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Original Article

Effect of Different Dentin Conditioning Agents on Release of Growth Factors from Radicular Dentin Discs: A Comparative In-Vitro Study

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Abstract

Aim: To evaluate and compare the effect of dentin conditioners; 10% citric acid, 7% maleic acid, and 17% EDTA on the release of vascular endothelial growth factor (VEGF) and transforming growth factor beta-1 (TGF-β1) from dentin discs.

Subjects and methods: Human dentin discs (n= 15) were disinfected and prepared to obtain a standardized size. The outer diameter is 4-5 mm, while the root canal diameter is 1-2 mm. 17% EDTA, 7% maleic acid (MA), and 10% citric acid (CA) were used as dentin conditioners. After conditioning, dentin discs were placed in PBS (1 mL) and kept at 37˚C for 28 days. An ELISA test was used to quantify VEGF and TGF-β1 in the released medium. ANOVA test was utilized to compare the mean value of growth factors released at a significance level of 0.05. For multiple comparisons between groups, a post hoc Tukey test was used.

Results: It was found that dentin conditioning using CA and MA released significantly more growth factors than EDTA. A statistically significant increase in VEGF and TGF-β1 release was observed in CA group than in MA group.

Conclusion: The release of growth factors by 7% MA and 10% CA was statistically more than 17% EDTA from dentin discs. CA was observed to be significantly more effective than other tested agents in growth factors release.

Keywords: EDTA; citric acid; maleic acid; vascular endothelial growth factor; transforming growth factor beta-1.

Introduction

Regenerative endodontic treatments (RETs) are complicated procedures. They need a direct interaction between bioactive molecules, stem cells, and three-dimensional collagen fibrous scaffolds (**Hargreaves et al., 2013**). The features of the newly generated tissue following REPs depend on the release of growth factors integrated into dentin during dentinogenesis (**Sloan and Smith, 2007**). These factors result in the migration of pulpal stem cells to the site of injury. They would also promote odontoblast secretory activity, and stem cell differentiation into osteodentin-producing cells (**Sloan and Smith, 2007**); (**Kim et al., 2013**).

One of the most well-investigated bioactive molecules is TGF-β1, which enhances chemotaxis, different cell type differentiation, and proliferation. It is therefore thought to be the crucial component for pulpal regeneration (**Niwa et al., 2018**).

On the other hand, VEGF has a very important role in the differentiation of stem cells into endothelial cells, their proliferation, migration, and survival. So, the sufficient release of VEGF is a vital factor in the success of RETs (**Ferrara, 2004**); (**Zhang et al., 2018**).

Several studies have demonstrated that the use of various dentin conditioners in regenerative endodontic treatments (RETs), would enhance the dentinogenesis process outcomes (**Graham et al., 2006**); (**Tomson et al., 2007**); (**Ferracane et al., 2013**); (**Galler et al., 2015**).

EDTA has been considered a gold standard chelator and one of the most commonly used materials. In endodontics, EDTA is frequently used to eliminate the smear layer due to its mode of action. It binds itself to calcium ions and dissolves hydroxyapatite crystals, thus permitting the release of the growth factors from the dentin matrix (**Gonçalves et al., 2016**); (**Hashimoto et al., 2018**). However, many studies claimed that it may weaken the mechanical integrity of the dentin (**Morago et al., 2016**).

Citric acid (CA) was mostly studied in vitro as a chelating agent. It enhanced cell behaviour and growth factors released from the dentin matrix during conditioning (**Chae et al., 2018**); (**Ivica et al., 2019**). Additionally, a recent systematic review evaluated the use of CA in various aspects of endodontic treatment, and it showed that CA results in a sufficient release of TGF-β1, and is more efficient in the removal of smear layer in comparison to other agents (**Gomez-Delgado et al., 2023**).

Maleic acid (MA) 7% is another chelator that was proven to be more effective at removing smear layer than 17% EDTA (**Balla et al., 2009**); (**Ulusoy and Görgùl, 2013**); (**Rao et al., 2021**). Additionally, it has been demonstrated to have less cytotoxicity and more efficient demineralization in contrast to EDTA (**Balla et al., 2011**); (**Balla et al., 2009**).

So, the main purpose of our current study was to evaluate and compare the effect of dentin conditioners; EDTA, maleic acid, and citric acid on growth factors release from radicular dentin discs.

Subjects and Methods

All procedures were approved by the ethics committee (EC), Faculty of Dentistry, Cairo University concerning scientific content compliance with applicable rehearse and human subjects and regulations. Approval number was (28-1-24).

Sample size determination

Based on a study conducted by **Atesci et al., 2020**, the values of the mean and standard deviation (SD) of the release of TGF- β1 from dentin discs was 102 ± 24.5 in control group, 227.7± 49 in EDTA group in comparison to 494.6 ± 58.7 in citric acid group, 284.08 ± 56.33 in phytic acid group and 335.51 ± 63.7 in phosphoric acid group.

The sample size was calculated according to Atesci et al. using G power statistical power analysis program (version 3.1.9.4), an overall sample size (n=9); divided into three groups, each group (n=3) was sufficient to detect a large effect size (f) =2.65, with an actual power (1-β error) of 0.9 (90%) and a level of significance (α error) 0.05 (5%) for two-sided hypothesis test. The total sample size was increased to 15 (5 samples in each group); to compensate for procedural errors.

Sample selection and preparation

From the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Cairo University, freshly extracted human teeth were collected. The teeth were mature, single-rooted, and sound (n=15). A radiographic examination was done to check the selected teeth for eligibility and to ensure the absence of any resorption or calcifications. Periodontal tissue was removed using an ultrasonic scaler, and periodontal curettes. After that, teeth were kept in a normal saline solution.

Preparation of dentin discs (n=15) was done using Isomet 1000 Precision Cutter (15HC blade, Isomet Buehler, Lake Bluff, IL) cooled with Phosphate buffered saline (PBS) solution. Below the cementoenamel junction, 2 mm thick discs were cut. The root lumen of all root discs was prepared using #3 gates-glidden drill under continuous sterile saline rinse.

The outer root canal diameter was standardized to be (4-5 mm), and the inner (1-2 mm) using a MarCal caliper (Mahr GmbH, Esslingen, Germany).

Root discs were then immersed in 2ml of 17% EDTA for 3 minutes followed by 2ml of 2.25% sodium hypochlorite and then washed with distilled water. 0.5% chloramine solution (Chloramine T trihydrate; BDH Chemicals, Poole, UK) was used to keep dentin discs until 24 hours before use. Then it was replaced with aqua bidets (Thermo Fischer Scientific, Waltham, MA) 24 hours before testing (**Khan et al., 2021**)

Dentin discs conditioning and growth factors release

Dentin discs were randomly assigned to one of the three groups. Group I; the dentin discs were immersd in 2 mL of 17% EDTA solution (n=5). Group II; 10% citric acid (n=5). Group III; 7% Maleic acid (n=5) for five minutes. Then rinsed immediately with deionized water to remove excess conditioned media.

After conditioning, dentin discs were placed into microcentrifuge tubes with 1mL of PBS, supplemented with a Halt™ Protease Inhibitor Cocktail (cat no: 78438, ThermoFisher Scientific, USA) and incubated at 37°C for 28 days.

Growth Factor Quantification

At the end of incubation time, the released medium was subjected to ELISA for measurement of VEGF and TGFβ1, using the Human VEGF ELISA Kit (Cat No.EH0327, Fine Biotech, Wuhan, Hubei, China), and the Human TGF-β1 ELISA Kit (Cat No.EH0287, Elabscience Biotechnology, USA).

The sample is added to the ELISA plate coated with the capture antibody and incubated for a specific period. The unbound sample is removed by washing, and the detection antibody is added to the plate.

After incubation and washing, the substrate is added, and the absorbance is measured at 450 nm using a microplate reader (Tecan Infinite F50 Absorbance Microplate Reader, Tecan Life Science, USA).

Statistical Analysis

Data was coded and entered using the statistical package SPSS version 22. Data were summarized using mean and standard deviation and were tested for normality using Kolmogrov-S mirnov test. Comparison of different outcomes for normally distributed data was done using analysis of variance ANOVA with multiple comparison post hoc Tukey test. P value less than or equal to 0.05 is considered statistically significant.

Results

The results of this study showed that the release of VEGF and TGFβ1 was detected in its highest levels in the citric acid (CA) group followed by the maleic acid (MA) group, while in EDTA group the release of the growth factors was the lowest (**Fig.1**).

A statistically significant increase in VEGF release was observed in MA and CA groups compared to EDTA group (P value <0.001, 0.002 respectively). CA resulted in a statistically significant increase in VEGF release than MA (P value <0.001).

Also, a statistically significant increase in TGFβ1 release was observed in MA and CA groups compared to EDTA group (P value <0.034, 0.001 respectively). TGFβ1 release was statistically significantly higher in CA group than in MA group (P value <0.001) (**Table 1**).

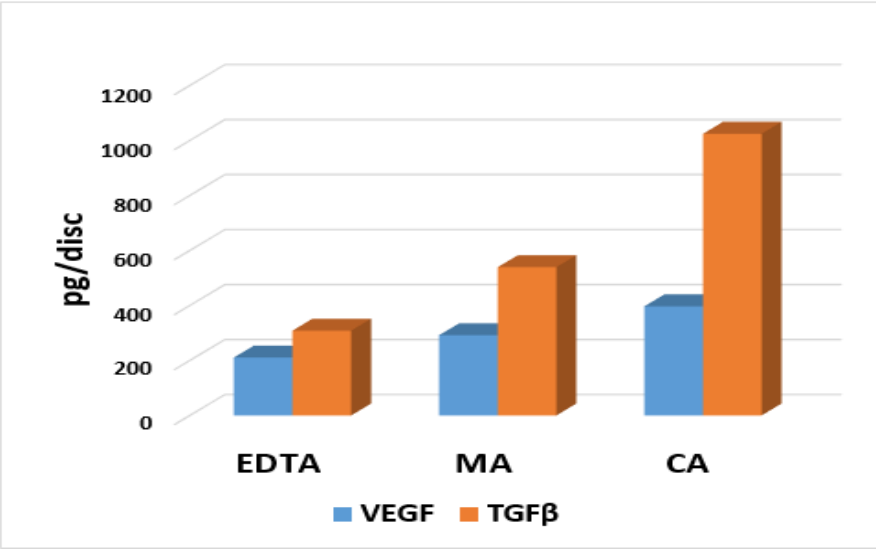

Figure (1): TGFβ1 and VEGF release from dentin discs after treatment with different conditioning agents

Table (1): Mean and standard deviation (SD) values of growth factors released from dentin discs in different groups

	EDTA	MA	CA	P value
VEGF	210.53±16.43	291.65±30.14	396.9±35.52	<0.001
TGFβ1	308.3±90.2	539.29±104.19	1024.42±170.81	<0.001

Discussion

Dental pulp regeneration techniques are of great importance, especially for non-vital immature permanent teeth. They provide a precious chance for maintaining those affected teeth for a long time with a better prognosis. Current methods include the use of stem cell therapy, where stem cells from dental pulp or other sources are utilized to promote healing; scaffold-based approaches that provide a structure for new tissue growth; and the application of bioactive materials that can stimulate the body's regenerative processes. Growth factors are examples of bioactive molecules that influence cell survival, proliferation, migration, and differentiation (**Barrientos et al., 2008**).

VEGF is one of these growth factors and is crucial for neovascularisation and angiogenesis (**Chu et al., 2004**). TGF-β1 stimulates proliferation, chemotaxis, and differentiation in various cell types. It is therefore thought to be the crucial component for pulpal regeneration (**Niwa et al., 2018**).

These growth factors are contained within the dentin, and various dentin conditioners affect how readily they are released.

EDTA is the most commonly used dentin conditioner after sodium hypochlorite irrigation due to its high efficacy in releasing growth factors from dentin and improving cell activity (**Dos Reis-Prado et al., 2022**).

Several studies in the literature used either dentin powder or dentin discs' models to investigate the effect of various conditioning solutions. In studies conducted by **Gallet et al., 2015** and **Sadaghiani et al., 2016**, the assessment of growth factors release was measured directly into the acidic solutions. In the current study, assessment of growth factors was done after 5 minutes of conditioning with the studied agents to simulate the clinical condition in REPs.

Human root discs may be regarded as a more trustworthy in vitro model for analyzing the release of growth factors in REPs. This is because the inner dentin surfaces of the roots

produce signaling molecules, and the volume of the root segment can influence the amount of growth factors released. On the other hand, using dentin powder, could maximize the amount of growth factors released which may compromise the validity of the findings (**Hancerliogullari et al., 2021**); (**Reis-Prado et al., 2024**).

The conditioning protocol that was followed in the present study was as follows; the chelators were applied to the dentin for 5 minutes. It's the same protocol that was followed by **Atesci et al., 2020**. However, another study examined various times of application and discovered that it is impractical to extend the exposure duration since the growth factors begin to release after five minutes. As well as a longer time of application increases the likelihood of dentin erosion and the destruction of cells needed for regeneration (**Gallet et al., 2015**); (**Baruwa et al., 2022**).

ELISA was used in the current study to quantify the amount of released growth factors because it is a sensitive diagnostic tool that allows the detection of different types of biological molecules at very low quantities and concentrations (**Gan and Patel, 2013**).

In the present study, both citric and maleic acids released significantly more growth factors than EDTA. These results are in agreement with other studies conducted by **Ivica et al., 2019**; **Atesci et al., 2020**; **Ballal et al., 2022**; and **Sadaghiani et al., 2022**.

However, our results contradict those of other studies conducted by **Gallet et al., 2015** and **Sadaghiani et al., 2016.** These variations could be attributed to the different methods used, which measured growth factor concentration released into acidic media. In the literature, many studies stated that the susceptibility of the ELISA method could be influenced by the acidity of the medium (**Lakshman et al., 2009**); **(Doucet et al., 2013**). To counteract these adverse events, the growth factors released from dentin discs were quantified into PBS.

Citric acid showed the higher growth factors release, this may be due to its high ability to eliminate the smear layer (**Turk et al., 2015**); (**Wilkònski et al., 2020**)**.** Besides its action of erosion/decalcification around intertubular and peritubular dentin compared to EDTA (**Gomez-Delgado et al., 2023**).

Our results also showed higher extraction of growth factors by MA compared to EDTA. This could be attributed to the higher smear layer removal capability of MA compared to EDTA (**Balla et al., 2009**); (**Ulusoy and Görgùl, 2013**); (**Rao et al., 2021**). It also results in considerable surface roughness of the intraradicular dentin (**Balla et al., 2010**).

Our study results also showed that the amount of VEGF produced from dentin discs was less in all examined groups when compared to TGF-β1, which agrees with the previous studies (**Galler et al., 2015**); (**Atesci et al., 2020**); (**Sadaghiani et al., 2016**). This is because dentin has a lower concentration of VEGF than other growth factors. In addition, it has a relatively short half-life compared to other bioactive molecules (**Eppler et al., 2002**); (**Roberts-Clark and Smith, 2020**). Moreover, a reduction in VEGF release from stromal cells has been demonstrated in irreversible pulpitis (**Artese et al., 2002**).

According to our results, alternative chelating agents to EDTA can effectively release a larger amount of growth factors required for pulp tissue regeneration. However, additional research is required to investigate the effect of these chelators on the release of other growth factors such as fibroblast growth factor, platelet-derived growth factor, and so on. As well as to assess how their application affects the stem cells' proliferation and adhesion to the conditioned root canal dentin.

It is worth mentioning that the results of in vitro studies have limited generalizability, so in vivo evaluations and clinical studies are strongly recommended.

Conclusion

Under the limitations of the present study, it could be concluded that using 10% CA, or 7% MA as dentin conditioners for 5 minutes would result in a higher release of growth factors compared to 17% EDTA. More studies are needed to have strong evidence about the effect of both solutions on cell behaviour and other growth factors release. In vivo studies would be of great importance as well.

Conflict of interest:

The authors declare no conflict of interest.

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Ethics:

This study protocol was approved by the ethical committee of the Faculty of Dentistry-Cairo University on 30 Jan 2024, approval number: 28-1-24.

Data Availability:

Data will be available upon request.

Credit statement:

Author 1: Conceptualization, Writing - original draft, Methodology, Data curation, Formal analysis, Writing - review & editing.

Author 2: Conceptualization, Methodology, Data curation, Formal analysis, Writing - review & editing, Investigation.

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