Influence of bone marrow stromal cell secreted molecules on pulpal and periodontal healing in replanted immature rat molars

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Abstract – *Aim*: To investigate the effect of paracrine factors secreted from human bone marrow stromal cell (BMSC-CM) on pulpal and periodontal healing following immediate replantation of maxillary rat first molars. *Material and Methods*: Fifty maxillary rat first molars were elevated and replanted after 2 min. The left teeth were replanted without treatment, whereas BMSC-CM was injected into the right socket prior to replantation. Twelve un-operated teeth served as reference teeth. The expression of vascular endothelial growth factor A, alkaline phosphatase, Runt-related transcription factor 2 and osteoclast stimulating factor 1 was studied by real-time reverse transcription polymerase chain reaction at day 3 and 14. The dentin thickness together with Laminin- and PGP 9.5-immunoreactivity were studied after 3, 14 and 90 days. *Results*: Real-Time qRT-PCR data showed up-regulated expression of ALP mRNA in the socket specimens of conditioned medium treated replanted teeth after 3 days. No morphological differences were found for the expression of Laminin and PGP 9.5 between control and conditioned medium treated replanted teeth. At day 14, external cervical and surface root resorption was found in one BMSC-CM and one control tooth. At 90 days, all control replanted teeth had external cervical and surface root resorptions, whereas only one sample was seen among the conditioned medium treated teeth. At day 90, more extensive dentine formation with narrowing of the pulpal space was observed in the control compared with conditioned medium treated teeth. *Conclusions*: The present findings showed that BMSC-CM treatment reduced the number of replanted teeth with external root resorption and resulted in a significant reduction in new dentin formation.

Traumatic dental injuries (TDI) are the most common facial injuries that affect the integrity of the natural dentition (1). After tooth avulsion, replantation may preserve the alveolar-ridge and orofacial development and growth. Despite attempts for immediate replantation of traumatically avulsed teeth, many factors may impair the healing process. Pulpal changes after rupture of blood vessels and nerve fibres, damaged odontoblast function and degree of attachment damage, may affect the healing outcomes after trauma (2, 3).

Several studies have focused on treatment of the root surface to control periodontal complications after replantation or transplantation. Despite attempts to control periodontal ligament (PDL) repair and tooth revascularization, uncertain healing outcome has been found (4–7). Bioactive soluble molecules, which are a group of signalling molecules able to control several endogenous activities, have shown promising results in some healing models. Bone morphogenic protein 7 (BMP-7) was shown to increase cementum formation, improve eruption and survival of transplanted teeth in immature mini-pigs (8). In addition, BMP-6 enhanced periodontal wound healing and cementogenesis in dogs (9). For enhanced pulpal revascularization, vascular endothelial factors (VEGF) has been suggested as a crucial angiogenic factor for traumatically avulsed teeth (10), whereas endothelial growth factor (EGF) improved revascularization of the pulp tissue after auto-transplantation in rat molars (11).

It has been shown that transplanted exogenous stem cells produce a variety of bioactive immunoregulatory, angiogenic and neurotrophic molecules that can be used for improving and accelerating wound healing (12). In particular, secreted factors derived from bone marrow mesenchymal stem cell (BMSC) or bone marrow mesenchymal stem cell conditioned medium

(BMSC-CM) has been shown to promote wound healing (13, 14). For dental tissues, BMSC-CM is able to induce osteo/odontogenic differentiation of dental pulp cells *in vitro* (15), and promote alveolar bone and cementum regeneration in infra-bony defects in rats (16). The existence of mesenchymal stem cells in pulpal and periodontal tissues may therefore participate and influence healing after tooth avulsion. Therefore, the aim of the presented study was to investigate the effect of human bone marrow stromal cell secreted molecules as a conditioned medium (BMSC-CM) to enhance periodontal and pulpal healing following replantation of rat molars.

Materials and methods

Animals and replantation procedures

The experimental protocol was approved by the Regional Committee for Animal Research Ethics, University of Bergen, under supervision of the Norwegian Experimental Animal Board.

Thirty-one female Sprague-Dawley rats, aged 3 weeks and approximately 100 g in weight were used (National Public Health Institute, Norway). The animals were fed standard pellet diets with water *ad libitum* and acclimatized for 7 days prior to the operation procedures. Anaesthesia was achieved with a subcutaneous injection of Hypnorm-Dormicum (1 ml fentanyl/fluansion diluted in 1 ml sterile water mixed with 1 ml midazolam diluted in 1 ml sterile water, 0.2 ml kg^{-1} body weight). Before the surgical procedure, the crown was cleaned with 0.3% chlorhexidine gel. The left and right first maxillary molars were selected in this study. The teeth were elevated with a sterile elevator placed at the distal aspect of the crown, according to an established protocol (17). The left tooth was first elevated for 2 min and replanted without any treatment (denoted as control replanted teeth). The contralateral right tooth was elevated in a similar manner, but received injection of BMSC-CM (lyophilized reagent diluted in 100 ml serum-free medium, 0.025 ml tooth^{-1}) in the socket before replantation (denoted as CM treated replanted teeth). No postoperative fixation was used. Postoperative pain killer (Temgesic 0.3 mg ml^{-1}, 0.05 ml 100 g^{-1} body weight) and antibiotic prophylaxis (benzylpenicillinprocaine 300 000 IE ml^{-1}), 0.020 ml 100 g^{-1} body weight, were given. The animals were kept on a soft diet after the operation to facilitate feeding and drinking. At the end of the observation periods (3, 14 and 90 days), all animals were anaesthetized subcutaneously with Hypnorm-Dormicum (approximately 0.4 ml rat^{-1}). Five rats at 3, 14 and 90 days were processed for immunohistological analysis. The rest of the rats at 3 and 14 days were euthanized with neck dislocation for gene analysis. Six reference teeth (three rats) were processed for the gene analysis, and six reference teeth (three rats) were processed for immunohistological analysis (Table 1).

Tissue preparation

For gene analysis, the upper jaw of the rats was dissected out and separated into left and right parts.

Table 1. Replanted and reference rat upper first molars

Endpoint (days)	IHC	Gene analysis	Rats No.
3 days	5 rats	5 rats (10 teeth and 10 sockets)	10 rats (20 teeth)
14 days	5 rats	5 rats (10 teeth and 10 sockets)	10 rats (20 teeth)
90 days	5 rats		5 rats (10 teeth)
Reference teeth	3 rats	3 rats (6 teeth and 6 sockets)	6 rats (12 teeth)
Total rats	18 rats	15 rats	31 rats (62 teeth)

Teeth and socket specimens were prepared and immediately submerged in RNA later and stored at −80°C. For the histological procedures, animals at day 3, 14 and 90 underwent transcardiac perfusion by heparinized saline (0.9% NaCl/0.03% heparin), followed by 10% ethylene-diamine-tetra acetate (EDTA), pH 7.4, at room temperature (Table 1). Briefly, after transverse abdominal incision, a fine needle was inserted into the descending aorta and immobilized by needle forceps before infusion of perfusion solution. The maxillary jaws were then dissected out and immersed in demineralizing solution (10% EDTA) at 4°C for 4 weeks. Decalcified specimens were rinsed in PBS for 24 h, subsequently soaked in 30% sucrose in 0.1 M phosphate buffer, pH 7.4 for 24 h, embedded in O.C.T. tissue-tech (Sakura Finetek, Tokyo, Japan), and immediately kept at −80°C.

Gene analysis

Total RNA was isolated using TRIZOL method and the RNA purity and quantification were determined by spectrophotometry (ThermoScientific NanoDrop Technologies, Wilmington, DE, USA). The reverse transcription reaction was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), using 1000 ng total RNA dissolved in 40 µl nuclease-free water mixed with reverse transcriptase (RT) buffer, random primers, dNTPs and MultiScribe RT. The cDNA corresponding to 10 ng of mRNA was used as a template in each PCR reaction of primers (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was conducted by a StepOnePlus real-time PCR system using TaqMan® gene expression assays: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn01749022-g1), Runt-related transcription factor 2 (RUNX 2, Rn01512298-m1), Vascular endothelial growth factors A (VEGFa, Rn01511601-m1) and Osteoclast stimulating factor 1 (OSTF1, Rn00686607-m1) (Applied Biosystems, Foster City, CA, USA). The data were analysed using the $2^{-\Delta\Delta CT}$ method and GAPDH served as house-keeping gene for normalization.

Immunohistochemical procedure (IHC) using Laminin and PGP 9.5

Cryosectioning was performed on a Leica CM 3050S (Leica Microsystems, Wetzlar, Germany) at −24°C into 25-µm sections. Sections were fixed with cold acetone

and treated with absolute methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. Sections were incubated with PBS containing 2.5% normal goat serum for 60 min at room temperature before incubation with rabbit polyclonal anti-Laminin (1:5000; StressGen Biotechnologies, Victoria, Canada) and rabbit polyclonal anti-PGP 9.5 (1:400; StressGen Biotechnologies, Victoria, BC, Canada) overnight. After several rinses in PBS, sections were incubated with biotinylated anti-rabbit IgG for 60 min, and finally with biotin/avidin solution (VECTASTAIN Elite ABC Kit, Vector Laboratories, Inc. Burlingame, CA, USA). Following washes with PBS, substrate solution-cell reactions (DAB Substrate Kit, Vector Laboratories, Inc., Burlingame, CA, USA) were added at room temperature. The expression of Laminin and PGP 9.5 was examined with an inverted microscope equipped with a digital camera (FV 500, Olympus, Tokyo, Japan).

Immunohistochemical evaluation

The immunostaining was evaluated according to the following parameters; pulp healing pattern including an analysis of angiogenic and neurogenic marker expression in the pulp chamber, and reparative or new dentin formation. Dentine formation was evaluated by calculating the dentine thickness from the furcal surface of the pulpal floor towards the internal pulp floor.

For analysis of root resorptions, several parameters were used; ESR, External surface resorption; IRR, Internal root resorption; EIRR, External inflammatory root resorption; ECR, External cervical resorption; Ankylosis (replacement resorption). All parameters were evaluated by a blinded observer.

Statistical analysis

The mRNA expression of the reference teeth was considered as the normal level of uninjured teeth. Quantitative data of dentin thickness after 3, 14 and 90 days and the mRNA expression levels were presented as mean ± standard deviation. For statistical analysis, one-way analysis of variance was followed by a multiple-comparison Tukey test, IBM® SPSS® Statistics 22.0 (IBM, USA). Statistical significance was determined at $P < 0.05$.

Results

Real-Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR)

The Real-Time qRT-PCR data revealed ALP, Cbfa1/Runx2 and OSTF1 (bone remodelling related genes), and VEGFa (angiogenic related gene) mRNA expression in all teeth and sockets samples after 3 and 14 days (Fig. 1).

Higher VEGFa mRNA expression was found in all tooth specimens of replanted teeth after 3 and 14 days compared with reference rat teeth. Although not statistically significant, the VEGFa mRNA level was slightly up-regulated in all socket specimens of replanted teeth after 3 and 14 days compared with reference rat teeth. No intergroup differences were found between the controls and CM treated tooth and socket specimens.

The ALP mRNA expression was significantly up-regulated in the tooth specimens of control and CM treated replanted teeth after 3 and 14 days compared with reference rat teeth. Compared to 3 days, the ALP mRNA expression was declined at 14 days. In the socket specimens, a significant up-regulation of ALP mRNA was detected in all replanted teeth after 3 days compared with reference rat teeth. A significant up-regulation of ALP mRNA expression in the socket specimens was also found for the CM treated replanted teeth compared with control replanted teeth after 3 days. Compared to 3 days, the ALP mRNA expression in the socket specimens was significantly down-regulated in the control and CM treated replanted after 14 days.

For the RUNX2 mRNA expression, up-regulation was found in the control replanted teeth compared with the reference rat teeth after 3 days. After 14 days, statistical down-regulation was found in the control replanted teeth compared with same group after 3 days. No intergroup time differences in the mRNA level of RUNX2 was found for the CM treated replanted teeth, although the expression after 14 days was declined. For the sockets, no differences were detected between replanted teeth and reference rat teeth at 3 and 14 days.

Increased mRNA expression of OSTF1 was found in all replanted teeth compared with the reference rat teeth, although not at a statistical level. On the other hand, the mRNA expression of OSTF1 in the sockets were down-regulated in all replanted compared to the reference rat teeth, and the expression of OSTF1 was statistically down-regulated in control replanted compared with reference rat teeth after 14 days.

Histological analysis

Reference rat teeth
The reference rat teeth showed normal distribution of Laminin and PGP 9.5 in pulp and periodontium (Fig. 2).

Three days after replantation
The pulp of control and CM treated replanted teeth demonstrated immunoreactivity to both Laminin and PGP 9.5 (Fig. 3). The PGP 9.5 staining was faint compared with reference rat teeth. The PDL was separated from the alveolar bone by organized connective tissue in all replanted teeth. No statistical differences were found between the experimental groups for the dentin thickness (Fig. 4).

Fourteen days after replantation
Increased immunoreactivity of Laminin was found in both control and CM treated replanted teeth, as compared with reference rat teeth (Fig. 5). Laminin- and PGP 9.5-immunoreactivity was densely distributed close to the odontoblast layer, with a pattern similar to reference rat teeth. The intensity of the Laminin- and
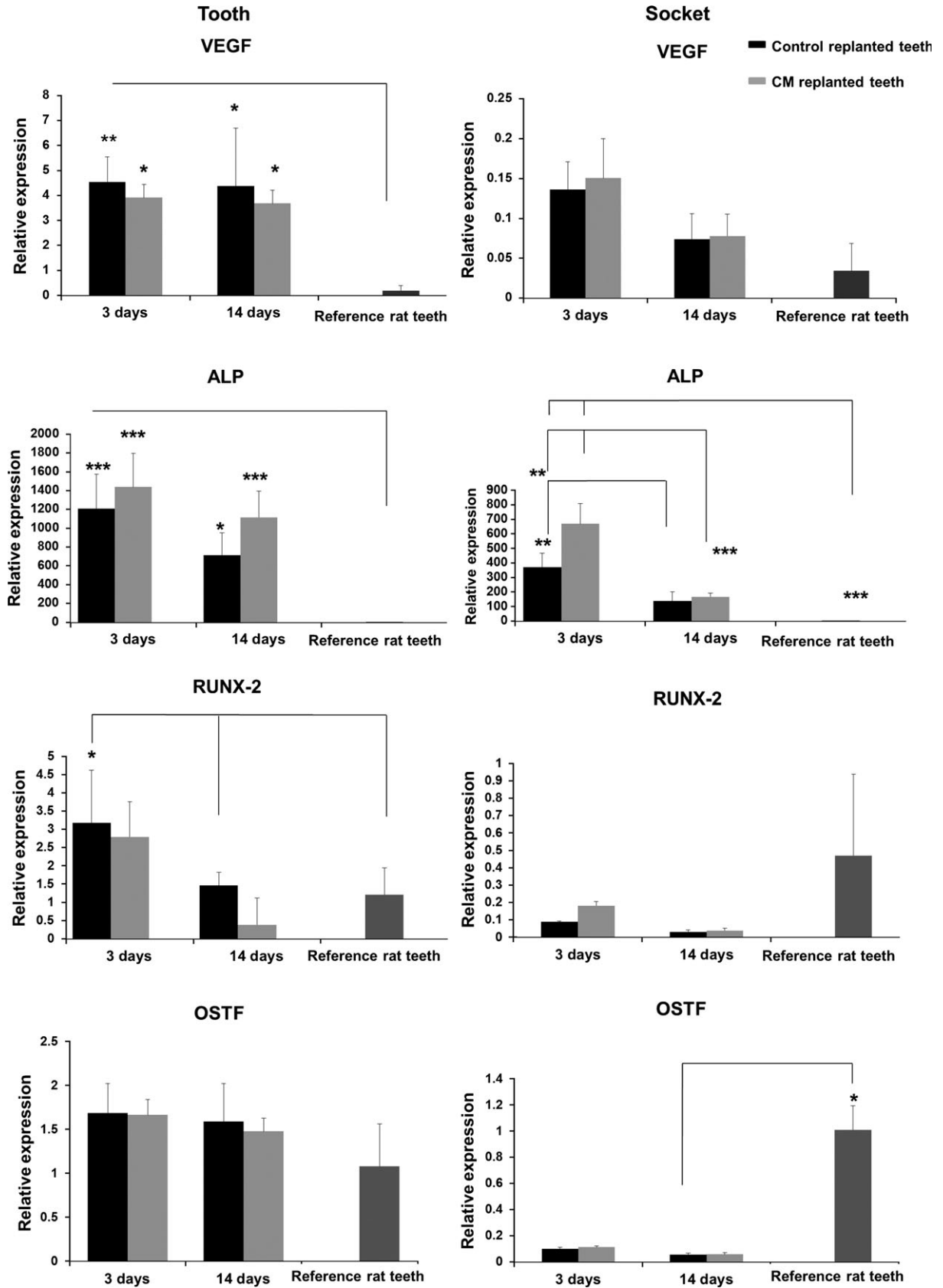
Fig. 1. Real-Time qRT-PCR analysis at day 3 and 14 in teeth and tooth socket specimens. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for data normalization. The data are presented as mean ± standard deviation (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).
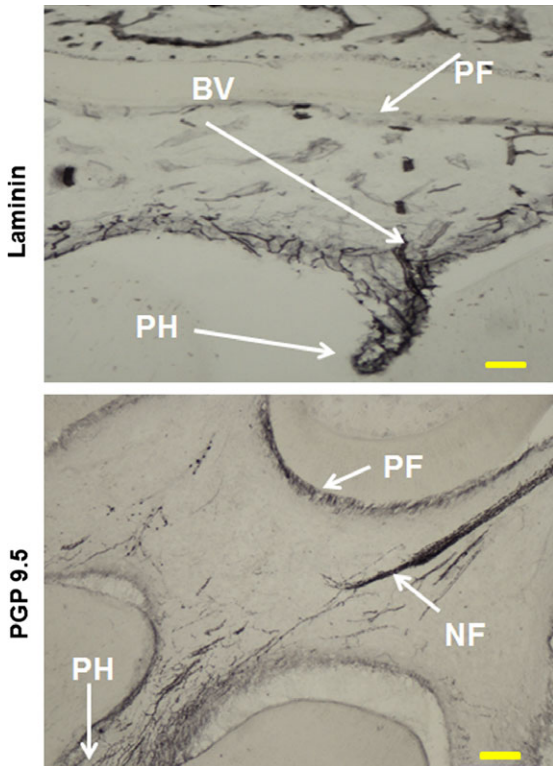
Fig. 2. Laminin- and PGP 9.5-immunoreactivity distribution, as demonstrated in reference rat teeth. BV, blood vessels; NF, nerve fibres; PH, pulp horn and PF, pulp floor (magnification, 10×; scale bar, 50 μm).

PGP 9.5-immunoreactivity was higher in the mesial root as compared with the distal root of the replanted teeth. For the dentin formation, statistically more dentin was formed in both replanted groups compared with day 3 groups and the reference rat teeth (Fig. 4, ***$P < 0,001$). However no intergroup difference was found between control and CM treated replanted teeth. Continued root development was observed in all replanted teeth comparable with the reference rat teeth. External cervical and surface root resorptions were noted in the mesial part of the mesial root of one specimen in each of the replanted groups (Fig. 6A).

Ninety days after replantation
Complete root development was shown with distinct PDL in the replanted teeth. Laminin and PGP 9.5-immunoreatctivity was observed in the pulp tissue, with a similar pattern as found for reference rat teeth. External cervical and surface root resorptions were noted in the replanted teeth (Fig. 6B). Resorptions were demonstrated in all control replanted teeth, whereas resorptions were only found in one CM treated replanted tooth. Extensive dentin formation was found at this time point, and was significantly higher in the control replanted teeth as compared with the CM treated replanted teeth (Fig. 4 and 7, ***$P < 0.001$). Dentin formation in both control and CM treated replanted teeth was significantly higher after 90 days as compared with 3 and 14 days, and to the reference rat teeth (Fig. 4, ***$P < 0.001$). Dentin formation was
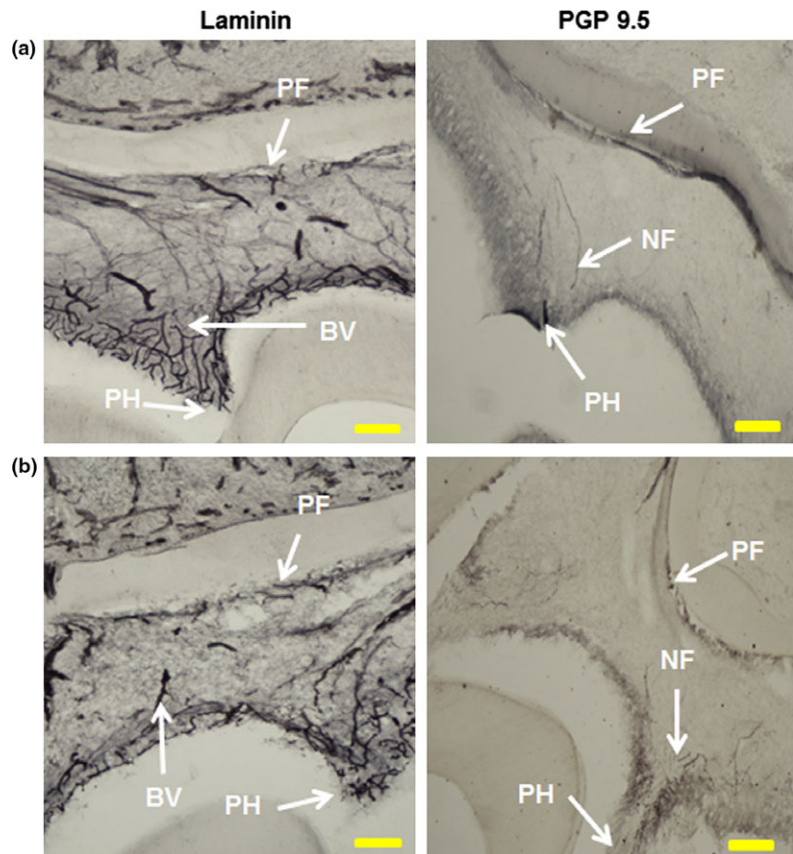

Fig. 3. Laminin- and PGP 9.5-immunoreactivity 3 days following tooth replantation. (a) control and (b) CM treated replanted teeth. Laminin and PGP 9.5-immunoreative distribution is demonstrated in both control and CM treated replanted teeth. BV, blood vessels; NF, nerve fibres; PH, pulp horn and PF, pulp floor (magnification, 10×; scale bar, 50 μm).
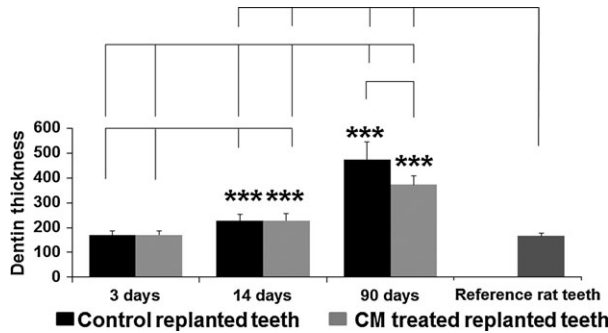
Fig. 4. Dentin thickness at day 3, 14 and 90. At day 3, no statistical differences were found among the experimental groups. At day 14, dentin thickness was significantly increased in all replanted teeth as compared to the same groups at day 3, and reference teeth at day 14. At day 90, CM treated replanted teeth exhibited less dentin thickness compared with the control replanted rat teeth. Both replanted groups had increased dentin thickness compared with the same groups at day 3 and 14. The data are presented as mean ± standard deviation (***$P < 0.001$).

characterized by narrowing of the pulp chamber and scattered calcified tissues was seen in one of the control replanted teeth (Fig. 7).

Discussion

The present study revealed that bone marrow stromal cell conditioned medium (BMSC-CM) reduced the number of teeth with external root resorption and prohibited excessive reactive dentin formation.

As angiogenesis is indicated as a critical step in pulp healing (18), this study used Laminin and VEGFa as markers for pulpal blood supply. Laminin is presents in the basement membrane of blood vessels and cell membranes (19). Laminin is a member of proteins and glycoproteins that has many biological functions including cell-cell interactions, and encourage neurite outgrowth after tissue damage (19). In the current study, gene and immunohistological data showed the expression of blood vessels markers even 3 days following replantation. At day 14, Laminin immunoreactivity appeared to be up-regulated in the pulp tissue of the replanted compared with reference rat teeth. This may reflect a role for Laminin in the tissue healing. The mesial root of the replanted teeth was characterized by intensive Laminin immunoreactivity compared to the distal root, indicating a more severe luxation trauma to the distal root during the procedure. These results might indicate better revascularization, possibly due to less sever luxation trauma and the fact that this root is bigger with a larger volume. Increased density of Laminin-positive blood vessels in the pulp after dentinal injury has been reported, reflecting localized increased blood flow (20).

Pulpal nerve fibres are shown to affect the pulp tissue homeostasis, blood flow and healing (21). In normal pulp tissue, nerve fibres are densely distributed in the odontoblast layer, predentin, and the inner part of dentin. In the root they are more centrally located along the blood vessels (22). During pulpal injury, nerve fibres react by sprouting of their terminal axons, indicating an important role in the healing process (23). PGP 9.5 has been used as neurochemical marker for intra-pulpal nerves during tooth development (24). In agreement with previous studies the odontoblasts also expressed PGP 9.5, possibly reflect-
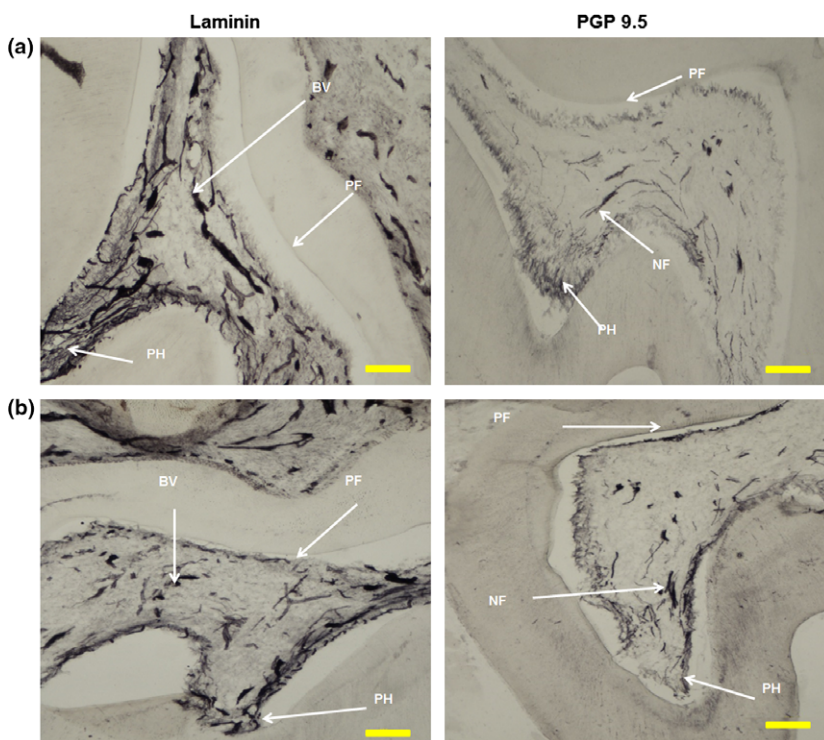

Fig. 5. Laminin- and PGP 9.5-immunoreactivity in the pulp chamber of replanted teeth at day 14. (a) Laminin- and PGP 9.5-immunoreative staining in a control replanted tooth. (b) Laminin- and PGP 9.5-immunoreative staining in a CM treated replanted tooth. BV, blood vessels; NF, nerve fibres; PH, pulp horn; PF, pulp floor (magnification, 10×; scale bar, 50 μm).

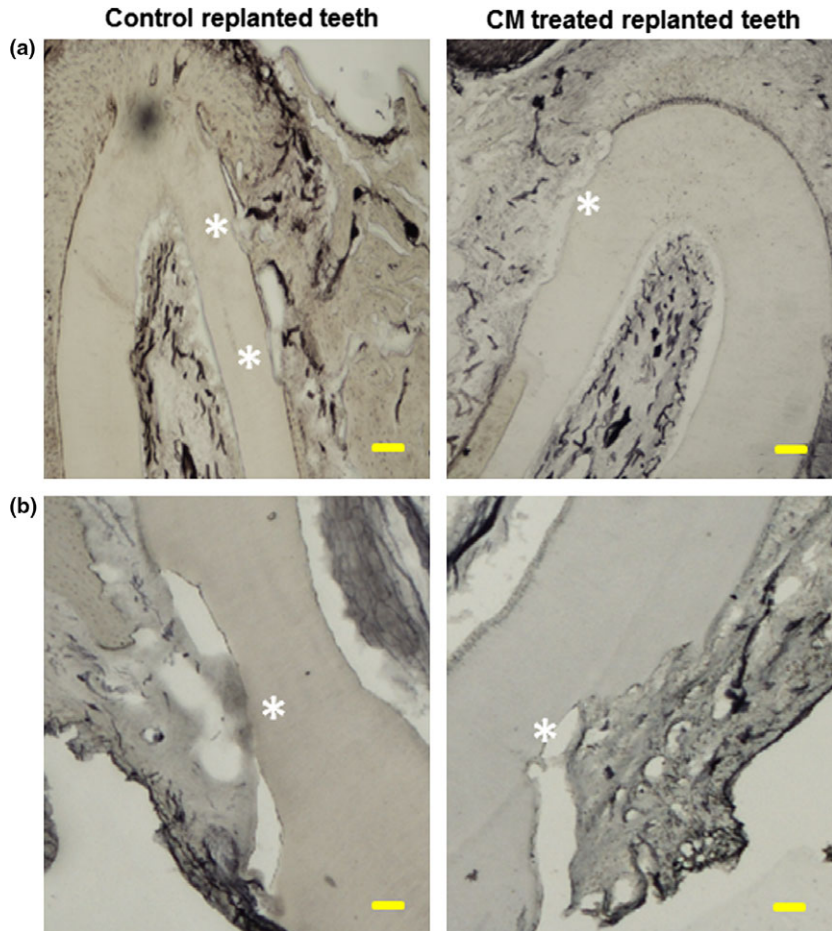**Control replanted teeth**   **CM treated replanted teeth**

Fig. 6. (a) External surface root resorptions on both control and CM treated replanted teeth at day 14 indicated by white asterisks (magnification, 4×; scale bar, 500 um). (b) External cervical root resorptions in both experimental groups at day 90 indicated by white asterisks (magnification, 10×; scale bar, 50 μm).
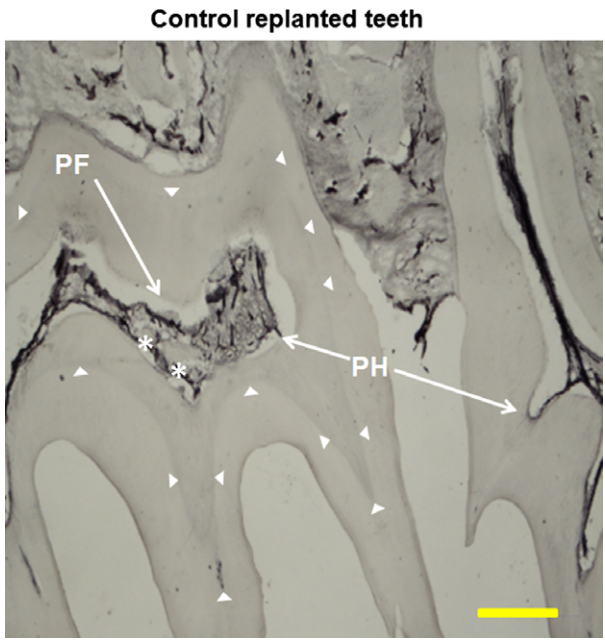
Control replanted teeth

Fig. 7. Extensive dentin formation with narrowing of the pulp space seen in a control replanted tooth at day 90. Pulp calcifications (white asterisks) occupy the pulp space beneath an extensive amount of tertiary dentin. Arrowheads indicate the new dentin formation. PH, pulp horn; PF, pulp floor (magnification, 20×; scale bar, 100 μm).

ing their neural crest origin (24). It has previously been documented that the PGP 9.5-immunoreactivity in pulpal nerves and odontoblasts disappeared 1–3 days following replantation, and that some fragmented immunoreative structures in the coronal pulp remained (25). That study also found that PGP 9.5-immunoreactive regenerating axons returned after 5 days (25). In the present study the PGP 9.5-immunoreactivity was weak or disappeared at day 3, possibly reflecting the presence of the protein and not functional nerve fibres.

Post-traumatic external root resorption is virtually a consequence of damage and inflammation in the PDL and the root cementum, and is considered as a self-limiting condition that is followed by repair (26). Cervical root resorption is also an inflammatory mediated external resorption of the root, but is more progressive in nature (27). Periodontal healing is strongly affected by the extent of damage to the PDL cells (28), and is mainly dependent on time and extra-oral storage (17). Numerous attempts to preserve the PDL cells and reduce the clastic activity have been suggested to minimize root resorption. For example, topical alendronate (ALN) treatment of dog teeth was found to inhibit the osteoclast activity, reflected by a reduction in the incidence of root resorption (29). Enamel matrix derivative (EMDOGAIN) was found to reduce the number of teeth with inflammatory root

resorption after replantation of permanent incisors in Beagle dogs (26). In the present study, we found a reduction in external surface resorption after BMSC-CM treatment at 90 days. These findings are in line with results showing that BMSC-CM treatment induced proliferation of PDL cells *in vitro*, and improved regeneration of cementum in a dog one-wall intrabony defect model (16). In addition, the newly regenerated bone and cementum in the dog one-wall intrabony defect model was associated with minimal inflammatory cell infiltration compared with the other treatment groups (16). Also, BMSC-CM prevent the activity of osteoclast cells, which is a prerequisite for bone resorption (30). Taken together these results indicate that BMSC-CM can promote periodontal healing of replanted teeth.

Damage to the neurovascular supply can induce pulp-dentin complex responses and accelerate deposition of hard-tissue. The severity of the damage, reflecting the extent of pulpal inflammation, influences the deposition of dentin along the periphery of the pulp space (31). A previous study has suggested that rupture of the blood vessels may cause pulpal ischaemia and degeneration of primary odontoblasts, thus triggering the underlining mesenchymal stem cells to form new odontoblast-like cells (32). However, three or more healing events with respect to the dentin-pulp reaction after tooth trauma have been presented. This includes dentin-like, bone-like, mixed tissue and/or fibrotic tissue healing (33, 34). The present study found dentine-like tissue with increased thickness in control replanted teeth compared with BMSC-CM treated and reference teeth. Evidence of calcified tissue in the central portion of the pulp chamber was found in one of the control replanted teeth at day 90. Central deposition of hard tissue may be produced by odontoblast-like cells originating from migrated and differentiated stem cells residing in the perivascular area. Based on the present results we are not able to conclude whether the dentin deposition in the replanted teeth is a result of surviving primary or newly differentiated secondary odontoblasts. It may be speculated that a reduced inflammation, as found for infrabony defects treated with BMSC-CM, may be responsible for reduced dentine thickness found in the present study.

Conclusions

Based on the current findings, BMSC-CM treatment was able to reduce the number of teeth with severe root resorptions and prevent excessive new dentin formation in the pulp chamber that was observed in untreated replanted maxillary immature teeth.

Acknowledgements

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