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Original Article

A comparative analysis of root surface biomodification with ethylene diamine tetra acetic acid and tetracycline hydrochloride: An in vitro scanning electron microscopic study

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ABSTRACT

Background: The outcome of periodontal regenerative therapy depends upon the of the biocompatibility root surfaces to the regenerating periodontal tissues. This in vitro scanning electron microscopic (SEM) study was designed to evaluate and compare the demineralizing efficacy of ethylene diamine tetra acetic acid (EDTA), with that of tetracycline hydrochloride applied on to the mechanically treated root surfaces of periodontally involved tooth.

Methods: Forty specimens were prepared from teeth extracted due to advanced periodontal disease and divided into two groups. The study group was treated with an EDTA solution (pH 7.4) and the control group was treated with a tetracycline hydrochloride solution (pH 1.8). The photomicrographs obtained were assessed for presence of smear layer, number of exposed dentinal tubules, area occupied by tubule orifices along with intertubular surface appearance. The results thus obtained were analyzed statistically.

Results: Both EDTA and tetracycline were effective in removing the smear layer and the exposure of the number of dentinal tubules. The diameters of the tubules and thereby the surface area occupied by the tubule orifices in the EDTA treated group were significantly greater than the tetracycline HCL treated group ($p < 0.05$).

Conclusion: The EDTA produced better effects than tetracycline by providing more demineralized area and collagen exposure at a neutral pH.

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Introduction

The final desired outcome of periodontal therapy is the arrest of progressive periodontal disease followed by the predictable regeneration of damaged periodontal tissues.

Periodontal diseases expose the root surfaces of teeth to the contents of the periodontal pocket which are contaminated with periodontal pathogens and endotoxins thereby triggering inflammatory reactions. Consequently, the root surfaces undergo changes in their physical, chemical and immuno-chemical properties as well as in their histologic appearances. All these changes alter the root surfaces, which become toxic and non-biocompatible to cells associated with periodontal healing and regeneration.¹

Root biomodification comprises of the alterations produced on the root surface by means of mechanical, chemical or combination of the two procedures to promote favorable root surface characteristics, as the decontamination of a periodontitis affected root surface by mechanotherapy alone is not possible.² A smear layer will cover the mechanically treated root surfaces, which contain remnants of calculus, contaminated cementum, subgingival plaque and the associated microorganisms. This smear layer impairs cell reattachment and can serve as a reservoir for microbial growth and thereby will inhibit new attachment.³

Demineralization treatment on affected root surface induces cementogenesis and enhances connective tissue attachment by an improved fibrin linkage and the periodontal ligament fibroblast migration. Demineralized dentin surfaces have a greater capacity to bind fibronectin than the surfaces which are not demineralized due to increased number of fibronectin binding sites on the exposed collagen matrix.⁴ The tufting of dentin or cementum matrix following demineralization will lead to an increase in the total surface area of the collagen and thereby the total number of binding sites for various proteins or growth factors which play an important role in wound healing and regeneration.⁵

The root treatment protocol earlier centered mainly on application of acids like citric acid and tetracycline hydrochloride.⁶ The use of low pH acting agents has been challenged, as their use might be detrimental to the surrounding vital periodontal tissues and may interfere with healing after the regenerative procedures.⁷ Studies have shown that ethylene diamine tetra acetic acid (EDTA), a chelating agent which can act at neutral pH is preferable over the other acid solutions because it preserves the integrity of surrounding periodontal tissues along with root surface demineralization and smear layer removal.⁸

With this background, a scanning electron microscopic (SEM) study was designed to evaluate and morphometrically analyze the demineralizing efficacy of an EDTA solution at lower concentration (15% and pH 7.4) with that of a tetracycline hydrochloride solution (pH 1.8), on root surfaces of periodontally involved tooth.

Material and methods

Twenty single rooted human teeth from patients with advanced periodontal disease were selected for use to

simulate the clinical use of root demineralizing agents and to account for the possible hypermineralization of diseased root surface.

The criteria for inclusion were unsavable, single rooted teeth which were periodontally compromised with clinical attachment loss of more than 5 mm. The general exclusion criteria were;

1. Carious teeth with periapical lesions
2. Root caries or erosions
3. Cervical restorations
4. Presence of enamel pearls or any cemental abnormalities
5. Recent periodontal therapy

The root surfaces were thoroughly planed and flattened using a fine diamond finishing bur in high speed hand piece under continuous water coolant to remove the diseased cementum and were re-examined under magnifying lenses to ensure complete calculus removal.

To obtain the experimental surface, the roots were then sectioned longitudinally into two equal halves which yielded a total number of forty specimens. Each of the specimens from the same root were assigned to different treatment groups and numbered. The specimens were cut into slabs of about $6 \times 6 \times 2$ mm in size. The slabs were then preserved in a mixture of anhydrous glycerol/absolute alcohol 1:1 by volume so as to preserve the tissues at the cellular organelle level and without denaturing the dentinal matrix, until the time of treatment as recommended by Wen et al.⁹

The prepared solutions were then applied with the help of applicator tips manually by a single operator to maintain the uniformity in the application pressure. Group I was treated with 15% EDTA solution in phosphate buffer solution (pH 7.4) and Group II were treated with 100 mg/ml tetracycline hydrochloride (pH 1.8) for 3 min.

The samples were prepared for SEM by first rinsing them in buffer solution and then washing with distilled water. They were dehydrated with a graded series of aqueous ethanol solution ranging from 70% to 90% and by air drying using a blower and then by keeping in desiccator jars containing silica gel overnight.

The specimens were then mounted on conductive carbon tape pasted on the standard specimen mounting SEM stubs. As the biological specimens have very low density and will lead to the absorption of electron beam resulting in low resolution, sputter coating was done with a thin layer of gold powder for 1 min using a Polaron SC7610 sputter coating device.

A Scanning Electron Microscope, Philips XL-20 manufactured by Philips Co. Netherlands with a standard computerized control system was used for the study. The electron beam parameters were kept constant while analyzing the samples and the SEM beam was maintained at a 0° tilt angle for achieving the standardization. The microscope accelerating voltage was kept between 15 kV and 20 kV for all the specimens at high vacuum and the photomicrographs were obtained at $1000\times$ and $2000\times$ magnifications. The specimens were then mounted under scanning electron microscope for surface topographical analysis. The specimens under the scanning electron microscope were examined for: -

- Presence or absence of smear layer
- Number of exposed dentinal tubules
- Diameter of individual tubules
- Individual and total surface area occupied by tubule orifices
- Depth of fibril tufting.

The recordings and the morphometrical calculations were compared and statistically analyzed. The Student's unpaired 't' test was used to compare the tubular diameters and the tubular surface areas between the EDTA group and the Tetracycline HCL group and using an SPSS 22.0 software.

Results

The appearances of each of the specimens within a treatment regimen were generally similar. All the samples except on specimen in each group showed incomplete removal of the smear layer (Fig. 1).

Most of the dentinal tubules in both the treatment group were patent (Fig. 2a & b and Fig. 3a & b). The mean number of patent dentinal tubules in Group I counted from photomicrograph of size 57.5×37.5 mm was 39.30 with a standard deviation of 4.105 and in Group II it was 41.00, with a standard deviation of 3.825. It was found that there were no significant differences between the groups for average number of tubules openings ($p > 0.05$).

The diameter of the tubule orifices were measured directly from microscope control computer software which had the facility for measurements in the original units. The mean tubule diameter in Group I was $2.678 \mu\text{m}$ with a standard deviation (SD) of 0.432. The mean tubule diameter of Group II was $2.360 \mu\text{m}$ with an SD of 0.431. Statistically it was found that the difference in average diameter in case of EDTA Group was significantly higher than the tetracycline HCL treated group ($p < 0.05$) [Table 1] (Fig. 4).

The next criterion was the surface area occupied by the individual tubule orifice. This was calculated using the formula $\pi d^2/4$ where 'd' is the diameter of the tubule orifice. The mean surface area of the EDTA treated group was $5.76 \mu\text{m}^2$ with an SD of 1.767 and in the tetracycline treated group it was $4.510 \mu\text{m}^2$ with an SD of 1.626. It was found that the area occupied

Fig. 1 – EDTA treated specimen showing minimal presence of smear layer at a magnification of 1000 $\times$.

Fig. 2 – (a): EDTA treated specimen showing patent tubules at a magnification of 1000 $\times$. (b): EDTA treated specimen showing patent tubules at a magnification of 2000 $\times$.

by an individual tubule in Group I was significantly higher than Group II ($p < 0.05$) [Table 2] (Fig. 5).

The total surface area occupied by the tubule orifices was calculated by multiplying the individual tubule surface area with total number of tubules. The mean total surface area in the EDTA treated Group I was $228.072 \mu\text{m}^2$ with an SD of 78.230 and in Group II was $186.211 \mu\text{m}^2$ with an SD of 71.584. The differences were found not to be statistically significant ($p > 0.05$).

The last criterion in the morphological analysis was the appearance of the intertubular surface and fibril tufting. It was found that all the specimens in both the groups had roughened appearance of the intertubular matrix while six out of twenty in the EDTA group and five out of twenty in tetracycline group demonstrated a fluffed appearance (Fig. 6).

Discussion

Periodontal diseases cause pathological alteration of periodontium, manifested as apical migration of the junctional epithelium, loss of connective tissue attachment to the tooth and loss of supporting alveolar bone. The treatment of this disease with multifactorial pathogenesis is directed at arresting the progression of these events which subsequently result

Fig. 3 – (a): Tetracycline HCL treated specimen showing patent tubules at a magnification of 1000×. (b): Tetracycline HCL treated specimen showing patent tubules at a magnification of 2000×.

Table 1 – Inter group comparison of mean tubule diameter ($d = \mu\text{m}$).

Group	EDTA group (20)	Tetracycline HCL group (20)
Mean	2.678	2.360
Standard Deviation	0.432	0.431
t – value 2.330279, df = 38,		p – value 0.025201
The difference in average diameter between the two groups was statistically significant ($p < 0.05$).		

in repair of the periodontal wounds. However, the healed tissue may not necessarily replicate the original architecture and function.¹⁰ Periodontitis affected root surfaces always exhibit the loss of collagen fiber insertion and are contaminated with bacteria and endotoxins.¹¹ Subsequently, the affected root surface becomes biologically incompatible with the adjacent periodontal cells, the proliferation of which is pivotal for new attachment and regeneration.¹²

The outcome of most of the regenerative periodontal therapies depends upon the modification of the contaminated root surfaces in order to restore its biocompatibility and to favor the attachment of the regenerated periodontal structures. Superficial demineralization of a periodontally involved root is an important step in the natural healing process and topical application of demineralizing agents to

the root surface during periodontal surgery shorten the biological sequence of events through producing artificial demineralization of root surface.¹³ Further, an SEM study on teeth with fluorosis and periodontal diseases observed hypermineralization of the cementum and mineralization of the periodontal fibers and cemental collagen was exposed more on application of super saturated solutions of EDTA.¹⁴

The concept of acid demineralization of root surfaces in periodontal therapy dates back to the turn of nineteenth century.¹⁵ Studies of Urist¹⁶ opened a new era in relation to inductive properties of demineralized dentin. It was evident from his studies that dentin following acid demineralization possessed inductive proteins which had the ability to induce differentiation of cells.

The mechanisms by which root surface modifiers can facilitate regeneration include effective removal of smear layer resulting from instrumentation, the removal of cementum-bound endotoxin and the exposure of the root associated collagen.¹⁷ All these mechanisms lead to a sequence of events which can be narrated as early fibrin linkage, increased fibronectin binding sites as well as accelerated mesenchymal cell adhesion, chemotaxis and growth. Simultaneously the apical growth of epithelium is inhibited followed by early cementogenesis.^{18,19}

Till recently, tetracycline HCL which acts in an acidic pH (1.8) was used as the root biomodification agent. Surface demineralization with tetracycline HCL enhances binding of matrix proteins to dentinal collagen matrix and stimulates fibroblast attachment and growth.²⁰

EDTA is the only agent in clinical use that exerts its demineralizing effect by chelating divalent cat ions like calcium of the mineralized tissue at neutral pH. In addition to smear layer removal and detoxification, it selectively removes the hydroxy apatite from the collagenous dentin matrix which is not achieved by other agents that act in acidic pH. Also, the optimal pH for vital fibroblast cell function varies between 7.2 and 7.4, though some cells may tolerate pH of 6.6–7.8. Thus the vitality of surrounding periodontal tissues with which the EDTA solution comes into contact is preserved.^{21,22}

The present study was undertaken after considering the current fact that the periodontally affected root surfaces does not favor regeneration of the tissues due to the altered surface characteristics and the recommendations for the application of EDTA which acts at a neutral pH, as an adjunct in regenerative therapies which involve various growth factors, bone morphogenetic proteins and enamel matrix derivatives.^{23,24}

The treated root surfaces were scanned with the beam of SEM which was standardized at a 0° tilt angle to assess the surface characteristics and to measure the other parameters accurately.

Studies conducted earlier to find out the capability of various root biomodifying agent have demonstrated the effectiveness of both EDTA and tetracycline HCL in surface demineralization and smear layer removal (Fig. 1). The observation in this study indicated that both EDTA and tetracycline effectively removed the smear layer and thus is in accordance with the earlier studies.^{21–23}

Fig. 4 – Inter Group comparison of tubule diameters.

Table 2 – Inter group comparison of mean individual tubule surface area ($d = \mu\text{m}^2$).

Group	EDTA group (20)	Tetracycline HCL(20)
Mean	5.769	4.510
Standard Deviation	1.767	1.626
t – value 2.343242	df = 38,	p – value 0.024452
The difference in surface areas occupied by individual tubules in Group I was significantly higher than Group II ($p < 0.05$).		

The mean number of tubule openings were 39 in the EDTA and 41 in the tetracycline HCL treated group and the differences were not statistically significant.

The average diameter of the exposed tubules of a specimen was calculated by dividing the total diameter of tubules in the

specimen by total number in the specimens. In the present study it was observed that the mean average of EDTA group (2.678 ± 0.432) was higher than the tetracycline treated group (2.360 ± 0.431). The difference in the tubule diameter was found statistically significant ($p < 0.05$) [Table 1] (Fig. 4). The calculated mean surface area of the tubules orifices in the EDTA treated group was significantly higher compared to that of TTC treated group [Table 2] (Fig. 5).

An increase in diameter of the tubule leads to an increase in surface area which in turn maximizes the number of binding sites for factors and proteins which stimulate the regeneration processes.⁵ As observed in the present study EDTA appears to be effective in promoting the process by providing the increased surface area.

The available relevant literature highlighted the fact that physiological tufting of intertubular collagen fibrils on the root

Fig. 5 – Inter Group comparison of surface area occupied by individual tubules.

Fig. 6 – EDTA treated specimen showing fibril tufting at a magnification of 2000 $\times$.

surface may optimize the biological mechanisms essential for successful new attachment and regeneration. These include fibronectin binding, fibroblast chemotaxis and proliferation, all of which lead to a more stable clot formation that is resistant to epithelial migration.^{1,5,6} It was observed that both the demineralizing solutions produced fibril tufting (Fig. 6), in an average graded scale of one which is in support of the previous studies.^{5,13}

The limitations of the present study are; being an in vitro study with a limited sample size, the findings cannot be extrapolated directly to an in vivo situation even though there is biological explanation or plausibility. The definitive way to ascertain the clinical efficacy of root biomodification is by conducting randomized controlled trials with adequate statistical power in a large population and with long term follow up as the most definitive method by human histological studies are ruled out due to ethical constraints. It may be worth mentioning that conflict between the thought processes will keep on arising, not because of the lack of biological reasoning but due to the lack of enough studies with sufficient statistical powers in the current literature.

Conclusion

Even though controversies exists on the role of root surface biomodification on the successful outcome of periodontal regeneration, it may be borne in mind that the initial wound healing is always better over a viable root surface devoid of any smear layer or biofilm. The demineralization causes increased total surface area available for the possible binding of extra cellular matrix proteins which play an important role in new attachment and regenerative therapies.

The results of the present study conclude that EDTA at lower concentrations widened the dentinal tubule openings on the treated surface which were comparable with other demineralizing agents. Moreover, as EDTA act at a neutral pH, the vitality of the surrounding periodontal tissues can be maintained thereby providing a suitable environment for periodontal healing.

Conflicts of interest

The authors have none to declare.

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IMPORTANT

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