Influence of Root Canal Disinfectants on Growth Factor Release from Dentin

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Abstract

Introduction: During dentinogenesis, growth factors become entrapped in the dentin matrix that can later be released by demineralization. Their effect on pulp stem cell migration, proliferation, and differentiation could be beneficial for regenerative endodontic therapies. However, precondition for success, as for conventional root canal treatment, will be sufficient disinfection of the root canal system. Various irrigation solutions and intracanal dressings are available for clinical use. The aim of this study was 2-fold: to identify a demineralizing solution suitable for growth factor release directly from dentin and to evaluate whether commonly used disinfectants for endodontic treatment will compromise this effect.

Methods: Dentin disks were prepared from extracted human teeth and treated with EDTA or citric acid at different concentrations or pH for different exposure periods. The amount of transforming growth factor-β1 (TGF-β1), fibroblast growth factor 2, and vascular endothelial growth factor were quantified via enzyme-linked immunosorbent assay and visualized by gold labeling. Subsequently, different irrigation solutions (5.25% sodium hypochloride, 0.12% chlorhexidine digluconate) and intracanal dressings (corticoid-antibiotic paste, calcium hydroxide: water-based and oil-based, triple antibiotic paste, chlorhexidine gel) were tested, and the release of TGF-β1 was measured after a subsequent conditioning step with EDTA.

Results: Conditioning with 10% EDTA at pH 7 rendered the highest amounts of TGF-β1 among all test solutions. Fibroblast growth factor 2 and vascular endothelial growth factor were detected after EDTA conditioning at minute concentrations. Irrigation with chlorhexidine before EDTA conditioning increased TGF-β1 release; sodium hypochloride had the opposite effect. All tested intracanal dressings interfered with TGF-β1 release except water-based calcium hydroxide.

Conclusions: Growth factors can be released directly from dentin via EDTA conditioning. The use of disinfecting solutions or medicaments can amplify or attenuate this effect. (J Endod 2015;41:363–368)

Key Words

Dentin, growth factors, intracanal dressing, irrigation solutions, regenerative endodontics

During tooth development, neural crest-derived cells of the dental papilla undergo terminal differentiation into dentin-forming odontoblasts. On completion of cytodifferentiation, the odontoblasts start their secretory phase and produce dentin, the major structural component of teeth. Similarly as in bone, dentin formation first requires an organic template that later mineralizes with hydroxyapatite crystals to form calcified tissue. During this synthesis process, the odontoblasts not only lay down the unmineralized predentin but also express various bioactive molecules, which are secreted into the extracellular space (1–5). During mineralization, these bioactive factors become embedded and immobilized in the dentin matrix. Because active proteins and growth factors have a short half-life, binding to extracellular matrix components may be required to maintain their bioactivity by protecting them from proteolytic degradation and thus prolonging their life span. Among growth factor–binding compounds are proteoglycans, mainly heparin sulfate (6, 7), also specific binding proteins (8), glycoproteins (9), and different types of collagen (10, 11).

Because there is no turnover in dentin extracellular matrix, regulatory molecules can be reactivated much later in life on release from their bond. During caries, bacterial lactate exposes the organic component of dentin and releases bioactive factors, which may modify immunoresponse, cell recruitment, and differentiation (12). Application of dental materials, namely calcium hydroxide or mineral trioxide aggregate but also self-etching dental adhesive, releases bioactive factors (13–15). Organic acids or chelating agents such as EDTA are also suitable for dentin demineralization. A variety of growth factors are present in the EDTA-soluble fraction of demineralized human dentin extracellular matrix, including transforming growth factor β1 (TGF-β1), fibroblast growth factor 2 (FGF-2), bone morphogenetic protein 2, platelet-derived growth factor, placenta growth factor, and epidermal growth factor, in addition to angiogenic factors such as vascular endothelial growth factor (VEGF) (1–3). These molecules are effective at very low concentrations and still elicit cellular responses at the picogram level, modifying immunodefense, angiogenesis, cell recruitment, proliferation, differentiation, and mineralization (16–18). Regenerative endodontic procedures are based on exactly these cellular reactions. After provocation of bleeding into the root canal, stem cells from the apical papilla are flushed in (19). For these cells to regenerate pulp tissue, they need to proliferate, differentiate, and produce dentin, and a vascular network is essential to maintain cellular metabolism. The exposure of growth factors from...
root canal dentin via dentin conditioning might be a contributing factor to successful regenerative endodontic treatment by directing cellular fate.

Previous studies have mainly used powdered dentin and the EDTA-soluble fraction to extract growth factors (1–5, 7, 13, 14, 17). In regenerative endodontic therapies, the direct release of growth factors from intact dentin would be more feasible. To our knowledge, this has not been investigated or quantified. Thus, the first objective of this study was to develop a protocol for growth factor release from human dentin, to quantify the amount of different growth factors, and to visualize their exposure on the dentin surface.

Yet another key factor to success of endodontic treatment is sufficient elimination of microorganisms from the root canal system (20). It can be assumed that this holds true also for regenerative approaches. In conventional root canal treatment, decontamination with antimicrobial solutions and intracanal dressings are essential. Commonly used disinfectants for irrigation during root canal preparation include sodium hypochlorite (21–25), chlorhexidine (22, 25, 26), or EDTA (21, 24, 25) in various concentrations. Intracanal medicaments, which remain in the root canal between visits, include calcium hydroxide–based, chlorhexidine-based, antibiotic-based, and corticosteroid-based gels and pastes (27). The second objective of this study was to test whether different irrigation solutions and intermediate intracanal dressings influence the amount of growth factor released from dentin.

**Materials and Methods**

**Quantification of TGF-β1 Release from Dentin**

For all experiments, dentin disks were prepared from human extracted molars with a wheel saw (SP/600; Leitz GmbH, Wetzlar, Germany) under constant water flow at 600 rpm (quantification of growth factors: 8-mm diameter, 200-μm thickness; gold labeling: 6-mm diameter, 500-μm thickness). Disks were stored in 0.5% chloramine solution (Chloramin T trihydrate; Merck, Darmstadt, Germany), which was exchanged with aqua bidest 24 hours before experimentation.

To test the ability of different solutions to release TGF-β1 from the dentin surface, each disk was immersed in 100 μL test solution for 5, 10, or 20 minutes: (group A) EDTA, 268 mol/L (10 w%), pH 6; (group B) EDTA, 268 mol/L (10 w%), pH 7; (group C) EDTA, 456 mol/L (17 w%), pH 7; (group D) citric acid, 0.476 mol/L (10 w%), pH 2; (group E) citrate buffer (citric acid 0.476 mol/L and trisodium citrate dihydrate 1.55 mol/L), pH 5; and (group F) citric acid phosphate buffer (citric acid 0.476 mol/L, trisodium citrate dihydrate 0.68 mol/L, trisodium phosphate 1.09 mol/L), pH 7.

The pH values were adjusted by using hydrochloric acid or sodium hydroxide. After dentin treatment, the irrigation solution was removed, immediately frozen in liquid nitrogen, and stored at −80°C. After completion of sample collection, all samples were thawed and subjected to growth factor quantification by using an enzyme-linked immunosorbent assay (ELISA) test system for TGF-β1 (Human TGF-beta 1 Quantikine ELISA Kit; R&D Systems, Wiesbaden, Germany). Three independent experiments were performed, each containing triplicate samples. Interference of the test solutions with antibody-binding on the ELISA plates was excluded in pilot experiments, where kit standards of known concentrations of recombinant TGF-β1 were dissolved in the solutions used in groups A–F. The resulting absorption values were in accordance with those of the standards delivered with the ELISA kit. Sodium hypochlorite (NaOCl) was also tested; this solution was not compatible with the ELISA test.

**Effect of EDTA on the Release of FGF-2 and VEGF**

To measure the release of the growth factors FGF-2 and VEGF, dentin disks were treated with EDTA, 10% at pH 7 for 5, 10, and 20 minutes. This irrigation solution was chosen on the basis of the results from experiments with TGF-β1 described above. Analog to the previous experiments, the amount of growth factor was determined by using ELISA test systems (Human FGF basic Quantikine ELISA Kit and Human VEGF Quantikine ELISA Kit; R&D Systems). Three independent experiments with triplicate samples were performed.

**Visualization of Growth Factor Exposure**

Growth factor exposure on the dentin surface after EDTA conditioning was visualized by gold labeling and subsequent scanning electron microscopy (SEM) imaging. Primary antibodies were used for collagen type I (Acris, Herford, Germany), as well as for TGF-β1, FGF-2, and VEGF (Abcam, Cambridge, UK). Dentin disks were immersed in EDTA (10%, pH 7) for 10 minutes, blocked with 2% goat serum for 2 hours, and incubated with primary antibodies overnight at 4°C. Secondary antibodies with gold nanoparticles (25 nm; Aurion, Wageningen, Netherlands) were applied for 2 hours at 4°C. Gold labeling with antibodies for collagen type I was performed for comparison. Negative controls included untreated dentin, which showed a smear layer, NaOCl-treated dentin, and EDTA-treated dentin without primary antibody treatment; none of the controls showed gold labeling.

Dentin disks were fixed in 2.5% glutaraldehyde at 4°C overnight, left to dry, mounted onto aluminum stubs by using self-adhesive carbon disks (Leit-Tabs; Provac, Oestrich-Winkel, Germany), and prepared for SEM analysis after carbon coating. An FEI Quanta 400 FEG environmental scanning electron microscope with a field emitter diode was used (FEI Europe B.V., Eindhoven, Netherlands) and operated in HVSEM imaging mode.

**TGF-β1 Release after the Use of Irrigation Solutions**

For further release studies, 10% EDTA at pH 7 was chosen for dentin conditioning and TGF-β1 as a representative growth factor. Two commonly used disinfection solutions were tested for interference with EDTA-induced growth factor release: chlorhexidine (Gum Paroex, chlorhexidine digluconate 0.12%; Sunstar, Etoy, Switzerland) and sodium hypochloride (5.25%; Speko, Münster, Germany).

Dentin disks were immersed in either solution for 5 or 10 minutes. Subsequently, the disks were treated with EDTA for 5, 10, or 20 minutes, and the release of TGF-β1 into the EDTA solution was quantified via ELISA.

**TGF-β1 Release after the Use of Intracanal Medicaments**

The following intracanal medicaments were tested: corticoid-antibiotic paste: Ledermix (Riemser Pharma GmbH, Greifswald, Germany); calcium hydroxide, water-based: Cabyl (OCO-Präparate, Dirmstein, Germany); calcium hydroxide, oil-based (sample from DMG, Hamburg, Germany); triple antibiotic paste: 20 mg ciprofloxacin, 20 mg cefuroxime, 40 mg metronidazole in macrogol propylene glycol (Apotheker Dr. Hörmann, Weinfelden, Switzerland); and chlorhexidinbis (D-gluconate) gel: Chlorhexamed 1% gel (GlaxoSmithKline, Brentford, UK).

Dentin disks were coated with a layer of intracanal dressing and incubated at 37°C in a humidified atmosphere for 48 hours. Subsequently, the disks were rinsed with phosphate-buffered saline 3 times and immersed in EDTA for 5, 10, or 20 minutes as described above, and TGF-β1 release was quantified via ELISA.
Graphical Depiction and Statistical Analysis

For all ELISA-based measurements, the detection limit (DL) was defined as the lowest concentration of the delivered kit standard divided by 2. Medians and 25% and 75% quantiles were computed and depicted graphically. Nonparametric Mann-Whitney test followed by application of the error-rates method (28) was used for statistical analyses ($\alpha = 0.05$).

Results

Quantification of TGF-$\beta1$ Release from Dentin

ELISA-based measurements of TGF-$\beta1$ release from dentin after demineralization are summarized in Figure 1. Conditioning with 10% EDTA, pH 7 for 20 minutes resulted in the release of the highest amounts of TGF-$\beta1$ (923 pg/mL), whereas 10% EDTA, pH 6 and 17% EDTA, pH 7 were less effective (449 pg/mL and 827 pg/mL, respectively). The release after treatment with citric acid and its variations was significantly lower compared with EDTA. Citric acid at pH 2 for 20 minutes released 57 pg/mL with citric acid–based solutions at pH 5 and 7, measurements ranged around the DL. Growth factor release showed a time-dependent profile, where increased exposure time yielded statistically significantly higher amounts.

Effect of EDTA on the Release of FGF-2 and VEGF

ELISA-based measurements of FGF-2 and VEGF from dentin after EDTA conditioning are depicted in Figure 2. The amounts of FGF-2 and VEGF liberated from dentin were also time-dependent but considerably lower compared with TGF-$\beta1$ (FGF-2, 10 pg/mL, VEGF, 32 pg/mL after 20-minute exposure). After exposure periods of 5 and 10 minutes, the release of FGF-2 ranged around the DL.

Visualization of Growth Factor Exposure after Dentin Conditioning

Representative images of growth factor visualization are compiled in Figure 3. SEM imaging after gold labeling for TGF-$\beta1$, FGF-2, and VEGF showed growth factor exposure on the dentin surface. Gold nanoparticles were detected both on peritubular and on intertubular dentin. Although it has to be noted that this method is not quantitative, a higher number of nanoparticles was observed for TGF-$\beta1$ compared with FGF-2 and VEGF. Gold labeling with collagen antibodies showed a high density of gold nanoparticles on the dentin surface (Supplemental Figure S1).

Influence of Irrigation Solutions on TGF-$\beta1$ Release

ELISA-based measurements of TGF-$\beta1$ after the use of different irrigation solutions and subsequent EDTA conditioning are depicted in Figure 4. Chlorhexidine for 5 minutes before EDTA conditioning increased TGF-$\beta1$ release (significant at 20 minutes), whereas release after 10-minute chlorhexidine and EDTA conditioning was similar to that of EDTA alone. The use of NaOCl before EDTA conditioning reduced TGF-$\beta1$ release significantly.

Influence of Intracanal Medicaments on TGF-$\beta1$ Release

The effects of intracanal medicaments on TGF-$\beta1$ release are summarized in Figure 5. The use of different intracanal medicaments before EDTA conditioning had different effects on TGF-$\beta1$ release. Water-based calcium hydroxide slightly increased the amounts of TGF-$\beta1$ compared with EDTA conditioning alone, but the effect was not statistically significant. However, all other tested medicaments led to a decrease in TGF-$\beta1$ release measured after 5-minute EDTA conditioning, which was significantly lower compared with the water-based calcium hydroxide group. Longer exposure to EDTA (10 and 20 minutes) after application of medicaments partially reversed this effect. Although the amounts of TGF-$\beta1$ were lower compared with water-based calcium hydroxide for all other medicaments, the differences were not statistically significant except for triple antibiotic paste after 10-minute EDTA conditioning and for oil-based calcium hydroxide and chlorhexidine gel after 20-minute EDTA conditioning.

Discussion

Regenerative endodontic procedures in vital immature teeth can lead to resolution of periapical inflammation and an increase of root length and thickness (29), which requires vital tissue with an intact immunodefense and differentiated cells capable of hard tissue formation inside the root canal. The origin of these cells is believed to be the apical papilla of the forming root, from which stem cells can migrate through the open apical foramen (19). The root canal will only provide an environment conducive for cell adhesion, proliferation, and differentiation if toxic solutions are restricted, and desired cellular responses will only
Gold labeling on dentin disks after EDTA conditioning (10%, pH 7, 10 minutes) of collagen (A), TGF-β1 (B), FGF-2 (C), and VEGF (D). Dots indicate gold nanoparticles after antibody binding, which show growth factor exposure on the dentin surface.

Release of TGF-β1 from dentin by EDTA conditioning after pretreatment with irrigation solutions chlorhexidine digluconate (CHX) and sodium hypochloride (NaOCl) for 5 or 10 minutes. Depicted are medians with 25%–75% quantiles. The narrow columns on the left are redundant and show TGF-β1 release after treatment with EDTA alone (pH 7, 0.268 mol/L; depicted in Fig. 1) for better comparability. α indicates statistically significant differences of EDTA conditioning alone (none) compared with the other test groups for the respective exposure period.

Release of TGF-β1 from dentin by EDTA conditioning after treatment of dentin disks with different intracanal dressings for 48 hours. Depicted are medians with 25%–75% quantiles. The narrow columns on the left are redundant and show TGF-β1 release after treatment with EDTA alone (pH 7, 0.268 mol/L; depicted in Figure 1) for better comparability. α indicates statistically significant differences between EDTA conditioning alone (none) compared with the other test groups; β shows statistically significant differences between water-based calcium hydroxide and the other test groups for the respective exposure period. CHX, chlorhexidine digluconate.
be achieved if the cells receive adequate cues. Taking advantage of dentin as a bioactive matrix by releasing dentin-bound signaling molecules could potentially increase the chance of success for regenerative procedures.

**Effective Growth Factor Release from Dentin**

The effects on dental pulp cells caused by TGF-β1, FGF-2, and VEGF, which were quantified in this study, have been demonstrated in different test systems. TGF-β1 acts as a chemoattractant (30) and drives cell differentiation and matrix synthesis (31, 32). FGF-2 increases cellular proliferation and exerts angiogenic effects, although less potent than VEGF (33, 34). Early studies on the use of dentin chips after pulp exposure in a canine model report cell differentiation in contact with dentin, which might be due to the presence of growth factors (35).

The present study demonstrates that EDTA or citric acid at different concentrations or pH release different amounts of TGF-β1. Both compounds are commonly used at the final step of root canal preparation because of their ability to remove the smear layer (36). EDTA, a hexadentate ligand, acts as a potent chelator that binds calcium ions and thus breaks down the hydroxyapatite crystal lattice. Citric acid at pH 2 and citrate buffer at pH 5 demineralize because of their acidity, whereas citric acid phosphate buffer at pH 7 acts as a chelating agent similar to EDTA, but as a tridentate ligand, it is less effective. The demineralizing effect is due to its calcium-binding ability, not its acidity.

Before experimentation, interference of the test solutions with the ELISA measurements was excluded. EDTA at pH 7 proved most effective of all test solutions regarding release of TGF-β1. An increase in concentration from 10% to 17% or change in pH did not render higher quantities of TGF-β1. On the contrary, exposure periods proved to be a critical factor. In a clinical setting, 20 minutes appear long and unpractical, but already after 5 minutes, EDTA released amounts at the low nanogram level. Citric acid–based solutions did not prove suitable for growth factor release, which is in accordance with a previous study where EDTA treatment followed by gold labeling provided visible exposure of TGF-β1 on the dentin surface, whereas citric acid treatment revealed lesser amounts (4). For both EDTA and citric acid, the amount of growth factor in solution might depend not only on the agent’s demineralization ability but also on the stability of growth factors in solution, because proteins are less suited to withstand acidic or alkaline rather than neutral pH.

The amounts of FGF-2 measured after EDTA conditioning for 5 or 10 minutes ranged around the DL of the ELISA kit; only after 20 minutes, release could be detected reliably. From the first 2 exposure periods, it might be due to its acidic pH (pH 5), where the demineralizing effect is enforced. If chlorhexidine is in contact with the dentin longer (10 minutes), protein stability might be negatively affected because of its acidity, or binding of cationic chlorhexidine to hydroxyapatite might hamper growth factor release.

Data from this study also indicate that intracanal medicaments interfere with growth factor release. The use of water-based calcium hydroxide enables the highest amounts of TGF-β1 release if followed by EDTA conditioning. After 5-minute exposure to EDTA, the release of growth factor is significantly higher compared with all other tested medicaments. Longer exposure to EDTA can level this effect out, likely because of increased dentin erosion. This can be explained by the difficulty of removing antibiotics or chlorhexidine from the dentin. More than 80% of triple antibiotic paste may be retained in the root canal regardless of the irrigation technique, whereas calcium hydroxide can be removed nearly completely (39). Chlorhexidine is likely to be retained because of its electrostatic binding to dentin, and an oil-based compound might also be retained more readily than its water-based equivalent.

**Influence of Disinfectants on Growth Factor Release**

If dentin conditioning were to be advocated for regenerative endodontic treatment to harness the bioactivity of dentin, it needs to be ensured that other materials used during the procedure, particularly disinfectants, do not counteract the release of bioactive molecules. The present study shows that the choice of irrigation solution for disinfection has a significant effect on growth factor release. NaOCl reduces subsequent growth factor release, which can be explained by its deleterious effect on proteins, which includes dentin-bound growth factors. Prolonged EDTA conditioning is necessary to enable TGF-β1 release after irrigation with NaOCl. On the contrary, short exposure to chlorhexidine before EDTA treatment increases the amounts of TGF-β1, which might be due to its acidic pH (pH 5), where the demineralizing effect is enforced. If chlorhexidine is in contact with the dentin longer (10 minutes), protein stability might be negatively affected because of its acidity, or binding of cationic chlorhexidine to hydroxyapatite might hamper growth factor release.

In conclusion, chances of success for procedures aiming at maintaining tooth vitality, such as direct pulp capping or pulpotomies, and recuperating tooth vitality, such as regenerative endodontic procedures, might increase after EDTA conditioning to release bioactive molecules from dentin. The choice of disinfectant irrigation solution and intracanal dressing should be considered carefully. The use of chlorhexidine appears unproblematic, whereas NaOCl reduces subsequent growth factor release and might thus be restricted to the first visit. Calcium hydroxide as intracanal dressing appears preferable in terms of subsequent growth factor release. These choices are supported by studies on the compatibility of disinfectants with the survival of stem cells from apical papilla. These show increased viability after contact with EDTA and calcium hydroxide, whereas high concentrations of NaOCl and antibiotic paste decrease survival (40, 41).
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The authors deny any conflicts of interest related to this study.

Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (http://dx.doi.org/10.1016/j.joen.2014.11.021).

References