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Manuscript Title

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Running title

Odontoblast markers in SPM and HSPM.

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Author contribution

SR, JS conceived idea. JS, DJM, MJM, SR designed the project. JS collected data, JS and SR analysis. JS and SR lead the writing. JS, DJM, MJM, SR approved the final manuscript.

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Conflict of interest

All authors deny any conflicts of interest in this study.

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TITLE

"Odontoblast markers and dentine reactions in carious primary molars with and without hypomineralised enamel defects."

SUMMARY

Background: Wnt/β-Catenin signalling and DMP1 have key roles in tertiary dentinogenesis.

Aim: To compare the relationship between remaining dentine thickness (RDT), tertiary dentine thickness (TDT), β-Catenin and dentine matrix protein 1 (DMP1) in carious second primary molar teeth with normal (SPM) and hypomineralised enamel (HSPM).

Design: Extracted carious SPM and HSPM were fixed, sectioned (5 μm), and stained with haematoxylin and eosin or with indirect immunofluorescence for β-Catenin and DMP1. Image analysis was performed to determine RDT, TDT, β-Catenin and DMP1 intensity in the odontoblast layer and dentine-pulp complex.

Results: Carious SPM (n=11; mean RDT=1536.1 μm) and HSPM (n=12; mean RDT=1179.9 μm) had mean TDT 248.6 μm and 518.1 μm respectively (P=0.02). There were no significant differences in intensity values in the odontoblast layer and dentine-pulp complex for β-Catenin and DMP1 for both groups.

Conclusion: There was no observable variation in Wnt/β-Catenin and DMP1 expression between HSPM and SPM despite a statistically significant two-fold increased TDT in HSPM compared to SPM that had similar RDT. Thus, the observed increased TDT in HSPM is more likely due to an earlier onset of repair processes rather than an amplified response to caries.

KEYWORDS

DMP1, Wnt/β-Catenin, hypomineralised enamel, odontoblasts, primary teeth

1. INTRODUCTION

Odontoblasts are the first pulpal cells encountered by dentine-invading pathogens and their released products. They defend the pulp against bacterial infection by releasing antibacterial agents (nitric oxide, β-defensins and liposaccharide-binding proteins) and secreting pro-inflammatory cytokines and chemokines[1]. However, their ability to produce tertiary dentine remains their most important response to carious lesion progression. A mild pulpal inflammatory response, combined with the leaching of signalling molecules and growth factors from the dentine, can stimulate tertiary dentine formation[2].

Carious lesion progression involves bacteria and their by-products, breaking down dentinal tubule walls and releasing signalling molecules, such as the Small-Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs)[3], matrix metalloproteinases[1], and growth factors such as tumour growth factor-β super family, insulin-like growth factor-1 and 2, fibroblast growth factor-2 and several angiogenic growth factors[2]. Dentine matrix protein 1 (DMP1), a member of the SIBLINGs family[3], is a highly phosphorylated extracellular matrix protein that has been detected in inflamed permanent pulps, both within the sub-odontoblast cell layer at the periphery of the pulp and within the core of the pulp[4]. DMP1 plays a key role in regulation of odontoblast differentiation and dentine mineralisation[5] and has been identified as a marker for odontoblast differentiation[6-9] and maturity[7, 10, 11]. The presence of DMP1 in inflamed pulps and absence in normal pulps indicates that DMP1 also plays an important role in the inflammatory process[4], increasing the release of proinflammatory mediators (Interleukin-6 and Interleukin-8) from dental pulp fibroblasts, which are responsible for recruiting inflammatory cells such as neutrophils to the site of injury. Therefore, DMP1 may be considered as a marker for assessing the pro-inflammatory activity of pulpal fibroblasts and angiogenesis[4].

In initial, slowly progressing carious lesions, signalling molecules interact with post-mitotic odontoblasts to upregulate their formation of reactionary dentine. Further lesion progression may result in necrosis of odontoblasts. The interaction of signalling molecules with dental pulp stem cells (DPSCs) can trigger their differentiation to odontoblast-like cells that produce reparative dentine[2]. Due to the cyclic nature of carious lesion progression, tertiary dentine is usually a combination of reactionary and reparative dentine[12]. The interaction of signalling molecules with odontoblasts and DPSCs can activate several signalling transduction pathways involved in tertiary dentinogenesis. These include p38 Mitogen-Activated Protein

Kinase, Transforming Growth Factor/Small Mother Against Decapentaplegic, Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinase, c-Jun N-terminal Kinase and Wingless/β-Catenin (Wnt/β-Catenin) Pathways[13].

The Wnt/β-Catenin signalling pathway plays an important role in tertiary dentinogenesis by promoting the proliferation and differentiation of DPSCs to odontoblast-like cells[14-17]. This is thought to occur via the activation of the Runt-related transcription factor 2[15]. The protein β-Catenin is the main component of the Wnt/β-Catenin pathway and is believed to be the bottleneck through which signals pass[15]. Odontoblasts are Wnt responsive[14] and the Wnt/β-Catenin pathway activates both post-mitotic and odontoblast-like cells[18]. Therefore β-Catenin, which indicates activation of the Wnt/β-Catenin pathway, can be considered a marker for assessing tertiary dentinogenesis[13-15]. Wnt/β-Catenin signalling also stimulates angiogenesis via the induction of angiogenic regulators such as Interleukin-8[19]. The upregulation of angiogenic signalling during pulpal inflammation contributes considerably to the pathogenesis associated with survival and differentiation of DPSCs into mature odontoblast-like cells[20, 21]. Therefore, angiogenic activity can be assessed through β-Catenin markers that indicate activation of the Wnt/β-Catenin signalling pathway[20].

There is limited research related to the role of Wnt/β-Catenin signalling and DMP1 in tertiary dentine formation in carious primary teeth with both normal and hypomineralised enamel. Therefore, the aim of this study is to compare the relationship between remaining dentine thickness (RDT), tertiary dentine thickness (TDT), and β-Catenin and DMP1 biomarkers for tertiary dentine in carious second primary molar teeth with normal (SPM) and hypomineralised enamel (HSPM).

2. MATERIALS AND METHODS

Ethical approval for the study was obtained from the University of Melbourne Human Ethics Research Committee (HREC number 1749762.1). Parental consent and child assent were obtained prior to tooth collection. A convenience sample of 23 extracted carious second primary molar teeth (SPM n=11, HSPM n=12) were collected from 23 healthy children (mean age 5.5 ± 1.6 y, range 3-7.5 y), requiring dental extractions under general anaesthesia at the Royal Dental Hospital Melbourne between April-December 2018. Teeth with clinical or radiographical signs of pulp pathosis were excluded. Collected teeth were deidentified

prior to laboratory processing. The percentage of total root length that had undergone any resorption was calculated using Kramer and Ireland's normative data[22].

Immediately following extraction, teeth were sectioned at the root apices and placed in fixative (10% neutral buffered formalin) for 24 h at room temperature prior to decalcification in 20% ethylene diamine tetra-acetic acid solution at pH 7.4. Decalcified teeth were cut longitudinally through the carious lesion. Tooth halves were embedded in paraffin wax and sectioned at 5 μm with a microtome through the deepest part of the carious lesion. Two sections per tooth were stained with either haematoxylin and eosin (n=46) or stained using indirect immunofluorescence for β-Catenin (n=46) and DMP1 (n=46).

Prior to immunostaining, slides were immersed in boiling antigen retrieval solution (10 mM sodium citrate) and quenched (0.25% potassium permanganate) for 20 min to reduce autofluorescence. Slides were then incubated in phosphate-buffered saline and triton containing 10% goat serum for 30 min at room temperature. Following this, sections were labelled with either monoclonal antibody to β-Catenin (rabbit anti-β-Catenin E247, dilution 1: 100, Abcam, Cambridge, UK (ab32572)) or polyclonal antibody to DMP1 (rabbit anti-DMP1, dilution 1:300, Abcam, Cambridge, UK (ab103203)) for 1 h at room temperature. Slides were subsequently incubated with polyclonal fluorescent secondary antibodies (goat anti-rabbit IgG H & L Alexa Fluor 488 preadsorbed, dilution 1:500, Abcam, Cambridge, UK (ab150081)) for 1h at room temperature. Nuclear counterstaining (Hoechst 33342, Abcam, Cambridge, UK (ab228551)) was performed for 10 min prior to cover slipping. No positive labelling was seen in any of the controls.

Haematoxylin and eosin-stained slides were viewed in Brightfield Standard Mode with the Nikon Eclipse TE2000-U microscope (Nikon Corporation, Japan) at magnifications of x1.5 and x20. Remaining dentine thickness (RDT/μm), the shortest distance between the cavity base and pulp-dentine border, and tertiary dentine thickness (TDT/μm), the longest distance between the cavity base and calciotraumatic line, were obtained using the measurement tool in the Fiji (Fiji Is Just ImageJ, open source project hosted on GitHub, https://fiji.sc/) analysis software. Immuno-stained slides were viewed in Wide-Field Fluorescent Mode with the Nikon Eclipse TE2000-U microscope at X20 magnification. A TG-2A long pass filter cube was used to view Alexa Fluor 488 fluorescence and a DAPI Bandpass filter cube was used to view the Hoechst 33342 fluorescence. 16-bit multi-channel colour images of pulp sections subjacent to the cavity base were captured using a high sensitivity Nikon Ds-Fi1 colour

camera (Nikon Corporation, Japan) with standardised exposure settings (333ms, Gamma=0, Offset=0). The colour images were converted to 16-bit grey images in Fiji, and subsequently the mean grey value (MGV) of β-Catenin and DMP1 within the odontoblast layer and the dentine-pulp complex were obtained using the measurement tool in Fiji.

A single examiner performed all the measurements and intra-examiner reproducibility was assessed by repeating measurements for RDT, TDT, β-Catenin and DMP1 intensities in the odontoblast layer and dentine-pulp complex on 22% of the samples 1 week after the initial measurement.

The data obtained had normal distribution and therefore parametric tests were used. Pearson's correlation was used to test for statistically significant correlations between RDT, TDT, and intensities of β-Catenin and DMP1. The t-test for independent samples determined whether there were any statistically significant differences between the two groups: SPM and HSPM. The statistical significance level was set at $p < 0.05$.

3. RESULTS

SPM (occlusal caries: n=9; proximal caries: n=2) were collected from children (4 boys, 7 girls) with mean age 5.6 ± 1.4 y, range 3-7.5 y. The degree of root resorption ranged between 7.4% and 63.2% (mean 27.5 ± 16.7%) and RDT (remaining dentine thickness) ranged between 880.9 μm and 2165.7 μm. HSPM (occlusal caries: n=9; proximal caries: n=3) were collected from children (8 boys, 4 girls) with mean age 5.4 ± 1.8 y, range 3-7.5 y. The degree of root resorption ranged between 9.4% and 57.0% (mean 28.7 ± 16.7%) and RDT ranged between 631.6 μm and 1957.2 μm.

Tertiary dentine was observed in nine of eleven SPM (mean RDT 1457.1 ± 459.0 μm; Figures 1A-C). The two SPM without tertiary dentine had RDT of 2051.6 μm and 1731.9 μm. While tertiary dentine was observed in all HSPM (n=12) (Figures 2A-C). TDT ranged between 0 μm and 579.8 μm in SPM, and between 150.6 μm and 1235.2 μm in HSPM.

DMP1 was detected within the peripheral odontoblast layer and the mineralised tertiary dentine subjacent to the base of the cavity, as well as deeper within the core of the pulp tissue (Figure 1D) in all SPM teeth. DMP1 was detected in supra- and intra-nuclear locations within the odontoblast-like cells (Figure 1F). The intensity of DMP1 ranged between 1290.5 MGV and 4457.8 MGV in the odontoblast layer, and between 123.7 MGV and 1164.1 MGV in the

dentine pulp complex of SPM. β-Catenin was detected within the peripheral odontoblast layer subjacent to the base of the cavity in all SPM teeth (Figure 1G). The accumulation was mainly localised within the nuclei of the odontoblast-like cells and DPCs (Figure 1I). The intensity of β-Catenin ranged between 649.4 MGV and 2640.2 MGV in the odontoblast layer, and between 1920.0 MGV and 12413.0 MGV in the dentine pulp complex of SPM.

Similarly, in all HSPM teeth, DMP1 was detected within the peripheral odontoblast layer and the mineralised tertiary dentine subjacent to the base of the cavity, as well as deeper within the core of the pulp tissue (Figure 2D). DMP1 was also detected in supra- and intra-nuclear locations within the odontoblast-like cells (Figure 2F). With HSPM, the intensity of DMP1 ranged between 2138.6 MGV and 5821.6 MGV in the odontoblast layer, and between 231.6 MGV and 811.7 MGV in the dentine pulp complex. β-Catenin was detected within the peripheral odontoblast layer subjacent to the base of the cavity in all HSPM samples (Figure 2G). The accumulation was mainly localised within the nuclei of the odontoblast-like cells and DPCs (Figure 2I). β-Catenin was also noted in the endothelium lining of nearby pulpal blood vessels (Figure 3B). With HSPM, the intensity of β-Catenin ranged between 501.1 MGV and 3220.0 MGV in the odontoblast layer, and between 1675.0 MGV and 14389.0 MGV in the dentine pulp complex. All measurements were within the 95% limits of agreement showing high intra-examiner reproducibility.

A moderately negative correlation was noted between RDT and TDT in SPM (Pearson's coefficient = -0.494, $p = 0.061$), and in HSPM (Pearson's coefficient = -0.461, $p = 0.066$). There was a significant difference between the mean TDT in SPM (248.6 ± 205.8 μm) and HSPM (518.1 ± 282.0 μm) with a 108.4% (approximately 2-fold) increase in the mean TDT in HSPM (Table 1). There were no statistically significant correlations between RDT and the intensity of β-Catenin and DMP-1 in the odontoblast layer and dentine pulp complex in both SPM and HSPM teeth. There was no statistically significant difference in the mean RDT in SPM and HSPM (Table 1). Although the mean intensity of β-Catenin and DMP1 in the odontoblast layer and dentine-pulp complex were greater in HSPM compared to SPM, this was not statistically significant (Table 1).

4. DISCUSSION

Tertiary dentine is deposited subjacent to the base of the carious lesion at pulp dentine interface and its thickness is proportional to the duration and intensity of the stimulus[12]. As

lesion progression becomes closer to pulp tissue, injury to pulp triggers the formation of tertiary dentine. Increased inflammatory changes in pulpal innervation, vascularity, and immune cell accumulation[23], as well as an increase in pulpal TRPV1 expression and vascular flow[24] have been reported in hypomineralised teeth compared to teeth with normal enamel. It has been suggested that sensitive intact hypomineralised first permanent molars have an underlying pulpal inflammation triggered by an ingress of biofilm-derived acids, bacteria[25], and their by-products into the dentinal tubules via the porosities in the hypomineralised enamel[23] leading to a cascade of events resulting in earlier tertiary dentine formation[25, 26]. In the present study, we found no statistically significant difference in the mean RDT in carious SPM and HSPM, but a statistically significant two-fold increase in mean TDT in carious HSPM when compared to carious SPM suggests an amplified or an earlier onset of dentinal repair processes in the pulps of HSPM.

Pulpal injury and inflammation activate the Wnt/β-catenin signalling pathway which plays an important role in tertiary dentinogenesis. Implantation of an exogenous liposome-reconstituted form of Wnt3A protein into mice molar pulps, activated Wnt/β-Catenin signalling, promoted differentiation of odontoblast-like cells and reduced apoptosis, culminating in a superior repair response[14]. Similarly, direct pulp capping of mice molars with glycogen synthase kinase 3 (GSK-3) inhibitors, Wnt/β-Catenin signalling agonists, resulted in greater reparative dentine formation at pulp exposure sites when compared with plain collagen sponge or mineral trioxide aggregate[27-29]. The reparative dentine was found to be close to native dentine compositions at defect sizes translatable to small human lesions[29]. Animal immunofluorescence studies performed on dental pulps have demonstrated the translocation of β-Catenin into nuclei of the odontoblast-like cells coating the reparative dentine[15-17]. The distribution of β-Catenin within the peripheral odontoblast layer was noted to be broader and deeper at the exposure site or base of the cavity, subjacent to the reparative dentine[15-17]. The findings of the present study are in agreement with the results of the animal immunofluorescence studies. The presence of β-Catenin in the peripheral odontoblast layer of pulps without tertiary dentine formation suggest that the Wnt/β-Catenin signalling pathway had been activated in the dental pulp cells activating stem cell differentiation to form odontoblast-like cells. An interesting finding was the presence of β-Catenin in the endothelial lining of nearby blood vessels. Angiogenic effects of the Wnt/β-Catenin pathway have been reported[19-21], and the findings of the present study suggest that the β-Catenin in the

endothelial cells was promoting endothelial cell proliferation and angiogenesis via the Wnt/β-Catenin signalling pathway.

DMP1 is another important biomarker for odontoblast differentiation that has been investigated in this study. Direct pulp capping of rat maxillary first molars with recombinant DMP1-impregnated collagen matrix[6], and with Delta like 1 homolog, a DMP1 agonist[30], stimulated the differentiation of mouse stem cells to odontoblast-like cells resulting in reparative dentine deposition. Immunohistochemical studies have localised DMP1 within the nuclei of undifferentiated DPSCs and odontoblast-like cells at pulp exposure sites in rat[7] and human primary[9] molars. DMP1 has a dual role in tertiary dentinogenesis[4]. Within the nucleus, DMP1 regulates specific genes that control odontoblast-like differentiation[7, 8]. Within the supranuclear (Golgi) region of secretory odontoblast-like cells and the mineralising dentine matrix[7-10], DMP1 is involved in dentine mineralisation. The findings of the present study indicate that DMP1 has a dual role in tertiary dentinogenesis. In addition to the peripheral odontoblast layer, DMP-1 has also been localised deeper within the pulp tissue of inflamed permanent pulps with normal enamel where it is thought to play an important role in inflammation by stimulating the release of Interleukin-6 and Interleukin-8 from pulp fibroblasts, which are responsible for recruiting inflammatory cells such as neutrophils to the site of injury[4]. To the best of our knowledge, this is the first study to localise DMP1 in inflamed pulps of human primary molars with normal and hypomineralised enamel, and the findings are in agreement with a previous study[11]. Therefore, in addition to its active involvement in the dentinal repair process in carious HSPM and SPM, DMP1 may also be participating in the development of inflammatory changes in the pulp by stimulating pro-inflammatory activity of pulp fibroblasts.

Research investigating clinical applications of pulp healing and repair mechanisms in primary teeth is still in its infancy and is currently limited to animal studies and histological outcomes. Due to the limited sample size and the findings being histological observations rather than experimental findings, it is difficult to make definitive remarks on the clinical implications of the results of the present study. Nevertheless, the results have provided further insights into the molecular processes involved in tertiary dentinogenesis in carious HSPM and SPM. A statistically significant two-fold increase in mean TDT in carious HSPM when compared to carious SPM suggests an amplified or an earlier onset of repair processes in the dentine-pulp complex of HSPM. However, the absence of a statistically significant difference between mean intensity of β-Catenin and DMP1 in the odontoblast layer and dentine-pulp complex in

carious HSPM and SPM, suggests that the intensity of molecular processes involved in tertiary dentinogenesis via the stimulation of Wnt/β-Catenin and DMP1 in HSPM and SPM are similar, and the increased TDT in HSPM pulps is more likely due to an earlier onset of repair processes rather than an amplified response to caries. An extension of the present project, with a larger sample size to observe interactions between the many variables, would be valuable and may identify further significant relationships that may have been lost to type II error.

5. CONCLUSION

Similar Wnt/β-Catenin and DMP1 activities were observed in the peripheral odontoblast layer and the dentine-pulp complex between HSPM and SPM. The pulps of HSPM appear to respond earlier to carious lesions, via activation of Wnt/β-Catenin and DMP1 signalling pathways, to produce a two-fold increased TDT compared to SPM with similar RDT.

Why this paper is important to paediatric dentists

- There appears to be no differences in the intensity of the molecular processes involved in the stimulation of Wnt/B-Catenin and DMP1 between HSPM and SPM
- With similar remaining dentine thickness, HSPM produce two-folds tertiary dentine compared to SPM
- HSPM may mount an earlier response to caries compared to SPM. This has an implication on clinical management of HSPM

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TABLES

TABLE 1 Difference between mean RDT, TDT and intensity of β-Catenin and DMP1 in HSPM and SPM teeth

	HSPM†		SPM‡		(t-test)
	Mean	**SD**	**Mean**	**SD**	**P**
RDT (µm)	1179.9	471.7	1536.1	443.5	0.08
TDT (µm)	518.1	282.0	248.6	205.8	0.02*
β-Catenin (Od) (MGV)	1931.4	928.5	1425.7	726.7	0.16
β-Catenin (DPC) (MGV)	8129.4	3912.0	6670.1	3085.2	0.34
DMP1 (Od) (MGV)	3360.1	1012.6	2865.6	897.4	0.23
DMP1 (DPC) (MGV)	541.4	187.6	495.1	296.5	0.66

Abbreviations: RDT, remaining dentine thickness; TDT, tertiary dentine thickness, Od, odontoblast-like cells; DPC, dentine-pulp complex; DMP1, dentine matrix protein 1; µm, micrometre; MGV, mean grey value (unit of measurement of intensity); HSPM, hypomineralised second primary molar; SPM, second primary molar; SD, Standard Deviation, P, two-tail significance.

† n = 12, ‡ n = 11, * $P < 0.05$

FIGURE LEGENDS

FIGURE 1 (A) Occlusal view of a second primary molar with an occlusal cavitated lesion (B) x1.5 Haematoxylin and eosin image (C) x 20 Haematoxylin and eosin image (D) DMP1 (green); localisation of DMP1 in peripheral odontoblast layer (white arrow), and deeper within pulp (gold arrow) (E) Nuclear staining with Hoechst 33342 (blue) (F) Merge of D & E; intranuclear (yellow arrow) and supranuclear (brown arrow) localisation of DMP1 (G) β-Catenin (red); localisation of β-Catenin in peripheral odontoblast layer (white arrow) (H) Nuclear staining with Hoechst 33342 (blue) (I) Merge of G & H; intranuclear localisation of β-catenin (yellow arrow).

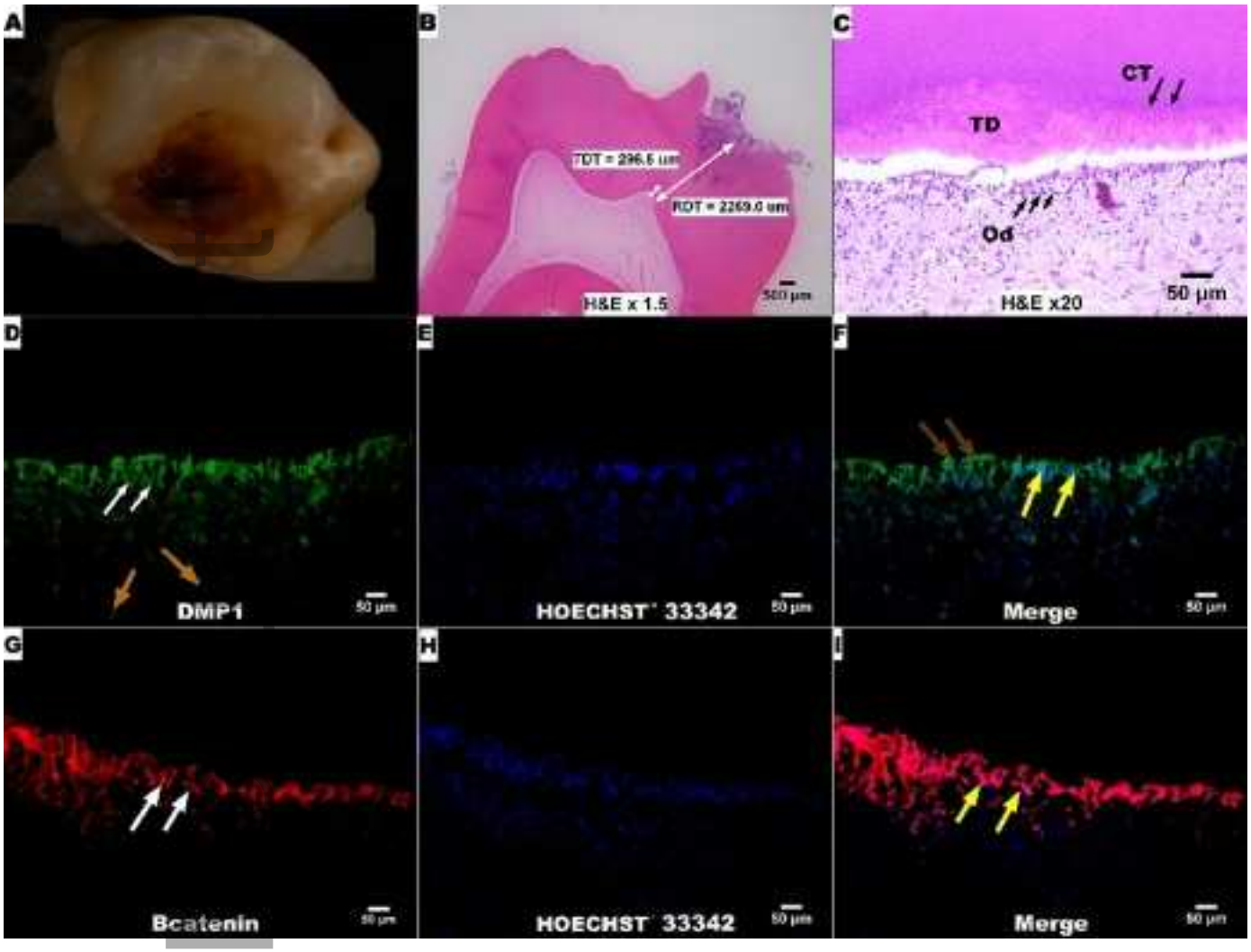
Abbreviations: TDT, tertiary dentine thickness; RDT, remaining dentine thickness; CT, calciotraumatic line; TD, tertiary dentine; Od, odontoblast-like cells; DMP1, dentine matrix protein 1; Bcatenin, β-Catenin.

FIGURE 2 (A) Occlusal view of a hypomineralised second primary molar with a disto-occlusal cavitated lesion (B) x1.5 Haematoxylin and eosin image (C) x 20 Haematoxylin and eosin image (D) DMP1 (green); localisation of DMP1 in peripheral odontoblast layer (white arrow), and deeper within pulp (gold arrow) (E) Nuclear staining with Hoechst 33342 (blue) (F) Merge of D & E; intranuclear (yellow arrow) and supranuclear (brown arrow) localisation of DMP1 (G) β-Catenin (red); localisation of β-Catenin in peripheral odontoblast layer (white arrow) (H) Nuclear staining with Hoechst 33342 (blue), (I) Merge of G & H; intranuclear localisation of β-Catenin (yellow arrow).

Abbreviations: TDT, tertiary dentine thickness; RDT, remaining dentine thickness; CT, calciotraumatic line; TD, tertiary dentine; Od, odontoblast-like cells; DMP1, dentine matrix protein 1; Bcatenin, β-Catenin.

FIGURE 3 Presence of β-Catenin in cells lining blood vessels in the pulp of a carious hypomineralised second primary molar (A) Haematoxylin and eosin staining x20 magnification (B) immunofluorescence (red) detection of β-Catenin within peripheral odontoblast layer and endothelial lining of blood vessels (C) Nuclear staining with Hoechst 33342 (blue) (D) β-Catenin and nuclear staining merged.

Abbreviations: Od, odontoblast-like cells; bv, blood vessels; B catenin, β-Catenin.



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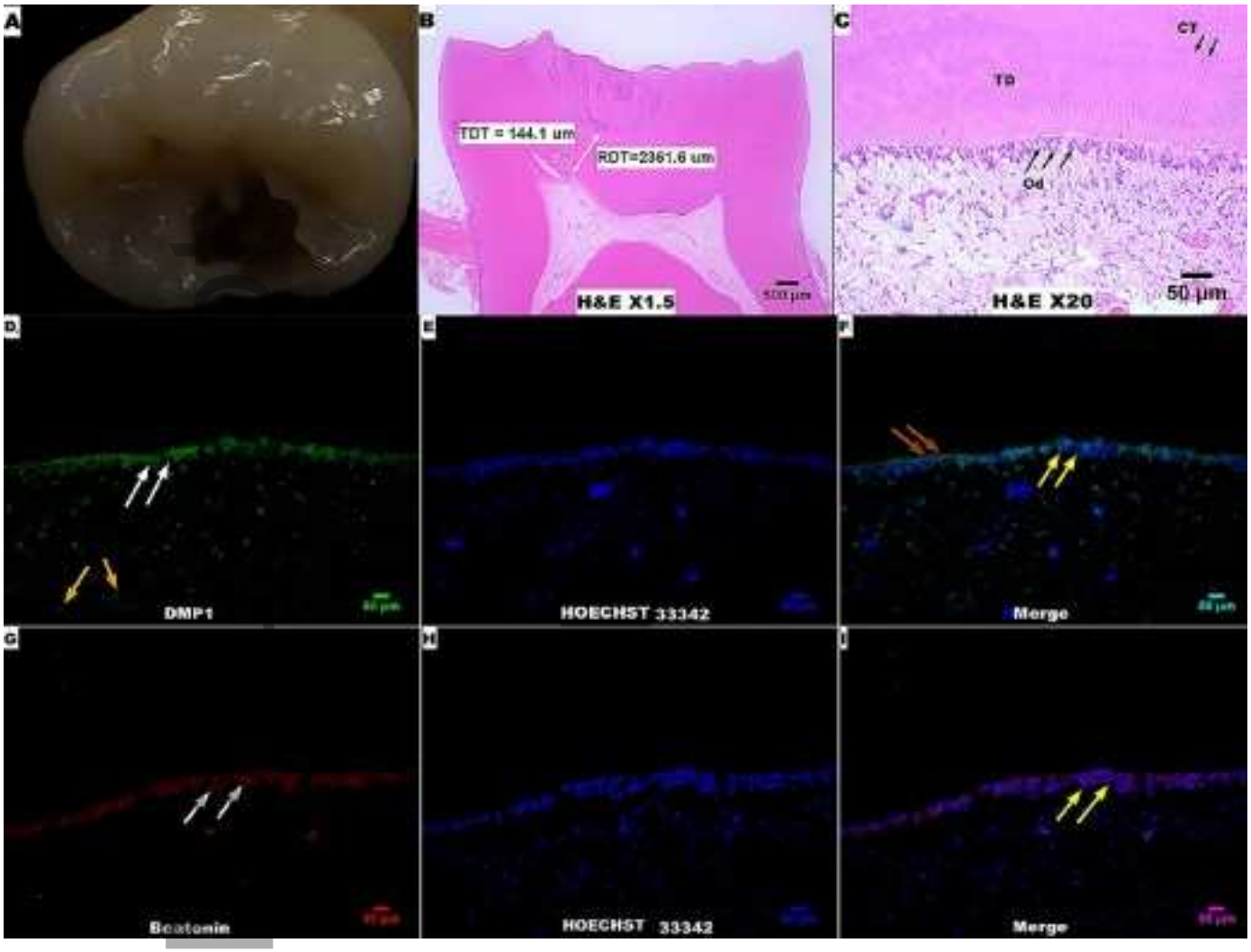

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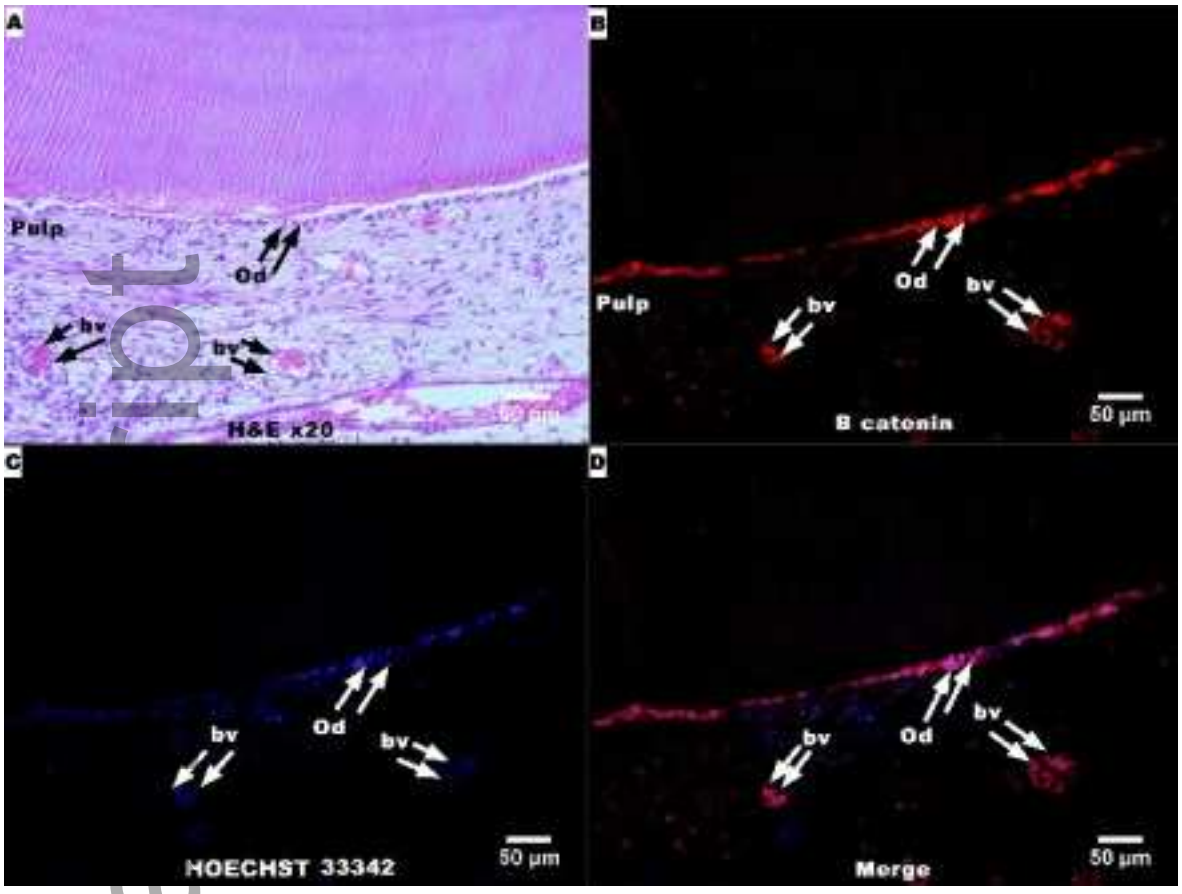

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