ORIGINAL ARTICLE

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Do variations in the concentrations of EDTA and citric acid affect the release of different growth factors from the dentin matrix?

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Purpose: To investigate the release levels of transforming growth factor beta-1 (TGF-β1), bone morphogenetic protein-7 (BMP-7), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor-A (VEGF-A) from the dentin matrix using 10% and 17% ethylenediaminetetraacetic acid (EDTA) and 10% and 20% citric acid during regenerative endodontic procedures.

Methods: Fifty mandibular single-rooted premolar teeth were used. 8-mm cylindrical roots were irrigated with 1.5% sodium hypochlorite. Root segments were divided into groups by 20 mL of final irrigants: saline, 10% or 17% EDTA, and 10% or 20% citric acid. The samples were placed into culture medium and kept in an incubator at 37°C for 24 h. The supernatants were collected to measure the levels of growth factors with an enzyme-linked immunosorbent assay. The data were evaluated using a one-way analysis of variance.

Results: EDTA or citric acid increased the release levels of all growth factors compared to the control group. The greatest amount of TGF-β1 release was measured with 17% EDTA; however, it was not significantly different from 10% citric acid. The lowest amount of BMP-7 released was with 10% citric acid, which was significantly different from the other groups.

Conclusion: The 17% EDTA and 10% citric acid solutions had a similar effect on the release of growth factors from the dentin matrix.

Keywords: Citric acid, ethylenediaminetetraacetic acid, growth factors, regenerative endodontics.

Introduction

Dental trauma and/or developmental anomalies may cause pulp inflammation and apical periodontitis in immature teeth, resulting in the degradation of odontoblast maturation and inhibition of root development (1). Apexification therapies that use traditional calcium hydroxide, Ca(OH)2, treatment (2) or mineral trioxide aggregate (MTA) (3) have no capability to repair the vitality of pulp tissue (4). Therefore, regenerative endodontics has become a popular approach for treating necrotic teeth. The target of regenerative endodontic applications is the regeneration of soft and hard tissue and blood vessels in the root canal (5). Much like tissue engineering technology, regenerative endodontic clinical applications include stem cells, growth factors, and scaffolds to achieve the revascularization of a functioning pulp-dentin complex (6).

Growth factors are sequestered in the dentin matrix after root development (7). Transforming growth factor-β1 (TGF-β1), which is expressed by pre-odontoblasts and

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odontoblasts, plays a key role in mineralization and differentiation (8). Bone morphogenetic protein-7 (BMP-7) can stimulate differentiation of stem cells, dentinogenesis, and mineralization in the pulp chamber (9). Insulin-like growth factor-I (IGF-1) regulates osteoblast regulates osteoblast and osteoclast differentiation as well as apposition-resorption balance in bone tissue through osteoblasts or osteoclast precursors (10). In addition, it has been shown that IGF-1, the crucial signaling molecule, participates in pulp regeneration by stimulating the proliferation and differentiation of dental stem cells (11). Vascular endothelial growth factor-A (VEGF-A), which is a potent pro-angiogenic factor, activates local angiogenesis by inducing endothelial cells at sites of inflammation in the wound healing process (12).

Previous studies have reported that these growth factors can participate in the process of repairing pulp damage, such as pulp injury or amputation (13,14). Conditioning the dentin matrix with chelating agents can reactivate and increase the release of growth factors from the dentin matrix (15-17). Studies have also shown that ethylenediaminetetraacetic acid (EDTA) and citric acid can remove the smear layer (18), expose dentin tubules, and release growth factors, providing an optimal environment for autologous stem cells in the pulp regeneration process (19).

Considering the explanations above, using a chelating agent that provides effective canal disinfection, does not affect the biological integrity of periapical tissues, does not weaken dentin structure, and also produces the highest rate of release of growth factors may increase the success of regenerative protocols (20-22). As far as we know, there are no studies investigating the effects of different concentrations of EDTA and citric acid solutions on the release of growth factors in endodontic literature.

Therefore, the aim of this study was to investigate the effect of 10%, 17% EDTA solutions, and 10%, 20% citric acid solutions on the release of four different growth factors that are thought to have a significant impact on endodontic regeneration, from the inner dentin matrix of the root canal. The null hypothesis was that the different concentrations of EDTA and citric acid would not affect the release of growth factors.

Materials and Methods

All procedures were certified by the Non-interventional Research Ethics Committee of Kirikkale University, Turkey (January 07, 2021/January 08, 2021). A total of 50 mandibular premolar teeth, extracted for periodontal indications and with no fractures or anatomical malformations were used in this study. All teeth were rinsed with phosphate-buffered saline solution (PBS) (Gündüz Kimya, Istanbul, Turkey) immediately after extraction and the remaining periodontal tissue was removed from the root surface with periodontal curettes.

The crowns and the apical fragments of the teeth were removed and samples with 8-mm cylindrical root shapes with large open apices were obtained. Each root segment was enlarged with size ≠5 Gates-Glidden drills (VDW, Munich, Germany) with saline (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) irrigation. All root segments were covered with nail varnish except for the inner canal dentine surfaces. The specimens were irrigated with 20 mL of 1.5% sodium hypochlorite (NaOCl) and saline to simulate the regenerative clinical protocol (23). Ten specimens were assigned to the control group. Forty specimens were randomly allocated to four groups according to which chelating agent was employed and the specimens were irrigated with 20 mL of either 10%, 17% EDTA solution (Werax;

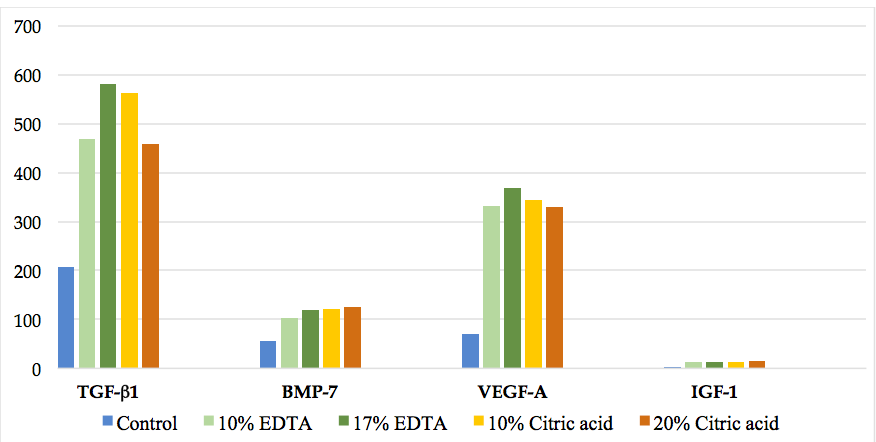

Fig. 1. The release of TGF-β1, BMP-7, IGF-1, and VEGF-A from inner dentin treated with EDTA and citric acid.

Table 1. The mean and standard deviations of growth factors release levels with different concentration chelating agents

	TGF-β1 (ng/mL)	BMP-7 (ng/mL)	IGF-1 (ng/mL)	VEGF-A (pg/mL)
Control	207.06±17.05[a]	56.05±4.00[a]	3.00±0.23[a]	70.59±2.56[a]
10% EDTA	468.27±57.69[b]	101.98±8.89[b]	12.54±2.01[b]	330.73±43.30[b]
17% EDTA	580.30±57.57[c]	118.53±8.84[c]	13.44±2.18[b]	367.61±35.40[b]
10% Citric acid	561.51±59.02[c]	122.17±9.21[c]	12.73±1.21[b]	343.51±27.53[b]
20% Citric acid	458.67±32.17[b]	124.42±7.46[c]	14.51±2.13[b]	329.32±26.82[b]

Values with the same superscript letters were not statistically different at p=0.05. EDTA: Ethylenediaminetetraacetic acid; TGF-β1: Transforming growth factor beta-1; BMP-7: Bone morphogenetic protein-7; IGF-1: Insulin-like growth factor-1.

Spot Dis Deposu A.Ş., Izmir, Turkey) or 10%, 20% citric acid (Gündüz Kimya, İstanbul, Turkey) solution for 5 min. Afterward, the specimens were rinsed with 20 mL of saline and dried with sterile paper points (Meta Dental Co., Ltd., Chongiu City, Korea).

After the final irrigation procedure, the samples were placed into sterile Eppendorf tubes with 1 mL alpha-mem (HyClone, Logan, UT, USA) containing 100 U mL-1 penicillin and 100 U mL-1 streptomycin and kept in an incubator at 37°C. After 24 h, the samples were collected and the release levels of TGF-β1, BMP-7, IGF-1, and VEGF-A were evaluated by enzyme-linked immunosorbent assay (ELISA) (SunRed Biotechnology Company).

An SPSS version 23.0 (IBM, Armonk, NY, USA) program was used for the statistical analysis. The results were analyzed by one-way analysis of variance (ANOVA) and Tukey post-hoc tests. $p< 0.05$ was considered significant.

Results

The mean and standard deviations of growth factor release levels with different concentrations of chelating agents are shown in Table 1. TGF-ß1, BMP-7, and IGF-1 were measured in ng/mL, and VEGF-A was measured in pg/mL. The levels of TGF-β1, BMP-7, IGF-1, and VEGF-A in all the experimental groups were significantly increased by the chelating agents compared to the control group ($p< 0.01$).

The use of 17% EDTA and 10% citric acid significantly increased TGF-β1 levels compared to 10% EDTA and 20% citric acid ($p< 0.01$). No significant difference was observed between the other chelating agents ($p> 0.05$).

The lowest amount of BMP-7 released was with 10% citric acid, which was significantly different from the other groups ($p< 0.01$). There were no statistically significant differences in BMP-7 release levels between the other chelating agents ($p> 0.05$).

EDTA or citric acid with different concentrations did not affect the release levels of IGF-1 and VEGF-A ($p> 0.05$).

Discussion

In current regenerative endodontic procedures (REPs), both the European Society of Endodontology (23) and the American Association of Endodontists (24) recommend using 17% EDTA solution (20 mL, 5 min) in the first and second appointments. This procedure may help to remove calcium hydroxide, double-triple antibiotic paste, and the inorganic content of infected dentin. As a chelating agent, EDTA also enables the release of growth factors embedded in the dentin matrix. Chelation agents can lead to cytotoxicity by causing changes in membrane permeability, cell metabolism, cell viability, and/or self-renewal abilities (25). The results of the studies in the literature indicate that 17% EDTA has more cytotoxic effects than 15% citric acid, and citric acid may be more biocompatible for fibroblasts than EDTA (25-27). Therefore, this study's aim was to evaluate two different concentrations of two chelating agents (10%, 17% EDTA, and 10%, 20% citric acid) used as the final irrigation agent in the release of growth factors. As a result of this study, the null hypothesis that the different concentrations of EDTA and citric acid would not affect the release of growth factors was partially rejected.

At the end of the mineralization phase of tooth development, bioactive factors get immobilized and embedded in the dentin matrix. Bacterial acids or dental materials can cause the release of TGF-β superfamily and angiogenic growth factors, thus contributing to dentin repair and regeneration (28-31). Previous studies reported that the TGF-β and BMP families provide apical closure and pulpal repair as a result of the differentiation of odontoblasts (32,33). VEGF-A, a pro-angiogenic factor, provides this effect by increasing the proliferation and migration of endothelial cells (34). IGF-1 plays a crucial role in mineralization and cell differentiation by increasing alkaline phosphatase activity (35). It has been shown that IGF-1 has a synergistic interaction with BMP-7 and stimulates the expression of VEGF-A (36,37).

TGF-β1 plays a significant role in dentinogenesis by regulating odontoblast growth and differentiation (8). This

study verified that root canal dentine irrigated with 17% EDTA and 10% citric acid significantly increased the release of TGF-β1, which was not significantly different in both experimental groups. This result contradicted a study by Chae et al. (15), which reported that TGF-β1 released after treatment with 10% citric acid was significantly higher compared to 17% EDTA. Although the test method used in this experiment was similar, this difference may be due to the number of samples and the different sensitivity ranges of the ELISA kits. Zeng et al. (38) stated that the highest level of release was in the experimental group that applied 1.5% NaOCl + 17% EDTA irrigation. In this study, while the highest TGF-β1 release levels were observed in root segments treated with 17% EDTA, no statistically significant difference was found when compared to 10% citric acid. Interestingly, despite the lower concentration, 10% citric acid significantly increased TGF-β1 release compared to 20% citric acid in our study. Similarly, Sadaghiani et al. (39) indicated that 37% phosphoric acid caused less TGF-β1 release than 10% citric acid. These results may be associated with the detrimental effect of highly acidic solutions on dentin by denaturing the protein structure of TGF-β1 (39).

BMP-7 is a secretory signal molecule that can be expressed in pre-odontoblasts and odontoblasts during the differentiation period of hard-tissue formation (40). There are no studies in the literature that compare BMP-7 release levels with the effect of chelating agents in regenerative endodontics. The results of this study revealed that no significant difference was observed in the samples treated with 17% EDTA or 10% 20% citric acid.

In regenerative medicine, the effects of IGF-1 and BMP-7 release levels on the osteogenic differentiation of mesenchymal stem cells (MSCs) have been investigated (35). When IGF-1, IGF-2, and TGF-β1 levels were evaluated in bone and dentin tissue, it was reported that IGF-1 was found in dentin tissue at lower concentrations compared to bone tissue (14). It was also stated that lower concentrations of IGF-1 compared to BMP-7 are sufficient for the osteogenic differentiation of MSCs, and IGF-1 may be a valuable option for improving the osteogenic differentiation of MSCs (35). The lower IGF-1 levels compared to BMP-7 obtained in this study are similar to the results of other researchers. Zeng et al. (38) revealed that TGF-β1 and VEGF-A release levels could be measured in dentin, but IGF-1 could not. According to the results of the present study, although IGF-1 is at a lower level compared to TGF-β1, it could be detected in all the experimental groups.

VEGF is a heparin-binding protein that provides neovascularization in the wound area by stimulating the proliferation of endothelial cells (41). Studies about regenerative endodontics carried out so far have observed that measurable VEGF-A levels are considerably lower than growth factors. (38,42-44). In line with previous studies, ELISA revealed the lowest level of VEGF-A compared with IGF-1, TGF-β1, and BMP-7, and no statistically significant difference was detected between all chelating agents in the present study.

It has been stated in previous studies that the proportion of macrophages, which play an important role in the inflammatory response, was decreased by 25% by citric acid and 95% by EDTA (25). Ivica et al. (16) also reported that stem cell viability and adhesion were higher in citric acid-applied dentin discs (16). Noting that all 10% dilutions of citric acid are more biocompatible than EDTA-T, it has been reported that cultures treated with citric acid have a higher percentage of viable cells and retain their self-renewal capacity (45). Considering the level of macrophages in the inflammatory response and stem cell viability, citric acid can be evaluated as an alternative chelating agent to EDTA.

There are methods, such as enzyme-linked immunosorbent assay (ELISA), fluorescent labeling, circular dichroism, and western blot (46-49), for measuring growth factor concentrations, but all of these methods have limitations (50). ELISA can detect non-bioactive protein, with the required binding groups, and if ELISA cannot bind with antibodies, it can detect bioactive forms of the protein (51). Insufficient blocking of the surface of the antigen-immobilized microtiter plate can result in a higher probability of false positive or negative results. Refrigerated storage and transfer of the antibody also lead to antibody instability (51). Since it is the method most commonly used in studies examining the release of growth factors, ELISA was also preferred in this study.

The limitation of the present study was our inability to measure the release levels of growth factors in the root dentin of mature teeth and the use of healthy dentin. Results from other clinical scenarios may differ from these results, as the biofilm layer in immature teeth with necrosis pulp might affect release levels. Since the root formation levels and dentin structures of each tooth might be different, average concentrations were evaluated in this study.

Conclusions

This study revealed that 17% EDTA and 10% citric acid used in regenerative endodontics procedures release similar amounts of IGF-1, TGF-β1, BMP-7, and VEGF-A. In REPs, chelating agent selection should be evaluated in terms of a broad profile, such as stem cell viability, migration, adhesion, differentiation, and inflammatory response,

as well as the release of growth factors. A complete understanding of the strategy for the selection of disinfecting solutions or medicaments may lead to improved release levels of growth factors in regenerative applications.

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