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# Cyclooxygenase 2 plays a role in Emdogain-induced proliferation

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*Background and Objective:* Enamel matrix proteins are involved in the development and regeneration of root cementum and in its attachment to dentin; however, the mechanisms through which this occurs have yet to be elucidated. The present study was therefore carried out to evaluate the mitogenic and proliferative responses of human periodontal fibroblast (HPLF) cells to Emdogain (EMD), and the potential role of cyclooxygenase 2 (COX-2) in this process.

*Material and Methods:* We investigated the effects of EMD on 5-bromo-2′-deoxyuridine (BrdU) incorporation, colchicine freezing of mitosis, XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction and Trypan Blue dye exclusion, with or without celecoxibe, a selective cyclooxygenase-2 (COX-2) inhibitor; we also evaluated the expression of COX-2 mRNA and COX-2 protein in response to EMD.

*Results:* EMD significantly enhanced mitosis in, and proliferation of, human periodontal ligament fibroblasts in a dose-dependent manner; however, there was a small increase of DNA synthesis only in response to a high dose of EMD (200 μg/mL). EMD (100 and 200 μg/mL) elicited an increase in COX-2 expression ($p \leq 0.05$). Celecoxibe (20 μM) diminished the EMD-induced mitosis and proliferation of HPLF cells ($p \leq 0.05$).

*Conclusion:* Celecoxibe hampered EMD-induced mitosis and proliferation, which, in association with EMD-increased COX-2 expression, indicates that COX-2 may be involved in the proliferative response of HPLF cells to EMD.

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Human periodontal ligament fibroblasts (HPLFs) play a remarkable role in the wound-healing process following periodontal disease or dental trauma; therefore, maintaining their viability is crucial in every case of avulsion (1–3). Growth factors have been extensively evaluated regarding the stimulation of growth and/or differentiation of periodontal ligament (PDL) cells; however, the lack of functional specificity has compromised their clinical application (4–6). An alternative approach is based on embryonic tooth formation and enamel matrix proteins. Emdogain (EMD) is accumulated in cells at the root surface and improves restoration of the PDLs of the transplanted teeth (7,8). Immunohistochemical staining of the periodontium for osteopontin and collagen types I and III revealed that treatment with EMD results in a higher expression of collagen and in the rapid development of connective tissue during the early phase of wound healing (9). The treatment of PDL, gingival fibroblasts and osteosarcoma cells (MG-63) by EMD was found to improve the wound-fill process *in vitro* (10,11).

Several clinical trials, as well as experimental research, have shown that EMD promotes the formation of root cementum, as well as PDL and tissue regeneration; however, the mechanisms by which it produces such effects have yet to be elucidated (4,12).

Prostaglandins (PGs) provoke mitosis and differentiation in osteoblasts, and stimulate periosteal and endosteal bone construction, resulting in alveolar

bone augmentation; however, matrix synthesis might be reduced as a result of the production of differentiated osteoblasts (13). EMD induces the transcription of fibroblast growth factor-2 in human osteoblastic (SaM-1) cells through increasing the synthesis of PGE2 and the expression of cyclooxygenase 2 (*COX2*) mRNA (14). COX-2 is considered to be an essential moderator of bone formation as well as of bone resorption and fracture healing. Moreover, the Core-binding factor alpha 1 transcription factor, which plays a crucial role in osteoblastic differentiation, is also modulated by COX-2 (15–17).

The purpose of this study was to evaluate the mitogenic and proliferative response of HPLF cells to EMD, and the potential role of COX-2 in this process.

## Material and methods

### Drugs and chemicals

Emdogain (which consists of enamel matrix derivative and propylene glycol alginate as the carrier) was obtained from Straumann (Basel, Switzerland) and dissolved (at a final concentration of 20 mg/mL) in 5% acetic acid to enhance EMD solubility. The final concentration of acetic acid and propylene glycol alginate in the medium was negligible and did not significantly alter HPLF proliferation in a pilot study (Khedmat S, unpublished data). Celecoxibe was purchased from Sigma (St Louis, MO, USA). All cell culture media and reagents were purchased from Gibco (Paisley, UK). The 5-bromo-2′-deoxyuridine (BrdU) assay kit and the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) proliferation kit were supplied by Roche (Berlin, Germany).

### Cell culture

HPLF cells were isolated and cultured based on the method described by Somerman *et al*. (18). Briefly, the healthy unerupted third molars from six healthy subjects (18–26 years of age) were extracted, with informed consent, atraumatically and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). The teeth were subsequently washed three times with Hanks' balanced salt solution under sterile conditions and the PDL tissue was scraped from the mid-third of the root surface and cultured in Dulbecco's modified Eagle's medium supplemented with L-glutamine, 10% FBS and 200 U/mL of penicillin/0.2 mg/mL of streptomycin at 37°C and 5% $CO_2$. The tissue-outgrowth cells were trypsinized after 1 wk and allowed to propagate to reach the desired confluence and then utilized to prepare the primary cell line for the study. Cells with passage numbers 3–6 were applied for the subsequent experiments (19).

### BrdU-incorporation assay

Newly synthesized nucleic acid was evaluated based on the incorporation of BrdU in the DNA of replicating cells. HPLFs ($1.5 \times 10^3$ cells) were plated on cover slips and treated with EMD and celecoxibe for 48 h. At the end of each treatment, the cells were incubated with BrdU for 60 min, fixed with ethanol and then exposed to anti-BrdU primary antibody (for 30 min at 37°C). Proliferating cells were finally visualized using an alkaline phosphatase-linked secondary antibody. The intensity of at least six microscopic shunts in each experiment was recorded using Olysia software in an Olympus microscope (Olympus, Center valley, PA, USA) and quantified using the ImageJ (National Institutes of Health, Bethesda, MD, USA) analysis tool (the results are expressed as mean ± SD, $n = 3$).

### Mitosis evaluation

HPLFs ($1.5 \times 10^3$ cells) were cultured on coverslips (Nunc, Roskilde, Denmark) and treated with EMD, with or without celecoxibe, for 48 h, and in the presence of colchicine (50 μM) for the second 24 h. At the end of the treatments, the cells were fixed in 10% formalin for 10 min at room temperature and were then subjected to Harris hematoxylin and eosin staining. The proportion of mitotic cells in at least six microscopic shunts were measured using Olysia software in an Olympus microscope (the results are expressed as mean ± SD, $n = 3$).

### XTT assay

Cell viability was investigated based on the reduction of XTT by mitochondrial dehydrogenases in proliferating cells. HPLFs ($2 \times 10^3$ cells) were cultured in 96-well plates (Nunc) and subjected to EMD and celecoxib treatments. The XTT labeling mixture (a final concentration of 0.2 mg/mL of XTT) was then added, and the cells were incubated at 37°C for another 3 h, after which the final absorbance was determined in a dual-beam spectrophotometer at 490 nm against 690 nm as the reference (the results are expressed as mean ± SD; $n = 6$).

### Trypan Blue dye exclusion

HPLFs ($50 \times 10^3$ cells) were cultured in six-well plates (Nunc) and subjected to different treatments. At the end of each experiment, the cells were trypsinized, mixed 1:1 with Trypan Blue (0.4% w/v) and then counted using a hemocytometer (the results are expressed as mean ± SD; $n = 6$).

### RT-PCR

The cells were plated in six-well plates and exposed to different concentrations of EMD for 48 h. Total RNA was extracted using Trizol (Roche), and 500 ng of RNA was used for the synthesis of complementary DNA with random hexamer primers and M-MuLV reverse transcriptase (Fermentas, Vilnius, Latvia). A semiquantitative PCR procedure for COX-2 (forward: 5′-AGCTGGGAAGCCTTCTCTAAC-3′ and reverse: 5′-AGATCATCTCTGCCTGAGTATCTT-3′; 35 cycles; annealing temperature: 54°C) and β-actin (forward: 5′-GATGATGATATCGCCGCGCT-3′ and reverse: 5′-CTTCTCGCGGTTGGCCTTGG-3′; 23 cycles; annealing temperature: 59°C) was applied to amplify a 412-bp or a 351-bp product, respectively. The cycle number and temperature chosen for each reaction was based on the exponential amplification of desired

molecules. The PCR product was then visualized by ethidium bromide staining of an agarose gel using a photo doc system, and the COX-2 band intensity was finally normalized to the corresponding β-actin band using TOTAL LAB TL120 (Newcastle, UK) image-analysis software.

### Western blotting

Cells were plated in six-well plates and exposed to different concentrations of EMD for 48 h. Total cell lysate was prepared with a lysis buffer containing 2% SDS, 100 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 0.5% Na-deoxycholate, 5 μg/mL of leupeptin and 62.5 mM Tris–HCl (pH 6.8). Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto a poly(vinylidene difluoride) membrane (Roche). After blocking with casein 1%, the membranes were exposed overnight at 4°C to COX-2 antibody (Cell Signaling, Beverly, MA, USA) or β-actin antibody (Santa Cruz, CA, USA). The protein bands were then revealed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescence substrates (Roche). Finally, the COX-2 band intensity was normalized to the corresponding β-actin band intensity using Total lab TL120 image-analysis software.

### Statistical analysis

Data were obtained from at least two independent experiments and are presented as mean ± SD relative to the data of the control group. Two-way analysis of variance, followed by Tukey's test, was applied to study the EMD-induced changes in BrdU, mitosis, XTT and the Trypan Blue assay, with respect to celecoxibe; one-way analysis of variance was utilized to evaluate the effects of EMD on COX-2 expression. A $p$-value of $\leq 0.05$ was considered statistically significant.

## Results

Celecoxibe elicited a dose-dependent reduction in HPLF cell viability, as measured using the XTT assay. The maximum dose of celecoxibe (20 μM) that did not significantly affect HPLF proliferation was selected for studying the effects of celecoxibe on EMD (Fig. 1).

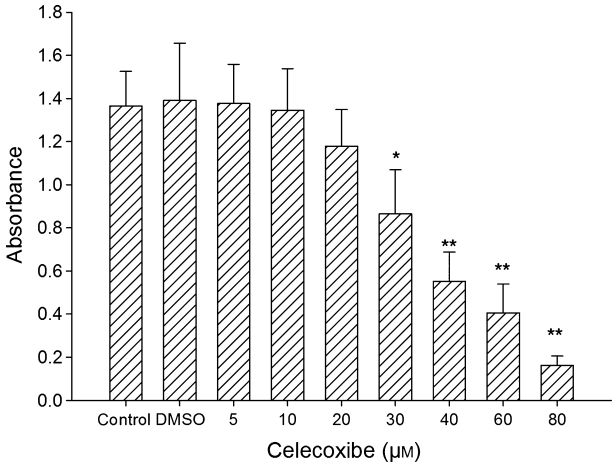


*Fig. 1.* Effects of celecoxibe on human periodontal ligament fibroblast (HPLF) proliferation. HPLF cells were plated and exposed to increasing doses of celecoxibe for 48 h, and the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay was applied to study cell proliferation. Celecoxibe elicited a dose-dependent reduction in HPLF proliferation. Dimethylsulfoxide (DMSO), at a final concentration of 0.5% in medium, was applied as vehicle. *$p < 0.05$; **$p < 0.01$.

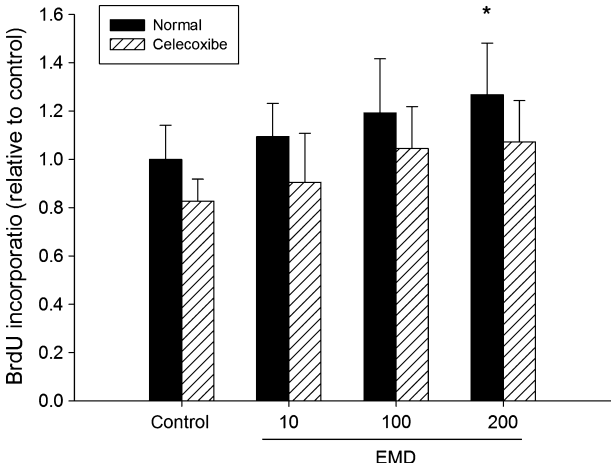


*Fig. 2.* DNA synthesis following treatment with Emdogain (EMD) and celecoxibe in human periodontal ligament fibroblasts (HPLFs). Following treatment of cells with EMD (10, 100 or 200 μg/mL), with or without celecoxibe (20 μM), for 48 h, a 5-bromo-2′-deoxyuridine (BrdU)-incorporation assay was performed. The only concentration of EMD that significantly enhanced BrdU incorporation was 200 μg/mL (*$p < 0.05$). Celecoxibe elicited a slight, but not statistically significant, decrease in both control and EMD groups.

BrdU incorporation by HPLF cells was enhanced only at higher doses of EMD (200 μg/mL; $p < 0.05$); treatment of cells with 20 μM celecoxibe did not significantly change DNA synthesis in either the control group or the EMD group (Fig. 2).

Exposure of HPLF cells to EMD for 48 h increased the percentage of mitotic cells, as evaluated by colchicine freezing of mitosis. As depicted in Fig. 3, 100 and 200 μg/mL of EMD significantly enhanced the proportion of mitotic cells, whereas 10 μg/mL of

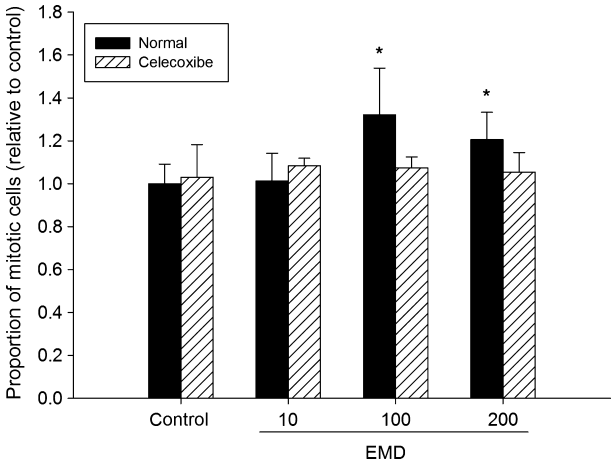


*Fig. 3.* The effect of Emdogain (EMD) and celecoxibe on the mitosis of human periodontal ligament fibroblasts (HPLFs). HPLF cells were exposed to EMD (10, 100 and 200 μg/mL), with or without celecoxibe (20 μM), for 48 h and in the presence of colchicine (50 μM) for the second 24 h. EMD (100 and 200 μg/mL) significantly enhanced the percentage of mitotic cells compared with the control. This effect was abolished during co-treatment of the cells with celecoxibe (*$p < 0.05$).

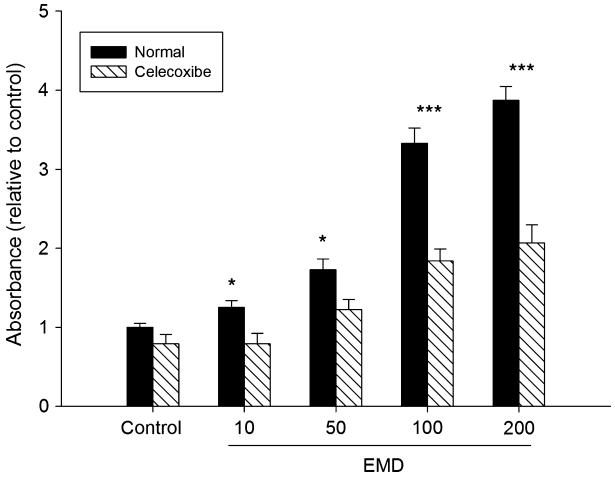


*Fig. 4.* The effect of Emdogain (EMD) and celecoxibe on the proliferation of human periodontal ligament fibroblasts (HPLFs). HPLF cells were exposed to EMD (10, 50, 100 and 200 μg/mL), with or without celecoxibe (20 μM), for 48 h. At the end of the treatments the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay was applied to study the proliferation of cells. EMD enhanced proliferation in a dose-dependent manner, which was abrogated at the presence of celecoxibe. *$p < 0.05$; ***$p < 0.001$.

EMD did not. Although 20 μM celecoxibe did not change the proportion of mitotic cells in the control group, it efficiently blocked the enhanced mitosis caused by EMD ($p < 0.05$).

Celecoxibe reversed the EMD-induced enhancement of HPLF cell proliferation. HPLF cells were exposed to EMD (10, 50, 100 and 200 μg/mL), with or without celecoxibe (20 μM), for 48 h, and the XTT assay was then performed. As illustrated in Fig. 4, EMD considerably enhanced the number of viable cells in a dose-dependent manner ($p < 0.05$). Celecoxibe (20 μM) did not significantly change HPLF proliferation; however, it efficiently hindered the elevated proliferation rate caused by exposure to EMD.

HPLF cells showed an increased cell number in response to treatment, for 48 h, with 50, 100 or 200 μg/mL of EMD; however, this amplification was not observed with 10 μg/mL of EMD ($p < 0.05$). The enhanced cell number occurring as a result of exposure to EMD was diminished when the cells were co-treated with EMD and celecoxibe (20 μM) (Fig. 5).

EMD significantly increased *COX2* transcription and expression of COX-2 protein. As depicted in Figs 6 and 7, the treatment of HPLF cells with 100 and 200 μg/mL of EMD resulted in a significant increase in the expression of *COX2* mRNA and COX-2 protein ($p < 0.05$); however, at lower doses of EMD, this was not observed.

## Discussion

Enamel matrix proteins are involved in the development and regeneration of root cementum and in its attachment to dentin (20,21). During PDL cell culture, EMD improves cell adherence, expansion and proliferation. It also enhances matrix synthesis and influences some growth and differentiation factors (22,23).

Celecoxibe elicited a dose-dependent reduction in HPLF proliferation, which was measured using the XTT assay. It has been reported that inhibition of COX-2 decreases interleukin (IL)-1β-stimulated IL-6 production in gingival cells and might be helpful in the treatment of periodontitis (24). Celecoxibe decreases the production of PGE2 in response to inflammatory mediators such as IL-1β and tumor necrosis factor-α (25). It has also been described that COX-2-dependent PGE2 down-regulates IL-1α-induced matrix metalloproteinase-3 production by cyclic AMP-dependent pathways in PDL cells and may have a role in periodontal regeneration and degradation (26,27).

We observed a slight increase in the incorporation of BrdU into DNA in the presence of 200 μg/mL of EMD; however, DNA synthesis was not altered at lower doses of EMD (10 and 100 μg/mL). Van der Pauw *et al.* (22) showed that EMD did not appreciably affect the incorporation of [$^3$H]thymi-

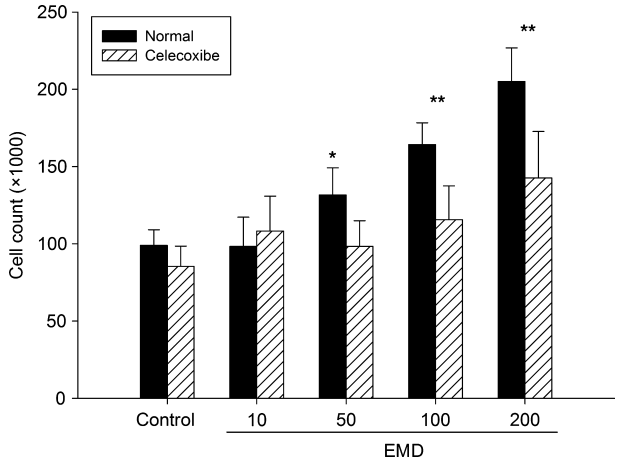


*Fig. 5.* The effect of Emdogain (EMD) and celecoxibe on the human periodontal ligament fibroblast (HPLF) cell count. HPLF cells were exposed to EMD (10, 50, 100 and 200 μg/mL), with or without celecoxibe (20 μM), for 48 h, and Trypan Blue staining was applied to study their effects on the cell count. EMD (50, 100 and 200 μg/mL) enhanced proliferation, which was then abolished in the presence of celecoxibe $*p < 0.05$; $**p < 0.01$.

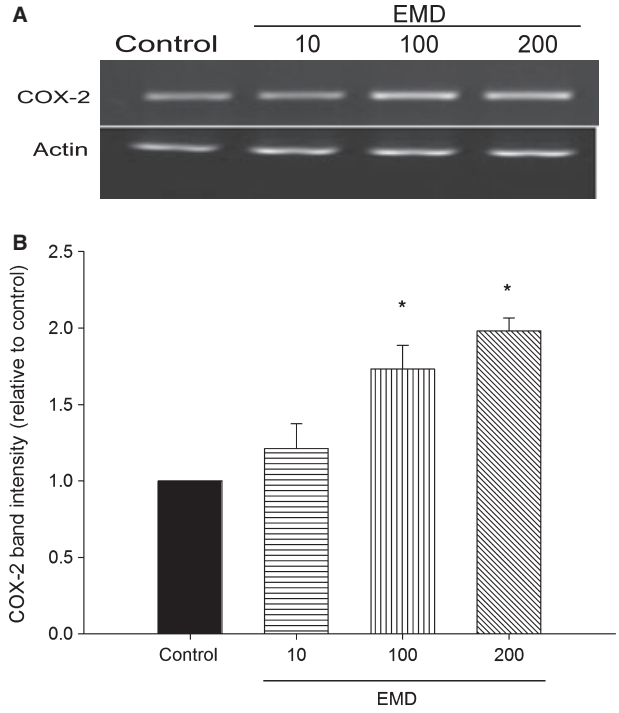


*Fig. 6.* The effects of Emdogain (EMD) on cyclooxygenase-2 (*COX2*) transcription. (A) Human periodontal ligament fibroblasts (HPLFs) were subjected to EMD (10, 100 and 200 μg/mL) for 48 h and a semiquantitative RT-PCR was performed. (B) The intensity of the *COX2* band (412 bp) relative to that of the beta-actin band (351 bp). EMD at 100 and 200 μg/mL enhanced the mRNA expression of *COX2* ($*p < 0.05$).

dine into HPLFs or human gingival fibroblasts. In the experiments of Pischon *et al.* (28), while EMD increased the incorporation of both BrdU and [$^3$H]proline into osteoblasts, there was an uncertain increase (mean 20% with a higher SD) in the incorporation of BrdU into PDL cells. Our results are appear to be controversial with those of Rincon *et al.* (10), who reported a significant increase in [$^3$H]thymidine incorporation by HPLF cells in the presence of 50, 100 and 150 μg/mL of EMD; however, it is noteworthy that they used 0.2% FBS as a negative control, while 10% FBS was applied as a negative control in our study; in addition, we performed BrdU assay to study DNA synthesis in HPLF cells, whereas they have used [3H]thymidine incorporation for this purpose.

We observed that EMD (100 and 200 μg/mL) induced mitosis in HPLF cells, and celecoxibe (20 μM) inhibited the mitogenic effect of EMD. Mastuda *et al.*, in two consequent studies, evaluated the mitogenic response of PDL cells; they described a rapid activation of ERK1/2 following exposure to EMD, which was reversed by receptor tyrosine kinase inhibitors. This indicates that receptor tyrosine kinase proteins are expressed on PDL membranes and might be involved in signal transfer from EMD components (29,30).

EMD significantly elevated the percentage of proliferating cells in the XTT assay and in the Trypan Blue dye-exclusion test; EMD was also able to increase *COX2* mRNA and COX-2 protein expression; celecoxibe (20 μM) decreased the EMD-enhanced proliferation of HPLF, which confirms the involvement of PGs and COX-2 on EMD-mediated HPLF replication. EMD induces the transcription of fibroblast growth factor-2 in human osteoblastic (SaM-1) cells through increasing the synthesis of PGE2 and expression of *COX2* mRNA (14). Our observation regarding the expression of COX-2 is consistent with the results of Takayanagi *et al.* (31), who described that EMD increased the transcription of the *COX2* gene in PDL cells. In line with this, Dean *et al.* (32) reported that EMD increases the proliferation and production of PGE2 in chondrocytes. By contrast, Ashkenazi *et al.* reported that the treatment of cells with EMD for 5 wk decreased the clonogenic capacity of HPLF in which the percentage of cells capable of developing into colonies with 75–100% confluency was significantly lower in the EMD group compared with the control group. The prolonged exposure

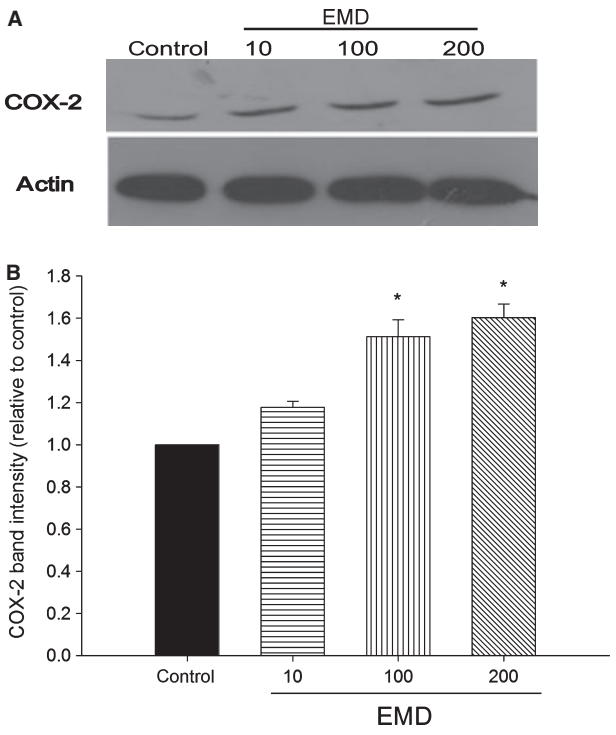


*Fig. 7.* The effects of Emdogain (EMD) on cyclooxygenase-2 (COX-2) protein expression. (A) Human periodontal ligament fibroblasts (HPLFs) were subjected to EMD (10, 100 and 200 μg/mL) for 48 h and a western blot was performed. (B) The intensity of the COX-2 band (412 bp) relative to that of the beta-actin band (351 bp). EMD at 100 and 200 μg/mL enhanced the expression of COX-2 protein (*$p < 0.05$).

of HPLF cells to EMD might result in their differentiation, rather than their replication, as proposed by the enhanced alkaline phosphatase activity in EMD-treated cells (33). Cataneo *et al*. (34), using a Burker's chamber, also reported that EMD treatment of PDL cells culminates in a significant increase in the cell count and that their morphology becomes much like that of cementoblasts even after 3 d.

## Conclusion

The COX-2 selective inhibitor, celecoxibe, effectively decreased the EMD-induced mitosis and proliferation of HPLF cells, which, in association with the increased expression of the *COX-2* gene stimulated by EMD, indicates a potential role for COX-2 in EMD-mediated proliferation.

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