

## Conditioned medium from human bone marrow stromal cells attenuates initial inflammatory reactions in dental pulp tissue

Niyaz Al-Sharabi<sup>1</sup>, Manal Mustafa<sup>2</sup>,  
Minoru Ueda<sup>3</sup>, Ying Xue<sup>1</sup>,  
Kamal Mustafa<sup>1</sup>, Inge Fristad<sup>1</sup>

<sup>1</sup>Department of Clinical Dentistry, University of Bergen; <sup>2</sup>Oral Health Centre of Expertise in Western Norway, Bergen, Norway; <sup>3</sup>Department of Oral and Maxillofacial Surgery, University of Nagoya, Nagoya, Japan

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Correspondence to: Niyaz Al-Sharabi, Department of Clinical Dentistry, University of Bergen, Årstadveien 19, Bergen N-5009, Norway

Tel.: + 47 45547118

Fax: +47 55 58 85 61/+97333032353

e-mails: nal030@iko.uib.no and

niazalhemiar@gmail.com

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**Abstract – Aim:** To evaluate the effect of MSC-conditioned medium (CM) on the secretion of pro- and anti-inflammatory cytokines from dental pulp cells (hDPC) *in vitro*, and on the gene expression *in vivo* after replantation of rat molars. **Materials and methods:** hDPC were cultured in CM for 24 h, and the concentration of interleukin IL-10, IL-4, IL-6, and IL-8, regulated on activation, normal T Cell expressed and secreted (RANTES), and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in the media were measured by multiplex assay and ELISA, respectively. Expression of cyclooxygenase-2 (COX-2) was also examined by Western blot analysis after 24 h. Left and right maxillary first rat molars (*n* = 20) were elevated for 2 min and then replanted with or without application of CM into the tooth sockets. Levels of IL-1 $\beta$ , IL-10, IL-4, IL-6, and IL-8, and tumor necrosis factor-alpha (TNF- $\alpha$ ) mRNA were evaluated by real-time qRT-PCR 3 and 14 days following tooth replantation. **Results:** The production of IL-8, IL-10, and IL-6, RANTES and PGE<sub>2</sub> by cells cultured in CM was significantly higher than production by cells cultured in standard medium (DMEM). At day 3 following replantation *in vivo*, the levels of IL-1 $\beta$  and IL-6, and TNF- $\alpha$  mRNA were significantly lower in the CM-treated replanted teeth compared with control teeth. Further, at day 3, the IL-6/IL-10 ratio was significantly lower in the CM-treated replanted teeth compared with control. At day 14 following replantation, no differences in the mRNA ratios were detected between the pulp tissues of replanted and control teeth.

**Conclusions:** These findings indicated that CM promotes secretion of pro- and anti-inflammatory cytokines from hDPC *in vitro* and attenuates the initial inflammatory response in the rat dental pulp *in vivo* following tooth replantation.

Teeth and supporting tissues are well equipped with tissue-specific stem cells/progenitor cells that are activated during tissue injury and inflammation (1). The fate of stem cells in terms of migration, self-renewal, and differentiation into specialized phenotypes is governed by the surrounding local microenvironment and soluble molecules (2). Exogenously transplanted mesenchymal stem cells (MSC) are shown to stimulate a wide range of biological activities in immune cells as well as endogenous resident stem cells/progenitor cells (3). It has been shown that MSC are potent cells for tissue healing and repair, an effect exerted either by cell-cell contact or via the secretion of soluble factors including growth factors, cytokines and chemokines (4, 5). Although bone marrow stromal/stem cells (BMSC) and dental stem cells differ in their osteo/odontogenic phenotype characteristics (6), bone marrow is widely used as a standard available source for adult stem cells. It has been shown that BMSC participate in regeneration

of tooth-like structures when transplanted into the alveolar socket together with scaffold and dental bud cells in mice (7). BMSC are also able to be reprogrammed to give rise to odontoblasts and ameloblast-like cells in proximity to embryonic epithelium (8, 9).

Conditioned medium (CM) from mesenchymal stem cells (MSC-CM) contains growth factors and cytokines and has been shown to mimic the regulatory effects of stem cells on immunocompetent cells (10, 11). CM from stem cells derived from adipose tissue has been shown to enhance secretion of anti-inflammatory IL-10 from T-helper cells *in vitro* (12). More recently, CM from bone marrow stromal cells (BMSC-CM) was found to induce formation of new bone and cementum in intrabony defects with minimal inflammatory cell infiltration (13).

Dental trauma is complex in nature and affects both hard and soft tissues. The healing outcome is thought to depend on a successful interplay and balance

between progenitor cells residing in different tissue compartments (14). Recent data have suggested that dental pulp resident stem cells/progenitor cells are involved in production of inflammatory mediators that promote cell differentiation and pulpal healing (15). Although replantation after tooth displacement can preserve the tooth and supporting tissues, minimizing inflammation is important after replantation (16).

As growth factors and cytokines play a role in healing events following injury (17), it was hypothesized that the growth factors and cytokines present in the BMSC-CM can induce production of cytokines with a protective function. Thus, the first aim of this study was to examine the effect of BMSC-CM on the release of interleukin IL-10, IL-4, IL-6, and IL-8, chemokine RANTES and PGE<sub>2</sub> from human dental pulp cells (hDPC) *in vitro*. Next, the *in vivo* effect of BMSC-CM on mRNA expression of IL-1 $\beta$ , IL-10, IL-4, IL-6, and IL-8, and TNF- $\alpha$  in pulp tissue of replanted teeth was evaluated.

## Materials and methods

### BMSC culture and collection of conditioned medium

Primary human bone marrow stromal cells (hBMSC) were purchased from European Service Center for Lonza Bioscience (Lonza, Verviers, Belgium). The BMSC were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells at passage 3 were seeded into T-75 cm<sup>2</sup> culture flasks at an initial density of  $2 \times 10^6$ . When the cells reached 80–90% confluence they were washed with PBS three times, supplemented with fresh serum-free DMEM containing 1% penicillin–streptomycin and collected after 48 h. The supernatants from different culture flasks were pooled, then centrifuged at 4°C at  $3000 \times g$  for 3 min followed by 5 min at  $1500 \times g$  and collected as a CM. DMEM serum-free medium incubated under the same conditions served as a control. The media were stored at –80°C for further experiments.

### Isolation of human dental pulp cells (hDPC) and cell culture

According to the protocol approved by the Regional Committees for Medical and Health Research Ethics (REC) at the University of Bergen, Norway (225.05, 3.2008.1750, 2009/610 and 2013/1248), human third molar teeth ( $n = 3$ ) from healthy adult patients aged 18–25 were extracted during routine operative procedures at the Dental Clinic at the University of Bergen, Norway. hDPC were enzymatically isolated and characterized by phenotype on the basis of positive expression for mesenchymal cell surface markers, such as CD90, CD105, STRO-1, and CD24 (18). Cells from passages 4–6 were used for the *in vitro* study, and the experiments were performed in triplicate. The isolated hDPC were cultured with DMEM containing 5% FBS and 1% penicillin–streptomycin (DMEM-5% FBS) at

an initial density of  $6 \times 10^5$  for 24 h. Next, the cells were incubated with either DMEM-1% FBS (control cells) or BMSC-CM-1% FBS (CM-treated cells).

### Multiplex assay of cytokine Levels

Supernatants from control or CM-treated hDPC, as well as samples of BMSC-CM or control medium, were tested for the presence of IL-10, IL-4, IL-6, and IL-8, and RANTES by use of Bio-Plex Pro Human Cytokine Group I 6-plex Assay (human multiplex bead-based immunoassay kit; Bio-Rad company, Hercules, CA, USA). after a 24-h incubation. The multiplex cytokine assay allows simultaneous quantification and was performed according to the manufacturer's instructions (Bio-Plex™ Cytokine Assay; Bio-Rad Company) (19). The level of released cytokines was measured in pg ml<sup>-1</sup>, using a Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad company, Hercules, CA, USA).

### PGE<sub>2</sub> determination by enzyme linked immunosorbent assay (ELISA)

The collected supernatants used for determination of cytokine level were also tested for the presence of PGE<sub>2</sub> using a commercial ELISA kit (Prostaglandin E<sub>2</sub> ELISA Kit, Monoclonal; Neogen Corporation, Lansing, MI, USA), according to the manufacturer's instruction. The PGE<sub>2</sub> content of the samples was quantified by microplate spectrophotometry (BMG Labtech, GmbH, Germany) at a wavelength of 450 nm, and the concentration was calculated in ng ml<sup>-1</sup> according to standards.

### Western blot analysis

To measure the protein expression of COX-2 in hDPC after BMSC-CM treatment,  $7 \times 10^5$  cells were seeded in 80-mm petri dishes and cultured for 24 h. Cell total protein fractions were extracted using RIPA buffer according to the manufacturer's instruction (RIPA buffer plus protease and phosphatase inhibitors; ThermoScientific, Waltham, MA USA). The concentration of total proteins was measured with the Pierce™ BCA Protein Assay Kit (ThermoScientific). Twenty microgram of total protein extracts were separated by electrophoresis and transferred as previously described (18). The membranes were blocked with 5% non-fat milk in TBS containing 0.1% Tween 20 for overnight at 4°C with rabbit anti-COX-2 antibody (1:1000 dilutions, Abcam®, Cambridge, UK). The membranes were then incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:2000 dilutions, Santa Cruz Biotechnology; Santa Cruz, CA, USA), in 5% non-fat milk in TBS containing 0.1% Tween 20 for 1 h. Rabbit anti-GAPDH served as an internal control for the total expression of COX-2 (1:1000 dilutions, Santa Cruz Biologicals, Santa Cruz, CA, USA). Protein detection was performed with an enhanced chemiluminescence (ECL) kit, and the bands were visualized by the Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA).

### Replantation model using rat maxillary first molars

To study the effect of BMSC-CM on the gene expression of IL-1 $\beta$ , IL-10, IL-4, IL-6 and IL-8, and TNF- $\alpha$  *in vivo*, a tooth replantation model using rat maxillary first molars was used. Ten female Sprague-Dawley rats, aged 3 weeks (approximately 100 g in weight), were included (National Public Health Institute, Norway). General anesthesia was achieved in each animal with 0.2 ml kg<sup>-1</sup> of Hypnorm–Dormicum (1 ml fentanyl/fluansion and 1 ml midazolam diluted in 2 ml sterile water). Before surgery, the teeth crowns were cleaned with 0.3% chlorhexidine gel. The left and right first maxillary molars ( $n = 20$ ) were then extracted as previously described (20). Briefly, the extraction procedure was performed by a small straight elevator inserted at the distal aspect of the first molar. The teeth were elevated 90 degrees mesially and left attached to the mesial gingival tissue for 2 min, and then replanted immediately to their original positions (Fig. 3a). The left first maxillary molars ( $n = 10$ ) were replanted without treatment (control replanted teeth), whereas the right first maxillary molars ( $n = 10$ ) were replanted immediately after application of 25  $\mu$ l of CM in the tooth socket (CM-treated replanted teeth). No postoperative splinting was used. Three and 14 days following replantation all animals were anaesthetized with Hypnorm–Dormicum and euthanized with neck dislocation. Next, the upper jaws of the rats were dissected out and the teeth were then extracted, submerged in RNAlater and stored at  $-80^{\circ}\text{C}$ . The animal experiments were approved by the Regional Committee for Animal Research Ethics at the University of Bergen, under regulations of the Norwegian Experimental Animal Board.

### Real-time quantitative reverse transcription PCR (Real-Time qRT-PCR)

To study the *in vivo* effects of BMSC-CM on gene expression of IL-1 $\beta$ , IL-10, IL-4, IL-6 and IL-8, and TNF- $\alpha$  in the rat tooth pulp 3 and 14 days following tooth replantation, total RNA was isolated using the TRIZOL (Invitrogen, Carlsbad, CA, USA). RNA purity and quantification were determined by spectrophotometry (ThermoScientific NanoDrop Technologies, Wilmington, DE, USA), and the reverse transcription reaction was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The cDNA corresponding to 1  $\mu$ g of mRNA was used as a template in each PCR reaction of the different primers (Table 1), and the RT-PCR

Table 1. Real-Time qRT-PCR probes used in *in vivo* study

Probes	Codes
GAPDH	Rn01749022-g1
IL1 $\beta$	Rn00563409-m1
IL-10	Rn00563409-m1
IL-4	Rn99999010-m1
IL-6	Rn99999011-m1
IL-8	Rn00567841-m1
TNF- $\alpha$	Rn00562055-m1

was performed on a StepOnePlus real-time PCR system using TaqMan<sup>®</sup> gene expression assays (Applied Biosystems, Foster City, CA, USA). Rat glyceraldehyde 3-phosphate dehydrogenase was used as a normalized reference gene, and the relative expression of each gene was analyzed using the  $2^{-\Delta\Delta\text{CT}}$  method.

### Data analysis

Quantitative results were expressed as mean  $\pm$  SD. For the *in vitro* experiments, a Student's *t*-test was used for comparison between CM and controls. A similar comparison was performed between CM-treated cells and control cells. For the *in vivo* experiments, the expression of each cytokine was calculated and then the ratios of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$  to the anti-inflammatory IL-10 were calculated. A Student's *t*-test was used for comparison to determine differences between CM-treated and control replanted teeth. The analysis was performed using IBM SPSS Statistics 22 (IBM<sup>®</sup> SPSS<sup>®</sup> Statistics 22.0, USA), and the differences between the means were considered statistical significant at  $P < 0.05$ .

### Results

#### Secretion of inflammatory mediators *in vitro*

The levels of IL-6 and IL-8, and RANTES were significantly higher in CM than the control medium (Fig. 1). When hDPC were cultured in CM, the levels of IL-10, IL-6, and IL-8, and RANTES were significantly higher in the supernatant compared with control cells (Fig. 1). IL-4 was detected in low levels in CM and control medium, and the levels were not enhanced in the supernatant from the control and CM-treated cells (data not shown).

#### The effect of CM on the expression of COX-2 and PGE<sub>2</sub> in hDPC

The effect of CM on PGE<sub>2</sub> release and expression of COX-2 was further examined at the protein level. CM contains significant higher level of PGE<sub>2</sub> compared with control, and induced significantly higher release of PGE<sub>2</sub> from hDPC compared with control cells (Fig. 2a). As CM resulted in upregulated production of PGE<sub>2</sub>, COX-2 expression was assessed in hDPC. The protein expression of COX-2 was greater in CM-treated cells compared with control cells (Fig. 2b).

#### Effect of CM on mRNA levels of IL-1 $\beta$ , IL-10, IL-6 and IL-8, and TNF- $\alpha$ in replanted rat teeth

Three and 14 days following tooth replantation *in vivo*, mRNA for IL-1 $\beta$ , IL-10, IL-6, and IL-8, and TNF- $\alpha$  was detected in all replanted teeth (Fig. 3b). No mRNA expression of IL-4 was detected in the tooth samples at all time points (data not shown).

At day 3, a significantly lower level of IL-1 $\beta$  and IL-6, and TNF- $\alpha$  mRNA was detected in the CM-treated replanted teeth than in the control (Fig. 3b). There was no statistical difference in the mRNA level of IL-10

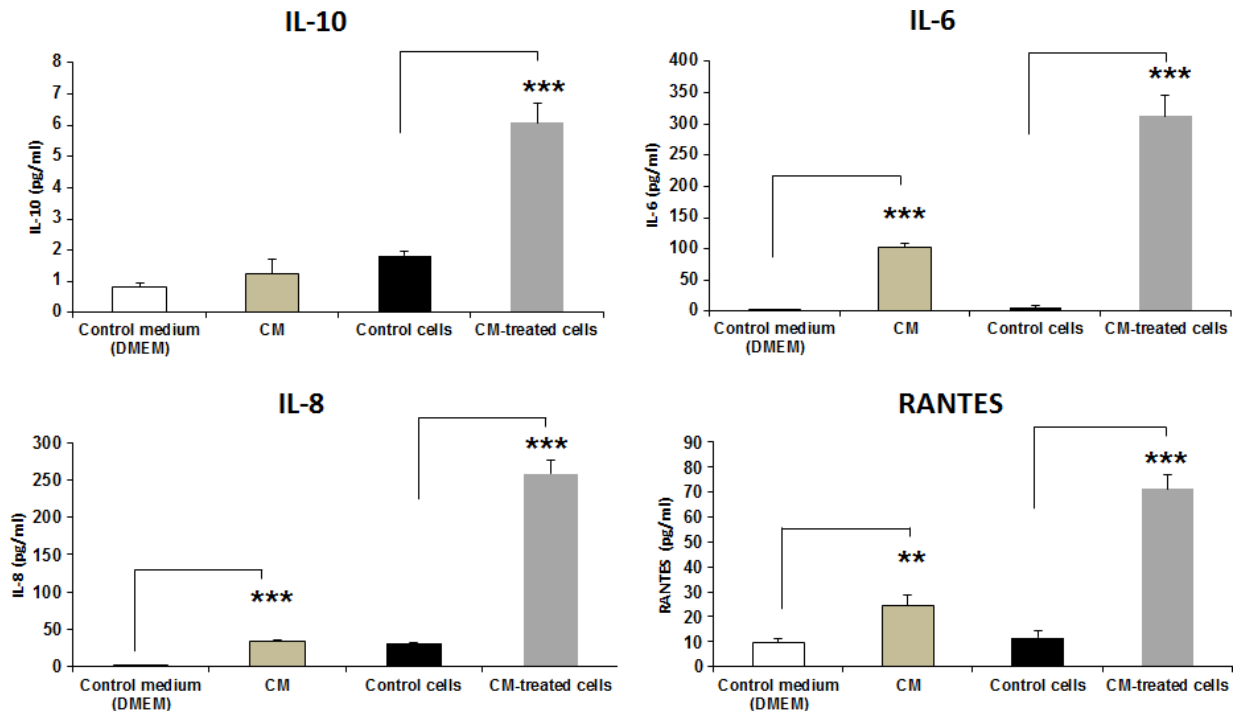


Fig. 1. Multiplex assay for IL-10, IL-6, and IL-8, and RANTES in control, CM, control cells, and CM-treated cells. The data are presented as mean ± standard deviation (\*\**P* < 0.01 and \*\*\**P* < 0.001).

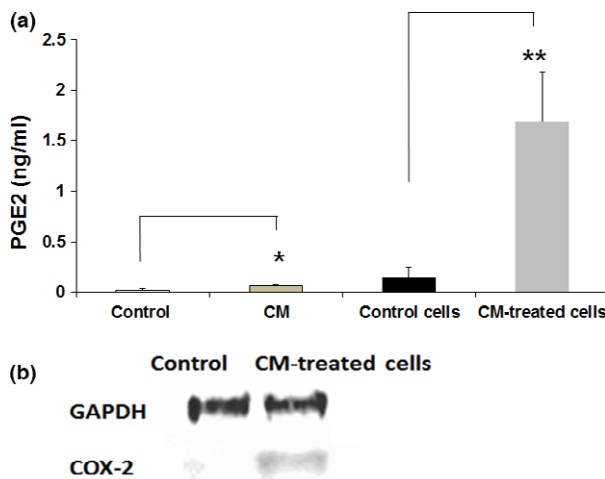


Fig. 2. Expression levels of PGE<sub>2</sub> and COX-2. (a) ELISA for PGE<sub>2</sub> and (b) Western blot analysis of COX-2. The intensity of each band of COX-2 expression was normalized to the internal control GAPDH. The data are presented as mean ± standard deviation (\**P* < 0.05 and \*\**P* < 0.01).

and IL-8 between the groups. At day 14, no-intergroup differences were found for the mRNA levels of IL-1β, IL-10, IL-6, and IL-8, and TNF-α between the CM-treated and control replanted teeth (Fig. 3b).

At day 3 following replantation, the IL-6/IL-10 ratio was significantly lower in CM-treated compared to control replanted teeth. The ratios of IL-1β/IL-10 and TNF-α/IL-10, however, did not differ between the groups (Fig. 4). By day 14 following replantation, the

ratios of pro-inflammatory cytokines IL-1β and IL-6, and TNF-α to anti-inflammatory cytokine IL-10 did not differ between the groups (Fig. 4).

### Discussion

The present study showed that BMSC-CM induced higher secretion of PGE<sub>2</sub>, IL-10, IL-8, and IL-6, and RANTES in hDPC *in vitro* and attenuated the gene expression of IL-1β, IL-6 and TNF-α in pulp tissues 3 days following tooth replantation *in vivo*. A limitation of the *in vivo* part of the current study was the single application of BMSC-CM. The use of single dose application was based on practical problems connected to trauma by use of repeated applications. The aim was thus to evaluate the effect on the initial inflammatory phase, as it was not expected that CM would have long-term effects. However, it has been previously shown that repeated administration of CM over time promote bone healing in a distraction osteogenesis model (21).

IL-6 is a pleiotropic cytokine that acts as a major mediator in the host response following tissue injury, with an effect on bone osteoclasts and bone resorption through an autocrine/paracrine manner (22). It has been reported that MSC secrete high levels of IL-6 (23), and the cytokine has previously been reported to be involved in the immunoregulatory effects exerted by MSC (24). In the pulp tissue, the mRNA level of IL-6 has been found to be significantly upregulated in teeth with irreversible pulpitis compared with healthy teeth (25). It has been reported that continued expression of IL-6 correlates with the degree of inflammation in the



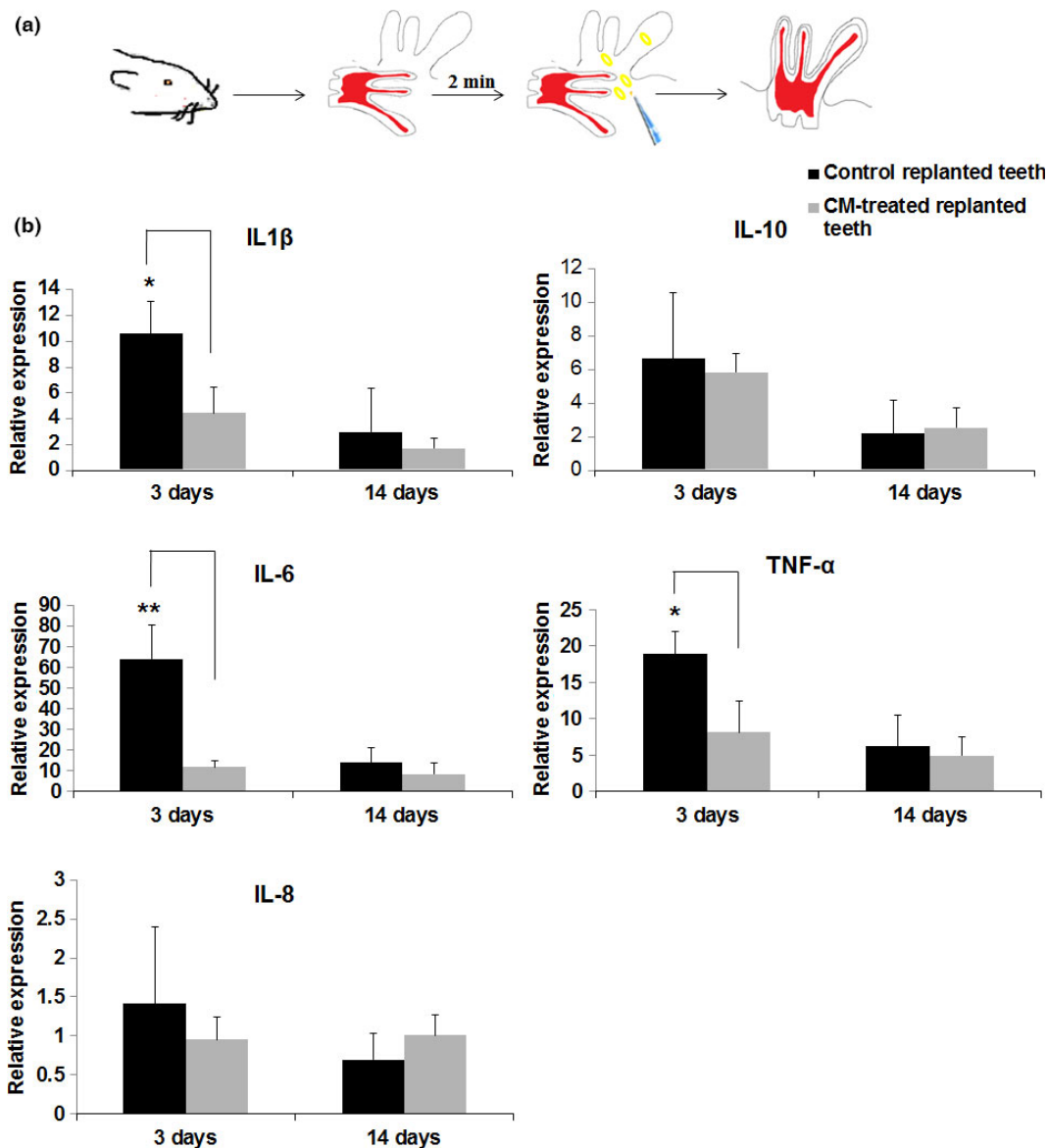


Fig. 3. (a) CM treatment of rat first maxillary molars in the rat replantation model. (b) Real-Time qRT-PCR data showing expression of IL-1 $\beta$ , IL-10, IL-6, and IL-8, and TNF- $\alpha$  3 and 14 days following replantation (\* $P$  < 0.05 and \*\* $P$  < 0.01).

pulp following tissue injury and infection (26). Three days following tooth replantation, IL-6 was significantly lower in the CM-treated than the control teeth, a finding indicating an effect of CM on the immediate inflammatory response. Notably, the mRNA level of IL-6 declined in pulp tissues of replanted teeth after 14 days in both groups, suggesting a normal resolution process.

In an *in vivo* inflammatory arthritic model, MSC secretion of the immunoregulatory factor PGE<sub>2</sub> showed a concomitant IL-6 upregulation (4). PGE<sub>2</sub> has been shown to induce osteoblast and cementoblast differentiation and mineralization *in vitro* (27, 28), and higher production of COX-2 and PGE<sub>2</sub> by hDPC was found after stimulation with mineral trioxide aggregate (MTA) (15). Low concentrations of exogenous PGE<sub>2</sub> were able to induce calcified nodule formation by

hDPC *in vitro* (29). It could therefore be speculated that the presence of PGE<sub>2</sub> in CM, and the subsequent increase after cultivation of hDPC in CM *in vitro*, could be involved in the differentiation and mineralization of endogenous dental pulp cells (18). Although the expression of PGE<sub>2</sub> was not examined *in vivo*, the finding that PGE<sub>2</sub> is present in CM suggests a possible role of CM during pulp healing following tissue injury.

IL-10 is a key anti-inflammatory cytokine that inhibits production of pro-inflammatory cytokines including IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (30), thereby having an immunoregulatory effect. Increased secretion of IL-10 has also been detected in rats with acute kidney injury treated with BMSC-CM, suggesting a significant role of IL-10 in survival and protection of the kidney (31). No difference was found in the levels of IL-10 between the CM and control medium, but an elevated level was

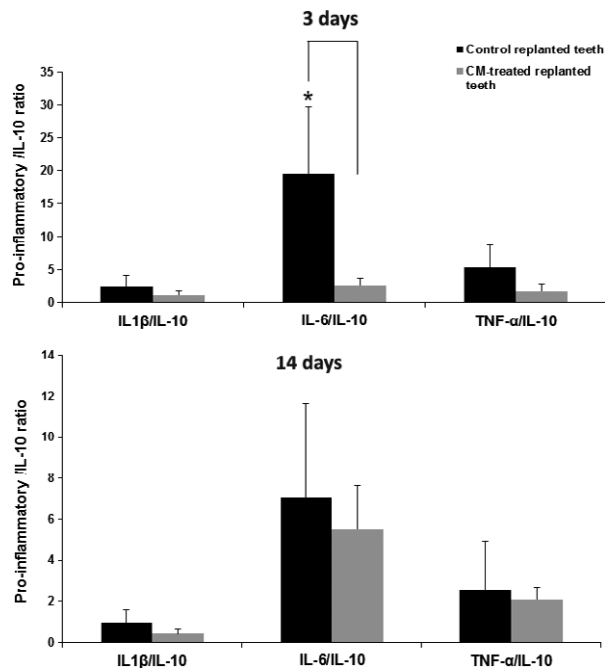


Fig. 4. Real-Time qRT-PCR results showing the pro-inflammatory cytokines/anti-inflammatory IL-10 ratios 3 and 14 days following tooth replantation (\* $P < 0.05$ ).

detected in the medium of CM-treated compared with the control hDPC, suggesting stimulatory effect/s of CM on hDPC for the production of the resolution molecules. This is in parallel with a previous finding showing that CM from adipose stem cells induces secretion of IL-10 from CD4 + FoxP3 + cells *in vitro* (12). It has been reported that the mRNA level of IL-10 is upregulated in the pulp beneath deep caries compared with shallow caries (32), and also in teeth with irreversible pulpitis (33). Following tooth replantation, no difference was found in the mRNA level of IL-10 between CM-treated and control replanted teeth. However, the expression of IL-10 in CM-treated replanted teeth was previously reported to be associated with a reduction in the mRNA level of IL-1β, IL-6, and TNF-α, suggesting an immunoregulatory effect of CM (34).

IL-4 is another anti-inflammatory cytokine, and plays a role in formation of TH-2 cells and in shifting the macrophage phenotypes into IL-10-producing cells (35). IL-4 is also known to stimulate fibroblast proliferation and inhibit osteoclast activities (36). The present *in vitro* experiments did not find an elevated level of IL-4 either before and/or after CM treatment. Moreover, mRNA level of IL-4 was not detected in the replanted teeth. The relevance of these findings is unclear, and further molecular investigations to examine this undetected gene are needed.

IL-1β is considered a strong inflammatory mediator, important in acute host responses (37). It is well known that IL-1β activates osteoclasts, resulting in bone destruction and root resorption (38). Continued release of IL-1β might thus impair tissue healing. As for IL-6, IL-1β mRNA declined after 14 days, indicating attenuation of inflammation over time.

In agreement with reports showing that chemokines are constitutively produced and secreted from stem cells (39), this study found high level of RANTES and IL-8 in CM, with a significant increase when hDPC were cultured in CM. In dental pulp tissue, both the mRNA level and protein expression of IL-8 have been found to increase in inflamed pulp (25, 40). As for IL-1β, continued expression of IL-8 may contribute to root and bone destruction of injured teeth (26). The present *in vivo* results found no differences in the mRNA level of IL-8 between the groups, suggesting a minor role for IL-8 in the current model.

TNF-α, a potent pro-inflammatory mediator, is able to induce secretion of other pro-inflammatory cytokines, including IL-6 (41). TNF-α has been detected during orthodontic tooth movement, indicating a role for TNF-α in inflammation and root resorption (42). TNF-α has also been found in teeth with symptomatic irreversible pulpitis (43) and in the gingival crevicular fluid after tooth injury (26). However, no difference in the mRNA level of TNF-α was found between teeth with healthy pulps and reversible pulpitis (44). Down-regulated mRNA level of TNF-α in CM-treated replanted teeth after 3 days compared with controls might suggest that CM treatment attenuates the initial inflammatory reaction after tooth replantation.

Collectively, the lower level of IL-1β, IL-6, and TNF-α in the CM-treated replanted teeth supports an immunoregulatory action of the stem cells paracrine factors. However, cytokines always act in a network and some cytokines can block the effects of others. In particular, it has been shown that increased IL-6/IL-10 ratio during pulpitis could be used as an indicator for pulp disease (33). Based on this, the ratio of each pro-inflammatory cytokine IL-1β and IL-6, and TNF-α to the anti-inflammatory cytokine IL-10 was investigated. The findings of the current study suggest an impact of CM on the inflammatory/resolution process through the IL-6 and IL-10 network. Although the pro/anti-inflammatory ratios were lowered after 14 days in all replanted teeth, the inflammatory reaction at early time points might affect the healing outcome negatively. In this context, it has been shown that pulp tissue calcification and inflammation, especially after replantation, are closely related mechanisms (45, 46).

## Conclusion

This study suggests that CM promotes secretion of inflammatory/resolution molecules from hDPC *in vitro*. Moreover, CM attenuates the initial inflammatory response in rat dental pulp tissue *in vivo* following tooth replantation.

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