Transplantation of Dental Pulp Stem Cells and Platelet-rich Plasma for Pulp Regeneration

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Abstract

Introduction: The loss of dental pulp may weaken teeth, rendering them susceptible to reinfection, fracture, and subsequent tooth loss. Therefore, regeneration of pulp is considered an ideal treatment to preserve teeth. The aim of this study was to explore the capacity of dental pulp stem cells (DPSCs) and platelet-rich plasma (PRP) to regenerate dental pulp in canine mature permanent teeth. **Methods:** Pulpectomy with apical foramen enlarged to a #80 file was performed in 16 upper premolars of 4 beagle dogs. Four experimental groups were randomly established: (1) the blood clot group, (2) the autologous DPSCs group, (3) the PRP group, and (4) the DP + PRP group (a mixture of DPSCs and PRP). Four lower premolars without any further treatment after pulpectomy were used as the control group. All teeth were sealed with mineral trioxide aggregate and composite. Twelve weeks after transplantation, the teeth were subjected to radiographic and histologic examination. **Results:** Twenty-four of 32 experimental root canals gained newly formed tissues. All canals with an introduction of a blood clot showed histologic evidence of vital tissue formation. Cementum-like and periodontal ligament–like tissues along the internal root canal walls were typical structures in most cases. There is no significant difference between groups with or without autologous DPSC transplantation (exact chi-square test, $P < .05$). **Conclusions:** New vital tissues can be regenerated in permanent canine teeth after pulpectomy and enlargement of the apical foramen. Histologically, transplantation of DPSCs and/or PRP into root canals showed no enhancement in new tissue formation compared with inducement of a blood clot into the root canals alone. *(J Endod 2012;38:1604–1609)*

Key Words

Dental pulp stem cells, permanent teeth, platelet-rich plasma, pulp regeneration, vital tissue growth

The emergence of regenerative medicine has prompted the interest in endodontics to test the possibility of dental pulp and dentin regeneration. It was considered that pulp/dentin regeneration is preferable to pulpectomy/gutta-percha obturation from the perspective of preserving tooth longevity (1, 2). Two approaches have been undertaken to test pulp/dentin regeneration. One is the revitalization approach (revascularization) to treat endodontically involved immature permanent teeth. Multiple clinical case reports have shown a certain level of success after revitalization treatment in terms of the absence of clinical symptoms, the healing of periapical lesions, and the increase of root thickness and length based on radiographic evidence (3, 4). Animal studies have provided both radiographic and histologic evidence of tissue generated in canals after the revitalization of immature permanent teeth with experimentally induced apical periodontitis (5, 6). It was found that there was no regeneration of pulp/dentin in the canals; instead, they were filled with periodontal tissues including cementum, periodontal ligament, and bone (6).

The other approach to regenerate pulp/dentin is to introduce exogenous stem cells into the canals. In an ectopic regeneration study model, human dental pulp/dentin-like tissues were formed in the emptied canals of human tooth fragments using a heterogeneous population of stem cells from apical papilla (SCAPs) or dental pulp stem cells (DPSCs) (7). Although heterologous DPSCs showed a relatively low immunologic response in animal studies, autologous DPSC transplantation is obviously less risky. Complete pulp regeneration also has been shown orthotopically in a large animal model by transplanting pulp $CD105^+$ cells with stromal cell–derived factor-1 into mature teeth with the apex enlarged only $\sim$0.7 mm (8).

The immature tooth has an open apex, large canal, and a short root. The new tissue can easily grow into the root canal space, reaching the coronal pulp chamber (9). A permanent tooth with a mature apex may have a limited blood supply to allow tissue ingrowth into the canal (10). Reimplantation of avulsed teeth with an apical opening of $\sim$1.0 mm in diameter showed a greater likelihood of revascularization (11, 12), which indicates that regeneration of dental pulp in teeth with mature apices might be achievable by enlarging the apex to 1–2 mm in diameter.

A recent clinical case report showed that revitalization of an endodontically compromised immature tooth by using platelet-rich plasma (PRP) may provide a desirable outcome (13). PRP contains many growth factors including platelet-derived growth factor, transforming growth factor β, and insulin-like growth factor (14) and may be a good supplement for cell-based pulp/dentin regeneration. PRP may be derived from patient's own blood, is easy to prepare, and is capable of forming a 3-dimensional fibrin matrix (that acts as a scaffold) (15–17). An *in vitro* study showed that PRP can

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enhance the proliferation and differentiation of human DPSCs (18). In this study, we aimed to investigate the potential synergistic effects of autologous DPSCs and PRP for *de novo* dental pulp regeneration in the root canals after pulpectomy in a dog study model.

Materials and Methods

Animals

Four beagles approximately 1 year old were obtained from the Experimental Animal Center of the Peking University Health Science Center, Beijing, China. Animal care and handling followed the guidelines of the Institutional Authority for Laboratory Animal Care, Peking University. This study was reviewed and approved by the Health Science Center, Peking University.

Isolation, Culturing, and Identification of Canine Dental Pulp Stem Cells

Autologous canine DPSCs (cDPSCs) were isolated from freshly extracted incisors (n = 4) of each beagle as previously described (19). Briefly, tooth surfaces of the freshly extracted teeth were cleaned and cut at the cementoenamel junction using a sterile fissure bur to reveal the pulp chamber. After separating the pulp tissue gently from the crown and root, it was digested in a 3-mg/mL collagenase type I (GIBCO-Invitrogen, Carlsbad, CA) and 4- mg/mL dispase (GIBCO-Invitrogen) solution for 1 hour at 37°C. Then, the cells were passed through a 70-μm strainer (BD Falcon, Franklin Lakes, NJ) to obtain single-cell suspensions. These cells were seeded in 75-cm^2 culture flasks containing α–minimum essential medium supplemented with 15% fetal bovine serum, L-ascorbic acid-2- phosphate, 100 U/mL penicillin-G, 100 mg/mL streptomycin, and 0.25 mg/mL Fungizone (Gemini Bio-Products, Woodland, CA) and cultured under 5% CO_2 at 37°C. Medium was replaced every 3 days, and cells were subcultured at 70% confluence. The expression of mesenchymal stem cell markers was analyzed on a fluorescence activated cell sorter caliber flow cytometer. cDPSCs were STRO-1(+), CD146(+), CD73(−), CD105(−), and CD45(−) as previously described (19). Multilineage differentiation potential into osteo/odontogenic, adipogenic, and neurogenic lineages was confirmed (data not shown). cDPSCs at passages 3 to 4 were harvested, centrifuged, and washed 3 times with physiological saline before transplanting into the root canals.

Preparation of Activated PRP

PRP was procured using a previously described method (20) with minor modification. Approximately 20 mL blood was drawn from each dog into a centrifuge tube containing 3 mL citrate solution. Collected blood was centrifuged for 10 minutes at 200g to obtain PRP without erythrocytes and leukocytes. A second centrifugation was performed for 15 minutes at 360g. PRP was taken, and the platelet-poor plasma was removed (18). Platelets in whole blood and PRP were counted with an automatic hematology analyzer to make sure that platelet concentration was more than $1{,}200 \times 10^9$/L. Bovine thrombin (Sigma, St Louis, MO) was combined with 10% calcium chloride in a proportion of 1,000 U thrombin/1 mL $CaCl_2$. The release of platelet products into the supernatant was induced by adding 200 IU activated thrombin into each PRP sample.

In Vivo Transplantation Study

Root Canal Preparation. The protocol of whole pulp removal from permanent premolars was established in beagle dogs as described previously (8). Briefly, under general anesthesia (ie, induction by pentothal 13.5 mg/kg intravenously and intubation and maintenance with isoflurane) supplemented with local anesthesia (ie, 4% articaine with 1:100,000 epinephrine), the pulp was mechanically exposed with a #2 round carbide bur in a high-speed handpiece and taken out by a barbed broach. A sterile #15 K-file (Dentsply Maillefer, Johnson City, TN) was used to negotiate to the apex as determined by preoperative radiographs. Then, the root canals were prepared followed by the enlargement of the apical foramen to 0.8 mm in diameter using a #80 K-file with 5.25% sodium hypochloride (NaOCl) and 17% EDTA irrigation. Each prepared canal received a final rinse with 5 mL physiological saline and was dried with sterile paper points before the subsequent procedures described later.

Experimental and Control Groups. Sixteen double-rooted upper premolar teeth (second and third upper premolar) in 4 beagles were divided into 4 experimental groups, and 4 lower premolars (1 from each dog) were used as the control group. Two canals in the same tooth were used for the same experimental procedures. The sample size (n) is referred to as the number of root canals.

For the blood clot (BC) group (n = 8 root canals), bleeding into canals was evoked by overinstrumentation into apical tissues with a #80 K-file allowing the formation of a blood clot. For the cDPSC (DP) group (n = 8 root canals), autologous cDPSCs (1×10^6 cells) were suspended in 20 μL vein blood and syringed into each root canal, whereas for the PRP group (n = 8 root canals), 20 μL PRP was injected into each root canal immediately after being activated. Finally, for the cDPSCs plus PRP (DP + PRP) group (n = 8 root canals), cDPSCs (1×10^6 cells) and 20 μL activated PRP were mixed and injected into each root canal. A patency check to the apex was performed with a sterile #15 K-file after transplantation in DP, PRP, and DP + PRP groups to make sure the root canal and the material transplanted connected with periapical tissue directly. For the control group, 4 lower premolars (1 from each dog) received the endodontic procedure, and the canals were left empty. The coronal portions of the root canals were double sealed by white mineral trioxide aggregate (MTA) (Dentsply Tulsa Dental, Johnson City, TN) and composite (P60; 3M Dental Products, St Paul, MN).

Preoperative and 3-month postoperative radiographs were taken. The presence or absence of apical radiolucency was evaluated by 2 independent examiners. The animals were sacrificed under general anesthesia (pentobarbital; Butler Company, Columbus, OH) at 30 mg/kg intravenously. The jaws with the involved teeth were resected and fixed for histologic processing.

Histologic Analysis

After fixation in 4% paraformaldehyde for 24 hours and decalcification in 10% EDTA for 6 months, the specimens were embedded in paraffin wax, sectioned longitudinally along the long axis of the teeth, and stained with hematoxylin-eosin. Each individual root was analyzed as an independent sample unit histologically under a light microscope for the presence or absence of regenerated pulp tissue and the structure of the generated vital tissues.

Data Analysis

The categoric data (whether regenerated tissue was present or not) were analyzed with exact chi-square tests, with the level of

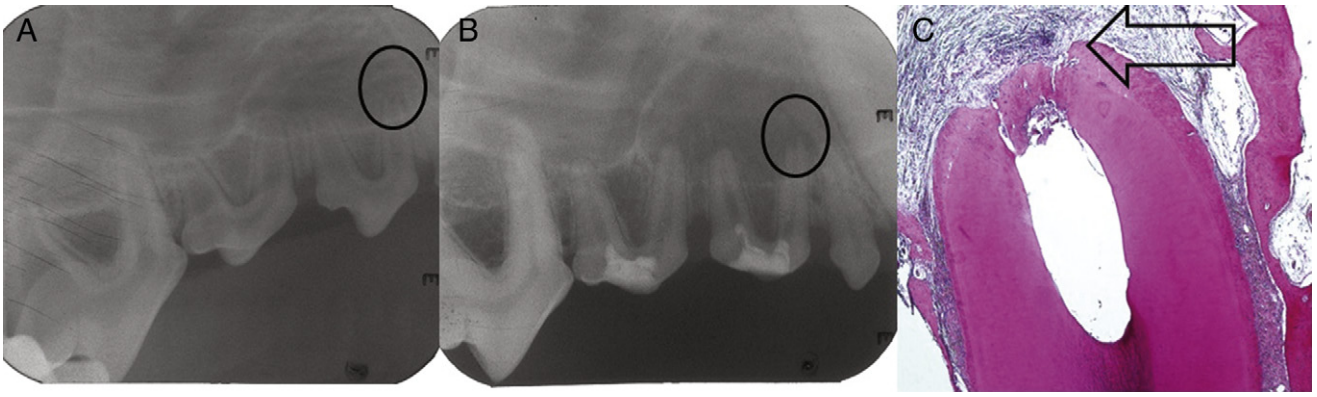

Figure 1. (*A*) A preoperative radiograph. (*B*) Twelve weeks after the regenerative endodontic procedure, periapical radiolucency was detected from the second upper premolar (*circle*). (*C*) Histologic examination showed the inflammatory cell infiltration and bone loss around the apex (*arrow*).

significance set at $P < .05$, to determine if there were any significant differences between the experimental groups with respect to the histologic criteria.

Results

Clinical and Radiologic Findings

All 16 experimental teeth showed no signs of mobility swelling or sinus tracts. All fillings were intact. Periapical radiolucency was detected in 8 teeth (2 teeth in the DP group, 1 tooth from the PRP and DP + PRP groups, and 4 teeth in the control group) and was confirmed as periapical periodontitis by histologic examination (Fig. 1*A–C*).

Histologic Findings of Vital Tissues in the Canal Space

Newly formed vital tissues were found in the canal space in 24 of 32 roots in the experimental groups. The data in Figure 2 present the number of root canals with newly generated tissue in different groups. Two roots (from the DP and DP + PRP groups) were excluded because of cracking during the histologic slice procedure. No tissue or only some inflammatory cells in apical portion were found in 6 roots (from 4 teeth), and these samples were sorted as necrotic (Fig. 2). Periapical periodontitis was also detected in these 6 roots. In the BC group, new vital tissue was present in all 8 roots. The absence of new vital tissue was found in 3 root canals in the DP group. The PRP and DP + PRP groups had 2 and 1 canals without new vital tissue, respectively. All 6 canals that had no new vital tissue inside presented inflammatory cell infiltration in the periapical area, which correlates with radiologic findings. The categoric data on the formation of vital tissues in the canal space from each group were 8 out of 8 teeth in the BC group, 4 out of 7 teeth in the DP group, 6 out of 8 teeth in the PRP group, and 6 out of 7 teeth in the DP + PRP group. There was no significant difference between groups (exact chi-square test, $P < .05$). Hard-tissue deposition along internal root canal walls was detected in 23 of 24 that had vital tissues generated. Twenty of these 24 root canals showed a bone-like structure inside the newly formed tissues.

Histologic Characteristics of Vital Tissues in the Root Canal Space

Eighteen cases of the newly generated tissues extended to the surface of the MTA and 6 cases to the midlevel of the root canals (Figs. 2 and 3*A* and *B*). In the coronal part in which tissues were in direct contact with MTA or in close promixity, osteoid hard tissue was observed. Some particles embedded in the newly formed osteoid tissues may have been the MTA; cells in the middle portion were stellate-like (processes radiating from the cell body giving them a spindle or stellate shape), whereas those in the apical third were more immature with larger and heavily stained nuclei (Fig. 4*B–D*). Among all specimens with vital tissues in the canals, no dentin-like tissue was detected, whereas cementum-like tissue and periodontal ligament–like tissue were observed along the dentinal wall (Fig. 4*E–G*).

Cementum-like Tissue Deposition along Internal Root Canal Walls

An aavascular mineralized tissue (cementum-like tissue) covering along the internal root canal walls was observed in nearly all root canals with vital tissues (except 1 canal from the PRP group). The cementum-like tissue showed no difference in structure among the 4 groups. Continuation of the cementum from the outer root surface into the inner canal surface was observed in most cases (Fig. 3*C* and *D*). The thickness of the cementum-like tissue decreased from the apical to the coronal area (Fig. 3*E* and *F*). Cementocyte-like cells were found embedded in this tissue (Fig. 3*J*), and cementoblast-like cells were seen on the surface of the cementum-like tissue (Fig. 3*G* and *I*). Immediately adjacent to the cementoblast-like layer in the canal, a layer of soft tissue resembling the periodontal ligament

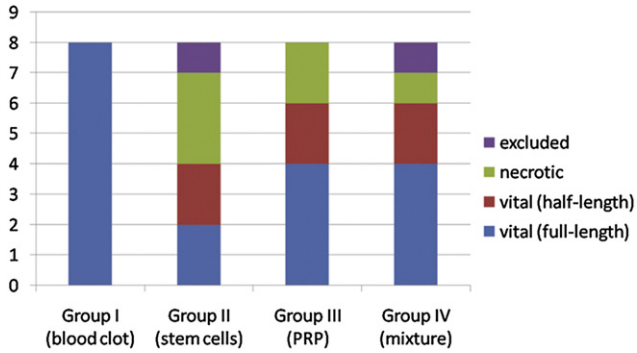

Figure 2. Twenty-four of 32 experimental roots got newly formed vital tissues. Two roots cracked during the histologic sectioning procedure and were excluded from the study. Periodontitis was detected in 6 roots. No significant difference on the rate of vital tissue formation in the canals can be found between groups (exact chi-square test, $P < .05$).

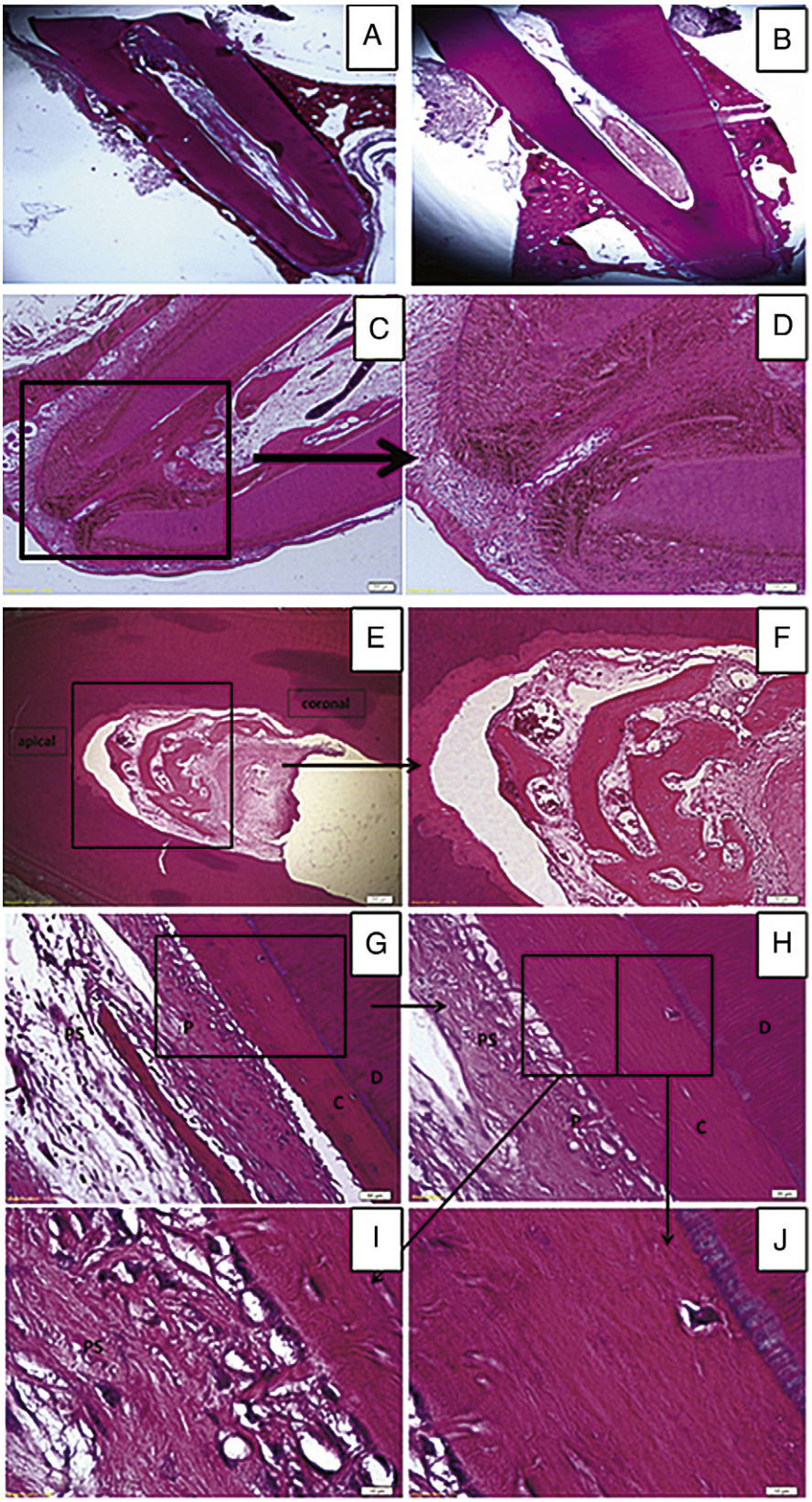

Figure 3. (*A*) A full-length regenerated case (from the DP + PRP group); the tissue has grown above the cervical level up to the pulp horns. (*B*) A half-length regenerated case (from the DP + PRP group); the tissue growth has stopped at the midlevel of the root. Some inflammatory cells can be seen above the tissue. (*C* and *D*) Ingrowth of cementum-like tissue from the outer root surface into the inner canal surface. (*E* and *F*) The thickness of cementum-like tissue has reduced from the apical to the coronal direction. Bone-like structure was shown in the section. (*G*) Cementum-like tissue deposition along the internal root canal walls. (*H*) A magnified view shows the combination of dentin (D), cementum-like tissue (C), and periodontal ligament–like tissue (P). PS, pulp space. (*I*) Cementoblast-like cells lie along cementum-like tissue, and some fibers from the PDL-like tissue were inserted into the cementum-like tissue, resembling Sharpey's fibers. (*J*) An embedded cementocyte-like cell (*arrow*).

was observed in 18 samples (Fig. 3*H*). Sharpey's fibers were found inserting from the PDL-like tissue into the cementum-like tissue (Figs. 3*I* and 4*G*).

Bone-like Tissue Formation in the Newly Regenerated Tissue

There was histologic evidence of islets of bone-like matrix with strong eosinophilic staining in hematoxylin-eosin–stained sections. Some cells were also seen embedded in the matrix. Twenty experimental roots showed bone-like tissue in the root canal space (2 roots from the PRP group and 2 roots from the DP + PRP group showed no evidence on bone-like structure). These structures were commonly present in the coronal part of the root canal, especially in the zone close to or directly connected with MTA (Figs. 3*E* and *F* and 4*A* and *D*). In some cases, bone-like tissue resembled cementum-like tissue without apparent bone marrow space.

Discussion

Several revitalization studies in humans and animals have shown that after disinfection and evoking blood into root canals, the root thickening and lengthening could be achieved (4, 5, 13, 21). However, it was shown that the newly grown tissues into the root canal space have little similarity to normal pulp tissue but with more resemblance to cementum, periodontal ligament, or bone. The cause of this outcome is possibly related to the lack of stem cells derived from remaining vital pulp and apical papilla, which are destroyed by severe endodontic infection. Stem cells responsible for newly regenerated tissues might be derived from several other sources, including systemic blood, local tissue such as bone, and the periodontal ligament (22). Furthermore, whether these newly formed tissues can function like normal pulp and stabilize the tooth without giving rise to further infection or canal obliteration still remains unclear (23). Therefore, more research is needed before the regenerative procedures can be routinely performed with a predictable long-term prognosis.

The triad of cells, growth factors, and scaffolds is necessary for appropriate tissue regeneration. It has been hypothesized that after disinfection stem cells from the remaining vital pulp or apical papilla may reconstitute the lost structure of pulp/dentin complex (6). However, endogenous DPSCs and SCAP might not be able to survive from severe endodontic infection. In the present study, we attempted to deliver DPSCs into the canal space filled with blood or DPSCs mixed with PRP and compared these approaches with a blood clot alone.

In contrast to our expectation, the histologic examination did not display any difference between the BC and DPSC transplantation groups. One possible reason may be that fractionation of stem cells and proper growth factors is a more favorable approach for dental pulp regeneration. According to studies by Iohara et al (8), they also found that transplantation of unfractionated total pulp cells into root canal showed less tissue formation followed by evidence of mineralization on day 90 compared with transplantation of $CD105^{+}$ pulp cells and stromal cell–derived factor-1. Although DPSCs are the most direct cell source in dental pulp regeneration, a number of other cell sources including SCAPs, periodontal ligament stem cells, and bone marrow mesenchymal stem cells may also contribute to dental pulp regeneration (24). This may be another reason why the transplantation of autologous DPSCs alone did not help dental pulp regeneration in the present study. Further studies are needed to identify the cell sources of the tissues formed in the canal space (ie, from periapical tissues or from the transplanted DPSCs).

Growth factors and a suitable scaffold are also essential considerations in tissue regeneration. PRP contains several growth factors including transforming growth factor beta 1, platelet-derived growth factor, fibroblast growth factor, vascular endothelial growth factor, and epidermal growth factor (25, 26) that support cell growth,

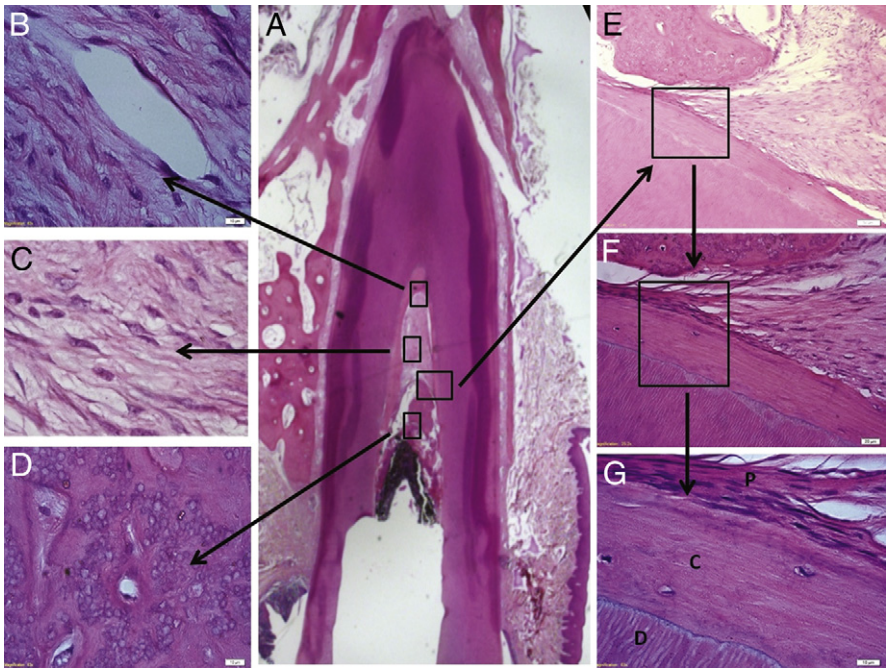

Figure 4. (*A*) A full-length regenerated case (from the PRP group). (*B*) The apical cells were heavily stained with large nuclei. (*C*) Cells in the middle portion showed stellate-like appearance resembling the normal pulp cells. (*D*) Cells at the coronal part differentiated into irregular bone-like tissue. (*E–G*) Cementum-like (C) and periodontal ligament–like (P) structures along the dentinal wall (D).

differentiation, and migration of DPSCs (27, 28). PRP is also capable of forming a 3-dimensional fibrin matrix, which acts as a scaffold (15, 16). Therefore, PRP has been suggested as a potential scaffold for regenerative endodontic procedure (15); 1 clinical case report appears to support this idea (13). Although PRP has been widely used in treatments of bone defect in periodontitis (29), some animal and clinical studies found that PRP does not appear to enhance bone regeneration (30, 31). Limited information is available on the use of PRP for dental pulp regeneration. In the present study, we found that PRP alone or the combination of PRP and DPSCs did not enhance the regeneration of pulp-like tissues. It is possible that the collagen scaffold used by Iohara et al (8) to carry DPSCs into the canals may provide a better condition for pulp regeneration compared with PRP used in the present study. The *in vitro* study showed that although PRP can enhance mineralization differentiation of DPSCs (18), it is not clear whether PRP enhances dentinogenesis (ie, PRP may not promote pulp-dentin regeneration).

Newly formed tissues in the canals could extend to the surface of MTA in some cases or occupy half of the canal space after 3 months. The growth of the tissue into the canal seemed not limited by the blood supply with an apical opening of 0.8 mm in diameter. When tissues engineered in the laboratory are implanted into the human body, only cells within 100–200 μm from the nearest capillary can attain sufficient diffusion of nutrients to survive. Thus, it was suggested that a voluminous tissue be prevascularized for achieving immediate and sufficient blood supply after implantation (32). It would be reasonable to infer that once the newly generated vasculature inside canals is connected with periapical tissues, the middle and coronal portion of pulp-like tissue could also be incrementally formed from the apical portion after 3 months.

The newly formed tissue showed no obvious difference among the 4 experimental groups in our study. Bone-like structures were found in most of the cases, especially in the coronal portion or close to the MTA. MTA is a good material for pulp capping and apexification, which can induce dental pulp cell differentiation and the secretion of mineralized tissue (33). Cells in new vital tissues in the apical canal were more immature with larger and deeply stained nuclei. These cells pertain more potential for multilineage differentiation. Cementum-like tissue and periodontal ligament-like tissue were seen along the internal root canal walls. In some cases, cementum-like tissue inside the root canal was connected with root surface cementum. The source of stem cells responsible for bone-like, periodontal ligament–like, and cementum-like tissues is not clear, possibly from the periapical tissues.

Acknowledgments

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

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