5-FU), and D (5-FU+H-MW-HA), with respectively n= 12, 12, 18, 18 mice per group (Figure 6.1). 5-FU was intravenously administered, as described above. Mice showed adverse signs after exposure to 5-FU. These included a dull ruffled coat, a change in temperament, and significant weight loss ($p<0.05$). A total of six unexpected euthanizations occurred within 5-FU treated groups, due to animals reaching intervention weight loss of $< 20\%$, before the endpoint (2 mice on day 10, 1 mouse on day 12, one mouse day 14, one mouse on day 15, and 1 mouse on day 17). In group D (5-FU+H-MW-HA), only four unexpected euthanizations happened due to animals reaching intervention weight loss of $< 20\%$, before the endpoint (one mouse on day 10, one mouse on day 11, and two mice on day 14). Control mice (Group A) consistently gained weight throughout the experiment. H-MW-HA-only treated mice (Group B) showed no signs of distress or adverse effects to the HA preparation and continued to gain weight for the duration of the trial.

6.4.2 H-MW-HA administration does not reverse 5-FU associated weight loss but increase overall survival rate of mice

To investigate the systemic adverse effects associated with mucositis, mice BW was initially measured every 24-48 hours and compared between groups. The mean baseline body weight of group A (control), group B (H-MW-HA), group C (5-FU), and group D (5-FU+H-MW-HA) was 17.5 ± 1.32 g, 17.35 ± 1.32 g, 17.4 ± 0.78 g, and 17.64 ± 1.28 g, respectively, with no significant ($p>0.05$) differences found between groups. The largest BW loss was observed in groups C and D, on day 16; on this day the average weight of the groups A, B, C, and D was 17.98 ± 1.4 g, 18.91 ± 1.2 g, 14.02 ± 1.41 g, and 14.68 ± 2.04 g, respectively. By the end of day 19, the final mean BW of the mice in groups A, B, C, and D was 18.15 ± 0.88 g, 19.05 ± 1.02 g, 14.5 ± 2.58 g, and 16.9 ± 2.86 g, respectively, with significant differences between group C and group B ($p=0.031$) (Table 6.2). Change in the BW percentage was apparent across the treatment groups. Mice in groups C and D had higher BW loss percentage compared with those in the group A, and group B. The BW loss was noted immediately after the first injection of 5-FU on day 2; this was persistent throughout the remainder of the study, however increased after day 16. BW loss was significant from day 6 to day 9 in group C and at day 6, and days 10 to 17 in group D, and

peaked at day 16 in both groups (Figure 6.6a). In contrast to the 5-FU treated mice, the mice treated with 5-FU+H-MW-HA exhibited more moderate BW loss at day 8, 10, 15, 17, 18, and 19, however this was not significant. Importantly, significant BW gain was observed during H-MW-HA administration in the group who received H-MW-HA alone, starting on the second day of the treatment administration and persistent throughout the remainder of the study (Figure 6.6a).

Since weight loss in excess of 20% was deemed unethical, death/drop out was recorded as those mice with <20% weight loss (Fig. 6.6 b). All mice in groups A and B survived throughout the experiment. Conversely, among the other groups the number of mice that survived until endpoint started to decrease after day 10. Even with this limitation, mortality in the 5-FU treated group was significantly higher ($p = 0.03$) (11.2%, 16.7%, 22.2%, 30%, and 44% on days 10, 12, 14, 15, and 19, respectively) than that of the H-MW-HA+5-FU group (5.6%, 11.2%, and 22.2% on days, 10, 11, and 19 respectively). An association between the 5-FU treatment groups and survival mice was observed, $\chi^2 (3) = 19.98$, $p=0.0002$ (Figure 6.6 b). In conclusion, H-MW-HA treatment improved the survival rate of mice in the 5-FU+H-MW-HA group compared to the 5-FU group.

Table 6.2 Treatment allocations and body weights from 5-Flurouracil (5-FU) and hyaluronic acid (HA) treated mice. Mice were treated with high molecular weight hyaluronic acid (H-MW-HA) 0.01% w/v orally in drinking water, from one day before the 5-FU injections until the endpoint day. Body weight was measured every 24-48 hrs. N=number. * P <.05 vs 5-FU; ** P<0.05 vs 5-FU+H-MW-HA.

Days	Groups	N	Mean ± SD	Days	Groups	N	Mean ± SD
Body weight (grams) at Day-0	Control	12	17.5±1.32	Body weight (grams) at Day-13	Control	12	17.97±1.18 *, **
	H-MW-HA	12	17.35±1.32		H-MW-HA	12	18.65±1.17 *, **
	5-FU	18	17.4±0.78		5-FU	15	15.85±1.09
	5-FU + H-MW-HA	18	17.64±1.28		5-FU + H-MW-HA	16	15.92±1.55
	Total	60	17.48±1.14		Total	55	16.94±1.75
Body weight (grams) at Day-2	Control	12	17.86±1.19	Body weight (grams) at Day-14	Control	12	18.05±1.23*, **
	H-MW-HA	12	17.81±1.07		H-MW-HA	12	8.43±1.11 *, **
	5-FU	18	17.52±0.86		5-FU	15	15.12±1.30
	5-FU + H-MW-HA	18	17.75±1.26		5-FU + H-MW-HA	16	15.16±1.82
	Total	60	17.72±1.08		Total	55	16.49±2.08
Body weight (grams) at Day-4	Control	12	17.64±1.27	Body weight (grams) at Day-15	Control	8	17.9±1.49 *, **
	H-MW-HA	12	18.01±1.04		H-MW-HA	8	18.55±1.23 *, **
	5-FU	18	17.23±0.86		5-FU	10	14.44±0.99
	5-FU + H-MW-HA	18	17.43±1.22		5-FU + H-MW-HA	8	15.83±1.48
	Total	60	17.53±1.11		Total	34	16.55±2.10
Body weight (grams) at Day-6	Control	12	17.77±1.28	Body weight (grams) at Day-16	Control	8	17.98±1.40 *, **
	H-MW-HA	12	18.1±1.16 *		H-MW-HA	8	18.91±1.20 *, **
	5-FU	18	16.82±0.83		5-FU	9	14.02±1.41
	5-FU + H-MW-HA	18	17.08±1.17		5-FU + H-MW-HA	8	14.68±2.04
	Total	60	17.35±1.18		Total	33	16.33±2.58
Body weight (grams) at Day-8	Control	12	17.48±1.18	Body weight (grams) at Day-17	Control	4	17.72±0.73 *
	H-MW-HA	12	18.15±1.12 *		H-MW-HA	4	18.37±1.04 *
	5-FU	18	16.78±0.77		5-FU	5	14.5±1.70
	5-FU + H-MW-HA	18	17.18±1.02		5-FU + H-MW-HA	3	16.83±1.62
	Total	60	17.32±1.10		Total	16	16.71±2.02
Body weight (grams) at Day-10	Control	12	17.68±1.19 *	Body weight (grams) at Day-18	5-FU	4	14.72±1.97
	H-MW-HA	12	18.24±1.20 *, **		5-FU + H-MW-HA	3	16.86±2.00
	5-FU	18	16.25±1.27		Total	7	15.64±2.14
	5-FU + H-MW-HA	18	16.72±1.34		Control	4	18.15±0.88
	Total	60	17.07±1.45		H-MW-HA	4	19.05±1.02 *
Body weight (grams) at Day-11	5-FU	16	16.81±1.12	Body weight (grams) at Day-19	5-FU	4	14.5±2.58
	5-FU + H-MW-HA	17	16.85±1.28		5-FU + H-MW-HA	3	16.9±2.86
	Total	33	16.83±1.19		Total	15	17.16±2.51
Body weight (grams) at Day-12	Control	12	18.01±1.35 *, **				
	H-MW-HA	12	18.57±1.16 *, **				
	5-FU	16	16.46±1.21				
	5-FU + H-MW-HA	16	16.51±1.19				
	Total	56	17.26±1.50				

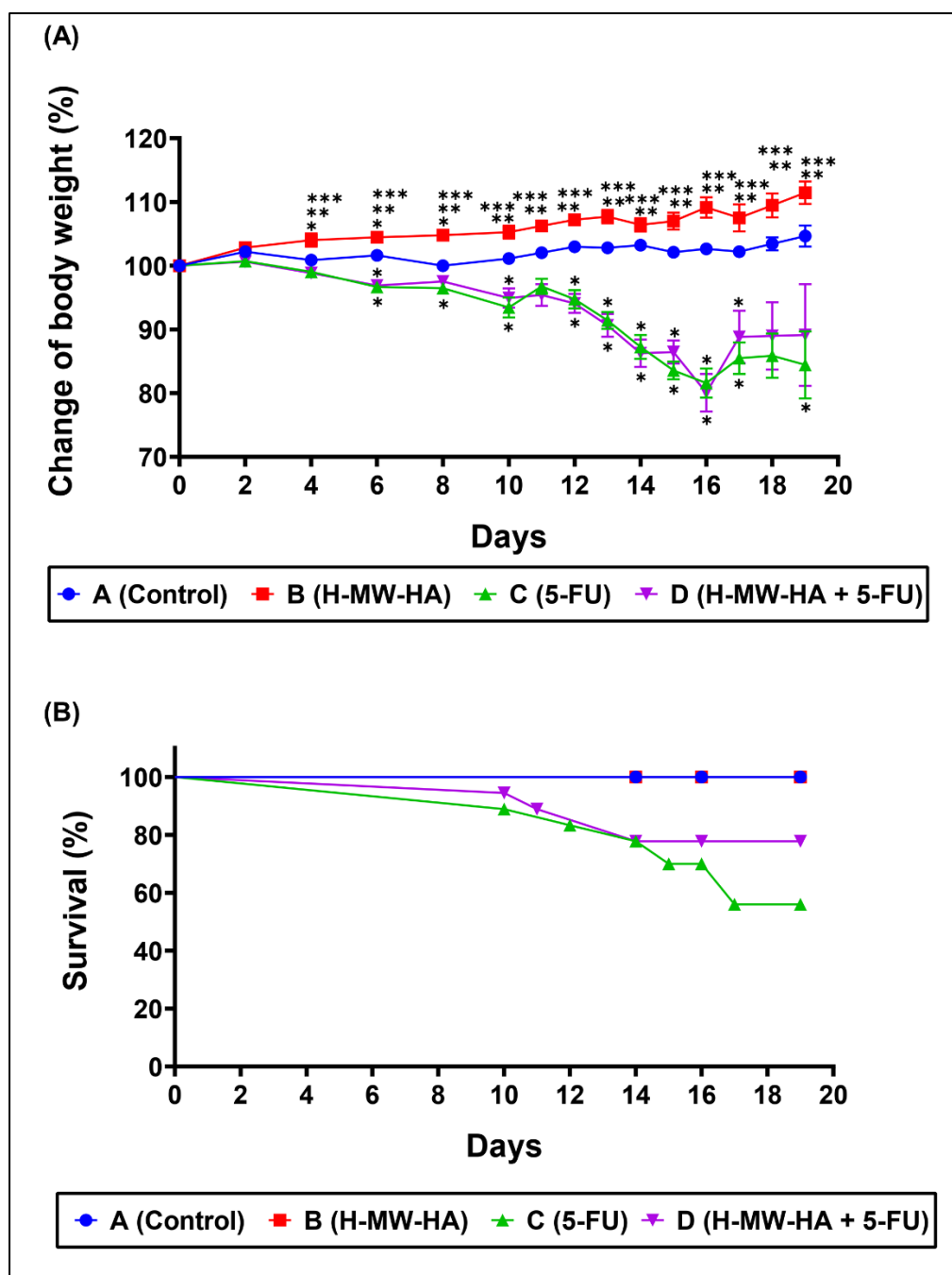


Figure 6.6 The effects of high molecular weight Hyaluronic acid (H-MW-HA) treatment on body weight and survival of mice. (A) Changes in mice body weight (BW) observed over time while receiving either no treatment (Control), HA, 5-FU, or co-treatment with 5-FU and HA; Mice BW was measured every 24-48 hrs. The BW (%) showed here represents the mean $\pm$ SEM of each group. The single mouse BW (%) on a specific day was calculated as difference from the BW of the same mouse on that day compared to the weight on day 0. The mean BW of each group at day-0 was defined as 100%. **(B)** Time-course of mice survival while receiving no treatment (control), or treatment with either HA, 5-FU, or co-treatment with 5-FU and HA. * $P < 0.05$ vs control group; ** $P < 0.05$ vs 5-FU; *** $P < 0.05$ vs 5-FU+H-MW-HA, (n = see table 2).

6.4.3 H-MW-HA ablates Chemotherapy-induced diarrhea

In order to assess the effects of H-MW-HA treatment on 5-FU induced diarrhea, we assessed this mucositis complication in all treatment groups (A-D). Diarrhea severity was assessed based on the consistency of mice stool according to Bowen's score system (Gibson et al., 2003), as previously described in "Materials and methods" section of this thesis. The control (A) and H-MW-HA only (B) treated groups exhibited no obvious diarrhea symptoms throughout the entire experiment. Group C treated with 5-FU alone, developed diarrhea from day 9, peaking at day 13 of 5-FU treatment, when 40% of mice had developed mild diarrhea and 6.6% of mice had moderate disease. After this time point, diarrhea improved and was entirely resolved by day 16. However, daily administration of H-MW-HA at (0.01% w/v) nearly completely prevented diarrheal events compared to the 5-FU alone treated group (Figure 6.8). In fact, mice receiving 0.01% w/v H-MW-HA before, during, and after CT treatment had a different pattern of diarrhea from those receiving CT alone. Diarrhea developed on day 11 and was transient, with mainly one mouse who developed mild diarrhea and this was resolved by 24hrs (Figure 6.8). Stool consistency score between group C and all other groups was statistically significantly different ($p < 0.01$) on day 12, 13, and 14 (Figure 6.7). Diarrhea developed in 72.2% of group C mice, which was reduced to 5.6% in mice also receiving H-MW-HA (Table 6.3). A correlation between diarrhea status (present/absent) and the treatment groups was found; $\chi^2 (1) = 16.831$, $p = 0.00004$, Cramer's $V = 0.684$, and H-MW-HA showed a decreased occurrence in 5-FU-induced diarrhea.

Table 6.3 Description of diarrheal status stratified by treatment received. Mice received a single intravenous (IV) 5-FU injection (50 mg/kg/day), every 48 hours, from day 1 to day 13, plus hyaluronic acid (H-MW-HA) compound (0.01% w/v) orally in drinking water from day 0 until the endpoint day. Diarrhea was assessed every 24-48 hrs. n=60. %= within treatment groups.

		Diarrhea status					
		Absent		Present		Total	
		Count	%	Count	%	Count	%
Treatment groups	Control	12	100%	0	0.0%	12	100%
	H-MW-HA	12	100%	0	0.0%	12	100%
	5-FU	5	27.8%	13	72.2%	18	100%
	5-FU+H-MW-HA	17	94.4%	1	5.6%	18	100%
	Total	46	76.7%	14	23.3%	60	100%

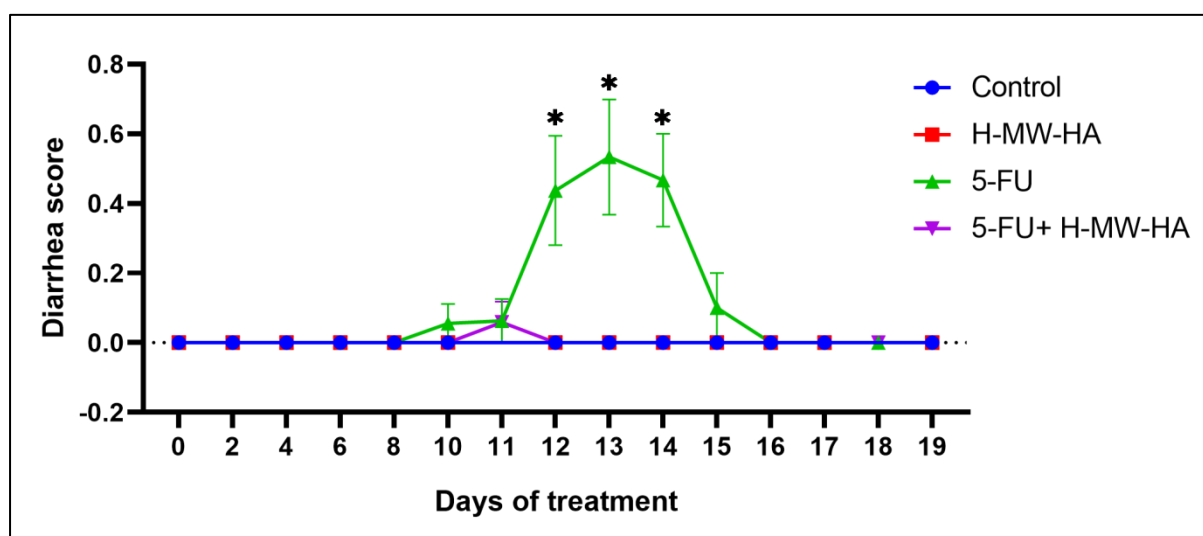


Figure 6.7 Effect of High molecular weight hyaluronic acid (H-MW-HA) on diarrhea induced by 5-Flurouracil (5-FU) in mice. Score: 0= no diarrhea; 1= mild diarrhea; 2= moderate diarrhea; 3= severe diarrhea. Administration of H-MW-HA in drinking water significantly ameliorated chemotherapy-induced diarrhea. Data represent the mean value of diarrhoea score between treated groups over a period of 19 days. Data are represented as mean $\pm$ SEM * p <0.01 vs control, H-MW-HA, and 5-FU+H-MW-HA group.

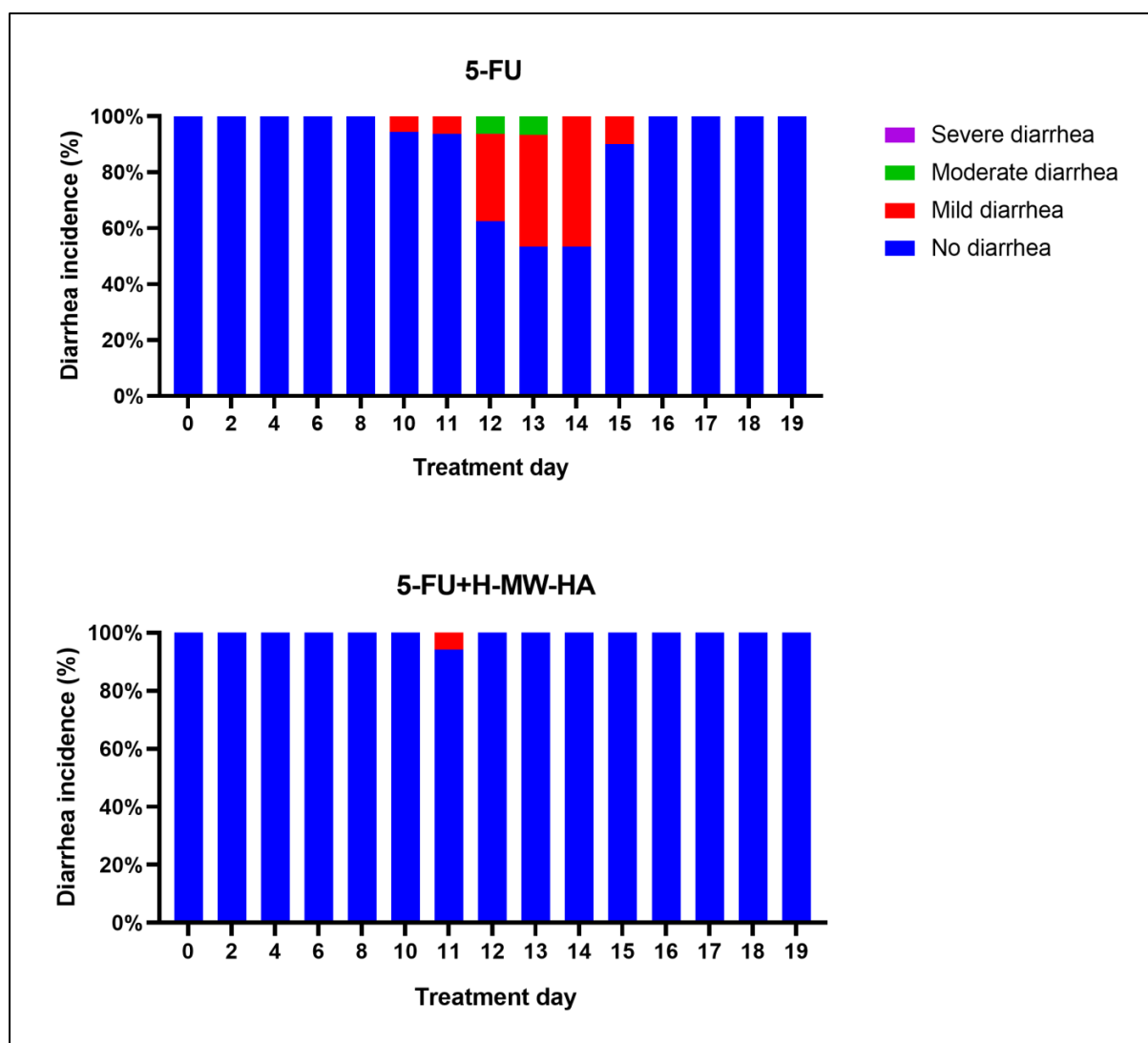


Figure 6.8 Incidence and severity of diarrhea overtime in group C and group D mice. The occurrence of severe and moderate diarrhea was prevented in mice treated with 5-FU by the co-administration of H-MW-HA.

6.4.4 H-MW-HA decreases the severity and duration of Chemotherapy-induced oral mucositis in mice

In order to assess the potential protective effects of HA against CIOM, the oral cavity was visually assessed in all mice treatment groups (A-D) (Figure 6.9a), using a well-established scoring system for OM, as described in Table 1. In agreement with the results of a previous study (Bertolini et al., 2017), i.v. administration of 5-FU induced the development of OM. In both groups treated with 5-FU, OM was not observed until day-8. For the entire duration of the experiment the 5-FU induced ulcers were not clinically visible on mice tongue during the conventional *in vivo* oral cavity inspection (Figure 6.9A). Conversely, the tongue ulcers became immediately detectable after the tissues were stained by toluidine blue. In 5-FU alone group, mucositis continued to increase in severity, reaching a peak at days 14 and 17. By the end of the experiment, the mean OM score was 0.75 ± 0.47 . In contrast, the mean OM score for the group D was significantly lower than that of group C on days-10, 12, 13, and 14 ($p < 0.01$). Thereafter, the score decreased overtime, and by day 16, the oral mucosa of the remaining mice was completely healed (Figure 6.9B). The total mean OM scores between day-0 and day-19 for animals treated with 5-FU+H-MW-HA were significantly ($p < 0.001$) lower than those treated with 5-FU alone (Figure 6.9C). Furthermore, we clinically evaluated the tongues stained with toluidine blue post-mortem at 3 different time points (days 14, 16, and 19) and integrated the lesions found on the tongue surface after post-mortem evaluation into our established OM scoring system. Then we correlated the post-mortem OM scoring with the one we did clinically in living animals. Consistent with our finding from the live clinical assessment of OM in mice, our results showed that H-MW-HA administration in 5-FU treated mice significantly reduced ($P < 0.001$) the OM severity compared to the mice only treated with 5-FU (Figure 6.9D). Specifically, on day 14, the OM scoring went down from 3 ± 0.00 (moderate) in the 5-FU treated mice to 1.5 ± 0.43 (mild) in the 5-FU treated mice that received H-MW-HA in the drinking water (Figure 6.9D). In summary, we demonstrated, for the first time, that H-MW-HA is capable of decreasing the incidence of CIOM and favourably impact its duration.

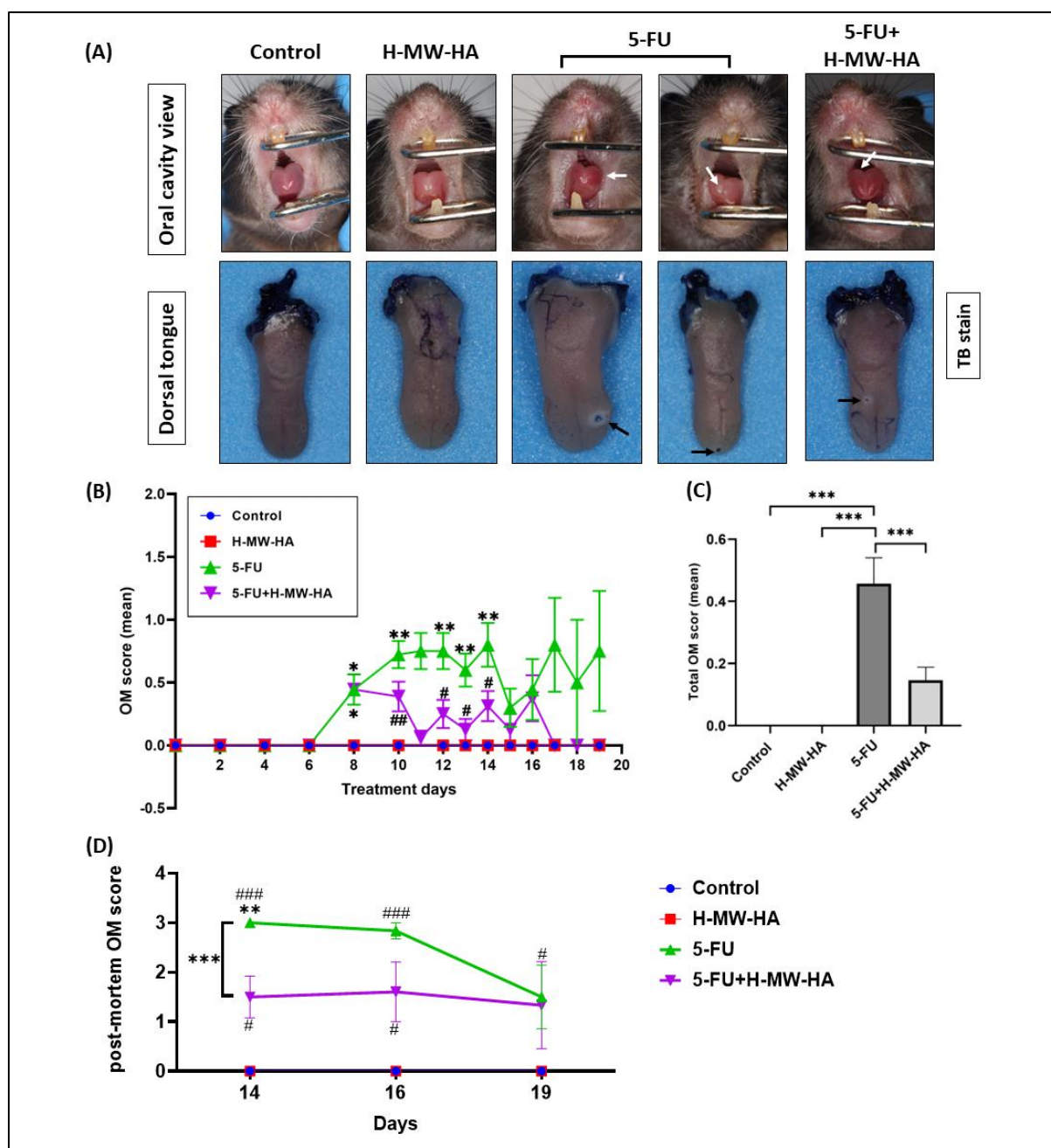


Figure 6.9 Effect of high molecular weight hyaluronic acid (H-MW-HA) co-treatment on oral mucositis severity in 5-FU treated mice. Intravenous (i.v.) 5-FU administration was accompanied by daily treatment with H-MW-HA 0.01%w/v administered orally in drinking water. Oral mucositis (OM) severity was scored using a six-point scale. Mice tongues were collected at different endpoints and were stained with 1% toluidine blue (TB) in 10% acetic acid. **(A)** Extra oral representative photographs of the mice showing the oral mucosa (tongue dorsum). The photographs show diffuse erythema on the anterodorsal surface of the tongue in treated mice. Below, representative photographs of mice tongues after excision and TB staining showing the epithelium ulceration (in blue) on the dorsal surface of the tongue. White arrows show areas of erythema, erosion, and ulceration; Black arrows show areas of ulceration in blue after TB staining. **(B)** Comparative oral mucositis scores. Animals treated with H-MW-HA demonstrated significantly less mucositis ($p < 0.01$) than mice treated with 5-FU alone on days-10, 12, 13, and 14. Data represent the mean value of OM score between treated groups over 19

days. Data are represented as mean $\pm$ SEM. * $p < 0.01$ versus control and H-MW-HA groups; ** $p < 0.01$ versus control, H-MW-HA, and 5-FU+H-MW-HA groups; # $p < 0.01$ versus 5-FU group; ## $p < 0.01$ versus control, H-MW-HA, and 5-FU groups (Tukey's post hoc test). **(C)** Mean value of the total score of oral mucositis between day-0 and day-19 for each group. Data are the mean $\pm$ SEM (n= see table 2). * $p = 0.0191$; *** $p < 0.0001$ (Tukey's post hoc test). **(D)** Comparative post-mortem oral mucositis score. Data represent OM score between treated groups at days 14, 16, and 19. Data are represented as mean $\pm$ SEM. ** $p < 0.01$ versus 5-FU groups; # $p < 0.05$ versus control and H-MW-HA groups; ### $p < 0.001$ versus control, H-MW-HA, (Two ways ANOVA followed by Tukey's post hoc test).

6.4.5 H-MW-HA reduces chemotherapy-induced oral ulcerations

CIOM ulceration was localized mainly to the tongue dorsolateral and ventral surfaces (Figure 6.10A). TB (deep blue colouration) positive areas of ulcerative mucositis corresponded to erosive or ulcerated lesions with extremely atrophic or absent epithelium. In the group that received 5-FU only, H&E staining showed an ulcerated lesion covered by necrotic fibrinoid tissue, with complete loss of the stratified squamous keratinized epithelium, corresponding to the marked deep blue coloration at the TB staining (Figure 6.10B). Furthermore, the sub-epithelial connective tissue showed a marked increase in infiltrating inflammatory cells.

The TB-stained ulceration on the tongues was evident in 85.71% of animals from group C. In contrast, ulceration was detectable on the tongue of only 68.75% of mice that received 5-FU+H-MW-HA (group D). Importantly, a strong association between tongue ulcer status (present/absent) and the treatment groups was observed; $\chi^2 (3) = 32.93$, $p < 0.0001$, Cramer's $V = 0.781$.

In an initial phase of this study, we attempted a quantification and analysis of the ulcerative lesions of the whole surface of the tongue. However, given that there is no real separation between dorsal and ventral surfaces, and lateral borders of the tongue, this analysis was not possible. Therefore, for the final analysis, only dorsal and ventral surfaces of the tongue were considered. Results were quantified as the mean $\pm$ SEM. After 5-FU treatment, animals showed the largest ulcer mean size % at day-14 (1.7 ± 0.36 %), and the ulcer size % started to decrease afterward. Furthermore, The CIOM ulcer size percentage compared to the total epithelialized upper surface of the tongue (the dorsal tongue) was also the highest, on day-14 (2.34 ± 0.45 % Figure 6.11C) and significantly elevated compared to all other treatment groups (groups A, B, D, Figure 6.11C). The total mean size percentage of CIOM ulcer relative to the total epithelialized surface of the tongue between day-14 and day-19 for animals treated with 5-FU+H-MW-HA was significantly lower than the ulcer percentage for those treated with 5-FU

alone (Figure 6.11A). Specifically, the significant difference was documented on day-14, but not days, 16, and 19 as compared to the 5-FU alone treatment group (figure 6.11A-C). Thus, H-MW-HA showed a mitigative effect against 5-Fu-induced OM.

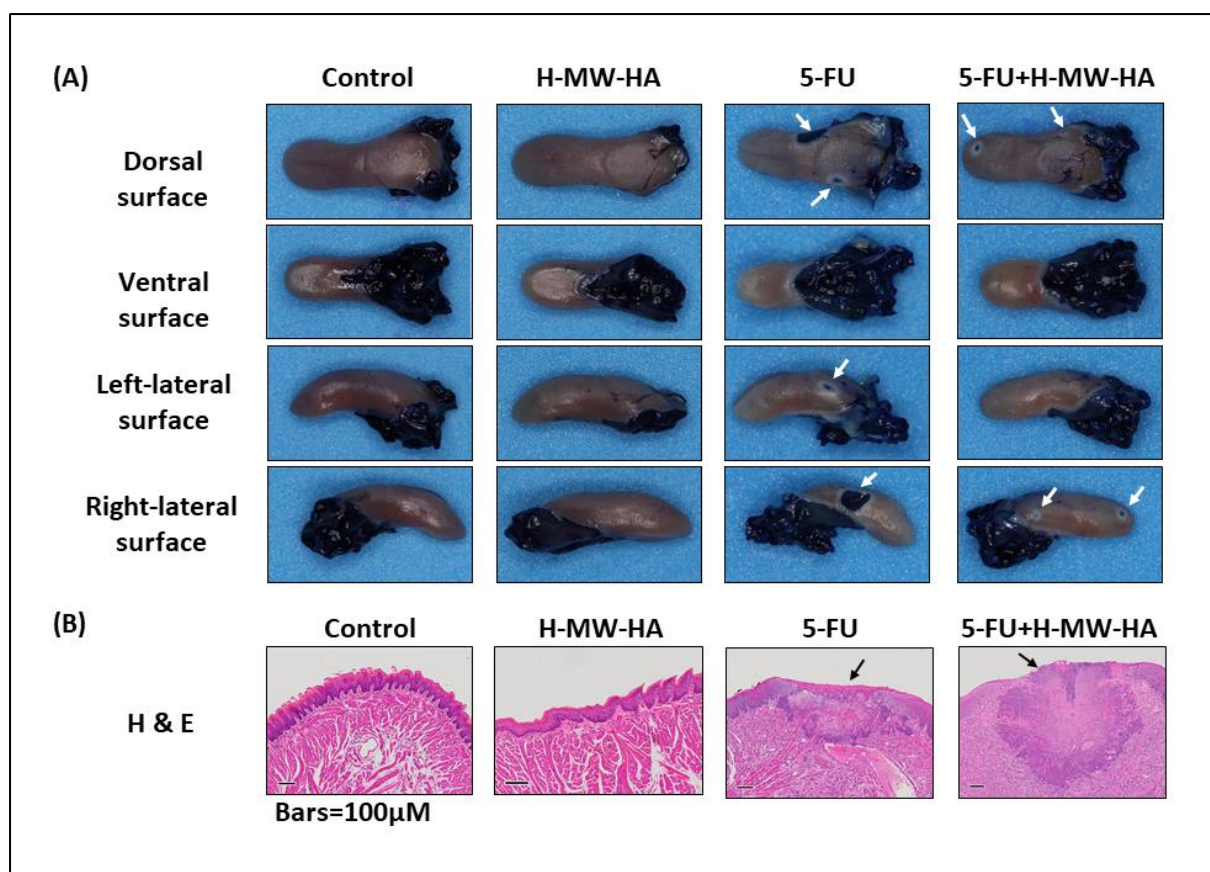


Figure 6.10 Chemotherapy-induced oral mucositis (CIOM) ulcers in (5-FU)-treated mice. Mice received saline i.v. (Control), H-MW-HA, 5-FU i.v., or 5-FU+H-MW-HA as described in the method. Animals were sacrificed at different time points, and tongues were collected. Tongues were stained with 1% TB in 10% acetic acid. **(A):** Excised animal tongues after TB staining showing the CIOM ulcers in blue (white arrows) involving different surfaces of the tongue. **(B):** Histopathological aspect of representative tongue samples from each group (H&E-stained tissue sections, 20x.). Black arrows show areas of erosion.

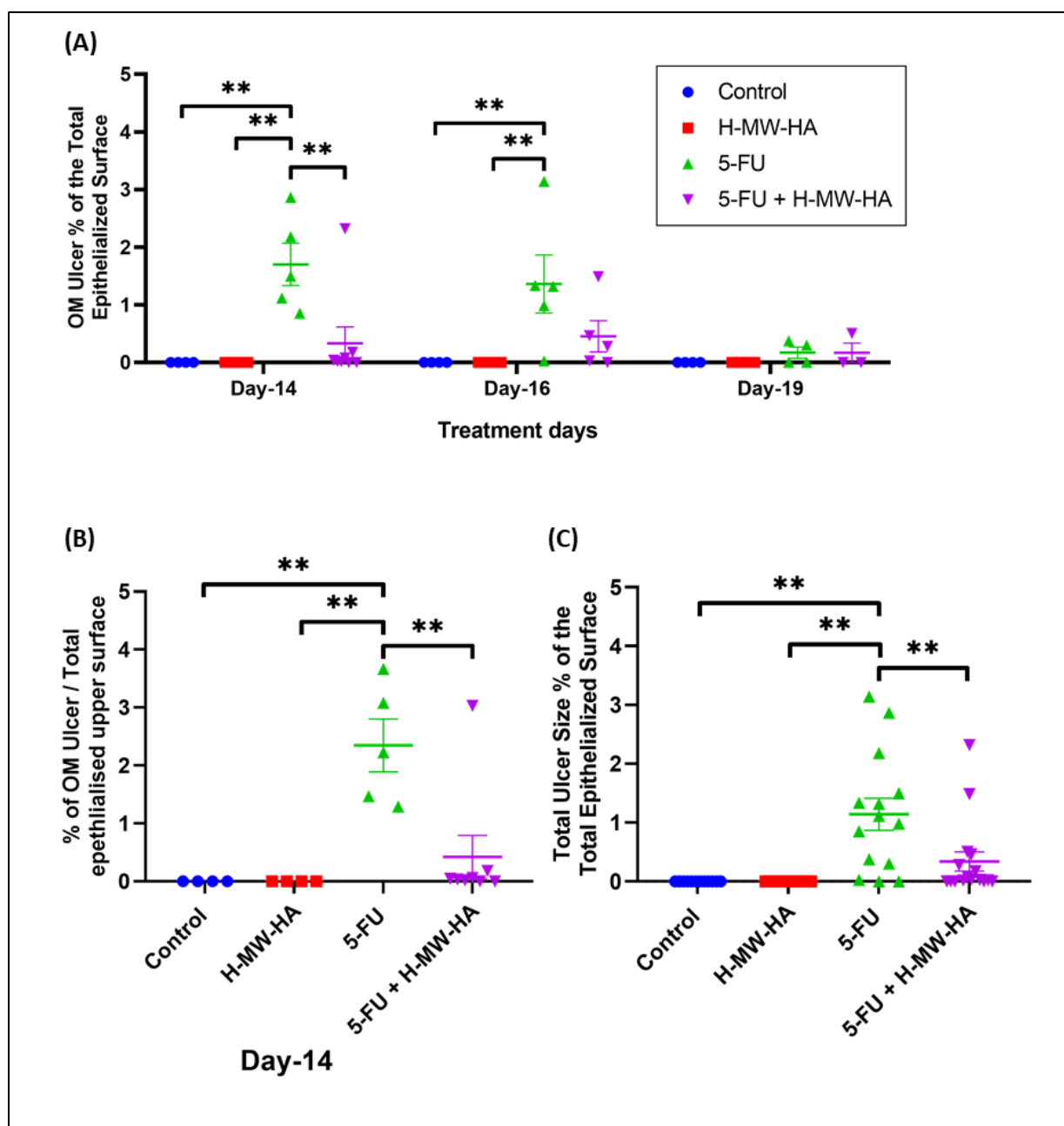


Figure 6.11 Chemotherapy-induced oral mucositis (CIOM) ulcer size percentage after HA treatment in the 5-fluorouracil (5-FU)-treated mice. Mice received saline i.v. (Control), H-MW-HA, 5-FU i.v., or 5-FU+H-MW-HA as described in the method. Animals were sacrificed at different time points, and tongues were collected. Tongues were stained with 1% TB in 10% acetic acid. After tongue imaging, the ulcer size and the total epithelialized upper surface of the tongue were measured in all groups. **(A)** Represents the mean percentage of oral mucositis (OM) ulcer size of the total epithelialized surface of the tongue at days 14, 16, and 19. **(B)** Represents the mean percentage of OM ulcer size to the total epithelialized upper surface of the tongue on day 14. **(C)** Represents the total mean percentage of OM ulcer size to the total epithelialized surface of the tongue between day-14 and day-19. Data from days 14, 16, and 19 (n = see table 2) are presented as mean $\pm$ SEM. **p-value <0.005.

6.4.6 Histological Evaluation of the Mice Tongue and Intestine in the control group

The histopathological aspect of tongue and jejunum from the control group was assessed through H & E staining of collected samples. Figure 6.12(A) shows a histologic section of the mucosa taken at the dorsal surface of the base of the tongue of a representative control animal. Histologically, a normal pluri-stratified squamous keratinized epithelium could be visualized. Basal layer cells were located just above the basement membrane. Superficially to the basal layer are the succeeding prickle, granular, and squamous layers. They consist of polygonal cells that turn flattened scales, and keratin is formed and shed from the surface following vertical maturation. The dorsal surface of the tongue showed hair-like filiform papillae formed by epithelium and keratin placement. The basement membrane is a thin mat of the ECM that separates epithelial sheets from connective tissue. Underlying stroma is composed of connective tissue that contains muscle fibres, blood vessels, nerves, fibroblasts, and can include minor salivary glands.

Histological examination of the representative photomicrographs of the transverse and longitudinal sections of mice small intestine showed that the innermost layer of intestinal mucosa of the control mice displayed tall villi lined by columnar epithelium, with the crypts open between the villi and extending to the muscularis mucosa. (Figure 13 A and B). The tunica propria formed the core of the villi and consists of connective tissue that contains elastic fibres, reticular fibres, and lymphocytes. Muscular mucosa follows, which consists of a thin layer of longitudinally arranged smooth muscles. Immediately beneath the mucosa lies the tunica submucosa, a layer of loose to dense connective tissue containing blood and lymphatic vessels. Tunica muscularis is the outer covering layer of the intestine and consists of two thick layers of smooth muscle.

6.4.7 Effects of H-MW-HA on Oral Histology in 5-FU-Treated Mice

Tissue samples were collected on days 14, 16, and 19 following treatment. Photomicrographs in Figure 6.12B show the representative histologic sections (H&E)_from the tongue of control mice, or mice treated with either H-MW-HA alone, 5-FU alone (seven i.v. doses of 5-FU) or %FU with concomitant H-MW-HA. Between day-14 and Day-16, the tongue of the mice in both control and H-MW-HA alone treated groups presented an overall preserved histological structure with no visible lesions. Analysis of the histological sections revealed that 5-FU administration caused epithelial atrophy as reflected by the significant reduction in epithelial

thickness compared to the control group (Figure 6.12B). In addition, i.v. administration of 5-FU also affected the structural integrity of the dorsal mucosa, with the filiform papillae being entirely damaged and the keratinized layer having a desquamative appearance in the great majority of the examined samples from this group. Additional, severe changes were also noted with the 5-FU administration, consisting of an overall breakdown of the epithelia, resulting in the formation of ulcerative lesions covered by a thin layer of necrotic fibrinoid material at the base and margins and consisting of regions containing inflammatory cells and active granulation tissue. Importantly, H-MW-HA administration protected the tongue epithelium of the mice from 5-FU-induced damage. Qualitatively, tongue mucosa of group D mice appeared less damaged; quantitatively, the mucosa preserved in its thickness to a degree almost resembling the control group (Figure 6.12B). Finally, the filiform papilla could be observed more frequently in the group that received H-MW-HA than in the 5-FU alone group. However, H-MW-HA did not show any effect on the mucosal thickness of the tongue of the mice in the H-MW-HA alone treated group. Significant differences were found in the total epithelial thickness of the tongue mucosa between day-14 and day-19 for mice exposed to 5-FU and treated with H-MW-HA in comparison to the 5-FU alone group. The mean epithelial thickness of the mucosa of the mice tongue in the 5-FU+H-MW-HA treated group ($105.27 \pm 2.56 \mu\text{m}$) were significantly higher than those in the 5-FU alone group ($84.81 \pm 9.14 \mu\text{m}$), $p=0.000$ (figure 6.12D). In particular, in terms of days, the significantly thicker mucosa was found in the 5-FU+H-MW-HA treated group ($102.78 \pm 3.55 \mu\text{m}$, and $107.91 \pm 12.25 \mu\text{m}$) compared to that of the 5-FU alone group ($81.66 \pm 4.92 \mu\text{m}$, and $77.65 \pm 4.79 \mu\text{m}$), $p<0.005$ on day-16, and day-19, respectively, but not on day 14. Furthermore, a significantly thinner mucosa was found in 5-FU alone group on days 16 and 19 compared to day-14 (Figure 6.12C).

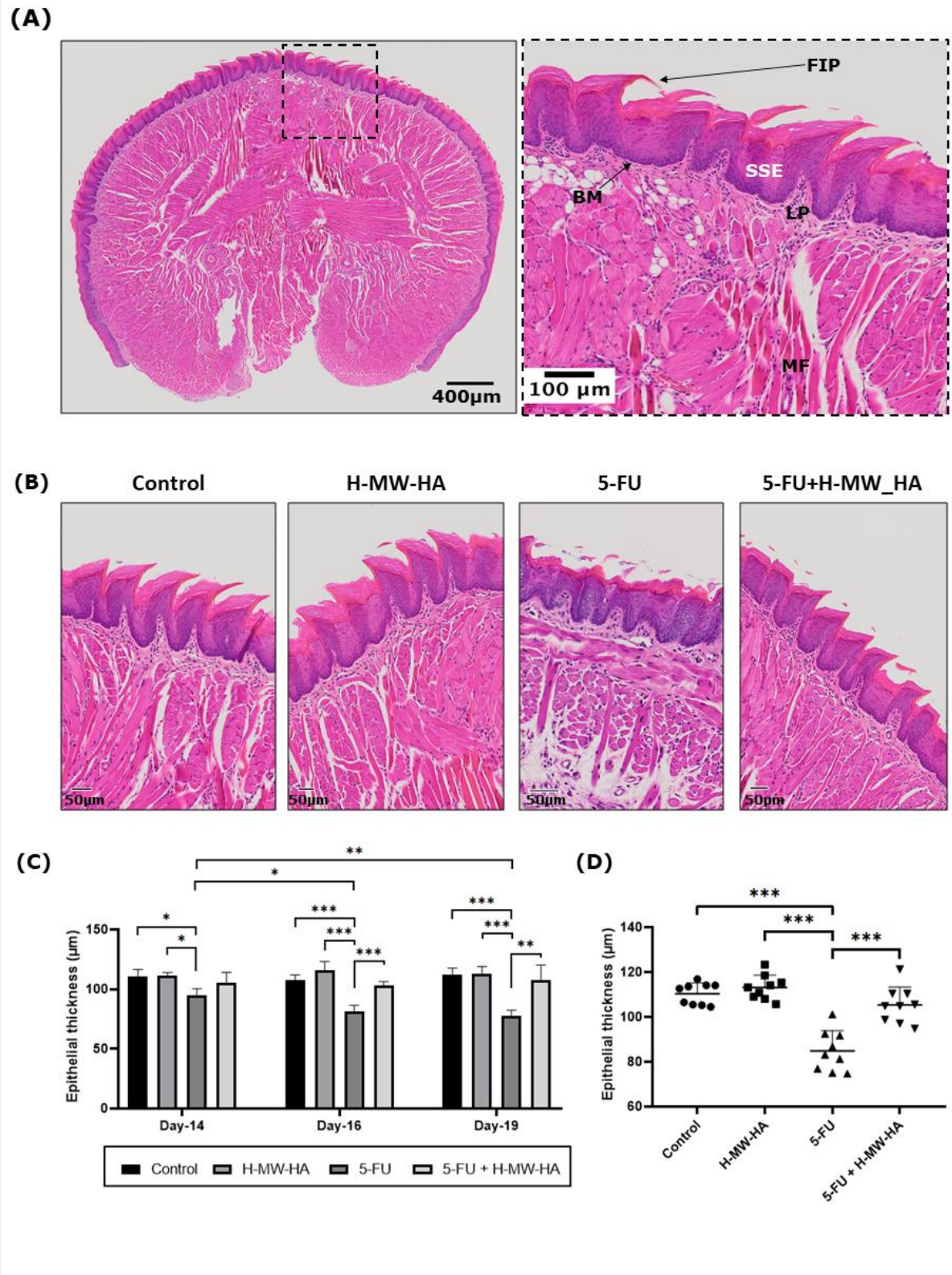


Figure 6.12 Effect of H-MW-HA on the 5-FU-induced tongue mucosa damage. Histological changes in the tongue were determined using hematoxylin and eosin (H&E) staining. **(A)** Representative microphotograph showing the histologic section of the base of the tongue (control group). H&E stain of the tongue sections (20x). Structural landmarks: filiform

papillae (FIP), stratified squamous keratinized epithelium (SSE), basement membrane (BM), lamina propria (LP), and muscle fibres (MF) . **(B)** Representative microphotograph showing histological appearances of the tongue mucosa of control, H-MW-HA-treated, 5-FU-treated, and 5-FU+H-MW-HA-treated mice. H&E stain of dorsal tongue sections in each group (20x). Compared with control mice, the superficial regions in 5-FU alone treated mice were largely destroyed, and epithelial atrophy was present, with the filiform papillae being entirely damaged. In addition, the connective tissue showed a marked increase in infiltrating cells as a sign of the inflammatory response. **(C)** The average epithelial thickness of the dorsal tongue at each time point (27 representative measurement/tissue (three representative mice/group)). **(D)** The total epithelial thickness of the dorsal tongue between Day-14 and Day-19; each dot is the average of 27 representative measurements/tissue. Data represented as $M \pm SD$. $*=p<0.05$. $**=p<0.005$. $***=p<0.001$.

6.4.8 H-MW-HA treatment decreases intestinal injury elicited by 5-FU

The jejunum of the mice in both control and H-MW-HA alone treated groups showed no histomorphologic changes. The structure of the intestinal mucosa was normal, intact epithelium was seen with tall and columnar epithelial cells, and the villi were aligned with intact crypts. 5-FU disrupted the integrity and the normal architecture of intestinal mucosa. In the jejunum, 5-FU treatment caused substantial changes, including epithelial atrophy, a reduction in villi length, and thinning of the lamina propria with inflammatory cell infiltration (Figure 6.13C).

Villi lengths were remarkably decreased in the jejunum of 5-FU group ($310.35 \pm 14.02 \mu\text{m}$) as compared to the control and H-MW-HA alone treated groups ($511.92 \pm 49.73 \mu\text{m}$, and $500.66 \pm 25.93 \mu\text{m}$; respectively), $p < 0.0001$ (Figure 6.14A). On the other hand, the villi lengths of the 5-FU+H-MW-HA treatment group were decreased less than that in the 5-FU group ($434.44 \pm 63.11 \mu\text{m}$; $p < 0.0001$), and the normal architecture was still preserved compared with 5-FU treated group. However, the shortening in the villi length was still significant compared to the control and H-MW-HA alone treated groups (Figure 6.14A). More precisely, and in terms of days' analysis, jejunum mucosa showed a significant reduction in villi length in the 5-FU group compared to control, and H-MW-HA alone treated groups on day-14, day-16, and day-19. In contrast, mice co-treated with H-MW-HA (group D) did not present tremendous intestinal damage on the fore mentioned days, and the villi were significantly longer in the 5-FU alone group (Table 6.4, and Figure 6.14A). Intestinal villi length reduction observed in 5-FU+H-MW-HA mouse group was significant compared with the control and H-MW-HA alone treated groups on days 14, and 16, but not day-19. Additionally, 5-FU mice treated with H-MW-HA showed a significant increase in villi length on day-19 compared to those on day 14, and day 16 (Figure 6.14B).

Intestinal mucosa also showed significant reduction in the thickness of the tunica mucosa in the 5-FU group compared to the control and H-MW-HA alone treated groups on day-14, day-16 and day-19, and in total between day-14 and day-19. Treatment with H-MW-HA alleviated the severity of mucositis, and reversed the decrease in the tunica mucosa thickness observed in the 5-FU group (Table 6.4, and Figure 6.14C and D). Crypts depth showed minimal reduction in the 5-FU alone and H-MW-HA alone treated groups compared to the control group, while 5-FU +H-MW-HA treatment did not show any significant difference compared to the other groups. A marked reduction in the depth of the intestinal crypts was observed in 5-FU group

between day-14 and day-19 compared to the control group. More precisely, the significant change was observed on day-19 (Table 6.4).

These findings provided evidence for the positive effects of H-MW-HA on the intestinal mucosa and suggested that H-MW-HA significantly protected the small intestinal mucosa against 5-FU induced damage.

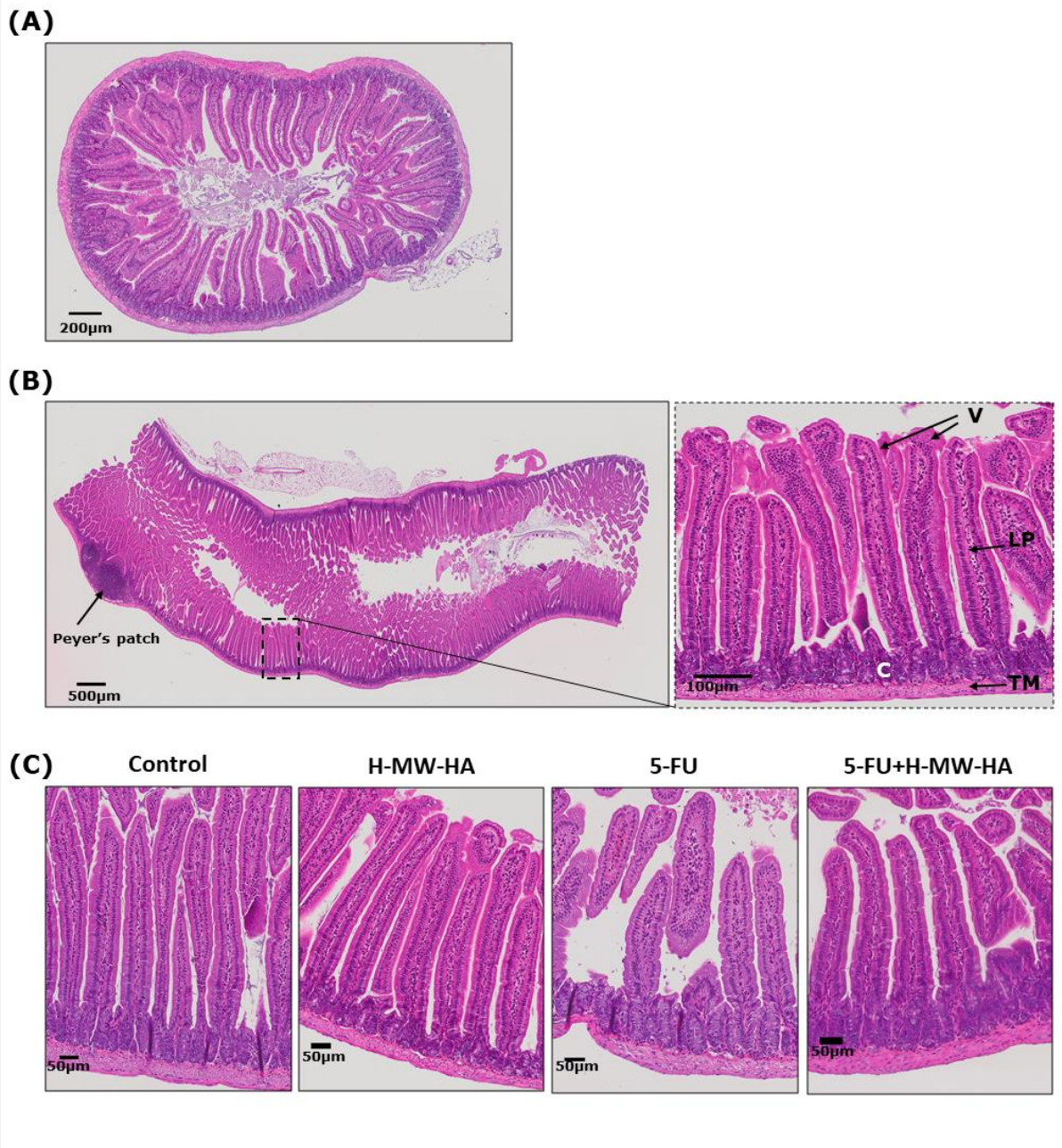


Figure 6.13 Effect of H-MW-HA on the 5-FU-induced intestinal mucosa damage. Histological changes in the intestinal villi were determined using hematoxylin and eosin (H&E) staining. **(A)** Representative microphotograph showing the histologic section of a transversely cut intestinal segment of the control animal. **(B)** Representative microphotograph showing the histologic section of a longitudinally cut intestinal segment of a control animal. Structural landmarks: Villi (V), Crypts (C), lamina propria (LP), Tunica muscularis (TM). **(C)** Representative microphotograph showing histological appearances of the intestinal mucosa of control, H-MW-HA-treated, 5-FU-treated, and 5-FU+H-MW-HA-treated mice. Compared with control mice, the innermost layer was largely destroyed, and the epithelial thickness was reduced (epithelial atrophy), with the villi length shortening and thinning of lamina propria with inflammatory cell infiltration. H&E stain of the jejunum sections (20x)

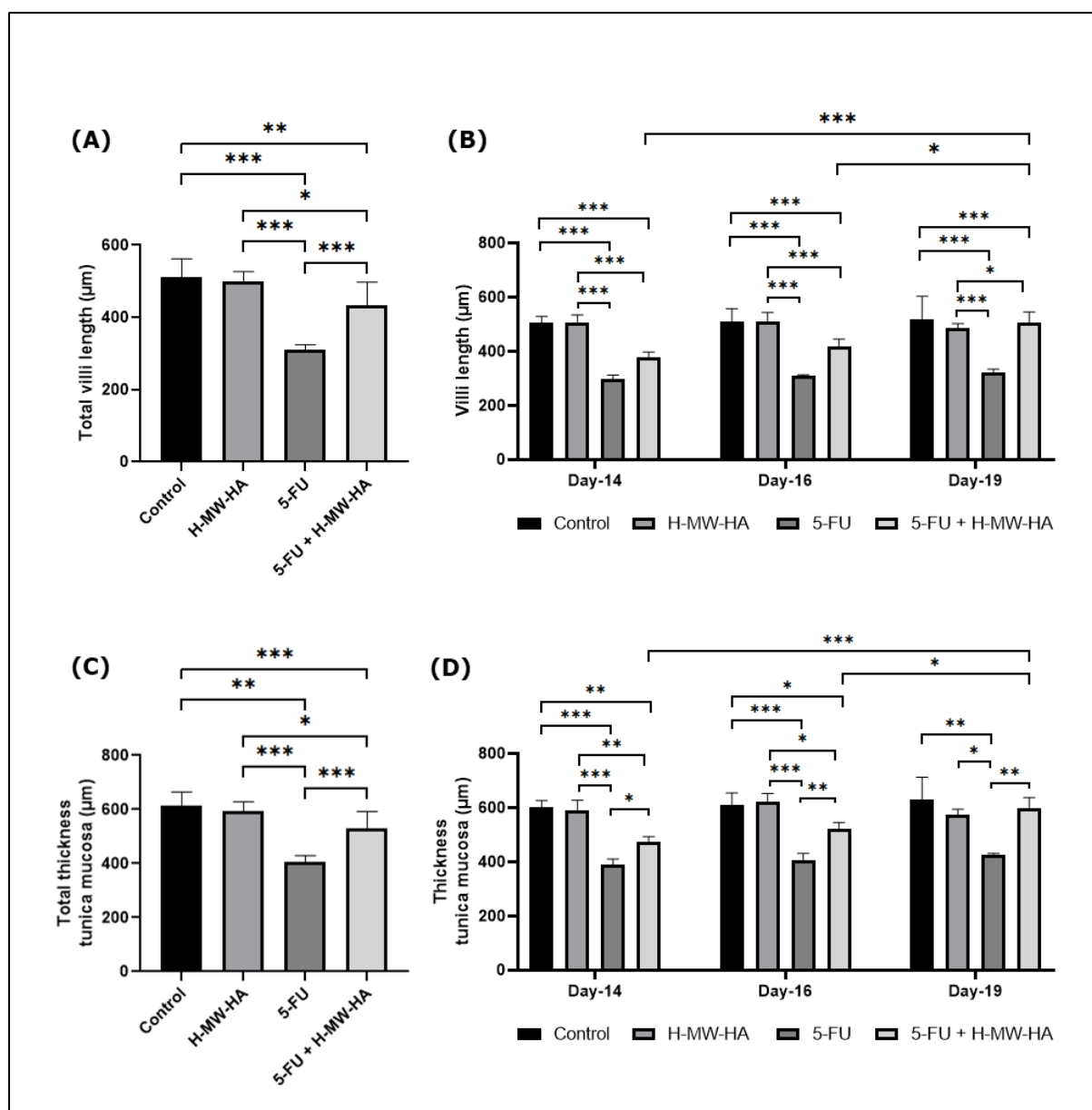


Figure 6.14 Effect of H-MW-HA on the 5-FU-induced intestinal mucosa damage. (A) Total villi length (average) of the mice jejunum between Day-14 and Day-19 (12 representative measurements/tissue (nine representative mice/group)). **(B)** The average villi length of the jejunum at each time point (12 representative measurements/tissue (three representative mice/group)). **(C)** Total thickness of tunica mucosa (average) of the mice jejunum between Day-14 and Day-19 (12 representative measurements/tissue (nine representative mice/group)). **(D)** The average tunica mucosa thickness of the jejunum at each time point (12 representative measurements/tissue (three representative mice/group)). Data are presented as $M \pm SD$. *= $p < 0.05$. **= $p < 0.005$. ***= $p < 0.001$.

Table 6.4 The effect of H-MW-HA on the 5-FU-induced intestinal injury. Descriptive statistics of morphometric variables of the jejunum wall of C57BL/6 mice following chemotherapy and Hyaluronic acid (HA) treatment. Groups were treated with saline via tail vein (i.v.) (control), H-MW-HA (0.01%) in the drinking water (H-MW-HA), 5-FU (50 mg/kg) i.v. (5-FU), or H-MW-HA/5-FU (5-FU+H-MW-HA). Data presented are means $\pm$ SD of 12 representative measurement/tissue (three representative mice/group). a =p<0.05 compared to control and H-MW-HA groups. b=p<0.05 compared to the 5-FU group. c=p<0.05 compared to the control group.

Treatment groups	Morphometric measurement (μm)		
	Tunica mucosa thickness	Villi length	Crypts depth
Control			
Day-14	602.43 $\pm$ 24.19	507.20 $\pm$ 21.72	95.09 $\pm$ 6.76
Day-16	611.92 $\pm$ 42.08	508.74 $\pm$ 48.67	114.09 $\pm$ 6.25
Day-19	631.06 $\pm$ 81.28	519.83 $\pm$ 83.13	118.37 $\pm$ 22.25
Total	615.14 $\pm$ 48.98	511.92 $\pm$ 49.73	109.18 $\pm$ 16.13
H-MW-HA			
Day-14	590.4 $\pm$ 37.41	507.7 $\pm$ 26.37	87.95 $\pm$ 6.96
Day-16	620.34 $\pm$ 31.93	509.6 $\pm$ 33.28	108.85 $\pm$ 1.74
Day-19	574.02 $\pm$ 20.26	484.7 $\pm$ 17.51	92.87 $\pm$ 4.52
Total	594.92 $\pm$ 33.48	500.66 $\pm$ 25.93	96.56 $\pm$ 10.37
5-FU			
Day-14	390.9 $\pm$ 19.99 ^a	298.8 $\pm$ 13.75 ^a	89.59 $\pm$ 13.45
Day-16	405.86 $\pm$ 25.04 ^a	308.7 $\pm$ 4.98 ^a	91.60 $\pm$ 11.78 ^c
Day-19	424.71 $\pm$ 7.09 ^a	323.5 $\pm$ 10.51 ^a	95.88 $\pm$ 11.67
Total	406.86 $\pm$ 22.26 ^a	310.35 $\pm$ 14.02 ^a	92.36 $\pm$ 11.03 ^c
5-FU+H-MW-HA			
Day-14	472.28 $\pm$ 21.61 ^{b;c}	377.23 $\pm$ 20.40 ^{a;b}	98.38 $\pm$ 8.97
Day-16	523.94 $\pm$ 21.59 ^{b;c}	418.23 $\pm$ 27.53 ^{a;b}	105.16 $\pm$ 8.90
Day-19	599.61 $\pm$ 37.09 ^b	507.87 $\pm$ 36.97 ^b	99.40 $\pm$ 5.76
Total	531.94 $\pm$ 60.44 ^{b;c}	434.44 $\pm$ 63.11 ^{a;b}	100.98 $\pm$ 7.63

6.4.9 Serum superoxide dismutase (SOD) activity is induced by H-MW-HA in 5-FU treated mice

Given the vital role played by ROS in chemotherapy-induced oral and gastrointestinal mucosal injury (Yoshino et al., 2013), we explored whether the reduction in both oral and gastrointestinal pathologies by H-MW-HA treatment could be explained by an associated antioxidant effect resulting from an increase in antioxidant defences. SOD enzyme activity was measured in the serum of mice blood at days 14, 16, and 19. As shown in Figure 6.14a, control group mice (group A) exhibited a low basal level of serum SOD activity, which was not significantly different from either the H-MW-HA (group B) or 5-FU (group C) alone treated mice serum levels. However, mice treated with 5-FU+H-MW-HA (Group D) displayed significantly higher levels of serum SOD enzyme activity on all days tested compared to the control group and on days 14 and 16 compared to the 5-FU treated mice (Figure 6.15 a). Total serum SOD activity between days 14 and 19 for animals treated with 5-FU+H-MW-HA was also significantly higher compared with those in control, H-MW-HA only, or 5-FU only groups (Figure 6.15b). Within the same group and between day 14 and day 19, there was an increase in the SOD activity, but it was nonsignificant. These results demonstrate that H-MW-HA exhibited antioxidant effect against 5-FU-induced oxidative damage and provide a possible mechanism for H-MW-HA mediated reduction in oral and intestinal mucositis.

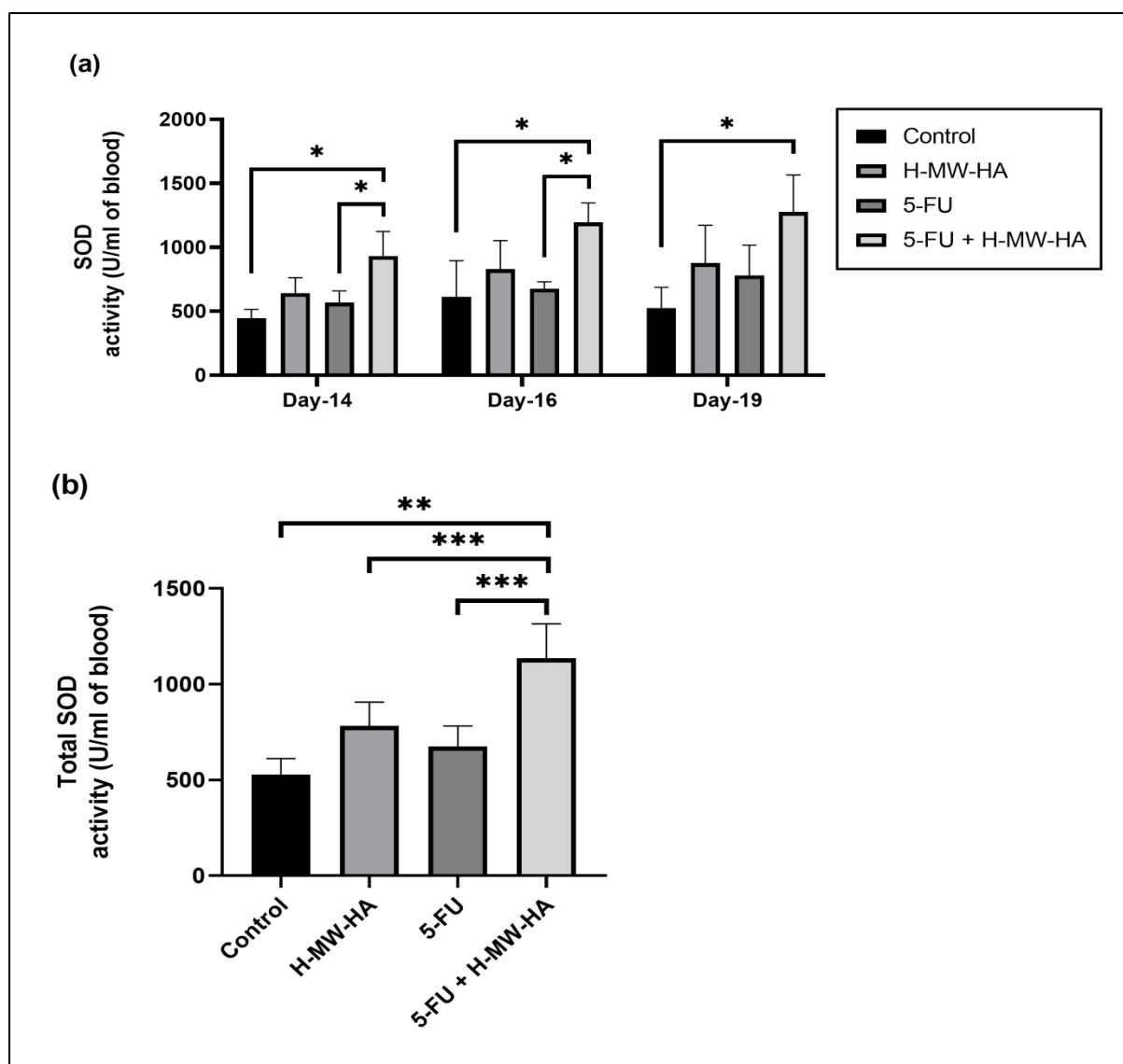


Figure 6.15 High molecular weight hyaluronic acid (H-MW-HA) increased activity superoxide dismutase (SOD) enzyme in 5-FU treated mice. Mice received 5-FU Intravenously (i.v.) with or without daily treatment of H-MW-HA (0.01% w/v) administered orally in the drinking water. Animals were sacrificed at days 14, 16, and 19, and blood samples were obtained. SOD activity was measured in serum. **(a)** SOD activity at days 14, 16, and 19. **(b)** Total SOD activity between days 14 and 19. Data are expressed as mean $\pm$ SD, $n=3$. * $p<0.05$. ** $p<0.005$. *** $p<0.0001$.

6.5 Discussion:

Oral and gastrointestinal mucositis are debilitating adverse effects observed in approximately 40-80% of cancer patients undergoing CT (Cheng et al., 2012; Elting et al., 2003; Sonis et al., 2001; Lalla and Peterson, 2006; Campos et al., 2014). In the present study, we used a dual murine model of oral and intestinal mucositis to demonstrate that HA administered in drinking water can successfully reduce the severity of mucositis.

Chemotherapy Induced Oral Mucositis (CIOM) is a painful side-effect that can reduce the quality of life of cancer patients resulting in the early discontinuation of CT, prolonged hospitalization, and death (Elting et al., 2008b; Sonis et al., 2004a). The complexity of mucositis's biological process has only been recognized recently (Sonis, 2004b; Logan et al., 2007b; Sonis et al., 2007; Bowen et al., 2019). It is now thought that mucositis represents a sequential series of biological and cellular events, including the interaction of oral mucosal cells and tissues, ROS, proinflammatory cytokines, apoptosis mediators, and local factors such as saliva and the oral microbiota, with the role of the microbiome has as the main contributor in all phases of mucositis pathogenesis has also been considered. Defects of the tight junctions (multiple protein complexes that regulate mucosal paracellular permeability) and epithelial barrier distortion have been highlighted as important factors in injury exacerbation (Bowen et al., 2019). According to the model described by Sonis the pathophysiology of mucositis can be divided into five sequential phases: (1) The initiation stage, where CT and radiation therapy induce direct cytotoxicity results in irreversible DNA-strand breaks in basal cell populations leading to apoptosis and mucosal atrophy in the oral cavity. Further, this phase includes ROS formation leading to indirect DNA damage. Bacterial ligands have also been approved to play a role in the initiation phase of mucositis by regulating the immune responses to radiation and CT treatments to modify ROS generation.; (2) ROS production activates downstream inflammatory signalling cascades, including NF- κ B causing upregulation of inflammatory and apoptotic genes, the release of proinflammatory cytokines such as tumour necrosis factor (TNF α), interleukin (IL)-1 β , and IL-6, whilst fibroblasts in the submucosa release metalloproteinases leading to the collapse the epithelial basement membrane; (3) Signal transduction induced by these cytokines lead to amplification of damage and inflammation; (4) During the ulceration phase, apoptosis occurs in many epithelial cells, and the patients feel pain during this phase; and (5) The ulcerative lesions typically spontaneously heal when the cancer therapy ends. Therefore, the production of ROS at an early preclinical stage activates oxidative

stress pathways, which play a critical role in mucositis pathogenesis. The use of agents that inhibit oxidative stress-induced damage could, therefore, provide an effective therapeutic approach. There is already support for this strategy in previous studies that demonstrate that inhibition of oxidative stress can alleviate chemotherapy-induced mucositis in animal models (Shiota et al., 2010; Atiq et al., 2019) and humans (Moslehi et al., 2014; Tsujimoto et al., 2015). It is likely that systemically administered H-MW-HA exerts antioxidant effects by working as a ROS scavenging agent (Hiramoto et al., 2012). Furthermore, our data show that HA when used in combination with 5-FU, increases blood SOD dramatically. Hence, in our model, HA works at least in part by potentiating the systemic antioxidant defence in mice.

5-FU is one of the foremost effective chemotherapeutic agents used in the treatment of advanced colorectal cancer and malignancies of head and neck (Grem, 2000; Curra et al., 2018). However, its use is limited by the severity of the side effects, including CIOM and diarrhea (Curra et al., 2018; Wilken et al., 2011). Despite various available treatment options, a need for effective therapies to alleviate or even prevent CIOM is still necessary and urgent. In the present study, 5-FU-induced mucosal damage in C57BL/6 mice was significantly limited by H-MW-HA treatment.

Several studies have reported that the administration of 5-FU to experimental animals causes oral and intestinal mucositis (Chen et al., 2013; Bertolini et al., 2017; Liu et al., 2017; Li et al., 2019). In agreement with existing literature, in the current study i.v. administration of 5-FU in mice resulted in ulcerative OM, moderate diarrhea, BW loss, damage to the intestinal mucosa, villi lengths shortening and loss of crypt architecture in the jejunum. OM started in mice of the 5-FU alone treated group from day-8, and the TB-stained CIOM ulceration on the tongues was evident in 12 mice. The oral ulcerative mucositis score reached a maximum value on day-14. Furthermore, we investigated the animal weight in relationship to chemotherapy-induced oral and intestinal mucositis phase and severity. We found a relation between OM severity and score, diarrhea severity, and mouse weight. The onset of OM in mice was similar to the onset of OM observed after CT in human subjects (Campos et al., 2014). For this reason, we considered our model to be a suitable *in vivo* model of CIOM. These findings are, in part, consistent with those of Bertolini et al. (2017), who also observed significant atrophy of the oral and jejunum epithelium in a mice model of 5-FU-induced OM. However, the study from Bertolini did not investigate the role of 5-FU in OM onset or the diarrhea incidence and severity in gastrointestinal mucositis. Specifically, it did not assess the incidence, development, and

extent of OM clinically, nor assess stool consistency. Our study differs from previous studies in that it: 1) used a live clinical scoring of mucositis in addition to post-mortem assessment; 2) introduced clinical parameters of intestinal mucositis; 3) reported systemic changes. To our knowledge, this is the most thorough simultaneous characterization of the oral and intestinal mucosal response to 5-FU published to date in a mouse model, including clinical monitoring of OM onset, diarrhea severity in animals as a surrogate marker of gastrointestinal mucositis, the oxidative stress markers, as well as assessment of the integrity of the mucosal barrier.

BW loss and anorexia are common side effects of treatment with 5-FU (Inui, 2002; Cao et al., 2014). To determine whether these adverse effects were associated with mucositis in our model, the weight of the mice was measured every 24-48 hours, and the results of all groups were compared. The results indicated that 5-FU treated animals also significantly lost weight. Besides, a close correlation between severity and the degree of the CIOM and animal weight was also observed. However, no protective effect against 5-FU-induced weight loss was found in the H-MW-HA treated mice. The reduction noted in BW could be partially explained by the physical obstruction of food and fluid intake due to ulceration. In addition, diarrhea and the systemic inflammatory reaction likely further exacerbated weight loss. Interestingly, the daily consumption of H-MW-HA in drinking water in the control group resulted in weight gain. We hypothesize that this might probably be because HA administration is associated with increased levels of EGF in the intestine and increased levels of insulin-like growth factor I (IGF-I) in the colon (Riehl et al., 2012). IGF-I is the growth factor most likely associated with intestinal growth and the promotion of epithelial cell proliferation (Kuemmerle and Zhou, 2002). EGF binds to EGF-receptor (EGFR), which is associated with increased intestinal and colonic epithelial proliferation and the epithelial response to injury (Cohen et al., 1980; Riegler et al., 1996). Furthermore, Ji et al. (2014) demonstrated that the downregulation of HA led to the suppression of abdominal fat accumulation in high-fat diet-feeding mice.

Large parts of the oral cavity including, the palate, the floor of the mouth, cheeks, the labial surface of the oral mucosa, and anterior two-thirds of the dorsal, ventral, and lateral surfaces of the tongue were clinically examined in mice using tailored oral cavity examination tools. The deep parts of the oral cavity and the posterior third of the tongue were also carefully examined. OM was evaluated every 24-48 hrs during the study. The severity of CIOM was assessed using the ulcer size percentage to the total epithelialized upper and lower surface of the tongue, in order to overcome the minimal individual variation in tongue size between mice

of the same age. Macroscopically, IV administration of 5-FU triggered diffuse erythema on the dorsal and ventral surface of the tongues. Consistent with these results, OM ulceration was localized mainly to the dorsolateral surface of the tongue, followed by the ventral surface, evident by tissue staining with toluidine blue. The results are consistent with the previous study, which shows that i.v. administration of 5-FU triggered diffuse erythema on the dorsal surface of the tongues, with the toluidine blue positive areas corresponding to erosive or ulcerative lesions (Bertolini et al., 2017). However, H-MW-HA was significantly able to limit the cytotoxic damage observed by 5-FU and reduced the CIOM ulcer size. There was a dramatic improvement in the development and progression of OM in the 5-FU treated animals. Fewer mice treated with H-MW-HA developed oral mucosal ulcers in addition to a significant decrease in mucositis scores compared to animals treated with 5-FU only.

Further, the peak disease period was shorter in H-MW-HA treated animals (Figure 9). Therefore, in this animal model of CIOM, H-MW-HA showed protection and acceleration in the healing of the injured oral mucosa following CT. As mentioned above, the antioxidant effect of H-MW-HA on ROS may partially account for this observation. Besides, the observation that HA stimulates migration and proliferation of keratinocytes might reflect its protective and healing effect against chemotherapy-induced injury (Oksala et al., 1995b; Kaya et al., 1997; Nyman et al., 2013). Finally, exogenous HA has a positive effect on re-epithelialization and accelerated wound healing in human *in vivo* deep dermal incisional skin wounds (Nyman et al., 2019). Analysis with immunohistochemistry is needed further to study cell proliferation and apoptosis in mucosal tissues, the cell infiltrates nature, and possible changes following the HA intervention. The present *in vivo* study focused on the clinical and histological changes in the oral and intestinal mucosa, with the initial focus of *in vitro* experiments being on the antioxidant effects of HA.

This study assessed the effect of H-MW-HA in ameliorating 5-FU-induced diarrhea, which is an important sign of IM induced by 5-FU and is often used as a surrogate marker (Chen et al., 2013; Borges et al., 2006). In our study, daily administration of H-MW-HA was able to delay the onset and reduce the duration and severity of 5-FU treated mice's diarrhea. Besides, animals receiving H-MW-HA had improved survival rates compared to the 5-FU-alone groups. We noticed that with (50 mg/kg/day) of i.v. 5-FU injection, the survival rate of mice beyond 14 days was low, which is likely be due to severe IM that resulted in irreversible weight loss and dehydration. However, a significant improvement in chemotherapy-associated animal survival

was evident when mice were treated with H-MW-HA. Thus, the daily administration of H-MW-HA effectively ameliorated the severity of diarrhea and improved the mortality associated with 5-FU administration. One limitation of this study was the monitoring of individual food and water intake, which couldn't be observed during the experiment because mice were housed in groups.

An essential feature of inflammation and tissue healing following 5-FU-induced mucositis is the leukocyte infiltration process (Soares et al., 2013). To assess whether H-MW-HA can prevent 5-FU-induced mucosal damage and alleviate leukocyte infiltration, the histology of tongue and jejunum samples were examined by H & E staining. In the 5-FU treated mice, the dorsal tongue showed a reduction in epithelial thickness, and a distortion in the structural integrity of the dorsal mucosa, with filiform papillae being completely effaced and the keratinized layer being reduced or absent. The connective tissue showed an acute inflammatory infiltrate among the muscle fibres. The morphological analysis of mice intestines after 5-FU administration demonstrated remarkable histological damage resulting in shortening of villi length, distortion of crypts, excessive inflammatory cell infiltration, and cellular damage in the intestinal mucosa of the mice after 5-FU administration. Epithelial damage and inflammatory cell infiltration in the mucosa during the inflammatory stage of mucositis have been reported in previous studies (Sonis et al., 2004a). In a mouse model of 5-FU-induced OM, Bertolini et al. (2017) observed important macroscopic and microscopic lesions and increased proinflammatory cytokines activity and neutrophil infiltration. Other studies revealed a significant increase in villous atrophy in mice intestinal mucosa after 5-FU treatment (Al-Asmari et al., 2015). Intestinal injury may include the decreased proliferation of epithelial cells, leading to blunted villus height and crypt distortion with increased crypt cell apoptosis, and abundant inflammatory cell infiltration (Chen et al., 2013; Justino et al., 2014). However, the damage in the tongue and jejunal samples induced by 5-FU was ameliorated by H-MW-HA administration. The thickness of the tongue epithelium and the villi length of the jejunum were able to recover spontaneously and significantly retained, and fewer inflammatory cells were observed. Importantly, the results of this study suggest the novel protective effects of H-MW-HA for attenuation of 5-FU-induced oral and intestinal mucositis. This data would be valuable for further assessing the influence of H-MW-HA in tumour models treated with 5-FU. Another limitation of this study was that tongue ulcerations were not detected in daily visual clinical inspections of living animals. The reason was that scoring based on naked-eye instructions made it difficult to identify the small lesions that formed inside mice's mouths. However, we

integrated the information gathered from the post-mortem assessment of the mice tongue at three different time points and correlated them with the one we gathered clinically from assessing the live mice.

It is well-established that oxidative/nitrosative stress, inflammation, and apoptosis are major contributors to the development of mucositis induced by 5-FU treatment (Criswell et al., 2003; Yoshino et al., 2013; Soares et al., 2008; Kim et al., 2015). Oxidative stress results when intracellular concentrations of free radicals exceed physiological values (Ma et al., 2009). Oxidative stress-induced damage of the tissue is attenuated by the enzymatic antioxidant defence system of the cell. It does so by neutralizing free radicals either indirectly by raising the activity of the antioxidant enzymes or directly by scavenging free radicals (Cui et al., 2010; Shal et al., 2018). In the current study, we focused on the antioxidant effects of H-MW-HA. Free radical scavenging activity and antioxidant properties of HA both *in vitro* and *in vivo* have been reported in several studies (Presti and Scott, 1994; Campo et al., 2004b; Halicka et al., 2009; Ke et al., 2011; Gocmen et al., 2015; Chernos et al., 2017; Cortivo et al., 1996). In a rat model of carbon tetrachloride-induced liver fibrosis, Campo et al. (2004b) demonstrated that treatment with HA successfully reduced lipid peroxidation and increased the endogenous antioxidants (glutathione peroxidase, GSH-Px, and superoxide dismutase, SOD) activities in the liver. Substantial evidence shows that ROS, including superoxide, H₂O₂, nitric oxide, peroxynitrite, and hypohalous acids, which are massively produced during inflammatory responses, are responsible for HA degradation in inflammatory diseases (Balogh et al., 2003). HA antioxidant scavenging activity towards ROS, such as hydroxyl radical (OH), has a possible role in the activation and modulation of the inflammatory response. In patients suffering from rheumatic diseases, the reduction of HA molar mass in the synovial fluid has led to investigating HA degradation *in vitro* by ROS (Soltés et al., 2006). It was also reported that HA is able to protect granulation tissue from ROS damage and accelerate skin wound healing in rats (Trabucchi et al., 2002). Furthermore, enzymatically digested HA revealed a better radical scavenging capacity than intact HA (El-Safory and Lee, 2010). However, minimal information is available about the efficacy of HA in preventing or reducing ROS-induced damage to oral mucosal tissues, both *in vitro* and *in vivo*. This information arose from using a hyaluronic acid-based compound (Mucosamin[®]) to inhibit fibroblast senescence and epithelial cytotoxicity induced by oxidative stress *in vitro* (Cirillo et al., 2015).

The first line of the antioxidant defence system against ROS-induced oxidative stress *in vivo* is antioxidant enzymes, such as SOD, catalase (CAT), and GSH-Px. Superoxide dismutase (SOD) catalyses the dismutation of the superoxide anion to form H₂O₂, which in turn is decomposed by GSH-Px and CAT to water and oxygen, thereby preventing hydroxyl radicals formation (Cui et al., 2010). Therefore, this enzyme acts cooperatively with other antioxidative enzymes at different sites in the metabolic pathway of free radicals.

In the present study, it was observed that the activity of SOD in serum slightly increased when the animals were treated with 5-FU compared to control mice. This increase most likely occurred in an attempt to counteract the ROS-induced damage. However, animals treated with both 5-FU and H-MW-HA exhibited a significant increase in SOD activity. The enhanced activity of SOD and other antioxidant enzymes activity can be very effective in scavenging the various types of oxygen free radicals and their products. Therefore, the protective effect of the H-MW-HA on 5-FU-induced damage might be, at least partially, attributed to its influence on the antioxidant enzymes and non-enzymatic system. It was observed that polysaccharides could upregulate the mRNA expression of antioxidant enzymes (Pang et al., 2000; Cheng et al., 2008). Therefore, the effect of the H-MW-HA on SOD was possibly associated with its induction on gene expression of SOD at the transcription level.

A feedforward loop in which exogenous HA induces the synthesis of endogenous HA has been previously demonstrated (O'Neill, 2009). In a mouse model, Riehl et al. (2012) reported that daily HA treatment of mice induced the expression of hyaluronic acid synthases (HAS1) in the intestine and colon and resulted in increased production of endogenous HA in lamina propria. Therefore, we suggest that changes in the amount and distribution of endogenous HA might be contributed, to some extent, to increase the antioxidant activity of HA. Further research focusing on the effect of HA on the antioxidant enzymes and mediated pathways that regulate antioxidant enzyme activity is necessary to clarify the mechanisms underlying HA ability to prevent or mitigate OM.

From what we know, this study is the first to assess the effect of H-MW-HA administration on the functional and inflammatory characteristics of the 5-FU-induced oral and intestinal mucositis. These results provide an experimental rationale to develop a novel HA-derived treatment as a therapeutic agent to protect against oral and intestinal toxicity associated with anticancer therapy. However, further in-depth investigations of the molecular mechanism are required to further strengthen the data for H-MW-HA preventive role by investigating cell

proliferation, several antioxidant enzymes, and several redox-sensitive transcription factors, anti-inflammatory mediators, and apoptotic markers.

6.6 Conclusion

The present work provides evidence that H-MW-HA can reverse 5-FU-induced damage on mice tongues and small intestines, recover the villi length of the jejunum, and the thickness of the tongue epithelium, suppress oxidative stress and reduce inflammatory infiltrates. Thus, based on the current findings, it is concluded that H-MW-HA could efficiently ameliorate 5-FU-induced oral and intestinal mucositis.

CHAPTER 7 DISCUSSION AND CONCLUSION

7.1 Discussion

Cancer is ranked as the leading cause of death in economically developed countries, and it ranks second in developing countries in the 21st century (Jemal et al., 2011; Bray et al., 2018). Based on the GLOBOCAN 2020 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer (IARC), about 19.3 million cancer cases and almost 10 million cancer deaths were reported in 2020 (Sung et al., 2021). CT and RT are highly effective cancer treatments. However, these treatments cause significant damage to healthy tissues. In particular, the rapidly dividing epithelial progenitor cells of the alimentary tract can be severely affected (Sonis, 1998; Duncan and Grant, 2003; Sonis, 2009; Campos et al., 2014; Bertolini et al., 2017). During the course of cancer treatment many adverse events occur both with acute and chronic onset (Carlotto et al., 2013). Mucosal inflammation and atrophy of the mouth and gastrointestinal tract, collectively known as alimentary tract mucositis, are debilitating adverse effects observed in cancer patients undergoing CT (Cheng et al., 2012; Elting et al., 2003; Sonis et al., 2001). Among these, OM has been reported as the most bothersome side effect that cancer patients experience (Scully et al., 2003; Sonis, 2011). It has been over 100 years since the first description of OM as a RT -related toxicity, and well over 50 years since its development was noted among the first CT users. Subsequently, millions of cancer patients have suffered from this pathological condition (Yuan and Sonis, 2014; Sonis, 2012a).

OM is a severely debilitating inflammatory condition clinically presenting as erythema, oedema, ulceration, and pseudomembrane formation of the oral mucosa resulting in severe function-altering pain and discomfort that can restrict patients' oral intake (Murphy and Gilbert, 2009; Rosenthal, 2007; Yeoh et al., 2006; Sonis et al., 2004a). The frequency of OM varies with around 20–40% reported incidence in patients receiving standard-dose chemotherapeutic treatment for solid tumours (Villa and Sonis, 2015; Lalla et al., 2014) to almost 100% in patients receiving high-dose CT prior to hematopoietic stem cell transplantation, as well as for patients being treated with RT for HNC (Campos et al., 2014; Lalla et al., 2008). In severe cases, this can lead to parenteral nutrition, loss of function, worsened quality of life, and ultimately culminating in dose reduction, delays, or even cessation in the delivery of optimal antineoplastic treatment (Sonis, 2007; Peterson and Lalla, 2010; Peterson et al., 2016). In addition, the lesions can also weaken the skin barrier resulting in local or systemic infections, and increasing patients morbidity (Sonis, 2007). The consequences of OM also can result in

new or extended hospital admissions, additional emergency room, unplanned physician office visits, gastrostomy reliance or total parenteral nutrition, pain and fluid management, with the consequent use of additional health resources with its accompanying costs. This sequel can adversely affect patients' quality of life and cost the healthcare system hundreds of millions of dollars (Carlotto et al., 2013). It has been calculated that the incremental cost of OM in patients afflicted by ulcerative lesions ranges from \$17,000 to more than \$40,000 (Sonis et al., 2001; Nonzee et al., 2008; Barasch et al., 2009). According to the Quintiles IMS Institute report, the amount of total global cost spent per year on supportive care treatments needed to relieve cancer treatment's side effects, including OM, is about \$23.4 billion (Aitken et al., 2017).

Despite its severity, frequency, and economic impact, for the vast majority of patients who are at risk of developing and who develop OM, the options to prevent, effectively mitigate the onset, or completely treat the disease are frustratingly insufficient (Pulito et al., 2020), leaving it a very important unmet medical need. While focusing on pain control and maintenance of nutrition, most of the current standard therapies for mucositis are predominantly palliative (Bowen et al., 2019). Cirillo et al. (2015) point out that there is currently no effective treatment that mechanistically targets the pathogenesis of the disease and surprisingly, so far there is no drug that has been approved to prevent the disease. Motivated by this urgent unmet clinical need, improving the quality of life of oncological patients, and potentially large economic return that has been estimated at well over US\$1 billion annually (Yuan and Sonis, 2014), the development of new and cost-effective therapeutic mechanism-based mucositis interventions that can prevent rather than treat OM with greater efficacy has become an urgent requirement.

Given the kinetics of regimen-induced mucosal injury, determining and targeting the pathophysiological mechanisms underlying OM development may provide fundamental knowledge for developing effective preventive and /or therapeutic strategies (Biswal, 2008; Cinausero et al., 2017). Yet, the pathogenesis of OM is the subject of great debate, and it was only recently when the complexity of mucositis's biological process was recognized (Sonis, 2004b; Logan et al., 2007b; Sonis et al., 2007; Bowen et al., 2019). It is now thought that mucositis actually represents a sequence of several biological and cellular events arising predominantly in the cells and tissues of the submucosa, and ending in degeneration and breakdown of the epithelial barrier with resultant mucositis ulcers. These events include the interaction of oral mucosal cells and tissues, ROS generation, tight junction defects and epithelial barrier dysfunction, inflammatory signalling cascades including NF- κ B and

proinflammatory cytokine production, innate and cellular immune responses, apoptosis mediators, and local factors such microbiome (Sonis et al., 2004a; Al-Dasooqi et al., 2013; Bowen et al., 2019). Biologically, mucositis develops in five phases, including initiation; upregulation/activation; signalling and amplification; ulceration; and healing (Sonis, 2004b). Nonetheless, the primary mode of action of many chemotherapeutic agents and ionizing radiation includes mucosal injury triggered by the excessive production of ROS, such as H₂O₂, superoxide, hydroxyl radicals, and consequent activation of oxidative stress pathway (Criswell et al., 2003; Yoshino et al., 2013; Zhao and Robbins, 2009). As such, there is a need to establish a new research model of oxidative stress-induced oral mucosal toxicity in which the new therapeutic interventions can be characterized.

Proceeding from this concept, and taking into account the oxidant-pathways involved in the OM pathogenesis, a study was conducted (chapter 3) with the aim to establish an *in vitro* model of oxidative stress-induced oral mucosal toxicity, to simulate what is thought to occur in radio-chemotherapy- induced OM. To achieve this we used three different monolayer cultures of oral mucosa keratinocytes (cancer cell lines H357 and H400, and immortalized normal human oral keratinocytes, OKF6) widely used to mimic specific parts of oral mucosal epithelial barrier (Abbott, 2003; Bierbaumer et al., 2018), and treated them with H₂O₂, a well-known inducer of oxidative stress (Gille and Joenje, 1992; Cross et al., 2015). Specifically, in the present study, we studied the cytotoxic effect of H₂O₂ on oral epithelial cells proliferation, viability, and apoptosis by performing a series of preliminary experiments using cell viability and growth assay (section 2.3.9), fluorescence-based live-dead assay (section 2.1.10), and FCA (section 2.3.11). In addition, the morphological changes of the cells were assessed using a fluid cell imaging system (FLoid™ Cell Imaging Station, Life Technologies Australia) (section 2.3.9.2). In the present study (chapter 3) we successfully developed a novel *in vitro* model of oxidative stress-induced oral mucosa toxicity using H₂O₂, based on monolayer cultures of oral keratinocytes (H400, 357, and OKF6) and evaluated cytotoxicity, and morphological changes induced by H₂O₂. This established *in vitro* model of H₂O₂-induced OM could allow for the study of OM's pathogenesis and the screening of potential anti-oxidant drugs.

The present study showed that H₂O₂ exerted cytotoxic effects and led to an anti-growth effect on H400, H357, and OKF6 cells by suppressing cell proliferation, viability, and the induction of cell apoptosis (chapter 3). The H₂O₂-induced cytotoxic effect on epithelial cells viability and growth was found to be time and dose-dependent. Based on FCA assays, the IC₅₀ value in the

H400, H357, and OKF6 cells was reached after 24 hours of exposure to 800 μM , 800 μM , and 400 μM of H_2O_2 , respectively. Our finding is consistent with other studies where H_2O_2 was found to promote damage in different normal and malignant cells lines in a time and concentration-dependent manner (Datta et al., 2002; Liu and Zhao, 2014; Cirillo et al., 2015; Zhou et al., 2012; Moll et al., 2007; Yiannis et al., 2020). However, there was a variation in the approximate IC_{50} value of H_2O_2 that may be attributable to the variation in H_2O_2 sensitivity for the studied cells.

The decrease in epithelial cell proliferation and viability observed in the present study may be attributed to the increase in the number of dead cells, as shown by the trypan blue exclusion assay and fluorescence-based live-dead assay. Reciprocally, morphological analysis of the oral keratinocyte cells through a phase-contrast inverted microscope showed morphological changes of apoptosis in H_2O_2 treated cells, including shrinkage, loss of cellular adhesion to their neighbours, membrane blebbing, and chromatin condensation and fragmentation, along with the creation of apoptotic bodies, when compared to untreated cells that retained their original polygonal morphology. These observed morphological changes suggest that H_2O_2 -induced oxidative stress may have induced an apoptotic process that lead to epithelial cell death. It can be postulated that the decrease in cell viability may be related to oxidative stress, suggested as one of the most important causative factors for the induction of cell apoptosis (Holbrook and Ikeyama, 2002). The observed reduction in keratinocyte cells proliferation may be attributed to senescence also postulated to have a role in OM pathogenesis (Cirillo et al., 2015). Incubation of the cells with potentially harmful molecules, such as H_2O_2 , induces sub-cytotoxic stress and brings the cells into a state similar to senescence, termed stress-induced premature senescence (SIPS) (Fripiat et al., 2002). This underscores the importance of continuing research to explore the role of cell senescence in the pathogenesis of mucositis. The finding of the present study supported the hypothesis that H_2O_2 treatment can exert a cytotoxic effect on oral epithelial cells *in vitro* and rejected the null hypothesis that H_2O_2 treatment does not exert a cytotoxic effect on oral epithelial cells.

HA is an unbranched non-sulfated glycosaminoglycan (GAG), with distinct physico-chemical properties, that are naturally occurring and diffused in the epithelial, connective and nervous tissues of vertebrates (Fallacara et al., 2018; Lapcák et al., 1998; Fraser et al., 1997). The past several years have seen increasing attention in HA and its derivatives for their potential effectiveness in treating and preventing OM and other inflammatory conditions. (Favia et al.,

2008; Colella et al., 2010; Cirillo et al., 2015; La Gatta et al., 2019). Several reports have demonstrated the potential efficacy of HA- based compound in treating OM both *in vitro* and *in vivo* (Favia et al., 2008; Colella et al., 2010; Ruggiero et al., 2016). Nevertheless, HA does not act as a standalone therapy universally recognized or accepted to treat or prevent OM (Elad et al., 2020; Villa and Sonis, 2020). This could be attributed to the variety of HA constituents and treatment protocols employed in various trials, including molecular weight, concentration, cross-linking, and mixing with other compounds (D'Agostino et al., 2017; La Gatta et al., 2019; La Gatta et al., 2020). Nevertheless, with several potential advantages of HA, including off-the-shelf availability and reduced cost (Lapcík et al., 1998; Rah, 2011), biocompatibility, biodegradability, non-immunogenicity (Laurent and Fraser, 1992), and antioxidant properties (Campo et al., 2004b; Balogh et al., 2003; Halicka et al., 2009; Trabucchi et al., 2002), the use of HA as a standalone material to inhibit oxidative stress-induced damage in oral keratinocytes could represent a valid therapeutic strategy to prevent OM. Given that molecular size is a discriminating factor in the HA biophysical characteristics (D'Agostino et al., 2017; La Gatta et al., 2019; La Gatta et al., 2020), an *in vitro* study was first conducted to assess whether different constituents of HA exert specific biological effects on normal human oral keratinocyte (OKF6) cells (chapter 4).

HA's direct effect on epidermal keratinocytes and mesenchymal cells has been shown is thus clear (Colella et al., 2012). However, Limited information regarding HA's effect on the oral epithelial cells is available (Cirillo et al., 2015). Accordingly, and with hypothesizing that HA and HA-derivatives directly affect normal oral epithelial cell proliferation, cell biological function of HA constituents was investigated *in vitro* to determine whether HA is involved in regulating normal oral epithelial cell proliferation during normal cell growth. Furthermore, this work aimed to establish if, and to what extent, the varying preparations of HA may directly affect normal epithelial cell proliferation. This was achieved by utilizing monolayer cultures of normal human oral keratinocytes (OKF6) and various HA products, including commercial formulation of HA enriched with amino acids (Mucosamin[®]), native H-MW-HA, and three cross-linked (xl-) HA's constituents (xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100). The effect of HA's constituents on cell proliferation was evaluated by employing a proliferation assay and using fluorometric fluorescein diacetate (FDA) for correlation between different concentrations of different sized HA products and relative cellular proliferation (section 2.3.12). In addition, the morphological changes of the cells were assessed using a fluid cell imaging system (FLoid™ Cell Imaging Station, Life Technologies Australia) (section 2.3.9.2).

The present study showed variation of oral epithelial cell proliferation for the various tested HA's preparations, inclusive of analyses of concentration and incubation time. Except for Mucosamin[®], all tested HA preparations were safe, biologically active, and directly affect oral keratinocyte through promoting cell proliferation. Specifically, both H-MW-HA at a concentration of up to 0.1% w/v, and xl-HA 100/100 0.01% v/v showed no cytotoxic effect and promoted significant oral epithelial cell proliferation. Similarly, xl-HA5/5 and xl-HA30/30 at 0.01% v/v were non-toxic to the oral epithelial cells and induced an increase in OKF6 cell proliferation, although not statistically significant. It has been previously demonstrated that the diverse pharma grade HA fragments could modulate cellular processes differently (D'Agostino et al., 2017; La Gatta et al., 2020). Consistent with the findings of the present study, previous studies highlighted the pro-proliferative effects of HA on several kinds of cells *in vitro*, including colonic epithelial (Caco-2) cells (Sammarco et al., 2019), prostate epithelial cell (PEC) (Damodarasamy et al., 2015), human corneal epithelial cell line (HCE-2) (Wu et al., 2013), human umbilical vein endothelial cells (hUVECs) (Mo et al., 2011), human amniotic mesenchymal stem cells (hAMSCs) (Liu et al., 2016), and human amniotic epithelial cells (hAECs) (Tian et al., 2020).

Unlike the other tester HA constituents, at a concentration of up to 5% v/v, Mucosamin[®] treatment was safe while not promoting oral epithelial cell proliferation. However, at a concentration higher than 5% (v/v) and after incubation of OKF 6 cells for 48 hours, Mucosamin[®] became cytotoxic, causing a significant reduction in oral epithelial cell proliferation. A previous study has shown that Aminogam[®] (HA-based compound that has the same composition of Mucosamin[®]) and at a concentration of 1% (v/v) accelerated keratinocyte migration and hastened wound closure of keratinocyte monolayers in an *in vitro* scratch wound healing assays. However, keratinocyte migration was impaired in the presence of 10% (v/v) Aminogam[®]. These results are consistent with the results in the present study, where Mucosamin[®] was observed to impair cell proliferation at concentrations higher than 5% (v/v).

Morphological analysis of the cells through a phase-contrast inverted microscope showed morphological changes of apoptosis in oral epithelial cells treated with higher than 5% (v/v) Mucosamin[®], suggesting that Mucosamin[®] may exert cytotoxic effects via inducing apoptosis in oral keratinocyte. However, it is not clear yet whether the cytotoxic effect of Mucosamin[®] is related to amino acids rather than HA. Some amino acids, like D-alanine, D-proline, and D-lysine, a D-amino are found to be cytotoxic when tested in several epithelial cell lines

(Bardaweel et al., 2013). In addition, in the relevant literature, H-MW-HA has been shown not to be cytotoxic on epithelial cells *in vitro* (D'Agostino et al., 2017; Riehl et al., 2012; Tian et al., 2020).

The mechanism underlying HA's ability to promote oral epithelial cell proliferation is not completely clear. It could be attributed to the biological role of HA in the regulation of cell proliferation via HA receptors, CD44 and RHAMM (Viola et al., 2015; Asari, 2004). In physiological conditions, HA is an essential component of the ECM (Fraser et al., 1997). In addition to its central role in the hydration of tissue, HA is involved in a number of cell biological phenomena including cell differentiation, growth, proliferation, migration, and adhesion, and thus consequently, processes such as inflammation, wound healing, angiogenesis, tissue development, morphogenesis, tumor progression, and metastasis (Lee and Spicer, 2000; D'Agostino et al., 2015; Dicker et al., 2014; Toole, 2004; Alho and Underhill, 1989; Slevin et al., 2002). These biological processes are mediated by the interaction of HA with cell surface receptors, such as cluster determinant 44 (CD44), the receptor for hyaluronate-mediated motility (RHAMM), and toll-like receptors (TLRs) (Vigetti et al., 2014; Viola et al., 2015; Tolg et al., 2006). As such, it has been postulated that the increase in oral epithelial cell proliferation observed with HA treatment could be attributed to the direct effect of HA on oral epithelial cells mediated by binding of HA to such receptors as CD44, TLRs, and RHAMM. Therefore, the results from the present supported our second specific hypothesis that HA directly affects normal oral epithelial cell proliferation *in vitro*, thus rejecting the null hypothesis that HA does not directly affect normal oral epithelial cell proliferation.

Surprisingly, despite the relatively low cost of commercial HA preparations, HA effectiveness in preventing, rather than treating, OM has been poorly investigated (Cirillo et al., 2015). Furthermore, no studies have explored the effect of HA's antioxidant properties on the oxidative stress-induced damage in oral keratinocyte cells. In this context, a study was conducted to assess the protective characteristics of different HA constituents *in vitro* (chapter 5) in our previously established oxidative stress-induced model of oral mucosal toxicity (chapter 3), with a main focus on the antioxidant effects of HA. We used H₂O₂ to induce oxidative damage in normal human oral keratinocytes (OKF6) *in vitro*. The protective effect of various HA products, including commercial formulation of HA enriched with amino acids (Mucosamin[®]), native H-MW-HA, and cross-linked (xl-) HA (xl-HA 5/5, xl-HA 30/30, and xl-HA 100/100) on oxidative stress were investigated by employing the FCA using the FDA

dye detection of relative cell viability (section 2.3.12). In addition, intracellular ROS production was evaluated by using CM-H₂DCFDA assay (section 2.3.13 and section 2.3.14).

The present study showed that OKF6 cells, when exposed to pathological levels (400 μ M) of H₂O₂, showed a significant decrease in cell viability accompanied by a rapid increase in intracellular ROS production that was apparent from the first few minutes of treatment with H₂O₂. It is well established that the mechanisms initiating chemoradiation-induced mucosal injury, including OM, are linked to the excessive production of ROS (Zhao and Robbins, 2009; Sonis, 2009). Oral keratinocytes, in response to H₂O₂, developed oxidative stress-induced DNA damage and generated ROS, which in turn, initiated oxidative cellular damage (Cross et al., 2015; Cross et al., 1987). As such, the results presented herein demonstrate the pivotal role of excessive production of ROS in initiating oxidative cellular damage (Iglesias-Bartolome et al., 2012; Yoshino et al., 2013; Chen et al., 2020). Strikingly, oxidative-induced cellular toxicity was massively reduced by pre-incubating the oral keratinocytes with HA constituents diluted in a culture medium. H-MW-HA demonstrated the most significant potential protection against oxidative stress-induced cellular damage, followed by Xl-HA 30/30, Xl-HA 100/100, Mucosamin[®], and Xl-HA 5/5. In addition, H-MW-HA and Mucosamin[®] exhibited a dose-dependent protective effect against H₂O₂-induced cellular damage in oral keratinocytes, with the most marked effects being obtained with H-MW-HA. Consistent with our earlier findings (Chapter 4), all tested HA products were safe, showing no cytotoxic effect against the treated cells. Besides, other than Mucosamin[®], which did not affect oral keratinocyte proliferation, all tested HA products effectively promoted OKF6 cell proliferation.

To further understand the cellular mechanism and the dynamics underlying the HA protection effect, we investigated the impact of H-MW-HA on redox status in oral keratinocytes cells. The CM-H₂DCFDA assay revealed that H-MW-HA acts as a scavenger of ROS during H₂O₂-induced oxidative stress. Pre-incubation the oral keratinocytes with H-MW-HA treatment significantly reduced the intracellular ROS levels induced by H₂O₂ in OKF6 cells. In particular, 0.01% w/v H-MW-HA exerted a moderate antioxidant activity, whereas a striking reduction in ROS levels was detected in cells exposed to the higher concentration of H-MW-HA (0.05% w/v). Current literature has demonstrated anti-oxidative properties of HA and its constituents, such as (H-MW-HA, (L-MW-HA, cross-linked HA, and HA-based compounds both *in vitro* and *in vivo* (Moseley et al., 2003; Krasinski et al., 2009; Ke et al., 2011; Mendoza et al., 2009; Trabucchi et al., 2002; Halicka et al., 2009). It has been found that there is a relation between

depolarization of HA by ROS and its ability to scavenge these species and thereby exerting an antioxidant effect (Balogh et al., 2003; Moseley et al., 2003; Trommer et al., 2003; Parsons et al., 2002; Pigman et al., 1961). This function is found to be both molecular weight- and dose-dependent (Presti and Scott, 1994; Kvam et al., 1995; Moseley et al., 2002; Cortivo et al., 1996). High molecular mass HA binding to free radicals catalyzes the breakdown of HA into smaller fragments, which in turn inactivates ROS (Mendoza et al., 2009). This is consistent with the results in the present study where H-MW-HA was found to have a greater protective effect towards oxidative stress-induced cellular damage in comparison with the other tested HA derivatives. Moreover, higher ROS scavenging activity was obtained with 0.05% w/v H-MW-HA, compared to 0.01% w/v H-MW-HA. It is therefore most likely that HA constituents prevented the propagation of oxidative stress by sequestering ROS. Studies have shown that in corneal epithelial cells, UVB irradiation leads to a deficiency or inactivation in antioxidant enzymes, a process prevented by HA (Li et al., 2013). Another recent study (Cirillo et al., 2015) has demonstrated that HA enriched with amino acids (Mucosamin[®]) has free radical scavenging antioxidant properties. Taken together, the present study demonstrated the protective effect of HA constituents against oxidative stress-induced damage on oral keratinocytes and that H-MW-HA 0.05% w/v exerts the most significant ROS scavenging activity. Furthermore, the present study further reinforces the notion that the protective effect of the HA tested in the present study depends, at least in part, on its antioxidant properties.

Thus, these findings on the protective effect and antioxidant properties of HA supported our third hypothesis that HA can effectively prevent and/or mitigate oxidative stress-induced damage *in vitro*. Moreover, this protective effect of HA constituents is likely to be antioxidant properties dependent. Therefore, we rejected the null hypothesis that HA cannot effectively prevent or mitigate oxidative stress-induced damage *in vitro* and that HA constituents do not exert antioxidant activity. Although further research is needed to confirm our hypothesis, the current data suggest that the tested HA, particularly H-MW-HA, may prevent mucosal injury initiated by ROS by inactivating the free radicals that are produced by chemoradiation.

Since our data demonstrated that HA constituents effectively prevented oxidative damage *in vitro*, with the most significant effect seen by using H-MW-HA, we wondered if it was possible to test the biological properties of this preparation in an *in vivo* pre-clinical studies. Animal models' predictive value in guiding clinical development has been demonstrated by correlative pre-clinical and clinical studies (Farrell et al., 1998; Rosen et al., 2006; Caluwaerts et al., 2010;

Limaye et al., 2013). Therefore, several chemotherapy-induced animal models for OM and IM have been used, including hamster, rat, and mouse models (Chang et al., 2015; Yamaguchi et al., 2016; Patricia et al., 2017). In this context, an *in vivo* study was conducted to assess the potential efficacy of H-MW-HA in preventing or mitigating oral and intestinal mucositis in a mouse model of chemotherapy-induced mucositis (Chapter 5). To this end, we first needed to establish an experimental mouse model of chemotherapy-induced mucositis, where we could concurrently assess both oral and gastrointestinal damage induced by CT. To achieve this, we used C57BL/6 female mice and intravenous (i.v.) administration of low and intermittent dose of 5-Fluorouracil (5-FU) CT for two weeks to induce simultaneous oral and intestinal mucositis in mice (section 2.4.2 to section 2.3.6). In addition, and simultaneously to 5-FU treatment, mice were treated daily with H-MW-HA (0.01% w/v) in the drinking water (section 2.4.3). Post initial injection, mice were monitored clinically for weight loss (Section 2.4.7) and diarrhea (Section 2.4.8), as a surrogates for intestinal injury. A thorough clinical assessment of the oral cavity was undertaken by systematic oral examination using a visual oral ulcerative mucositis score (Section 2.4.9). Mice were sacrificed at 3 interim time points during the experimental period (Section 2.4.10). Macroscopic and histomorphometric analyses of the tongue and intestinal tissues were conducted, and superoxide dismutase (SOD) enzyme activity was evaluated in blood serum taken at each timepoint (Section 2.4.10. to Section 2.4.16).

The present study has shown that repeated i.v. administration of 5-FU in mice resulted in successfully establishing a mouse model of mucositis that replicates the features observed in both oral and gastrointestinal tract mucositis (Yasuda et al., 2012; Yasuda et al., 2013; Bertolini et al., 2017), including oral epithelial layer loss, ulcerative OM, moderate diarrhea, bodyweight loss, damaged intestinal mucosa, villi lengths shortening, and loss of crypt architecture in the jejunum. Furthermore, a close correlation was observed between the severity and score of OM and diarrhea with both mouse BW loss and survival rate. Several pre-clinical studies have used diverse experimental animal designs of chemotherapy-induced mucositis (CIM) (Tancharoen et al., 2018; Sonis et al., 1990; Kajiwara et al., 2015; Chang et al., 2015; Wu et al., 2011b). Among animal models, the i.v 5-FU-induced mucositis mouse model has been the most representative method to study OM. It faithfully reproduced macroscopic, histopathological, and inflammatory responses of the human oral mucosa to 5-FU treatment (Bertolini et al., 2017). In addition, research has extensively reported that the administration of 5-FU to experimental animals causes both oral and intestinal mucositis (Chen et al., 2013; Bertolini et al., 2017; Liu et al., 2017; Li et al., 2019). However, mechanical-, chemical- or radiation-

induced injury of the oral mucosal surface is additionally required in most existing CIOM models (Yamaguchi et al., 2016; Wardill et al., 2019). Given that, most of the existing CIM murine model research has predominantly focused on either oral or intestinal mucosal damage as single entities, shifting away from what is the real pathological clinical scenario faced by the great majority of cancer patients. (Takeuchi et al., 2018; Shimamura et al., 2018; Tancharoen et al., 2018; de Koning et al., 2007; Zhang et al., 2018; Huang et al., 2019). Our model is a significant improvement over existing models because it: i) does not require additional oral mucosal “irritation” to induce macroscopically and histologically recognizable oral erosions and ulcers; ii) triggers a significant clinical ulcerative response in oral epithelium without severe loss of intestinal function; and iii) resembles most of the current human anti-cancer 5-FU regimens (Meulendijks et al., 2016). The present study findings are consistent with the literature, where i.v. administration of 5-FU in mice induced OM and triggered diffuse erythema and erosive and ulcerative lesions on the dorsal surface of the tongues without the need for additional oral mucosal irritation (Bertolini et al., 2017). In addition, other literature has reported that the 5-FU administration to experimental animals produced diarrhea and BW loss and caused severe IM (Wadler et al., 1998; Benson et al., 2004; Stein et al., 2010). As such, we have suggested the mouse model established in the present study to be a suitable *in vivo* model of both oral and intestinal mucositis, which may be useful for studying chemotherapy-induced mucositis and developing preventative therapies.

Incidence, severity, and duration of mucositis were always considered an important endpoint to assessing prophylactic and therapeutic effects of treatments on chemotherapy-induced mucositis, based on the lack of erythema, grade of mucositis, the severity of diarrhea, and the absence of ulceration or erosions (Chen et al., 2013; Ogata et al., 2016; Elad et al., 2020). The clinical parameters collected from the present study showed that the prophylactic use of H-MW-HA (0.01% w/) in our mouse model was able to reduce the development of 5-FU-induced oral and intestinal mucositis. This prophylactic efficacy of H-MW-HA was apparent by effectively reducing the occurrence, onset, severity, and duration of OM, and ameliorating the severity and onset of diarrhea. Furthermore, the development and size of OM erosive or ulcerative lesions on the tongue were significantly reduced when the 5-FU treated mice simultaneously co-treated with H-MW-HA. The prophylactic effect of H-MW-HA is likely to be due to preventing the 5-FU-induced damage to the tongue epithelium and intestinal mucosa and preserving their thickness (Bertolini et al., 2017). Early studies of 5-FU-related oral and intestinal mucositis animal models showed reduced proliferation of the basal layers of tongue

mucosa and intestinal crypts cell (Siber et al., 1980; Kato et al., 2015; Bertolini et al., 2017), resulting in a reduced relative fraction of tongue epithelium and villous enterocytes and eventually reduced the intestinal surface area of absorption, leading to diarrhea (Bhanja et al., 2009). Histological analysis in the present study indicated that H-MW-HA treatment reduced the 5-FU-induced damage to the tongue epithelium and intestinal mucosa and preserved their thickness, to a degree almost resembling the aspect of the mice in the control group. HA is known to play a role in intracellular functions, including cell adhesion, migration, and proliferation (Zhu et al., 2006; Etscheid et al., 2005). Exogenous HA has been shown to increase epithelial cell proliferation and villus height in the intestine and crypt depth in the intestine and colon (Riehl et al., 2012). We consider that based on these suppositions, the administration of H-MW-HA in the present experimental model might have negated, at least in part, the adverse effects of 5-FU by facilitating cell proliferation in tongue and intestinal mucosa, and thus reduced the occurrence of oral and intestinal mucositis and associated diarrhea. This likely resulted in a reduction in the BW lost closely related to 5-FU-induced diarrhea (Symonds, 1998) and increased the survival rate of H-MW-HA treated mice.

Although we have demonstrated in the present study that H-MW-HA effectively prevented 5-FU-induced oral and intestinal mucositis, the mechanisms by which HA prevents or mitigates mucositis, however, are poorly understood (Cirillo et al., 2015; La Gatta et al., 2019). In particular, ROS induction is the initial trigger of cell injury in 5-FU-induced oral and intestinal mucositis (Yoshino et al., 2013; Bhattacharyya et al., 2014). Oxidative stress and cellular damage result when the production of ROS exceeds physiological values and cannot be scavenged in time (Ma et al., 2009). Furthermore, excessive production of ROS activates cascades of inflammatory responses leading to impair cell proliferation, inducing cell senescence/apoptosis, and inhibiting cell regeneration in mucosal and submucosal compartments, which ultimately cause ulcerations (Elting et al., 2007; Cinausero et al., 2017; Maria et al., 2017; Lotze et al., 2007; Subramaniam and Muthukrishnan, 2019; Bowen et al., 2019). Our *in vitro* data support this view as we have demonstrated that in response to H₂O₂, oral keratinocytes developed oxidative stress-induced damage and generated ROS, which, in turn, reduced cell viability and proliferation (Chapter 5). Once again, pre-treatment of OKF6 cells with H-MW-HA almost abolished these effects on oral keratinocytes (Chapter 5). It is also interesting to note that H-MW-HA in the present study triggered the antioxidant pathways and increased SOD enzyme activity *in vivo*. Free radical binding to H-MW-HA catalyses the breakdown of HA into smaller fragments and thus inactivates ROS (Mendoza et al., 2007;

Mendoza et al., 2009). Furthermore, studies have shown HA's ability to upregulate gene expression of antioxidant enzymes at the transcription level (Pang et al., 2000; Cheng et al., 2008). Thus, it is possible that H-MW-HA prevented the propagation of oxidative stress either directly by sequestering ROS produced by CT (Cui et al., 2010) or indirectly by raising the activity of the antioxidant enzymes (Shal et al., 2018). Studies also demonstrate that oxidative burst leads to an increase in the intracellular level of reactive oxidants and DNA damage in human blood leukocyte cells, which was prevented by HA (Halicka et al., 2009). A pilot study (Lockington et al., 2014) has demonstrated the free radical scavenging antioxidant properties of HA-rich amniotic membrane. Taking together, these findings further reinforces the notion that the prophylactic effectiveness of the H-MW-HA tested in the present study depends, at least in part, on its antioxidant properties.

In the present study, we provide evidence for the efficacy of H-MW-HA as a preventive measure for both oral and intestinal mucositis. Thus, these findings on the prophylactic use of H-MW-HA in a 5-FU-induced mice model of oral and intestinal mucositis support our hypothesis that H-MW-HA could completely prevent or effectively attenuate the chemotherapy-induced mucositis *in vivo* in a mice model of 5-FU-induced oral and intestinal mucositis. Thus, the null hypothesis that H-MW-HA does not prevent chemotherapy-induced mucositis *in vivo* was rejected.

7.2 Conclusion and future studies

The present study findings have supported the overall hypothesis that HA can effectively prevent and/or attenuate oxidative stress-induced cellular damage *in vitro* and prevent or ameliorate oral and intestinal mucositis *in vivo* in a mice model. In the present study, we demonstrate that the detrimental effects of oxidative stress in human oral keratinocytes can be prevented by HA *in vitro*. We provide evidence for the prophylactic effect of five HA constituents, namely Mucosamin[®], H-MW-HA, XI-HA 5/5, XI-HA 30/30, and XI-HA 100/100, effect, with the most marked effects obtained with H-MW-HA. We then translated the results into pre-clinical *in vivo* practice to provide the evidence that prophylactic use of HA constituent (H-MW-AH) may prevent the onset of oral and intestinal mucositis in a mice model of 5-FU-induced mucositis. Moreover, this protective effect of HA against chemotherapy-induced mucositis is more likely related to its antioxidant effect at the initial stage of mucosal injury. These results provide an experimental rationale to develop a novel HA-derived treatment as a therapeutic agent to protect against oral and intestinal mucositis associated with CT used for cancer treatment. Nevertheless, HA's preventive or therapeutic promotion of oral and intestinal mucositis has profound clinical implications and requires further elucidation of the exact mechanism by which this occurs, given the unique molecular structure and physicochemical properties of HA.

OM is a complex multifactorial disease whose pathogenesis cannot be entirely optimized *in vitro* so as to include local and systemic factors. The role of the cellular environment is of great importance in the expression of cellular phenotype. Ideally, the complete OM microenvironment should be concurrently analyzed, and yet such an experimental situation is entirely hypothetical. Furthermore, an understanding cell signalling pathways and the molecular events leading to OM pathogenesis will provide more insight into the identification of the molecular mechanism underlying HA's ability to target these pathways. Future cell culture studies should be integrated with studies *in vivo*, especially in ongoing clinical trials, to evaluate the applicability of these mechanisms in OM prevention in humans.

Cell lines provide a model where phenotype and genotype OM evolution can be studied (Bierbaumer et al., 2018). Cultured cells lose expression of genes involved in unnecessary functions, thus providing a simplified model for testing the efficacy of potential therapeutic compounds (van Staveren et al., 2009). Cell culture medium allows the optimal environment for cell proliferation to be maintained, and formulated to prevent undesired cell growth and

prevent terminal differentiation. However, experiments conducted using monolayer cultured human cells may not be reflective of the *in vivo* processes where the cells exist in a complex three dimension network; cells grown in monolayers on tissue culture plates lack complex cell-to-cell interaction, as well as cell-to-matrix communications. On the other hand, formalin fixed paraffin embedded (FFPE) samples provide a picture of the OM at a single time point, and so are more like a “snap shot” of one stage in the OM evolution. Thus, a highly reproducible organotypic model of the oral mucosa is likely to provide rich information in future studies as it provides a 3D model of the oral mucosa, where the epithelial cells compose multiple stratified layers resembling the upper alimentary tract mucosa both morphologically and functionally, and where the functional tissue response as close as possible to normal human response.

In the present study, the exact molecular mechanism for the observed reduction in oral epithelial cell proliferation when exposed to exogenous H₂O₂ remains unknown. Future investigation into the understanding of the cell apoptosis signalling pathways would assist in furthering our understanding of the toxicological effect of exogenous H₂O₂ on epithelial cells. This is likely to include assessing caspase proteins and pathway proteins, such as P43 protein, utilizing mRNA assays and western blot analysis.

To further understand the mechanism behind H-MW-HA's ability to prevent or attenuate the oxidative stress-induced cellular damage *in vitro*, high-throughput proteomic analysis of normal oral epithelial cells compared to OM cells, or oral epithelial cells exposed to H₂O₂-induced oxidative stress damage are likely to have a profound impact. These studies could identify OM biologically associated proteins, including post-translational important modifications. Furthermore, the explorer antibody array findings are likely to be useful for identifying how H-MW-HA regulates the OM-associated proteins. It is now possible to assess the profiles of the proteins of significance in normal and OM tissue by *in silico* analysis using publically available databases, such as Protein Atlas, to characterize the properties and structure of the protein, results of which could profoundly inform our understanding of this complex system (Dana et al., 2020).

An understanding of the biological effects of HA-based formulations cannot be achieved without due consideration of HA's variable effects that are entirely dependent on its size, and molecular and biophysical properties (Ferguson et al., 2011). Therefore, this is recommended as a necessary requirement to determine the biophysical properties (hydrodynamic and rheological) of HA constituents when testing their biological activity. In addition, further

studies are required to assess the biological response of the cells to specific HA sizes in an assay that can correlate HA binding to specific receptors, such as CD44, RHAMM, and TLRs, while concurrently demonstrating the specific signalling associated with underlying HA direct effects on oral epithelial cell proliferation.

The mechanism underlying the protective role and antioxidant properties of HA against oxidative stress-induced damage in OKF6 cells has yet to be fully elucidated. Further investigations are needed to evaluate if H-MW-HA is able to regulate the transcriptional activity of Nuclear factor-erythroid-2-related factor 2 (Nrf2), a key transcription factor for both the inducible and constitutive expression of antioxidant and phase II enzymes (Li et al., 2004). The role of other upstream signalling pathways to intervene in the antioxidant activity of H-MW-HA in normal human oral keratinocytes is unknown. In addition, more investigations are required to identify the effect of H-MW-HA on bioavailability and pharmacokinetics *in vivo* and in 3D-OM organotypic models. Further in-depth investigations of the molecular profile utilizing *in vivo* using the FFPE mice samples would clearly elucidate the data for H-MW-HA postulated preventive role by investigating cell proliferation and apoptosis markers, inflammatory mediators, several redox-sensitive transcription factors, and anti-inflammatory mediators. Indeed, Treatment with H-MW-HA would be clearly unjustified without assessing the H-MW-HA effect on the tumoricidal action of the CT. Thus, of primary importance is further *in vitro* studies to assess whether HA has the potential to effect the chemoresistance of cancer cells. We consider that findings from these further studies might suggest additional information, including the route of administration and metabolome analysis of H-MW-HA, that might have the potential for the development of HA-based formulation that counteracts chemotherapy-associated toxicity and aid in the prevention of oral and intestinal mucositis, with potentially profound suitability for clinical application.

Finally, To our knowledge, this is the first study to evaluate the effects of systemic administration of H-MW-HA on the inflammatory and functional aspects of 5-FU-induced oral and intestinal mucositis. This study provides support that systemic administration of H-MW-HA as a preventative or therapeutic tool in oral and intestinal mucositis may have notable clinical implications in the management of cancer patients undergoing CT. In addition, these results provide an experimental rationale to develop a novel HA-derived treatment as a preventative or therapeutic agent to protect against alimentary tract mucosal toxicity associated

with anticancer therapy. However, any potential interference of HA with the antitumor effect of cancer treatment would require further studies.

REFERENCES

- Abbott, A. 2003. Biology's new dimension. Nature Publishing Group.
- Abdel-Rahman, O., ElHalawani, H. & Essam-Eldin, S. 2016. S-1-based regimens and the risk of oral and gastrointestinal mucosal injury: a meta-analysis with comparison to other fluoropyrimidines. *Expert Opinion on Drug Safety*, 15(1), pp 5-20.
- Abdel-Rahman, O. & Fouad, M. 2015. Risk of oral and gastrointestinal mucosal injury in patients with solid tumors treated with everolimus, temsirolimus or ridaforolimus: a comparative systematic review and meta-analysis. *Expert Rev Anticancer Ther*, 15(7), pp 847-58.
- Abdel Moneim, A. E., Guerra-Librero, A., Florido, J., Shen, Y. Q., Fernández-Gil, B., Acuña-Castroviejo, D. & Escames, G. 2017. Oral Mucositis: Melatonin Gel an Effective New Treatment. *Int J Mol Sci*, 18(5), pp.
- Acebedo, A. R., Amor, E. C. & Jacinto, S. D. 2014. Apoptosis Inducing Activity of HPLC Fraction from *Voacanga globosa* (Blanco) Merr. on the Human Colon Carcinoma Cell Line, HCT116. *Asian Pacific Journal of Cancer Prevention*, 15(2), pp 617-622.
- Adebahr, S., Schimek-Jasch, T., Nestle, U. & Brunner, T. B. 2016. Oesophagus side effects related to the treatment of oesophageal cancer or radiotherapy of other thoracic malignancies. *Best Practice & Research Clinical Gastroenterology*, 30(4), pp 565-580.
- Adelstein, D., Gillison, M. L., Pfister, D. G., Spencer, S., Adkins, D., Brizel, D. M., Burtneess, B., Busse, P. M., Caudell, J. J., Cmelak, A. J., Colevas, A. D., Eisele, D. W., Fenton, M., Foote, R. L., Gilbert, J., Haddad, R. I., Hicks, W. L., Hitchcock, Y. J., Jimeno, A., Leizman, D., Lydiatt, W. M., Maghami, E., Mell, L. K., Mittal, B. B., Pinto, H. A., Ridge, J. A., Rocco, J., Rodriguez, C. P., Shah, J. P., Weber, R. S., Witek, M., Worden, F., Yom, S. S., Zhen, W., Burns, J. L. & Darlow, S. D. 2017. NCCN Guidelines Insights: Head and Neck Cancers, Version 2.2017. *Journal of the National Comprehensive Cancer Network J Natl Compr Canc Netw*, 15(6), pp 761-770.
- Adler, H. I. & Hardigree, A. A. 1965. Postirradiation growth, division, and recovery in bacteria. *Radiation Research*, 25(1), pp 92-102.
- Afonso, V., Champy, R., Mitrovic, D., Collin, P. & Lomri, A. 2007. Reactive oxygen species and superoxide dismutases: role in joint diseases. *Joint Bone Spine*, 74(4), pp 324-329.
- Agra, I. M., Filho, J. G., Martins, E. P. & Kowalski, L. P. 2010. Second salvage surgery for re-recurrent oral cavity and oropharynx carcinoma. *Head Neck*, 32(8), pp 997-1002.

- Ahmed, T., Engelking, C., Szalyga, J., Helson, L., Coombe, N., Cook, P., Corbi, D., Puccio, C., Chun, H. & Mittelman, A. 1993. Propantheline prevention of mucositis from etoposide. *Bone marrow transplantation*, 12(2), pp 131-132.
- Aisa, Y., Mori, T., Kudo, M., Yashima, T., Kondo, S., Yokoyama, A., Ikeda, Y. & Okamoto, S. 2005. Oral cryotherapy for the prevention of high-dose melphalan-induced stomatitis in allogeneic hematopoietic stem cell transplant recipients. *Supportive Care in Cancer*, 13(4), pp 266-269.
- Aitken, M., Kleinrock, M. & Kumar, S. 2017. Quintiles IMS Institute. Global Oncology Trends 2017: advances, complexity and cost. 2017: IQVIA, Retrieved from <https://www.iqvia.com/insights/the-iqvia-institute/reports/global-oncology-trends-2017-advances-complexity-and-cost>,
- Aksungur, P., Sungur, A., Unal, S., Iskit, A. B., Squier, C. A. & Senel, S. 2004. Chitosan delivery systems for the treatment of oral mucositis: in vitro and in vivo studies. *J Control Release*, 98(2), pp 269-79.
- Al-Ansari, S., Zecha, J. A., Barasch, A., de Lange, J., Rozema, F. R. & Raber-Durlacher, J. E. 2015. Oral Mucositis Induced By Anticancer Therapies. *Curr Oral Health Rep*, 2(4), pp 202-211.
- Al-Asmari, A. K., Khan, A. Q., Al-Qasim, A. M. & Al-Yousef, Y. 2015. Ascorbic acid attenuates antineoplastic drug 5-fluorouracil induced gastrointestinal toxicity in rats by modulating the expression of inflammatory mediators. *Toxicology Reports*, 2(908-916).
- Al-Assaf, S., Phillips, G. O., Deeble, D., Parsons, B., Starnes, H. & Von Sonntag, C. 1995. The enhanced stability of the cross-linked hylan structure to hydroxyl (OH) radicals compared with the uncross-linked hyaluronan. *Radiation Physics and Chemistry*, 46(2), pp 207-217.
- Al-Dasooqi, N., Bowen, J. M., Gibson, R. J., Logan, R. M., Stringer, A. M. & Keefe, D. M. 2011. Irinotecan-induced alterations in intestinal cell kinetics and extracellular matrix component expression in the dark agouti rat. *International Journal of Experimental Pathology*, 92(5), pp 357-365.
- Al-Dasooqi, N., Gibson, R. J., Bowen, J. M., Logan, R. M., Stringer, A. M. & Keefe, D. M. 2010. Matrix metalloproteinases are possible mediators for the development of alimentary tract mucositis in the dark agouti rat. *Experimental Biology and Medicine*, 235(10), pp 1244-1256.
- Al-Dasooqi, N., Sonis, S. T., Bowen, J. M., Bateman, E., Blijlevens, N., Gibson, R. J., Logan, R. M., Nair, R. G., Stringer, A. M., Yazbeck, R., Elad, S., Lalla, R. V. & Mucositis Study Group of Multinational Association of Supportive Care in Cancer/International Society of Oral, O. 2013. Emerging evidence on the pathobiology of mucositis. *Support Care Cancer*, 21(7), pp 2075-83.

- Al-Rudayni, A. H. M., Gopinath, D., Maharajan, M. K., Veettil, S. K. & Menon, R. K. 2021. Efficacy of Oral Cryotherapy in the Prevention of Oral Mucositis Associated with Cancer Chemotherapy: Systematic Review with Meta-Analysis and Trial Sequential Analysis. *Curr Oncol*, 28(4), pp 2852-2867.
- Al-Taie, A., Al-Shohani, A. D., Albasry, Z. & Altaee, A. 2020. Current topical trends and novel therapeutic approaches and delivery systems for oral mucositis management. *J Pharm Bioallied Sci*, 12(2), pp 94-101.
- Al-Tikriti, U., Martin, M. V. & Bramley, P. A. 1984. A pilot study of the clinical effects of irradiation on the oral tissues. *British Journal of Oral and Maxillofacial Surgery*, 22(2), pp 77-86.
- Alexander, J. L., Wilson, I. D., Teare, J., Marchesi, J. R., Nicholson, J. K. & Kinross, J. M. 2017. Gut microbiota modulation of chemotherapy efficacy and toxicity. *Nat Rev Gastroenterol Hepatol*, 14(6), pp 356-365.
- Alho, A. M. & Underhill, C. B. 1989. The hyaluronate receptor is preferentially expressed on proliferating epithelial cells. *J Cell Biol*, 108(4), pp 1557-65.
- Allen, G., Logan, R., Revesz, T., Keefe, D. & Gue, S. 2018. The Prevalence and Investigation of Risk Factors of Oral Mucositis in a Pediatric Oncology Inpatient Population; a Prospective Study. *Journal of Pediatric Hematology/Oncology*, 40(1), pp 15-21.
- Allison, D. D. & Grande-Allen, K. J. 2006. Review. Hyaluronan: a powerful tissue engineering tool. *Tissue Eng*, 12(8), pp 2131-40.
- Allison, R. R., Ambrad, A. A., Arshoun, Y., Carmel, R. J., Ciuba, D. F., Feldman, E., Finkelstein, S. E., Gandhavadi, R., Heron, D. E., Lane, S. C., Longo, J. M., Meakin, C., Papadopoulos, D., Pruitt, D. E., Steinbrenner, L. M., Taylor, M. A., Wisbeck, W. M., Yuh, G. E., Nowotnik, D. P. & Sonis, S. T. 2014. Multi-institutional, randomized, double-blind, placebo-controlled trial to assess the efficacy of a mucoadhesive hydrogel (MuGard) in mitigating oral mucositis symptoms in patients being treated with chemoradiation therapy for cancers of the head and neck. *Cancer*, 120(9), pp 1433-40.
- Almond, A. 2007. Hyaluronan. *Cellular and Molecular Life Sciences*, 64(13), pp 1591-1596.
- Alongi, F., Bignardi, M., Garassino, I., Pentimalli, S., Cavina, R., Mancosu, P., Reggiori, G., Poletti, A., Ferrari, D. & Foa, P. 2012. Prospective phase II trial of cetuximab plus VMAT-SIB in locally advanced head and neck squamous cell carcinoma. *Strahlentherapie und Onkologie*, 188(1), pp 49-55.
- Alterio, D., Jereczek-Fossa, B. A., Fiore, M. R., Piperno, G., Ansarin, M. & Orecchia, R. 2007. Cancer treatment-induced oral mucositis. *Anticancer research*, 27(2), pp 1105-1125.

- Amarnath, L. P., Srinivas, A. & Ramamurthi, A. 2006. In vitro hemocompatibility testing of UV-modified hyaluronan hydrogels. *Biomaterials*, 27(8), pp 1416-1424.
- Ambrosio, L., Borzacchiello, A., Netti, P. & Nicolais, L. 1999. Rheological study on hyaluronic acid and its derivative solutions. *Journal of Macromolecular Science—Pure and Applied Chemistry*, 36(7-8), pp 991-1000.
- Amstutz, U., Henricks, L. M., Offer, S. M., Barbarino, J., Schellens, J. H. M., Swen, J. J., Klein, T. E., McLeod, H. L., Caudle, K. E., Diasio, R. B. & Schwab, M. 2018. Clinical Pharmacogenetics Implementation Consortium (CPIC) Guideline for Dihydropyrimidine Dehydrogenase Genotype and Fluoropyrimidine Dosing: 2017 Update. *Clin Pharmacol Ther*, 103(2), pp 210-216.
- Anderson, G., Ebadi, M., Vo, K., Novak, J., Govindarajan, A. & Amini, A. 2021. An Updated Review on Head and Neck Cancer Treatment with Radiation Therapy. *Cancers*, 13(19), pp 4912.
- Andrieu-Abadie, N. & Levade, T. 2002. Sphingomyelin hydrolysis during apoptosis. *Biochim Biophys Acta*, 1585(2-3), pp 126-34.
- Angel, P., Szabowski, A. & Schorpp-Kistner, M. 2001. Function and regulation of AP-1 subunits in skin physiology and pathology. *Oncogene*, 20(19), pp 2413-23.
- Antin, J. H., Lee, S. J., Neuberg, D., Alyea, E., Soiffer, R. J., Sonis, S. & Ferrara, J. L. 2002. A phase I/II double-blind, placebo-controlled study of recombinant human interleukin-11 for mucositis and acute GVHD prevention in allogeneic stem cell transplantation. *Bone Marrow Transplant*, 29(5), pp 373-7.
- Aprile, G., Rihawi, K., De Carlo, E. & Sonis, S. T. 2015. Treatment-related gastrointestinal toxicities and advanced colorectal or pancreatic cancer: a critical update. *World journal of gastroenterology*, 21(41), pp 11793.
- Argiris, A., Karamouzis, M. V., Raben, D. & Ferris, R. L. 2008. Head and neck cancer. *Lancet*, 371(9625), pp 1695-709.
- Ariyawardana, A., Cheng, K. K. F., Kandwal, A., Tilly, V., Al-Azri, A. R., Galiti, D., Chiang, K., Vaddi, A., Ranna, V., Nicolatou-Galitis, O., Lalla, R. V., Bossi, P. & Elad, S. 2019. Systematic review of anti-inflammatory agents for the management of oral mucositis in cancer patients and clinical practice guidelines. *Support Care Cancer*, 27(10), pp 3985-3995.
- Artis, D. & Spits, H. 2015. The biology of innate lymphoid cells. *Nature*, 517(7534), pp 293-301.

- Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B. & Seed, B. 1990. CD44 is the principal cell surface receptor for hyaluronate. *Cell*, 61(7), pp 1303-13.
- Asari, A. 2004. Chapter 21 - Medical Application of Hyaluronan. *In: Garg, H. G. & Hales, C. A. (eds.) Chemistry and Biology of Hyaluronan*. Oxford: Elsevier Science Ltd, pp 457-473.
- Asayama, S., Nogawa, M., Takei, Y., Akaike, T. & Maruyama, A. 1998. Synthesis of novel polyampholyte comb-type copolymers consisting of a poly(L-lysine) backbone and hyaluronic acid side chains for a DNA carrier. *Bioconjug Chem*, 9(4), pp 476-81.
- Atiq, A., Shal, B., Naveed, M., Khan, A., Ali, J., Zeeshan, S., Al-Sharari, S. D., Kim, Y. S. & Khan, S. 2019. Diadzein ameliorates 5-fluorouracil-induced intestinal mucositis by suppressing oxidative stress and inflammatory mediators in rodents. *Eur J Pharmacol*, 843(292-306).
- Aust, S. D., Morehouse, L. A. & Thomas, C. E. 1985. Role of metals in oxygen radical reactions. *Journal of free radicals in biology & medicine*, 1(1), pp 3-25.
- Bachour, P. C. & Sonis, S. T. 2018. Predicting mucositis risk associated with cytotoxic cancer treatment regimens: rationale, complexity, and challenges. *Curr Opin Support Palliat Care*, 12(2), pp 198-210.
- Bae, S., Lee, E.-J., Lee, J. H., Park, I.-C., Lee, S.-J., Hahn, H. J., Ahn, K. J., An, S., An, I.-S. & Cha, H. J. 2014. Oridonin protects HaCaT keratinocytes against hydrogen peroxide-induced oxidative stress by altering microRNA expression. *Int J Mol Med*, 33(1), pp 185-193.
- Bahri, R., Saidane-Mosbahi, D. & Rouabhia, M. 2010. Candida famata modulates toll-like receptor, beta-defensin, and proinflammatory cytokine expression by normal human epithelial cells. *J Cell Physiol*, 222(1), pp 209-18.
- Baker, D. G. 1982. The radiobiological basis for tissue reactions in the oral cavity following therapeutic x-irradiation: a review. *Archives of Otolaryngology*, 108(1), pp 21-24.
- Balazs, E. A., Watson, D., Duff, I. F. & Roseman, S. 1967. Hyaluronic acid in synovial fluid. I. Molecular parameters of hyaluronic acid in normal and arthritic human fluids. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, 10(4), pp 357-376.
- Balis, F. M., Holcenberg, J. & Poplack, D. 2002. General principles of chemotherapy. *Principles and practice of pediatric oncology*, 4(237-308).
- Balogh, G. T., Illés, J., Székely, Z., Forrai, E. & Gere, A. 2003. Effect of different metal ions on the oxidative damage and antioxidant capacity of hyaluronic acid. *Archives of Biochemistry and Biophysics*, 410(1), pp 76-82.

- Bamba, S., Andoh, A., Yasui, H., Araki, Y., Bamba, T. & Fujiyama, Y. 2003. Matrix metalloproteinase-3 secretion from human colonic subepithelial myofibroblasts: role of interleukin-17. *Journal of Gastroenterology*, 38(6), pp 548-554.
- Banerji, S., Wright, A. J., Noble, M., Mahoney, D. J., Campbell, I. D., Day, A. J. & Jackson, D. G. 2007. Structures of the Cd44–hyaluronan complex provide insight into a fundamental carbohydrate-protein interaction. *Nature structural & molecular biology*, 14(3), pp 234-239.
- Bankhead, P., Loughrey, M. B., Fernández, J. A., Dombrowski, Y., McArt, D. G., Dunne, P. D., McQuaid, S., Gray, R. T., Murray, L. J. & Coleman, H. G. 2017. QuPath: Open source software for digital pathology image analysis. *Scientific reports*, 7(1), pp 1-7.
- Bansil, R. & Turner, B. S. 2006. Mucin structure, aggregation, physiological functions and biomedical applications. *Current Opinion in Colloid & Interface Science*, 11(2), pp 164-170.
- Banzato, A., Bobisse, S., Rondina, M., Renier, D., Bettella, F., Esposito, G., Quintieri, L., Meléndez-Alafort, L., Mazzi, U. & Zanovello, P. 2008. A paclitaxel-hyaluronan bioconjugate targeting ovarian cancer affords a potent in vivo therapeutic activity. *Clinical cancer research*, 14(11), pp 3598-3606.
- Barasch, A., Elad, S., Altman, A., Damato, K. & Epstein, J. 2006. Antimicrobials, mucosal coating agents, anesthetics, analgesics, and nutritional supplements for alimentary tract mucositis. *Supportive Care in Cancer*, 14(6), pp 528-532.
- Barasch, A., Epstein, J. & Tilashalski, K. 2009. Palifermin for management of treatment-induced oral mucositis in cancer patients. *Biologics*, 3(111-6).
- Barasch, A. & Peterson, D. E. 2003. Risk factors for ulcerative oral mucositis in cancer patients: unanswered questions. *Oral Oncology*, 39(2), pp 91-100.
- Barasch, A., Peterson, D. E., Tanzer, J. M., D'Ambrosio, J. A., Nuki, K., Schubert, M. M., Franquin, J. C., Clive, J. & Tutschka, P. 1995. Helium-neon laser effects on conditioning-induced oral mucositis in bone marrow transplantation patients. *Cancer*, 76(12), pp 2550-2556.
- Barbucci, R., Lamponi, S., Borzacchiello, A., Ambrosio, L., Fini, M., Torricelli, P. & Giardino, R. 2002. Hyaluronic acid hydrogel in the treatment of osteoarthritis. *Biomaterials*, 23(23), pp 4503-13.
- Bardaweel, S. K., Abu-Dahab, R. & Almomani, N. F. 2013. An in vitro based investigation into the cytotoxic effects of D-amino acids. *Acta Pharmaceutica*, 63(4), pp 467-478.

- Basile, D., Di Nardo, P., Corvaja, C., Garattini, S. K., Pelizzari, G., Lisanti, C., Bortot, L., Da Ros, L., Bartoletti, M., Borghi, M., Gerratana, L., Lombardi, D. & Puglisi, F. 2019. Mucosal Injury during Anti-Cancer Treatment: From Pathobiology to Bedside. *Cancers*, 11(6), pp 857.
- Beg, A. A., Finco, T. S., Nantermet, P. V. & Baldwin, A. S., Jr. 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Molecular And Cellular Biology*, 13(6), pp 3301-3310.
- Beighton, D., Carr, A. D. & Oppenheim, B. A. 1994. Identification of viridans streptococci associated with bacteraemia in neutropenic cancer patients. *Journal of Medical Microbiology*, 40(3), pp 202-204.
- Bell, S., Howard, A., Wilson, J., Abbot, E., Smith, W., Townes, C., Hirst, B. & Hall, J. 2012. Streptococcus pyogenes infection of tonsil explants is associated with a human β -defensin 1 response from control but not recurrent acute tonsillitis patients. *Molecular oral microbiology*, 27(3), pp 160-171.
- Bellm, L. A., Epstein, J. B., Rose-Ped, A., Martin, P. & Fuchs, H. J. 2000. Patient reports of complications of bone marrow transplantation. *Supportive Care in Cancer*, 8(1), pp 33-39.
- Beneš, L., Ďuračková, Z. & Ferencik, M. 1999. Chemistry, physiology and pathology of free radicals. *Life sciences*, 65(18-19), pp 1865-1874.
- Bensadoun, R.-J. & Ciais, G. 2002. Radiation- and Chemotherapy-induced Mucositis in Oncology: Results of Multicenter Phase III Studies. *Journal of Oral Laser Applications*, 2(2), pp 115.
- Bensinger, W., Schubert, M., Ang, K.-K., Brizel, D., Brown, E., Eilers, J. G., Elting, L., Mittal, B. B., Schattner, M. A. & Spielberger, R. 2008. NCCN task force report: prevention and management of mucositis in cancer care. *Journal of the National Comprehensive Cancer Network*, 6(S1), pp S-1-S-21.
- Benson, A. B., 3rd, Ajani, J. A., Catalano, R. B., Engelking, C., Kornblau, S. M., Martenson, J. A., Jr., McCallum, R., Mitchell, E. P., O'Dorisio, T. M., Vokes, E. E. & Wadler, S. 2004. Recommended guidelines for the treatment of cancer treatment-induced diarrhea. *J Clin Oncol*, 22(14), pp 2918-26.
- Bentzen, S. M., Saunders, M. I., Dische, S. & Bond, S. J. 2001. Radiotherapy-related early morbidity in head and neck cancer: quantitative clinical radiobiology as deduced from the CHART trial. *Radiotherapy and Oncology*, 60(2), pp 123-135.
- Bergman, K., Elvingson, C., Hilborn, J., Svensk, G. & Bowden, T. 2007. Hyaluronic acid derivatives prepared in aqueous media by triazine-activated amidation. *Biomacromolecules*, 8(7), pp 2190-2195.

- Bernier-Latmani, J. & Petrova, T. V. 2017. Intestinal lymphatic vasculature: structure, mechanisms and functions. *Nature Reviews Gastroenterology & Hepatology*, 14(9), pp 510-526.
- Bertolini, M., Sobue, T., Thompson, A. & Dongari-Bagtzoglou, A. 2017. Chemotherapy induces oral mucositis in mice without additional noxious stimuli. *Translational oncology*, 10(4), pp 612-620.
- Bhanja, P., Saha, S., Kabarriti, R., Liu, L., Roy-Chowdhury, N., Roy-Chowdhury, J., Sellers, R. S., Alfieri, A. A. & Guha, C. 2009. Protective role of R-spondin1, an intestinal stem cell growth factor, against radiation-induced gastrointestinal syndrome in mice. *PloS one*, 4(11), pp e8014.
- Bhattacharyya, A., Chattopadhyay, R., Mitra, S. & Crowe, S. E. 2014. Oxidative Stress: An Essential Factor in the Pathogenesis of Gastrointestinal Mucosal Diseases. *Physiological Reviews*, 94(2), pp 329-354.
- Bialkowska, A. B., Ghaleb, A. M., Nandan, M. O. & Yang, V. W. 2016. Improved Swiss-rolling technique for intestinal tissue preparation for immunohistochemical and immunofluorescent analyses. *JoVE (Journal of Visualized Experiments)*, 113, pp e54161.
- Bian, L., Han, G., Zhao, C. W., Garl, P. J. & Wang, X. J. 2015. The role of Smad7 in oral mucositis. *Protein Cell*, 6(3), pp 160-9.
- Bierbaumer, L., Schwarze, U. Y., Gruber, R. & Neuhaus, W. 2018. Cell culture models of oral mucosal barriers: A review with a focus on applications, culture conditions and barrier properties. *Tissue Barriers*, 6(3), pp 1479568.
- Biju, P. G., Garg, S., Wang, W., Choudhry, M. A., Kovacs, E. J., Fink, L. M. & Hauer-Jensen, M. 2012. Procalcitonin as a predictive biomarker for total body irradiation-induced bacterial load and lethality in mice. *Shock*, 38(2), pp 170-6.
- Billis, W., Fuks, Z. & Kolesnick, R. 1998. Signaling in and regulation of ionizing radiation-induced apoptosis in endothelial cells. *Recent progress in hormone research*, 53(85-92; discussion 93).
- Biswal, B. M. 2008. Current trends in the management of oral mucositis related to cancer treatment. *The Malaysian journal of medical sciences : MJMS*, 15(3), pp 4-13.
- Blijlevens, N., Schwenkglenks, M., Bacon, P., D'Addio, A., Einsele, H., Maertens, J., Niederwieser, D., Rabitsch, W., Roosaar, A. & Ruutu, T. 2008. Prospective oral mucositis audit: oral mucositis in patients receiving high-dose melphalan or BEAM conditioning chemotherapy—European Blood and Marrow Transplantation Mucositis Advisory Group. *Journal of clinical oncology*, 26(9), pp 1519-1525.

- Block, K. I., Koch, A. C., Mead, M. N., Tothy, P. K., Newman, R. A. & Gyllenhaal, C. 2007. Impact of antioxidant supplementation on chemotherapeutic efficacy: a systematic review of the evidence from randomized controlled trials. *Cancer treatment reviews*, 33(5), pp 407-418.
- Bloomer, W. D. & Hellman, S. 1975. Normal tissue responses to radiation therapy. *New England Journal of Medicine*, 293(2), pp 80-83.
- Blumenthal, R. D. 2005. An overview of chemosensitivity testing. *Chemosensitivity: Volume 1 In Vitro Assays*, 3-18.
- Bockel, S., Vallard, A., Levy, A., Francois, S., Bourdis, M., Le Gallic, C., Riccobono, D., Annede, P., Drouet, M. & Tao, Y. 2018. Pharmacological modulation of radiation-induced oral mucosal complications. *Cancer/Radiothérapie*, 22(5), pp 429-437.
- Bolwell, B. J., Kalaycio, M., Sobecks, R., Andresen, S., Kuczkowski, E., Bernhard, L., Lomax, R., Kohuth, J., Mendiola, S., Rybicki, L. & Pohlman, B. 2002. A multivariable analysis of factors influencing mucositis after autologous progenitor cell transplantation. *Bone Marrow Transplantation*, 30(9), pp 587-591.
- Bonnaure-Mallet, M., Bunetel, L., Tricot-Doleux, S., Guérin, J., Bergeron, C. & LeGall, E. 1998. Oral complications during treatment of malignant diseases in childhood: effects of tooth brushing. *Eur J Cancer*, 34(10), pp 1588-91.
- Bonner, J. A., Harari, P. M., Giralt, J., Azarnia, N., Shin, D. M., Cohen, R. B., Jones, C. U., Sur, R., Raben, D., Jassem, J., Ove, R., Kies, M. S., Baselga, J., Yousoufian, H., Amellal, N., Rowinsky, E. K. & Ang, K. K. 2006. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med*, 354(6), pp 567-78.
- Bonner, J. A., Harari, P. M., Giralt, J., Cohen, R. B., Jones, C. U., Sur, R. K., Raben, D., Baselga, J., Spencer, S. A., Zhu, J., Yousoufian, H., Rowinsky, E. K. & Ang, K. K. 2010. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *Lancet Oncol*, 11(1), pp 21-8.
- Borges, L., Rex, K. L., Chen, J. N., Wei, P., Kaufman, S., Scully, S., Pretorius, J. K. & Farrell, C. L. 2006. A protective role for keratinocyte growth factor in a murine model of chemotherapy and radiotherapy-induced mucositis. *International Journal of Radiation Oncology*Biophysics*, 66(1), pp 254-262.
- Borzacchiello, A., Mayol, L., Schiavinato, A. & Ambrosio, L. 2010. Effect of hyaluronic acid amide derivative on equine synovial fluid viscoelasticity. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, 92(3), pp 1162-1170.

- Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z. & Kolesnick, R. 1995. Ceramide synthase mediates daunorubicin-induced apoptosis: An alternative mechanism for generating death signals. *Cell*, 82(3), pp 405-414.
- Bossi, P., Lucchesi, M. & Antonuzzo, A. 2015. Gastrointestinal toxicities from targeted therapies: measurement, duration and impact. *Curr Opin Support Palliat Care*, 9(2), pp 163-7.
- Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A. & Fusenig, N. E. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *The Journal of cell biology*, 106(3), pp 761-771.
- Boussemart, L., Routier, E., Mateus, C., Opletalova, K., Seville, G., Kamsu-Kom, N., Thomas, M., Vagner, S., Favre, M., Tomasic, G., Wechsler, J., Lacroix, L. & Robert, C. 2013. Prospective study of cutaneous side-effects associated with the BRAF inhibitor vemurafenib: a study of 42 patients. *Ann Oncol*, 24(6), pp 1691-7.
- Bowen, J., Al-Dasooqi, N., Bossi, P., Wardill, H., Van Seville, Y., Al-Azri, A., Bateman, E., Correa, M., Raber-Durlacher, J. & Kandwal, A. 2019. The pathogenesis of mucositis: updated perspectives and emerging targets. *Supportive Care in Cancer*, 27(10), pp 4023-4033.
- Bowen, J. M., Gibson, R. J., Keefe, D. M. & Cummins, A. G. 2005. Cytotoxic chemotherapy upregulates pro-apoptotic Bax and Bak in the small intestine of rats and humans. *Pathology*, 37(1), pp 56-62.
- Bowie, A. & O'Neill, L. A. 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *Journal of Leukocyte Biology*, 67(4), pp 508-14.
- Brach, M. A., Hass, R., Sherman, M. L., Gunji, H., Weichselbaum, R. & Kufe, D. 1991. Ionizing radiation induces expression and binding activity of the nuclear factor kappa B. *Journal of Clinical Investigation*, 88(2), pp 691-695.
- Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A. & Jemal, A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, 68(6), pp 394-424.
- Brizuela, M. & Winters, R. 2021. Oral Mucosa. 2021 Jun 9. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan—. PMID: 34283481.
- Brown, C. G. & Wingard, J. 2004. Clinical consequences of oral mucositis. *Semin Oncol Nurs*, 20(1), pp 16-21.

- Brown, M. F., Leibowitz, B. J., Chen, D., He, K., Zou, F., Sobol, R. W., Beer-Stolz, D., Zhang, L. & Yu, J. 2015. Loss of Caspase-3 sensitizes colon cancer cells to genotoxic stress via RIP1-dependent necrosis. *Cell Death & Disease*, 6(e1729).
- Buchanan, E. P., Longaker, M. T. & Lorenz, H. P. 2009. Fetal skin wound healing. *Advances in clinical chemistry*, 48(137-161).
- Buchsel, P. C. 2003. Gelclair oral gel. *Clinical Journal Of Oncology Nursing*, 7(1), pp 109-110.
- Buchsel, P. C. 2008. Polyvinylpyrrolidone-sodium hyaluronate gel (Gelclair): a bioadherent oral gel for the treatment of oral mucositis and other painful oral lesions. *Expert Opin Drug Metab Toxicol*, 4(11), pp 1449-54.
- Bulpitt, P. & Aeschlimann, D. 1999. New strategy for chemical modification of hyaluronic acid: preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels. *J Biomed Mater Res*, 47(2), pp 152-69.
- Burdelya, L. G., Gleiberman, A. S., Toshkov, I., Aygun-Sunar, S., Bapardekar, M., Manderscheid-Kern, P., Bellnier, D., Krivokrysenko, V. I., Feinstein, E. & Gudkov, A. V. 2012. Toll-like receptor 5 agonist protects mice from dermatitis and oral mucositis caused by local radiation: implications for head-and-neck cancer radiotherapy. *Int J Radiat Oncol Biol Phys*, 83(1), pp 228-34.
- Burdick, J. A. & Prestwich, G. D. 2011. Hyaluronic acid hydrogels for biomedical applications. *Advanced materials*, 23(12), pp H41-H56.
- Burgoyne, J. R., Oka, S.-i., Ale-Agha, N. & Eaton, P. 2012. Hydrogen Peroxide Sensing and Signaling by Protein Kinases in the Cardiovascular System. *Antioxidants & Redox Signaling*, 18(9), pp 1042-1052.
- Çakmak, S. & Nural, N. 2019. Incidence of and risk factors for development of oral mucositis in outpatients undergoing cancer chemotherapy. *International Journal of Nursing Practice*, 25(1), pp e12710.
- Caluwaerts, S., Vandenbroucke, K., Steidler, L., Neirynck, S., Vanhoenacker, P., Corveleyn, S., Watkins, B., Sonis, S., Coulie, B. & Rottiers, P. 2010. AG013, a mouth rinse formulation of *Lactococcus lactis* secreting human Trefoil Factor 1, provides a safe and efficacious therapeutic tool for treating oral mucositis. *Oral Oncology*, 46(7), pp 564-570.
- Campo, G., Avenoso, A., Campo, S., D'Ascola, A., Traina, P. & Calatroni, A. 2008a. Chondroitin-4-sulphate inhibits NF- κ B translocation and caspase activation in collagen-induced arthritis in mice. *Osteoarthritis and cartilage*, 16(12), pp 1474-1483.

- Campo, G. M., Avenoso, A., Campo, S., D'Ascola, A., Ferlazzo, A. M. & Calatroni, A. 2004a. Reduction of DNA fragmentation and hydroxyl radical production by hyaluronic acid and chondroitin-4-sulphate in iron plus ascorbate-induced oxidative stress in fibroblast cultures. *Free radical research*, 38(6), pp 601-611.
- Campo, G. M., Avenoso, A., Campo, S., D'Ascola, A., Traina, P., Samà, D. & Calatroni, A. 2008b. NF-kB and caspases are involved in the hyaluronan and chondroitin-4-sulphate-exerted antioxidant effect in fibroblast cultures exposed to oxidative stress. *Journal of Applied Toxicology: An International Journal*, 28(4), pp 509-517.
- Campo, G. M., Avenoso, A., Campo, S., D'Ascola, A., Ferlazzo, A. M. & Calatroni, A. 2004b. The antioxidant and antifibrogenic effects of the glycosaminoglycans hyaluronic acid and chondroitin-4-sulphate in a subchronic rat model of carbon tetrachloride-induced liver fibrogenesis. *Chemico-biological interactions*, 148(3), pp 125-138.
- Campo, G. M., Avenoso, A., Campo, S., D'Ascola, A., Nastasi, G. & Calatroni, A. 2010. Small hyaluronan oligosaccharides induce inflammation by engaging both toll-like-4 and CD44 receptors in human chondrocytes. *Biochemical Pharmacology*, 80(4), pp 480-490.
- Campoccia, D., Doherty, P., Radice, M., Brun, P., Abatangelo, G. & Williams, D. F. 1998. Semisynthetic resorbable materials from hyaluronan esterification. *Biomaterials*, 19(23), pp 2101-2127.
- Campos, M. I., Campos, C. N., Aarestrup, F. M. & Aarestrup, B. J. 2014. Oral mucositis in cancer treatment: Natural history, prevention and treatment. *Mol Clin Oncol*, 2(3), pp 337-340.
- Cao, Z., Zhang, Z., Huang, Z., Wang, R., Yang, A., Liao, L. & Du, J. 2014. Antitumor and immunomodulatory effects of low-dose 5-FU on hepatoma 22 tumor-bearing mice. *Oncol Lett*, 7(4), pp 1260-1264.
- Cario, E. 2016. Toll-like receptors in the pathogenesis of chemotherapy-induced gastrointestinal toxicity. *Current Opinion in Supportive and Palliative Care*, 10(2), pp 157-164.
- Carlotto, A., Hogsett, V. L., Maiorini, E. M., Razulis, J. G. & Sonis, S. T. 2013. The economic burden of toxicities associated with cancer treatment: review of the literature and analysis of nausea and vomiting, diarrhoea, oral mucositis and fatigue. *Pharmacoeconomics*, 31(9), pp 753-66.
- Carrard, V. C., Pires, A. S., Badauy, C. M., Rados, P. V., Lauxen, I. S. & Sant'Ana Filho, M. 2008. Effects of aging on mouse tongue epithelium focusing on cell proliferation rate and morphological aspects. *The Bulletin of Tokyo Dental College*, 49(4), pp 199-205.
- Cawley, M. M. & Benson, L. M. 2005. Current trends in managing oral mucositis. *Clinical journal of oncology nursing*, 9(5), pp.

- Celentano, A., McCullough, M. & Cirillo, N. 2019. Glucocorticoids reduce chemotherapeutic effectiveness on OSCC cells via glucose-dependent mechanisms. *J Cell Physiol*, 234(3), pp 2013-2020.
- Celentano, A., Yiannis, C., Paolini, R., Zhang, P., Farah, C. S., Cirillo, N., Yap, T. & McCullough, M. 2020. Kava constituents exert selective anticancer effects in oral squamous cell carcinoma cells in vitro. *Scientific Reports*, 10(1), pp 1-11.
- Chang, C.-T., Hsiang, C.-Y., Ho, T.-Y., Wu, C.-Z., Hong, H.-H. & Huang, Y.-F. 2015. Comprehensive Assessment of Host Responses to 5-Fluorouracil-Induced Oral Mucositis through Transcriptomic Analysis. *PLOS ONE*, 10(8), pp e0135102.
- Chansky, K., Benedetti, J. & Macdonald, J. S. 2005. Differences in toxicity between men and women treated with 5-fluorouracil therapy for colorectal carcinoma. *Cancer*, 103(6), pp 1165-1171.
- Chapko, M. K., Syrjala, K. L., Bush, N., Jedlow, C. & Yanke, M. R. 1991. Development of a behavioral measure of mouth pain, nausea, and wellness for patients receiving radiation and chemotherapy. *Journal of pain and symptom management*, 6(1), pp 15-23.
- Chaudhry, H. M., Bruce, A. J., Wolf, R. C., Litzow, M. R., Hogan, W. J., Patnaik, M. S., Kremers, W. K., Phillips, G. L. & Hashmi, S. K. 2016. The Incidence and Severity of Oral Mucositis among Allogeneic Hematopoietic Stem Cell Transplantation Patients: A Systematic Review. *Biology of Blood and Marrow Transplantation*, 22(4), pp 605-616.
- Chaveli-Lopez, B. & Bagan-Sebastian, J. V. 2016. Treatment of oral mucositis due to chemotherapy. *J Clin Exp Dent*, 8(2), pp e201-9.
- Chen, C., Tian, L., Zhang, M., Sun, Q., Zhang, X., Li, X., Cao, X., Liu, Q., Li, X. & Hao, L. 2013. Protective effect of amifostine on high-dose methotrexate-induced small intestinal mucositis in mice. *Digestive diseases and sciences*, 58(11), pp 3134-3143.
- Chen, C., Zhang, Q., Yu, W., Chang, B. & Le, A. D. 2020. Oral Mucositis: An Update on Innate Immunity and New Interventional Targets. *Journal of Dental Research*, 99(10), pp 1122-1130.
- Chen, C. F., Wang, R. H., Cheng, S. N. & Chang, Y. C. 2004. Assessment of chemotherapy-induced oral complications in children with cancer. *J Pediatr Oncol Nurs*, 21(1), pp 33-9.
- Chen, H.-M. 2008. Patients' experiences and perceptions of chemotherapy-induced oral mucositis in a day unit. *Cancer Nursing*, 31(5), pp 363-369.
- Chen, H., Wu, M., Li, G., Hua, L., Chen, S. & Huang, H. 2017. Association between XRCC1 single-nucleotide polymorphism and acute radiation reaction in patients with nasopharyngeal carcinoma: A cohort study. *Medicine (Baltimore)*, 96(44), pp e8202.

- Chen, J., Zhang, T., Jiang, B., Mu, W. & Miao, M. 2012. Characterization and antioxidant activity of Ginkgo biloba exocarp polysaccharides. *Carbohydrate Polymers*, 87(1), pp 40-45.
- Chen, P., Lingen, M., Sonis, S. T., Walsh-Reitz, M. M. & Toback, F. G. 2011. Role of AMP-18 in oral mucositis. *Oral oncology*, 47(9), pp 831-839.
- Chen, P., Mancini, M., Sonis, S. T., Fernandez-Martinez, J., Liu, J., Cohen, E. E. & Toback, F. G. 2016. A Novel Peptide for Simultaneously Enhanced Treatment of Head and Neck Cancer and Mitigation of Oral Mucositis. *PLoS One*, 11(4), pp e0152995.
- Chen, W. Y. & Abatangelo, G. 1999. Functions of hyaluronan in wound repair. *Wound Repair Regen*, 7(2), pp 79-89.
- Chen, Y., Peng, F., Song, X., Wu, J., Yao, W. & Gao, X. 2018. Conjugation of paclitaxel to C-6 hexanediamine-modified hyaluronic acid for targeted drug delivery to enhance antitumor efficacy. *Carbohydrate polymers*, 181(150-158).
- Chen, Y., Williams, J., Ding, I., Hernady, E., Liu, W., Smudzin, T., Finkelstein, J. N., Rubin, P. & Okunieff, P. 2002. Radiation pneumonitis and early circulatory cytokine markers. *Semin Radiat Oncol*, 12(1 Suppl 1), pp 26-33.
- Cheng, A., Wan, F., Jin, Z., Wang, J. & Xu, X. 2008. Nitrite oxide and inducible nitric oxide synthase were regulated by polysaccharides isolated from Glycyrrhiza uralensis Fisch. *Journal of ethnopharmacology*, 118(1), pp 59-64.
- Cheng, K., Molassiotis, A., Chang, A., Wai, W. & Cheung, S. 2001. Evaluation of an oral care protocol intervention in the prevention of chemotherapy-induced oral mucositis in paediatric cancer patients. *European journal of cancer*, 37(16), pp 2056-2063.
- Cheng, K. K.-F., Leung, S. F., Liang, R. H. S., Tai, J. W. M., Yeung, R. M. W. & Thompson, D. R. 2010. Severe oral mucositis associated with cancer therapy: impact on oral functional status and quality of life. *Supportive Care in Cancer*, 18(11), pp 1477-1485.
- Cheng, K. K. F., Lee, V., Li, C. H., Yuen, H. L. & Epstein, J. B. 2012. Oral mucositis in pediatric and adolescent patients undergoing chemotherapy: the impact of symptoms on quality of life. *Supportive Care in Cancer*, 20(10), pp 2335-2342.
- Chernos, M., Grecov, D., Kwok, E., Bebe, S., Babsola, O. & Anastassiades, T. 2017. Rheological study of hyaluronic acid derivatives. *Biomedical engineering letters*, 7(1), pp 17-24.
- Cheung-Ong, K., Giaever, G. & Nislow, C. 2013. DNA-damaging agents in cancer chemotherapy: serendipity and chemical biology. *Chem Biol*, 20(5), pp 648-59.

- Cheung, W. F., Cruz, T. F. & Turley, E. A. 1999. Receptor for hyaluronan-mediated motility (RHAMM), a hyaladherin that regulates cell responses to growth factors. *Biochem Soc Trans*, 27(2), pp 135-42.
- Cho, H.-J., Park, Y. S., Kang, W. K., Kim, J.-W. & Lee, S.-Y. 2007. Thymidylate Synthase (TYMS) and Dihydropyrimidine Dehydrogenase (DPYD) Polymorphisms in the Korean Population for Prediction of 5-Fluorouracil-Associated Toxicity. *Therapeutic Drug Monitoring*, 29(2), pp.
- Chrzanowski, K., Bielawska, A., Bielawski, K., Wołczyński, S. & Pałka, J. 2001. Cytotoxicity and effect on collagen biosynthesis of proline analogue of melphalan as a prolidase-convertible prodrug in cultured human skin fibroblasts. *Il Farmaco*, 56(9), pp 701-706.
- Chun, C., Lee, D. Y., Kim, J.-T., Kwon, M.-K., Kim, Y.-Z. & Kim, S.-S. 2016. Effect of molecular weight of hyaluronic acid (HA) on viscoelasticity and particle texturing feel of HA dermal biphasic fillers. *Biomaterials Research*, 20(1), pp 1-7.
- Cinausero, M., Aprile, G., Ermacora, P., Basile, D., Vitale, M. G., Fanotto, V., Parisi, G., Calvetti, L. & Sonis, S. T. 2017. New Frontiers in the Pathobiology and Treatment of Cancer Regimen-Related Mucosal Injury. *Frontiers in Pharmacology*, 8(354), pp.
- Cirillo, N., Vicidomini, A., McCullough, M., Gambardella, A., Hassona, Y., Prime, S. S. & Colella, G. 2015. A Hyaluronic Acid-Based Compound Inhibits Fibroblast Senescence Induced by Oxidative Stress In Vitro and Prevents Oral Mucositis In Vivo. *Journal of Cellular Physiology*, 230(7), pp 1421-1429.
- Clarkson, J. E., Worthington, H. V., Furness, S., McCabe, M., Khalid, T. & Meyer, S. 2010. Interventions for treating oral mucositis for patients with cancer receiving treatment. *Cochrane Database Syst Rev*, 8), pp CD001973.
- Cohen, E. E. W., Ahmed, O., Kocherginsky, M., Shustakova, G., Kistner-Griffin, E., Salama, J. K., Yefremenko, V. & Novosad, V. 2013. Study of functional infrared imaging for early detection of mucositis in locally advanced head and neck cancer treated with chemoradiotherapy. *Oral Oncology*, 49(10), pp 1025-1031.
- Cohen, S., Carpenter, G. & King, L. 1980. Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. *Journal of Biological Chemistry*, 255(10), pp 4834-4842.
- Colella, G., Cannavale, R., Vicidomini, A., Rinaldi, G., Compilato, D. & Campisi, G. 2010. Efficacy of a Spray Compound Containing a Pool of Collagen Precursor Synthetic Amino Acids (L-Proline, L-Leucine, L-Lysine and Glycine) Combined with Sodium Hyaluronate to Manage Chemo/Radiotherapy-Induced Oral Mucositis: Preliminary Data of an Open Clinical Trial. *International journal of immunopathology and pharmacology*, 23(1), pp 143-151.

- Colella, G., Vicidomini, A., Soro, V., Lanza, A. & Cirillo, N. 2012. Molecular insights into the effects of sodium hyaluronate preparations in keratinocytes. *Clinical and experimental dermatology*, 37(5), pp 516-520.
- Collins, L. K., Chapman, M. S., Carter, J. B. & Samie, F. H. 2017. Cutaneous adverse effects of the immune checkpoint inhibitors. *Curr Probl Cancer*, 41(2), pp 125-128.
- Collins, L. M. C. & Dawes, C. 1987. The Surface Area of the Adult Human Mouth and Thickness of the Salivary Film Covering the Teeth and Oral Mucosa. *Journal of Dental Research*, 66(8), pp 1300-1302.
- Collins, M. N. & Birkinshaw, C. 2013. Hyaluronic acid based scaffolds for tissue engineering—A review. *Carbohydrate polymers*, 92(2), pp 1262-1279.
- Conner, E. M. & Grisham, M. B. 1996. Inflammation, free radicals, and antioxidants. *Nutrition*, 12(4), pp 274-277.
- Cool, J. C., Dyer, J. L., Xian, C. J., Butler, R. N., Geier, M. S. & Howarth, G. S. 2005. Pre-treatment with insulin-like growth factor-I partially ameliorates 5-fluorouracil-induced intestinal mucositis in rats. *Growth hormone & IGF research*, 15(1), pp 72-82.
- Coracin, F. L., Santos, P. S. d. S., Gallottini, M. H., Saboya, R., Musqueira, P. T., Barban, A., Chamone, D. d. A. F., Dulley, F. L. & Nunes, F. D. 2013. Oral health as a predictive factor for oral mucositis. *Clinics*, 68(792-796).
- Correa, M. E. P., Cheng, K. K. F., Chiang, K., Kandwal, A., Loprinzi, C. L., Mori, T., Potting, C., Rouleau, T., Toro, J. J., Ranna, V., Vaddi, A., Peterson, D. E., Bossi, P., Lalla, R. V. & Elad, S. 2020. Systematic review of oral cryotherapy for the management of oral mucositis in cancer patients and clinical practice guidelines. *Support Care Cancer*, 28(5), pp 2449-2456.
- Cortivo, R., Brun, P., Cardarelli, L., O'Regan, M., Radice, M. & Abatangelo, G. Antioxidant effects of hyaluronan and its α -methyl-prednisolone derivative in chondrocyte and cartilage cultures. *Seminars in arthritis and rheumatism*, 1996. Elsevier, 492-501.
- Cowman, M. K., Lee, H.-G., Schwertfeger, K. L., McCarthy, J. B. & Turley, E. A. 2015. The content and size of hyaluronan in biological fluids and tissues. *Frontiers in immunology*, 6(261).
- Cowman, M. K. & Matsuoka, S. 2005. Experimental approaches to hyaluronan structure. *Carbohydrate research*, 340(5), pp 791-809.

- Cox, J. D., Stetz, J. & Pajak, T. F. 1995. Toxicity criteria of the Radiation Therapy Oncology Group (RTOG) and the European Organization for Research and Treatment of Cancer (EORTC). *Int J Radiat Oncol Biol Phys*, 31(5), pp 1341-6.
- Criswell, T., Leskov, K., Miyamoto, S., Luo, G. & Boothman, D. A. 2003. Transcription factors activated in mammalian cells after clinically relevant doses of ionizing radiation. *Oncogene*, 22(37), pp 5813-5827.
- Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M. & Harman, D. 1987. Oxygen radicals and human disease. *Ann Intern Med*, 107(4), pp 526-45.
- Cross, C. E., Tolba, M. F., Rondelli, C. M., Xu, M. & Abdel-Rahman, S. Z. 2015. Oxidative stress alters miRNA and gene expression profiles in villous first trimester trophoblasts. *BioMed research international*, 2015(
- Cui, J.-J., Yuan, J.-F. & Zhang, Z.-Q. 2010. Anti-oxidation activity of the crude polysaccharides isolated from *Polygonum cillinerve* (Nakai) Ohwi in immunosuppressed mice. *Journal of ethnopharmacology*, 132(2), pp 512-517.
- Cunningham, D., Humblet, Y., Siena, S., Khayat, D., Bleiberg, H., Santoro, A., Bets, D., Mueser, M., Harstrick, A., Verslype, C., Chau, I. & Van Cutsem, E. 2004. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med*, 351(4), pp 337-45.
- Curra, M., Junior, S., Valente, L. A., Martins, M. D. & Santos, P. S. d. S. 2018. Chemotherapy protocols and incidence of oral mucositis. An integrative review. *Einstein (Sao Paulo)*, 16(1), pp.
- Cyphert, J. M., Trempus, C. S. & Garantziotis, S. 2015. Size Matters: Molecular Weight Specificity of Hyaluronan Effects in Cell Biology. *International Journal of Cell Biology*, 2015(563818.
- D'Agostino, A., Stellavato, A., Busico, T., Papa, A., Tirino, V., Papaccio, G., La Gatta, A., De Rosa, M. & Schiraldi, C. 2015. In vitro analysis of the effects on wound healing of high- and low-molecular weight chains of hyaluronan and their hybrid H-HA/L-HA complexes. *BMC Cell Biol*, 16(19.
- D'Agostino, A., Stellavato, A., Corsuto, L., Diana, P., Filosa, R., La Gatta, A., De Rosa, M. & Schiraldi, C. 2017. Is molecular size a discriminating factor in hyaluronan interaction with human cells? *Carbohydr Polym*, 157(21-30.
- da Silva Santos, P. S., Coracin, F. L., Barros, J. C. d. A., Dulley, F. L., Nunes, F. D. & Magalhães, M. G. 2011. Impact of oral care prior to HSCT on the severity and clinical outcomes of oral mucositis. *Clinical transplantation*, 25(2), pp 325-328.

- Dale, R. A., Harrison, J. S. & Redding, S. W. 2004. Oral complications in cancer chemotherapy, cancer incidence, and mortality in the US. *General dentistry*, 52(1), pp 64-71.
- Dalley, A. J., Pitty, L. P., Major, A. G., AbdulMajeed, A. A. & Farah, C. S. 2014. Expression of ABCG 2 and B mi-1 in oral potentially malignant lesions and oral squamous cell carcinoma. *Cancer medicine*, 3(2), pp 273-283.
- Damodarasamy, M., Vernon, R. B., Chan, C. K., Plymate, S. R., Wight, T. N. & Reed, M. J. 2015. Hyaluronan in aged collagen matrix increases prostate epithelial cell proliferation. *In Vitro Cellular & Developmental Biology - Animal*, 51(1), pp 50-58.
- Dana, H., Mahmoodi Chalbatani, G., Gharagouzloo, E., Miri, S. R., Memari, F., Rasoolzadeh, R., Zinatizadeh, M. R., Kheirandish Zarandi, P. & Marmari, V. 2020. In silico Analysis, Molecular Docking, Molecular Dynamic, Cloning, Expression and Purification of Chimeric Protein in Colorectal Cancer Treatment. *Drug design, development and therapy*, 14(309-329).
- Danishefsky, I. & Siskovic, E. 1971. Conversion of carboxyl groups of mucopolysaccharides into amides of amino acid esters. *Carbohydrate Research*, 16(1), pp 199-205.
- Datta, K., Babbar, P., Srivastava, T., Sinha, S. & Chattopadhyay, P. 2002. p53 dependent apoptosis in glioma cell lines in response to hydrogen peroxide induced oxidative stress. *The International Journal of Biochemistry & Cell Biology*, 34(2), pp 148-157.
- David-Raoudi, M., Tranchepain, F., Deschrevel, B., Vincent, J. C., Bogdanowicz, P., Boumediene, K. & Pujol, J. P. 2008. Differential effects of hyaluronan and its fragments on fibroblasts: relation to wound healing. *Wound Repair and Regeneration*, 16(2), pp 274-287.
- Davis, R. J. 2000. Signal Transduction by the JNK Group of MAP Kinases. *Cell*, 103(2), pp 239-252.
- Day, A. & Sheehan, J. 2001. Hyaluronan: Polysaccharide chaos to protein organisation. *Current Opinion in Structural Biology*, 11(617-622).
- de Farias Gabriel, A., Silveira, F. M., Curra, M., Schuch, L. F., Wagner, V. P., Martins, M. A. T., da Silveira Matte, U., Siebert, M., Botton, M. R., Brunetto, A. T., Gregianin, L. J. & Martins, M. D. 2022. Risk factors associated with the development of oral mucositis in pediatric oncology patients: Systematic review and meta-analysis. *Oral Diseases*, 28(4), pp 1068-1084.
- de Freitas Cuba, L., Salum, F. G., Cherubini, K. & de Figueiredo, M. 2015. Antioxidant agents: a future alternative approach in the prevention and treatment of radiation-induced oral mucositis. *Altern Ther Health Med*, 21(2), pp 36-41.

- de Koning, B. A. E., Sluis, M. v. d., Lindenberg-Kortleve, D. J., Velcich, A., Pieters, R., Büller, H. A., Einerhand, A. W. C. & Renes, I. B. 2007. Methotrexate-induced mucositis in mucin 2-deficient mice. *Journal of Cellular Physiology*, 210(1), pp 144-152.
- De Ryck, T., Grootaert, C., Jaspaert, L., Kerckhof, F. M., Van Gele, M., De Schrijver, J., Van den Abbeele, P., Swift, S., Bracke, M., Van de Wiele, T. & Vanhoecke, B. 2014. Development of an oral mucosa model to study host-microbiome interactions during wound healing. *Appl Microbiol Biotechnol*, 98(15), pp 6831-46.
- De Ryck, T., Van Impe, A., Vanhoecke, B. W., Heyerick, A., Vakaet, L., De Neve, W., Müller, D., Schmidt, M., Dörr, W. & Bracke, M. E. 2015. 8-prenylnaringenin and tamoxifen inhibit the shedding of irradiated epithelial cells and increase the latency period of radiation-induced oral mucositis. *Strahlentherapie und Onkologie*, 191(5), pp 429-436.
- De Sousa, S. O. M., Mesquita, R. A., Pinto, D. S. & Gutkind, S. 2002. Immunolocalization of c-Fos and c-Jun in human oral mucosa and in oral squamous cell carcinoma. *Journal of Oral Pathology & Medicine*, 31(2), pp 78-81.
- de Thonel, A. & Eriksson, J. E. 2005. Regulation of death receptors—Relevance in cancer therapies. *Toxicology and Applied Pharmacology*, 207(2, Supplement), pp 123-132.
- Debela, D. T., Muzazu, S. G. Y., Heraro, K. D., Ndalama, M. T., Mesele, B. W., Haile, D. C., Kitui, S. K. & Manyazewal, T. 2021. New approaches and procedures for cancer treatment: Current perspectives. *SAGE Open Medicine*, 9(20503121211034366).
- Denham, J. W. & Hauer-Jensen, M. 2002. Review article: The radiotherapeutic injury – a complex ‘wound’. *Radiotherapy and Oncology*, 63(129-145).
- Dhar, S., Nygren, P., Csoka, K., Botling, J., Nilsson, K. & Larsson, R. 1996. Anti-cancer drug characterisation using a human cell line panel representing defined types of drug resistance. *British journal of cancer*, 74(6), pp 888-896.
- Di Gennaro, E., Bruzzese, F., Pepe, S., Leone, A., Delrio, P., Subbarayan, P., Avallone, A. & Budillon, A. 2009. Modulation of thymidilate synthase and p53 expression by HDAC inhibitor vorinostat resulted in synergistic antitumor effect in combination with 5FU or raltitrexed. *Cancer biology & therapy*, 8(9), pp 782-791.
- Di Liberto, C., Caroprese, M., Pizzo, G., Compilato, D., Fulfaro, F., Rinaldi, T., Lo Muzio, L. & Campisi, G. 2007. [Oral complications in patients with head and neck cancer after radio-chemotherapy. Mucositis and xerostomia]. *Recenti progressi in medicina*, 98(5), pp 302-314.
- Diao, L., Shen, A., Yang, Y., Tao, J. & Hu, Y. 2019. CD44-targeted hyaluronic acid-curcumin reverses chemotherapeutics resistance by inhibiting P-gp and anti-apoptotic pathways. *RSC Adv*, 9(70), pp 40873-40882.

- Diaz-Sanchez, R. M., Pachón-Ibáñez, J., Marín-Conde, F., Rodríguez-Caballero, Á., Gutierrez-Perez, J. L. & Torres-Lagares, D. 2015. Double-blind, randomized pilot study of bioadhesive chlorhexidine gel in the prevention and treatment of mucositis induced by chemoradiotherapy of head and neck cancer. *Med Oral Patol Oral Cir Bucal*, 20(3), pp e378-85.
- Dicker, K. T., Gurski, L. A., Pradhan-Bhatt, S., Witt, R. L., Farach-Carson, M. C. & Jia, X. 2014. Hyaluronan: a simple polysaccharide with diverse biological functions. *Acta Biomater*, 10(4), pp 1558-70.
- Dickler, M. N., Tolaney, S. M., Rugo, H. S., Cortés, J., Diéras, V., Patt, D., Wildiers, H., Hudis, C. A., O'Shaughnessy, J., Zamora, E., Yardley, D. A., Frenzel, M., Koustenis, A. & Baselga, J. 2017. MONARCH 1, A Phase II Study of Abemaciclib, a CDK4 and CDK6 Inhibitor, as a Single Agent, in Patients with Refractory HR(+)/HER2(-) Metastatic Breast Cancer. *Clin Cancer Res*, 23(17), pp 5218-5224.
- Dickson, M. A., Hahn, W. C., Ino, Y., Ronfard, V., Wu, J. Y., Weinberg, R. A., Louis, D. N., Li, F. P. & Rheinwald, J. G. 2000. Human Keratinocytes That Express hTERT and Also Bypass a p16INK4a-Enforced Mechanism That Limits Life Span Become Immortal yet Retain Normal Growth and Differentiation Characteristics. *Molecular and Cellular Biology*, 20(4), pp 1436-1447.
- Dimanche-Boitrel, M. T. & Rebillard, A. 2013. Sphingolipids and response to chemotherapy. *Handb Exp Pharmacol*, 216), pp 73-91.
- Dirix, P., Nuyts, S. & Van den Bogaert, W. 2006. Radiation-induced xerostomia in patients with head and neck cancer: a literature review. *Cancer*, 107(11), pp 2525-34.
- Dodd, M. J. 2004. The Pathogenesis and Characterization of Oral Mucositis Associated With Cancer Therapy. *Oncology Nursing Forum*, 31(5-11).
- Dongari-Bagtzoglou, A. & Kashleva, H. 2003. Candida albicans triggers interleukin-8 secretion by oral epithelial cells. *Microbial pathogenesis*, 34(4), pp 169-177.
- Dongari-Bagtzoglou, A. & Kashleva, H. 2006. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. *Nature protocols*, 1(4), pp 2012.
- Dongari-Bagtzoglou, A., Kashleva, H. & Villar, C. C. 2004. Bioactive interleukin-1 α is cytolytically released from Candida albicans-infected oral epithelial cells. *Medical mycology*, 42(6), pp 531-541.

- Dongari-Bagtzoglou, A. & Kashleva, H. 2003. Granulocyte-macrophage colony-stimulating factor responses of oral epithelial cells to *Candida albicans*. *Oral microbiology and immunology*, 18(3), pp 165-170.
- Donnelly, J. P., Bellm, L. A., Epstein, J. B., Sonis, S. T. & Symonds, R. P. 2003. Antimicrobial therapy to prevent or treat oral mucositis. *The Lancet Infectious Diseases*, 3(7), pp 405-412.
- Doonan, F. & Cotter, T. G. 2008. Morphological assessment of apoptosis. *Methods*, 44(3), pp 200-204.
- Dovedytis, M., Liu, Z. J. & Bartlett, S. 2020. Hyaluronic acid and its biomedical applications: A review. *Engineered Regeneration*, 1(102-113).
- Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., A. Van De Putte, L. B. & Lipsky, P. E. 1998. Cyclooxygenase in biology and disease. *The FASEB Journal*, 12(12), pp 1063-1073.
- Duncan, M. & Grant, G. 2003. Oral and intestinal mucositis — causes and possible treatments. *Alimentary Pharmacology & Therapeutics*, 18(9), pp 853-874.
- Dwivedy, A. & Aich, P. 2011. Importance of innate mucosal immunity and the promises it holds. *International journal of general medicine*, 4(299-311).
- Eenschooten, C., Guillaumie, F., Kontogeorgis, G. M., Stenby, E. H. & Schwach-Abdellaoui, K. 2010. Preparation and structural characterisation of novel and versatile amphiphilic octenyl succinic anhydride–modified hyaluronic acid derivatives. *Carbohydrate Polymers*, 79(3), pp 597-605.
- Eilenberger, C., Kratz, S. R. A., Rothbauer, M., Ehmoser, E. K., Ertl, P. & Küpcü, S. 2018. Optimized alamarBlue assay protocol for drug dose-response determination of 3D tumor spheroids. *MethodsX*, 5(781-787).
- Eilers, J. 2004. Nursing Interventions and Supportive Care for the Prevention and Treatment of Oral Mucositis Associated With Cancer Treatment. *Oncology Nursing Forum*, 31(13-23).
- Eilers, J., Berger, A. M. & Petersen, M. C. 1988. Development, testing, and application of the oral assessment guide. *Oncol Nurs Forum*, 15(3), pp 325-30.
- Eilers, J. & Million, R. Prevention and management of oral mucositis in patients with cancer. *Seminars in oncology nursing*, 2007. Elsevier, 201-212.
- El-Safory, N. S. & Lee, C.-K. 2010. Cytotoxic and antioxidant effects of unsaturated hyaluronic acid oligomers. *Carbohydrate polymers*, 82(4), pp 1116-1123.

- Elad, S. 2019. The MASCC/ISOO Mucositis Guidelines 2019 Update: introduction to the first set of articles. *Support Care Cancer*, 27(10), pp 3929-3931.
- Elad, S. 2020. The MASCC/ISOO mucositis guidelines 2019: the second set of articles and future directions. *Support Care Cancer*, 28(5), pp 2445-2447.
- Elad, S., Cheng, K. K. F., Lalla, R. V., Yarom, N., Hong, C., Logan, R. M., Bowen, J., Gibson, R., Saunders, D. P., Zadik, Y., Ariyawardana, A., Correa, M. E., Ranna, V., Bossi, P., Cancer, f. t. M. G. L. G. o. t. M. A. o. S. C. i. & Oncology, I. S. o. O. 2020. MASCC/ISOO clinical practice guidelines for the management of mucositis secondary to cancer therapy. *Cancer*, 126(19), pp 4423-4431.
- Elad, S., Raber-Durlacher, J. E., Brennan, M. T., Saunders, D. P., Mank, A. P., Zadik, Y., Quinn, B., Epstein, J. B., Blijlevens, N. M., Waltimo, T., Passweg, J. R., Correa, M. E., Dahlöf, G., Garming-Legert, K. U., Logan, R. M., Potting, C. M., Shapira, M. Y., Soga, Y., Stringer, J., Stokman, M. A., Vokurka, S., Wallhult, E., Yarom, N. & Jensen, S. B. 2015. Basic oral care for hematology-oncology patients and hematopoietic stem cell transplantation recipients: a position paper from the joint task force of the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO) and the European Society for Blood and Marrow Transplantation (EBMT). *Support Care Cancer*, 23(1), pp 223-36.
- Elad, S., Yarom, N., Zadik, Y., Kuten-Shorrer, M. & Sonis, S. T. 2022. The broadening scope of oral mucositis and oral ulcerative mucosal toxicities of anticancer therapies. *CA Cancer J Clin*, 72(1), pp 57-77.
- Elting, L. S., Avritscher, E. B., Cooksley, C. D., Cardenas-Turanzas, M., Garden, A. S. & Chambers, M. S. 2008a. Psychosocial and economic impact of cancer. *Dent Clin North Am*, 52(1), pp 231-52, x.
- Elting, L. S., Bodey, G. P. & Keefe, B. H. 1992. Septicemia and Shock Syndrome Due to Viridans Streptococci: A Case-Control Study of Predisposing Factors. *Clinical Infectious Diseases*, 14(6), pp 1201-1207.
- Elting, L. S. & Chang, Y. C. 2019. Costs of Oral Complications of Cancer Therapies: Estimates and a Blueprint for Future Study. *J Natl Cancer Inst Monogr*, 2019(53), pp.
- Elting, L. S., Chang, Y. C., Parelkar, P., Boers-Doets, C. B., Michelet, M., Hita, G., Rouleau, T., Cooksley, C., Halm, J., Vithala, M., Bossi, P., Escalante, C. & Brennan, M. T. 2013. Risk of oral and gastrointestinal mucosal injury among patients receiving selected targeted agents: a meta-analysis. *Support Care Cancer*, 21(11), pp 3243-54.
- Elting, L. S., Cooksley, C., Chambers, M., Cantor, S. B., Manzullo, E. & Rubenstein, E. B. 2003. The burdens of cancer therapy. Clinical and economic outcomes of chemotherapy-induced mucositis. *Cancer*, 98(7), pp 1531-9.

- Elting, L. S., Cooksley, C. D., Chambers, M. S. & Garden, A. S. 2007. Risk, outcomes, and costs of radiation-induced oral mucositis among patients with head-and-neck malignancies. *International Journal of Radiation Oncology* Biology* Physics*, 68(4), pp 1110-1120.
- Elting, L. S., Keefe, D. M., Sonis, S. T., Garden, A. S., Spijkervet, F. K., Barasch, A., Tishler, R. B., Canty, T. P., Kudrimoti, M. K., Vera-Llonch, M., Burden of Illness, H. & Neck Writing, C. 2008b. Patient-reported measurements of oral mucositis in head and neck cancer patients treated with radiotherapy with or without chemotherapy: demonstration of increased frequency, severity, resistance to palliation, and impact on quality of life. *Cancer*, 113(10), pp 2704-13.
- Elting, L. S. & Shih, Y. C. 2004. The economic burden of supportive care of cancer patients. *Support Care Cancer*, 12(4), pp 219-26.
- Endo, Y., Yoshida, H., Ota, Y., Akazawa, Y., Sayo, T., Hanai, U., Imagawa, K., Sasaki, M. & Takahashi, Y. 2021. Accelerated human epidermal turnover driven by increased hyaluronan production. *J Dermatol Sci*, 101(2), pp 123-133.
- Engels-Deutsch, M., Pini, A., Yamashita, Y., Shibata, Y., Haikel, Y., Schöller-Guinard, M. & Klein, J.-P. 2003. Insertional Inactivation of pac and rmlB Genes Reduces the Release of Tumor Necrosis Factor Alpha, Interleukin-6, and Interleukin-8 Induced by Streptococcus mutans in Monocytic, Dental Pulp, and Periodontal Ligament Cells. *Infection and Immunity*, 71(9), pp 5169-5177.
- Entwistle, J., Hall, C. L. & Turley, E. A. 1996. HA receptors: regulators of signalling to the cytoskeleton. *J Cell Biochem*, 61(4), pp 569-77.
- Epstein, J. B., Beaumont, J. L., Gwede, C. K., Murphy, B., Garden, A. S., Meredith, R., Le, Q. T., Brizel, D., Isitt, J. & Cella, D. 2007. Longitudinal evaluation of the oral mucositis weekly questionnaire-head and neck cancer, a patient-reported outcomes questionnaire. *Cancer*, 109(9), pp 1914-22.
- Epstein, J. B., Silverman, S., Jr., Paggiarino, D. A., Crockett, S., Schubert, M. M., Senzer, N. N., Lockhart, P. B., Gallagher, M. J., Peterson, D. E. & Leveque, F. G. 2001. Benzydamine HCl for prophylaxis of radiation-induced oral mucositis: results from a multicenter, randomized, double-blind, placebo-controlled clinical trial. *Cancer*, 92(4), pp 875-85.
- Etscheid, M., Beer, N. & Dodt, J. 2005. The hyaluronan-binding protease upregulates ERK1/2 and PI3K/Akt signalling pathways in fibroblasts and stimulates cell proliferation and migration. *Cell Signal*, 17(12), pp 1486-94.
- Fadda, G., Campus, G. & Lugliè, P. 2006. Risk factors for oral mucositis in paediatric oncology patients receiving alkylant chemotherapy. *BMC Oral Health*, 6(1), pp 13.

- Fallacara, A., Baldini, E., Manfredini, S. & Vertuani, S. 2018. Hyaluronic Acid in the Third Millennium. *Polymers*, 10(7), pp 701.
- Fanning, S., Rybicki, L., Kalaycio, M., Andresen, S., Kuczkowski, E., Pohlman, B., Sobecks, R., Sweetenham, J. & Bolwell, B. 2006. Severe mucositis is associated with reduced survival after autologous stem cell transplantation for lymphoid malignancies. *British journal of haematology*, 135(3), pp 374-381.
- Farrell, C. L., Bready, J. V., Rex, K. L., Chen, J. N., DiPalma, C. R., Whitcomb, K. L., Yin, S., Hill, D. C., Wiemann, B. & Starnes, C. O. 1998. Keratinocyte growth factor protects mice from chemotherapy and radiation-induced gastrointestinal injury and mortality. *Cancer research*, 58(5), pp 933-939.
- Farwell, D. G., Reilly, D. F., Weymuller Jr, E. A., Greenberg, D. L., Staiger, T. O. & Futran, N. A. 2002. Predictors of perioperative complications in head and neck patients. *Archives of Otolaryngology–Head & Neck Surgery*, 128(5), pp 505-511.
- Favia, G., Mariggio, M. A., Maiorano, F., Cassano, A., Capodiferro, S. & Ribatti, D. 2008. Accelerated wound healing of oral soft tissues and angiogenic effect induced by a pool of aminoacids combined to sodium hyaluronate (AMINOGAM). *Journal of biological regulators and homeostatic agents*, 22(2), pp 109-116.
- Ferguson, E. L., Roberts, J. L., Moseley, R., Griffiths, P. C. & Thomas, D. W. 2011. Evaluation of the physical and biological properties of hyaluronan and hyaluronan fragments. *International journal of pharmaceutics*, 420(1), pp 84-92.
- Ferrer, E., Juan-García, A., Font, G. & Ruiz, M. 2009. Reactive oxygen species induced by beauvericin, patulin and zearalenone in CHO-K1 cells. *Toxicology in Vitro*, 23(8), pp 1504-1509.
- Feucht, E., DeSanti, C. & Weinberg, A. 2003. Selective induction of human beta-defensin mRNAs by *Actinobacillus actinomycetemcomitans* in primary and immortalized oral epithelial cells. *Oral microbiology and immunology*, 18(6), pp 359-363.
- Filicko, J., Lazarus, H. & Flomenberg, N. 2003. Mucosal injury in patients undergoing hematopoietic progenitor cell transplantation: new approaches to prophylaxis and treatment. *Bone marrow transplantation*, 31(1), pp 1-10.
- Filippi, A. R., Franco, P. & Ricardi, U. 2006. Is clinical radiosensitivity a complex genetically controlled event? *Tumori*, 92(2), pp 87.
- Follain, N., Montanari, S., Jeacomine, I., Gambarelli, S. & Vignon, M. R. 2008. Coupling of amines with polyglucuronic acid: Evidence for amide bond formation. *Carbohydrate Polymers*, 74(3), pp 333-343.

- Frank, M., Hennenberg, E. M., Eyking, A., Runzi, M., Gerken, G., Scott, P., Parkhill, J., Walker, A. W. & Cario, E. 2015. TLR signaling modulates side effects of anticancer therapy in the small intestine. *J Immunol*, 194(4), pp 1983-95.
- Fraser, J. R. E., Laurent, T. C. & Laurent, U. 1997. Hyaluronan: its nature, distribution, functions and turnover. *Journal of internal medicine*, 242(1), pp 27-33.
- Freitas, A. C., Campos, L., Brandao, T. B., Cristofaro, M., Eduardo Fde, P., Luiz, A. C., Marques, M. M., Eduardo Cde, P. & Simoes, A. 2014. Chemotherapy-induced oral mucositis: effect of LED and laser phototherapy treatment protocols. *Photomed Laser Surg*, 32(2), pp 81-7.
- Frenkel, J. S. 2014. The role of hyaluronan in wound healing. *International wound journal*, 11(2), pp 159-163.
- Frippiat, C., Dewelle, J., Remacle, J. & Toussaint, O. 2002. Signal transduction in H₂O₂-induced senescence-like phenotype in human diploid fibroblasts. *Free Radic Biol Med*, 33(10), pp 1334-46.
- Fuks, Z., Haimovitz-Friedman, A. & Kolesnick, R. N. 1995. The role of the sphingomyelin pathway and protein kinase C in radiation-induced cell kill. *Important Adv Oncol*, 19-31.
- Fukuda, K., Takayama, M., Ueno, M., Oh, M., Asada, S., Kumano, F. & Tanaka, S. 1997. Hyaluronic acid inhibits interleukin-1-induced superoxide anion in bovine chondrocytes. *Inflammation Research*, 46(3), pp 114-117.
- Funk, G. F., Karnell, L. H., Robinson, R. A., Zhen, W. K., Trask, D. K. & Hoffman, H. T. 2002. Presentation, treatment, and outcome of oral cavity cancer: a National Cancer Data Base report. *Head Neck*, 24(2), pp 165-80.
- Fusco, S., Borzacchiello, A., Miccio, L., Pesce, G., Rusciano, G., Sasso, A. & Netti, P. A. 2007. High frequency viscoelastic behaviour of low molecular weight hyaluronic acid water solutions. *Biorheology*, 44(5-6), pp 403-418.
- Galbiatti, A. L. S., Padovani-Junior, J. A., Maníglia, J. V., Rodrigues, C. D. S., Pavarino, É. C. & Goloni-Bertollo, E. M. 2013. Head and neck cancer: causes, prevention and treatment. *Brazilian Journal of Otorhinolaryngology*, 79(2), pp 239-247.
- Garden, A. S. 2001. Altered fractionation for head and neck cancer. *Oncology (Williston Park)*, 15(10), pp 1326-32, 1334; discussion 1334, 1339-41.
- Garg, H. G. & Hales, C. A. 2004. Chemistry and biology of hyaluronan / edited by Hari G. Garg and Charles A. Hales. 1st ed. ed. Amsterdam ;Boston :: Elsevier.

- Gate, L., Paul, J., Ba, G. N., Tew, K. & Tapiero, H. 1999. Oxidative stress induced in pathologies: the role of antioxidants. *Biomedicine & Pharmacotherapy*, 53(4), pp 169-180.
- Geara, F. B., Peters, L. J., Ang, K. K., Garden, A. S., Tucker, S. L., Levy, L. B. & Brown, B. W. 1996. Comparison between normal tissue reactions and local tumor control in head and neck cancer patients treated by definitive radiotherapy. *International Journal of Radiation Oncology* Biology* Physics*, 35(3), pp 455-462.
- Geesman, G., Gesiotto, Q. J., Lalani, Z. & Tejani, N. 2021. Anatomy of the gastrointestinal system. *Surgical and perioperative management of patients with anatomic anomalies*, 145-177.
- Gelberg, H. B. 2014. Comparative Anatomy, Physiology, and Mechanisms of Disease Production of the Esophagus, Stomach, and Small Intestine. *Toxicologic Pathology*, 42(1), pp 54-66.
- Gençler, B. & Gönül, M. 2016. Cutaneous Side Effects of BRAF Inhibitors in Advanced Melanoma: Review of the Literature. *Dermatol Res Pract*, 2016(5361569).
- Genestra, M. 2007. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cellular signalling*, 19(9), pp 1807-1819.
- Georgiou, M., Patapatiou, G., Domoxoudis, S., Pistevou-Gompaki, K. & Papanikolaou, A. 2012. Oral Mucositis: understanding the pathology and management. *Hippokratia*, 16(3), pp 215-216.
- Geyer, C. E., Forster, J., Lindquist, D., Chan, S., Romieu, C. G., Pienkowski, T., Jagiello-Gruszfeld, A., Crown, J., Chan, A., Kaufman, B., Skarlos, D., Campone, M., Davidson, N., Berger, M., Oliva, C., Rubin, S. D., Stein, S. & Cameron, D. 2006. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med*, 355(26), pp 2733-43.
- Ghalayani, P., Emami, H., Pakravan, F. & Nasr Isfahani, M. 2017. Comparison of triamcinolone acetonide mucoadhesive film with licorice mucoadhesive film on radiotherapy-induced oral mucositis: A randomized double-blinded clinical trial. *Asia Pac J Clin Oncol*, 13(2), pp e48-e56.
- Gibson, R. J., Bowen, J. M., Inglis, M. R., Cummins, A. G. & Keefe, D. M. 2003. Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer. *J Gastroenterol Hepatol*, 18(9), pp 1095-100.
- Gibson, R. J. & Keefe, D. M. 2006. Cancer chemotherapy-induced diarrhoea and constipation: mechanisms of damage and prevention strategies. *Support Care Cancer*, 14(9), pp 890-900.

- Gierut, J. J., Mathur, P. S., Bie, W., Han, J. & Tyner, A. L. 2012. Targeting Protein Tyrosine Kinase 6 Enhances Apoptosis of Colon Cancer Cells following DNA Damage. *Molecular Cancer Therapeutics*, 11(11), pp 2311-2320.
- Giles, F. J. 2001. The Vascular Endothelial Growth Factor (VEGF) Signaling Pathway: A Therapeutic Target in Patients with Hematologic Malignancies. *The Oncologist*, 6(suppl 5), pp 32-39.
- Gille, J. & Joenje, H. 1992. Cell culture models for oxidative stress: superoxide and hydrogen peroxide versus normobaric hyperoxia. *Mutation Research/DNAging*, 275(3-6), pp 405-414.
- Ginsberg, M. H., Partridge, A. & Shattil, S. J. 2005. Integrin regulation. *Current Opinion in Cell Biology*, 17(5), pp 509-516.
- Giri, S., Takada, A., Paudel, D., Yoshida, K., Furukawa, M., Kuramitsu, Y., Matsushita, K., Abiko, Y. & Furuichi, Y. 2022. An in vitro senescence model of gingival epithelial cell induced by hydrogen peroxide treatment. *Odontology*, 110(1), pp 44-53.
- Girish, K. & Kemparaju, K. 2007. The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. *Life sciences*, 80(21), pp 1921-1943.
- Girmenia, C., Lo Coco, F., Breccia, M., Latagliata, R., Spadea, A., D'Andrea, M., Gentile, G., Micozzi, A., Alimena, G., Martino, P. & Mandelli, F. 2003. Infectious complications in patients with acute promyelocytic leukaemia treated with the AIDA regimen. *Leukemia*, 17(5), pp 925-30.
- Goa, K. L. & Benfield, P. 1994. Hyaluronic Acid. *Drugs*, 47(3), pp 536-566.
- Gocmen, G., Gonul, O., Oktay, N. S., Yarat, A. & Goker, K. 2015. The antioxidant and anti-inflammatory efficiency of hyaluronic acid after third molar extraction. *Journal of Cranio-Maxillofacial Surgery*, 43(7), pp 1033-1037.
- Gonzalez-Reyes, S., Guzman-Beltran, S., Medina-Campos, O. N. & Pedraza-Chaverri, J. 2013. Curcumin pretreatment induces Nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats. *Oxid Med Cell Longev*, 2013(801418).
- Gori, E., Arpinati, M., Bonifazi, F., Errico, A., Mega, A., Alberani, F., Sabbi, V., Costazza, G., Leanza, S., Borrelli, C., Berni, M., Feraut, C., Polato, E., Altieri, M. C., Pirola, E., Loddo, M. C., Banfi, M., Barzetti, L., Calza, S., Brignoli, C., Bandini, G., De Vivo, A., Bosi, A. & Baccarani, M. 2007. Cryotherapy in the prevention of oral mucositis in patients receiving low-dose methotrexate following myeloablative allogeneic stem cell transplantation: a prospective randomized study of the Gruppo Italiano Trapianto di Midollo Osseo nurses group. *Bone Marrow Transplant*, 39(6), pp 347-352.

- Goutham, H. V., Mumbrekar, K. D., Vadhiraaja, B. M., Fernandes, D. J., Sharan, K., Kanive Parashiva, G., Kapaettu, S. & Bola Sadashiva, S. R. 2012. DNA Double-Strand Break Analysis by γ -H2AX Foci: A Useful Method for Determining the Overreactors to Radiation-Induced Acute Reactions Among Head-and-Neck Cancer Patients. *International Journal of Radiation Oncology, Biology, Physics*, 84(5), pp e607-e612.
- Graham, K. M., Pecoraro, D. A., Ventura, M. & Meyer, C. C. 1993. Reducing the incidence of stomatitis using a quality assessment and improvement approach. *Cancer nursing*, 16(2), pp 117-122.
- Grazziutti, M., Dong, L., Miceli, M., Krishna, S., Kiwan, E., Syed, N., Fassas, A., Van Rhee, F., Klaus, H. & Barlogie, B. 2006. Oral mucositis in myeloma patients undergoing melphalan-based autologous stem cell transplantation: incidence, risk factors and a severity predictive model. *Bone marrow transplantation*, 38(7), pp 501-506.
- Grem, J. L. 2000. 5-Fluorouracil: forty-plus and still ticking. A review of its preclinical and clinical development. *Investigational new drugs*, 18(4), pp 299-313.
- Groeger, S. & Meyle, J. 2019. Oral Mucosal Epithelial Cells. *Front Immunol*, 10(208).
- Gruber, J. V., Holtz, R. & Riemer, J. 2022. Hyaluronic acid (HA) stimulates the in vitro expression of CD44 proteins but not HAS1 proteins in normal human epidermal keratinocytes (NHEKs) and is HA molecular weight dependent. *J Cosmet Dermatol*, 21(3), pp 1193-1198.
- Guggenheimer, J., Verbin, R. S., Appel, B. N. & Schmutz, J. 1977. Clinicopathologic effects of cancer chemotherapeutic agents on human buccal mucosa. *Oral Surgery, Oral Medicine, And Oral Pathology*, 44(1), pp 58-63.
- Guha, M. & Mackman, N. 2001. LPS induction of gene expression in human monocytes. *Cell Signal*, 13(2), pp 85-94.
- Gupta, A., De Felice, K. M., Loftus, E. V., Jr. & Khanna, S. 2015. Systematic review: colitis associated with anti-CTLA-4 therapy. *Aliment Pharmacol Ther*, 42(4), pp 406-17.
- Gurujeyalakshmi, G., Wang, Y. & Giri, S. N. 2000. Taurine and Niacin Block Lung Injury and Fibrosis by Down-Regulating Bleomycin-Induced Activation of Transcription Nuclear Factor- κ B in Mice. *Journal of Pharmacology and Experimental Therapeutics*, 293(1), pp 82-90.
- Haagen, J., Krohn, H., Röhlig, S., Schmidt, M., Wolfram, K. & Dörr, W. 2009. Effect of selective inhibitors of inflammation on oral mucositis: Preclinical studies. *Radiotherapy and Oncology*, 92(3), pp 472-476.

- Hafner, M., Niepel, M., Chung, M. & Sorger, P. K. 2016. Growth rate inhibition metrics correct for confounders in measuring sensitivity to cancer drugs. *Nat Methods*, 13(6), pp 521-7.
- Haines, R. J., Beard, R. S., Jr., Eitner, R. A., Chen, L. & Wu, M. H. 2016. TNF α /IFN γ Mediated Intestinal Epithelial Barrier Dysfunction Is Attenuated by MicroRNA-93 Downregulation of PTK6 in Mouse Colonic Epithelial Cells. *PLOS ONE*, 11(4), pp e0154351.
- Haley, R. M. & von Recum, H. A. 2019. Localized and targeted delivery of NSAIDs for treatment of inflammation: A review. *Exp Biol Med (Maywood)*, 244(6), pp 433-444.
- Halicka, H. D., Mitlitski, V., Heeter, J., Balazs, E. A. & Darzynkiewicz, Z. 2009. Attenuation of the oxidative burst-induced DNA damage in human leukocytes by hyaluronan. *International journal of molecular medicine*, 23(5), pp 695.
- Hall, J. E. & Guyton, A. C. 2011. *Guyton and Hall textbook of medical physiology*, 12th ed.: Saunders/Elsevier.
- Hall, P. D., Benko, H., Hogan, K. R. & Stuart, R. K. 1995. The influence of serum tumor necrosis factor- α and interleukin-6 concentrations on nonhematologic toxicity and hematologic recovery in patients with acute myelogenous leukemia. *Experimental Hematology*, 23(12), pp 1256-1260.
- Halliwell, B., Gutteridge, J. M. & Cross, C. E. 1992. Free radicals, antioxidants, and human disease: where are we now? *The Journal of laboratory and clinical medicine*, 119(6), pp 598-620.
- Hamann, W., Floter, A., Schmutzler, W. & Zwadlo-Klarwasser, G. 1995. Characterization of a novel anti-inflammatory factor produced by RM3/1 macrophages derived from glucocorticoid treated human monocytes. *Inflamm Res*, 44(12), pp 535-40.
- Han, N.-K., Shin, D. H., Kim, J. S., Weon, K. Y., Jang, C.-Y. & Kim, J.-S. 2016. Hyaluronan-conjugated liposomes encapsulating gemcitabine for breast cancer stem cells. *International journal of nanomedicine*, 11(14)13.
- Han, X. Y., Kamana, M. & Rolston, K. V. 2006. Viridans streptococci isolated by culture from blood of cancer patients: clinical and microbiologic analysis of 50 cases. *J Clin Microbiol*, 44(1), pp 160-5.
- Handschel, J., Prott, F. J., Sunderkötter, C., Metze, D., Meyer, U. & Joos, U. 1999. Irradiation induces increase of adhesion molecules and accumulation of beta2-integrin-expressing cells in humans. *International Journal Of Radiation Oncology, Biology, Physics*, 45(2), pp 475-481.

- Handsichel, J., Sunderkötter, C., Prott, F. J., Meyer, U., Kruse-Lösler, B. & Joos, U. 2001. Increase of RM3/1-positive macrophages in radiation-induced oral mucositis. *The Journal Of Pathology*, 193(2), pp 242-247.
- Hannun, Y. A. 1997. Apoptosis and the Dilemma of Cancer Chemotherapy. *Blood*, 89(6), pp 1845-1853.
- Hargittai, I. & Hargittai, M. 2008. Molecular structure of hyaluronan: an introduction. *Structural Chemistry*, 19(5), pp 697-717.
- Hartmann, J. T. & Lipp, H.-P. 2003. Toxicity of platinum compounds. *Expert Opinion on Pharmacotherapy*, 4(6), pp 889-901.
- Hassona, Y., Cirillo, N., Heesom, K., Parkinson, E. K. & Prime, S. S. 2014. Senescent cancer-associated fibroblasts secrete active MMP-2 that promotes keratinocyte dis-cohesion and invasion. *Br J Cancer*, 111(6), pp 1230-1237.
- Hassona, Y., Cirillo, N., Lim, K. P., Herman, A., Mellone, M., Thomas, G. J., Pitiyage, G. N., Parkinson, E. K. & Prime, S. S. 2013. Progression of genotype-specific oral cancer leads to senescence of cancer-associated fibroblasts and is mediated by oxidative stress and TGF- β . *Carcinogenesis*, 34(6), pp 1286-1295.
- Hatzis, C., Bedard, P. L., Birkbak, N. J., Beck, A. H., Aerts, H. J., Stem, D. F., Shi, L., Clarke, R., Quackenbush, J. & Haibe-Kains, B. 2014. Enhancing reproducibility in cancer drug screening: how do we move forward? *Cancer Res*, 74(15), pp 4016-23.
- Hawkins, C. L. & Davies, M. J. 1998. Degradation of hyaluronic acid, poly- and monosaccharides, and model compounds by hypochlorite: evidence for radical intermediates and fragmentation. *Free Radic Biol Med*, 24(9), pp 1396-410.
- Helander, H. F. & Fändriks, L. 2014. Surface area of the digestive tract - revisited. *Scand J Gastroenterol*, 49(6), pp 681-9.
- Heldin, P. & Pertoft, H. 1993. Synthesis and assembly of the hyaluronan-containing coats around normal human mesothelial cells. *Experimental cell research*, 208(2), pp 422-429.
- Herbers, A. H., Feuth, T., Donnelly, J. P. & Blijlevens, N. M. 2010. Citrulline-based assessment score: first choice for measuring and monitoring intestinal failure after high-dose chemotherapy. *Ann Oncol*, 21(8), pp 1706-1711.
- Hernández-Moreno, A., Vidal-Casariago, A., Calleja-Fernández, A., Kyriakos, G., Villar-Taibo, R., Urioste-Fondo, A., Cano-Rodríguez, I. & Ballesteros-Pomar, M. D. 2015. CHRONIC ENTERITIS

IN PATIENTS UNDERGOING PELVIC RADIOTHERAPY: PREVALENCE, RISK FACTORS AND ASSOCIATED COMPLICATIONS. *Nutr Hosp*, 32(5), pp 2178-83.

Hess, J., Angel, P. & Schorpp-Kistner, M. 2004. AP-1 subunits: quarrel and harmony among siblings. *Journal of Cell Science*, 117(25), pp 5965-5973.

Hinoi, E., Takarada, T., Ueshima, T., Tsuchihashi, Y. & Yoneda, Y. 2004. Glutamate signaling in peripheral tissues. *European Journal of Biochemistry*, 271(1), pp 1-13.

Hiramoto, K., Kobayashi, H., Yamate, Y., Ishii, M. & Sato, E. F. 2012. Intercellular pathway through hyaluronic acid in UVB-induced inflammation. *Exp Dermatol*, 21(12), pp 911-4.

Hofmann, L., Forschner, A., Loquai, C., Goldinger, S. M., Zimmer, L., Ugurel, S., Schmidgen, M. I., Gutzmer, R., Utikal, J. S., Göppner, D., Hassel, J. C., Meier, F., Tietze, J. K., Thomas, I., Weishaupt, C., Leverkus, M., Wahl, R., Dietrich, U., Garbe, C., Kirchberger, M. C., Eigentler, T., Berking, C., Gesierich, A., Krackhardt, A. M., Schadendorf, D., Schuler, G., Dummer, R. & Heinzerling, L. M. 2016. Cutaneous, gastrointestinal, hepatic, endocrine, and renal side-effects of anti-PD-1 therapy. *Eur J Cancer*, 60(190-209).

Holbrook, N. J. & Ikeyama, S. 2002. Age-related decline in cellular response to oxidative stress: links to growth factor signaling pathways with common defects. *Biochem Pharmacol*, 64(5-6), pp 999-1005.

Hong, C. H. L., Gueiros, L. A., Fulton, J. S., Cheng, K. K. F., Kandwal, A., Galiti, D., Fall-Dickson, J. M., Johansen, J., Ameringer, S., Kataoka, T., Weikel, D., Eilers, J., Ranna, V., Vaddi, A., Lalla, R. V., Bossi, P. & Elad, S. 2019. Systematic review of basic oral care for the management of oral mucositis in cancer patients and clinical practice guidelines. *Support Care Cancer*, 27(10), pp 3949-3967.

Horiot, J.-C., Le Fur, R., N'guyen, T., Chenal, C., Schraub, S., Alfonsi, S., Gardani, G., Van den Bogaert, W., Danczak, S. & Bolla, M. 1992. Hyperfractionation versus conventional fractionation in oropharyngeal carcinoma: final analysis of a randomized trial of the EORTC cooperative group of radiotherapy. *Radiotherapy and Oncology*, 25(4), pp 231-241.

Horiot, J., Le Fur, R., N'guyen, T., Chenal, C., Schraub, S., Alfonsi, S., Gardani, G., Van den Bogaert, W., Danczak, S. & Bolla, M. 1990. Hyperfractionated compared with conventional radiotherapy in oropharyngeal carcinoma: an EORTC randomized trial. *European Journal of Cancer and Clinical Oncology*, 26(7), pp 779-780.

Hseu, Y. C., Yang, T. Y., Li, M. L., Rajendran, P., Mathew, D. C., Tsai, C. H., Lin, R. W., Lee, C. C. & Yang, H. L. 2019. Chalcone flavokawain A attenuates TGF- β 1-induced fibrotic pathology via inhibition of ROS/Smad3 signaling pathways and induction of Nrf2/ARE-mediated antioxidant genes in vascular smooth muscle cells. *Journal of cellular and molecular medicine*, 23(2), pp 775-788.

- Huang, L., Chiang Chiau, J.-S., Cheng, M.-L., Chan, W.-T., Jiang, C.-B., Chang, S.-W., Yeung, C.-Y. & Lee, H.-C. 2019. SCID/NOD mice model for 5-FU induced intestinal mucositis: Safety and effects of probiotics as therapy. *Pediatrics & Neonatology*, 60(3), pp 252-260.
- Huang, Z. 2000. Bcl-2 family proteins as targets for anticancer drug design. *Oncogene*, 19(56), pp 6627.
- Hubrecht, R. 2013. Revised Australian Code for the care and use of animals for scientific purposes. *Animal Welfare*, 22(4), pp 491-491.
- Hwang, D., Popat, R., Bragdon, C., O'Donnell, K. E. & Sonis, S. T. 2005. Effects of ceramide inhibition on experimental radiation-induced oral mucositis. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 100(3), pp 321-329.
- Hyslop, P. A., Zhang, Z., Pearson, D. V. & Phebus, L. A. 1995. Measurement of striatal H₂O₂ by microdialysis following global forebrain ischemia and reperfusion in the rat: correlation with the cytotoxic potential of H₂O₂ in vitro. *Brain Research*, 671(2), pp 181-186.
- Iglesias-Bartolome, R., Patel, V., Cotrim, A., Leelahavanichkul, K., Molinolo, A. A., Mitchell, J. B. & Gutkind, J. S. 2012. mTOR inhibition prevents epithelial stem cell senescence and protects from radiation-induced mucositis. *Cell stem cell*, 11(3), pp 401-414.
- Inui, A. 2002. Cancer anorexia-cachexia syndrome: current issues in research and management. *CA Cancer J Clin*, 52(2), pp 72-91.
- Isacke, C. M. & Yarwood, H. 2002. The hyaluronan receptor, CD44. *The international journal of biochemistry & cell biology*, 34(7), pp 718-721.
- Itano, N., Atsumi, F., Sawai, T., Yamada, Y., Miyaishi, O., Senga, T., Hamaguchi, M. & Kimata, K. 2002. Abnormal accumulation of hyaluronan matrix diminishes contact inhibition of cell growth and promotes cell migration. *Proceedings of the National Academy of Sciences*, 99(6), pp 3609-3614.
- Itano, N., Sawai, T., Atsumi, F., Miyaishi, O., Taniguchi, S. i., Kannagi, R., Hamaguchi, M. & Kimata, K. 2004. Selective expression and functional characteristics of three mammalian hyaluronan synthases in oncogenic malignant transformation. *Journal of Biological Chemistry*, 279(18), pp 18679-18687.
- Jacobs, S., Baggott, C., Agarwal, R., Hesser, T., Schechter, T., Judd, P., Tomlinson, D., Beyene, J. & Sung, L. 2013. Validation of the Children's International Mucositis Evaluation Scale (ChIMES) in paediatric cancer and SCT. *Br J Cancer*, 109(10), pp 2515-22.

- Jacox, A., Carr, D. B. & Payne, R. 1994. New clinical-practice guidelines for the management of pain in patients with cancer. *N Engl J Med*, 330(9), pp 651-5.
- Jaenke, R. S., Robbins, M. E., Bywaters, T., Whitehouse, E., Rezvani, M. & Hopewell, J. W. 1993. Capillary endothelium. Target site of renal radiation injury. *Lab Invest*, 68(4), pp 396-405.
- Jamieson, E. R. & Lippard, S. J. 1999. Structure, recognition, and processing of cisplatin-DNA adducts. *Chemical reviews*, 99(9), pp 2467-2498.
- Janeway, C. A., Jr. & Medzhitov, R. 2002. Innate immune recognition. *Annual Review Of Immunology*, 20(197-216).
- Jankovic, S., Aleksic, Z., Klokkevold, P., Lekovic, V., Dimitrijevic, B., Barrie Kenney, E. & Camargo, P. 2012. Use of platelet-rich fibrin membrane following treatment of gingival recession: a randomized clinical trial. *International Journal of Periodontics and Restorative Dentistry*, 32(2), pp 165.
- Jansen, K., Van Der Werff, J., Van Wachem, P., Nicolai, J. A., De Leij, L. & Van Luyn, M. 2004. A hyaluronan-based nerve guide: in vitro cytotoxicity, subcutaneous tissue reactions, and degradation in the rat. *Biomaterials*, 25(3), pp 483-489.
- Jaresova, I., Rožková, D., Špišek, R., Janda, A., Brázová, J. & Šedivá, A. 2007. Kinetics of Toll-like receptor-4 splice variants expression in lipopolysaccharide-stimulated antigen presenting cells of healthy donors and patients with cystic fibrosis. *Microbes and Infection*, 9(11), pp 1359-1367.
- Jedrzejewski, M. J. & Stern, R. 2005. Structures of vertebrate hyaluronidases and their unique enzymatic mechanism of hydrolysis. *Proteins*, 61(2), pp 227-38.
- Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E. & Forman, D. 2011. Global cancer statistics. *CA: a cancer journal for clinicians*, 61(2), pp 69-90.
- Jennings, L. R., Colley, H. E., Ong, J., Panagakos, F., Masters, J. G., Trivedi, H. M., Murdoch, C. & Whawell, S. 2016. Development and Characterization of In Vitro Human Oral Mucosal Equivalents Derived from Immortalized Oral Keratinocytes. *Tissue Eng Part C Methods*, 22(12), pp 1108-1117.
- Jenq, R. R., Taur, Y., Devlin, S. M., Ponce, D. M., Goldberg, J. D., Ahr, K. F., Littmann, E. R., Ling, L., Gobourne, A. C. & Miller, L. C. 2015. Intestinal *Blautia* is associated with reduced death from graft-versus-host disease. *Biology of Blood and Marrow Transplantation*, 21(8), pp 1373-1383.

- Jensen, S. B., Jarvis, V., Zadik, Y., Barasch, A., Ariyawardana, A., Hovan, A., Yarom, N., Lalla, R. V., Bowen, J., Elad, S. & For the Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society of Oral, O. 2013. Systematic review of miscellaneous agents for the management of oral mucositis in cancer patients. *Supportive Care in Cancer*, 21(11), pp 3223-3232.
- Ji, E., Jung, M., Park, J., Kim, S., Seo, C., Park, K., Lee, E., Yeom, C. & Lee, S. 2014. Inhibition of adipogenesis in 3T3-L1 cells and suppression of abdominal fat accumulation in high-fat diet-feeding C57BL/6J mice after downregulation of hyaluronic acid. *International journal of obesity*, 38(8), pp 1035-1043.
- Jia, X., Cai, X., Chen, Y., Wang, S., Xu, H., Zhang, K., Ma, M., Wu, H., Shi, J. & Chen, H. 2015. Perfluoropentane-encapsulated hollow mesoporous prussian blue nanocubes for activated ultrasound imaging and photothermal therapy of cancer. *ACS applied materials & interfaces*, 7(8), pp 4579-4588.
- Jiang, D., Liang, J., Fan, J., Yu, S., Chen, S., Luo, Y., Prestwich, G. D., Mascarenhas, M. M., Garg, H. G., Quinn, D. A., Homer, R. J., Goldstein, D. R., Bucala, R., Lee, P. J., Medzhitov, R. & Noble, P. W. 2005. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat Med*, 11(11), pp 1173-9.
- Jiang, D., Liang, J. & Noble, P. W. 2007. Hyaluronan in tissue injury and repair. *Annu. Rev. Cell Dev. Biol.*, 23(435-461).
- Jiang, Z., Hu, Z., Zeng, L., Lu, W., Zhang, H., Li, T. & Xiao, H. 2011. The role of the Golgi apparatus in oxidative stress: is this organelle less significant than mitochondria? *Free Radical Biology and Medicine*, 50(8), pp 907-917.
- Johnston, T. P. 2015. Anatomy and Physiology of the Oral Mucosa. In: Rathbone, M. J., Senel, S. & Pather, I. (eds.) *Oral Mucosal Drug Delivery and Therapy*. Boston, MA: Springer US; pp 1-15.
- Jones, K. H. & Senft, J. A. 1985. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *J Histochem Cytochem*, 33(1), pp 77-9.
- Jones, R. M., Sloane, V. M., Wu, H., Luo, L., Kumar, A., Kumar, M. V., Gewirtz, A. T. & Neish, A. S. 2011. Flagellin administration protects gut mucosal tissue from irradiation-induced apoptosis via MKP-7 activity. *Gut*, 60(5), pp 648-57.
- Jones, S. A., Richards, P. J., Scheller, J. & Rose-John, S. 2005. Review: IL-6 transsignaling: the in vivo consequences. *Journal of interferon & cytokine research*, 25(5), pp 241.
- Justino, P. F., Melo, L. F., Nogueira, A. F., Costa, J. V., Silva, L. M., Santos, C. M., Mendes, W. O., Costa, M. R., Franco, A. X. & Lima, A. A. 2014. Treatment with *Saccharomyces boulardii*

reduces the inflammation and dysfunction of the gastrointestinal tract in 5-fluorouracil-induced intestinal mucositis in mice. *British journal of nutrition*, 111(9), pp 1611-1621.

Kaanders, J. H. & Ang, K. K. Early reactions as dose-limiting factors in radiotherapy. *Seminars in radiation oncology*, 1994. Elsevier, 55-67.

Kajiwara, T., Miura, K., Ohnuma, S., Shimada, M., Komura, T., Toshima, M., Kohyama, A., Kudoh, K., Haneda, S., Musha, H., Naitoh, T., Shirasaka, T. & Unno, M. 2015. Gastrointestinal toxicities of 5-fluorouracil increase the proportion of regulatory T cells in intestinal tract: advantages of alternate-day S-1 administration. *International Journal of Clinical Oncology*, 20(5), pp 913-921.

Kanetaka, K., Enjoji, A., Furui, J., Nagata, Y., Fujioka, H., Shiogama, T., Miyata, A., Kishikawa, H., Matsuo, S., Iwata, T., Kanematsu, T., Eguchi, S. & Nagasaki Study Group for Digestive Organ Cancer, C. 2012. Effects of intermittent 5-fluorouracil and low-dose cisplatin therapy on advanced and recurrent gastric cancer. *Anticancer Res*, 32(8), pp 3495-9.

Kansanen, E., Jyrkkänen, H. K. & Levonen, A. L. 2012. Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. *Free Radic Biol Med*, 52(6), pp 973-82.

Karamanos, N. K., Axelsson, S., Vanky, P., Tzanakakis, G. N. & Hjerpe, A. 1995. Determination of hyaluronan and galactosaminoglycan disaccharides by high-performance capillary electrophoresis at the attomole level. Applications to analyses of tissue and cell culture proteoglycans. *Journal of Chromatography A*, 696(2), pp 295-305.

Kashiwazaki, H., Matsushita, T., Sugita, J., Shigematsu, A., Kasashi, K., Yamazaki, Y., Kanehira, T., Yamamoto, S., Kondo, T. & Endo, T. 2012. Professional oral health care reduces oral mucositis and febrile neutropenia in patients treated with allogeneic bone marrow transplantation. *Supportive Care in Cancer*, 20(2), pp 367-373.

Kasiappan, R. & Safe, S. 2016. ROS-Inducing Agents for Cancer Chemotherapy. *Reactive Oxygen Species*, 1(1), pp.

Kato, S., Hayashi, S., Kitahara, Y., Nagasawa, K., Aono, H., Shibata, J., Utsumi, D., Amagase, K. & Kadowaki, M. 2015. Saireito (TJ-114), a Japanese Traditional Herbal Medicine, Reduces 5-Fluorouracil-Induced Intestinal Mucositis in Mice by Inhibiting Cytokine-Mediated Apoptosis in Intestinal Crypt Cells. *PLOS ONE*, 10(1), pp e0116213.

Kawaguchi, Y., Tanaka, H., Okada, T., Konishi, H., Takahashi, M., Ito, M. & Asai, J. 1997. Effect of reactive oxygen species on the elastin mRNA expression in cultured human dermal fibroblasts. *Free Radical Biology and Medicine*, 23(1), pp 162-165.

Kaya, G., Rodriguez, I., Jorcano, J. L., Vassalli, P. & Stamenkovic, I. 1997. Selective suppression of CD44 in keratinocytes of mice bearing an antisense CD44 transgene driven by a tissue-

- specific promoter disrupts hyaluronate metabolism in the skin and impairs keratinocyte proliferation. *Genes & development*, 11(8), pp 996-1007.
- Ke, C., Sun, L., Qiao, D., Wang, D. & Zeng, X. 2011. Antioxidant activity of low molecular weight hyaluronic acid. *Food Chem Toxicol*, 49(10), pp 2670-5.
- Ke, C., Wang, D., Sun, Y., Qiao, D., Ye, H. & Zeng, X. 2013. Immunostimulatory and antiangiogenic activities of low molecular weight hyaluronic acid. *Food and Chemical Toxicology*, 58(401-407).
- Keefe, D., Brealey, J., Goland, G. & Cummins, A. 2000. Chemotherapy for cancer causes apoptosis that precedes hypoplasia in crypts of the small intestine in humans. *Gut*, 47(5), pp 632-637.
- Keefe, D. M. 2004. Gastrointestinal mucositis: a new biological model. *Supportive Care in Cancer*, 12(1), pp 6-9.
- Keefe, D. M., Schubert, M. M., Elting, L. S., Sonis, S. T., Epstein, J. B., Raber-Durlacher, J. E., Migliorati, C. A., McGuire, D. B., Hutchins, R. D. & Peterson, D. E. 2007. Updated clinical practice guidelines for the prevention and treatment of mucositis. *Cancer*, 109(5), pp 820-831.
- Kenny, S. A. 1990. Effect of two oral care protocols on the incidence of stomatitis in hematology patients. *Cancer Nurs*, 13(6), pp 345-53.
- Kerr, J. F., Wyllie, A. H. & Currie, A. R. 1972. Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. *British journal of cancer*, 26(4), pp 239-257.
- Kersun, L. S., Propert, K. J., Lautenbach, E., Bunin, N. & Demichele, A. 2005. Early bacteremia in pediatric hematopoietic stem cell transplant patients on oral antibiotic prophylaxis. *Pediatr Blood Cancer*, 45(2), pp 162-9.
- Keston, A. S. & Brandt, R. 1965. The fluorometric analysis of ultramicro quantities of hydrogen peroxide. *Analytical Biochemistry*, 11(1), pp 1-5.
- Kierszenbaum, A. L. 2020. *Histology and cell biology : an introduction to pathology*, Fifth edition.: Elsevier.
- Kim, H., Park, H. T., Tae, Y. M., Kong, W. H., Sung, D. K., Hwang, B. W., Kim, K. S., Kim, Y. K. & Hahn, S. K. 2013. Bioimaging and pulmonary applications of self-assembled Flt1 peptide–hyaluronic acid conjugate nanoparticles. *Biomaterials*, 34(33), pp 8478-8490.
- Kim, H. J., Kim, J. H., Moon, W., Park, J., Park, S. J., Song, G. A., Han, S. H. & Lee, J. H. 2015. Rebamipide Attenuates 5-Fluorouracil-Induced Small Intestinal Mucositis in a Mouse Model. *Biological and Pharmaceutical Bulletin*, 38(2), pp 179-183.

- Kim, J. W., Cha, Y., Kim, S.-J., Han, S.-W., Oh, D.-Y., Lee, S.-H., Kim, D.-W., Im, S.-A., Kim, T.-Y. & Heo, D. S. 2012. Association of oral mucositis with quality of life and symptom clusters in patients with solid tumors receiving chemotherapy. *Supportive Care in Cancer*, 20(2), pp 395-403.
- King, S. R., Hickerson, W. L. & Proctor, K. G. 1991. Beneficial actions of exogenous hyaluronic acid on wound healing. *Surgery*, 109(1), pp 76-84.
- Kinhult, S., Albertsson, M., Eskilsson, J. & Cwikiel, M. 2003. Effects of probucol on endothelial damage by 5-fluorouracil. *Acta Oncologica*, 42(4), pp 304-308.
- Kishimoto, T. 2005. IL-6: from laboratory to bedside. *Clinical reviews in allergy & immunology*, 28(3), pp 177-186.
- Kiyoshima, T., Enoki, N., Kobayashi, I., Sakai, T., Nagata, K., Wada, H., Fujiwara, H., Ookuma, Y. & Sakai, H. 2012. Oxidative stress caused by a low concentration of hydrogen peroxide induces senescence-like changes in mouse gingival fibroblasts. *Int J Mol Med*, 30(5), pp 1007-1012.
- Klausner, M., Handa, Y. & Aizawa, S. 2021. In vitro three-dimensional organotypic culture models of the oral mucosa. *In Vitro Cellular & Developmental Biology - Animal*, 57(2), pp 148-159.
- Kobayashi, H. & Terao, T. 1997. Hyaluronic acid-specific regulation of cytokines by human uterine fibroblasts. *American Journal of Physiology-Cell Physiology*, 273(4), pp C1151-C1159.
- Kogan, G., Šoltés, L., Stern, R. & Gemeiner, P. 2007. Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnology letters*, 29(1), pp 17-25.
- Kolbus, A., Herr, I., Schreiber, M., Debatin, K.-M., Wagner, E. F. & Angel, P. 2000. c-Jun-Dependent CD95-L Expression Is a Rate-Limiting Step in the Induction of Apoptosis by Alkylating Agents. *Molecular and Cellular Biology*, 20(2), pp 575-582.
- Kopjar, N., Garaj-Vrhovac, V. & Milas, I. 2002. Assessment of chemotherapy-induced DNA damage in peripheral blood leukocytes of cancer patients using the alkaline comet assay. *Teratogenesis, Carcinogenesis, and Mutagenesis*, 22(1), pp 13-30.
- Köstler, W. J., Hejna, M., Wenzel, C. & Zielinski, C. C. 2001. Oral mucositis complicating chemotherapy and/or radiotherapy: options for prevention and treatment. *CA: A Cancer Journal For Clinicians*, 51(5), pp 290-315.
- Krasiński, R., Tchórzewski, H. & Lewkowicz, P. 2009. Antioxidant effect of hyaluronan on polymorphonuclear leukocyte-derived reactive oxygen species is dependent on its molecular weight and concentration and mainly involves the extracellular space. *Postepy Hig Med Dosw (Online)*, 63(205-12).

- Krishna, S. G., Zhao, W., Graziutti, M. L., Sanathkumar, N., Barlogie, B. & Anaissie, E. J. 2011. Incidence and risk factors for lower alimentary tract mucositis after 1529 courses of chemotherapy in a homogenous population of oncology patients: clinical and research implications. *Cancer*, 117(3), pp 648-55.
- Kuemmerle, J. F. & Zhou, H. 2002. Insulin-like growth factor-binding protein-5 (IGFBP-5) stimulates growth and IGF-I secretion in human intestinal smooth muscle by Ras-dependent activation of p38 MAP kinase and Erk1/2 pathways. *Journal of Biological Chemistry*, 277(23), pp 20563-20571.
- Kuenen, B. C., Levi, M., Meijers, J. C., van Hinsbergh, V. W., Berkhof, J., Kakkar, A. K., Hoekman, K. & Pinedo, H. M. 2003. Potential role of platelets in endothelial damage observed during treatment with cisplatin, gemcitabine, and the angiogenesis inhibitor SU5416. *J Clin Oncol*, 21(11), pp 2192-8.
- Kuiken, N. S. S., Rings, E. H. H. M., Blijlevens, N. M. A. & Tissing, W. J. E. 2017. Biomarkers and non-invasive tests for gastrointestinal mucositis. *Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer*, 25(9), pp 2933-2941.
- Kuiken, N. S. S., Rings, E. H. H. M. & Tissing, W. J. E. 2015. Risk analysis, diagnosis and management of gastrointestinal mucositis in pediatric cancer patients. *Critical Reviews in Oncology/Hematology*, 94(1), pp 87-97.
- Kumar, B., Cordell, K. G., Lee, J. S., Worden, F. P., Prince, M. E., Tran, H. H., Wolf, G. T., Urba, S. G., Chepeha, D. B. & Teknos, T. N. 2008. EGFR, p16, HPV Titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, 26(19), pp 3128.
- Kuo, J. 2005. *Practical aspects of hyaluronan based medical products*: CRC Press, P 240.
- Kuo, J. & Prestwich, G. 2010. Materials of biological origin—Materials analysis and implant uses. *Comprehensive Biomaterials; Ducheyne, P., Healy, K., Hutmacher, D., Kirkpatrick, J., Eds.*
- Kuo, J. W., Swann, D. A. & Prestwich, G. D. 1991. Chemical modification of hyaluronic acid by carbodiimides. *Bioconjugate chemistry*, 2(4), pp 232-241.
- Kusiak, A., AlicjaJereczek-Fossa, B., Cichońska, D. & Alterio, D. 2020. Oncological-Therapy Related Oral Mucositis as an Interdisciplinary Problem—Literature Review. *International Journal of Environmental Research and Public Health*, 17(7), pp 2464.
- Kuten-Shorrer, M., Zadik, Y. & Elad, S. 2021. Oral Mucositis Following Cancer Therapy. *Diseases of the Oral Mucosa: Study Guide and Review*, 389-399.

- Kvam, B., Fragonas, E., Degrassi, A., Kvam, C., Matulova, M., Pollesello, P., Zanetti, F. & Vittur, F. 1995. Oxygen-derived free radical (ODFR) action on hyaluronan (HA), on two HA ester derivatives, and on the metabolism of articular chondrocytes. *Experimental cell research*, 218(1), pp 79-86.
- Kwon, Y. 2016. Mechanism-based management for mucositis: option for treating side effects without compromising the efficacy of cancer therapy. *OncoTargets and therapy*, 9(2007).
- Kydd, W. L. & Daly, C. H. 1982. The biologic and mechanical effects of stress on oral mucosa. *J Prosthet Dent*, 47(3), pp 317-29.
- La Gatta, A., D'Agostino, A., Schiraldi, C., Colella, G. & Cirillo, N. 2019. A biophysically-defined hyaluronic acid-based compound accelerates migration and stimulates the production of keratinocyte-derived neuromodulators. *Cell Adh Migr*, 13(1), pp 23-32.
- La Gatta, A., Salzillo, R., Catalano, C., Pirozzi, A. V. A., D'Agostino, A., Bedini, E., Cammarota, M., De Rosa, M. & Schiraldi, C. 2020. Hyaluronan-based hydrogels via ether-crosslinking: Is HA molecular weight an effective means to tune gel performance? *International Journal of Biological Macromolecules*, 144(94-101).
- Lacouture, M. E., Anadkat, M. J., Bensadoun, R. J., Bryce, J., Chan, A., Epstein, J. B., Eaby-Sandy, B. & Murphy, B. A. 2011. Clinical practice guidelines for the prevention and treatment of EGFR inhibitor-associated dermatologic toxicities. *Support Care Cancer*, 19(8), pp 1079-95.
- Lacouture, M. E., Duvic, M., Hauschild, A., Prieto, V. G., Robert, C., Schadendorf, D., Kim, C. C., McCormack, C. J., Myskowski, P. L., Spleiss, O., Trunzer, K., Su, F., Nelson, B., Nolop, K. B., Grippo, J. F., Lee, R. J., Klimek, M. J., Troy, J. L. & Joe, A. K. 2013. Analysis of dermatologic events in vemurafenib-treated patients with melanoma. *Oncologist*, 18(3), pp 314-22.
- Lacy, F., Kailasam Mala, T., O'Connor Daniel, T., Schmid-Schönbein Geert, W. & Parmer Robert, J. 2000. Plasma Hydrogen Peroxide Production in Human Essential Hypertension. *Hypertension*, 36(5), pp 878-884.
- Laheij, A. M. & de Soet, J. J. 2014. Can the oral microflora affect oral ulcerative mucositis? *Current opinion in supportive and palliative care*, 8(2), pp 180-187.
- Laheij, A. M. G. A., de Soet, J. J., von dem Borne, P. A., Kuijper, E. J., Kraneveld, E. A., van Loveren, C. & Raber-Durlacher, J. E. 2012. Oral bacteria and yeasts in relationship to oral ulcerations in hematopoietic stem cell transplant recipients. *Supportive Care in Cancer*, 20(12), pp 3231-3240.
- Lai, S. K., Wang, Y.-Y., Wirtz, D. & Hanes, J. 2009. Micro- and macrorheology of mucus. *Advanced Drug Delivery Reviews*, 61(2), pp 86-100.

- Lalla, R. V., Bowen, J., Barasch, A., Elting, L., Epstein, J., Keefe, D. M., McGuire, D. B., Migliorati, C., Nicolatou-Galitis, O. & Peterson, D. E. 2014. MASCC/ISOO clinical practice guidelines for the management of mucositis secondary to cancer therapy. *Cancer*, 120(10), pp 1453-1461.
- Lalla, R. V., Brennan, M. T., Gordon, S. M., Sonis, S. T., Rosenthal, D. I. & Keefe, D. M. 2019. Oral Mucositis Due to High-Dose Chemotherapy and/or Head and Neck Radiation Therapy. *J Natl Cancer Inst Monogr*, 2019(53), pp.
- Lalla, R. V. & Peterson, D. E. 2006. Treatment of mucositis, including new medications. *The Cancer Journal*, 12(5), pp 348-354.
- Lalla, R. V., Schubert, M. M., Bensadoun, R.-J. & Keefe, D. 2006. Anti-inflammatory agents in the management of alimentary mucositis. *Supportive Care in Cancer*, 14(6), pp 558-565.
- Lalla, R. V., Sonis, S. T. & Peterson, D. E. 2008. Management of oral mucositis in patients who have cancer. *Dental Clinics of North America*, 52(1), pp 61-77.
- Lambros, M. P., Parsa, C., Mulamalla, H., Orlando, R., Lau, B., Huang, Y., Pon, D. & Chow, M. 2011. Identifying cell and molecular stress after radiation in a three-dimensional (3-D) model of oral mucositis. *Biochemical and biophysical research communications*, 405(1), pp 102-106.
- Lamson, D. W. & Brignall, M. 1999. Antioxidants in cancer therapy; their actions and interactions with oncologic therapies. *Alternative Medicine Review*, 4(304-329).
- Langer, C. J. 2008. Targeted therapy in head and neck cancer: state of the art 2007 and review of clinical applications. *Cancer*, 112(12), pp 2635-45.
- Lapcık, L., Jr., Lapcık, L., De Smedt, S., Demeester, J. & Chabreck, P. 1998. Hyaluronan: Preparation, Structure, Properties, and Applications. *Chem Rev*, 98(8), pp 2663-2684.
- Larsen, N. E., Lombard, K. M., Parent, E. G. & Balazs, E. A. 1992. Effect of hylan on cartilage and chondrocyte cultures. *Journal of orthopaedic research*, 10(1), pp 23-32.
- Larson, P. J., Miaskowski, C., MacPhail, L., Dodd, M. J., Greenspan, D., Dibble, S. L., Paul, S. M. & Ignoffo, R. 1998. The PRO-SELF Mouth Aware program: an effective approach for reducing chemotherapy-induced mucositis. *Cancer Nurs*, 21(4), pp 263-8.
- Larsson, P., Engqvist, H., Biermann, J., Werner Rönnerman, E., Forssell-Aronsson, E., Kovács, A., Karlsson, P., Helou, K. & Parris, T. Z. 2020. Optimization of cell viability assays to improve replicability and reproducibility of cancer drug sensitivity screens. *Scientific Reports*, 10(1), pp 5798.

- Larsson, R., Kristensen, J., Sandberg, C. & Nygren, P. 1992. Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia, using a fluorometric microculture cytotoxicity assay (FMCA). *International journal of cancer*, 50(2), pp 177-185.
- Larsson, R., Nygren, P., Ekberg, M. & Slater, L. 1990. Chemotherapeutic drug sensitivity testing of human leukemia cells in vitro using a semiautomated fluorometric assay. *Leukemia*, 4(8), pp 567-571.
- Lasheen, S., Shohdy, K. S., Kassem, L. & Abdel-Rahman, O. 2017. Fatigue, alopecia and stomatitis among patients with breast cancer receiving cyclin-dependent kinase 4 and 6 inhibitors: a systematic review and meta-analysis. *Expert Rev Anticancer Ther*, 17(9), pp 851-856.
- Laurent, T. C. 1998. Biology and Medical Applications of Hyaluronan and its Derivatives. In: *The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives*. (TC Laurent, ed.). Wenner-Gren Symp. Series, Portland Press.
- Laurent, T. C. & Fraser, J. R. 1992. Hyaluronan. *Faseb j*, 6(7), pp 2397-404.
- Leach, J. K., Van Tuyle, G., Lin, P. S., Schmidt-Ullrich, R. & Mikkelsen, R. B. 2001. Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. *Cancer Research*, 61(10), pp 3894-3901.
- Lee, H., Kim, J. B., Park, S. Y., Kim, S. S. & Kim, H. 2013. Combination effect of paclitaxel and hyaluronic acid on cancer stem-like side population cells. *J Biomed Nanotechnol*, 9(2), pp 299-302.
- Lee, H., Yeom, M., Shin, S., Jeon, K., Park, D. & Jung, E. 2019. Protective Effects of Aqueous Extract of *Mentha suaveolens* against Oxidative Stress-Induced Damages in Human Keratinocyte HaCaT Cells. *Evidence-Based Complementary and Alternative Medicine*, 2019(5045491).
- Lee, J.-J., Ng, S.-C., Hsu, J.-Y., Liu, H., Chen, C.-J., Huang, C.-Y. & Kuo, W.-W. 2022. Galangin Reverses H₂O₂-Induced Dermal Fibroblast Senescence via SIRT1-PGC-1 α /Nrf2 Signaling. *International Journal of Molecular Sciences*, 23(3), pp 1387.
- Lee, J. Y. & Spicer, A. P. 2000. Hyaluronan: a multifunctional, megaDalton, stealth molecule. *Current opinion in cell biology*, 12(5), pp 581-586.
- Lepperdinger, G., Fehrer, C. & Reitingner, S. 2004. *Biodegradation of hyaluronan*: Elsevier Press: Amsterdam.
- Levy-Polack, M. P., Sebelli, P. & Polack, N. L. 1998. Incidence of oral complications and application of a preventive protocol in children with acute leukemia. *Spec Care Dentist*, 18(5), pp 189-93.

- Levy, A., Blanchard, P., Bellefqih, S., Brahimi, N., Guigay, J., Janot, F., Temam, S., Bourhis, J., Deutsch, E. & Daly-Schveitzer, N. 2014. Concurrent use of cisplatin or cetuximab with definitive radiotherapy for locally advanced head and neck squamous cell carcinomas. *Strahlentherapie und Onkologie*, 190(9), pp 823-831.
- Li, C.-H., Ko, J.-L., Ou, C.-C., Lin, W.-L., Yen, C.-C., Hsu, C.-T. & Hsiao, Y.-P. 2019. The Protective Role of GMI, an Immunomodulatory Protein From *Ganoderma microsporum*, on 5-Fluorouracil-Induced Oral and Intestinal Mucositis. *Integrative Cancer Therapies*, 18(1534735419833795).
- Li, C., Hong, Y., Tao, X.-A., Xia, J. & Cheng, B. 2011. The correlation between the severity of radiotherapy-induced glossitis and endothelial cell injury in local tissues in a rat model. *Med Oral Patol Oral Cir Bucal*, 16(6), pp e711-5.
- Li, G., Huang, Z., Chen, X. & Wei, Q. 2010. Role of human papillomavirus and cell cycle-related variants in squamous cell carcinoma of the oropharynx. *Journal of Biomedical Research*, 24(5), pp 339-346.
- Li, J. M., Chou, H. C., Wang, S. H., Wu, C. L., Chen, Y. W., Lin, S. T., Chen, Y. H. & Chan, H. L. 2013. Hyaluronic acid-dependent protection against UVB-damaged human corneal cells. *Environmental and molecular mutagenesis*, 54(6), pp 429-449.
- Li, N., Alam, J., Venkatesan, M. I., Eiguren-Fernandez, A., Schmitz, D., Di Stefano, E., Slaughter, N., Killeen, E., Wang, X., Huang, A., Wang, M., Miguel, A. H., Cho, A., Sioutas, C. & Nel, A. E. 2004. Nrf2 Is a Key Transcription Factor That Regulates Antioxidant Defense in Macrophages and Epithelial Cells: Protecting against the Proinflammatory and Oxidizing Effects of Diesel Exhaust Chemicals. *The Journal of Immunology*, 173(5), pp 3467-3481.
- Lim, J. Y., Lim, Y. C., Kim, S. H., Kim, J. W., Jeong, H. M. & Choi, E. C. 2010. Predictive factors of isolated distant metastasis after primary definitive surgery without systemic treatment for head and neck squamous cell carcinoma. *Oral Oncol*, 46(7), pp 504-8.
- Lima, V., Brito, G. A. C., Cunha, F. Q., Rebouças, C. G., Falcão, B. A. A., Augusto, R. F., Souza, M. L. P., Leitão, B. T. & Ribeiro, R. A. 2005. Effects of the tumour necrosis factor-alpha inhibitors pentoxifylline and thalidomide in short-term experimental oral mucositis in hamsters. *European Journal of Oral Sciences*, 113(3), pp 210-217 8p.
- Limaye, S. A., Haddad, R. I., Cilli, F., Sonis, S. T., Colevas, A. D., Brennan, M. T., Hu, K. S. & Murphy, B. A. 2013. Phase 1b, multicenter, single blinded, placebo-controlled, sequential dose escalation study to assess the safety and tolerability of topically applied AG013 in subjects with locally advanced head and neck cancer receiving induction chemotherapy. *Cancer*, 119(24), pp 4268-4276.

- Lin, B., Williams-Skipp, C., Tao, Y., Schleicher, M. S., Cano, L. L., Duke, R. C. & Scheinman, R. I. 1999. NF- κ B functions as both a proapoptotic and antiapoptotic regulatory factor within a single cell type. *Cell Death Differ*, 6(6), pp 570-582.
- Lin, K.-Y., Chung, C.-H., Ciou, J.-S., Su, P.-F., Wang, P.-W., Shieh, D.-B. & Wang, T.-C. 2019. Molecular damage and responses of oral keratinocyte to hydrogen peroxide. *BMC Oral Health*, 19(1), pp 1-10.
- Lin, X. B., Dieleman, L. A., Ketabi, A., Bibova, I., Sawyer, M. B., Xue, H., Field, C. J., Baracos, V. E. & Gänzle, M. G. 2012. Irinotecan (CPT-11) Chemotherapy Alters Intestinal Microbiota in Tumour Bearing Rats. *PLOS ONE*, 7(7), pp e39764.
- Lin, Y.-K., Matsumoto, Y., Kuroyanagi, Y. & Kagawa, S. 2009. A Bilayer Hyaluronic Acid Wound Dressing to Promote Wound Healing in Diabetic Ulcer. *Journal of Bioactive and Compatible Polymers*, 24(5), pp 424-443.
- Lindhagen, E., Nygren, P. & Larsson, R. 2008. The fluorometric microculture cytotoxicity assay. *Nat Protoc*, 3(8), pp 1364-9.
- Lindhagen, E., Rickardson, L., Elliott, G., Leoni, L., Nygren, P., Larsson, R. & Åleskog, A. 2007. Pharmacological profiling of novel non-COX-inhibiting indole-pyran analogues of etodolac reveals high solid tumour activity of SDX-308 in vitro. *Investigational new drugs*, 25(4), pp 297-303.
- List, M. A., D'Antonio, L. L., Cella, D. F., Siston, A., Mumby, P., Haraf, D. & Vokes, E. 1996. The Performance Status Scale for Head and Neck Cancer Patients and the Functional Assessment of Cancer Therapy-Head and Neck Scale. A study of utility and validity. *Cancer*, 77(11), pp 2294-301.
- Liu, R.-M., Sun, R.-G., Zhang, L.-T., Zhang, Q.-F., Chen, D.-X., Zhong, J.-J. & Xiao, J.-H. 2016. Hyaluronic acid enhances proliferation of human amniotic mesenchymal stem cells through activation of Wnt/ β -catenin signaling pathway. *Experimental Cell Research*, 345(2), pp 218-229.
- Liu, T. & Zhao, H. 2014. [Setting up the oxidative cell mode with hippocampal cell of primary culture induced by H₂O₂]. *Wei Sheng Yan Jiu = Journal Of Hygiene Research*, 43(5), pp 719.
- Liu, Y.-Y., Lee, C.-H., Dedaj, R., Zhao, H., Mrabat, H., Sheidlin, A., Syrkina, O., Huang, P.-M., Garg, H. G. & Hales, C. A. 2008. High-molecular-weight hyaluronan—a possible new treatment for sepsis-induced lung injury: a preclinical study in mechanically ventilated rats. *Critical Care*, 12(4), pp R102.
- Liu, Z., Xie, W., Li, M., Teng, N., Liang, X., Zhang, Z., Yang, Z. & Wang, X. 2017. Oral Administration of Polaprezinc Attenuates Fluorouracil-induced Intestinal Mucositis in a Mouse Model. *Basic & clinical pharmacology & toxicology*, 121(6), pp 480-486.

- Lliakis, G. 1991. The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. *BioEssays*, 13(12), pp 641.
- Lockhart, P. B. & Sonis, S. T. 1979. Relationship of oral complications to peripheral blood leukocyte and platelet counts in patients receiving cancer chemotherapy. *Oral Surgery, Oral Medicine, Oral Pathology*, 48(1), pp 21-28.
- Lockhart, P. B. & Sonis, S. T. 1981. Alterations in the oral mucosa caused by chemotherapeutic agents. A histologic study. *The Journal Of Dermatologic Surgery And Oncology*, 7(12), pp 1019-1025.
- Lockington, D., Agarwal, P., Young, D., Caslake, M. & Ramaesh, K. 2014. Antioxidant properties of amniotic membrane: novel observations from a pilot study. *Can J Ophthalmol*, 49(5), pp 426-30.
- Logan, R. M., Al-Azri, A. R., Bossi, P., Stringer, A. M., Joy, J. K., Soga, Y., Ranna, V., Vaddi, A., Raber-Durlacher, J. E., Lalla, R. V., Cheng, K. K. F. & Elad, S. 2020. Systematic review of growth factors and cytokines for the management of oral mucositis in cancer patients and clinical practice guidelines. *Support Care Cancer*, 28(5), pp 2485-2498.
- Logan, R. M., Gibson, R. J., Sonis, S. T. & Keefe, D. M. K. 2007a. Nuclear factor- κ B (NF- κ B) and cyclooxygenase-2 (COX-2) expression in the oral mucosa following cancer chemotherapy. *Oral Oncology*, 43(4), pp 395-401.
- Logan, R. M., Stringer, A. M., Bowen, J. M., Gibson, R. J., Sonis, S. T. & Keefe, D. M. 2008. Serum levels of NFkappaB and pro-inflammatory cytokines following administration of mucotoxic drugs. *Cancer Biol Ther*, 7(7), pp 1139-45.
- Logan, R. M., Stringer, A. M., Bowen, J. M., Gibson, R. J., Sonis, S. T. & Keefe, D. M. K. 2009. Is the pathobiology of chemotherapy-induced alimentary tract mucositis influenced by the type of mucotoxic drug administered? *Cancer Chemotherapy and Pharmacology*, 63(2), pp 239-251.
- Logan, R. M., Stringer, A. M., Bowen, J. M., Yeoh, A. S., Gibson, R. J., Sonis, S. T. & Keefe, D. M. 2007b. The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: pathobiology, animal models and cytotoxic drugs. *Cancer Treat Rev*, 33(5), pp 448-60.
- Lokeshwar, V. B., Mirza, S. & Jordan, A. 2014. Targeting hyaluronic acid family for cancer chemoprevention and therapy. *Advances in cancer research*, 123(35-65).
- Longley, D. B., Harkin, D. P. & Johnston, P. G. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. *Nature reviews cancer*, 3(5), pp 330-338.

- Lotze, M. T., Zeh, H. J., Rubartelli, A., Sparvero, L. J., Amoscato, A. A., Washburn, N. R., DeVera, M. E., Liang, X., Tör, M. & Billiar, T. 2007. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunological reviews*, 220(1), pp 60-81.
- Luissint, A.-C., Parkos, C. A. & Nusrat, A. 2016. Inflammation and the Intestinal Barrier: Leukocyte–Epithelial Cell Interactions, Cell Junction Remodeling, and Mucosal Repair. *Gastroenterology*, 151(4), pp 616-632.
- Luo, Y., Kirker, K. R. & Prestwich, G. D. 2000. Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of controlled release*, 69(1), pp 169-184.
- Lurie, Z., Offer, T., Russo, A., Samuni, A. & Nitzan, D. 2003. Do stable nitroxide radicals catalyze or inhibit the degradation of hyaluronic acid? *Free Radic Biol Med*, 35(2), pp 169-78.
- Lym, H. S., Suh, Y. & Park, C. K. 2004. Effects of hyaluronic acid on the polymorphonuclear leukocyte (PMN) release of active oxygen and protection of bovine corneal endothelial cells from activated PMNs. *Korean Journal of Ophthalmology*, 18(1), pp 23-28.
- Ma, B. & Hottiger, M. O. 2016. Crosstalk between Wnt/ β -catenin and NF- κ B signaling pathway during inflammation. *Frontiers in immunology*, 7(378).
- Ma, C., Yue, Q.-X., Guan, S.-H., Wu, W.-Y., Yang, M., Jiang, B.-H., Liu, X. & Guo, D.-A. 2009. Proteomic analysis of possible target-related proteins of cyclophosphamide in mice thymus. *Food and chemical toxicology*, 47(8), pp 1841-1847.
- MacDonald, T. T., Bajaj-Elliott, M. & Pender, S. L. F. 1999. T cells orchestrate intestinal mucosal shape and integrity. *Immunology Today*, 20(11), pp 505-510.
- Madara, J. L. 1998. Regulation of the movement of solutes across tight junctions. *Annual review of physiology*, 60(1), pp 143-159.
- Maddalo, M., Borghetti, P., Tomasini, D., Corvò, R., Bonomo, P., Petrucci, A., Paiar, F., Lastrucci, L., Bonù, M. L., Greco, D., Costa, L., Pegurri, L., Triggiani, L., Belgioia, L., Desideri, I., Grisanti, S., Buglione, M. & Magrini, S. M. 2020. Cetuximab and Radiation Therapy Versus Cisplatin and Radiation Therapy for Locally Advanced Head and Neck Cancer: Long-Term Survival and Toxicity Outcomes of a Randomized Phase 2 Trial. *Int J Radiat Oncol Biol Phys*, 107(3), pp 469-477.
- Maddens, S., Charruyer, A., Plo, I., Dubreuil, P., Berger, S., Salles, B., Laurent, G. & Jaffrézou, J.-P. 2002. Kit signaling inhibits the sphingomyelin-ceramide pathway through PLC γ 1: implication in stem cell factor radioprotective effect. *Blood*, 100(4), pp 1294-1301.

- Maggioni, D., Garavello, W., Rigolio, R., Pignataro, L., Gaini, R. & Nicolini, G. 2013. Apigenin impairs oral squamous cell carcinoma growth in vitro inducing cell cycle arrest and apoptosis. *International Journal of Oncology*, 43(5), pp 1675-1682.
- Magrini, S. M., Buglione, M., Corvò, R., Pirtoli, L., Paiar, F., Ponticelli, P., Petrucci, A., Bacigalupo, A., Crociani, M., Lastrucci, L., Vecchio, S., Bonomo, P., Pasinetti, N., Triggiani, L., Cavagnini, R., Costa, L., Tonoli, S., Maddalo, M. & Grisanti, S. 2016. Cetuximab and Radiotherapy Versus Cisplatin and Radiotherapy for Locally Advanced Head and Neck Cancer: A Randomized Phase II Trial. *J Clin Oncol*, 34(5), pp 427-35.
- Maharjan, A. S., Pilling, D. & Gomer, R. H. 2011. High and low molecular weight hyaluronic acid differentially regulate human fibrocyte differentiation. *PloS one*, 6(10), pp e26078.
- Maiya, G. A., Sagar, M. & Fernandes, D. 2006. Effect of low level helium-neon (He-Ne) laser therapy in the prevention & treatment of radiation induced mucositis in head & neck cancer patients. *Indian Journal of medical research*, 124(4), pp 399.
- Maleki, A., Kjøniksen, A.-L. & Nyström, B. 2008. Effect of pH on the Behavior of Hyaluronic Acid in Dilute and Semidilute Aqueous Solutions. *Macromolecular Symposia*, 274(1), pp 131-140.
- Malson, T. & Lindqvist, B. L. 1987. Gel of crosslinked hyaluronic acid for use as a vitreous humor substitute. Google Patents.
- Man, W., Ming, D., Fang, D., Chao, L. & Jing, C. 2014. Dimethyl sulfoxide attenuates hydrogen peroxide-induced injury in cardiomyocytes via heme oxygenase-1. *Journal of cellular biochemistry*, 115(6), pp 1159-1165.
- Maňáková, Š., Puttonen, K. A., Raasmaja, A. & Männistö, P. T. 2003. Ara-C induces apoptosis in monkey fibroblast cells. *Toxicology in Vitro*, 17(3), pp 367-373.
- Manzano, M., Bueno, P., Rueda, R., Ramirez-Tortosa, C. L., Prieto, P. A. & Lopez-Pedrosa, J. M. 2007. Intestinal toxicity induced by 5-fluorouracil in pigs: a new preclinical model. *Chemotherapy*, 53(5), pp 344-355.
- Manzi, N. d. M., Silveira, R. C. d. C. P. & Reis, P. E. D. d. 2016. Prophylaxis for mucositis induced by ambulatory chemotherapy: systematic review. *Journal of advanced nursing*, 72(4), pp 735-746.
- Maree, J. E., Combrink, M. J., De Lange, T., Toerien, A. S. & Bedeker, M. 2012. Incidence, severity and management of cancer chemotherapy related oral mucositis in Eastern Cape and Western Cape. *Health SA Gesondheid (Online)*, 17(1), pp 1-7.

- Maria, O. M., Eliopoulos, N. & Muanza, T. 2017. Radiation-induced oral mucositis. *Frontiers in oncology*, 7(89).
- Mariggiò, M. A., Cassano, A., Vinella, A., Vincenti, A., Fumarulo, R., Muzio, L. L., Maiorano, E., Ribatti, D. & Favia, G. 2009. Enhancement of fibroblast proliferation, collagen biosynthesis and production of growth factors as a result of combining sodium hyaluronate and aminoacids. *International journal of immunopathology and pharmacology*, 22(2), pp 485-492.
- Marthey, L., Mateus, C., Mussini, C., Nachury, M., Nancey, S., Grange, F., Zallot, C., Peyrin-Biroulet, L., Rahier, J. F., Bourdier de Beauregard, M., Mortier, L., Coutzac, C., Soularue, E., Lanoy, E., Kapel, N., Planchard, D., Chaput, N., Robert, C. & Carbonnel, F. 2016. Cancer Immunotherapy with Anti-CTLA-4 Monoclonal Antibodies Induces an Inflammatory Bowel Disease. *J Crohns Colitis*, 10(4), pp 395-401.
- Maselli, R., Grembiale, R. D., Pelaia, G. & Cuda, G. 2002. Oxidative stress and lung diseases. *Monaldi Arch Chest Dis*, 57(3-4), pp 180-1.
- Matsumura, G., Herp, A. & Pigman, W. 1966. Depolymerization of hyaluronic acid by autoxidants and radiations. *Radiation research*, 28(4), pp 735-752.
- Mattsson, T., Heimdahl, A., Dahllof, G., Nilsson, B., Lonnqvist, B. & Ringdén, O. 1991. Variables predicting oral mucosal lesions in allogenic bone marrow recipients. *Head & neck*, 13(3), pp 224-229.
- Mattsson, T., Sundqvist, K. G., Heimdahl, A., Dahllöf, G., Ljungman, P. & Ringdén, O. 1992. A comparative immunological analysis of the oral mucosa in chronic graft-versus-host disease and oral lichen planus. *Archives of Oral Biology*, 37(7), pp 539-547.
- Mayo, B. J., Stringer, A. M., Bowen, J. M., Bateman, E. H. & Keefe, D. M. 2017. Irinotecan-induced mucositis: the interactions and potential role of GLP-2 analogues. *Cancer Chemotherapy and Pharmacology*, 79(2), pp 233-249.
- Mazzucco, A. 2019. Hyaluronic acid: evaluation of efficacy with different molecular weights. *Int. J. Chem. Res*, 1(13-18).
- McCarthy, G. M., Awde, J. D., Ghandi, H., Vincent, M. & Kocha, W. I. 1998. Risk factors associated with mucositis in cancer patients receiving 5-fluorouracil. *Oral Oncology*, 34(6), pp 484-490.
- McCarthy, K. M., Skare, I. B., Stankewich, M. C., Furuse, M., Tsukita, S., Rogers, R. A., Lynch, R. D. & Schneeberger, E. E. 1996. Occludin is a functional component of the tight junction. *Journal of cell science*, 109(9), pp 2287-2298.

- McDonald, J. T., Kim, K., Norris, A. J., Vlashi, E., Phillips, T. M., Lagadec, C., Della Donna, L., Ratikan, J., Szelag, H., Hlatky, L. & McBride, W. H. 2010. Ionizing Radiation Activates the Nrf2 Antioxidant Response. *Cancer Research*, 70(21), pp 8886-8895.
- McGuire, D., Altomonte, V., Peterson, D., Wingard, J., Jones, R. J. & Grochow, L. B. Patterns of mucositis and pain in patients receiving preparative chemotherapy and bone marrow transplantation. *Oncology nursing forum*, 1992. 1493-1502.
- McGuire, D., Owen, D. & Peterson, D. Nursing interventions for acute oral pain and mucositis. *Oncol Nurs Forum*, 1998. 341.
- McGuire, D. B., Correa, M. E., Johnson, J. & Wienandts, P. 2006. The role of basic oral care and good clinical practice principles in the management of oral mucositis. *Support Care Cancer*, 14(6), pp 541-7.
- McGuire, D. B., Peterson, D. E., Muller, S., Owen, D. C., Slemmons, M. F. & Schubert, M. M. 2002. The 20 item oral mucositis index: reliability and validity in bone marrow and stem cell transplant patients. *Cancer Invest*, 20(7-8), pp 893-903.
- McKee, C. M., Lowenstein, C. J., Horton, M. R., Wu, J., Bao, C., Chin, B. Y., Choi, A. M. & Noble, P. W. 1997. Hyaluronan fragments induce nitric-oxide synthase in murine macrophages through a nuclear factor kappaB-dependent mechanism. *J Biol Chem*, 272(12), pp 8013-8.
- McKee, C. M., Penno, M. B., Cowman, M., Burdick, M. D., Strieter, R. M., Bao, C. & Noble, P. W. 1996. Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J Clin Invest*, 98(10), pp 2403-13.
- McManus, L. M., Ostrom, K. K., Lear, C., Luce, E. B., Gander, D. L., Pinckard, R. N. & Redding, S. W. 1993. Radiation-induced increased platelet-activating factor activity in mixed saliva. *Lab Invest*, 68(1), pp 118-24.
- Melichar, B., Dvořák, J., Hyšpler, R. & Zadák, Z. 2005. Intestinal permeability in the assessment of intestinal toxicity of cytotoxic agents. *Chemotherapy*, 51(6), pp 336-338.
- Melo, M. L., Brito, G. A., Soares, R. C., Carvalho, S. B., Silva, J. V., Soares, P. M., Vale, M. L., Souza, M. H., Cunha, F. Q. & Ribeiro, R. A. 2008. Role of cytokines (TNF-alpha, IL-1beta and KC) in the pathogenesis of CPT-11-induced intestinal mucositis in mice: effect of pentoxifylline and thalidomide. *Cancer Chemother Pharmacol*, 61(5), pp 775-84.
- Mendonça, R. M. H. d., Araújo, M. d., Levy, C. E., Morari, J., Silva, R. A., Yunes, J. A. & Brandalise, S. R. 2015. Oral Mucositis in Pediatric Acute Lymphoblastic Leukemia Patients: Evaluation of Microbiological and Hematological Factors. *Pediatric Hematology and Oncology*, 32(5), pp 322-330.

- Mendoza, G., Alvarez, A. I., Pulido, M. M., Molina, A. J., Merino, G., Real, R., Fernandes, P. & Prieto, J. G. 2007. Inhibitory effects of different antioxidants on hyaluronan depolymerization. *Carbohydr Res*, 342(1), pp 96-102.
- Mendoza, G., Prieto, J. G., Real, R., Perez, M., Merino, G. & Alvarez, A. I. 2009. Antioxidant Profile of Hyaluronan: Physico-Chemical Features and its Role in Pathologies. *Mini Reviews in Medicinal Chemistry*, 9(13), pp 1479-1488.
- Menezes-Garcia, Z., Arifa, N., Fagundes, C. T. & Souza, D. G. Mechanisms Underlying Chemotherapy-Associated Mucositis : The Role of Inflammatory Mediators and Potential Therapeutic Targets. 2018.
- Mercadante, S., Aielli, F., Adile, C., Ferrera, P., Valle, A., Fusco, F., Caruselli, A., Cartoni, C., Massimo, P., Masedu, F., Valenti, M. & Porzio, G. 2015. Prevalence of oral mucositis, dry mouth, and dysphagia in advanced cancer patients. *Support Care Cancer*, 23(11), pp 3249-55.
- Meredith, J. E., Fazeli, B. & Schwartz, M. A. 1993. The extracellular matrix as a cell survival factor. *Molecular Biology of the Cell*, 4(9), pp 953-961.
- Merlano, M., Corvo, R., Margarino, G., Benasso, M., Rosso, R., Sertoli, M., Cavallari, M., Scala, M., Guenzi, M. & Santi, L. 1991. Combined chemotherapy and radiation therapy in advanced inoperable squamous cell carcinoma of the head and neck. The final report of a randomized trial. *Cancer*, 67(4), pp 915-921.
- Mero, A. & Campisi, M. 2014. Hyaluronic acid bioconjugates for the delivery of bioactive molecules. *Polymers*, 6(2), pp 346-369.
- Meulendijks, D., Cats, A., Beijnen, J. H. & Schellens, J. H. M. 2016. Improving safety of fluoropyrimidine chemotherapy by individualizing treatment based on dihydropyrimidine dehydrogenase activity – Ready for clinical practice? *Cancer Treatment Reviews*, 50(23-34).
- Meulendijks, D., Henricks, L. M., Sonke, G. S., Deenen, M. J., Froehlich, T. K., Amstutz, U., Largiadèr, C. R., Jennings, B. A., Marinaki, A. M., Sanderson, J. D., Kleibl, Z., Kleiblova, P., Schwab, M., Zanger, U. M., Palles, C., Tomlinson, I., Gross, E., van Kuilenburg, A. B. P., Punt, C. J. A., Koopman, M., Beijnen, J. H., Cats, A. & Schellens, J. H. M. 2015. Clinical relevance of *DPYD* variants c.1679T>G, c.1236G>A/HapB3, and c.1601G>A as predictors of severe fluoropyrimidine-associated toxicity: a systematic review and meta-analysis of individual patient data. *The Lancet Oncology*, 16(16), pp 1639-1650.
- Migliorati, C., Hewson, I., Lalla, R. V., Antunes, H. S., Estilo, C. L., Hodgson, B., Lopes, N. N. F., Schubert, M. M., Bowen, J. & Elad, S. 2013. Systematic review of laser and other light therapy for the management of oral mucositis in cancer patients. *Supportive Care in Cancer*, 21(1), pp 333-341.

- Migliorati, C. A., Oberle-Edwards, L. & Schubert, M. 2006. The role of alternative and natural agents, cryotherapy, and/or laser for management of alimentary mucositis. *Supportive Care in Cancer*, 14(6), pp 533-540.
- Mihandoost, E., Shirazi, A., Mahdavi, S. R. & Aliasgharzadeh, A. 2014. Can melatonin help us in radiation oncology treatments? *Biomed Res Int*, 2014(578137).
- Milas, M. & Rinaudo, M. 2004. Characterization and properties of hyaluronic acid (hyaluronan). In: *Polysaccharides: Structural Diversity and Functional Versatility*. 2nd ed. Sherbrooke(Quebec): Marcel Dekker Inc., pp. 535-550.
- Miller, M. M., Donald, D. V. & Hagemann, T. M. 2012. Prevention and treatment of oral mucositis in children with cancer. *The Journal of Pediatric Pharmacology and Therapeutics*, 17(4), pp 340-350.
- Miranda-Silva, W., Gomes-Silva, W., Zadik, Y., Yarom, N., Al-Azri, A. R., Hong, C. H. L., Ariyawardana, A., Saunders, D. P., Correa, M. E., Arany, P. R., Bowen, J., Cheng, K. K. F., Tissing, W. J. E., Bossi, P. & Elad, S. 2021. MASCC/ISOO clinical practice guidelines for the management of mucositis: sub-analysis of current interventions for the management of oral mucositis in pediatric cancer patients. *Support Care Cancer*, 29(7), pp 3539-3562.
- Mironov, V., Kasyanov, V., Shu, X. Z., Eisenberg, C., Eisenberg, L., Gonda, S., Trusk, T., Markwald, R. R. & Prestwich, G. D. 2005. Fabrication of tubular tissue constructs by centrifugal casting of cells suspended in an in situ crosslinkable hyaluronan-gelatin hydrogel. *Biomaterials*, 26(36), pp 7628-7635.
- Misra, S., Hascall, V. C., Markwald, R. R. & Ghatak, S. 2015. Interactions between hyaluronan and its receptors (CD44, RHAMM) regulate the activities of inflammation and cancer. *Frontiers in immunology*, 6(201).
- Mitic, L. L., Van Itallie, C. M. & Anderson, J. M. 2000. Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 279(2), pp G250-G254.
- Mittmann, N., Verma, S., Koo, M., Alloul, K. & Trudeau, M. 2010. Cost effectiveness of TAC versus FAC in adjuvant treatment of node-positive breast cancer. *Curr Oncol*, 17(1), pp 7-16.
- Mizuno, H., Miyai, H., Yokoi, A., Kobayashi, T., Inabu, C., Maruyama, T., Ekuni, D., Mizukawa, N., Kariya, S. & Nishizaki, K. 2019. Relationship between renal dysfunction and oral mucositis in patients undergoing concurrent chemoradiotherapy for pharyngeal cancer: a retrospective cohort study. *in vivo*, 33(1), pp 183-189.

- Mo, W., Yang, C., Liu, Y., He, Y., Wang, Y. & Gao, F. 2011. The influence of hyaluronic acid on vascular endothelial cell proliferation and the relationship with ezrin/merlin expression. *Acta Biochimica et Biophysica Sinica*, 43(12), pp 930-939.
- Moharamzadeh, K., Brook, I., Van Noort, R., Scutt, A. & Thornhill, M. 2007. Tissue-engineered oral mucosa: a review of the scientific literature. *Journal of dental research*, 86(2), pp 115-124.
- Moll, S. J., Jones, C. J., Crocker, I. P., Baker, P. N. & Heazell, A. E. 2007. Epidermal growth factor rescues trophoblast apoptosis induced by reactive oxygen species. *Apoptosis*, 12(9), pp 1611-1622.
- Monheit, G. D. & Coleman, K. M. 2006. Hyaluronic acid fillers. *Dermatol Ther*, 19(3), pp 141-50.
- Montagner, I. M., Merlo, A., Carpanese, D., Zuccolotto, G., Renier, D., Campisi, M., Pasut, G., Zanovello, P. & Rosato, A. 2015. Drug conjugation to hyaluronan widens therapeutic indications for ovarian cancer. *Oncoscience*, 2(4), pp 373-381.
- Montassier, E., Batard, E., Massart, S., Gastinne, T., Carton, T., Caillon, J., Le Fresne, S., Caroff, N., Hardouin, J. B., Moreau, P., Potel, G., Le Vacon, F. & de La Cochetière, M. F. 2014. 16S rRNA Gene Pyrosequencing Reveals Shift in Patient Faecal Microbiota During High-Dose Chemotherapy as Conditioning Regimen for Bone Marrow Transplantation. *Microbial Ecology*, 67(3), pp 690-699.
- Montassier, E., Gastinne, T., Vangay, P., Al-Ghalith, G. A., Bruley des Varannes, S., Massart, S., Moreau, P., Potel, G., de La Cochetière, M. F., Batard, E. & Knights, D. 2015. Chemotherapy-driven dysbiosis in the intestinal microbiome. *Aliment Pharmacol Ther*, 42(5), pp 515-28.
- Moreland, L. W. 2003. Intra-articular hyaluronan (hyaluronic acid) and hylans for the treatment of osteoarthritis: mechanisms of action. *Arthritis Res Ther*, 5(2), pp 54.
- Mori, M., Yamaguchi, M., Sumitomo, S. & Takai, Y. 2004. Hyaluronan-based biomaterials in tissue engineering. *Acta Histochemica et Cytochemica*, 37(1), pp 1-5.
- Moriguchi, T., Toyoshima, F., Masuyama, N., Hanafusa, H., Gotoh, Y. & Nishida, E. 1997. A novel SAPK/JNK kinase, MKK7, stimulated by TNF α and cellular stresses. *The EMBO Journal*, 16(23), pp 7045-7053.
- Mortensen, H. R., Overgaard, J., Specht, L., Overgaard, M., Johansen, J., Evensen, J. F., Andersen, L. J., Andersen, E. & Grau, C. 2012. Prevalence and peak incidence of acute and late normal tissue morbidity in the DAHANCA 6&7 randomised trial with accelerated radiotherapy for head and neck cancer. *Radiother Oncol*, 103(1), pp 69-75.

- Moseley, R., Leaver, M., Walker, M., Waddington, R., Parsons, D., Chen, W. & Embury, G. 2002. Comparison of the antioxidant properties of HYAFF®-11p75, AQUACEL® and hyaluronan towards reactive oxygen species in vitro. *Biomaterials*, 23(10), pp 2255-2264.
- Moseley, R., Walker, M., Waddington, R. J. & Chen, W. Y. J. 2003. Comparison of the antioxidant properties of wound dressing materials--carboxymethylcellulose, hyaluronan benzyl ester and hyaluronan, towards polymorphonuclear leukocyte-derived reactive oxygen species. *Biomaterials*, 24(9), pp 1549-1557.
- Moslehi, A., Taghizadeh-Ghehi, M., Gholami, K., Hadjibabaie, M., Jahangard-Rafsanjani, Z., Sarayani, A., Javadi, M., Esfandbod, M. & Ghavamzadeh, A. 2014. N-acetyl cysteine for prevention of oral mucositis in hematopoietic SCT: a double-blind, randomized, placebo-controlled trial. *Bone Marrow Transplant*, 49(6), pp 818-23.
- Moslemi, D., Nokhandani, A. M., Otaghsaraei, M. T., Moghadamnia, Y., Kazemi, S. & Moghadamnia, A. A. 2016. Review: Management of chemo/radiation-induced oral mucositis in patients with head and neck cancer: A review of the current literature. *Radiotherapy and Oncology*.
- Moss, R. W. 2007. Do antioxidants interfere with radiation therapy for cancer? *Integrative cancer therapies*, 6(3), pp 281-292.
- Mowat, A. M. & Agace, W. W. 2014. Regional specialization within the intestinal immune system. *Nature Reviews Immunology*, 14(10), pp 667-685.
- Muanza, T. M., Cotrim, A. P., McAuliffe, M., Sowers, A. L., Baum, B. J., Cook, J. A., Feldchtein, F., Amazeen, P., Coleman, C. N. & Mitchell, J. B. 2005. Evaluation of radiation-induced oral mucositis by optical coherence tomography. *Clin Cancer Res*, 11(14), pp 5121-7.
- Muller, S., Yeager, K., Woodruff, N. H., Peterson, D. & McGuire, D. 1999. Factors Influencing The Development And Severity Of Oral Mucositis In Bone Marrow And Stem Cell Transplant Patients. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, & Endodontics*, 87(4), pp 458.
- Murata, M., Fukushima, K., Takao, T., Seki, H., Takeda, S. & Wake, N. 2013. Oxidative stress produced by xanthine oxidase induces apoptosis in human extravillous trophoblast cells. *J Reprod Dev*, 59(1), pp 7-13.
- Murata, Y., Kofuji, K., Nishida, N. & Kamaguchi, R. 2012. Development of film dosage form containing allopurinol for prevention and treatment of oral mucositis. *ISRN Pharm*, 2012(764510).
- Murphy, B. A. 2007. Clinical and economic consequences of mucositis induced by chemotherapy and/or radiation therapy. *The Journal Of Supportive Oncology*, 5(9 Suppl 4), pp 13-21.

- Murphy, B. A., Beaumont, J. L., Isitt, J., Garden, A. S., Gwede, C. K., Trotti, A. M., Meredith, R. F., Epstein, J. B., Le, Q. T., Brizel, D. M., Bellm, L. A., Wells, N. & Cella, D. 2009. Mucositis-related morbidity and resource utilization in head and neck cancer patients receiving radiation therapy with or without chemotherapy. *J Pain Symptom Manage*, 38(4), pp 522-32.
- Murphy, B. A. & Gilbert, J. Dysphagia in head and neck cancer patients treated with radiation: assessment, sequelae, and rehabilitation. *Seminars in radiation oncology*, 2009. Elsevier, 35-42.
- Mytar, B., Siedlar, M., Woloszyn, M., Colizzi, V. & Zembala, M. 2001. Cross-talk between human monocytes and cancer cells during reactive oxygen intermediates generation: The essential role of hyaluronan. *International journal of cancer*, 94(5), pp 727-732.
- Naidu, M. U. R., Ramana, G. V., Rani, P. U., Suman, A. & Roy, P. 2004. Chemotherapy-induced and/or radiation therapy-induced oral mucositis-complicating the treatment of cancer. *Neoplasia*, 6(5), pp 423-431.
- Nakajima, N., Watanabe, S., Kiyoi, T., Tanaka, A., Suemaru, K. & Araki, H. 2015. Evaluation of edaravone against radiation-induced oral mucositis in mice. *J Pharmacol Sci*, 127(3), pp 339-43.
- Nam, Y.-D., Kim, H. J., Seo, J.-G., Kang, S. W. & Bae, J.-W. 2013. Impact of Pelvic Radiotherapy on Gut Microbiota of Gynecological Cancer Patients Revealed by Massive Pyrosequencing. *PLOS ONE*, 8(12), pp e82659.
- Napeñas, J. J., Brennan, M. T., Bahrani-Mougeot, F. K., Fox, P. C. & Lockhart, P. B. 2007. Relationship between mucositis and changes in oral microflora during cancer chemotherapy. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, 103(1), pp 48-59.
- Napeñas, J. J., Brennan, M. T., Coleman, S., Kent, M. L., Noll, J., Frenette, G., Nussbaum, M. L., Mougeot, J.-L., Paster, B. J., Lockhart, P. B. & Bahrani-Mougeot, F. K. 2010. Molecular methodology to assess the impact of cancer chemotherapy on the oral bacterial flora: a pilot study. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, 109(4), pp 554-560.
- National Cancer Institute. 2011. National Cancer Institute. Common Terminology Criteria for Adverse Events (CTCAE). Version 4.0. Published: May 28, 2009 (v4. 03: June 14, 2010).
- National Cancer Institute. 2017. National Cancer Institute. Common Terminology Criteria for Adverse Events (CTCAE). Version 5.0. National Cancer Institute, National Institutes of Health, US Department of Health and Human Services; Published: November 27, 2017

- Navarrete, J., Vásquez, B. & del Sol, M. 2015. Morphoquantitative analysis of the Ileum of C57BL/6 mice (*Mus musculus*) fed with a high-fat diet. *International journal of clinical and experimental pathology*, 8(11), pp 14649.
- Negi, L. M., Jaggi, M., Joshi, V., Ronodip, K. & Talegaonkar, S. 2015. Hyaluronan coated liposomes as the intravenous platform for delivery of imatinib mesylate in MDR colon cancer. *International journal of biological macromolecules*, 73(222-235).
- Neuman, M. G., Nanau, R. M., Oruña-Sanchez, L. & Coto, G. 2015. Hyaluronic acid and wound healing. *J Pharm Pharm Sci*, 18(1), pp 53-60.
- Nguyen, H., Sangha, S., Pan, M., Shin, D. H., Park, H., Mohammed, A. I. & Cirillo, N. 2022. Oxidative Stress and Chemoradiation-Induced Oral Mucositis: A Scoping Review of In Vitro, In Vivo and Clinical Studies. *International Journal of Molecular Sciences*, 23(9), pp 4863.
- Nguyen, T., Nioi, P. & Pickett, C. B. 2009. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *Journal of biological chemistry*, 284(20), pp 13291-13295.
- Nicolatou-Galitis, O., Dardoufas, K., Markoulatos, P., Sotiropoulou-Lontou, A., Kyprianou, K., Kolitsi, G., Pissakas, G., Skarleas, C., Kouloulis, V., Papanicolaou, V., Legakis, N. J. & Velegraki, A. 2001. Oral pseudomembranous candidiasis, herpes simplex virus-1 infection, and oral mucositis in head and neck cancer patients receiving radiotherapy and granulocyte-macrophage colony-stimulating factor (GM-CSF) mouthwash. *Journal of Oral Pathology & Medicine*, 30(8), pp 471-480.
- Nicolatou-Galitis, O., Sarri, T., Bowen, J., Di Palma, M., Kouloulis, V. E., Niscola, P., Riesenbeck, D., Stokman, M., Tissing, W. & Yeoh, E. 2013. Systematic review of anti-inflammatory agents for the management of oral mucositis in cancer patients. *Supportive Care in Cancer*, 21(11), pp 3179-3189.
- Nicolatou-Galitis, O., Velegraki, A., Sotiropoulou-Lontou, A., Dardoufas, K., Kouloulis, V., Kyprianou, K., Kolitsi, G., Skarleas, C., Pissakas, G., Papanicolaou, V. S. & Kouvaris, J. 2006. Effect of fluconazole antifungal prophylaxis on oral mucositis in head and neck cancer patients receiving radiotherapy. *Support Care Cancer*, 14(1), pp 44-51.
- Niepel, M., Hafner, M., Mills, C. E., Subramanian, K., Williams, E. H., Chung, M., Gaudio, B., Barrette, A. M., Stern, A. D., Hu, B., Korkola, J. E., Gray, J. W., Birtwistle, M. R., Heiser, L. M. & Sorger, P. K. 2019. A Multi-center Study on the Reproducibility of Drug-Response Assays in Mammalian Cell Lines. *Cell Syst*, 9(1), pp 35-48.e5.
- Nishii, M., Soutome, S., Kawakita, A., Yutori, H., Iwata, E., Akashi, M., Hasegawa, T., Kojima, Y., Funahara, M., Umeda, M. & Komori, T. 2020. Factors associated with severe oral mucositis and candidiasis in patients undergoing radiotherapy for oral and oropharyngeal carcinomas:

- a retrospective multicenter study of 326 patients. *Supportive Care in Cancer*, 28(3), pp 1069-1075.
- Nishimura, N., Nakano, K., Ueda, K., Kodaira, M., Yamada, S., Mishima, Y., Yokoyama, M., Terui, Y., Takahashi, S. & Hatake, K. 2012. Prospective evaluation of incidence and severity of oral mucositis induced by conventional chemotherapy in solid tumors and malignant lymphomas. *Supportive Care in Cancer*, 20(9), pp 2053-2059.
- Nishina, H., Wada, T. & Katada, T. 2004. Physiological Roles of SAPK/JNK Signaling Pathway. *Journal of Biochemistry*, 136(2), pp 123-126.
- Nobuhiko, Y., Teruo, O. & Yasuhisa, S. 1992. Inflammation responsive degradation of crosslinked hyaluronic acid gels. *Journal of Controlled release*, 22(2), pp 105-116.
- Nonzee, N. J., Dandade, N. A., Markossian, T., Agulnik, M., Argiris, A., Patel, J. D., Kern, R. C., Munshi, H. G., Calhoun, E. A. & Bennett, C. L. 2008. Evaluating the supportive care costs of severe radiochemotherapy-induced mucositis and pharyngitis. *Cancer*, 113(6), pp 1446-1452.
- Noronha, V. R., Araujo, G. S., Gomes, R. T., Iwanaga, S. H., Barbosa, M. C., Abdo, E. N., Ferreira e Ferreira, E., Viana Campos, A. C., Souza, A. A., Abreu, S. R. & Santos, V. R. 2014. Mucoadhesive propolis gel for prevention of radiation-induced oral mucositis. *Curr Clin Pharmacol*, 9(4), pp 359-64.
- Nottage, M., McLachlan, S. A., Brittain, M. A., Oza, A., Hedley, D., Feld, R., Siu, L. L., Pond, G. & Moore, M. J. 2003. Sucralfate mouthwash for prevention and treatment of 5-fluorouracil-induced mucositis: a randomized, placebo-controlled trial. *Support Care Cancer*, 11(1), pp 41-7.
- Nyman, E., Henricson, J., Ghafouri, B., Anderson, C. D. & Kratz, G. 2019. Hyaluronic Acid Accelerates Re-epithelialization and Alters Protein Expression in a Human Wound Model. *Plastic and reconstructive surgery. Global open*, 7(5), pp e2221-e2221.
- Nyman, E., Huss, F., Nyman, T., Junker, J. & Kratz, G. 2013. Hyaluronic acid, an important factor in the wound healing properties of amniotic fluid: in vitro studies of re-epithelialisation in human skin wounds. *Journal of plastic surgery and hand surgery*, 47(2), pp 89-92.
- O'Neill, L. A. 2009. A feed-forward loop involving hyaluronic acid and toll-like receptor-4 as a treatment for colitis? *Gastroenterology*, 137(6), pp 1889-1891.
- O'toole, E. A., Goel, M. & Woodley, D. T. 1996. Hydrogen peroxide inhibits human keratinocyte migration. *Dermatologic Surgery*, 22(6), pp 525-529.

- Oblon, D., Paul, S., Oblon, M. & Malik, S. 1997. Propantheline protects the oral mucosa after high-dose ifosfamide, carboplatin, etoposide and autologous stem cell transplantation. *Bone marrow transplantation*, 20(11), pp 961-963.
- Ogata, Y., Ishibashi, N., Yamaguchi, K., Uchida, S., Kamei, H., Nakayama, G., Hirakawa, H., Tanigawa, M. & Akagi, Y. 2016. Preventive effects of amino-acid-rich elemental diet Elental® on chemotherapy-induced oral mucositis in patients with colorectal cancer: A prospective pilot study. *Supportive Care in Cancer*, 24(2), pp 783-789.
- Oh, E. J., Kang, S. W., Kim, B. S., Jiang, G., Cho, I. H. & Hahn, S. K. 2008. Control of the molecular degradation of hyaluronic acid hydrogels for tissue augmentation. *Journal of Biomedical Materials Research Part A*, 86(3), pp 685-693.
- Oh, E. J., Park, K., Kim, K. S., Kim, J., Yang, J.-A., Kong, J.-H., Lee, M. Y., Hoffman, A. S. & Hahn, S. K. 2010. Target specific and long-acting delivery of protein, peptide, and nucleotide therapeutics using hyaluronic acid derivatives. *Journal of Controlled Release*, 141(1), pp 2-12.
- Oksala, O., Salo, T., Tammi, R., Häkkinen, L., Jalkanen, M., Inki, P. & Larjava, H. 1995a. Expression of proteoglycans and hyaluronan during wound healing. *J Histochem Cytochem*, 43(2), pp 125-35.
- Oksala, O., Salo, T., Tammi, R., Häkkinen, L., Jalkanen, M., Inki, P. & Larjava, H. 1995b. Expression of proteoglycans and hyaluronan during wound healing. *Journal of Histochemistry & Cytochemistry*, 43(2), pp 125-135.
- Ong, Z. Y., Gibson, R. J., Bowen, J. M., Stringer, A. M., Darby, J. M., Logan, R. M., Yeoh, A. S. & Keefe, D. M. 2010. Pro-inflammatory cytokines play a key role in the development of radiotherapy-induced gastrointestinal mucositis. *Radiat Oncol*, 5(22).
- Onodera, Y., Teramura, T., Takehara, T. & Fukuda, K. 2015. Hyaluronic acid regulates a key redox control factor Nrf2 via phosphorylation of Akt in bovine articular chondrocytes. *FEBS Open Bio*, 5(1), pp 476-484.
- Oprenyész, F., Chausson, M., Maquet, V., Dubuc, J. E. & Henrotin, Y. 2013. Protective effect of a new biomaterial against the development of experimental osteoarthritis lesions in rabbit: a pilot study evaluating the intra-articular injection of alginate-chitosan beads dispersed in an hydrogel. *Osteoarthritis and Cartilage*, 21(8), pp 1099-1107.
- Oryan, A., Alemzadeh, E. & Moshiri, A. 2018. Potential role of propolis in wound healing: Biological properties and therapeutic activities. *Biomed Pharmacother*, 98(469-483).
- Pahl, H. L. 1999. Activators and target genes of Rel/NF- κ B transcription factors. *Oncogene*, 18(49), pp 6853.

- Pallone, F. & Monteleone, G. 2001. Mechanisms of tissue damage in inflammatory bowel disease. *Current Opinion in Gastroenterology*, 17(4), pp 307-312.
- Palm, N. W. & Medzhitov, R. 2009. Pattern recognition receptors and control of adaptive immunity. *Immunol Rev*, 227(1), pp 221-33.
- Pang, Z.-J., Chen, Y. & Zhou, M. 2000. Polysaccharide krestin enhances manganese superoxide dismutase activity and mRNA expression in mouse peritoneal macrophages. *The American journal of Chinese medicine*, 28(03n04), pp 331-341.
- Pangtey, B., Kaul, J. M. & Mishra, S. 2017. Histogenesis of Muscularis Mucosa and Muscularis Externa of Stomach: A Human Foetal Study. *J Clin Diagn Res*, 11(8), pp Ac01-ac03.
- Papakonstantinou, E., Roth, M. & Karakiulakis, G. 2012. Hyaluronic acid: A key molecule in skin aging. *Dermato-endocrinology*, 4(3), pp 253-258.
- Paris, F., Fuks, Z., Kang, A., Capodiecici, P., Juan, G., Ehleiter, D., Haimovitz-Friedman, A., Cordon-Cardo, C. & Kolesnick, R. 2001. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *science*, 293(5528), pp 293-297.
- Park, K., Tan, E. H., O'Byrne, K., Zhang, L., Boyer, M., Mok, T., Hirsh, V., Yang, J. C., Lee, K. H., Lu, S., Shi, Y., Kim, S. W., Laskin, J., Kim, D. W., Arvis, C. D., Kölbeck, K., Laurie, S. A., Tsai, C. M., Shahidi, M., Kim, M., Massey, D., Zazulina, V. & Paz-Ares, L. 2016. Afatinib versus gefitinib as first-line treatment of patients with EGFR mutation-positive non-small-cell lung cancer (LUX-Lung 7): a phase 2B, open-label, randomised controlled trial. *Lancet Oncol*, 17(5), pp 577-89.
- Park, W. H. 2013. The effects of exogenous H₂O₂ on cell death, reactive oxygen species and glutathione levels in calf pulmonary artery and human umbilical vein endothelial cells. *Int J Mol Med*, 31(2), pp 471-476.
- Parsons, B., Al-Assaf, S., Navaratnam, S. & Phillips, G. 2002. Comparison of the reactivity of different reactive oxidative species (ROS) towards hyaluronan. *Hyaluronan*. Elsevier, pp 141-150.
- Parsons, J. T. 1994. The effect of radiation on normal tissues of the head and neck. *Management of head and neck cancer: a multidisciplinary approach*, 245-289.
- Patricia, M. P., Francisco, G. G., Francisco, M. D., Rui, A. M. & Pia, L.-J. 2017. The therapeutic effects of apigenin and dexamethasone on 5-fluorouracil-induced oral mucositis – a pilot study using a Syrian hamster model. *Journal of Oral Pathology & Medicine*, 46(2), pp 142-147.
- Patton, K. T. 2019. *Anatomy & physiology / Kevin T. Patton ; author emeritus, Gary A. Thibodeau*, [Adapted International edition]. Elsevier.

- Patton, K. T. & Thibodeau, G. A. 2019. Upper digestive tract. *Anatomy and Physiology*. [Adapted International edition]. ed.: Elsevier, pp 860-881.
- Pauloin, T., Dutot, M., Joly, F., Warnet, J. M. & Rat, P. 2009a. High molecular weight hyaluronan decreases UVB-induced apoptosis and inflammation in human epithelial corneal cells. *Mol Vis*, 15(577-83).
- Pauloin, T., Dutot, M., Liang, H., Chavinier, E., Warnet, J. M. & Rat, P. 2009b. Corneal protection with high-molecular-weight hyaluronan against in vitro and in vivo sodium lauryl sulfate-induced toxic effects. *Cornea*, 28(9), pp 1032-41.
- Pauloin, T., Dutot, M., Warnet, J.-M. & Rat, P. 2008. In vitro modulation of preservative toxicity: high molecular weight hyaluronan decreases apoptosis and oxidative stress induced by benzalkonium chloride. *European Journal of Pharmaceutical Sciences*, 34(4-5), pp 263-273.
- Pauloski, B. R., Rademaker, A. W., Logemann, J. A., Lundy, D., Bernstein, M., McBreen, C., Santa, D., Campanelli, A., Kelchner, L., Klaben, B. & Discekici-Harris, M. 2011. Relation of mucous membrane alterations to oral intake during the first year after treatment for head and neck cancer. *Head Neck*, 33(6), pp 774-9.
- Pedersen, A., Hougen, H. P. & Kenrad, B. 1992. T-lymphocyte subsets in oral mucosa of patients with recurrent aphthous ulceration. *Journal Of Oral Pathology & Medicine: Official Publication Of The International Association Of Oral Pathologists And The American Academy Of Oral Pathology*, 21(4), pp 176-180.
- Pedroso, S., Vieira, A. T., Bastos, R. W., Oliveira, J. S., Cartelle, C. T., Arantes, R. M. E., Soares, P. M. G., Generoso, S. V., Cardoso, V. N., Teixeira, M. M., Nicoli, J. R. & Martins, F. S. 2015. Evaluation of mucositis induced by irinotecan after microbial colonization in germ-free mice. *Microbiology (Reading)*, 161(10), pp 1950-1960.
- Peer, D. & Margalit, R. 2004. Loading mitomycin C inside long circulating hyaluronan targeted nano-liposomes increases its antitumor activity in three mice tumor models. *International Journal of Cancer*, 108(5), pp 780-789.
- Pelle, E., Mammone, T., Maes, D. & Frenkel, K. 2005. Keratinocytes act as a source of reactive oxygen species by transferring hydrogen peroxide to melanocytes. *Journal of investigative dermatology*, 124(4), pp 793-797.
- Peracchia, G. & Salti, C. 1981. Radiotherapy with thrice-a-day fractionation in a short overall time: clinical experiences. *International Journal of Radiation Oncology* Biology* Physics*, 7(1), pp 99-104.

- Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., Castoria, G. & Migliaccio, A. 2020. ROS in cancer therapy: the bright side of the moon. *Exp Mol Med*, 52(2), pp 192-203.
- Peterman, A., Cella, D., Glandon, G., Dobrez, D. & Yount, S. 2001. Mucositis in head and neck cancer: economic and quality-of-life outcomes. *J Natl Cancer Inst Monogr*, 29(45-51).
- Peterson, D. E. 2006. New strategies for management of oral mucositis in cancer patients. *The journal of supportive oncology*, 4(2 Suppl 1), pp 9-13.
- Peterson, D. E., Bensadoun, R. J., Roila, F. & Group, E. G. W. 2011. Management of oral and gastrointestinal mucositis: ESMO Clinical Practice Guidelines. *Ann Oncol*, 22 Suppl 6(suppl 6), pp vi78-84.
- Peterson, D. E., Keefe, D. M. & Sonis, S. T. 2012. New frontiers in mucositis. *Am Soc Clin Oncol Educ Book*, 32(545-551).
- Peterson, D. E. & Lalla, R. V. 2010. Oral mucositis: the new paradigms. *Curr Opin Oncol*, 22(4), pp 318-22.
- Peterson, D. E., O'Shaughnessy, J. A., Rugo, H. S., Elad, S., Schubert, M. M., Viet, C. T., Campbell-Baird, C., Hronek, J., Seery, V. & Divers, J. 2016. Oral mucosal injury caused by mammalian target of rapamycin inhibitors: emerging perspectives on pathobiology and impact on clinical practice. *Cancer medicine*, 5(8), pp 1897-1907.
- Peterson, L. W. & Artis, D. 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature Reviews Immunology*, 14(3), pp 141-153.
- Pfister, D. G., Spencer, S., Adelstein, D., Adkins, D., Anzai, Y., Brizel, D. M., Bruce, J. Y., Busse, P. M., Caudell, J. J., Cmelak, A. J., Colevas, A. D., Eisele, D. W., Fenton, M., Foote, R. L., Galloway, T., Gillison, M. L., Haddad, R. I., Hicks, W. L., Hitchcock, Y. J., Jimeno, A., Leizman, D., Maghami, E., Mell, L. K., Mittal, B. B., Pinto, H. A., Ridge, J. A., Rocco, J. W., Rodriguez, C. P., Shah, J. P., Weber, R. S., Weinstein, G., Witek, M., Worden, F., Yom, S. S., Zhen, W., Burns, J. L. & Darlow, S. D. 2020. Head and Neck Cancers, Version 2.2020, NCCN Clinical Practice Guidelines in Oncology. *Journal of the National Comprehensive Cancer Network J Natl Compr Canc Netw*, 18(7), pp 873-898.
- Piccinini, A. M. & Midwood, K. S. 2010. DAMPening Inflammation by Modulating TLR Signalling. *Mediators of Inflammation*, 2010(672395).
- Picciolo, G., Mannino, F., Irrera, N., Minutoli, L., Altavilla, D., Vaccaro, M., Oteri, G., Squadrito, F. & Pallio, G. 2022. Reduction of oxidative stress blunts the NLRP3 inflammatory cascade in LPS stimulated human gingival fibroblasts and oral mucosal epithelial cells. *Biomedicine & Pharmacotherapy*, 146(112525).

- Pico, J.-L., Avila-Garavito, A. & Naccache, P. 1998. Mucositis: its occurrence, consequences, and treatment in the oncology setting. *The Oncologist*, 3(6), pp 446-451.
- Pigman, W., Rizvi, S. & Holley, H. L. 1961. Depolymerization of hyaluronic acid by the ORD reaction. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, 4(3), pp 240-252.
- Pisoschi, A. M. & Pop, A. 2015. The role of antioxidants in the chemistry of oxidative stress: A review. *Eur J Med Chem*, 97(55-74).
- Platt, V. M. & Szoka Jr, F. C. 2008. Anticancer therapeutics: targeting macromolecules and nanocarriers to hyaluronan or CD44, a hyaluronan receptor. *Molecular pharmaceutics*, 5(4), pp 474-486.
- Pontoppidan, P. E., Shen, R. L., Cilieborg, M. S., Jiang, P., Kissow, H., Petersen, B. L., Thymann, T., Heilmann, C., Müller, K. & Sangild, P. T. 2015. Bovine Colostrum Modulates Myeloablative Chemotherapy-Induced Gut Toxicity in Piglets. *The Journal of Nutrition*, 145(7), pp 1472-1480.
- Porock, D., Nikoletti, S. & Cameron, F. 2004. The Relationship Between Factors That Impair Wound Healing and the Severity of Acute Radiation Skin and Mucosal Toxicities in Head and Neck Cancer. *Cancer Nursing*, 27(1), pp 71-78.
- Prado, C. M., Baracos, V. E., McCargar, L. J., Mourtzakis, M., Mulder, K. E., Reiman, T., Butts, C. A., Scarfe, A. G. & Sawyer, M. B. 2007. Body composition as an independent determinant of 5-fluorouracil-based chemotherapy toxicity. *Clin Cancer Res*, 13(11), pp 3264-8.
- Pratesi, N., Mangoni, M., Mancini, I., Paiar, F., Simi, L., Livi, L., Cassani, S., Buglione, M., Grisanti, S., Almici, C., Polli, C., Saieva, C., Magrini, S. M., Biti, G., Pazzagli, M. & Orlando, C. 2011. Association between single nucleotide polymorphisms in the XRCC1 and RAD51 genes and clinical radiosensitivity in head and neck cancer. *Radiother Oncol*, 99(3), pp 356-61.
- Prehm, P. 1984. Hyaluronate is synthesized at plasma membranes. *Biochemical Journal*, 220(2), pp 597-600.
- Prehm, P. & Schumacher, U. 2004. Inhibition of hyaluronan export from human fibroblasts by inhibitors of multidrug resistance transporters. *Biochemical pharmacology*, 68(7), pp 1401-1410.
- Presti, D. & Scott, J. E. 1994. Hyaluronan-mediated protective effect against cell damage caused by enzymatically produced hydroxyl (OH·) radicals is dependent on hyaluronan molecular mass. *Cell Biochemistry and Function: Cellular biochemistry and its modulation by active agents or disease*, 12(4), pp 281-288.

- Prestwich, G. D. & Kuo, J.-w. 2008. Chemically-modified HA for therapy and regenerative medicine. *Current pharmaceutical biotechnology*, 9(4), pp 242-245.
- Price, R. D., Berry, M. & Navsaria, H. A. 2007. Hyaluronic acid: the scientific and clinical evidence. *Journal of Plastic, Reconstructive & Aesthetic Surgery*, 60(10), pp 1110-1119.
- Price, T. J., Peeters, M., Kim, T. W., Li, J., Cascinu, S., Ruff, P., Suresh, A. S., Thomas, A., Tjulandin, S., Zhang, K., Murugappan, S. & Sidhu, R. 2014. Panitumumab versus cetuximab in patients with chemotherapy-refractory wild-type KRAS exon 2 metastatic colorectal cancer (ASPECCT): a randomised, multicentre, open-label, non-inferiority phase 3 study. *Lancet Oncol*, 15(6), pp 569-79.
- Prime, S. S., Nixon, S. V., Crane, I. J., Stone, A., Matthews, J. B., Maitland, N. J., Remnant, L., Powell, S. K., Game, S. M. & Scully, C. 1990. The behaviour of human oral squamous cell carcinoma in cell culture. *J Pathol*, 160(3), pp 259-69.
- Pryor, D., Burmeister, E., Burmeister, B., Poulsen, M. & Porceddu, S. 2011. Distinct patterns of stomatitis with concurrent cetuximab and radiotherapy for head and neck squamous cell carcinoma. *Oral oncology*, 47(10), pp 984-987.
- Pugazhenth, S., Zhang, Y., Bouchard, R. & Mahaffey, G. 2013. Induction of an Inflammatory Loop by Interleukin-1 β and Tumor Necrosis Factor- α Involves NF- κ B and STAT-1 in Differentiated Human Neuroprogenitor Cells. *PLoS ONE*, 8(7), pp 1-12.
- Pulito, C., Cristaudo, A., Porta, C. L., Zapperi, S., Blandino, G., Morrone, A. & Strano, S. 2020. Oral mucositis: the hidden side of cancer therapy. *Journal of Experimental & Clinical Cancer Research*, 39(1), pp 210.
- Quinn, B., Potting, C. M., Stone, R., Blijlevens, N. M., Fliedner, M., Margulies, A. & Sharp, L. 2008. Guidelines for the assessment of oral mucositis in adult chemotherapy, radiotherapy and haematopoietic stem cell transplant patients. *Eur J Cancer*, 44(1), pp 61-72.
- Raber-Durlacher, J. E., Barasch, A., Peterson, D. E., Lalla, R. V., Schubert, M. M. & Fibbe, W. E. 2004. Oral complications and management considerations in patients treated with high-dose chemotherapy. *Support Cancer Ther*, 1(4), pp 219-29.
- Raber-Durlacher, J. E., Elad, S. & Barasch, A. 2010. Oral mucositis. *Oral Oncology*, 46(6), pp 452-456.
- Raber-Durlacher, J. E., von Bültzingslöwen, I., Logan, R. M., Bowen, J., Al-Azri, A. R., Everaus, H., Gerber, E., Gomez, J. G., Pettersson, B. G., Soga, Y., Spijkervet, F. K. L., Tissing, W. J. E., Epstein, J. B., Elad, S. & Lalla, R. V. 2013. Systematic review of cytokines and growth factors for the management of oral mucositis in cancer patients. *Supportive Care in Cancer*, 21(1), pp 343-355.

- Rah, M. J. 2011. A review of hyaluronan and its ophthalmic applications. *Optometry*, 82(1), pp 38-43.
- Ramalingam, S. S., Jänne, P. A., Mok, T., O'Byrne, K., Boyer, M. J., Von Pawel, J., Pluzanski, A., Shtivelband, M., Docampo, L. I., Bennouna, J., Zhang, H., Liang, J. Q., Doherty, J. P., Taylor, I., Mather, C. B., Goldberg, Z., O'Connell, J. & Paz-Ares, L. 2014. Dacomitinib versus erlotinib in patients with advanced-stage, previously treated non-small-cell lung cancer (ARCHER 1009): a randomised, double-blind, phase 3 trial. *Lancet Oncol*, 15(12), pp 1369-78.
- Rampino, M., Bacigalupo, A., Russi, E., Schena, M., Lastrucci, L., Iotti, C., Reali, A., Musu, A., Balcet, V. & Piva, C. 2012. Efficacy and feasibility of induction chemotherapy and radiotherapy plus cetuximab in head and neck cancer. *Anticancer research*, 32(1), pp 195-199.
- Rangaswamy, V. & Jain, D. 2008. An efficient process for production and purification of hyaluronic acid from *Streptococcus equi* subsp. *zooepidemicus*. *Biotechnology letters*, 30(3), pp 493-496.
- Reagan-Shaw, S., Nihal, M. & Ahmad, N. 2008. Dose translation from animal to human studies revisited. *The FASEB Journal*, 22(3), pp 659-661.
- Reichenbach, S., Blank, S., Rutjes, A. W., Shang, A., King, E. A., Dieppe, P. A., Jüni, P. & Trelle, S. 2007. Hyalran versus hyaluronic acid for osteoarthritis of the knee: A systematic review and meta-analysis. *Arthritis care & research*, 57(8), pp 1410-1418.
- Remberger, M., Ringden, O. & Markling, L. 1995. TNF alpha levels are increased during bone marrow transplantation conditioning in patients who develop acute GVHD. *Bone Marrow Transplant*, 15(1), pp 99-104.
- Reyes-Gibby, C. C., Melkonian, S. C., Wang, J., Yu, R. K., Shelburne, S. A., Lu, C., Gunn, G. B., Chambers, M. S., Hanna, E. Y., Yeung, S. J. & Shete, S. 2017. Identifying novel genes and biological processes relevant to the development of cancer therapy-induced mucositis: An informative gene network analysis. *PLoS One*, 12(7), pp e0180396.
- Reyna-Figueroa, J., Garcia-Beristain, J. C., Galindo-Delgado, P., Limon-Rojas, A. E. & Madrid-Marina, V. 2015. Antibiotic Use Before Chemotherapy: A Risk Factor for Developing Neutropenic Colitis in Children With Leukemia. *Journal of Pediatric Hematology/Oncology*, 37(2), pp 121-127.
- Rhee, S. G., Kang, S. W., Jeong, W., Chang, T.-S., Yang, K.-S. & Woo, H. A. 2005. Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Current opinion in cell biology*, 17(2), pp 183-189.
- Rice-Evans, C., Halliwell, B., Lunt, G. & Davies, K. J. Oxidative stress: the paradox of aerobic life. *Biochemical Society Symposia*, 1995. Portland Press, 1-31.

- Riegler, M., Sedivy, R., Sogukoglu, T., Cosentini, E., Bischof, G., Teleky, B., Feil, W., Schiessel, R., Hamilton, G. & Wenzl, E. 1996. Epidermal growth factor promotes rapid response to epithelial injury in rabbit duodenum in vitro. *Gastroenterology*, 111(1), pp 28-36.
- Riehl, T., Cohn, S., Tessner, T., Schloemann, S. & Stenson, W. F. 2000. Lipopolysaccharide is radioprotective in the mouse intestine through a prostaglandin-mediated mechanism. *Gastroenterology*, 118(6), pp 1106-1116.
- Riehl, T. E., Ee, X. & Stenson, W. F. 2012. Hyaluronic acid regulates normal intestinal and colonic growth in mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 303(3), pp G377-G388.
- Ringden, O., Erkers, T., Aschan, J., Garming-Legert, K., Le Blanc, K., Hägglund, H., Omazic, B., Svenberg, P., Dahllöf, G. & Mattsson, J. 2013. A prospective randomized toxicity study to compare reduced-intensity and myeloablative conditioning in patients with myeloid leukaemia undergoing allogeneic haematopoietic stem cell transplantation. *Journal of internal medicine*, 274(2), pp 153-162.
- Ro, S.-H., Nam, M., Jang, I., Park, H.-W., Park, H., Semple, I. A., Kim, M., Kim, J. S., Park, H. & Einat, P. 2014. Sestrin2 inhibits uncoupling protein 1 expression through suppressing reactive oxygen species. *Proceedings of the National Academy of Sciences*, 111(21), pp 7849-7854.
- Robbins, M. E. C. & Zhao, W. 2004. Chronic oxidative stress and radiation-induced late normal tissue injury: a review. *International Journal of Radiation Biology*, 80(4), pp 251-259.
- Robert, L., Robert, A. M. & Renard, G. 2010. Biological effects of hyaluronan in connective tissues, eye, skin, venous wall. Role in aging. *Pathol Biol (Paris)*, 58(3), pp 187-98.
- Roberts, N. A. 1988. Free radicals, immunoglobulins and complement as mediators of inflammation. *Bailliere's clinical rheumatology*, 2(1), pp 211-232.
- Robien, K., Schubert, M. M., Bruemmer, B., Lloid, M. E., Potter, J. D. & Ulrich, C. M. 2004. Predictors of oral mucositis in patients receiving hematopoietic cell transplants for chronic myelogenous leukemia. *J Clin Oncol*, 22(7), pp 1268-75.
- Rodríguez-Caballero, A., Torres-Lagares, D., Robles-García, M., Pachón-Ibáñez, J., González-Padilla, D. & Gutiérrez-Pérez, J. L. 2012. Cancer treatment-induced oral mucositis: a critical review. *Int J Oral Maxillofac Surg*, 41(2), pp 225-38.
- Roopashri, G., Jayanthi, K. & Guruprasad, R. 2011. Efficacy of benzydamine hydrochloride, chlorhexidine, and povidone iodine in the treatment of oral mucositis among patients undergoing radiotherapy in head and neck malignancies: A drug trail. *Contemp Clin Dent*, 2(1), pp 8-12.

- Rose-Ped, A. M., Bellm, L. A., Epstein, J. B., Trotti, A., Gwede, C. & Fuchs, H. J. 2002. Complications of radiation therapy for head and neck cancers: the patient's perspective. *Cancer nursing*, 25(6), pp 461-467.
- Rosen, L. S., Abdi, E., Davis, I. D., Gutheil, J., Schnell, F. M., Zalcberg, J., Cesano, A., Gayko, U., Chen, M.-G. & Clarke, S. 2006. Palifermin Reduces the Incidence of Oral Mucositis in Patients With Metastatic Colorectal Cancer Treated With Fluorouracil-Based Chemotherapy. *Journal of Clinical Oncology*, 24(33), pp 5194-5200.
- Rosenthal, D. I. 2007. Consequences of mucositis-induced treatment breaks and dose reductions on head and neck cancer treatment outcomes. *J Support Oncol*, 5(9 Suppl 4), pp 23-31.
- Rosenthal, D. I., Lewin, J. S. & Eisbruch, A. 2006. Prevention and treatment of dysphagia and aspiration after chemoradiation for head and neck cancer. *J Clin Oncol*, 24(17), pp 2636-43.
- Rosenthal, D. I. & Trotti, A. 2009. Strategies for managing radiation-induced mucositis in head and neck cancer. *Semin Radiat Oncol*, 19(1), pp 29-34.
- Roshak, A. K., Jackson, J. R., McGough, K., Chabot-Fletcher, M., Mochan, E. & Marshall, L. A. 1996. Manipulation of Distinct NF κ B Proteins Alters Interleukin-1 β -induced Human Rheumatoid Synovial Fibroblast Prostaglandin E2 Formation. *Journal of Biological Chemistry*, 271(49), pp 31496-31501.
- Roux, P. P. & Blenis, J. 2004. ERK and p38 MAPK-Activated Protein Kinases: a Family of Protein Kinases with Diverse Biological Functions. *Microbiology and Molecular Biology Reviews*, 68(2), pp 320-344.
- Royack, G., Nguyen, M., Tong, D., Poot, M. & Oda, D. 2000. Response of human oral epithelial cells to oxidative damage and the effect of vitamin E. *Oral Oncology*, 36(1), pp 37-41.
- Rtibi, K., Selmi, S., Grami, D., Amri, M., Sebai, H. & Marzouki, L. 2018. Contribution of oxidative stress in acute intestinal mucositis induced by 5 fluorouracil (5-FU) and its pro-drug capecitabine in rats. *Toxicology mechanisms and methods*, 28(4), pp 262-267.
- Rubenstein, E. B., Peterson, D. E., Schubert, M., Keefe, D., McGuire, D., Epstein, J., Elting, L. S., Fox, P. C., Cooksley, C., Sonis, S. T., Mucositis Study Section of the Multinational Association for Supportive Care in, C. & International Society for Oral, O. 2004. Clinical practice guidelines for the prevention and treatment of cancer therapy-induced oral and gastrointestinal mucositis. *Cancer*, 100(9 Suppl), pp 2026-46.
- Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., Scudiero, D. A., Monks, A. & Boyd, M. R. 1990. Comparison of in vitro anticancer-drug-screening data

- generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J Natl Cancer Inst*, 82(13), pp 1113-8.
- Ruescher, T. J., Sodeifi, A., Scrivani, S. J., Kaban, L. B. & Sonis, S. T. 1998. The impact of mucositis on alpha-hemolytic streptococcal infection in patients undergoing autologous bone marrow transplantation for hematologic malignancies. *Cancer*, 82(11), pp 2275-2281.
- Ruggiero, T., Pol, R., Camisassa, D., Arata, V., Martino, I., Giaccone, L. & Carossa, S. 2016. Use of sodium hyaluronate and synthetic amino acid precursors of collagen for the symptomatic treatment of mucositis in patients undergoing haematopoietic stem cell transplants. *J Biol Regul Homeost Agents*, 30(3), pp 889-894.
- Russi, E. G., Raber-Durlacher, J. E. & Sonis, S. T. 2014. Local and Systemic Pathogenesis and Consequences of Regimen-Induced Inflammatory Responses in Patients with Head and Neck Cancer Receiving Chemoradiation. *Mediators of Inflammation*, 2014(518261).
- Saadeh, C. E. 2005. Chemotherapy- and radiotherapy-induced oral mucositis: review of preventive strategies and treatment. *Pharmacotherapy*, 25(4), pp 540-54.
- Saari, H., Konttinen, Y. T., Friman, C. & Sorsa, T. 1993. Differential effects of reactive oxygen species on native synovial fluid and purified human umbilical cord hyaluronate. *Inflammation*, 17(4), pp 403-415.
- Sadrzadeh-Afshar, M.-S., Gholizadeh, N. & Sheykhbahaei, N. 2016. New Treatment Approaches of Oral Mucositis: A Review of Literature. *Advances in Human Biology*, 6(2), pp 66-72.
- Sakamoto, K., Oka, M., Yoshino, S., Hazama, S., Abe, T., Okayama, N. & Hinoda, Y. 2006. Relation between cytokine promoter gene polymorphism and toxicity of 5-fluorouracil plus cisplatin chemotherapy. *Oncol Rep*, 16(2), pp 381-7.
- Sakurai, K., Miyazaki, K., Kodera, Y., Nishimura, H., Shingu, M. & Inada, Y. 1997. Anti-inflammatory activity of superoxide dismutase conjugated with sodium hyaluronate. *Glycoconjugate journal*, 14(6), pp 723-728.
- Salvo Romero, E., Alonso Cotoner, C., Pardo Camacho, C., Casado Bedmar, M. & Vicario, M. 2015. The intestinal barrier function and its involvement in digestive disease. *Rev Esp Enferm Dig*, 107(11), pp 686-96.
- Sammarco, G., Shalaby, M., Elangovan, S., Petti, L., Roda, G., Restelli, S., Arena, V., Ungaro, F., Fiorino, G. & Day, A. J. 2019. Hyaluronan Accelerates Intestinal Mucosal Healing through Interaction with TSG-6. *Cells*, 8(9), pp 1074.

- Sanguineti, G., Sormani, M. P., Marur, S., Gunn, G. B., Rao, N., Cianchetti, M., Ricchetti, F., McNutt, T., Wu, B. & Forastiere, A. 2012. Effect of Radiotherapy and Chemotherapy on the Risk of Mucositis During Intensity-Modulated Radiation Therapy for Oropharyngeal Cancer. *International Journal of Radiation Oncology, Biology, Physics*, 83(1), pp 235-242.
- Santini, D., Vincenzi, B., Perrone, G., Rabitti, C., Borzomati, D., Caricato, M., La Cesa, A., Grilli, C., Verzi, A., Coppola, R. & Tonini, G. 2004. Thymidylate synthase expression in normal colonic mucosa: a predictive marker of toxicity in colorectal cancer patients receiving 5-fluorouracil-based adjuvant chemotherapy. *Oncology*, 67(2), pp 135-42.
- Sato, T., Van Es, J. H., Snippert, H. J., Stange, D. E., Vries, R. G., Van Den Born, M., Barker, N., Shroyer, N. F., Van De Wetering, M. & Clevers, H. 2011. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature*, 469(7330), pp 415-418.
- Sato, T., Vries, R. G., Snippert, H. J., Van De Wetering, M., Barker, N., Stange, D. E., Van Es, J. H., Abo, A., Kujala, P. & Peters, P. J. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*, 459(7244), pp 262-265.
- Saunders, D. P., Epstein, J. B., Elad, S., Allemanno, J., Bossi, P., Van De Wetering, M. D., Rao, N. G., Potting, C., Cheng, K. K. & Freidank, A. 2013. Systematic review of antimicrobials, mucosal coating agents, anesthetics, and analgesics for the management of oral mucositis in cancer patients. *Supportive Care in Cancer*, 21(11), pp 3191-3207.
- Saunders, D. P., Rouleau, T., Cheng, K., Yarom, N., Kandwal, A., Joy, J., Bektas Kayhan, K., van de Wetering, M., Brito-Dellan, N., Kataoka, T., Chiang, K., Ranna, V., Vaddi, A., Epstein, J., Lalla, R. V., Bossi, P. & Elad, S. 2020. Systematic review of antimicrobials, mucosal coating agents, anesthetics, and analgesics for the management of oral mucositis in cancer patients and clinical practice guidelines. *Support Care Cancer*, 28(5), pp 2473-2484.
- Scaffidi, P., Misteli, T. & Bianchi, M. E. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*, 418(6894), pp 191-5.
- Schack, L. M. H., Naderi, E., Fachal, L., Dorling, L., Luccarini, C., Dunning, A. M., Barnett, G., Barrera, M. E. A., Burnet, N. G., Calvo, L. M., Diergaarde, B., Dudding, T., Dunning, A., Duprez, F., Kerns, S. L., Kiang, M. C. L., Langendijk, H., Mehanna, H., Ness, A., Sim, A. Y. L., Spiessens, A., Summersgill, H. R., Tajés, J. F., Vega, A., Welsh, C., Wen, E. O. H., West, C., Ong, E. H. W., Chua, M. L. K., Langendijk, J. A., Alizadeh, B. Z., Overgaard, J., Eriksen, J. G., Andreassen, C. N., Alsner, J., The, H., Neck Group of the Radiogenomics, C., The Danish, H. & Neck Cancer, G. 2022. A genome-wide association study of radiotherapy induced toxicity in head and neck cancer patients identifies a susceptibility locus associated with mucositis. *British Journal of Cancer*, 126(7), pp 1082-1090.
- Schanté, C. E., Zuber, G., Herlin, C. & Vandamme, T. F. 2011. Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications. *Carbohydrate Polymers*, 85(3), pp 469-489.

Schieber, M. & Chandel, N. S. 2014. ROS function in redox signaling and oxidative stress. *Current biology*, 24(10), pp R453-R462.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S. & Schmid, B. 2012. Fiji: an open-source platform for biological-image analysis. *Nature methods*, 9(7), pp 676-682.

Schledzewski, K., Falkowski, M., Moldenhauer, G., Metharom, P., Kzhyshkowska, J., Ganss, R., Demory, A., Falkowska-Hansen, B., Kurzen, H., Ugurel, S., Geginat, G., Arnold, B. & Goerdts, S. 2006. Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumours and wound healing tissue in vivo and in bone marrow cultures in vitro: implications for the assessment of lymphangiogenesis. *J Pathol*, 209(1), pp 67-77.

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature methods*, 9(7), pp 671-675.

Schubert, M. M., Williams, B. E., Lloid, M. E., Donaldson, G. & Chapko, M. K. 1992. Clinical assessment scale for the rating of oral mucosal changes associated with bone marrow transplantation. Development of an oral mucositis index. *Cancer*, 69(10), pp 2469-77.

Schwab, M., Zanger, U. M., Marx, C., Schaeffeler, E., Klein, K., Dippon, J., Kerb, R., Blievernicht, J., Fischer, J., Hofmann, U., Bokemeyer, C. & Eichelbaum, M. 2008. Role of Genetic and Nongenetic Factors for Fluorouracil Treatment-Related Severe Toxicity: A Prospective Clinical Trial by the German 5-FU Toxicity Study Group. *Journal of Clinical Oncology*, 26(13), pp 2131-2138.

Scott, J. E. 1992. The chemical morphology of the vitreous. *Eye*, 6(6), pp 553-555.

Scott, J. E., Cummings, C., Brass, A. & Chen, Y. 1991. Secondary and tertiary structures of hyaluronan in aqueous solution, investigated by rotary shadowing-electron microscopy and computer simulation. Hyaluronan is a very efficient network-forming polymer. *Biochemical Journal*, 274(3), pp 699-705.

Scully, C., Epstein, J. & Sonis, S. 2003. Oral mucositis: a challenging complication of radiotherapy, chemotherapy, and radiochemotherapy: part 1, pathogenesis and prophylaxis of mucositis. *Head & neck*, 25(12), pp 1057-1070.

Scully, C., Epstein, J. & Sonis, S. 2004. Oral mucositis: a challenging complication of radiotherapy, chemotherapy, and radiochemotherapy. Part 2: diagnosis and management of mucositis. *Head Neck*, 26(1), pp 77-84.

Scully, C., Sonis, S. & Diz, P. D. 2006. Oral mucositis. *Oral Diseases*, 3), pp 229.

- Sen, C. K. & Packer, L. 1996. Antioxidant and redox regulation of gene transcription. *The FASEB Journal*, 10(7), pp 709-20.
- Senel, O., Cetinkale, O., Ozbay, G., Ahçioğlu, F. & Bulan, R. 1997. Oxygen free radicals impair wound healing in ischemic rat skin. *Annals of plastic surgery*, 39(5), pp 516-523.
- Seong, S.-Y. & Matzinger, P. 2004. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol*, 4(6), pp 469-478.
- Seto, K., Shoda, J., Horibe, T., Warabi, E., Ishige, K., Yamagata, K., Kohno, M., Yanagawa, T., Bukawa, H. & Kawakami, K. 2013. Interleukin-4 receptor α -based hybrid peptide effectively induces antitumor activity in head and neck squamous cell carcinoma. *Oncology reports*, 29(6), pp 2147-2153.
- Seton-Rogers, S. 2012. Multitasking hyaluronic acid. *Nature Reviews Cancer*, 12(4), pp 228-228.
- Shal, B., Ding, W., Ali, H., Kim, Y. S. & Khan, S. 2018. Anti-neuroinflammatory potential of natural products in attenuation of Alzheimer's disease. *Frontiers in pharmacology*, 9(548).
- Shameem, R., Lacouture, M. & Wu, S. 2015. Incidence and risk of high-grade stomatitis with mTOR inhibitors in cancer patients. *Cancer Invest*, 33(3), pp 70-7.
- Shankar, A., Roy, S., Bhandari, M., Rath, G. K., Biswas, A. S., Kanodia, R., Adhikari, N. & Sachan, R. 2017. Current Trends in Management of Oral Mucositis in Cancer Treatment. *Asian Pac J Cancer Prev*, 18(8), pp 2019-2026.
- Shen, Z., Wang, J., Huang, Q., Shi, Y., Wei, Z., Zhang, X., Qiu, Y., Zhang, M., Wang, Y. & Qin, W. 2018. Genetic modification to induce CXCR2 overexpression in mesenchymal stem cells enhances treatment benefits in radiation-induced oral mucositis. *Cell death & disease*, 9(2), pp 1-14.
- Shepherd, F. A., Rodrigues Pereira, J., Ciuleanu, T., Tan, E. H., Hirsh, V., Thongprasert, S., Campos, D., Maoleekoonpiroj, S., Smylie, M., Martins, R., van Kooten, M., Dediu, M., Findlay, B., Tu, D., Johnston, D., Bezjak, A., Clark, G., Santabárbara, P. & Seymour, L. 2005. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med*, 353(2), pp 123-32.
- Sherman, M. L., Datta, R., Hallahan, D. E., Weichselbaum, R. R. & Kufe, D. W. 1990. Ionizing radiation regulates expression of the c-jun protooncogene. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 87(15), pp 5663-5666.
- Shi, V. J., Levy, L. L. & Choi, J. N. 2016. Cutaneous manifestations of nontargeted and targeted chemotherapies. *Seminars in Oncology*, 43(3), pp 419-425.

- Shiedlin, A., Bigelow, R., Christopher, W., Arbabi, S., Yang, L., Maier, R. V., Wainwright, N., Childs, A. & Miller, R. J. 2004. Evaluation of Hyaluronan from Different Sources: Streptococcus z ooepidemicus, Rooster Comb, Bovine Vitreous, and Human Umbilical Cord. *Biomacromolecules*, 5(6), pp 2122-2127.
- Shimamura, Y., Takeuchi, I., Terada, H. & Makino, K. 2018. A Mouse Model for Oral Mucositis Induced by Cancer Chemotherapy. *Anticancer Res*, 38(1), pp 307-312.
- Shin, D. M. & Khuri, F. R. 2013. Advances in the management of recurrent or metastatic squamous cell carcinoma of the head and neck. *Head & neck*, 35(3), pp 443-453.
- Shiota, A., Hada, T., Baba, T., Sato, M., Yamanaka-Okumura, H., Yamamoto, H., Taketani, Y. & Takeda, E. 2010. Protective effects of glycolipids extracted from spinach on 5-fluorouracil induced intestinal mucosal injury. *J Med Invest*, 57(3-4), pp 314-20.
- Shouval, R., Kouniavski, E., Fein, J., Danylesko, I., Shem-Tov, N., Geva, M., Yerushalmi, R., Shimoni, A. & Nagler, A. 2019. Risk factors and implications of oral mucositis in recipients of allogeneic hematopoietic stem cell transplantation. *Eur J Haematol*, 103(4), pp 402-409.
- Shrivastava, R. & Deshmukh, S. 2013. A new therapeutic approach to treat oral mucositis using specific MMP blockers in an osmotically active solution. *Journal of Cancer Research and Treatment*, 1(1), pp 4-11.
- Sibaud, V., Niec, R. E., Schindler, K., Busam, K. J., Roché, H., Modi, S., Delord, J. P. & Lacouture, M. E. 2014. Ado-trastuzumab emtansine-associated telangiectasias in metastatic breast cancer: a case series. *Breast Cancer Res Treat*, 146(2), pp 451-6.
- Sibaud, V., Vigarios, E., Combemale, P., Lamant, L., Lacouture, M. E., Lacaze, J. L., Dalenc, F. & Delord, J. P. 2015. T-DM1-related telangiectasias: a potential role in secondary bleeding events. *Ann Oncol*, 26(2), pp 436-7.
- Siber, G. R., Mayer, R. J. & Levin, M. J. 1980. Increased Gastrointestinal Absorption of Large Molecules in Patients after 5-Fluorouracil Therapy for Metastatic Colon Carcinoma. *Cancer Research*, 40(10), pp 3430-3436.
- Silverman, S., Jr. 2007. Diagnosis and management of oral mucositis. *J Support Oncol*, 5(2 Suppl 1), pp 13-21.
- Sims, G. P., Rowe, D. C., Rietdijk, S. T., Herbst, R. & Coyle, A. J. 2010. HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol*, 28(367-88).
- Singer, S., Amdal, C. D., Hammerlid, E., Tomaszewska, I. M., Castro Silva, J., Mehanna, H., Santos, M., Inhestern, J., Brannan, C., Yarom, N., Fullerton, A., Pinto, M., Arraras, J. I., Kiyota, N.,

- Bonomo, P., Sherman, A. C., Baumann, I., Galalae, R., Fernandez Gonzalez, L., Nicolatou-Galitis, O., Abdel-Hafeez, Z., Raber-Durlacher, J., Schmalz, C., Zotti, P., Boehm, A., Hofmeister, D., Krejovic Trivic, S., Loo, S., Chie, W. C., Bjordal, K., Brokstad Herlofson, B., Grégoire, V. & Licitra, L. 2019. International validation of the revised European Organisation for Research and Treatment of Cancer Head and Neck Cancer Module, the EORTC QLQ-HN43: Phase IV. *Head Neck*, 41(6), pp 1725-1737.
- Sixou, J. L., de Medeiros-Batista, O. & Bonnaure-Mallet, M. 1996. Modifications of the microflora of the oral cavity arising during immunosuppressive chemotherapy. *European Journal of Cancer Part B: Oral Oncology*, 32(5), pp 306-310.
- Skof, E., Rebersek, M., Hlebanja, Z. & Ocvirk, J. 2009. Capecitabine plus Irinotecan (XELIRI regimen) compared to 5-FU/LV plus Irinotecan (FOLFIRI regimen) as neoadjuvant treatment for patients with unresectable liver-only metastases of metastatic colorectal cancer: a randomised prospective phase II trial. *BMC Cancer*, 9(1), pp 120.
- Slade, D. 2020. PARP and PARG inhibitors in cancer treatment. *Genes & development*, 34(5-6), pp 360-394.
- Slaoui, M. & Fiette, L. 2011. Histopathology procedures: from tissue sampling to histopathological evaluation. *Drug safety evaluation*. Springer.
- Sledge, G. W., Jr., Toi, M., Neven, P., Sohn, J., Inoue, K., Pivot, X., Burdaeva, O., Oker, M., Masuda, N., Kaufman, P. A., Koh, H., Grischke, E. M., Frenzel, M., Lin, Y., Barriga, S., Smith, I. C., Bourayou, N. & Llombart-Cussac, A. 2017. MONARCH 2: Abemaciclib in Combination With Fulvestrant in Women With HR+/HER2- Advanced Breast Cancer Who Had Progressed While Receiving Endocrine Therapy. *J Clin Oncol*, 35(25), pp 2875-2884.
- Sleijfer, S., Vujaskovic, Z., Limburg, P. C., Schraffordt Koops, H. & Mulder, N. H. 1998. Induction of tumor necrosis factor-alpha as a cause of bleomycin-related toxicity. *Cancer*, 82(5), pp 970-974.
- Slevin, M., Kumar, S. & Gaffney, J. 2002. Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses. *J Biol Chem*, 277(43), pp 41046-59.
- Sloan, J. A., Goldberg, R. M., Sargent, D. J., Vargas-Chanes, D., Nair, S., Cha, S. S., Novotny, P. J., Poon, M. A., O'Connell, M. J. & Loprinzi, C. L. 2002. Women Experience Greater Toxicity With Fluorouracil-Based Chemotherapy for Colorectal Cancer. *Journal of Clinical Oncology*, 20(6), pp 1491-1498.
- Sloan, P., Picardo, M. & Schor, S. L. 1991. The structure and function of oral mucosa. *Dental update*, 18(5), pp 208-212.

- Soares, P. M. G., Mota, J. M. S. C., Gomes, A. S., Oliveira, R. B., Assreuy, A. M. S., Brito, G. A. C., Santos, A. A., Ribeiro, R. A. & Souza, M. H. L. P. 2008. Gastrointestinal dysmotility in 5-fluorouracil-induced intestinal mucositis outlasts inflammatory process resolution. *Cancer Chemotherapy and Pharmacology*, 63(1), pp 91-98.
- Soares, P. M. G., Mota, J. M. S. C., Souza, E. P., Justino, P. F. C., Franco, A. X., Cunha, F. Q., Ribeiro, R. A. & Souza, M. H. L. P. 2013. Inflammatory intestinal damage induced by 5-fluorouracil requires IL-4. *Cytokine*, 61(1), pp 46-49.
- Sobue, T., Bertolini, M., Thompson, A., Peterson, D. E., Diaz, P. I. & Dongari-Bagtzoglou, A. 2018. Chemotherapy-induced oral mucositis and associated infections in a novel organotypic model. *Molecular oral microbiology*, 33(3), pp 212-223.
- Soga, Y., Yamasuji, Y., Kudo, C., Matsuura-Yoshimoto, K., Yamabe, K., Sugiura, Y., Maeda, Y., Ishimaru, F., Tanimoto, M. & Nishimura, F. 2009. Febrile neutropenia and periodontitis: lessons from a case periodontal treatment in the intervals between chemotherapy cycles for leukemia reduced febrile neutropenia. *Supportive care in cancer*, 17(5), pp 581-587.
- Šoltés, L. & Mendichi, R. 2003. Molecular characterization of two host–guest associating hyaluronan derivatives. *Biomedical chromatography*, 17(6), pp 376-384.
- Soltés, L., Mendichi, R., Kogan, G., Schiller, J., Stankovska, M. & Arnhold, J. 2006. Degradative action of reactive oxygen species on hyaluronan. *Biomacromolecules*, 7(3), pp 659-68.
- Šoltés, L., Stankovská, M., Brezová, V., Schiller, J., Arnhold, J., Kogan, G. & Gemeiner, P. 2006. Hyaluronan degradation by copper (II) chloride and ascorbate: rotational viscometric, EPR spin-trapping, and MALDI–TOF mass spectrometric investigations. *Carbohydrate research*, 341(17), pp 2826-2834.
- Sonis, S. 2012a. *Oral mucositis*: Springer Science & Business Media.
- Sonis, S., Andreotta, P. & Lyng, G. 2017. On the pathogenesis of mTOR inhibitor-associated stomatitis (mIAS)—studies using an organotypic model of the oral mucosa. *Oral Diseases*, 23(3), pp 347-352.
- Sonis, S., Edwards, L. & Lucey, C. 1999a. The biological basis for the attenuation of mucositis: the example of interleukin-11. *Leukemia*, 13(831-834).
- Sonis, S., Haddad, R., Posner, M., Watkins, B., Fey, E., Morgan, T. V., Mookanamparambil, L. & Ramoni, M. 2007. Gene expression changes in peripheral blood cells provide insight into the biological mechanisms associated with regimen-related toxicities in patients being treated for head and neck cancers. *Oral Oncology*, 3), pp 289.

- Sonis, S. T. 1998. Mucositis as a biological process: a new hypothesis for the development of chemotherapy-induced stomatotoxicity. *Oral Oncology*, 34(1), pp 39-43.
- Sonis, S. T. 2002. The Biologic Role for Nuclear Factor-KappaB in Disease and its Potential Involvement in Mucosal Injury Associated with Anti-neoplastic Therapy. *Critical Reviews in Oral Biology & Medicine*, 13(5), pp 380-389.
- Sonis, S. T. 2004a. Oral mucositis in cancer therapy. *J Support Oncol*, 2(6 Suppl 3), pp 3-8.
- Sonis, S. T. 2004b. The pathobiology of mucositis. *Nat Rev Cancer*, 4(4), pp 277-84.
- Sonis, S. T. 2007. Pathobiology of oral mucositis: novel insights and opportunities. *The Journal Of Supportive Oncology*, 5(9 Suppl 4), pp 3-11.
- Sonis, S. T. 2009. Mucositis: The impact, biology and therapeutic opportunities of oral mucositis. *Oral Oncol*, 45(12), pp 1015-20.
- Sonis, S. T. 2010a. New thoughts on the initiation of mucositis. *Oral Diseases*, 7), pp 597.
- Sonis, S. T. 2010b. Regimen-related gastrointestinal toxicities in cancer patients. *Current Opinion in Supportive & Palliative Care*, 4(1), pp 26.
- Sonis, S. T. 2011. Oral mucositis. *Anticancer Drugs*, 22(7), pp 607-12.
- Sonis, S. T. 2012b. The epidemiology and risk assessment of mucositis. *Oral mucositis*, 15-24.
- Sonis, S. T. 2013. Oral mucositis in head and neck cancer: risk, biology, and management. *American Society of Clinical Oncology Educational Book*, 33(1), pp e236-e240.
- Sonis, S. T. 2015. Genomics, personalized medicine, and supportive cancer care. *Am Soc Clin Oncol Educ Book*, 9-16.
- Sonis, S. T., Eilers, J. P., Epstein, J. B., LeVeque, F. G., Liggett, W. H., Jr., Mulagha, M. T., Peterson, D. E., Rose, A. H., Schubert, M. M., Spijkervet, F. K. & Wittes, J. P. 1999b. Validation of a new scoring system for the assessment of clinical trial research of oral mucositis induced by radiation or chemotherapy. Mucositis Study Group. *Cancer*, 85(10), pp 2103-13.
- Sonis, S. T., Elting, L. S., Keefe, D., Peterson, D. E., Schubert, M., Hauer-Jensen, M., Bekele, B. N., Raber-Durlacher, J., Donnelly, J. P., Rubenstein, E. B., Mucositis Study Section of the Multinational Association for Supportive Care in, C. & International Society for Oral, O. 2004a. Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. *Cancer*, 100(9 Suppl), pp 1995-2025.

- Sonis, S. T. & Fey, E. G. 2002. Oral complications of cancer therapy. *Oncology (Williston Park, N.Y.)*, 16(5), pp 680-6; discussion 686, 691-2, 695.
- Sonis, S. T., Lieberman, A. & Sonis, A. L. 1978. Oral complications in patients receiving treatment for malignancies other than of the head and neck. *The Journal of the American Dental Association*, 97(3), pp 468-472.
- Sonis, S. T., O'Donnell, K. E., Popat, R., Bragdon, C., Phelan, S., Cocks, D. & Epstein, J. B. 2004b. The relationship between mucosal cyclooxygenase-2 (COX-2) expression and experimental radiation-induced mucositis. *Oral Oncology*, 40(170-176).
- Sonis, S. T., Oster, G., Fuchs, H., Bellm, L., Bradford, W. Z., Edelsberg, J., Hayden, V., Eilers, J., Epstein, J. B. & LeVeque, F. G. 2001. Oral mucositis and the clinical and economic outcomes of hematopoietic stem-cell transplantation. *Journal of clinical oncology*, 19(8), pp 2201-2205.
- Sonis, S. T., Peterson, R. L., Edwards, L. J., Lucey, C. A., Wang, L., Mason, L., Login, G., Ymamkawa, M., Moses, G., Bouchard, P., Hayes, L. L., Bedrosian, C. & Dorner, A. J. 2000. Defining mechanisms of action of interleukin-11 on the progression of radiation-induced oral mucositis in hamsters. *Oral Oncology*, 36(4), pp 373-381.
- Sonis, S. T., Scherer, J., Phelan, S., Lucey, C. A., Barron, J. E., O'Donnell, K. E., Brennan, R. J., Pan, H., Busse, P. & Haley, J. D. 2002. The gene expression sequence of radiated mucosa in an animal mucositis model. *Cell Prolif*, 35 Suppl 1(93-102).
- Sonis, S. T., Tracey, C., Shklar, G., Jenson, J. & Florine, D. 1990. An animal model for mucositis induced by cancer chemotherapy. *Oral Surg Oral Med Oral Pathol*, 69(4), pp 437-43.
- Soria, J. C., Felip, E., Cobo, M., Lu, S., Syrigos, K., Lee, K. H., Göker, E., Georgoulas, V., Li, W., Isla, D., Guclu, S. Z., Morabito, A., Min, Y. J., Ardizzoni, A., Gadgeel, S. M., Wang, B., Chand, V. K. & Goss, G. D. 2015. Afatinib versus erlotinib as second-line treatment of patients with advanced squamous cell carcinoma of the lung (LUX-Lung 8): an open-label randomised controlled phase 3 trial. *Lancet Oncol*, 16(8), pp 897-907.
- Spielberger, R., Stiff, P., Bensinger, W., Gentile, T., Weisdorf, D., Kewalramani, T., Shea, T., Yanovich, S., Hansen, K., Noga, S., McCarty, J., LeMaistre, C. F., Sung, E. C., Blazar, B. R., Elhardt, D., Chen, M.-G. & Emmanouilides, C. 2004. Palifermin for Oral Mucositis after Intensive Therapy for Hematologic Cancers. *New England Journal of Medicine*, 351(25), pp 2590-2598.
- Spijkervet, F., Saene, H. v., Panders, A., Vermey, A. & Mehta, D. 1989. Scoring irradiation mucositis in head and neck cancer patients. *Journal of Oral Pathology & Medicine*, 18(3), pp 167-171.

- Spijkervet, F. K., Van Saene, H. K., Van Saene, J. J., Panders, A. K., Vermey, A., Mehta, D. M. & Fidler, V. 1991. Effect of selective elimination of the oral flora on mucositis in irradiated head and neck cancer patients. *Journal of surgical oncology*, 46(3), pp 167-173.
- Squier, C. & Hill, M. 1989. Oral mucosa. In: Ten Cate, A. R. (ed.) *Oral histology : development, structure, and function*. 3rd ed. ed.: Mosby. p. 341–81.
- Squier, C. A. & Kremer, M. J. 2001. Biology of Oral Mucosa and Esophagus. *JNCI Monographs*, 2001(29), pp 7-15.
- Stefanelli, C., Tantini, B., Fattori, M., Stanic, I., Pignatti, C., Clo, C., Guarnieri, C., Caldarera, C. M., Mackintosh, C. A. & Pegg, A. E. 2002. Caspase activation in etoposide-treated fibroblasts is correlated to ERK phosphorylation and both events are blocked by polyamine depletion. *FEBS letters*, 527(1-3), pp 223-228.
- Stein, A., Voigt, W. & Jordan, K. 2010. Chemotherapy-induced diarrhea: pathophysiology, frequency and guideline-based management. *Therapeutic advances in medical oncology*, 2(1), pp 51-63.
- Stellavato, A., Corsuto, L., D'Agostino, A., La Gatta, A., Diana, P., Bernini, P., De Rosa, M. & Schiraldi, C. 2016. Hyaluronan hybrid cooperative complexes as a novel frontier for cellular bioprocesses re-activation. *PLoS One*, 11(10), pp e0163510.
- Stern, R. 2004. Hyaluronan catabolism: a new metabolic pathway. *European journal of cell biology*, 83(7), pp 317-325.
- Stern, R., Asari, A. A. & Sugahara, K. N. 2006. Hyaluronan fragments: an information-rich system. *European journal of cell biology*, 85(8), pp 699-715.
- Stern, R., Kogan, G., Jedrzejewski, M. J. & Šoltés, L. 2007. The many ways to cleave hyaluronan. *Biotechnology advances*, 25(6), pp 537-557.
- Stiff, P. J., Erder, H., Bensinger, W. I., Emmanouilides, C., Gentile, T., Isitt, J., Lu, Z. J. & Spielberger, R. 2006. Reliability and validity of a patient self-administered daily questionnaire to assess impact of oral mucositis (OM) on pain and daily functioning in patients undergoing autologous hematopoietic stem cell transplantation (HSCT). *Bone Marrow Transplant*, 37(4), pp 393-401.
- Stokman, M. A., Spijkervet, F. K. L., Boezen, H. M., Schouten, J. P., Roodenburg, J. L. N. & de Vries, E. G. E. 2006. Preventive intervention possibilities in radiotherapy- and chemotherapy-induced oral mucositis: results of meta-analyses. *Journal Of Dental Research*, 85(8), pp 690-700.

- Stringer, A. M., Al-Dasooqi, N., Bowen, J. M., Tan, T. H., Radzuan, M., Logan, R. M., Mayo, B., Keefe, D. M. K. & Gibson, R. J. 2013. Biomarkers of chemotherapy-induced diarrhoea: a clinical study of intestinal microbiome alterations, inflammation and circulating matrix metalloproteinases. *Supportive Care in Cancer*, 21(7), pp 1843-1852.
- Stringer, A. M., Gibson, R. J., Bowen, J. M. & Keefe, D. M. 2009a. Chemotherapy-induced modifications to gastrointestinal microflora: evidence and implications of change. *Curr Drug Metab*, 10(1), pp 79-83.
- Stringer, A. M., Gibson, R. J., Bowen, J. M., Logan, R. M., Ashton, K., Yeoh, A. S., Al-Dasooqi, N. & Keefe, D. M. 2009b. Irinotecan-induced mucositis manifesting as diarrhoea corresponds with an amended intestinal flora and mucin profile. *Int J Exp Pathol*, 90(5), pp 489-99.
- Stringer, A. M., Gibson, R. J., Logan, R. M., Bowen, J. M., Yeoh, A. S.-J., Burns, J. & Keefe, D. M. K. 2007. Chemotherapy-Induced Diarrhea Is Associated with Changes in the Luminal Environment in the DA Rat. *Experimental Biology and Medicine*, 232(1), pp 96-106.
- Stringer, A. M. & Logan, R. M. 2015. The role of oral flora in the development of chemotherapy-induced oral mucositis. *Journal of Oral Pathology & Medicine*, 44(2), pp 81-87.
- Subramaniam, N. & Muthukrishnan, A. 2019. Oral mucositis and microbial colonization in oral cancer patients undergoing radiotherapy and chemotherapy: A prospective analysis in a tertiary care dental hospital. *Journal of investigative and clinical dentistry*, 10(4), pp e12454.
- Sudha, P. N. & Rose, M. H. 2014. Beneficial effects of hyaluronic acid. *Advances in food and nutrition research*, 72(137-176).
- Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. 2021. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*, 71(3), pp 209-249.
- Sung, L., Robinson, P., Treister, N., Baggott, T., Gibson, P., Tissing, W., Wiernikowski, J., Brinklow, J. & Dupuis, L. L. 2017. Guideline for the prevention of oral and oropharyngeal mucositis in children receiving treatment for cancer or undergoing haematopoietic stem cell transplantation. *BMJ Support Palliat Care*, 7(1), pp 7-16.
- Sung, L., Tomlinson, G. A., Greenberg, M. L., Koren, G., Judd, P., Ota, S. & Feldman, B. M. 2007. Validation of the oral mucositis assessment scale in pediatric cancer. *Pediatr Blood Cancer*, 49(2), pp 149-53.
- Suzuki, Y. & Yamaguchi, T. 1993. Effects of hyaluronic acid on macrophage phagocytosis and active oxygen release. *Agents and actions*, 38(1), pp 32-37.

- Svanberg, A., Birgegård, G. & Ohrn, K. 2007. Oral cryotherapy reduces mucositis and opioid use after myeloablative therapy--a randomized controlled trial. *Supportive Care In Cancer: Official Journal Of The Multinational Association Of Supportive Care In Cancer*, 15(10), pp 1155-1161.
- Svanberg, A., Öhrn, K. & Birgegård, G. 2010. Oral cryotherapy reduces mucositis and improves nutrition -- a randomised controlled trial. *Journal of Clinical Nursing*, 19(15/16), pp 2146-2151.
- Swafford, D. & Manicassamy, S. 2015. Wnt signaling in dendritic cells: its role in regulation of immunity and tolerance. *Discov Med*, 19(105), pp 303-10.
- Symonds, R. P. 1998. Treatment-induced mucositis: an old problem with new remedies. *Br J Cancer*, 77(10), pp 1689-95.
- Takahashi, K., Soga, Y., Murayama, Y., Udagawa, M., Nishimoto, H., Sugiura, Y., Maeda, Y., Tanimoto, M. & Takashiba, S. 2010. Oral mucositis in patients receiving reduced-intensity regimens for allogeneic hematopoietic cell transplantation: comparison with conventional regimen. *Supportive care in cancer*, 18(1), pp 115-119.
- Takei, Y., Maruyama, A., Ferdous, A., Nishimura, Y., Kawano, S., Ikejima, K., Okumura, S., Asayama, S., Nogawa, M., Hashimoto, M., Makino, Y., Kinoshita, M., Watanabe, S., Akaike, T., Lemasters, J. J. & Sato, N. 2004. Targeted gene delivery to sinusoidal endothelial cells: DNA nanoassociate bearing hyaluronan-glycocalyx. *Faseb j*, 18(6), pp 699-701.
- Takeuchi, I., Kamiki, Y. & Makino, K. 2018. Therapeutic efficacy of rebamipide-loaded PLGA nanoparticles coated with chitosan in a mouse model for oral mucositis induced by cancer chemotherapy. *Colloids and Surfaces B: Biointerfaces*, 167(468-473).
- Talwar, S., House, R., Sundaramurthy, S., Balasubramanian, S., Yu, H. & Palanisamy, V. 2014. Inhibition of caspases protects mice from radiation-induced oral mucositis and abolishes the cleavage of RNA-binding protein HuR. *J Biol Chem*, 289(6), pp 3487-500.
- Tancharoen, S., Shakya, P., Narkpinit, S., Dararat, P. & Kikuchi, K. 2018. Anthocyanins Extracted from *Oryza sativa* L. Prevent Fluorouracil-Induced Nuclear Factor- κ B Activation in Oral Mucositis: In Vitro and In Vivo Studies. *International Journal of Molecular Sciences*, 19(10), pp 2981.
- Tao, X.-a., Li, C.-y., Rhodus, N. L., Xia, J., Yang, X.-p. & Cheng, B. 2008. Simultaneous detection of IFN- γ and IL-4 in lesional tissues and whole unstimulated saliva from patients with oral lichen planus. *Journal of Oral Pathology & Medicine*, 37(2), pp 83-87.
- Tao, Z., Gao, J., Qian, L., Huang, Y., Zhou, Y., Yang, L., He, J., Yang, J., Wang, R. & Zhang, Y. 2017. Factors associated with acute oral mucosal reaction induced by radiotherapy in head and

neck squamous cell carcinoma: A retrospective single-center experience. *Medicine (Baltimore)*, 96(50), pp e8446.

Tardieu, C., Cowen, D., Thirion, X. & Franquin, J. C. 1996. Quantitative scale of oral mucositis associated with autologous bone marrow transplantation. *Eur J Cancer B Oral Oncol*, 32b(6), pp 381-7.

Tavianatou, A. G., Caon, I., Franchi, M., Piperigkou, Z., Galesso, D. & Karamanos, N. K. 2019. Hyaluronan: molecular size-dependent signaling and biological functions in inflammation and cancer. *Febs j*, 286(15), pp 2883-2908.

Tebbutt, N. C., Norman, A. R., Cunningham, D., Allen, M., Chau, I., Oates, J. & Hill, M. 2003. Analysis of the time course and prognostic factors determining toxicity due to infused fluorouracil. *British Journal of Cancer*, 88(10), pp 1510-1515.

Termeer, C., Sleeman, J. P. & Simon, J. C. 2003. Hyaluronan—magic glue for the regulation of the immune response? *Trends in immunology*, 24(3), pp 112-114.

Theis, V. S., Sripadam, R., Ramani, V. & Lal, S. 2010. Chronic Radiation Enteritis. *Clinical Oncology*, 22(1), pp 70-83.

Tian, Y. B., Wang, N. X., Xu, Y., Yu, C. Y., Liu, R. M., Luo, Y. & Xiao, J. H. 2020. Hyaluronic acid ameliorates the proliferative ability of human amniotic epithelial cells through activation of TGF- β /BMP signaling. *PeerJ*, 8(e10104).

Tobita, T., Izumi, K. & Feinberg, S. E. 2010. Development of an in vitro model for radiation-induced effects on oral keratinocytes. *International journal of oral and maxillofacial surgery*, 39(4), pp 364-370.

Tolg, C., Hamilton, S. R., Nakrieko, K. A., Kooshesh, F., Walton, P., McCarthy, J. B., Bissell, M. J. & Turley, E. A. 2006. Rhamm-/- fibroblasts are defective in CD44-mediated ERK1,2 mitogenic signaling, leading to defective skin wound repair. *J Cell Biol*, 175(6), pp 1017-28.

Tomlinson, D., Ethier, M. C., Judd, P., Doyle, J., Gassas, A., Naqvi, A. & Sung, L. 2011. Reliability and construct validity of the oral mucositis daily questionnaire in children with cancer. *Eur J Cancer*, 47(3), pp 383-8.

Toole, B. P. 2004. Hyaluronan: from extracellular glue to pericellular cue. *Nature Reviews Cancer*, 4(7), pp 528-539.

Toole, B. P., Ghatak, S. & Misra, S. 2008. Hyaluronan oligosaccharides as a potential anticancer therapeutic. *Current pharmaceutical biotechnology*, 9(4), pp 249-252.

- Toole, B. P., Wight, T. N. & Tammi, M. I. 2002. Hyaluronan-cell interactions in cancer and vascular disease. *J Biol Chem*, 277(7), pp 4593-6.
- Touchefeu, Y., Montassier, E., Nieman, K., Gastinne, T., Potel, G., Bruley des Varannes, S., Le Vacon, F. & de La Cochetière, M. F. 2014. Systematic review: the role of the gut microbiota in chemotherapy- or radiation-induced gastrointestinal mucositis – current evidence and potential clinical applications. *Alimentary Pharmacology & Therapeutics*, 40(5), pp 409-421.
- Trabucchi, E., Pallotta, S., Morini, M., Corsi, F., Franceschini, R., Casiraghi, A., Pravettoni, A., Foschi, D. & Minghetti, P. 2002. Low molecular weight hyaluronic acid prevents oxygen free radical damage to granulation tissue during wound healing. *International journal of tissue reactions*, 24(2), pp 65-71.
- Trommer, H., Wartewig, S., Böttcher, R., Pöpl, A., Hoentsch, J., Ozegowski, J. H. & Neubert, R. H. 2003. The effects of hyaluronan and its fragments on lipid models exposed to UV irradiation. *International journal of pharmaceutics*, 254(2), pp 223-234.
- Trotti, A., Bellm, L. A., Epstein, J. B., Frame, D., Fuchs, H. J., Gwede, C. K., Komaroff, E., Nalysnyk, L. & Zilberberg, M. D. 2003a. Mucositis incidence, severity and associated outcomes in patients with head and neck cancer receiving radiotherapy with or without chemotherapy: a systematic literature review. *Radiotherapy and Oncology*, 66(3), pp 253-262.
- Trotti, A., Byhardt, R., Stetz, J., Gwede, C., Corn, B., Fu, K., Gunderson, L., McCormick, B., Morris, M. & Rich, T. 2000. Common toxicity criteria: version 2.0. an improved reference for grading the acute effects of cancer treatment: impact on radiotherapy. *International Journal of Radiation Oncology* Biology* Physics*, 47(1), pp 13-47.
- Trotti, A., Colevas, A. D., Setser, A., Rusch, V., Jaques, D., Budach, V., Langer, C., Murphy, B., Cumberlin, R., Coleman, C. N. & Rubin, P. 2003b. CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. *Semin Radiat Oncol*, 13(3), pp 176-81.
- Trucci, V. M., Veeck, E. B. & Morosolli, A. R. 2009. Current strategies for the management of oral mucositis induced by radiotherapy or chemotherapy. *Revista Odonto Ciência*, 24(3), pp 309-314.
- Tschachojan, V., Schroer, H., Averbek, N. & Mueller-Klieser, W. 2014. Carbon ions and X-rays induce pro-inflammatory effects in 3D oral mucosa models with and without PBMCs. *Oncology reports*, 32(5), pp 1820-1828.
- Tsujimoto, T., Yamamoto, Y., Wasa, M., Takenaka, Y., Nakahara, S., Takagi, T., Tsugane, M., Hayashi, N., Maeda, K., Inohara, H., Uejima, E. & Ito, T. 2015. L-glutamine decreases the severity of mucositis induced by chemoradiotherapy in patients with locally advanced head and neck cancer: a double-blind, randomized, placebo-controlled trial. *Oncol Rep*, 33(1), pp 33-9.

- Turley, E. A., Noble, P. W. & Bourguignon, L. Y. 2002. Signaling properties of hyaluronan receptors. *J Biol Chem*, 277(7), pp 4589-92.
- Turner, J. R. 2009. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol*, 9(11), pp 799-809.
- Uchida, K., Veeramachaneni, R., Huey, B., Bhattacharya, A., Schmidt, B. L. & Albertson, D. G. 2014. Investigation of HOXA9 promoter methylation as a biomarker to distinguish oral cancer patients at low risk of neck metastasis. *BMC cancer*, 14(1), pp 1-8.
- Urba, S., van Herpen, C. M. L., Sahoo, T. P., Shin, D. M., Licitra, L., Mezei, K., Reuter, C., Hitt, R., Russo, F., Chang, S.-C., Hossain, A. M., Frimodt-Moller, B., Koustenis, A. & Hong, R.-L. 2012. Pemetrexed in combination with cisplatin versus cisplatin monotherapy in patients with recurrent or metastatic head and neck cancer. *Cancer*, 118(19), pp 4694-4705.
- Urbain, P., Raynor, A., Bertz, H., Lambert, C. & Biesalski, H.-K. 2012. Role of antioxidants in buccal mucosa cells and plasma on the incidence and severity of oral mucositis after allogeneic haematopoietic cell transplantation. *Supportive Care in Cancer*, 20(8), pp 1831-1838.
- Valeh, M., Kargar, M., Mansouri, A., Kamranzadeh, H., Gholami, K., Heidari, K. & Hajibabaei, M. 2018. Factors Affecting the Incidence and Severity of Oral Mucositis Following Hematopoietic Stem Cell Transplantation. *Int J Hematol Oncol Stem Cell Res*, 12(2), pp 142-152.
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. & Telser, J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, 39(1), pp 44-84.
- van der Beek, M. T., Laheij, A. M. G. A., Raber-Durlacher, J. E., von dem Borne, P. A., Wolterbeek, R., van der Blij-de Brouwer, C. S., van Loveren, C., Claas, E. C. J., Kroes, A. C. M., de Soet, J. J. & Vossen, A. C. T. M. 2012. Viral loads and antiviral resistance of herpesviruses and oral ulcerations in hematopoietic stem cell transplant recipients. *Bone Marrow Transplantation*, 47(9), pp 1222-1228.
- van Staveren, W. C., Solís, D. Y., Hébrant, A., Detours, V., Dumont, J. E. & Maenhaut, C. 2009. Human cancer cell lines: Experimental models for cancer cells in situ? For cancer stem cells? *Biochim Biophys Acta*, 1795(2), pp 92-103.
- Vane, J. R., Bakhle, Y. S. & Botting, R. M. 1998. Cyclooxygenases 1 and 2. *Annual Review Of Pharmacology And Toxicology*, 38(97-120).
- Vanhoecke, B., De Ryck, T., Stringer, A., Van de Wiele, T. & Keefe, D. 2015. Microbiota and their role in the pathogenesis of oral mucositis. *Oral Diseases*, 21(1), pp 17-30.

- Vanhoecke, B. W., De Ryck, T. R., De boel, K., Wiles, S., Boterberg, T., Van de Wiele, T. & Swift, S. 2016. Low-dose irradiation affects the functional behavior of oral microbiota in the context of mucositis. *Experimental Biology and Medicine*, 241(1), pp 60-70.
- Vanlancker, E., Vanhoecke, B., Smet, R., Props, R. & Van de Wiele, T. 2016. 5-Fluorouracil sensitivity varies among oral micro-organisms. *Journal of Medical Microbiology*, 65(8), pp 775-783.
- Vara, J. Á. F., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C. & González-Barón, M. 2004. PI3K/Akt signalling pathway and cancer. *Cancer Treatment Reviews*, 30(2), pp 193-204.
- Vasconcelos, R. M., Sanfilippo, N., Paster, B. J., Kerr, A. R., Li, Y., Ramalho, L., Queiroz, E. L., Smith, B., Sonis, S. T. & Corby, P. M. 2016. Host-Microbiome Cross-talk in Oral Mucositis. *Journal of Dental Research*, 95(7), pp 725-733.
- Velayutham, M., Guru, A., Arasu, M. V., Al-Dhabi, N. A., Choi, K. C., Elumalai, P., Harikrishnan, R., Arshad, A. & Arockiaraj, J. 2021. GR15 peptide of S-adenosylmethionine synthase (SAME) from *Arthrospira platensis* demonstrated antioxidant mechanism against H₂O₂ induced oxidative stress in in-vitro MDCK cells and in-vivo zebrafish larvae model. *Journal of Biotechnology*, 342(79-91).
- Venkatesh, G. H., Manjunath, V. B., Mumbreakar, K. D., Negi, H., Fernandes, D. J., Sharan, K., Banerjee, S. & Bola Sadashiva, S. R. 2014. Polymorphisms in radio-responsive genes and its association with acute toxicity among head and neck cancer patients. *PLoS One*, 9(3), pp e89079.
- Vera-Llonch, M., Oster, G., Ford, C. M., Lu, J. & Sonis, S. 2007. Oral mucositis and outcomes of autologous hematopoietic stem-cell transplantation following high-dose melphalan conditioning for multiple myeloma. *The journal of supportive oncology*, 5(5), pp 231-235.
- Vera-Llonch, M., Oster, G., Hagiwara, M. & Sonis, S. 2006. Oral mucositis in patients undergoing radiation treatment for head and neck carcinoma. *Cancer*, 106(2), pp 329-36.
- Vera-Llonch, M., Oster, G., Hagiwara, M. & Sonis, S. 2006. Oral mucositis in patients undergoing radiation treatment for head and neck carcinoma. *Cancer*, 106(2), pp 329-336.
- Vertuani, S., Angusti, A. & Manfredini, S. 2004. The antioxidants and pro-antioxidants network: an overview. *Current pharmaceutical design*, 10(14), pp 1677-1694.
- Vessey, D. A., Lee, K.-H. & Blacker, K. L. 1992. Characterization of the oxidative stress initiated in cultured human keratinocytes by treatment with peroxides. *Journal of investigative dermatology*, 99(6), pp 859-863.

- Vigarios, E., Epstein, J. B. & Sibaud, V. 2017. Oral mucosal changes induced by anticancer targeted therapies and immune checkpoint inhibitors. *Support Care Cancer*, 25(5), pp 1713-1739.
- Vigetti, D., Karousou, E., Viola, M., Deleonibus, S., De Luca, G. & Passi, A. 2014. Hyaluronan: biosynthesis and signaling. *Biochim Biophys Acta*, 1840(8), pp 2452-9.
- Vilhardt, F. & van Deurs, B. 2004. The phagocyte NADPH oxidase depends on cholesterol-enriched membrane microdomains for assembly. *The EMBO journal*, 23(4), pp 739-748.
- Villa, A. & Sonis, S. 2016a. Toxicities associated with head and neck cancer treatment and oncology-related clinical trials. *Current problems in cancer*, 40(5-6), pp 244-257.
- Villa, A. & Sonis, S. T. 2015. Mucositis: pathobiology and management. *Curr Opin Oncol*, 27(3), pp 159-64.
- Villa, A. & Sonis, S. T. 2016b. Pharmacotherapy for the management of cancer regimen-related oral mucositis. *Expert Opin Pharmacother*, 17(13), pp 1801-7.
- Villa, A. & Sonis, S. T. 2020. An update on pharmacotherapies in active development for the management of cancer regimen-associated oral mucositis. *Expert Opin Pharmacother*, 21(5), pp 541-548.
- Villa, A., Vollemans, M., De Moraes, A. & Sonis, S. 2021. Concordance of the WHO, RTOG, and CTCAE v4.0 grading scales for the evaluation of oral mucositis associated with chemoradiation therapy for the treatment of oral and oropharyngeal cancers. *Support Care Cancer*, 29(10), pp 6061-6068.
- Viola, M., Vigetti, D., Karousou, E., D'Angelo, M. L., Caon, I., Moretto, P., De Luca, G. & Passi, A. 2015. Biology and biotechnology of hyaluronan. *Glycoconjugate Journal*, 32(3-4), pp 93-103.
- Vistejnova, L., Safrankova, B., Nesporova, K., Slavkovsky, R., Hermannova, M., Hosek, P., Velebny, V. & Kubala, L. 2014. Low molecular weight hyaluronan mediated CD44 dependent induction of IL-6 and chemokines in human dermal fibroblasts potentiates innate immune response. *Cytokine*, 70(2), pp 97-103.
- Vokurka, S., Bystrická, E., Koza, V., Scudlová, J., Pavlicová, V., Valentová, D., Visokaiová, M. & Misaniová, L. 2006. Higher incidence of chemotherapy induced oral mucositis in females: a supplement of multivariate analysis to a randomized multicentre study. *Supportive care in cancer*, 14(9), pp 974-976.
- Vokurka, S., Steinerova, K., Karas, M. & Koza, V. 2009. Characteristics and risk factors of oral mucositis after allogeneic stem cell transplantation with FLU/MEL conditioning regimen in context with BU/CY2. *Bone marrow transplantation*, 44(9), pp 601-605.

- Waddington, R. J., Moseley, R. & Embery, G. 2000. Periodontal Disease Mechanisms: Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. *Oral diseases*, 6(3), pp 138-151.
- Wadler, S., Benson, A. B., Engelking, C., Catalano, R., Field, M., Kornblau, S. M., Mitchell, E., Rubin, J., Trotta, P. & Vokes, E. 1998. Recommended guidelines for the treatment of chemotherapy-induced diarrhea. *Journal of Clinical Oncology*, 16(9), pp 3169-3178.
- Walsh, L., Gillham, C., Dunne, M., Fraser, I., Hollywood, D., Armstrong, J. & Thirion, P. 2011. Toxicity of cetuximab versus cisplatin concurrent with radiotherapy in locally advanced head and neck squamous cell cancer (LAHNSCC). *Radiotherapy and Oncology*, 98(1), pp 38-41.
- Walsh, L. J., Hill, G., Seymour, G. & Roberts, A. 1990. A scoring system for the quantitative evaluation of oral mucositis during bone marrow transplantation. *Special Care in Dentistry*, 10(6), pp 190-195.
- Wang, A., Ling, Z., Yang, Z., Kiela, P. R., Wang, T., Wang, C., Cao, L., Geng, F., Shen, M., Ran, X., Su, Y., Cheng, T. & Wang, J. 2015. Gut microbial dysbiosis may predict diarrhea and fatigue in patients undergoing pelvic cancer radiotherapy: a pilot study. *PLoS One*, 10(5), pp e0126312.
- Wang, J., Albertson, C. M., Zheng, H., Fink, L. M., Herbert, J.-M. & Hauer-Jensen, M. 2002. Short-term inhibition of ADP-induced platelet aggregation by clopidogrel ameliorates radiation-induced toxicity in rat small intestine. *Thrombosis And Haemostasis*, 87(1), pp 122-128.
- Wang, W., Chen, J., Li, M., Jia, H., Han, X., Zhang, J., Zou, Y., Tan, B., Liang, W., Shang, Y., Xu, Q., A, S., Wang, W., Mao, J., Gao, X., Fan, G. & Liu, W. 2019. Rebuilding Postinfarcted Cardiac Functions by Injecting TIIA@PDA Nanoparticle-Cross-linked ROS-Sensitive Hydrogels. *ACS Applied Materials & Interfaces*, 11(3), pp 2880-2890.
- Wardill, H. R., Bowen, J. M., Al-Dasooqi, N., Sultani, M., Bateman, E., Stansborough, R., Shirren, J. & Gibson, R. J. 2014. Irinotecan disrupts tight junction proteins within the gut : implications for chemotherapy-induced gut toxicity. *Cancer Biol Ther*, 15(2), pp 236-44.
- Wardill, H. R., Gibson, R. J., Van Seville, Y. Z., Secombe, K. R., Coller, J. K., White, I. A., Manavis, J., Hutchinson, M. R., Staikopoulos, V., Logan, R. M. & Bowen, J. M. 2016. Irinotecan-Induced Gastrointestinal Dysfunction and Pain Are Mediated by Common TLR4-Dependent Mechanisms. *Mol Cancer Ther*, 15(6), pp 1376-86.
- Wardill, H. R., Sonis, S. T., Blijlevens, N. M. A., Van Seville, Y. Z. A., Ciorba, M. A., Loeffen, E. A. H., Cheng, K. K. F., Bossi, P., Porcello, L., Castillo, D. A., Elad, S., Bowen, J. M. & On behalf of The Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society of Oral, O. 2020. Prediction of mucositis risk secondary to cancer therapy: a systematic review of current evidence and call to action. *Supportive Care in Cancer*, 28(11), pp 5059-5073.

- Wardill, H. R., Tissing, W. J., Kissow, H. & Stringer, A. M. 2019. Animal models of mucositis: critical tools for advancing pathobiological understanding and identifying therapeutic targets. *Current opinion in supportive and palliative care*, 13(2), pp 119-133.
- Wardley, A. M., Jayson, G. C., Swindell, R., Morgenstern, G. R., Chang, J., Bloor, R., Fraser, C. & Scarffe, J. H. 2000. Prospective evaluation of oral mucositis in patients receiving myeloablative conditioning regimens and haemopoietic progenitor rescue. *British journal of haematology*, 110(2), pp 292-299.
- Watanabe, S., Suemaru, K., Yamaguchi, T., Hidaka, N., Sakanaka, M. & Araki, H. 2009. Effect of oral mucosal adhesive films containing ginsenoside Rb1 on 5-fluorouracil-induced oral mucositis in hamsters. *Eur J Pharmacol*, 616(1-3), pp 281-6.
- Watts, M., Woodcock, M., Arnold, S. & Chaplin, D. 1997. Effects of novel and conventional anti-cancer agents on human endothelial permeability: influence of tumour secreted factors. *Anticancer research*, 17(1A), pp 71-75.
- WCCNR, W. C. f. C. N. R. 1991. Development of a staging system for chemotherapy-induced stomatitis. *Cancer Nurs*, 14(1), pp 6-12.
- Webb, G. J., Brooke, R. & De Silva, A. N. 2013. Chronic radiation enteritis and malnutrition. *Journal of Digestive Diseases*, 14(7), pp 350-357.
- Weng, Z., Fluckiger, A.-C., Nisitani, S., Wahl, M. I., Le, L. Q., Hunter, C. A., Fernal, A. A., Le Beau, M. M. & Witte, O. N. 1998. A DNA damage and stress inducible G protein-coupled receptor blocks cells in G2/M. *Proceedings of the National Academy of Sciences*, 95(21), pp 12334-12339.
- Wenk, J., Sabiwalsky, A., Dissemond, J., Meewes, C., Brenneisen, P., Wlaschek, M., Scharffetter-Kochanek, K., Foitzik, A., Achterberg, V. & Reitz, A. 2001. Selective pick-up of increased iron by deferoxamine-coupled cellulose abrogates the iron-driven induction of matrix-degrading metalloproteinase 1 and lipid peroxidation in human dermal fibroblasts in vitro: a new dressing concept. *Journal of investigative dermatology*, 116(6), pp 833-839.
- West, D. C. & Kumar, S. 1989. Hyaluronan and angiogenesis. *Ciba Found Symp*, 143(187-201; discussion 201-7, 281-5).
- West, D. C., Shaw, D. M., Lorenz, P., Adzick, N. S. & Longaker, M. T. 1997. Fibrotic healing of adult and late gestation fetal wounds correlates with increased hyaluronidase activity and removal of hyaluronan. *Int J Biochem Cell Biol*, 29(1), pp 201-10.

- Wickström, M., Haglund, C., Lindman, H., Nygren, P., Larsson, R. & Gullbo, J. 2008. The novel alkylating prodrug J1: diagnosis directed activity profile ex vivo and combination analyses in vitro. *Investigational new drugs*, 26(3), pp 195-204.
- Widelitz, R. 2005. Wnt signaling through canonical and non-canonical pathways: Recent progress. *Growth Factors*, 23(2), pp 111-116.
- Wilberg, P., Hjermstad, M. J., Ottesen, S. & Herlofson, B. B. 2014. Chemotherapy-associated oral sequelae in patients with cancers outside the head and neck region. *Journal of pain and symptom management*, 48(6), pp 1060-1069.
- Wilken, R., Veena, M. S., Wang, M. B. & Srivatsan, E. S. 2011. Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. *Molecular cancer*, 10(1), pp 12.
- Wilkes, J. D. 1998. Prevention and treatment of oral mucositis following cancer chemotherapy. *Semin Oncol*, 25(5), pp 538-51.
- Williams, J. M., Duckworth, C. A., Vowell, K., Burkitt, M. D. & Pritchard, D. M. 2016. Intestinal preparation techniques for histological analysis in the mouse. *Current protocols in mouse biology*, 6(2), pp 148-168.
- Wingard, J. R., Niehaus, C. S., Peterson, D. E., Jones, R. J., Piantadosi, S., Levin, L. S., Saral, R. & Santos, G. W. 1991. Oral mucositis after bone marrow transplantation: a marker of treatment toxicity and predictor of hepatic veno-occlusive disease. *Oral surgery, oral medicine, oral pathology*, 72(4), pp 419-424.
- Winqvist, E., Al-Rasheedy, I., Nichols, A. C., Palma, D. A. & Stitt, L. 2014. Temporal changes in the efficacy of chemotherapy for recurrent or metastatic squamous cell carcinoma of the head and neck: a systematic review and meta-analysis. *Cancer treatment reviews*, 40(9), pp 1073-1079.
- Wisniewski, H. G., Hua, J. C., Poppers, D. M., Naime, D., Vilcek, J. & Cronstein, B. N. 1996. TNF/IL-1-inducible protein TSG-6 potentiates plasmin inhibition by inter-alpha-inhibitor and exerts a strong anti-inflammatory effect in vivo. *The Journal of Immunology*, 156(4), pp 1609-1615.
- Wong, D. V. T., Lima-Júnior, R. C. P., Carvalho, C. B. M., Borges, V. F., Wanderley, C. W. S., Bem, A. X. C., Leite, C. A. V. G., Teixeira, M. A., Batista, G. L. P., Silva, R. L., Cunha, T. M., Brito, G. A. C., Almeida, P. R. C., Cunha, F. Q. & Ribeiro, R. A. 2015. The Adaptor Protein Myd88 Is a Key Signaling Molecule in the Pathogenesis of Irinotecan-Induced Intestinal Mucositis. *PLOS ONE*, 10(10), pp e0139985.

- Wong, V. & Gumbiner, B. M. 1997. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *The Journal of cell biology*, 136(2), pp 399-409.
- Woo, S.-B., Sonis, S. T. & Sonis, A. L. 1990. The role of herpes simplex virus in the development of oral mucositis in bone marrow transplant recipients. *Cancer*, 66(11), pp 2375-2379.
- Woo, S. B., Sonis, S. T., Monopoli, M. M. & Sonis, A. L. 1993. A longitudinal study of oral ulcerative mucositis in bone marrow transplant recipients. *Cancer*, 72(5), pp 1612-1617.
- World Health Organization. 1979. WHO handbook for reporting results of cancer treatment. Geneva: World Health Organization, WHO Offset publication, (48), p.82.
- World Health Organization 1996. *Cancer pain relief : with a guide to opioid availability*, 2nd ed, Geneva: World Health Organization.
- Worthington, H. V., Clarkson, J. E., Bryan, G., Furness, S., Glenny, A. M., Littlewood, A., McCabe, M. G., Meyer, S. & Khalid, T. 2011. Interventions for preventing oral mucositis for patients with cancer receiving treatment. *Cochrane Database Syst Rev*, 4), pp CD000978.
- Worthington, H. V., Clarkson, J. E. & Eden, O. B. 2004. Interventions for treating oral mucositis for patients with cancer receiving treatment. *Cochrane Database Syst Rev*, 2), pp CD001973.
- Worthington, H. V., Clarkson, J. E. & Eden, O. B. 2007. Interventions for preventing oral mucositis for patients with cancer receiving treatment. *Cochrane Database Syst Rev*, 4), pp CD000978.
- Wu, C. L., Chou, H. C., Li, J. M., Chen, Y. W., Chen, J. H., Chen, Y. H. & Chan, H. L. 2013. Hyaluronic acid-dependent protection against alkali-burned human corneal cells. *Electrophoresis*, 34(3), pp 388-96.
- Wu, H., Zhang, H., Wang, C., Wu, Y., Xie, J., Jin, X., Yang, J. & Ye, J. 2011a. Genoprotective effect of hyaluronic acid against benzalkonium chloride-induced DNA damage in human corneal epithelial cells. *Mol Vis*, 17(3364-70).
- Wu, X., Chen, P., Sonis, S. T., Lingen, M. W., Berger, A. & Toback, F. G. 2012. A novel peptide to treat oral mucositis blocks endothelial and epithelial cell apoptosis. *International Journal of Radiation Oncology* Biology* Physics*, 83(3), pp e409-e415.
- Wu, Z. Q., Han, X. D., Wang, Y., Yuan, K. L., Jin, Z. M., Di, J. Z., Yan, J., Pan, Y., Zhang, P., Huang, X. Y., Wang, Z. G. & Zheng, Q. 2011b. Interleukin-1 receptor antagonist reduced apoptosis and attenuated intestinal mucositis in a 5-fluorouracil chemotherapy model in mice. *Cancer Chemother Pharmacol*, 68(1), pp 87-96.

- Wuketich, S., Hienz, S. A. & Marosi, C. 2012. Prevalence of clinically relevant oral mucositis in outpatients receiving myelosuppressive chemotherapy for solid tumors. *Supportive Care in Cancer*, 20(1), pp 175-183.
- Wymenga, A. N., van der Graaf, W. T., Spijkervet, F. L., Timens, W., Timmer-Bosscha, H., Sluiter, W. J., de Vries, E. G. & Mulder, N. H. 1997. A new in vitro assay for quantitation of chemotherapy-induced mucositis. *Br J Cancer*, 76(8), pp 1062-6.
- Xin, Y.-F., Wan, L.-L., Peng, J.-L. & Guo, C. 2011. Alleviation of the acute doxorubicin-induced cardiotoxicity by Lycium barbarum polysaccharides through the suppression of oxidative stress. *Food and Chemical Toxicology*, 49(1), pp 259-264.
- Xu, B., Guo, Y., Chen, Y., Lu, H., Tang, T., Yue, Z., Guan, G., Chi, P. & Lin, C. 2015. Is the irradiated small bowel volume still a predictor for acute lower gastrointestinal toxicity during preoperative concurrent chemo-radiotherapy for rectal cancer when using intensity-modulated radiation therapy? *Radiation Oncology*, 10(1), pp 257.
- Xu, H., Yu, S., Liu, Q., Yuan, X., Mani, S., Pestell, R. G. & Wu, K. 2017. Recent advances of highly selective CDK4/6 inhibitors in breast cancer. *J Hematol Oncol*, 10(1), pp 97.
- Xu, Q., Izumi, K., Tobita, T., Nakanishi, Y. & Feinberg, S. E. 2009. Constitutive release of cytokines by human oral keratinocytes in an organotypic culture. *Journal of oral and maxillofacial surgery*, 67(6), pp 1256-1264.
- Xu, Q., Venet, M., Wang, W., Creagh-Flynn, J., Wang, X., Li, X., Gao, Y., Zhou, D., Zeng, M. & Lara-Sáez, I. 2018. Versatile hyperbranched poly (β -hydrazide ester) macromers as injectable antioxidative hydrogels. *ACS applied materials & interfaces*, 10(46), pp 39494-39504.
- Yamaguchi, K., Ono, K., Hitomi, S., Ito, M., Nodai, T., Goto, T., Harano, N., Watanabe, S., Inoue, H., Miyano, K., Uezono, Y., Matoba, M. & Inenaga, K. 2016. Distinct TRPV1- and TRPA1-based mechanisms underlying enhancement of oral ulcerative mucositis-induced pain by 5-fluorouracil. *Pain*, 157(5), pp 1004-20.
- Yamamoto, H., Ishihara, K., Takeda, Y., Koizumi, W. & Ichikawa, T. 2013. Changes in the Mucus Barrier during Cisplatin-Induced Intestinal Mucositis in Rats. *BioMed Research International*, 2013(276186).
- Yamamoto, K., Arakawa, T., Ueda, N. & Yamamoto, S. 1995. Transcriptional Roles of Nuclear Factor B and Nuclear Factor-Interleukin-6 in the Tumor Necrosis Factor -Dependent Induction of Cyclooxygenase-2 in MC3T3-E1 Cells. *Journal of Biological Chemistry*, 270(52), pp 31315-31320.

- Yang, C., Cao, M., Liu, H., He, Y., Xu, J., Du, Y., Liu, Y., Wang, W., Cui, L. & Hu, J. 2012. The high and low molecular weight forms of hyaluronan have distinct effects on CD44 clustering. *Journal of Biological Chemistry*, 287(51), pp 43094-43107.
- Yang, D., Tournier, C., Wysk, M., Lu, H.-T., Xu, J., Davis, R. J. & Flavell, R. A. 1997. Targeted disruption of the MKK4 gene causes embryonic death, inhibition of c-Jun NH2-terminal kinase activation, and defects in AP-1 transcriptional activity. *Proceedings of the National Academy of Sciences*, 94(7), pp 3004-3009.
- Yang, T. J., Oh, J. H., Son, C. H., Apte, A., Deasy, J. O., Wu, A. & Goodman, K. A. 2013. Predictors of acute gastrointestinal toxicity during pelvic chemoradiotherapy in patients with rectal cancer. *Gastrointest Cancer Res*, 6(5-6), pp 129-36.
- Yarom, N., Hovan, A., Bossi, P., Ariyawardana, A., Jensen, S. B., Gobbo, M., Saca-Hazboun, H., Kandwal, A., Majorana, A., Ottaviani, G., Pentenero, M., Nasr, N. M., Rouleau, T., Lucas, A. S., Treister, N. S., Zur, E., Ranna, V., Vaddi, A., Barasch, A., Lalla, R. V., Cheng, K. K. F. & Elad, S. 2020. Systematic review of natural and miscellaneous agents, for the management of oral mucositis in cancer patients and clinical practice guidelines - part 2: honey, herbal compounds, saliva stimulants, probiotics, and miscellaneous agents. *Support Care Cancer*, 28(5), pp 2457-2472.
- Yarom, N., Hovan, A., Bossi, P., Ariyawardana, A., Jensen, S. B., Gobbo, M., Saca-Hazboun, H., Kandwal, A., Majorana, A., Ottaviani, G., Pentenero, M., Nasr, N. M., Rouleau, T., Lucas, A. S., Treister, N. S., Zur, E., Ranna, V., Vaddi, A., Cheng, K. K. F., Barasch, A., Lalla, R. V. & Elad, S. 2019. Systematic review of natural and miscellaneous agents for the management of oral mucositis in cancer patients and clinical practice guidelines-part 1: vitamins, minerals, and nutritional supplements. *Support Care Cancer*, 27(10), pp 3997-4010.
- Yasuda, M., Kato, S., Yamanaka, N., Iimori, M., Matsumoto, K., Utsumi, D., Kitahara, Y., Amagase, K., Horie, S. & Takeuchi, K. 2013. 5-HT₃ receptor antagonists ameliorate 5-fluorouracil-induced intestinal mucositis by suppression of apoptosis in murine intestinal crypt cells. *Br J Pharmacol*, 168(6), pp 1388-400.
- Yasuda, M., Kato, S., Yamanaka, N., Iimori, M., Utsumi, D., Kitahara, Y., Iwata, K., Matsuno, K., Amagase, K., Yabe-Nishimura, C. & Takeuchi, K. 2012. Potential role of the NADPH oxidase NOX1 in the pathogenesis of 5-fluorouracil-induced intestinal mucositis in mice. *Am J Physiol Gastrointest Liver Physiol*, 302(10), pp G1133-42.
- Ye, J., Wu, H., Wu, Y., Wang, C., Zhang, H., Shi, X. & Yang, J. 2012. High molecular weight hyaluronan decreases oxidative DNA damage induced by EDTA in human corneal epithelial cells. *Eye*, 26(7), pp 1012-1020.
- Ye, Y., Carlsson, G., Agholme, M. B., Wilson, J. A., Roos, A., Henriques-Normark, B., Engstrand, L., Modeer, T. & Putsep, K. 2013. Oral bacterial community dynamics in paediatric patients with malignancies in relation to chemotherapy-related oral mucositis: a prospective study. *Clin Microbiol Infect*, 19(12), pp E559-67.

- Yeager, K. A., Webster, J., Crain, M., Kasow, J. & McGuire, D. B. 2000. Implementation of an oral care standard for leukemia and transplantation patients. *Cancer Nurs*, 23(1), pp 40-7; quiz 47-8.
- Yeoh, A., Gibson, R., Yeoh, E., Bowen, J., Stringer, A., Giam, K., Logan, R. & Keefe, D. 2006. Radiation therapy-induced mucositis: relationships between fractionated radiation, NF- κ B, COX-1, and COX-2. *Cancer treatment reviews*, 32(8), pp 645-651.
- Yeoh, A. S., Bowen, J. M., Gibson, R. J. & Keefe, D. M. 2005. Nuclear factor κ B (NF κ B) and cyclooxygenase-2 (Cox-2) expression in the irradiated colorectum is associated with subsequent histopathological changes. *International Journal of Radiation Oncology* Biology* Physics*, 63(5), pp 1295-1303.
- Yiannis, C., Huang, K., Tran, A. N., Zeng, C., Dao, E., Baselyous, O., Mithwani, M. A., Paolini, R., Cirillo, N. & Yap, T. 2020. Protective effect of kava constituents in an in vitro model of oral mucositis. *Journal of Cancer Research and Clinical Oncology*, 146(7), pp 1801-1811.
- Ying, G., Tang, Z., Zhang, J., Zeng, J., Zheng, Z., Zhang, W., Ding, L., Wen, T. & Yi, D. 2022. Long noncoding RNA CASC2 protect ROS-induced oxidative stress in myocardial infarction by miR-18a/SIRT2. *Biotechnology and Applied Biochemistry*, 69(5), pp 1857-1866.
- Yoneda, S., Imai, S., Hanada, N., Yamazaki, T., Senpuku, H., Ota, Y. & Uematsu, H. 2007. Effects of oral care on development of oral mucositis and microorganisms in patients with esophageal cancer. *Japanese journal of infectious diseases*, 60(1), pp 23-28.
- Yoshino, F., Yoshida, A., Nakajima, A., Wada-Takahashi, S., Takahashi, S.-s. & Lee, M. C.-i. 2013. Alteration of the redox state with reactive oxygen species for 5-fluorouracil-induced oral mucositis in hamsters. *Plos One*, 8(12), pp e82834-e82834.
- Yu, Y. T., Deng, J. L., Jin, X. R., Zhang, Z. Z., Zhang, X. H. & Zhou, X. 2020. Effects of 9 oral care solutions on the prevention of oral mucositis: a network meta-analysis of randomized controlled trials. *Medicine (Baltimore)*, 99(16), pp e19661.
- Yuan, A., Kurtz, S. L., Barysaukas, C. M., Pilotte, A. P., Wagner, A. J. & Treister, N. S. 2015. Oral adverse events in cancer patients treated with VEGFR-directed multitargeted tyrosine kinase inhibitors. *Oral Oncol*, 51(11), pp 1026-1033.
- Yuan, A. & Sonis, S. 2014. Emerging therapies for the prevention and treatment of oral mucositis. *Expert Opinion On Emerging Drugs*, 19(3), pp 343-351.
- Zadik, Y., Arany, P. R., Fregnani, E. R., Bossi, P., Antunes, H. S., Bensadoun, R. J., Gueiros, L. A., Majorana, A., Nair, R. G., Ranna, V., Tissing, W. J. E., Vaddi, A., Lubart, R., Migliorati, C. A., Lalla, R. V., Cheng, K. K. F. & Elad, S. 2019. Systematic review of photobiomodulation for the

management of oral mucositis in cancer patients and clinical practice guidelines. *Support Care Cancer*, 27(10), pp 3969-3983.

- Zalcberg, J., Kerr, D., Seymour, L. & Palmer, M. 1998. Haematological and non-haematological toxicity after 5-fluorouracil and leucovorin in patients with advanced colorectal cancer is significantly associated with gender, increasing age and cycle number¹. *European Journal of Cancer*, 34(12), pp 1871-1875.
- Zerbe, M. B., Parkerson, S. G., Ortlieb, M. L. & Spitzer, T. 1992. Relationships between oral mucositis and treatment variables in bone marrow transplant patients. *Cancer nursing*, 15(3), pp 196-205.
- Zhang, S., Liu, Y., Xiang, D., Yang, J., Liu, D., Ren, X. & Zhang, C. 2018. Assessment of dose-response relationship of 5-fluorouracil to murine intestinal injury. *Biomedicine & Pharmacotherapy*, 106(910-916).
- Zhao, H., Tanaka, T., Mitlitski, V., Heeter, J., Balazs, E. A. & Darzynkiewicz, Z. 2008. Protective effect of hyaluronate on oxidative DNA damage in WI-38 and A549 cells. *International journal of oncology*, 32(6), pp 1159-1167.
- Zhao, W. & Robbins, M. E. 2009. Inflammation and chronic oxidative stress in radiation-induced late normal tissue injury: therapeutic implications. *Current medicinal chemistry*, 16(2), pp 130-143.
- Zheng, J., Piao, M. J., Keum, Y. S., Kim, H. S. & Hyun, J. W. 2013. Fucoxanthin Protects Cultured Human Keratinocytes against Oxidative Stress by Blocking Free Radicals and Inhibiting Apoptosis. *Biomolecules & Therapeutics*, 21(4), pp 270-276.
- Zhou, X., Zhang, G.-y., Wang, J., Lu, S.-l., Cao, J. & Sun, L.-z. 2012. A novel bridge between oxidative stress and immunity: the interaction between hydrogen peroxide and human leukocyte antigen G in placental trophoblasts during preeclampsia. *American journal of obstetrics and gynecology*, 206(5), pp 447. e7-447. e16.
- Zhu, H., Mitsuhashi, N., Klein, A., Barsky, L. W., Weinberg, K., Barr, M. L., Demetriou, A. & Wu, G. D. 2006. The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. *Stem Cells*, 24(4), pp 928-35.
- Zhu, W., Mow, V. C., Rosenberg, L. C. & Tang, L.-H. 1994. Determination of kinetic changes of aggrecan-hyaluronan interactions in solution from its rheological properties. *Journal of biomechanics*, 27(5), pp 571-579.
- Zhu, X., Wang, K., Zhang, K., Zhu, L. & Zhou, F. 2014. Ziyuglycoside II induces cell cycle arrest and apoptosis through activation of ROS/JNK pathway in human breast cancer cells. *Toxicology Letters*, 227(1), pp 65-73.

- Zoico, E., Nori, N., Darra, E., Tebon, M., Rizzatti, V., Policastro, G., De Caro, A., Rossi, A. P., Fantin, F. & Zamboni, M. 2021. Senolytic effects of quercetin in an in vitro model of pre-adipocytes and adipocytes induced senescence. *Scientific Reports*, 11(1), pp 23237.
- Zunt, S. L., Burton, L. V., Goldblatt, L. I., Dobbins, E. E. & Srinivasan, M. 2009. Soluble forms of Toll-like receptor 4 are present in human saliva and modulate tumour necrosis factor-alpha secretion by macrophage-like cells. *Clinical And Experimental Immunology*, 156(2), pp 285-293.
- Zuo, T., Cao, L., Li, X., Zhang, Q., Xue, C. & Tang, Q. 2015. The squid ink polysaccharides protect tight junctions and adherens junctions from chemotherapeutic injury in the small intestinal epithelium of mice. *Nutrition and cancer*, 67(2), pp 364-371.