

Regeneration of dental pulp tissue in immature teeth with apical periodontitis using platelet-rich plasma and dental pulp cells

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Abstract

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Aim To investigate the new tissues growing into the pulp space of immature dog teeth that were infected, disinfected and filled with blood clot (BC), dental pulp cells (DPCs), platelet-rich plasma (PRP) or a combination of DPCs and PRP in immature dog teeth with apical periodontitis.

Methodology Fifty-six immature roots from mandibular premolars of four beagles were divided into four experimental groups ($n = 40$) and two control groups. After the induction of apical periodontitis, the root canals of experimental groups were disinfected with NaOCl irrigation and a tri-antibiotic paste medication. The canals were then filled with different materials according to the experimental group: BC group, DPCs group, PRP group or DPCs + PRP group. Access cavities were sealed with MTA and composite. Radiographs were taken after 90 days, and the jaws

including the teeth were processed for histologic analysis. The data were statistically analysed using chi-square evaluation and Student's *t*-test.

Results Radiographic analyses demonstrated no significant difference between experimental groups in periradicular bone healing ($P > 0.05$), whilst those groups that used DPCs produced a significantly greater root thickening ($P < 0.01$). The histologic evaluation showed that the groups with PRP formed more tissues in the canals ($P = 0.01$). The groups with DPCs had substantially more mineralized tissue formation in the canal than those without DPCs, especially in the apical third. In DPCs + PRP group, bone-like tissue grew into the canal space from the periapical tissue.

Conclusions A combination of DPCs + PRP increased vital tissue regeneration within the root canals of immature teeth associated with apical periodontitis.

Keywords: dental pulp cells, immature teeth, platelet-rich plasma, regenerative endodontics.

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Introduction

Pulp necrosis arrests further root development of an immature permanent tooth. Such a tooth has thin and weak canal walls and is susceptible to fracture. Traditional treatment for these teeth is long-term calcium hydroxide application to induce the formation of an apical hard tissue barrier, that is apexification.

However, because of its high pH, calcium hydroxide not only weakens the root but may also inhibit new tissue formation in the canal (Andreasen *et al.* 2002). Over the last decade, the possibility of vital tissue regeneration in the root canal space with continuous increase in root thickness and length has been demonstrated for immature teeth (Iwaya *et al.* 2001, Banchs & Trope 2004, Thibodeau *et al.* 2007, Ding *et al.* 2009, Torabinejad & Turman 2011, Yamauchi *et al.* 2011). Biologic tissues, such as blood clot, collagen and platelet-rich plasma (PRP), have been used to fill root canals after disinfection with an antibiotic paste which may provide a conducive environment for new tissue ingrowth into the canals (Iwaya *et al.* 2001, Banchs & Trope 2004, Thibodeau *et al.* 2007, Ding *et al.* 2009, Torabinejad & Turman 2011, Yamauchi *et al.* 2011).

There are three major components in tissue engineering: (i) cells that are capable of hard tissue formation, (ii) scaffolds that can support cell growth and differentiation and (iii) molecules that provide signaling for cell growth, proliferation and differentiation (Hargreaves *et al.* 2008). Although cells from dental pulp, apical papilla and possibly other tissues can form odontoblast-like cells under appropriate conditions, it is a clinical challenge to find a cell source that fulfils all the following criteria. That is, autogenous to avoid tissues rejection or introduction of foreign pathogens, easy to harvest and capable of differentiating into odontoblasts (Murray *et al.* 2007). Dental pulp cells (DPCs) have gained much attention as a promising population of cells for pulp/dentine regeneration, because they contain dental pulp stem cells (DPSCs), and they can be isolated readily from the freshly extracted teeth (Gronthos *et al.* 2000, Huang 2009). Dental pulp stem cells are able to differentiate into functional odontoblast-like cells with an active mineralization potential and may be used in dental tissue engineering via stem cell-based approaches (Dissanayaka *et al.* 2011).

Platelet-rich plasma has been suggested as a potentially ideal scaffold for regenerative endodontic treatment (Hargreaves *et al.* 2008, Torabinejad & Turman 2011). It is autologous and relatively easy to prepare in a dental clinic. It forms a three-dimensional fibrin network and resorbs overtime (Anitua *et al.* 2006). Platelet-rich plasma can enhance the recruitment, proliferation and differentiation of cells involved in bone regeneration and soft tissue healing (Sanchez-Gonzalez *et al.* 2012). Mesenchymal stem cells (MSCs) can migrate effectively into and through PRP. It has

been shown that the MSCs/PRP mixture induces bone formation (Yamada *et al.* 2004) and significantly improves periodontal bone regeneration (Simsek *et al.* 2012).

A literature search reveals an absence of reports on combined use of DPCs and PRP in regenerative endodontics. In this study, the pulp regeneration capacity using PRP, DPCs or the combination of both for treating immature teeth with apical periodontitis in a dog model was tested.

Materials and methods

Animals

Four male beagle dogs approximately 6 months old were selected from the Experimental Animal Center of the Peking University Health Science Center, Beijing, China. Care and handling of the animals were performed according to the guidelines of the Institutional Authority for Laboratory Animal Care, Peking University. The study was reviewed and approved by the Health Science Center, Peking University. Eight mandibular first premolars (eight root canals) were assigned as positive controls (no treatment was performed). The other 24 mandibular premolars (48 root canals) were randomly divided into 40 experimental root canals and eight negative control root canals (teeth were infected only). Radiographs were taken to confirm incomplete root formation and parallel root canal walls with open apices.

Cell culture of dental pulp cells

Unsorted autologous DPCs were isolated from freshly extracted incisors ($n = 4$) of each beagle as previously described (Dissanayaka *et al.* 2011). Tooth surfaces of the freshly extracted teeth were cleaned and cut at the cemento-enamel junction using a sterile fissure bur to expose pulp chambers. The pulp tissue was digested in a 3-mg mL⁻¹ collagenase type I (GIBCO-Invitrogen, Carlsbad, CA, USA) and 4 mg mL⁻¹ dispase (GIBCO-Invitrogen) solution for 1 h at 37 °C. The digested mixture was passed through a 70- μ m strainer (BD Falcon, Franklin Lakes, NJ, USA) to obtain single-cell suspensions that were then seeded in 75-cm² culture flasks containing α -minimum essential medium supplemented with 15% foetal bovine serum (FBS), L-ascorbic acid-2-phosphate, 100 U mL⁻¹ penicillin-G, 100 mg mL⁻¹ streptomycin, and 0.25 mg mL⁻¹ fungizone (Gemini Bio-Products, Woodland, CA, USA) and

cultured under 5% CO₂ at 37 °C. Medium was replaced every 3 days, and cells were subcultured at 70% confluence. Expression of mesenchymal stem cell markers was analysed using a FACS calibre flow cytometer and real-time PCR. Dental pulp cells expressed mesenchymal stem cell markers STRO-1 and CD146. However, they were negative (<2%) for CD73, CD105 and CD45 as described previously (Dissanayaka *et al.* 2011). Multilineage differentiation potential into osteo/odontogenic, adipogenic and neurogenic lineages were also confirmed (Dissanayaka *et al.* 2011). Cell passages at 3–4 were used. Dental pulp cells were harvested, centrifuged and washed three times with physiological saline before transplanting into the root canals.

Preparation of activated platelet-rich plasma

Platelet-rich plasma was prepared as previously described with minor modifications (Nikolidakis & Jansen 2008). Approximately 20 mL blood was drawn from each dog into a centrifuge tube containing 3 mL citrate solution. Collected blood was centrifuged in a standard laboratory centrifuge for 10 min at 200 g to obtain PRP without erythrocytes and leucocytes. A second centrifugation was performed for 15 min at 360 g, and the PRP was taken, whereas the platelet-poor plasma was removed. Platelets in whole blood and PRP were counted with an automatic haematology analyser to make sure that platelet concentration was more than $1\,200 \times 10^9 \text{ L}^{-1}$. Bovine thrombin (Sigma, St. Louis, MO, USA) was combined with 10% calcium chloride in a proportion of 1 000 units thrombin per 1 mL CaCl₂. Release of platelet products into the supernatant was induced by adding activated-thrombin into PRP samples at a final concentration of 100 IU mL⁻¹.

Tooth preparation

The animals were anaesthetized during all experimental procedures. Anaesthesia was obtained by intravenous administration of 2.5% Pentothal (13.5 mg kg⁻¹) and maintained under 2% Isoflurane. The animals also received local anaesthetics (4% articaine with 1 : 100 000 epinephrine, 0.5 mL per quadrant) to provide regional nerve block anaesthesia. The pulp chambers of all teeth from the experimental groups and negative control were accessed with a size two round carbide bur in a high-speed handpiece with irrigation, and the pulp tissue was disrupted by an endodontic file. Supragingival plaque removed from the dog's teeth was

placed and sealed temporarily in the pulp chamber with Cavit (ESPE, 3M, Seefeld, Germany). At 4 weeks, all teeth were radiographed to confirm the development of apical periodontitis. All teeth in the experimental groups were re-entered, irrigated with 10 mL 1.25% sodium hypochlorite (NaOCl) and disinfected with a triple antibiotic paste consisting of metronidazole, minocycline and ciprofloxacin at concentration of 10 mg mL⁻¹ each. After 2 weeks, the antibiotic mixture was irrigated out from each canal with 10 mL of 1.25% NaOCl and sterile saline.

Experimental groups ($n = 10$ for each group) were as follows: BC group: bleeding into canals was evoked by overinstrumentation through the apex gently with a size 30 K-file to irritate the apical tissue leading to formation of a blood clot.

Dental pulp cells group: autologous transplantation of 1×10^6 DPCs into each canal mixed with blood induced from apex. The harvested DPCs (5×10^6 approximately) were in a pellet form after centrifuge. Twenty-five microlitres of vein blood was used to suspend the cell pellet. Five microlitres of the blood with DPCs was injected into each root canal with a syringe.

Platelet-rich plasma group: Fifty microlitres of PRP was injected into the root canals immediately after being activated.

DPCs + PRP group: 5×10^6 DPCs pellet was suspended in 25 µL of PRP and 5µL of the mixture was injected into the root canals immediately after being activated.

The coronal portions of the root canals were double-sealed by white MTA (Dentsply Tulsa Dental, Johnson City, TN, USA) and composite (P60; 3M Dental Products, St Paul, MN, USA). The canals of positive controls ($n = 8$) were left untouched to develop naturally for comparison with the experimental teeth. The canals of negative controls ($n = 8$) were infected and sealed without further disinfection.

Dental pulp cells were isolated and cultured for 2 weeks before being transplanted back to their donor, as described above. Platelet-rich plasma was prepared just before the transplantation.

Radiographic analysis

After 90 days, radiographs were taken for all teeth involved. Each individual root was taken as a unit for assessment. Radiographic healing, which included no radiolucency or decreased periapical lesion and root canal wall thickening based on the comparison of pre- and postoperative radiographs, was evaluated by

two blinded evaluators. The data were analysed using chi-square tests, with the level of significance set at $P < 0.05$.

Histologic evaluation

The animals were sacrificed under general anaesthesia (Pentobarbital; Butler Company, Columbus, OH, USA) at 30 mg kg^{-1} intravenously. The jaws with the involved teeth were resected and placed in 4% paraformaldehyde for 24 h and decalcification in 10% EDTA for 6 months. The specimens were processed, embedded, longitudinally sectioned along the long axis of the root and stained by haematoxylin and eosin. Each sample was analysed under a light microscope for the presence or absence of vital tissues in the canal space. The percentage of new vital tissue formation was calculated as $V(\text{vital tissues area})/T(\text{total canal area}) \times 100\%$ for every longitudinal section which contained the root apex. Images were imported in Jpg format to the Digimizer 4.2.0.0 (Medcalc Software bvba, Ostend, Belgium), and the V area and T area were drawn with the area selecting tool of the software. The number of pixels was obtained automatically. Statistical analysis was performed using Student's *t*-test (independent-sample test) with $P < 0.05$ considered significant.

Results

Teeth in the experimental groups had no sign of swelling, mobility or sinus tracts, and all restorations were intact. Two teeth were lost in the negative control group because of abscess and excessive mobility, and the others had apical periodontitis. All teeth from positive control group developed naturally. Mature apex and normal pulp tissue were observed using radiographic and histologic analyses.

Radiographic analyses

The results demonstrated that the periradicular healing rates of the experimental groups – BC, DPCs, PRP and DPCs + PRP – were 90%, 80%, 100% and 90%, respectively (Table 1). Chi-square evaluation showed no significant difference between the experimental groups ($P > 0.05$).

After disinfection and treatment, the numbers of specimens with root thickening in BC, DPCs, PRP and DPCs+PRP groups were 6, 10, 3 and 9,

Table 1 Radiographic results

	PH (n)	RWT (n)
Positive group (n = 8)	–	8
Negative group (n = 8)	0	0
BC (n = 10)	9 (90%)	6
DPCs (n = 10)	8 (80%)	10*
PRP (n = 10)	10 (100%)	3
DPCs + PRP (n = 10)	9 (90%)	9*
Statistic analysis(chi-square)	$P > 0.05$	$P < 0.01$

*Significant statistics difference.

BC, blood clot; DPCs, dental pulp cells; PRP, platelet-rich plasma; DPCs+PRP, dental pulp cells combined with platelet-rich plasma; PH, Periradicular healing; RWT, Root wall thickening.

respectively (Table 1). Chi-square evaluation revealed a significant difference between the groups with DPCs and the other two groups ($P < 0.01$). No increased root thickness was observed in the negative control group.

Histologic analyses

The negative control teeth showed no signs of newly formed tissues, and the positive control teeth showed normal root development and maturation. Newly formed tissues were found in all experimental root canals.

Regenerated vital tissue in the root canal space

The histologic evaluation revealed that the regenerated vital tissues in the canal space consisted of new hard tissues and blood vessel in a matrix of fibrous connective tissue. The new tissues extended to the coronal third in 12 canals (Fig. 1) and to the middle third in 13 canals. The average percentages of vital tissue area in terms of the total canal area were 34%, 48%, 75%, and 70%, respectively, in BC, DPCs, PRP and DPCs + PRP groups (Table 2). There was a significant difference between the groups in that the use of PRP formed more fibrous connective tissue with blood vessels, which was considered as soft tissues, in the canals ($P = 0.01$). In the PRP group, all canals were filled with well-structured connective tissue. This connective tissue ingrowth from the periapical tissue was densely infiltrated with young fibroblasts and fibrocytes and well vascularized. Of all the canals in PRP and DPCs + PRP groups, 7 had the tissue reaching the coronal third (Fig. 1a-4,b-4), with three reaching the middle third.

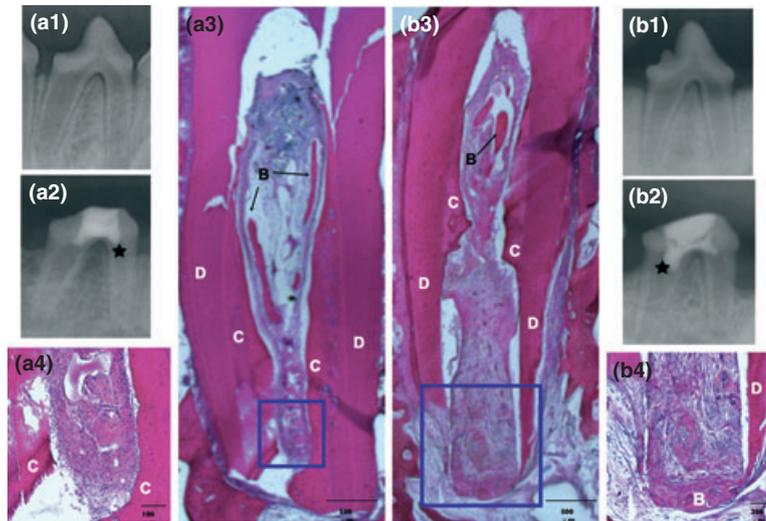


Figure 1 Typical radiographic and histologic images of tooth root in Dental pulp cells + platelet-rich plasma group (a) and platelet-rich plasma group (b). (a-1) Preoperative (a-2), postoperative radiographs (90 days follow-up). (a-3) Haematoxylin–eosin stain shows new tissues extending to the cervical third, and the apex has developed. D, dentine; C, cementum-like tissue; B, bone-like tissue. Cementum-like tissue is present on the inner dentine wall. Bone-like tissue is formed within the connective tissue. (a-4) Magnified view from the low boxed region in a-3. (b-1) Preoperative (b-2), postoperative radiographs (90 days follow-up). (b-3) Haematoxylin–eosin stain shows well-structured connective tissue grew into the root canal from the wide apical opening. (b-4) Magnified view from the boxed regions in b-3. ★: Studied roots.

Table 2 Histologic results

	CT (n)	BT (n)	The percentage of VT area		The percentage of ST area	
			Mean	SD	Mean	SD
Positive group (n = 8)	0	0	0	0	0	0
Negative group (n = 8)	0	0	0	0	0	0
BC (n = 10)	5	5	34	15	15	4
DPCs (n = 10)	10	6	48	30	17	8
PRP (n = 10)	6	6	75*	16	70*	16
DPCs + PRP (n = 10)	10	7	70*	21	21	8
Statistic analysis	$P > 0.05$ (chi-square)	$P > 0.05$ (chi-square)	$P = 0.01$ (t-test)		$P = 0.01$ (t-test)	

CT, Cementum-like tissue formation; BT, Bone-like tissue formation; VT, vital tissue; ST, soft tissue; BC, blood clot; DPCs, dental pulp cells; PRP, platelet-rich plasma.

*Significant statistics difference.

Cementum-like tissue formed on canal walls

An avascular mineralized tissue (cementum-like tissue) covering the internal root canal walls was observed in all root canals (100%) of DPCs and DPCs + PRP groups, 50% in the BC group and 60% in the PRP group (Table 2). The tissue grew from the root surface via the apical region into the canal walls with various thicknesses and was devoid of blood vessels within the tissue. It also extended apically increasing the root length (Figs 1a-4 and 2a-4).

There were relatively few cells embedded in the mineralized matrix. The cementum-like tissue was not different in structure amongst the four groups, but the groups with DPCs had more mineralized tissue formation than those without DPCs, especially in the apical third ($P < 0.01$) (Figs 1 and 2).

Bone-like tissue formed in the canal space

There was histologic evidence using H&E staining of islets of bone-like matrix with strong eosinophilic

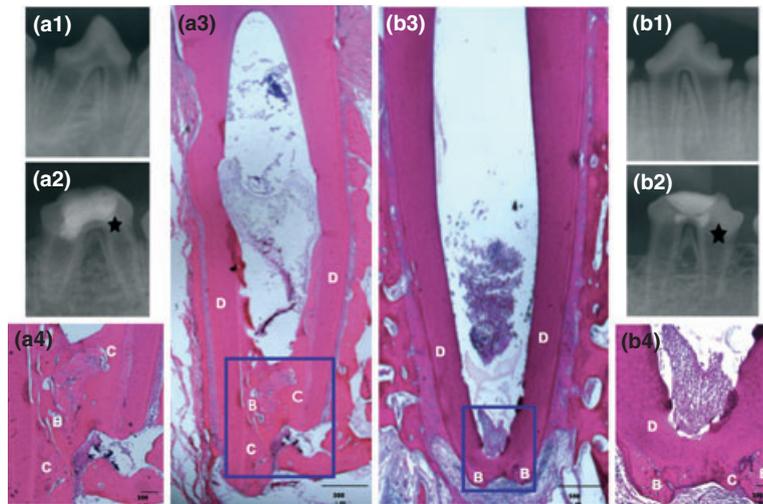


Figure 2 Typical radiographic and histologic images of tooth root in dental pulp cells group (a) and blood clot group (b). (a-1 & b-1) Preoperative (a-2 & b-2), postoperative radiographs (90 days follow-up). Dental pulp cells group had much more mineralized tissue formation in apical third. (a-4 & b-4) Magnified views from the boxed regions in a-3 and b-3. ★: Studied roots.

staining, which were considered as bone-like tissue. Some cells were also embedded in the matrix. Twenty-four of 40 root canals had bone-like structures inside the newly formed tissues. In each group – BC, DPCs, PRP and DPCs + PRP groups – there were 50%, 60%, 60% and 70% bone-like tissue in the canal space, respectively (Table 2). These structures appeared mostly as mineralized islands that had many embedded cells and blood vessels. In the DPCs + PRP group, some bone-like tissue grew into the canals from the apical region and formed a bridge (Fig. 3a) or merged with cementum-like tissue that occluded the apex (Fig. 3b,c).

Discussion

The present study demonstrated newly formed tissues in all experimental root canals. The histologic

characteristics of regenerated tissues were cementum-like tissue grown along the canal dentinal wall and bone-like tissue scattered in the canal space that was filled with soft connective tissue. No dentine-like tissue was observed in the experimental samples. These results are similar to other animal studies on pulp regeneration using blood clot, a type I collagen solution or a cross-linked collagen scaffold to fill root canals (da Silva *et al.* 2010, Wang *et al.* 2010, Yamauchi *et al.* 2011).

Dental pulp stem cells were able to differentiate into odontoblast-like cells as shown by increased alkaline phosphatase activity, dentine sialoprotein expression and formation of mineralized nodules (Dissanayaka *et al.* 2011). Addition of DPSCs and growth factors to the tooth chamber improves tissue and vessel formation (Srisuwan *et al.* 2012). In the present study, there was more mineralized hard tissue formation in

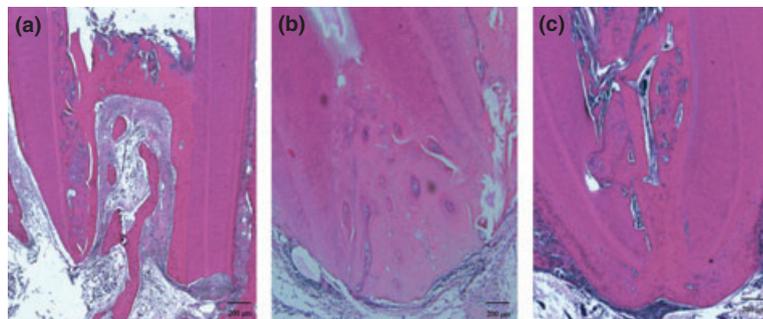


Figure 3 Different apical closure types in group dental pulp cells + platelet-rich plasma. (a) Some bone-like tissue grew into the canals from apex to form a bridge. (b) and (c) bone-like tissue merged with cementum-like tissue to close the apex.

the groups containing DPCs, especially in the apical third. The results provide evidence that implanted DPCs might promote mineralized tissue formation in root canals of immature teeth. However, there was a lack of dentine formation on canal dentinal walls, which may be due to several reasons: (i) the osteogenic potential of DPSCs. Evidence suggests that human DPSCs differentiate into osteoblasts and endotheliocytes and produce bone tissue along the implant surfaces (Mangano *et al.* 2010). (ii) Osteogenic/cementogenic changes of dental pulp cells: It has been reported that osteogenic/cementogenic, not dentinogenic, markers were expressed in the newly formed matrix in pulp tissue after being removed from the rat tooth and transplanted into subcutaneous space of rats (Hosoya *et al.* 2007). It is possible that DPSCs were transduced into osteo/cementogenic cells under the experimental design. This may explain in part why transplanted DPCs in this study did not regenerate dentine but formed cementum- and bone-like tissues in the canal. Further *in vitro* experiments are needed to verify this speculation. (iii) The migration of pre-osteoblasts and pre-cementoblasts into the canal space. Because haemorrhage was induced at the periapex and allowed a blood clot to form, this may have brought along these cells into the canals (Shimizu *et al.* 2000). Periodontal studies show cells may proliferate and migrate from adjacent undamaged PDL into the wounded area (King *et al.* 1997, King & Hughes 2001). This suggests stem cells present within the PDL and alveolar bone marrow might be able to be stimulated at a distance and migrate towards the immature tooth apex (Friedlander *et al.* 2009). (iv) Loss of transplanted DPCs. Although unlikely, there was a possibility that the transplanted cells migrated to other tissues. Future work by labelling the transplanted cells may help in following their migration to the host tissues.

Platelet-rich plasma is a natural reservoir of various growth factors that can be collected autologously, unlike the chemically processed molecules or recombinant proteins that may cause undesired side effects and unnecessary risks (Sanchez-Gonzalez *et al.* 2012). PRP should have a minimum of five times the number of platelets compared with whole blood as is considered 'platelet rich' (Yamada *et al.* 2004). It contains growth factors, stimulates collagen production, recruits other cells to the site of injury, produces anti-inflammatory agents, initiates vascular ingrowth, induces cell differentiation and controls the local inflammatory response (Hiremath *et al.* 2008). The use of PRP for enhancing

bone regeneration and soft tissue maturation has increased dramatically in the fields of orthopaedics, periodontics, maxillofacial surgery, urology and plastic surgery over the last decade (Frechette *et al.* 2005). However, controversies exist in the literature regarding the added benefit of this procedure. Whilst some publications have reported positive results in either bone or soft tissue healing (Marx *et al.* 1998), others did not observe any improvement when PRP was used alone or added to the grafts for bone regeneration (Froum *et al.* 2002, Choi *et al.* 2004, Sanchez-Gonzalez *et al.* 2012, Simsek *et al.* 2012). Platelet-rich plasma has been suggested as a scaffold for regenerative endodontic procedure. Torabinejad & Turman (2011) demonstrated in a case report that PRP is potentially an ideal scaffold for this procedure, and the advantages of using PRP include its relative ease of application and shorter time to induce vital tissues within the root canal system.

The radiographic and histologic results of the present experiments, however, showed that the rate of root canal wall thickening was lower and mineralized tissue formation was less when PRP was used alone. There was more soft connective tissue formed in the canal space in the PRP group than in other groups. This finding is in accordance with a previous dog study reporting that filling a mandibular bone defect with PRP alone does not allow osteogenesis to occur in the affected area (Yamada *et al.* 2004). This is likely due to a faster degradation of growth factors in PRP. Platelet degranulation and release of growth factors occur within 3–5 days, and the growth factor activity may end in as early as 7–10 days (Froum *et al.* 2002). Some authors suggest a sustained release form of PRP to be used, so that the effects of PRP can be optimized (Froum *et al.* 2002, Choi *et al.* 2004).

Previous work has demonstrated that MSCs alone can be used in repairing bone (Maxson *et al.* 2012). However, a large volume of MSCs is needed if they are used alone, and it is difficult to maintain MSCs to stay in the bone defect. The tissue-engineered bone using the combination of MSCs and PRP performed better, suggesting a positive influence of PRP on the MSCs (Froum *et al.* 2002). There is a positive dose–response relationship between platelet concentration and proliferation of human mesenchymal stem cells (hMSCs) (Liu *et al.* 2002). The PRP scaffold induces MSCs adhesion, proliferation and differentiation to elicit bone formation, and the implanted scaffold becomes vascularized (Froum *et al.* 2002). The MSCs/PRP group vascularized well and could

elicit true bone formation (Yamada *et al.* 2004). DPSCs are a type of MSCs and can be used in dental tissue engineering via stem cell-based approaches. Platelet-rich plasma in 0.5 and 1% can enhance proliferation and differentiation of human dental stem cells to form mineralized tissues (Lee *et al.* 2011). In the present study, PRP mixed with DPCs appeared to facilitate more tissue formation including hard tissue and connective tissue than using DPCs alone. Therefore, in this study system, the use of PRP in combination of DPCs may be beneficial for new tissue formation. However, further investigation is needed to understand the reason why there was a lack of dentine-like tissue formation on the root canal dentinal walls.

Conclusions

A combination of DPCs + PRP may increase vital tissue regeneration of immature teeth with apical periodontitis.

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The authors deny any conflicts of interest related to this study.

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