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Regenerative Endodontic Therapy Using Platelet-Rich Plasma (PRP) and Amelogenin for the Treatment of Non-vital Immature Permanent Teeth with Apical Periodontitis: An Animal Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Platelet-rich plasma (PRR) was proposed to serve as a possible scaffold model for regenerative endodontic therapies. However, its treatment outcomes are still controversial. Amelogenin protein has been shown to induce stem cell proliferation and differentiation. Thus, the aim of this study was to investigate whether the addition of amelogenin to PRP in non-vital immature permanent teeth with apical periodontitis can improve their treatment outcomes.

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Methods: Root canals of both maxillary and mandibular premolars in 8 mongrel dogs (n= 128) were instrumented and left open. After 14 days, in a second surgical procedure, canals were cleaned, irrigated, and treated with PRP (n=64) and PRP + amelogenin (n=64). After 1 and 3 months, animals were killed, and treated teeth were evaluated for histological and immune detection for nestin markers. Results: After 1 and 3 months post-surgery, PRP-treated canals showed full closure of opened apexes but minimal cementum, periodontal ligament (PDL), and bone regeneration. Importantly, no pulp regeneration was recognized. In contrast, PRP + amelogenin-treated canals at 1 and 3 months showed full closure of opened apexes, significant deposition of cementum, bone, regeneration of PDL, and pulp-like tissue regeneration compared to PRP-treated groups (p<0.001). By the 3-month period, full regeneration of all the lost dental-pulp complex tissues was seen, including vascular pulp-like tissue. Conclusions: PRP alone did not achieve the desired treatment outcomes, but after addition of amelogenin protein, it induced pulpal regeneration and regenerated the whole attachment apparatus. This combination could serve as a novel approach for regenerative endodontic therapy in non-vital immature permanent teeth with apical periodontitis. However, additional research is warranted to further evaluate the effect of such a combination in different animal settings before administrating this approach in clinical cases.

Keywords: Regenerative endodontic therapy; platelet-rich Plasma; PRP; Amelogenin; Nestin.

1. INTRODUCTION

Conventional endodontic therapy is usually designed for teeth with completed or nearly developed roots. It presents with great survival rates if the proper standards of care are followed. The average survival rates for endodontic treatment after 1-10 years are 86-98% [1,2]. However, permanent teeth with incomplete root formation can have poor prognosis when treated by conventional root canal therapy. Therefore, immature permanent teeth with necrotic pulp and apical periodontitis represent a challenge for endodontists. Thus, regenerative endodontic therapy (RET) should be considered for teeth with caries and traumatized incompletely developed teeth to ensure better success rates [3]. RET allows continual tooth development, which decreases the fracture rate and premature tooth loss related to the use of conventional root canal approaches, including apexification treatment [4]. According to the American Association of Endodontists, RET is known as “Biologically-based processes designed to physiologically restore harmed structures involving the dentin, root, and cells of the pulp-dentin complex [5] The basis of this approach is the interplay between progenitor stem cells, regulatory signals, and scaffold known as the tissue engineering triad [6].

Tissue engineering for dental pulp using progenitor stem cells is either a *cell-based* approach, which is depended upon the transplantation of stem cells into the root canal system or *cell homing,* which relies on the presence and stimulation of the resident tissue-specific host stem cells in the root canal system [7,8]. The tissue-specific stem cells from apical papilla (SCAP) actively participate in regenerative endodontic therapies in endodontically treated primary teeth with opened apexes because stem cells can survive in punitive environments such as necrotic tissue infected with bacteria [9]. Growth factors (GFs) are requested for odontoblastic differentiation and the adjustment of stem cells, and scaffolds supply an organized structure for stem cells to be distributed and arranged properly to regenerate the dental-pulp complex [10].

Platelet-rich plasma (PRP) is plasma with enriched levels of platelets relative to whole blood and has been suggested as a prospect model scaffold for endodontic regeneration therapies. However, compared to conventionally induced blood clots, its treatment outcomes are still controversial and need to be further elucidated [11-15]. PRP is produced from blood acquired by phlebotomy that is centrifuged to reach a high concentration of platelets in a small volume of plasma. The PRP is then re-injected or prepared as a gel or other biomaterial to finally be inserted at a site of injury [16]. The regenerative characteristics of PRP may be explained by its large stores of cytokines and GFs, which are normally released during clot formation at wound sites [17]. GFs such as PDGF, VEGF, TGF-β, and EGF improve healing by attracting undifferentiated cells to the newly formed matrix, trigger cell division, increase angiogenesis, and enhance the organization of collagen and rapid differentiation [18].

Amelogenin comprises about 90% of the enamel organic matrix proteins. It plays an important role

during teeth differentiation and provides signals that modulate the inflammatory response and regulate gene expression during the repair mechanisms of odontoblasts and cementoblasts [19,20]. Leucine-rich amelogenin peptide (LRAP) is a 59-amino-acid-long amelogenin protein isoform that has been proposed for induction stem cell proliferation, differentiation, and regulation by activating the Wnt beta-catenin signaling pathway following injury. In this way, it contributes to the repair or regeneration of damaged soft and hard dental tissues in necrotic pulps in immature permanent teeth with apical periodontitis [19,21,22]. The regenerative capacity of amelogenins is reported to be due to the promotion of regeneration by not only by induction of native MSC/progenitor cells, but also by direct or indirect recruitment of apoptosis for metabolic reinforce of developing tissues [23,24]. Amelogenin seems to possess a promising ability to support regeneration and production of all tooth-supporting tissues, thus implicating its use as a novel regenerative material in the fields of endodontics, implantology, bone regeneration, and others [24-26].

Nestin is a marker for stem/progenitor cells and also a superior angiogenic marker that has been used to detect neovascularity [27,28]. Nestin is also expressed during tooth development and tissue repair [29, 30].

Recently, our research group and collaborations used amelogenin as a material for RET to treat immature teeth with necrotic pulps and apical periodontitis. This resulted in thickening and strengthening of the canal walls and significantly better apical barriers compared to conventional calcium hydroxide treatment (CH) [31]. In addition, amelogenin induced better apical closure and enhanced apical tissue characteristics with functionally attached periodontal ligament and pulpal regeneration with recognized A- and C-fibers compared to CH treatment [32,33]. Thus, due to the controversial opinions and inconsistency of treatment outcomes, the aim of this study was to investigate whether the addition of amelogenin to PRP in non-vital immature permanent teeth with apical periodontitis can improve the treatment outcomes for future use in RET.

2. MATERIALS AND METHODS

2.1 Preparation of Amelogenin

About 1 mL of propylene glycol (PGA) alginate was mixed with 30 mg of recombinant amelogenin protein (RAP) M180 (180 amino acid mouse amelogenin) powder, which was pre-weighed utilizing aseptic technique. Then leave it to precipitate for 15 minutes prior utilize. Once mixed, the amelogenin was utilized up during 2-3 hrs.

2.2 PRP Preparation and Activation

A total of 10 mL of blood was collected from experimental animals in sterile tubes with anticoagulant. Centrifugation was performed at 1200 revolutions per minute (rpm) for 10 minutes. Following the upper layer was collected, the sample was further centrifuged at 2400 rpm for 10 minutes at room temperature. The upper layer was getting rid of accurately employing a pipette, and the remaining 1 mL of liquid contained PRP. The PRP was then activated by the addition of heparin. Following, centrifugation was achieved at 3000 rpm for 20 minutes at room temperature to eliminate platelet membrane fractions. The outcome supernatant was utilized in the experiment.

2.3 Preparation of Experimental Animals

Approval of the described animal studies was given by an Institutional Review Board charged with the safety and protection of vertebrate animals at Pharos University. This study included 8 mongrel dogs that were 6 months of age. The animals were maintained and observed for health assessment for one week under veterinarian surveillance prior surgeries for the study of estimating their health before any endodontic intervention was performed. The animals were anaesthetized utilizing sodium pentobarbital intravenous injection (30 mg/kg body weight). Pre-operative radiographs were obtained to prove the existence of open apices in all the premolars included in the study.

A total of 8 premolars were used for each dog (4 maxillary and 4 mandibular premolars adding up to 16 root canals for each dog). The total number of treated canals was 128 canals. Following endodontic access, canal length was recorded, and the pulp tissue was fully eliminated with K-files. Files were inserted to the radiographic apex and utilized to fully eliminate all pulp tissue residues. The same operator achieved all the processes with aid by a support team. All the canals were irrigated with distilled water, and following performing hemostasis, teeth were left without coronal restoration for 14 days to become infected with oral microbes.

In the second operative procedure, after the 14 days, the canals were cleaned under aseptic condition using rubber dam isolation, filled and irrigated with 2.5% sodium hypochlorite. Half of the 128 canals were filled with recombinant amelogenin added to PRP (64 canals), and the other half (64 canals) were filled with PRP only. The teeth were sealed with intermediate restorative material (IRM), and the access cavity was restored. Following each operation, the animals received an intravenously delivered painkiller (Voltaren, 25 mg/kg; Novartis Pharma Egypt, under license from Novartis Pharma, Switzerland). Amoxycilin (Cid Co, Egypt) was administered intramuscularly on the first day and then mixed with food thereafter for 7 additional days at a dose of 15 mg/kg. The dogs were placed on a soft diet throughout the post-surgery interval to decrease the prospect of local trauma to the operated teeth.

2.4 Histology and Immunohistochemistry

After 1 and 3 months postoperatively, animals were euthanized using an intravenous overdose injection of thiopental sodium. Water-cooled diamond disc was used to remove teeth and surrounding bone as a block, and samples were then evaluated at each time interval. Samples were demineralized, then standard histologic methods were used to prepare tissue sections at 5-μm thickness, which were stained with hematoxylin and eosin stain (H&E) or nestin for immunodetection. Antibodies for nestin were purchased from Abcam (Cambridge, UK, ab105389). Conjugated secondary antibodies were purchased from Thermo Fisher Scientific (Fremont, CA) and utilized at a dilution of 1:2000. All steps were followed according to the manufacturer's recommendations.

Digital sections images were prepared using a Nikon Eclipse 80i microscope (Tokyo, Japan) with a BX 51 digital camera (Tokyo, Japan).

3. RESULTS

3.1 PRP-treated Canal Cohort

At 1-month post-treatment, all canals treated with PRP cleared closure of the opened apex by cellular mineralized tissue bridging (MTB) on the opened apex forming an apical barrier in 100% of roots. Proliferating dense apical papilla (AP) filled the previously destroyed and empty periapical space in 100% of the teeth. Cementum was seen in 18.7% of the roots, and periodontal ligament (PDL) and bone were seen in 12.5% of the roots. Pulp tissue was not regenerated in the root canal system (Fig. 1A; Table 2). In addition, dense immune reactivity to nestin antibody was found in the cellular MTB of the opened apex and in the regenerated cementum. Moderate to dense immune reactivity was seen in the AP only (Fig. 1C; Table 2).

At 3 months post-treatment, all canals treated with PRP showed closure of the opened apex by cellular MTB of the opened apex in 100% of teeth. Proliferating AP was persistent in the periapical space in 87.5% of teeth. Cementum was seen in 31.2% of roots, while PDL and bone were seen in 18.7% of roots. There was still no pulp regenerated in the root canal system (Fig. 2A; Table 2). In addition, there was dense immune reactivity to nestin antibody in the cellular MTB of the opened apex, the regenerated cementum, and the regenerated PDL. Moderate to dense immune reactivity was seen in the AP (Fig. 2C; Table 2).

3.2 PRP + amelogenin-treated Canal Cohort

At 1 month post-treatment, all canals treated with PRP + amelogenin showed closure of the opened apex by MTB composed of mineralized deltas in 100% of roots, proliferating AP filled the periapical space in 100% of teeth. Cementum was seen in 87.5% of roots, PDL was seen in 43.7%, and bone was seen in 50% of roots. Vascular pulp-like tissue regenerated in 31.2% of root canal systems (Fig. 1B; Table 2). In addition, dense immune reactivity to nestin antibody was seen in the MTB closing the opened apex and in the regenerated cementum. Moderate to dense immune reactivity was seen in the AP pulp space and bone (Fig. 1D; Table 2).

Table 1. Total Canals distributions per treated group

Group/Time	PRP	PRP + amelogenin
1 month	16 premolars = 32 root canals	16 premolars = 32 root canals
3 months	16 premolars = 32 root canals	16 premolars = 32 root canals

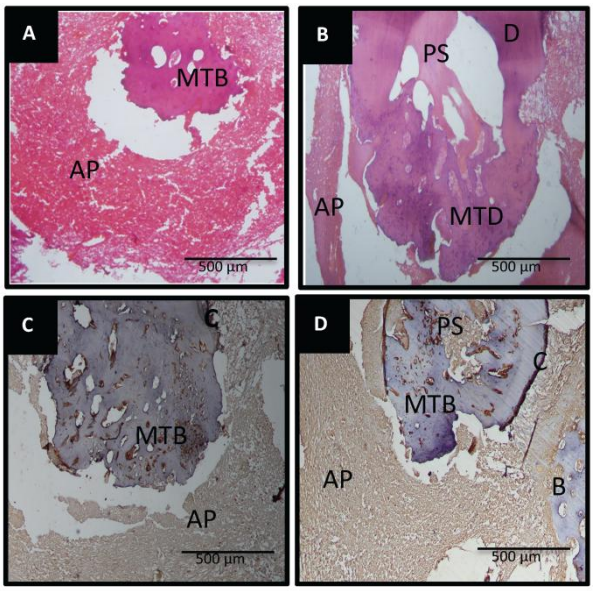

Fig. 1. Apices of premolar roots at 1 month post-operative period
(A) and (C) PRP treatment showing: root closure with cellular mineralized tissue bridging the opened apex (MTB) and dense immune-reactivity to Nestin in MTB and regenerated cementum (C), respectively. Apical papilla (AP)
(B) and (D) PRP+ amelogenin treatment showing: root closure with cellular mineralized tissue deltas (MTD) and dense immune-reactivity to Nestin in MTB and regenerated cementum (C), respectively. Apical papilla (AP). Dentin (D). Pulp space (PS) shows soft tissue regeneration in (B); Moderate to dense immune-reactivity in AP and PS in (D). Bone (B). H&E staining in A & B; Immune-reactivity to Nestin in C & D

At 3 months post-treatment, all canals treated with PRP + amelogenin showed closure of the opened apex by dentin-associated mineralized tissue (DAMT) in 100% of teeth, and proliferating AP was persistent in the periapical space in 18.7% of teeth. Cementum, PDL, and bone were seen in 100%, 90%, and 100% of roots, respectively. Interestingly, vascular pulp-like tissue regeneration was significantly increased, reaching 81.2% of root canal systems (Fig. 2B; Table 2). In addition, dense immune reactivity to nestin antibody was seen in the regenerated odontoblasts, cementum, and cellular cementum. Moderate to dense immune reactivity was seen in the persistent AP, pulp space, and regenerating PDL (Fig. 2D; Table 2). The data of all treatment outcomes of this study are described in Table 2.

3.3 Statistical Analysis

The current investigation was depended upon a total sample size of n= 128 canals divided into groups, as presented in Table 1. Samples were harvested and observed over 2 intervals of 1 and 3 months. A chi-squared test was employed to compare the two categorical differences with significant variations set at the $p<0.05$ level.

4. DISCUSSION

RET in immature teeth with opened apexes and apical periodontitis is based on the potential to regenerate the dentin-pulp complex in the canal space, restoring the continuation of the tooth root development by both lengthening and thickening of the channel walls or apical closing, and resolving apical gingivitis, thus eliminating the patient's clinical signs and symptoms. In regenerative endodontics, cell homing appears to be the most promising strategy [32-34]. In the cell homing concept, endogenous cells of the organism are recruited to the empty canal via different biomaterials and biomolecules, which can achieve tissue repair/regeneration [35]. This concept also depends on scaffold and GFs to promote host stem cell migration into the pulp space to regenerate the lost tissues [34]. Therefore, successful RET is based on the achievement of all three components of tissue engineering technology: stem cells, bioactive GFs, and biomimetic scaffolds [36].

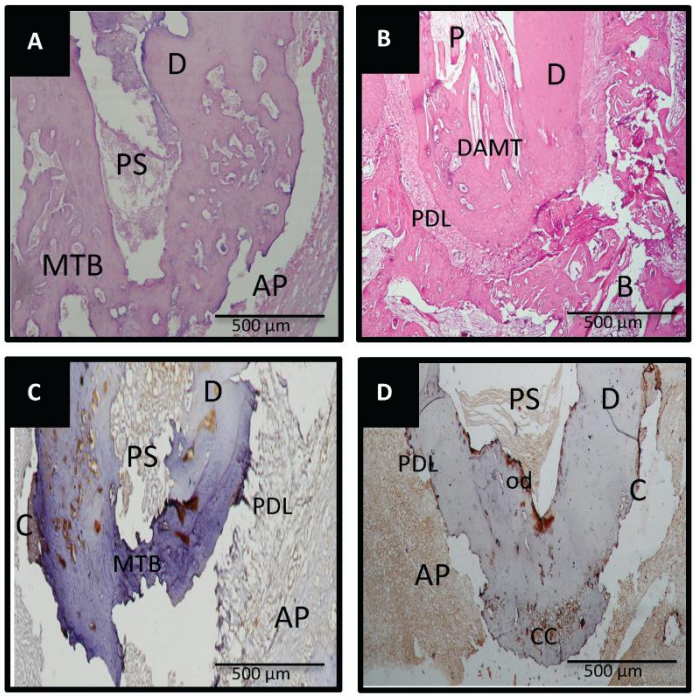

Fig. 2. Apices of premolar roots at 3 months post-operative period: (A) and (C) PRP treatment showing: root closure with cellular mineralized tissue bridging (MTB) in (A); and dense immune-reactivity to Nestin in MTB, regenerated cementum (C) and regenerated periodontal ligament (PDL) in (C). Dentin (D). Remaining proliferation of (AP). (B) and (D) PRP+ amelogenin treatment showing: root closure with dentin associated mineralized tissue (DAMT) and Pulp (P) shows vascular pulp-like tissue regeneration in (B); and dense immune-reactivity to Nestin in regenerated odontoblasts (od), cementum (C) and cellular cementum (CC). Bone (B). H&E staining in A & B; Immune-reactivity to Nestin in C & D

Table 2. Statistical analysis of treatment outcomes after 1 and 3 months

Group/Tooth area	PRP (1 month)	PRP+ amelogenin (1 months)	PRP 3 (3 month)	PRP+ amelogenin (3 months)	*p*-value
Apical papilla	32(100%)^{A}	32(100%)^{A}	28(87.5%)^{B}	6(18.7%)^{c}	<0.001^{a}
Root closure	32(100%)^{A}	32(100%)^{A}	32(100%)^{A}	32(100%)^{A}	-
Cementum	6(18.7%)^{A}	28(87.5%)^{B}	10(31.2%)^{A}	32(100%)^{B}	<0.001^{a}
Periodontal ligament	4(12.5%)^{A}	14(43.7%)^{B}	6(18.7%)^{A}	29(90%)^{C}	<0.001^{a}
Bone	4(12.5%)^{A}	16(50%)^{B}	6(18.7%)^{A}	32(100%)^{C}	<0.001^{a}
Pulp	(0.00)	10(31.2%)	(0.00)	26(81.2%)	<0.001^{a}

- *Uppercase super script letter indicates relation between categories within the variables*
- *Different uppercase is significant using Chi-Square Test @<0.05 level* $^{(a)}$

The role of PRP in RET has been studied in comparison to other materials such as platelet-rich fibrin (PRF), induced blood clot, and biodentine, which have had some promising but inconsistent treatment outcomes [12-15,37]. To our knowledge, this is the first study to report and assess the use of a combination of PRP and amelogenin as a novel RET approach in the treatment of non-vital immature permanent teeth with apical periodontitis. Our results are in agreement with other studies that investigated the use of PRP as a scaffold, which reported that it has potentially regenerative properties but with limited ability to regenerate the full dentin-pulp

complex [38-40]. In our study, PRP promoted cellular and hard tissue proliferation in the early time periods with AP proliferation and apical root closure but with limited production of cementum, PDL, bone, and importantly, no pulpal regeneration in the root canal system. In addition, this spurt of tissue regeneration seems to slow down with no significant improvement. In contrast to other observations, PRP alone in our study was able to induce apical closure in all of the samples after 1 month [41]. On the other hand, when PRP was used in combination with amelogenin, significant increases in both cellular and hard tissue proliferation of all dentin-pulp complexes was noticed, including pulpal regeneration. Thus, apical closure was achieved in all teeth with organized deposition and regeneration of cementum, PDL, bone, and importantly, vascular pulpal-like tissue regeneration. In addition, nestin was present in most of the regenerated tissues and pulp space.

The percentages of generated tissues were significantly different between the one and three-month of evaluation periods (p<0.001). Our results support our previous studies where amelogenin showed enhanced regenerative ability compared to traditionally used CH treatment, with the advantage of regenerating pulp-like structure similar to the authentic pulp innervation, the expression of peripherin neuronal intermediate filaments (A-fibers) in the pulp periphery, CGRP-reactive central nerves (C-fibers) in the pulp core, the expression of Sox2 by pluripotent SCAP surrounding the growing roots, and regenerated stem cells inside the root canals [31-33] .

5. CONCLUSION

PRP alone did not achieve the desired treatment outcomes of RET, whereas amelogenin protein added to PRP did fulfill the goals by inducing pulpal regeneration in addition to the regeneration of the whole attachment apparatus. This implies that this combination could be used as a novel approach for RET in non-vital immature permanent teeth with apical periodontitis. However, additional research is warranted to further evaluate the effects of such a combination in different animal settings before administrating this approach in clinical cases.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The approval of the described animal studies was given by an Institutional Review Board charged with the safety and protection of vertebrate animals at Pharos University, and was in full accordance with the World Medical Association Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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