

Release of Growth Factors into Root Canal by Irrigations in Regenerative Endodontics

Qian Zeng, PhD,* Sean Nguyen, DMD,[†] Hongming Zhang, PhD,[†] Hari Priya Chebrolu, BDS, MDS, DMD,[†] Dalia Alzebeleh, PhD,[‡] Mustafa A. Badi, DDS, MS,[§] Jong Ryul Kim, DMD, MSD, PhD,[†] Junqi Ling, PhD,^{||} and Maobin Yang, DMD, MDS, PhD[†]

Abstract

Introduction: The aim of this study was to investigate the release of growth factors into root canal space after the irrigation procedure of regenerative endodontic procedure. **Methods:** Sixty standardized root segments were prepared from extracted single-root teeth. Nail varnish was applied to all surfaces except the root canal surface. Root segments were irrigated with 1.5% NaOCl + 17% EDTA, 2.5% NaOCl + 17% EDTA, 17% EDTA, or deionized water. The profile of growth factors that were released after irrigation was studied by growth factor array. Enzyme-linked immunosorbent assay was used to validate the release of transforming growth factor (TGF)- β 1 and basic fibroblast growth factor (bFGF) at 4 hours, 1 day, and 3 days after irrigation. The final concentrations were calculated on the basis of the root canal volume measured by cone-beam computed tomography. Dental pulp stem cell migration on growth factors released from root segments was measured by using Transwell assay. **Results:** Total of 11 of 41 growth factors were detected by growth factors array. Enzyme-linked immunosorbent assay showed that TGF- β 1 was released in all irrigation groups. Compared with the group with 17% EDTA (6.92 ± 4.49 ng/mL), the groups with 1.5% NaOCl + 17% EDTA and 2.5% NaOCl + 17% EDTA had significantly higher release of TGF- β 1 (69.04 ± 30.41 ng/mL and 59.26 ± 3.37 ng/mL, respectively), with a peak release at day 1. The release of bFGF was detected at a low level in all groups (0 ng/mL to 0.43 ± 0.22 ng/mL). Migration assay showed the growth factors released from root segments induced dental pulp stem cell migration. **Conclusions:** The root segment model in present study simulated clinical scenario and indicated that the current irrigation protocol released a significant amount of TGF- β 1 but not

bFGF. The growth factors released into root canal space induced dental pulp stem cell migration. (*J Endod* 2016;42:1760–1766)

Key Words

Cell migration, growth factors, irrigation, regenerative endodontics, root canal surface

Endodontic therapy for an immature permanent tooth with pulp necrosis and apical periodontitis is a challenge. Conventional root canal therapy is limited because of the thin dentinal walls and open apex (1). Apexification provides an alternative treatment modality by inducing the formation of apical barrier for later obturation of the canal. In the 1960s, Nygaard-Ostby (2) raised the concept of tissue regeneration inside the root canal. In 2004, Banchs and Trope (3) introduced a modified clinical protocol that involved minimal instrumentation, copious irrigation, and placement of antimicrobial intracanal medicament, followed by inducing bleeding inside the root canal. In the last decade, numerous case series and several retrospective and prospective studies showed that continued root development and apical closure were achieved in regenerative endodontic cases (4–8). However, the success rate of regenerative endodontic treatment compared with apexification is still inconclusive because of the variable study designs and limited sample sizes (9–13). In addition, the histologic results from animal studies and human case reports showed that the regenerated tissues inside the root canal were not pulp tissues but periodontal tissues instead (14, 15).

To improve the outcome of regenerative endodontic therapy and regenerate a functional pulp-dentin complex, tissue engineering technology has been applied in the field of regenerative endodontics (16–18). The successful regeneration of a pulp-dentin complex needs all components of the tissue engineering triad: stem cells, growth factors, and scaffolds. Studies have proved that stem cells were delivered into the

Significance

Growth factors play an important role in regenerative endodontics. Until now, few studies have explored the release of growth factors into root canal by irrigations used in regenerative endodontic treatment. Our study provides the direct evidence that multiple growth factors are released from dentin after irrigations, and the study furthers the current knowledge of growth factors in regenerative endodontics.

From the *Operative Dentistry and Endodontics, Guanghua School of Stomatology, Affiliated Stomatological Hospital, Guangdong Province Key Laboratory of Stomatology, Sun Yat-Sen University, Guangzhou, Guangdong, China; [†]Department of Endodontology, Kornberg School of Dentistry, Temple University, Philadelphia, Pennsylvania; [‡]Regenerative Health Laboratory, Kornberg School of Dentistry, Temple University, Philadelphia, Pennsylvania; [§]Division of Oral and Maxillofacial Radiology, Kornberg School of Dentistry, Temple University, Philadelphia, Pennsylvania; and ^{||}Guanghua School of Stomatology, Affiliated Stomatological Hospital, Guangdong Province Key Laboratory of Stomatology, Sun Yat-Sen University, Guangzhou, Guangdong, China.

Address requests for reprints to Dr Maobin Yang, Department of Endodontology, Kornberg School of Dentistry, Temple University, Philadelphia, PA 19140; or Dr Junqi Ling, Guanghua School of Stomatology, Affiliated Stomatological Hospital, Guangdong Province Key Laboratory of Stomatology, Sun Yat-Sen University, Guangzhou, Guangdong, China. E-mail address: myang@temple.edu, lingjq@mail.sysu.edu.cn 0099-2399/\$ - see front matter

Copyright © 2016 American Association of Endodontists.
<http://dx.doi.org/10.1016/j.joen.2016.04.029>

root canal with bleeding in regenerative endodontic treatment (19, 20). These stem cells could be stem cells from apical papilla (SCAPs), inflamed periapical progenitor cells, periodontal ligament cells, and bone marrow stromal cells (21). The blood clot, platelet-rich plasma (PRP), platelet-rich fibrin (PRF), and various bioscaffolds have also been studied and tested (22–24). The remaining question is whether the growth factors are present in this regeneration system.

Growth factors play a critical role in dental stem cell recruitment, migration, proliferation, and differentiation (25–27). In regenerative endodontics, growth factors may come from different sources: blood clot, PRP, or PRF (28, 29), and dentin matrix has been found to be a reservoir of growth factors. During tooth development, a variety of bioactive molecules are secreted by odontoblasts and embedded in dentin matrix (30, 31), including transforming growth factor (TGF), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (32, 33). It has been shown that these growth factors could be released on the damage to dentin or during repair processes to promote dentin regeneration (34–36). It is important to know whether these growth factors could also be released from dentin matrix into the root canal space after the canal is treated with current regenerative endodontic protocol, which involves minimal instrumentation and copious irrigation with NaOCl and EDTA. Up to now, there has been no study directly addressing this question. In some previous studies, growth factors were either extracted from dentin powder (32) or extracted from EDTA in which the dentin disks were submerged (37). None of these studies simulated clinical scenario of regenerative endodontics in which the growth factors released after irrigation could contribute to the regeneration process.

The aim of this study was to investigate the release of growth factors into the root canal space after the irrigation procedure following current American Association of Endodontists (AAE) regenerative endodontic protocol (38). The type and amount of growth factors were evaluated and validated by using a root segment model. Functionality of growth factors released from root segments was also studied with migration of dental pulp stem cells (DPSCs).

Materials and Methods

Preparation of Root Segment Model

Total of 60 extracted teeth were collected from the oral surgery clinic. The teeth met the following criteria: permanent teeth, single root, and teeth without fractures, artificial alterations, and anatomic aberrations. The study was approved by the Institutional Review Board of Temple University.

The freshly extracted teeth were rinsed with phosphate-buffered saline, and periodontal tissues were removed by scrapping the root surface with a scalpel blade (Fig. 1A). After the coronal portions of teeth were removed, root segments were prepared and standardized by cutting 12 mm from apex (Fig. 1B). Because previous studies indicated that 1 mm was the critical apical size for revascularization (39, 40), all root segments were instrumented with hand files up to size 100 to achieve a standardized truncated cone-shaped canal with open apex of 1 mm in diameter (Fig. 1C). The external root surfaces were covered with nail varnish, and only the inner root canal surface was left uncovered (Fig. 1D and E).

Growth Factor Array

Three root segment samples were irrigated with 1.5% NaOCl (20 mL/5 min), followed by 17% EDTA (20 mL/5 min). Medium was collected at day 1 and subjected to growth factor array (Human 41 Growth Factor Glass Factor Antibody Array; Creative Proteomics, Shir-

ley, NY). Total of 41 targets were measured (Table 1). The negative control was the medium collected from 1 root segment that had all surfaces of root segment (including internal root canal surface) wrapped with nail varnish. The fluorescent signals of the target growth factors were imaged and captured by using GenePix4000 B Microarray Scanner (Axon Instruments, Sunnyvale, CA). The expression of targets was normalized to the control group in consideration of the background effect from each target. The targets with detectable expression were those that had higher expression than threshold according to the manufacturer's manual (Creative Proteomics).

Irrigation Protocols, Sample Collection, and Enzyme-linked Immunosorbent Assay

Forty-eight prepared root segments were randomly allocated to 4 groups with different irrigation protocols:

1. 1.5% NaOCl (20 mL/5 min) followed by 17% EDTA (20 mL/5 min),
2. 2.5% NaOCl (20 mL/5 min) followed by 17% EDTA (20 mL/5 min),
3. 17% EDTA (20 mL/5 min), and
4. deionized water (20 mL/5 min). After the irrigation, the segments were placed into 1 mL alpha-minimum essential medium (α -MEM) (HyClone, Logan, UT) supplemented with 100 U/mL penicillin and 100 U/mL streptomycin (HyClone). The samples were kept at 37°C for 4 hours, 1 day, or 3 days. At each time point, medium from 4 samples was collected and filtered. The amount of TGF- β 1 and bFGF released into collecting medium was quantified by using enzyme-linked immunosorbent assay (ELISA) following the protocol provided by the manufacturer (R&D Systems, Minneapolis, MN).

Calculation of Growth Factor Concentration in Root Canal

Considering the significant difference between the volume of root canal space and the volume of medium used in ELISA, previous studies that used the volume of solution in ELISA to calculate growth factor concentration may have underestimated the actual concentration of growth factors inside the canal. The volume of prepared root canal space in each root segment was measured by cone-beam computed tomography (CBCT) (OP300; Instrumentarium Imaging Dental, Milwaukee, WI), and the volume of canal space was calculated as a truncated cone. The parameters including the length (L), coronal diameter (D), and apical diameter (d) were measured by using software Invivo Version 5.2 (Anatomage Inc, San Jose, CA). Each measurement was repeated 3 times. The volume (V) of canal space was calculated as the following:

$$V_{(canal)} = \pi L \left\{ (D/2)^2 + (D/2)(d/2) + (d/2)^2 \right\} / 3$$

The final concentration of growth factors in root canal space (C_{canal}) was calculated as the following:

$$C_{(canal)} = C_{(ELISA)} \times V_{(collecting\ Medium)} / V_{(canal)}$$

Migration of DPSCs

The capability of growth factors inducing DPSC migration was measured by using Transwell assay (Corning, Kennebunk, ME). The third passage of human DPSCs (AllCells, Alameda, CA) were cultured in α -MEM supplemented with 15% fetal bovine serum (FBS) (HyClone), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a moist environment with 5% CO₂ atmosphere. These DPSCs expressed

Regenerative Endodontics

surface markers including CD73⁺, CD90⁺, CD105⁺, CD166⁺, CD34⁻, CD45⁻, and CD133⁻. For the migration study, DPSCs were starved in α -MEM containing 1% FBS for 3 hours, and 1×10^4 DPSCs were placed in the upper chamber of inserts for 2 hours to allow attachment. The upper chamber was then placed into lower chamber containing either 600 μ L commercial growth factors or prepared root segments. The pore size of the membrane between upper chamber and lower chamber was 8 μ m. The following migration studies were performed. First, commercial TGF- β 1 and bFGF (catalog no. 14-8348-62 and 68-8785-63; eBioscience Inc, San Diego, CA) were reconstituted into different concentrations (TGF- β 1: 0, 2, 10, 40, and 90 ng/mL; bFGF: 0, 0.05, 0.3, 0.7, and 10 ng/mL). Among them, concentration of 0 ng/mL served as negative control, and 10 ng/mL TGF- β 1 and bFGF served as positive controls according to previous study (27). DPSCs were allowed to migrate for 24 hours. In the second study, 8 prepared root segments that were irrigated with 1.5% NaOCl (20 mL/5 min) followed by 17% EDTA (20 mL/5 min) were placed in the lower chamber with 600 μ L medium (Fig. 2C) to measure whether the growth factors released from root segments could induce DPSC migration. In the positive control group, 600 μ L medium containing 20% FBS was added in the lower chamber. In the negative control groups, neither tooth segment nor growth factor was added. DPSCs were allowed to migrate for 24 hours and 72 hours before the final analysis.

After migration, cells on the upper membrane were carefully removed with a cotton swab, and the cells migrating into the lower chamber side were fixed with 4% neutral-buffered formalin (Fisher Scientific, Nepean, Canada) for 10 minutes. Cells were stained with crystal violet (ACROS, Livingston, NJ) and observed under the microscope (EVOS FL; Life Technologies, Rockville, MD). The number of cells in 5 random fields was counted, and average cell number in each field was calculated.

Data Analysis

Data points for each experimental group were expressed as average \pm standard deviation of the mean. Data were analyzed with one-way analysis of variance followed by Bonferroni post hoc tests. $P < .05$ was considered significant.

Results

Growth Factor Array

To investigate the profile of growth factors that were released into the root canal space, root segments were first irrigated with 1.5% NaOCl followed by 17% EDTA and then placed in medium for 1 day. The medium was collected and analyzed with the growth factor array. Total of 41 targets were measured with growth factor array. The expressions of



Figure 1. Preparation of root segment model. (A) Extracted tooth with single root; (B) tooth was decoronated, and root segment was prepared and standardized by cutting 12 mm from apex; (C) root canal was instrumented up to size #100; (D and E) external surfaces were covered with nail varnish, and only inner root canal surface was uncovered.

11 targets were above detectable threshold, including EGFR, CSF1, CSF3, IGFBP1, IGFBP3, PDGF-AB, TGF- α , TGF- β 1, TGF- β 2, VEGF-A, and VEGF-D as shown in Table 1 and representative maps in Figure 3A and B. The expressions of other 30 targets, including bFGF, were below the threshold (Table 1).

ELISA and Root Canal Space Measurement

On the basis of the results of growth factor array, 1 detectable target (TGF- β 1) and 1 undetectable target (bFGF) were selected for quantitative validation by using ELISA. To convert the concentration of growth factors from ELISA to that in canal space, the length (L), coronal diameter (D), and apical diameter (d) of canal space were measured by CBCT (Fig. 3C–E), and the volume of canal space was calculated as a truncated cone shape. The average volume of the prepared root canal space was $20.39 \pm 3.35 \text{ mm}^3$.

Growth Factor Concentration in Root Canal

The final concentrations of growth factors were calculated according to the volume of canal space. The samples irrigated with deionized water released TGF- β 1 at a very low level ($0.78 \pm 0.4 \text{ ng/mL}$). The group irrigated with 17% EDTA released TGF- β 1 at a level ranging from 4 ng/mL to 16 ng/mL (Fig. 3F). At 4 hours after irrigation, 3 groups (1.5% NaOCl + 17% EDTA, 2.5% NaOCl + 17% EDTA, and 17% EDTA group) had no statistical difference in the release of TGF- β 1. At day 1, the concentrations of TGF- β 1 in 1.5% NaOCl + 17% EDTA group ($69.04 \pm 30.41 \text{ ng/mL}$) and 2.5% NaOCl + 17% EDTA group ($59.26 \pm 3.37 \text{ ng/mL}$) reached the peak, and both groups were significantly higher than the 17% EDTA group ($6.92 \pm 4.49 \text{ ng/mL}$) ($P .05$). At day 3, the concentration of TGF- β 1 decreased in both 1.5% NaOCl + 17% EDTA group ($15.16 \pm 6.4 \text{ ng/mL}$) and 2.5% NaOCl + 17% EDTA group ($13.04 \pm 1.05 \text{ ng/mL}$) and became similar to that in 17% EDTA group ($16.25 \pm 9.56 \text{ ng/mL}$) (Fig. 3F). In contrast, the release of bFGF in all groups was very low at all time points, ranging from 0 ng/mL to $0.43 \pm 0.22 \text{ ng/mL}$ (Fig. 3G).

Migration of DPSCs

Transwell assay was used to measure the migration of DPSCs on the growth factors. On the basis of the concentrations found throughout groups (ranging from 2 ng/mL to 90 ng/mL), commercial TGF- β 1 was reconstituted to concentration of 2 ng/mL , 10 ng/mL , 40 ng/mL , and 90 ng/mL . TGF- β 1 promoted DPSC migration with a dose-dependent pattern (Fig. 2A). The commercial bFGF was also reconstituted to concentrations from 0.05 ng/mL to 0.7 ng/mL according to above ELISA results. None of these could induce significant DPSC migration when compared with positive control (10 ng/mL) (Fig. 2B). For migration, the root segments that were irrigated with 1.5% NaOCl + 17% EDTA were placed in the lower chamber for 24 and 72 hours. At both time points, the condition medium containing root segment induced DPSC

migration, and 72-hour group had a significant increase of cell migration compared with 24-hour group ($P .05$) (Fig. 2D).

Discussion

It has been long established that dentin matrix contains a variety of growth factors such as TGF- β , bFGF, VEGF, and IGF-1 (32, 33). These growth factors could promote the recruitment of dental stem cells to the site of injury, stimulate stem cell differentiation, and promote the process of regeneration (41). It has been shown that EDTA irrigation promotes the release of growth factors from dentin matrix (42, 43).

Although several previous studies measured the release of growth factors from dentin matrix (32, 33, 37), these studies had the following limitations regarding interpretation of results into clinical scenarios of regenerative endodontics. In some studies, dentin was ground into powders for the measurement, which maximized the release of growth factors (32, 33). Other studies used coronal dentin disks model, in which growth factors could be released from all surfaces of disks (37). This contrasts to clinical regenerative endodontic scenario in which only the growth factors released into canal space contribute to the regeneration process inside the canal. In the present study, standardized root segments were prepared, and all external root surfaces were covered with nail varnish; thus the growth factors could only be released via canal surface. In our preliminary study, the root segment models were covered with nail varnish on all surfaces. ELISA assay and growth factor array indicated that no growth factors were released from the nail varnish (data not shown).

In previous studies, dentin disks were submerged in EDTA, and EDTA was then collected to measure growth factor concentration (33, 37). This is different from the clinical scenario in which EDTA is removed from the canal after irrigation. The growth factors released after EDTA irrigation rather than during EDTA irrigation would be more critical for tissue regeneration because apical bleeding will be induced after EDTA was removed. In this study, the medium containing released growth factors was collected at different time points after the EDTA irrigation, and this method would represent the clinical scenario of regenerative endodontics more closely.

Another drawback of previous studies was that when the concentrations of growth factors were calculated by ELISA, the volume of solution collected for ELISA (at least $0.1 \text{ mL} = 100 \text{ mm}^3$) was used (32, 33, 37) instead of the volume of root canal space. Because the volume of root canal space is significantly smaller ($20.39 \pm 3.35 \text{ mm}^3$ in present study), the concentration of growth factors in the canal system was underestimated in previous studies. By using the volume of root canal from CBCT, the resulting concentration was higher than those from previously reported studies and could be more relevant to clinical situation.

The profile of growth factors was analyzed by an array containing total of 41 targets. These growth factor targets were chosen because they represent the key growth factors in stem cell migration, proliferation, and differentiation. For example, TGF- β 1 and PDGF are important in

TABLE 1. Expression of 41 Target Growth Factors

Growth factor detection	Target growth factors					
Detectable targets	EGFR	CSF1	CSF3	IGFBP1	IGFBP3	PDGF-AB
	TGF- α	TGF- β 1	TGF- β 2	VEGF-A	VEGF-D	
Undetectable targets	IGFBP2	PDGF-BB	PLGF	AREG	bFGF	β -NGF
	FGF6	FGF7	GDNF	CSF2	HB-EGF	HGF
	SCFR	IGFBP4	IGFBP6	IGF1	IGF1R	IGF2
	NT-4	PDGF R α	PDGF R β	PDGF-AA	VEGF R2	VEGF R3
	EGF	FGF4	TGF- β 3	SCF	CSF1R	NT-3

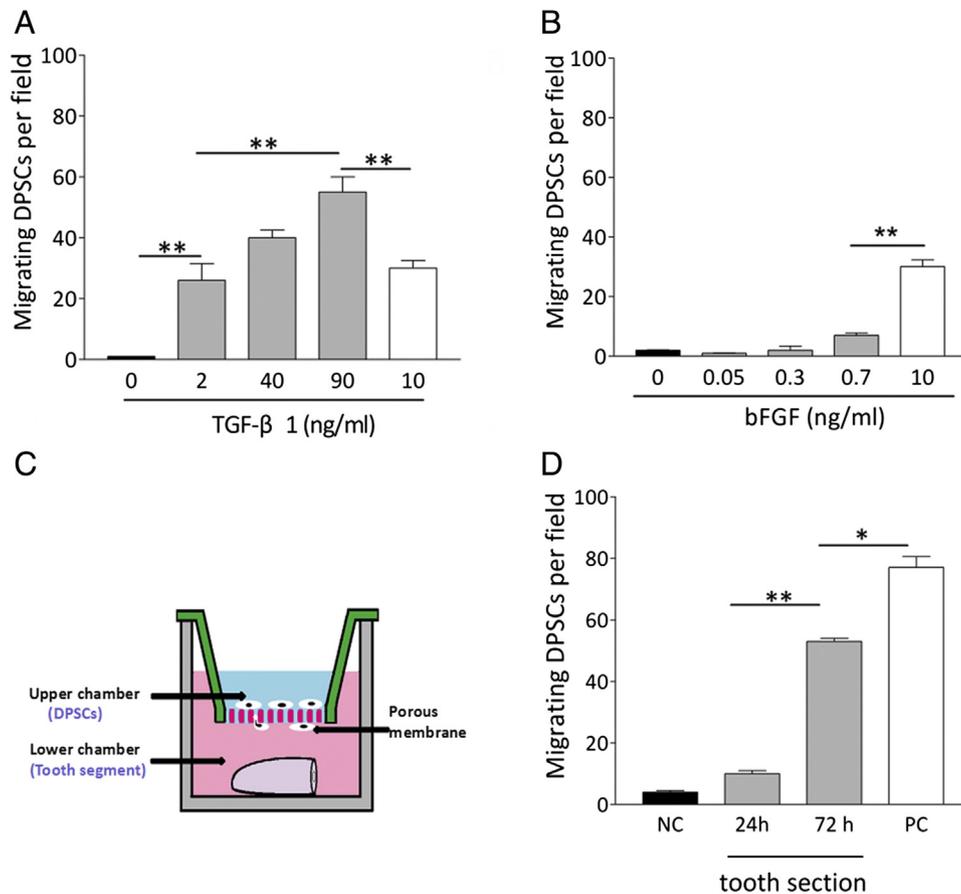


Figure 2. DPSC migration on growth factors. (A) DPSC migration on commercial TGF- β 1 constituted to concentrations ranging from 2 to 90 ng/mL; 10 ng/mL served as positive control. (B) DPSC migration on commercial bFGF constituted to concentrations ranging from 0.05 to 0.7 ng/mL; 10 ng/mL served as positive control. (C) Schematic illustration of Transwell assay with root segment. The root segment was placed on the lower chamber and submerged into the medium. DPSCs were cultured on the upper chamber and allowed migration for 24 and 72 hours. (D) DPSC migration on the root segments. In the negative control group (NC), neither tooth segment nor commercial growth factor was added. Cell migration on 20% FBS for 24 hours served as positive control (PC). Each value was mean \pm standard deviation. One-way analysis of variance and Bonferroni post hoc tests were used for statistical analysis. * $P < .05$, ** $P < .01$.

cell mobilization and homing as well as cell proliferation and differentiation (44–46). FGF, VEGF, and PDGF are important for angiogenesis (47, 48). The results showed that growth factors including EGFR, CSF1, CSF3, IGFBP1, IGFBP3, PDGF-AB, TGF- α , TGF- β 1, TGF- β 2, VEGF-A, and VEGF-D were detectable, whereas other 30 growth factors including bFGF and IGF-1 could not be detected. The results indicated multiple growth factors were released into canal space after the regenerative endodontic procedure. However, it should be noted that the array is only a qualitative assay, and the level of individual growth factor needs to be further validated by ELISA. The expressions of TGF- β 1 and bFGF were validated in the study, and more targets will be validated in future study.

Effect of different concentrations of NaOCl combined with EDTA on growth factor release was evaluated. Compared with the 17% EDTA group, groups that used NaOCl before EDTA enhanced the release of TGF- β 1, and both groups reached peak of release at day 1. In contrast, the release of bFGF was at a lower level compared with TGF- β 1. The results were consistent with the previous study by Finkelman et al (32) and Galler et al (37) in which release of TGF- β 1 was much more than bFGF. NaOCl is capable of removing the organic components of smear layer, leading to open dentinal tubules (49). This may be one possible reason for the higher release of TGF- β 1 after the application of NaOCl and EDTA. According to current regenerative endodontic pro-

ocol recommended by AAE, 17% EDTA is used for irrigation in the second visit (38). Our results showed that either 1.5% NaOCl + 17% EDTA or 2.5% NaOCl + 17% EDTA significantly increased the release of TGF- β 1 when compared with the group irrigated only with 17% EDTA, suggesting that NaOCl irrigation may need to be added to the second visit. It should be noted that multiple steps in current AAE regenerative endodontic protocol could affect the release of growth factors. For example, current protocol includes the application of calcium hydroxide or antibiotic paste as intracanal medicament. Galler et al have shown that calcium hydroxide increased TGF- β 1 release from dentin disks, whereas antibiotic paste decreased the release, indicating that intracanal medicament affected the release of growth factors.

In regenerative endodontics, after stem cells enter into the root canal, their further migration and differentiation are critical for the regeneration of a functional pulp-dentin complex. Howard et al (27) stated that 10 ng/mL TGF- β 1 and FGF could effectively induce DPSC migration. Our results showed the concentration of TGF- β 1 released into the canal space was in the range of 2–90 ng/mL, suggesting that it was capable of inducing the cell migration. This capability was confirmed by applying different concentrations of TGF- β 1 in migration study. The results showed that TGF- β 1 induced DPSC migration with a dose-dependent pattern. More importantly, the prepared root segments induced significant cell migration and proved that the growth factors released from root

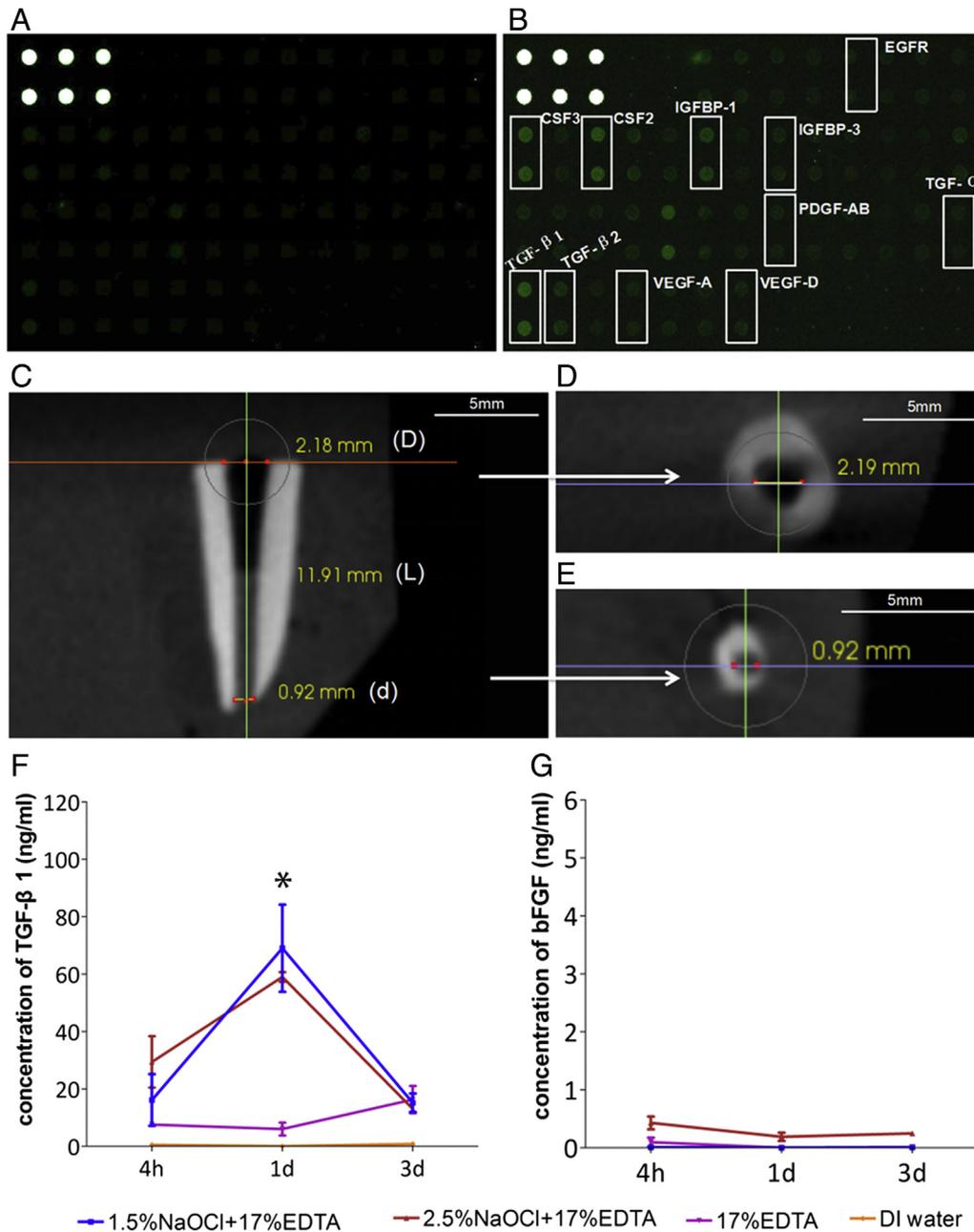


Figure 3. Release of growth factors into root canal space. (A) Map of growth factors array for negative control. (B) One representative map from 3 biological replicates of the experimental group; total of 11 of 41 targets were detectable. (C) Measurement of root canal volume. The length (L), coronal radius (D), and apical radius (d) were measured on the sagittal plane by CBCT. (D) Coronal radius was measured on axial view. (E) Apical radius was measured on axial view. (F) Quantitative measurement of release of TGF-β1 into root canal space after irrigation. One-way analysis of variance and Bonferroni post hoc tests were used for statistical analysis. *Different than 17% EDTA group and deionized (DI) water group. **P* < .05. (G) Quantitative measurement of release of bFGF into root canal space after irrigation. One-way analysis of variance was applied for analysis, and no significant difference was found.

segments were functional. The root segment wrapped with nail varnish on all surfaces only induced minimal DPSC migration, which was similar to the negative control in Figure 2D (data not shown), indicating that nail varnish itself had no significant effect on cell migration. It should be noted that the growth factors in the chamber of Figure 2C were also relatively diluted compared with the concentration of those in the root canal, suggesting even more cell migration would occur in clinical scenario.

In conclusion, this study provided the most direct evidence of growth factor release and showed that significant amount of TGF-β1

was released into root canal space after the regenerative endodontic procedure. Moreover, the growth factors released into canal space were functional, which was evidenced by the DPSC migration on the growth factors released from root segments. In current study, the average concentration of growth factors released into the root canal was measured. In clinical scenario, the growth factors released into canal may not be evenly concentrated. It would be interesting to study the spatial distribution of growth factors inside canal after their release. Our next step will also include using SCAPs to further simulate clinical

scenario, because SCAPs are suggested to be the main cell source of regenerative endodontics.

Acknowledgments

The authors thank Dr Xuanmao Jiao for his support in the cell migration study.

This research was supported in part by a research grant from the American Association of Endodontists Foundation and the Start-up fund from Temple University Kornberg School of Dentistry.

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

References

1. Al Ansary MA, Day PF, Duggal MS, Brunton PA. Interventions for treating traumatized necrotic immature permanent anterior teeth: inducing a calcific barrier & root strengthening. *Dent Traumatol* 2009;25:367–79.
2. Nygaard-Östby BN. The role of the blood clot in endodontic therapy: an experiential histologic study. *Acta Odontol Scand* 1961;19:323–53.
3. Banchs F, Trope M. Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? *J Endod* 2004;30:196–200.
4. Nosrat A, Homayounfar N, Oloomi K. Drawbacks and unfavorable outcomes of regenerative endodontic treatments of necrotic immature teeth: a literature review and report of a case. *J Endod* 2012;38:1428–34.
5. Kim DS, Park HJ, Yeom JH, et al. Long-term follow-ups of revascularized immature necrotic teeth: three case reports. *Int J Oral Sci* 2012;4:109–13.
6. Nosrat A, Seifi A, Asgary S. Regenerative endodontic treatment (revascularization) for necrotic immature permanent molars: a review and report of two cases with a new biomaterial. *J Endod* 2011;37:562–7.
7. Shah N, Logani A, Bhaskar U, Aggarwal V. Efficacy of revascularization to induce apexification/apexogenesis in infected, nonvital, immature teeth: a pilot clinical study. *J Endod* 2008;34:919–25.
8. Yang J, Zhao Y, Qin M, Ge L. Pulp revascularization of immature dens invaginatus with periapical periodontitis. *J Endod* 2013;39:288–92.
9. Jeeruphan T, Jantarat J, Yanpiset K, et al. Mahidol study I: comparison of radiographic and survival outcomes of immature teeth treated with either regenerative endodontic or apexification methods—a retrospective study. *J Endod* 2012;38:1330–6.
10. Alobaid AS, Cortes LM, Lo J, et al. Radiographic and clinical outcomes of the treatment of immature permanent teeth by revascularization or apexification: a pilot retrospective cohort study. *J Endod* 2014;40:1063–70.
11. Kahler B, Mistry S, Moule A, et al. Revascularization outcomes: a prospective analysis of 16 consecutive cases. *J Endod* 2014;40:333–8.
12. Bose R, Nummikoski P, Hargreaves K. A retrospective evaluation of radiographic outcomes in immature teeth with necrotic root canal systems treated with regenerative endodontic procedures. *J Endod* 2009;35:1343–9.
13. Saoud TM, Zaazou A, Nabil A, et al. Clinical and radiographic outcomes of traumatized immature permanent necrotic teeth after revascularization/revitalization therapy. *J Endod* 2014;40:1946–52.
14. Wang X, Thibodeau B, Trope M, et al. Histologic characterization of regenerated tissues in canal space after the revitalization/revascularization procedure of immature dog teeth with apical periodontitis. *J Endod* 2010;36:56–63.
15. Shimizu E, Jong G, Partridge N, et al. Histologic observation of a human immature permanent tooth with irreversible pulpitis after revascularization/regeneration procedure. *J Endod* 2012;38:1293–7.
16. Ferroni L, Gardin C, Sivolella S, et al. A hyaluronan-based scaffold for the in vitro construction of dental pulp-like tissue. *Int J Mol Sci* 2015;16:4666–81.
17. Nevins J, Finkelstein F, Borden BG, Laporta R. Revitalization of pulpless open apex teeth in rhesus monkeys, using collagen-calcium phosphate gel. *J Endod* 1976;2:159–65.
18. Yang J, Zhang Y, Sun Z, et al. Dental pulp tissue engineering with bFGF-incorporated silk fibroin scaffolds. *J Biomater Appl* 2015;30:221–9.
19. Chrepa V, Henry MA, Daniel BJ, Diogenes A. Delivery of apical mesenchymal stem cells into root canals of mature teeth. *J Dent Res* 2015;94:1653–9.
20. Lovelace TW, Henry M, Hargreaves KM, Diogenes A. Evaluation of the delivery of mesenchymal stem cells into the root canal space of necrotic immature teeth after clinical regenerative endodontic procedure. *J Endod* 2011;37:133–8.
21. Hargreaves KM, Diogenes A, Teixeira FB. Treatment options: biological basis of regenerative endodontic procedures. *J Endod* 2013;39:830–43.
22. Torabinejad M, Milan M, Shabahang S, et al. Histologic examination of teeth with necrotic pulps and periapical lesions treated with 2 scaffolds: an animal investigation. *J Endod* 2015;41:846–52.
23. Rodríguez-Benítez S, Stambolsky C, Gutiérrez-Pérez JL, et al. Pulp revascularization of immature dog teeth with apical periodontitis using triantibiotic paste and platelet-rich plasma: a radiographic study. *J Endod* 2015;41:1299–304.
24. Chen Y-J, Zhao Y-H, Zhao Y-J, et al. Potential dental pulp revascularization and odonto-osteogenic capacity of a novel transplant combined with dental pulp stem cells and platelet-rich fibrin. *Cell Tissue Res* 2015;361:439–55.
25. Del Angel-mosqueda C, Gutiérrez-puente Y, López-lozano AP, et al. Epidermal growth factor enhances osteogenic differentiation of dental pulp stem cells in vitro. *Head Face Med* 2015;11:29.
26. Arany PR, Cho A, Hunt TD, et al. Photoactivation of endogenous latent transforming growth factor- β 1 directs dental stem cell differentiation for regeneration. *Sci Transl Med* 2014;6:238ra69.
27. Howard C, Murray PE, Namerow KN. Dental pulp stem cell migration. *J Endod* 2010;36:1963–6.
28. Martínez CE, Smith PC, Palma Alvarado VA. The influence of platelet-derived products on angiogenesis and tissue repair: a concise update. *Front Physiol* 2015;6:290.
29. Wang SZ, Chang Q, Lu J, Wang C. Growth factors and platelet-rich plasma: promising biological strategies for early intervertebral disc degeneration. *Int Orthop* 2015;39:927–34.
30. Musson DS, McLachlan JL, Sloan AJ, et al. Adrenomedullin is expressed during rodent dental tissue development and promotes cell growth and mineralization. *Biol Cell* 2010;102:145–57.
31. Baker SM, Sugars RV, Wendel M, et al. TGF- β /extracellular matrix interactions in dentin matrix: a role in regulating sequestration and protection of bioactivity. *Calcif Tissue Int* 2009;85:66–74.
32. Finkelman RD, Mohan S, Jennings JC, et al. Quantitation of growth factors IGF-I, SGE/IGF-II, and TGF- β in human dentin. *J Bone Miner Res* 1990;5:717–23.
33. Roberts-Clark DJ, Smith AJ. Angiogenic growth factors in human dentine matrix. *Arch Oral Biol* 2000;45:1013–6.
34. Smith AJ, Tobias RS, Plant CG, et al. In vivo morphogenetic activity of dentine matrix proteins. *J Biol Buccale* 1990;18:123–9.
35. Ziafias D, Smith AJ, Lesot H. Designing new treatment strategies in vital pulp therapy. *J Dent* 2000;28:77–92.
36. Cassidy N, Fahey M, Prime SS, Smith AJ. Comparative analysis of transforming growth factor-beta isoforms 1-3 in human and rabbit dentine matrices. *Arch Oral Biol* 1997;42:219–23.
37. Galler KM, Buchalla W, Hiller K-A, et al. Influence of root canal disinfectants on growth factor release from dentin. *J Endod* 2015;41:363–8.
38. American Association of Endodontists. AAE clinical considerations for a regenerative procedure. Revised 6-8-16. 2016. Available at http://www.aae.org/uploadedfiles/publications_and_research/research/currentregenerativeendodonticconsiderations.pdf. Accessed October 21, 2016.
39. Huang GT. Dental pulp and dentin tissue engineering and regeneration: advancement and challenge. *Front Biosci (Elite Ed)* 2011;3:788–800.
40. Andreasen JO, Paulsen HU, Yu Z, Bayer T. A long-term study of 370 autotransplanted premolars: part IV—root development subsequent to transplantation. *Eur J Orthod* 1990;12:38–50.
41. Smith AJ, Scheven BA, Takahashi Y, et al. Dentine as a bioactive extracellular matrix. *Arch Oral Biol* 2011;57:109–21.
42. Khedmat S, Shokouhinejad N. Comparison of the efficacy of three chelating agents in smear layer removal. *J Endod* 2008;34:599–602.
43. Teixeira CS, Felipe MCS, Felipe WT. The effect of application time of EDTA and NaOCl on intracanal smear layer removal: an SEM analysis. *Int Endod J* 2005;38:285–90.
44. He H, Yu J, Liu Y, et al. Effects of FGF2 and TGF β 1 on the differentiation of human dental pulp stem cells in vitro. *Cell Biol Int* 2008;32:827–34.
45. Melin M, Joffre-Romeas A, Farges JC, et al. Effects of TGF β 1 on dental pulp cells in cultured human tooth slices. *J Dent Res* 2000;79:1689–96.
46. Denholm IA, Moule AJ, Bartold PM. The behaviour and proliferation of human dental pulp cell strains in vitro, and their response to the application of platelet-derived growth factor-BB and insulin-like growth factor-1. *Int Endod J* 1998;31:251–8.
47. Hellberg C, Östman A, Heldin CH. PDGF and vessel maturation. *Recent Results Cancer Res* 2010;180:103–14.
48. Mullane EM, Dong Z, Sedgley CM, et al. Effects of VEGF and FGF2 on the revascularization of severed human dental pulps. *J Dent Res* 2008;87:1144–8.
49. Gowda L, Mohan Das U. Effect of various concentrations of sodium hypochlorite on primary dentin: an in vitro scanning electron microscopic study. *J Clin Pediatr Dent* 2012;37:37–43.