Preliminary Evaluation of Platelet Rich Fibrin-Mediated Tissue Repair in Immature Canine Pulpless Teeth

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Objective: *To evaluate the use of platelet-rich fibrin (PRF) in the regenerative therapy of immature canine permanent teeth.*
Methods: *Eight immature premolars of beagle dogs were pulp extracted and cleaned with irrigation, then divided into two groups of empty root canals and those filled with a PRF clot. All of the eight premolars were sealed with mineral trioxide aggregate and glass ionomer cement. Two premolars were left naturally grown as a positive control. The root development was assessed radiographically and histologically after 12 weeks.*
Results: *The radiological findings showed greater increases in the thickness of lateral dentinal wall in the PRF group than in the vacant group. Histologically, dental-associated mineral tissue, connective tissue, and bone-like mineral tissue grew into the root canals independent of PRF clot use. The PRF was able to increase the thickness of dental-associated mineral tissue. However, the vital tissue differed from the pulp dentin complex.*
Conclusion: *Our study demonstrated the feasibility of using PRF-mediated regenerative therapy in pulpless immature teeth for improving tissue repair.*
Key words: *histological results, immature canine teeth, platelet- rich fibrin, pulp repair*
Chin J Dent Res 2016;19(1):49–54; doi: 10.3290/j.cjdr.a35697

A growing body of evidence suggests that revascularization/regenerative endodontic treatment might be possible after pulpal necrosis, even for apical periodontitis in immature teeth with open apexes. Blood clot-mediated treatment has demonstrated radiographic signs of continued thickening of the dentinal walls and subsequent apical closure with periapical lesion healing, which is important to the long-term prognosis and the enhancement of root intensity[1-3].

To promote tissue healing, the local application of growth factors and host modulating agents is used to maximise the body's healing potential. Platelet-rich plasma (PRP) has been shown to successfully enhance the endodontic regeneration of periapical inflammatory lesions in non-vital immature teeth[4,5]. Zhu et al found that PRP could improve the hard tissue volume in the canals of immature canine teeth with chronic periapical periodontitis after disinfection[6]. As a second-generation platelet concentration, platelet-rich fibrin (PRF) is a kind of bio-scaffold that contains a variety of biofactors[7]. In the guided-tissue regeneration treatment of chronic periodontitis, PRF can improve bone healing and regain the attachment[8]. In a previous study, PRF has been proven to accelerate the proliferation and migration of canine dental pulp cells at the appropriate concentration by the Transwell assay[9]. However, little research has been done using PRF in the field of revascularization or pulp regeneration treatment.

Here we focused on tissue regeneration and repair of the pulpless immature tooth using the application of PRF.

Materials and methods

Animals

Two inbred 26-week-old male beagle dogs weighing 17 kg each were obtained from Marshall Biotechnology (Beijing, China). Animal care and handling was performed according to the guidelines of the Institutional

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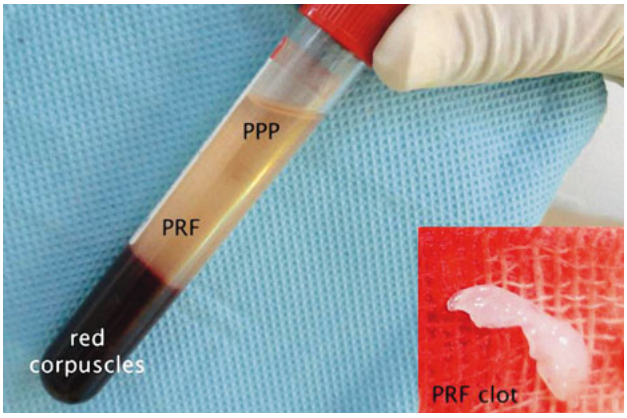

Fig 1 Venous blood was centrifuged to obtain the PRF portion in the middle of the tube. The PRF clot is shown in the bottom right panel: PPP- platelet-poor plasma; PRF- platelet-rich fibrin.

Authority for Laboratory Animal Care of Peking University.

This study was reviewed and approved by the Animal Care and Use Committee of Peking University Health Science Center (No. LA2011-042).

Preparation of PRF

PRF was procured by Dohan's method with minor modifications[7]. Briefly, approximately 9 ml of venous blood was drawn from each beagle dog vein using glass-coated plastic tubes without anticoagulant (Vacuette, Greiner, Austria). The collected blood was centrifuged at 3,000 rpm for 10 min at -4°C (Allegra X15-R; Brea, California, USA). Three layers were naturally formed in the tube: red corpuscles at the bottom, serum platelet-poor plasma on the surface, and a PRF clot in the middle. The PRF clot was easily separated from the other parts, for the treatment, after root canal preparation (Fig 1) .

Root canal preparation and treatment

The 26-week-old beagle dog with 10 immature bi-root premolars each was used to establish the model of pulpless immature permanent teeth. The dog was generally anaesthetised by 13.5 mg/kg intravenous sodium thiopental (Sinopharm Chemical Reagent, Shanghai, China) and maintained with isoflurane (Sinopharm Chemical Reagent). The pulp was mechanically exposed with a sterilised No. 2 round carbide bur in a high-speed handpiece and taken out using a barbed broach. The root canals were manually cleaned and shaped using a No. 25 sterilised file (Dentsply Maillefer, Tennessee, USA), alternately irrigated with 20 ml of 5.25% sodium hypochloride and 5 ml of 17% ethylenediaminetetraacetic acid (EDTA; PULPDENT, Massachusetts, USA), finally rinsed with 5 ml of physiological saline, and dried with sterilised paper points (Dayading, Liuyuan, Beijing, China).

After the pulpectomy, for each of the eight bi-root premolars, one root was filled with PRF clot and the other was left empty. The coronal portions of the root canals were sealed with mineral trioxide aggregate (MTA) (Dentsply, Pennsylvania, USA) and glass ionomer cement (GC Fuji IX, Tokyo, Japan). All of the treatments were delivered under a rubber dam and supplemented with local anesthesia (Lidocaine hydrochloride, China Otsuka Pharmaceutical Tianjin, China). Two bi-root premolars were left to grow naturally as a positive control.

Clinical, radiographic and histological evaluations

Clinical assessments including evaluations of tooth mobility, gingival condition and restoration retentions were carried out every 2 weeks after the operation. Preoperative, immediate postoperative, and 12-week postoperative radiographs were taken with extra care to produce the same angle and magnification. (Focus; Instrumentarium Imaging, Wisconsin, USA). The increment of the lateral root wall was judged by two independent evaluators who performed the stability test (paired t-test, $P = 0.22 > 0.05$).

The animals were sacrificed under general anaesthesia after 12 weeks. The jaws with the involved teeth were resected and fixed in 4% paraformaldehyde for 24 h and decalcified with 10% EDTA for approximately 8 months at room temperature.

After being decalcified, 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 analysed as an independent sample unit histologically under a light microscope (BX51; Olympus, Tokyo, Japan) to assess the structure of the generated vital tissues in the root canals.

Data analysis

The data were analysed with SPSS 20.0 (IBM, New York, USA) and the level of significance was set at $P < 0.05$ to determine whether there were any significant differences between the groups.

Results

Clinical and radiological findings

None of the experimental teeth showed signs of mobility, swelling or sinus tracts. All of the filling materials were left intact. No periapical radiolucency was detected in the 12 weeks of follow-up.

The radiological findings showed the number of lateral dentinal wall-increased roots in the PRF group (a total of eight roots) to be seven, while two roots in the negative group (a total of eight roots) increased ($P < 0.05$, Table 1). The density of the apical third of the root canal showed a diffuse increase in the PRF group compared with the vacant root (Fig 2). All of the untreated teeth showed normal root development as root thickening and elongation without the canal image changing.

Histological results

Newly formed vital tissues were found in all of the treated canal spaces. Two types of newly formed mineralised tissues were observed in the canal space, one adhering to the dentin wall and another forming bone-like mineral tissue (BLMT) in the connective tissue (CT), which filled the root canal with blood vessels and fibroblast-like cells (Fig 3).

Dentin-associated mineral tissue (DAMT)

DAMT is a mineral-rich tissue with uniform thickness and multiple layers of cells that settle along the inner dentin wall. The irregular tubes of DAMT appear to be disconnected from the inherent dentin tubes, and their inner surfaces are rough compared with those of the regenerative dentin. With its disorganised structure and embedded cells, DAMT was completely different from the regenerative dentin. The thickness of the root wall in seven roots in the PRF group and the vacant group were found to be increased compared with the postoperative root wall. The results were shown in Figure 4.

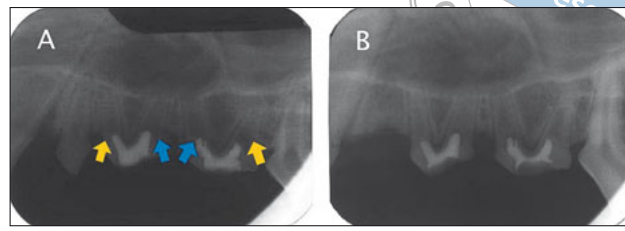

Fig 2 Postoperative (A) and 12-week follow-up (B) radiographs of the premolars. The root canals that were filled with platelet-rich fibrin clot (yellow arrow) showed greater increases in lateral wall thickness than the empty roots (blue arrow).

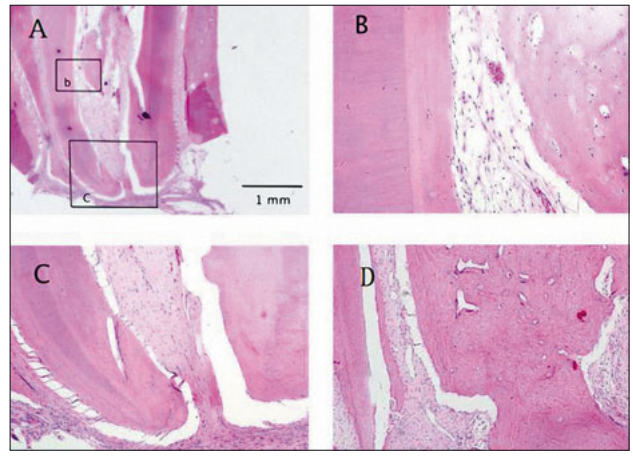

Fig 3 (A) Histological results of the immature root treated by PRF after 12 weeks (hematoxyline-eosin, original magnification 20×). (B) Detail of the boxed region b showed the DAMT attached to dentine; connective tissue was with blood vessels and bone-like mineral tissue (hematoxyline-eosin, original magnification 200×). (C) A magnified view of the boxed region c in part A showed the periodontal ligament-like connective tissue grew through the apical foreman and dentin-associated mineral tissue was deposited along the inner dentine (hematoxyline-eosin, original magnification 100×). (D) The apical region of immature root without PRF after pulp extraction for 12 weeks, which showed thinner mineral tissue along the inner dentine (hematoxyline-eosin, original magnification 100×).

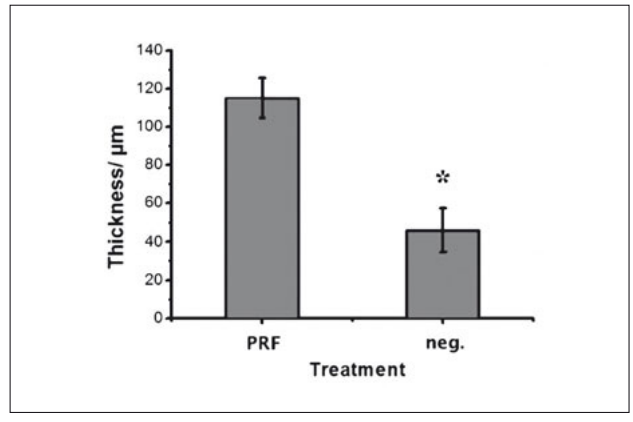

Fig 4 The thickness of the dentin-associated mineral tissue (DAMT) at the middle of the root canal in platelet-rich fibrin (PRF; n = 7) and empty (n = 7) groups. The mean thickness of the PRF group was 115 ± 21 μm, while that of the empty group was 46 ± 23 μm. Independent sample t-test: $^*P < 0.05$.

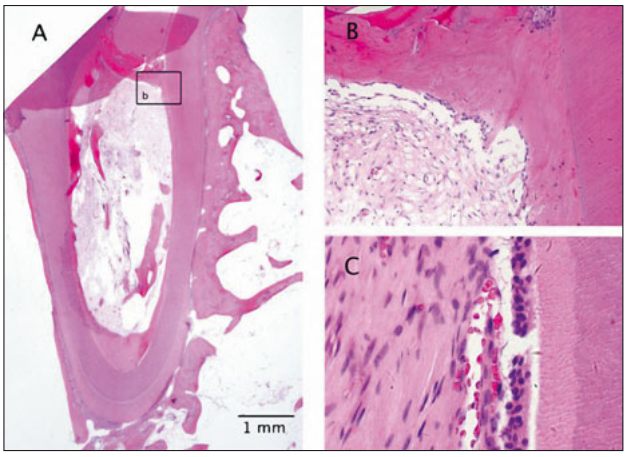

Fig 5 (A) The histological results of the platelet-rich fibrin group showed a 'bridge' structure formed by the dentin-associated mineral tissue (DAMT) under mineral trioxide aggregate. (B) Higher magnification of the boxed region b (hematoxylin-eosin, original magnification 200×). (C) Normal pulp-dentin complex in beagle dogs (hematoxylin-eosin, original magnification 400×).

Connective tissue (CT) and bone-like mineral tissue (BLMT)

In the normal roots, the odontoblasts were characterised by being highly polarised with the nuclei positioned away from the inner dentin[10]. The cells were fusiform in the fibroblast-like connective tissue with blood vessels. The cells that settled along the DAMT differed from the odontoblasts in their poorly polarised and non-palisade pattern-like arrangements. Typical bone mineral tissue was distributed around the apical foramen and continued to the outside of the bone tissue. Bone island mineral tissues were found in almost all of the roots, the structure of which was scattered in the canal space with trabecular formation, and cells were found trapped in the mineral tissue (Fig 5).

Discussion

Although the local transplantation or injection of mesenchymal stem cells (MSCs) represents a potential approach that may be useful in certain settings such as myocardial infarction and graft vs host disease[11], its potential for cell homing-based regenerative treatment is of particular interest. In the field of hematopoietic stem cells, homing is defined as the process in which cells actively cross the endothelial barrier and lodge in the bone marrow[12]. As a kind of MSC, DPCs can migrate with the help of chemotactic cytokines (e.g. stromal cell-derived factor-1 and fibroblast growth factor [FGF]) and act as endogenous cell sources for tissue regeneration[13]. Additionally, researchers have developed dental films containing collagen and chemotactic factors (e.g. FGF, vascular endothelial growth factor [VEGF] and platelet-derived growth factor [PDGF]) subcutaneously in immune-tolerant mice and successfully regenerated dental pulp-like tissues by enriching blood-sourced MSCs[14]. Our previous work evaluated the chemotactic effect of platelet-derived growth factors on canine dental pulp cells and the results have shown that PRF was a biocompatible material with cDPCs and the extract contains abundant chemokines that can enrich cDPCs *in vitro*[9], which corresponded well with those of an earlier study of Huang et al and Suzuki et al[13-15]. The present study was based on the hypothesis that PRF can improve the tissue regeneration and repair processes within the pulpless immature teeth.

Histological data showed that regardless of the presence or absence of the PRF clot, tissue neogenesis occurred within the root canal. Our histological results were consistent with histological data in the research of revascularisation using canine teeth with chronic periapical inflammation[16,17]. Generally, two types of mineral tissues, including DAMT and BLMT, were detected in the root canal.

The DAMT demonstrated internally embedded cells but had no dentinal canaliculi, which indicates that it is not any kind of regenerative or secondary dentin. Additionally, sclerous tissue was found to be tightly fitted to the original dentin, possibly due to opening of the dentinal canaliculi after ethylenediaminetetraacetic acid (EDTA) solution, which was in accordance with the findings of Yamauchi et al[17]. In some cases, a mineralised bridge was found beneath MTA and continuous with the DAMT, which may suggest a protective effect in the newborn tissues below. The imaging and histological data indicated that the sidewall thickening of the tooth roots observed in our experiment was caused by continuous thickening of the DAMT in the canal space. In the histological sections, nearly the entire dentinal wall was covered by DAMT. A possible mechanism for this is that the unique microstructure and the excreted active factors (e.g. bone morphogenetic protein and TGF-β), on the surface of the dentin induced DAMT formation[18,19]. Our results showed that PRF could improve DAMT thickness, possibly due to its rich growth factors such as PDGF, transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), endothelial growth factor and VEGF, which enhance the proliferation and differentiation of MSCs at proper concentrations[20].

Table 1 The number of the increased lateral dentinal walls in different groups.

	Positive group	PRF group	Vacant group
Increased roots / total	4/4	7/8	2/8 *

Chi-square test: * $P < 0.05$

The structure of the BLMT found inside the root canal was similar to that of bone tissue, and the tissues around the foramen were continuous with the alveolar bone outside the apical foramen in some sections. This phenomenon could be related to the diffuse increase in density throughout the root canal found on the radiographic results. Additionally, these findings can to some extent explain the clinical findings such as calcification in the root canal and rapid root canal obliteration after pulp revascularisation treatment in earlier case reports[3,21]. With regard to the histology results, we speculate that the precursor cells from the dental follicle or periodontal ligament may enter the root canal after the treatment and further differentiate and form the soft and hard tissues. In other words, the root canal space may be repaired by the cells in the dental follicle or periodontal tissue, which can differentiate into cementoblasts or osteoblasts, instead of being regenerated by the DPCs[22].

Demonstrating the diversified growth factors of PRF, more DAMT was generated in the PRF-treated root canals compared to the vacant root canals. Similar results were found in the Zhu et al study focusing on PRP[6]. Additionally, due to its tough fibrin structure and excellent physical properties (e.g. plasticity), the PRF clot can be easily placed in the root canal and can support the coronal MTA, showing excellent clinical operating performance features.

The previous literature shows that the apical foramen width is one of the important parameters contributing to pulp tissue regeneration that allows for the ingrowth of new tissue and blood vessels[23]. However, the apical diameter finding is contradictory. The results of several studies showed that the critical diameter to obtain regeneration of the pulp tissue is 0.32 to 1.00 mm[24,25]. In the present study, the diameter of the foramen was 0.4 to 1.4 mm and the tissue neogenesis did not differ amongst the groups. It is possible that a wider apical foramen is more conducive to the growth of new tissue. More research is needed to determine the narrowest apical radius that can be considered a clinical indicator for regenerative therapy.

Although PRF had a certain enrichment effect on the DPCs[9,13], it remained difficult for immature permanent teeth after pulpectomy to achieve real pulp regeneration by PRF treatment *in vivo* according to our limited study. Our results indicated that PRF could improve the regeneration rate of DAMT, but whether such a structure can improve the breaking resistance of roots requires further investigation. Additionally, whether the mineralised material within the root canal continues to precipitate over time and results in calcification, degeneration or avascular necrosis of the repaired tissue requires long-term observation. Hence, since residual healthy dental pulp tissue exists despite chronic periapical periodontitis of immature permanent teeth[26], protecting the healthy pulp is the key factor for maximising regenerative endodontic therapeutic success.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Dr Qilin Wang for the study design, for carrying out the experiments, for the data analysis and writing the manuscript; Dr Panpan Yang for the experiment; Dr Lihong Ge for the project instructions; Dr He Liu for the design and instructions of the experiment, and for approving the manuscript.

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