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TITLE:

Functional and molecular effects of a green tea constituent on oral cancer cells

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KEY WORDS:

oral cancer, oral squamous cell carcinoma, epidermal growth factor receptor, green tea

ABSTRACT:

Background:

Green tea is heavily consumed on a global basis for its health benefits. The active ingredient, (-)-epigallocatechin gallate (EGCG), is a major polyphenol demonstrated to inhibit the growth of various non-oral cancer cell lines and interfere with the carcinogenic process, including downregulation of the epidermal growth factor receptor (EGFR). Our aim was to determine the phenotypic changes of oral cancer cells treated with EGCG, and concurrently assess the effect on EGFR expression and activation.

Methods:

Oral cancer cells (H400 and H357) were treated with 10 µg/mL and 20 µg/mL of EGCG for up to 72 hours. Phenotypic changes were assessed by performing cell proliferation analysis and cell migration (Transwell) assays. Expression of EGFR and its phosphorylated form (p-EGFR) was determined by Western blotting.

Results:

Cell proliferation of both cell lines was significantly reduced at 48hrs when treated with 20 µg/mL EGCG. However, after 72 hours of treatment the effect of

EGCG on cell proliferation ceased. Treatment of both cell lines with 10 µg/mL and 20 µg/mL of EGCG resulted in significant reduction in cell migration. Mechanistically, EGFR expression did not change significantly after treatment with EGCG, however there was a reduction of its phosphorylated form.

Conclusion:

EGCG transiently inhibits both cell proliferation and migration of oral cavity cancer cells. This effect is associated with a decrease in the expression of phosphorylated EGFR. It is possible that more frequent bursts of EGCG could result in a persistent and sustained cancer inhibition, but this requires further research for clarification.

Introduction

Epidermal growth factor receptor (EGFR) overexpression has been identified in a number of malignancies, playing an important oncogenic role in the initial stages of head and neck cancer ¹. Invasive forms of cancers prone to metastasis are associated with overexpression of EGFR ². Furthermore, abnormal EGFR signalling, mostly triggered by receptor autophosphorylation, is associated with poor prognosis of head and neck squamous cell carcinoma (HNSCC) and resistance to radiation therapy ³. Pre-clinical research supports the efficacy of targeting the EGFR pathway to intercept the progression of oral carcinogenesis ⁴. At present, EGFR is the only molecular target in HNSCC for which there are regulatory approved pharmacological therapeutic agents to treat advanced cancers.

EGFR is a member of the Erb/HER family of receptor kinases ⁵. EGFR is a plasma membrane glycoprotein with an extracellular ligand-binding domain, a single transmembrane region, and an intracellular cytoplasmic domain, that exhibits tyrosine kinase activity ⁶. Binding of ligands that include the peptide ligands epidermal growth factor (EGF) and tumor necrosis factor alpha (TNF- α) to EGFR

triggers homo-dimerization of EGFR or hetero-dimerization of EGFR with other Erb/HER family members ⁷. This results in auto-phosphorylation (p-EGFR) that activates the receptor ⁸. After auto-phosphorylation the EGFR phosphorylates intracellular proteins that activate multiple downstream signaling pathways resulting in different events which include mitogenesis, cell proliferation, differentiation, migration, adhesion and inhibition of apoptosis ⁷. In addition, activation also modulates different aspects of the tumor microenvironment that includes angiogenesis and immune system responses ¹.

Aberrant expression and/or activity of EGFR play a critical role in cancer cell proliferation and migration, and in HNSCC has been identified to be associated with reduced overall survival ⁹. Grandis et al ¹⁰ were the first to identify the prognostic role of EGFR by finding that disease-free and cause-specific survival were reduced among HNSCC cases that displayed EGFR overexpression (both $p = 0.0001$). The significance of EGFR overexpression in relation to patient outcomes still holds true today, with Monteiro et al ¹¹ recently finding that EGFR overexpression in OSCC patients was significantly associated with poor overall survival ($p = 0.003$). EGFR is therefore a potential candidate for targeted OSCC therapy.

As oral carcinogenesis develops slowly and requires multiple events ¹², dietary flavonoids, such as those found in green tea, may prove to be an option for chemoprevention. Tea (*Camellia sinensis*) is the most popular beverage worldwide. The most bioactive compound (–)-epigallocatechin gallate (EGCG) is a polyphenol and one of several other catechins or flavonoids, accounting for more than 50% of total catechins found in tea ¹³. In vitro studies involving cancer cell lines have demonstrated that polyphenols found in tea affect several signalling pathways and molecular events, which result in inhibition of cancer cell proliferation and invasion ¹⁴. EGCG has been shown to target multiple signaling pathways involved with the carcinogenic process, including the downregulation of EGFR and associated downstream signaling molecules ². Hence, application of EGCG can provide an effective strategy for inhibition of cancer cells that display an overexpression of EGFR ¹⁵. Furthermore, inhibition of

the growth of several cancer cell lines by treatment with EGCG has been shown to occur via its inhibition of phosphorylation and activation of EGFR¹⁶⁻¹⁸. EGCG is a promising natural compound due to its safe toxicology profile, making it a candidate for cancer prevention and therapy¹⁹.

The aim of the present study was to determine if EGCG treatment could affect the expression of EGFR/p-EGFR in oral cancer cell lines, and concurrently assess any induced phenotypic changes of oral cancer cells in culture. Human cancer cell lines are still one of the most common in vitro models to pre-clinically assess potential target-specific therapeutic agents. The hypothesis is that EGFR inhibition in oral cancer cells would occur with EGCG treatment and result in changes in cell growth and migration.

Materials and Methods

Cell culture and treatments

Two human oral malignant keratinocyte cell lines H400 (ECACC 06092006) and H357 (ECACC 06092004) were selected for this study. The H400 cell line was supplied by the European Collection of Cell Cultures (ECACC; Salisbury, United Kingdom) as catalogue number 06092006, and purchased from CellBank Australia (NSW, Australia). The H357 cell line was donated from the Bristol Dental School, University of Bristol, UK. Both cell lines were reported to be human papillomavirus negative and were derived in the UK prior to 2001, thus not requiring ethics committee approval.

The cell lines were maintained in complete growth medium consisting of Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL) and hydrocortisone (0.5 µg/mL) in an atmosphere humidified with 5% CO₂ at 37°C. EGCG (>95% purity; Sigma-Aldrich, USA) was dissolved in DMEM to create a stock solution of 2 mg/mL before being diluted to the desired working concentration of 10 µg/mL or 20 µg/mL immediately prior to all experiments.

Western blot analysis

Cells were grown to 40-50% confluency in 6 well tissue culture-plates (Corning® Costar® TC-Treated Multiple Well Plates, CLS3516, Sigma-Aldrich, USA) in serum-free medium for 24 hrs and then incubated in complete medium or complete medium supplemented with 10 µg/mL or 20 µg/mL EGCG for an additional 24 hrs. Proteins (whole cell lysates) were extracted from the cells using the Extraction buffer provided in the Protein Extraction kit (Full Moon BioSystems, USA) supplemented with 1:100 cocktail protease inhibitor (Sigma-Aldrich) according to manufacturer's instructions. Protein concentration was determined using the Bradford colorimetric assay (Sigma-Aldrich).

Whole cell lysate proteins (40 µg) were separated by electrophoresis on Any kD™ Mini-PROTEAN® TGX™ Precast SDS-PAGE gels (Bio-Rad, AUS) and proteins transferred to Immun-Blot® polyvinylidene difluoride membranes (PVDF) (Bio-Rad). The following primary antibodies were used for immunoblotting: EGFR (sc-71034, Santa Cruz Biotechnology, USA), phosphorylated EGFR (p-EGFR) (sc-57542, Santa Cruz Biotechnology), actin (sc-8432, Santa Cruz Biotechnology) and β-actin (sc-47778, Santa Cruz Biotechnology). The p-EGFR antibody reacts only with the tyrosine auto-phosphorylated activated form of the EGFR.

Following overnight incubation of the membranes at 4°C, a horse anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Cell Signalling Technology, USA) and chemiluminescent substrate (Immobilon™ Western kit, Millipore, USA) was used to detect binding of the primary antibody and analysed by the LAS-3000 Imaging System (Fujifilm Life Science, Japan). Band intensity was semi-quantified using Image J software.

Viable cell proliferation assay

Cells were seeded in 12-well cell-culture plates (Corning® Costar® TC-Treated Multiple Well Plates, CLS3513, Sigma-Aldrich) at 1.0×10^5 cells/well in 2 mL of complete growth medium. Twenty-four hours after seeding, cells were

equilibrated in complete growth medium or treated with complete growth medium supplemented with 10 µg/mL or 20 µg/mL EGCG, which was replenished every 24 hrs for up to 72 hrs. For counting cells, the trypan blue exclusion assay was employed, in order to enable the exclusion of dead cells from the count. Briefly, cells were harvested with trypsin, a 10 µL volume of the harvested suspension was added to 10 µL of 4% Trypan Blue Dye (Bio-Rad) and cells counted using the TC10 Automated cell counter (Bio-Rad).

Transwell migration assay

The transwell migration assay was performed using the 24-well Corning® Transwell® permeable inserts containing 6.5 mm diameter polycarbonate membranes with a 8 µm pore size (CLS3422, Sigma-Aldrich) according to the manufacturer's instructions. Briefly, cells were trypsinized and 2.5×10^4 cells of the recovered cells resuspended in serum free medium (final volume 100 µL) without or with 10 µg/mL or 20 µg/mL EGCG. The cell mixture was then dispensed into the permeable insert (upper chamber), and 600 µL of complete medium was dispensed into each well (lower chamber). The Transwell® plate was then incubated in a humidified atmosphere with 5% CO₂ at 37°C for 16 hours. After 16 hrs of incubation medium was removed from the inserts and cells in the membranes were washed twice in 1X PBS, fixed to the membrane in 4% formalin followed by ice-cold methanol, and stained with crystal violet. A cotton swab was then applied to the membrane to remove non-migrated cells. Quantification was done by imaging five non-overlapping random high-power fields using a phase contrast microscope, and the number of migrated cells was counted using Image J software.

Statistical analysis

All cell culture and western blotting experiments were performed in biological triplicate. Data was expressed as a mean \pm SD (error bars) of three independent experiments, unless stated otherwise. The data results from the cell proliferation growth curve were normalized at logarithmic scale. To relatively quantify

protein bands from western blotting experiments the intensity of the bands were measured using Image J software, and a normalized value was calculated relative to the band intensity of the loading control (actin or β -actin) that enabled semi-quantitative calculation of the expression of the target protein. The statistical significance of the data was evaluated using the Student's t-test. A p value < 0.05 (two-tailed) was considered as statistically significant.

Results

EGCG treatment inhibits OSCC cell proliferation

In both H357 and H400 cell lines, cell proliferation was significantly reduced when treated with 10 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$ EGCG for 48 hrs ($p = 0.0087$, $p = 0.0054$ respectively) compared to the no treatment control (Figure 1).

Furthermore, proliferation of the H357 cells remained reduced after 48 hrs of exposure to 20 $\mu\text{g}/\text{mL}$ EGCG ($p = 0.00096$). After 72 hrs of EGCG treatment there were no differences of the cell proliferation rate of either cell lines demonstrating that the effect of EGCG on cell proliferation was time dependent (Figure 1).

EGCG treatment inhibits OSCC cell migration

Next, we investigated whether EGCG had any effect on the migratory phenotype of OSCC cells. Treatment with 10 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$ of EGCG for 16 hours resulted in a significant reduction in cell migration through Transwell® membranes by both oral cancer cell lines (Figure 2). This effect was unlikely to reflect changes in cell number given that there was no significant difference in cell proliferation 24 hrs after treatment. H357 cell migration decreased from 161.5 ± 13.5 cells for the no EGCG exposure control to 63 ± 15.7 cells ($p = 0.003$) (61% decrease) following 10 $\mu\text{g}/\text{mL}$ EGCG treatment and to 60.5 ± 9.8 cells ($p = 0.002$) after 20 $\mu\text{g}/\text{mL}$ EGCG treatment (a 63% decrease) (Figure 2). Similarly, for the H400 cells, migration through the Transwell® membrane decreased from 294.3 ± 18.2 cells for the no EGCG exposure control to 58.5 ± 12.3 cells ($p =$

0.0002) for 10 µg/mL of EGCG treatment (80% decrease), and to 45.7 ± 8.1 cells ($p = 0.0006$) for 20 µg/mL of EGCG treatment, an 84% decrease. Together, the results show that EGCG affects proliferation and migration of OSCC cells in a time- and dose-dependent manner.

Effect of EGCG on total and phosphorylated EGFR in OSCC cells

To understand whether the functional alterations induced by EGCG were associated with molecular changes, expression and phosphorylation of EGFR were investigated. Western blotting revealed that relative to the no treatment controls EGCG did not reduce the abundance of EGFR in detected in both H400 and H357 cell line extracts (Figure 3). When normalised against β -actin, EGCG did not reduce the level of p-EGFR in both H400 and H357 cell lines except for H400 cells treated with 10 µg/mL of EGCG for 24 hrs ($p = 0.05$) (Figure 4). Interestingly, when p-EGFR expression was normalised against total EGFR, p-EGFR was reduced by a factor of 4.87 and 5.76 in H400 cells treated with 10 and 20 µg/mL of EGCG respectively for 24 hrs. Relative to total EGFR, p-EGFR expression was also reduced in the H357 cell lines, by a factor of 2.31 when cells were treated with 10 µg/mL EGCG, and a factor of 1.51 when cells were treated with 20 µg/mL EGCG (Figure 4) for 24 hrs. Taken together, these data suggest that exposure of human OSCC cells to EGCG leads to altered phosphorylation of EGFR.

Discussion

In the present study, we demonstrate that the polyphenol EGCG, a chief constituent of green tea, reduces proliferation and migration of OSCC cells in a time- and dose-dependent manner. At a molecular level, this is associated with a reduction of EGFR phosphorylation, as indicated by the ratio between total and phosphorylated EGFR expression. These results suggest that green tea, a widely consumed beverage, has potential for use as an anti-cancer or chemopreventative agent.

Flavonoids are polyphenolic compounds derived from plants that have been reported to have a broad spectrum of biological activities, including anticancer properties¹⁹. The inhibitory effects of EGCG on carcinogenesis have been attributed to the regulation of protein function, which include those involved in the EGFR signaling pathway¹³. EGCG has been shown to inhibit binding of EGF to EGFR followed by inhibition of the signaling pathways, reducing phosphorylation of EGFR²⁰.

In various cancer cell lines, including those originating from HNSCC, EGCG has been shown to inhibit EGFR at various concentrations. Masuda et al¹⁶ found that EGFR expression was inhibited in two head and neck cancer cell lines derived from the hypopharynx and nasopharynx when treated with 10 µg/mL EGCG for 24 hours. Adachi et al¹⁸ found that deregulation of total cellular EGFR occurs at approximately 12 hrs of treatment with 25-50 µM (11.5-23 µg/mL) EGCG in colon cancer cells; with treatment with 25 µM EGCG for 24 hrs significantly reducing EGFR expression and 50 µM causing complete deregulation¹⁸. Liang et al²¹ found that as little as 0.5-1 µg/mL (approximately 1-2 µM) EGCG directly inhibited EGF binding to EGFR, which subsequently inhibited the auto-phosphorylation of EGFR in epidermoid carcinoma cell lines. Time course studies in human colon cancer cells have shown that EGCG (10-20 µg/mL) inhibited activation of EGFR and multiple downstream signaling pathways within 6 hrs¹⁷.

Due to these findings in the literature 10 µg/mL and 20 µg/mL EGCG were chosen to treat oral cancer cell lines for 24 hrs to determine the effect of EGCG on EGFR. After exposure to EGFR for 24 hrs western-blotting results demonstrated there were no alterations to EGFR however, p-EGFR decrease was detected in H400 cells when treated with 10 µg/mL of EGCG ($p = 0.05$), suggesting that exposure to EGCG can result in alteration to the phosphorylation state of EGFR and that these effects are cell line-dependent.

The present study has shown that EGCG has a predominantly anti-proliferative effect on oral cancer cells in vitro, particularly after 48 hrs of treatment with 20

$\mu\text{g}/\text{mL}$ EGCG for the H357 cell line and 10 and 20 $\mu\text{g}/\text{mL}$ EGCG for the H400 cell line. However by 72 hrs of EGCG treatment cell proliferation rate recovered to resemble that of the control, indicating that the cells had adapted to overcome the inhibitory effects of EGCG exposure. After 72 hrs of treatment with EGCG there were no statistically significant difference between treatment and control groups, potentially indicating that there may have been an adjustment of the signaling network to counteract the effects of EGCG on growth inhibition, but this needs further clarification. Other signaling pathways involved in cell proliferation have been reported to be altered by EGCG causing inhibition of proliferation in HNSCC cell lines ²². This may be responsible for the effects of EGCG on cell proliferation found in the H400 and H357 cell lines.

Inhibition of cancer cell proliferation by EGCG has been observed in cell lines derived from various origins ²³. Our results have confirmed that EGCG can inhibit OSCC cell growth in vitro at concentrations compatible with other studies ²⁴⁻²⁷. Pianetti et al ²⁴ found that breast cancer cell lines treatment with 20 - 80 $\mu\text{g}/\text{mL}$ of EGCG decreased the rate of cell proliferation. Nihal et al ²⁷ found that melanoma cells treatment with EGCG 10 $\mu\text{g}/\text{mL}$ resulted in a significant reduction in the number of proliferating cells after 48 hrs of treatment. Lim et al ²⁵ found that in HNSCC cell lines, treatment with EGCG (10 and 50 μM , approximately 4.6 and 23 $\mu\text{g}/\text{mL}$) resulted in a significant inhibition of cell proliferation. Similarly Elattar et al ²⁶ previously found that in an OSCC cell line, there was a significant dose-dependent inhibition in cell proliferation when treated with EGCG. More recently Braicu et al ²⁸ confirmed this finding from Elattar et al observing that EGCG treatment suppressed cell proliferation of an OSCC cell line, the effect being dose- and time-dependent.

From the current study we found that EGCG significantly reduced the migration of both oral cancer cell lines tested. At a concentration of 10 $\mu\text{g}/\text{mL}$ EGCG inhibited H357 cell migration by 61% and H400 cell migration by 80%. At a concentration of 20 $\mu\text{g}/\text{mL}$ EGCG inhibited H357 cell migration by 63% and H400 cell migration by 84%. EGCG has also been shown to exhibit anti-migratory action on oral cancer cells with Ho et al ²⁹ finding that EGCG exhibited a dose-

dependent inhibitory effect on migration of OC2 cells, with EGCG at a concentration of 60 μM (approximately 30 $\mu\text{g}/\text{mL}$) decreasing migration by 40%. Furthermore, Chang et al³⁰ found that 25 μM (approximately 11.5 $\mu\text{g}/\text{mL}$) of EGCG inhibited CAL-27 oral cancer cell migration by 22%. The percentage decrease in cell migration found in the present study is higher than that reported in the literature^{29,30}. Although in the absence of EGCG H400 cells migrate through the Transwell® membrane at a faster rate than H357 cells, in the presence of EGCG migration was repressed for the cell lines and was reduced to the same rate. This could be explained by EGCG having a greater physical impact on H400 cells or that migration has a constitutive basal level in viable cells and that each cell line has been repressed to this point.

The observed reduction in cell proliferation and migration in oral cancer cells when treated with EGCG could not be attributed to EGFR abundance, but may be influenced by reduced EGFR activation at least in the H400 cell line. In fact, there was no significant change in EGFR abundance in both cell lines when exposed to EGCG. However, reduction of p-EGFR abundance is indicative of reduced EGFR activation, which correlates with the observed reduction in proliferation and migration. It is plausible that changes in p-EGFR at 24 hrs were revealed in a temporal manner by reduced migration and proliferation at 48 hrs post EGCG exposure. The adaption of the cells to EGCG by 72 hrs would indicate that prolonged (therapeutic) exposure to EGCG may not be sufficient per se in preventing tumor progression. However, if the short-term inhibitory effect can be repeated by use of pulsed doses of EGCG adaption induced by prolonged exposure may be avoided. This warrants further investigation.

The precise identification of EGCG's direct and critical cellular targets that are responsible for the effect on cancer cell proliferation and migration remain to be elucidated in these oral cancer cell lines. Further in vitro experiments are required to establish the molecular mechanisms involved in the inhibition of oral cancer cell proliferation and migration following EGCG exposure. More definitive information into the application of EGCG for cancer treatment regimens warrants further research and clarification.

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Conflict of interest: None declared

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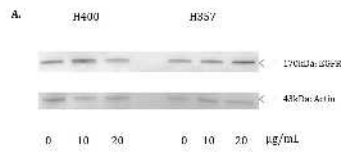
List of Figures

Figure 1: EGCG inhibited growth of oral cancer cell lines. H400 and H357 oral cancer cells were seeded in triplicate at a density of 1.0×10^5 cells/well. After 24 hrs incubation EGCG was dissolved in DMEM at a concentration of 10 $\mu\text{g}/\text{mL}$ or 20 $\mu\text{g}/\text{mL}$. The cell cultures were incubated for an additional 24, 48 and 72 hrs. Cell proliferation was semi-quantified by trypan blue exclusion assay.

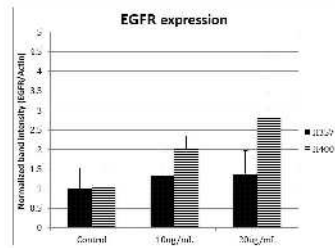
Figure 2: EGCG inhibited cell migration of oral cancer cell lines. H357 and H400 oral cancer cells were seeded in triplicate at a density of 2.5×10^4 cells in serum free medium with or without EGCG dissolved in DMEM at a concentration of 0 (control), 10 or 20 $\mu\text{g}/\text{mL}$. The cell cultures were incubated for 16 hrs. The number of migrated cells were analysed using Image J software, counting cells from 5 high-powered fields.

Figure 3: Expression of EGFR in H400 and H357 cell lines when treated with 0 (control), 10 or 20 $\mu\text{g}/\text{mL}$ EGCG for 24 hrs. (A) Western blot analysis for EGFR. Actin indicates equal loading of whole cell lysates. (B) Semi-quantified normalized band intensity of EGFR expression relative to the actin loading control band intensities. The mean and standard deviation of three independent experiments are displayed.

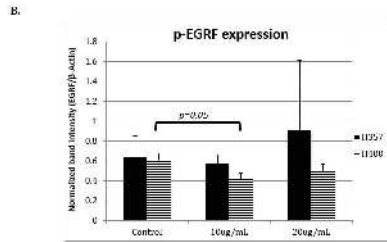
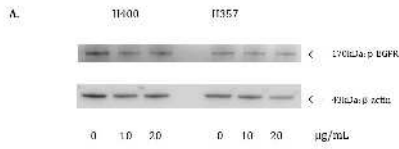
Figure 4: Expression of p-EGFR in H400 and H357 cell lines when treated with 0 (control), 10 or 20 $\mu\text{g}/\text{mL}$ EGCG for 24 hrs. (A) Western blot analysis for p-EGFR. β -actin indicates equal loading of whole cell lysates. (B) Semi-quantified normalized band intensity of p-EGFR expression relative to β -actin loading control band intensity. The mean and standard deviation of three independent experiments are displayed.



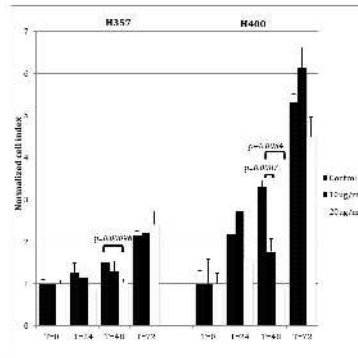
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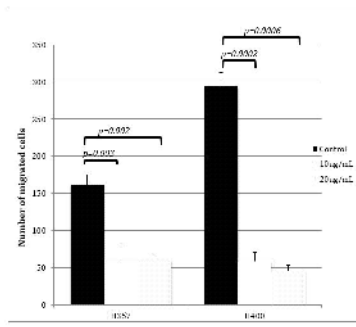
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