Original Article

Quantitative assessment of transforming growth factor-β1 release from dentin matrix upon conditioning with ethylene diamine tetra-acetate, doxycycline hydrochloride, and propolis: An *in vitro* study

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Abstract

Background: Transforming growth factor-beta 1 (TGF-β1) is a key morphogen in regenerative endodontics that plays a central role in regulating cellular functions. Various chelating agents have been shown to release this growth factor upon conditioning. The objective of the study was to evaluate TGF-β1 release from the dentin matrix upon conditioning with ethylene diamine tetra-acetate (EDTA), doxycycline hydrochloride, and propolis.

Materials and Methods: Forty-two human 3rd molar teeth were collected and coronal portion of the teeth was sectioned to obtain dentin blocks with dimensions 2 mm × 2 mm × 2 mm. The blocks were then randomly divided into three groups depending on the conditioning agent used; Group 1: 17% EDTA, Group 2: doxycycline hydrochloride (100 mg/mL), and Group 3: propolis (250 μg/mL). Conditioned blocks were placed in 0.5 mL of phosphate buffered saline and incubated for 1 week for quantification.

Results: Highest TGF-β1 release was noted for propolis (0.21 ng/mL), followed by doxycycline hydrochloride (0.18 ng/mL) and 17% EDTA (0.14 ng/mL).

Conclusion: Doxycycline hydrochloride and propolis significantly enhanced the release of TGF-β1 from the dentin matrix compared to EDTA ($P < 0.05$). No significant difference was observed between doxycycline hydrochloride and propolis ($P > 0.05$). Doxycycline and propolis can be used as effective alternatives to EDTA during regenerative endodontic procedures.

Keywords: Dentin matrix; doxycycline; propolis; transforming growth factor-beta 1

INTRODUCTION

Regeneration of pulp dentin complex depends upon highly coordinated interaction between three key factors, that often constitute the regenerative endodontic triad, which includes dental stem cells, growth factors, and scaffold.[1] Growth factors are bioactive polypeptides or signalling molecules that interact with specific receptors on the cell surface to bring about differentiation, proliferation, migration, and growth of the cell. These include factors like cytokines, chemokines, and various trophic factors which actively stimulate the intracellular biological cascades to

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bring about the cellular response.[2] Transforming growth factor beta (TGF-β) is a morphogen that plays an important role in the proliferation, differentiation, regulation, and maintenance of cellular functions. There are three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) of which TGF-β1 is most abundantly expressed in the mammalian tissues. It is synthesized either as a mature TFG-β dimer that is noncovalently linked to latency-associated protein (LAP) or covalently cross-linked to LAP through disulfide linkage as latent TGF-β binding protein which needs proteolytic cleavage for its activation.[3] The dentin matrix is a reservoir of soluble cytokines and growth factors secreted by the odontoblasts and pulp fibroblasts. During dentinogenesis, various growth factors such as vascular endothelial growth factor, platelet-derived growth factor, and bone morphogenic proteins, expressed by the odontoblasts, get embedded or fossilized within the matrix which is released upon demineralization of dentin. The surrounding extracellular matrix protects these fossilized growth factors from degradation and helps in retaining their bioactivity.[4]

Ethylene Diamine Tetra-acetate (EDTA), an amino polycarboxylic acid, has been widely used in endodontics due to its ability to chelate the inorganic component of the smear layer by forming stable complexes with bi-and tri-cationic metal ions such as calcium and iron.[5] In regenerative endodontics however, the major emphasis for the use of EDTA is due to its ability to release growth factors from the dentin matrix by selectively binding to the calcium ions through to its chelation action.[6] Doxycycline is a tetracycline antibiotic with a broad range of activities and is used in endodontics as a component of Mixture of Tetracycline, Acid, and Detergent (MTAD) and Tetraclean for its effectiveness against a wide range of microbes. It has also been used for the removal of the smear layer from the dentin, as an irrigant during apical surgeries along with its use as a medicament.[7] The chelating ability of doxycycline is well documented and is known to chelate and form complexes with iron and calcium.[8] However there is a lack of evidence supporting the release of TGF-β1 from the dentin matrix upon the action of doxycycline.

Propolis is an organic, resinous mixture that is collected and harvested by honeybees and is mainly used to close the cracks, strengthen the wax combs, and maintain a homeostatic environment within the hive. Propolis is well known for its superior antioxidant, immunomodulatory, antimicrobial, anti-inflammatory, and wound-healing properties.[9] The chemical composition of the raw propolis is variable and depends greatly on the geographical region from which it is obtained, i.e. diversity of the flora, environmental conditions, season, and the species of bees from which it is obtained. Various biologically active compounds have been identified and isolated, most of which are secondary floral metabolites. Main organic compounds include polyphenols, flavonoids, and terpenes along with aromatic acids, waxes, essential oils, amino acids, vitamins, and sugars. Such complexity also provides an advantage of each compound interacting synergistically with each other to bring about the biological response.[10] Propolis also possesses the property of chelation and the chelating activity of propolis is found to be around 70% of that of EDTA.[11] However, its effect on the release of growth factors from the dentin matrix has not been evaluated.

Thus, the aim of the present study was to quantitatively assess the release of TGF-β1 from the dentin matrix upon conditioning with EDTA, doxycycline hydrochloride, and propolis.

MATERIALS AND METHODS

Collection of teeth and preparation of dentin blocks

Forty-two human 3rd molars, free of caries, restorations, or any other developmental defects, were collected after obtaining written consent from the patients. After extraction, the soft tissue around the teeth was removed using a periodontal curette (GDC Universal) and washed with normal saline for 5 min. Teeth were then embedded in acrylic blocks, covering the root portion. The coronal portion of the teeth was then subjected to horizontal and vertical sectioning in a precision cutting machine (Minitom, Struers) with a diamond-edged blade under low speed (150 rpm) and with continuous cooling with phosphate buffered saline (PBS) (1X). Using low speed followed by constant cooling with PBS was done to prevent the denaturation of organic and protein content of the dentin matrix. The resultant dentin blocks obtained were 2 mm × 2 mm × 2 mm in dimension. The obtained dentin blocks were then washed with 1.5% sodium hypochlorite (NaOCl) (Hyposol™) for 5 min to disinfect and remove organic debris. Following this, the blocks were stored in sterile PBS until further surface treatment and growth factor quantification.

Preparation of ethanolic extract of propolis

Dried propolis was obtained from a local apiary (Graduate Beekeepers farm, Faridkot, India) with geographical coordinates of, Latitude: 30.6774° N and Longitude: 74.7539° E, in the month of February 2022. It was collected from the honeybee species, *Apis mellifera*. Prior to analysis, the sample was kept at room temperature in the dark. Ethanolic extract of propolis was obtained through the Soxhlet extraction process as described by Cunha *et al*.[12] Crude propolis was pulverized and 20 g of pulverized propolis was packed in a timber paper. It was then mixed with 200 mL of Ethanol solvent (1:10 weight/volume ratio) and was subjected to the Soxhlet extraction process for 8 h, with a maximum temperature of 60°C. The resultant solution (tincture) obtained was subjected to centrifugation at 3000 rpm for 15 min and filtered using

no 4 Whatman filter paper to eliminate waxes and other insoluble compounds. The filtrate obtained was evaporated using a rotary evaporator (Rotavap, Superfit) at reduced pressure (175 mBar) and temperature of 60°C at 100 rpm for 30 min. The remaining unevaporated filtrate (50 mL) was poured into a petri-dish and left for 12 h at room temperature for the ethanol to completely evaporate. After complete evaporation, the final product obtained was a semi-solid form of ethanolic extract of propolis (2.2 g). Five Milligram of the obtained ethanolic extract of propolis was dissolved in 20 mL of Dimethyl sulfoxide (Biobalance) solution to adjust the concentration at 250 µg/mL.

Conditioning of dentin blocks

Based on the conditioning agent used the obtained samples were randomly divided into three groups, ($n = 14$).

Group 1: Conditioning with 17% ethylene diamine tetra-acetate

Here the dentin blocks were conditioned with 17% EDTA (Waldent) solution for 1 min. Blocks were then washed with PBS for 1 min. The conditioned blocks were then transferred to graduated centrifugation tubes (Spinwin™, Tarsons) and were stored in 0.5 mL of PBS solution. It was then incubated at 37°C (ALPHA) for 7 days. The tooth slice conditioned medium was then collected for enzyme-linked immunosorbent assay (ELISA) quantification of the growth factor.

Group 2: Conditioning with doxycycline hydrochloride (100 mg/mL)

The solution was prepared by dissolving the constituents of the doxycycline hydrochloride capsule (Doxicip-100, Cipla) containing 100 mg of the active ingredient in 1 mL of distilled water. The dentin blocks were then conditioned with 100 mg/mL doxycycline hydrochloride solution for 1 min and washed with PBS for 1 min. It was then stored and processed as mentioned in the above group.

Group 3: Conditioning with propolis (250 µg/mL)

The dentin blocks were conditioned with 1 mL propolis solution for 1 min and washed with PBS for 1 min. It was then stored and processed as mentioned in the above group.

Growth factor quantification

TGF-β1 ELISA kit (Elabscience, USA) was used to quantify the growth factor released. A standard curve was determined with the serially diluted concentrations of TGF-β1 provided in the kit. Standards and samples were added to 96-well plates and the procedure was performed according to the manufacturer's instructions. The optical density was then measured immediately using a microplate reader (Sunrise, TECAN) at 450 nm. Each sample was assayed in triplicate, and the result was obtained through Magellan data analysis software.

Statistical analysis

SPSS (Statistical Package for Social Sciences) version 20 IBM SPSS, Chicago, Illinois. was used for statistical analysis. ANOVA test was applied to compare the TGF-β1 release among the groups and *post hoc* Scheffe's test for intergroup comparison ($P < 0.05$).

RESULTS

ELISA-based measurements of TGF-β1 release from the dentin following conditioning with different agents are summarized in Table 1 and Graph 1. The highest release of TGF-β1 was noted for propolis (0.2045 nm/mL) followed by doxycycline hydrochloride (0.1858 ng/mL) and the least value was observed for EDTA (0.1401 ng/mL). ANOVA test revealed a significant difference between the groups regarding TGF-β1 release. *Post hoc* test revealed a significant difference between EDTA and doxycycline hydrochloride ($P = 0.0016$), EDTA and propolis ($P = 0.0002$), but there was no significant difference between doxycycline hydrochloride and propolis [$P = 0.1766$, Table 2 and Graph 2].

Compared to EDTA and doxycycline hydrochloride, conditioning the dentin samples with propolis resulted in a better release of TGF-β1 from the dentin matrix.

DISCUSSION

The collagen matrix of dentin acts not only as a structural backbone but also as an important reservoir of various growth factors. The release of these embedded growth factors can be triggered during pathological processes such as dental caries or during biochemical conditioning of dentin with various bioactive agents.[13] The basic underlying mechanism involves demineralization of the

Table 1: Comparison of the mean transforming growth factor-beta 1 release among the groups using ANOVA

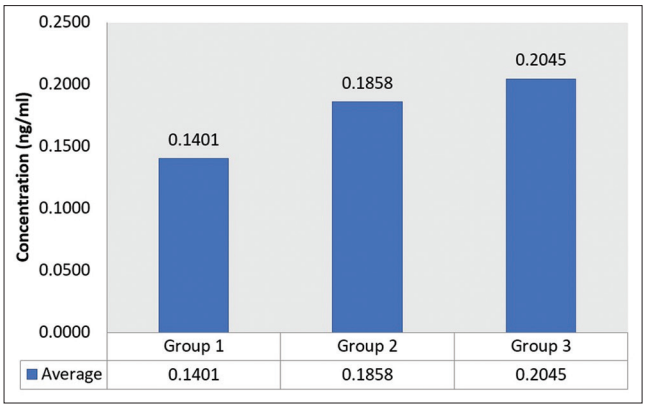
Groups	Count	Sum	Average	SD	One-way ANOVA	
					F	*P*
Group 1	14	0.980	0.1401	0.0261	9.4	0.001
Group 2	14	1.301	0.1858	0.0289		
Group 3	14	1.431	0.2045	0.0345		

SD: Standard deviation

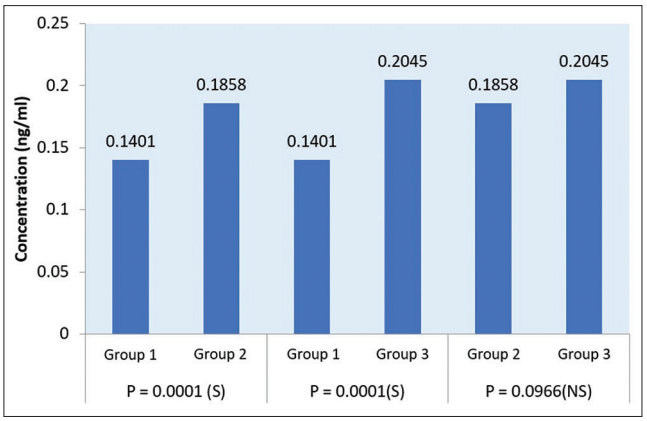
Table 2: Intergroup comparison using *post hoc* Scheffe's test

Groups	Count	Sum	Average	SD	*Post hoc* Scheffe's test	
					t	*P*
Group 1	14	0.980	0.1401	0.0261	5.612	0.0001
Group 2	14	1.301	0.1858	0.0289		
Group 1	14	0.980	0.1401	0.0261	6.01	0.0001
Group 3	14	1.431	0.2045	0.0345		
Group 2	14	1.301	0.1858	0.0289	1.85	0.0966
Group 3	14	1.431	0.2045	0.0345		

SD: Standard deviation



Graph 1: Comparison of the mean transforming growth factor-beta 1 release among the groups using One-way Analysis of Variance (ANOVA)



Graph 2: Intergroup comparison using *post hoc* scheffe's test. S: Statistically significant, NS: Not significant

inorganic component, which exposes the underlying organic extracellular matrix from which these growth factors can leach out.[4] The literature supports the evidence regarding the effect of EDTA on the release of this growth factor, however, there are no studies reporting the effect of doxycycline and propolis. Being proven to have the property of chelation, the present study was designed to evaluate and compare the effect of EDTA, doxycycline, and propolis in the release of TGF-β1 from the dentin matrix.

EDTA is often used as a final irrigant during regenerative endodontic procedures.[14] Being proven to influence the growth factor release, EDTA was used as a control group with which the other groups could be compared. Teixeira *et al*. showed that irrigation with EDTA and NaOCl for 1, 3, and 5 min were equally effective in eliminating the smear layer from root canal walls.[15] Accordingly, Calt and Serper evaluated the effect of EDTA on the smear layer removal after 1- and 10-min application time and concluded that a 1-min application was adequate and efficient in the removal of the smear layer.[16] Hence in the present study, the conditioning time was kept at 1 min for all three groups.

The smear layer removal ability and chelation property of doxycycline are well documented, however, its role in the release of TGF-β1 from the dentin matrix has not been established. The present study revealed a significant increase in the release of TGF-β1 following conditioning with doxycycline compared to EDTA. Barkhordar *et al*. evaluated the effect of doxycycline hydrochloride on the removal of the smear layer from the instrumented root canal walls. Compared to 15% EDTA, doxycycline was more effective in eliminating the smear layer in a concentration-dependent manner, with 100 mg/mL doxycycline being the most effective.[17] Santos *et al*. reported that doxycycline was effective in the removal of the smear layer completely from the coronal and middle third of the root canal.[18] Chae *et al*. suggest that using a more acidic irrigant than EDTA may have a benefit on the activation of latent TGF-β1. This can be explained by the fact that a more acidic environment causes denaturation of Latency associated peptide (LAP) resulting in significant activation of TGF-β1.[13] One hundred milligram/milliliter doxycycline HCl being acidic has a pH of 2 which can effectively cause activation of TGF-β1.[19] Cameron *et al*. report the detrimental effect of residual biofilm on the release of TGF-β and its consequent effect on the outcome of regenerative endodontic procedures.[20] Doxycycline also being bacteriostatic has added benefit of acting on residual biofilm without the release of bacterial byproducts. It has also got antiresorptive properties.[7]

In endodontics, propolis has been used as an irrigant, medicament, a storage media for avulsed teeth, in the management of resorption, and in vital pulp therapy.[21] In regenerative endodontics, propolis has been shown to be as effective as triple antibiotic paste when used as an intracanal medicament. In addition, it induces hard and soft tissue formation within the root canal when used as an orifice plug.[22]

Though the chelating ability of propolis is reported to be 70% as that of EDTA the present study showed a significantly higher TGF-β1 release for the propolis group compared to EDTA and doxycycline groups. Propolis is known to induce TGF-β1 release from odontoblast-like cells and human immune cells.[23] However, the release of TGF-β1 from dentin is attributed to the exposure of the dentin matrix which can be achieved by chelating the inorganic component of the matrix.[14] As an endodontic irrigant the antibacterial efficacy of propolis has been well established, but studies regarding its interaction with the smear layer are limited. Yuanita report that the enhanced smear layer removal ability of 8% propolis is due to the presence of "saponins" a natural detergent.[24] Moreover, Matochek *et al*., in their study on the effect of propolis on the bond strength of fiber post to root dentin, speculated that the aqueous-based propolis could remove the smear

layer from the dentin surface and also from within the tubules.[25] Moreover, flavonoids and polyphenols have known to have the property of chelation.[10] Similarly, the results obtained in the present study could be due to the chelating property and the synergistic interaction between the various biologically active substances found in propolis. Further analysis is required to verify its ability to chelate root dentin and to determine the active compounds involved specifically in the interaction with dentin.

CONCLUSION

Doxycycline and propolis can be used as effective alternatives to EDTA as final irrigant, especially during regenerative endodontic procedures. With the added benefits of having antibacterial and antiresorptive properties, doxycycline and propolis could provide an environment that is more favorable for the regenerative approaches and can contribute to the overall success of the therapy.

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Conflicts of interest
There are no conflicts of interest.

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