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RESEARCH ARTICLE

EFFECTS OF VARIOUS ROOT SURFACE BIOMODIFICATION AGENTS ON PERIODONTITIS-AFFECTED TEETH: A SCANNING ELECTRON MICROSCOPIC STUDY

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ABSTRACT

Background & objections: Changes in root surfaces in the environment of periodontal pocket hinder regeneration of the periodontal tissues. Periodontal treatment, therefore, must create a root surface compatible for the cells that mediate repair and regeneration of the periodontal tissue. To achieve this, a procedure called root biomodification is recommended as an adjunct to scaling and root planing. Various agents, namely citric acid, phosphoric acid, ethylene diamine tetraacetic acid (EDTA), tetracycline hydrochloride, hydrogen peroxide, fibronectin, enamel matrix proteins, recombinant human growth factors, dentin bonding conditioners, *etc.*, have been tried for root biomodification. However, the role of root biomodification in regeneration of the periodontal tissues is questionable. Considering this, an *in vitro* study was carried out to evaluate the morphologic characteristics of the periodontitis-affected roots after biomodification using citric acid, ethylene diamine tetraacetic acid (EDTA) and tetracycline hydrochloride (HCl) solutionunder scanning electron microscope (SEM). Since first step in periodontal regeneration is the adherence of blood clot to the root surfaces, the study is also intended to evaluate the attachment behaviour of blood clot to those biomodified roots. **Materials and Methods**: The present study was carried out on periodontally compromised human anterior teeth (n=40) comprising of maxillary (n=20) and mandibular (n=20). They were randomly divided into four groups (comprising ten teeth in each group) based on the biomodification agent used: Group 1 (control group): treated with normal saline, Group 2: treated with citric acid, Group 3: treated with EDTA and Group 4: treated with tetracycline HCl solution. Two specimens were prepared from each tooth. One of the specimens was evaluated for the patency of dentinal tubules after treating with the allotted biomodification agent, while other was evaluated for clot adhesion following treating with blood after biomodification under SEM. **Results**: Numbers of the patent dentinal tubules and scores of the blood clot adhesion were found to be significantly higher in all the three test groups compared to that of the control group. **Conclusions**: Biomodification of root surfaces may play a significant role in accomplishment of periodontal new attachment by increasing the patency of dentinal tubules and making the root surfaces more conducive for blood clot adhesion.

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INTRODUCTION

Periodontitis is an inflammatory disease of the supporting tissues of teeth caused by specific or group of specific microorganisms resulting in progressive destruction of periodontal ligament and alveolar bone with pocket formation, recession or both. Substantial alterations have been observed in the root surfaces exposed to the environment of the periodontal disease. The changes include loss of collagen fiber insertion, alterations in mineral density and composition, contamination of the root surface by bacteria and or endotoxins, and may lack the necessary chemotactic stimuli for migration of cells.

These changes in the root surfaces make them non-compatible to the periodontal cells that play the fundamental role in periodontal wound healing and subsequently, interrupt the healing process and interfere in attainment of the ultimate goal of the periodontal therapy (Newman *et al.,* 2007). The goal of the periodontal therapy includes the arrest of disease progression and regeneration of the lost periodontium due to periodontal disease as well as the prevention of disease recurrence (Polson and Caton, 1982). The first step of the periodontal regeneration is the adherence of blood clot to the root surface, which is stabilized by a network of fibrin through which undifferentiated mesenchymal cells from the periodontal

ligament migrate towards the root surface and forms the collagen fibers and allows regeneration of the cementum, periodontal ligament and alveolar bone by preventing apical migration of the junctional epithelium (Theodoro *et al.*, 2006; Polimeni *et al.*, 2009). Therefore, periodontal treatment must create a root surface which is compatible for the cells that mediate repair and regeneration of the periodontal tissue (Wikesjo and Nilveus, 1990). So as a part of treatment modality, scaling and root planing is performed to remove the residual embedded calculus and portions of cementum from the roots to produce a smooth, hard, clean surface (Newman *et al.*, 2007). On the other hand, a saline-resistant smear layer consisting of organic and mineralized debris has been demonstrated over the mechanically debrided root surfaces (Labahn *et al*., 1992), which inhibits the attachment of cells and thus, forms a physical barrier between the periodontal tissues and root surface. Again, this smear layer hides the dentinal collagen which takes active part in periodontal healing by serving as chemoattractant for the periodontal fibroblasts. Thus, it impairs the periodontal wound healing (Polson *et al.*, 1984). Therefore, a procedure for removal of this smear layer to facilitate exposure of the dentinal collagen fibers as an adjunct to mechanical root surface debridement is recommended, referred to as root biomodification (Blomlof and Lindskog, 1995). Various agents have been anticipated for root biomodification, such as citric acid and phosphoric acid, ethylene diamine tetraacetic acid (EDTA), tetracycline hydrochloride (HCl), hydrogen peroxide, fibronectin, enamel matrix proteins, recombinant human growth factors namely platelet-derived growth factor and dentin bonding conditioner (Abitbol *et al.*, 1996; Shewale *et al.*, 2016).

In addition to these chemicals, different laser systems, namely CO_2, Nd:YAG, diode and Er:YAG have also been used for the purpose of root biomodification (Pant *et al*., 2004; Schwarz *et al*., 2003). However, EDTA, tetracycline HCl and citric acid have gained popularity and have become the most commonly used agents. Since root biomodification provides a biocompatible surface for attachment of the cells, matrix deposition and also improves the mechanical interfacial bonding, it may be employed as a part of periodontal regenerative procedures (Bosshardt and Sculean, 2009). Thus, it becomes a recommended step in the treatment of intrabony defects, in cosmetic gingival reconstruction prior to placement of implants, and as primary treatment for class II furcation defects with or without bone grafts (McClain and Schallhorn, 1993). However, a systematic meta-analysis review stated that use of citric acid, tetracycline or EDTA to modify the root surface provides no benefit of clinical significance in regeneration of chronic periodontitis (Shewale *et al.*, 2016). Thus, root biomodification is still a debatable issue and its application in regenerative periodontal therapy is still questionable. Moreover, it is not yet clear which of the chemical agents is more effective in root biomodification. So, an *in vitro* study was planned to evaluate the morphologic characteristics and attachment behaviour of blood clot to periodontally compromised human root surfaces after root biomodification using citric acid, EDTA and tetracycline HCl under scanning electron microscope (SEM).

MATERIALS AND METHODS

The study was conducted partly in the Department of Periodontics and Oral Implantology, Regional Dental College and Hospital, Guwahati and partly in the Directorate of Forensic Sciences, Guwahati. The experiment was carried out on a total number of 40 periodontitis-affected human anterior teeth comprising of maxillary (n=20) and mandibular (n=20), which were either of 2 or 3 mobile and extracted due to poor prognosis. The freshly extracted teeth were used. Immediately after extraction, teeth were thoroughly rinsed under running tap water to cleanse the surface being covered with blood or debris. The teeth were then stored in normal saline at room temperature to maintain the hydration until use. The teeth were randomly divided into four groups, comprising ten teeth in each, based on the test solution used for root biomodification:

- **Group 1** (Control group): Normal saline
- **Group 2:** Citric acid solution
- **Group 3:** EDTA solution
- **Group 4:** Tetracycline HCl solution

Preparation of the root biomodification solutions

- **Normal Saline:** 0.9% w/v normal saline, pH 7 (Pareneteral Drugs Ltd., Jalandhar)
- **Citric Acid:** Fresh citric acid solution was prepared by slowly adding anhydrous citric acid powder (Butterfly Co. Pvt. Ltd., Gujrat) to the distilled water in a sterile dappen dish under continuous stirring motion until saturated at pH 1, measured using pH paper (Thermo Fisher Scientific India Pvt. Ltd., Mumbai).
- **EDTA:** 17% EDTA, pH 1 (Endo Canal Prep, Ramen Research Products, Kolkata, India)
- **Tetracycline HCl:** The content of tetracycline HCl capsules (500 mg) (Piramal Healthcare Ltd., Gujrat) was thoroughly mixed with 5 ml of distilled water in a sterile dappen dish under continuous stirring motion for 5 minutes until a viscous solution is obtained at pH 1.

Preparation of the specimen: In the present study, root portions of the periodontitis-affected teeth were used. The extracted teeth were first thoroughly washed with distilled water and then root planing was carried out using sharp Gracey Curettes (Hu-Friedy, Chicago) with 6 to 8 stroke per area to achieve a smooth surface. Two parallel horizontal grooves were then made on proximal surface of the root of each tooth using a diamond tapered fissure bur with air rotor handpiece under copious irrigation. The first groove was made at the cementoenamel junction and the second groove was made approximately 5 mm away from the first groove on root. Then both coronal and apical portions of the teeth were discarded, keeping cervical 5 mm of the root portions for the study. The root portion was sectioned longitudinally through the pulp canal using a double-sided diamond disc with a slow-speed hand piece under copious water irrigation. Thus two specimens (approximately 5 mm x 5 mm x 1 mm) were obtained from a single tooth and these were stored separately in order to avoid the mismatch amongst the specimens.

Biomodification of the specimens: All the specimens (n = 80) obtained from the extracted teeth (n = 40) were treated by allotted agents in the different groups (n = 4) using a camel brush. The agents were applied every 30 seconds for a total of five minutes to ensure consistent application. Then the

specimens were thoroughly rinsed with distilled water and air dried and stored separately.

After this, one of the two sections made from each root was evaluated morphologically under SEM for patent dentinal tubules (n = 40), while the other sections were treated with blood after biomodification (n = 40) and then evaluated under SEM for blood clot adhesion. Thus, each group was having 2 subgroups:

- a = only biomodification (namely, 1a, 2a, 3a and 4a)
- b = biomodification followed by treatment with blood (namely, 1b, 2b, 3b and 4b)

Preparation for adhesion of blood components: Blood was obtained from a 27 years old healthy female with no systemic diseases and habits. It was collected from the peripheral circulation using a 2 ml disposable syringe with 26 gauge needle (Dispovan, Hindustan Syringes & Medical Devices, Faridabad). Blood was then deposited on the specimens following treating them with their respective root biomodification agents and maintained for 20 minutes. They were then washed three times for five minutes in normal saline (0.9% NaCl w/v, pH 7) using gentle swirling motion. Specimens were then fixed in 2.5% glutaraldehyde for 30 minutes and followed by washing with normal saline. The specimens were then dehydrated overnight.

The scanning electron microscope: The SEM used in the present study was JSM 6380LA Analytical Scanning Electron Microscope (JEOL, Japan). It records the electrons emitted from the surface of the sample and gives three-dimensional image. Since tooth is nonconductive material, samples were made conductive by coating with a thin layer of carbon using vacuum Ion-sputtering device (JEOL JEC-560). These coated samples were then inserted in the SEM. As beam of electron falls on the specimen, the secondary electrons generated were captured. With the help of detection and signal processing system these were converted into the image, which was further transferred to the display unit. In the present study, the surface of the specimen was observed on the computer screen at magnification of 3000 x and photographs were obtained randomly.

Examination of Photographs: Using a single blind method, the photographs obtained using SEM were analyzed by four previously trained operators. The photographs obtained from the specimens in subgroup 'a' were analyzed for the total number of patent dentinal tubules, while those in subgroup 'b' were analyzed for the adhesion of blood clot using the criteria set by Theodoro *et al*., (2003):

- Score 0: Absence of fibrin network and blood cells
- Score 1: Scarce fibrin network and/or blood cells
- Score 2: Moderate fibrin network and moderate quantity of blood cells
- Score 3: Dense fibrin network and trapped blood cells

The data collected from the study was compiled by single blind operator and statistical analysis was performed.

RESULTS

Patency of dentinal tubules: The patency of the dentinal tubules was evaluated in the photographs obtained using SEM. The effects of normal saline (group 1a), citric acid (group 2a), EDTA (group 3a) and tetracycline HCl (group 4a) solution on the patency of dentinal tubules are shown in Figure 1. As shown in the Figure 1, an amorphous irregular surface with no evidence of patent dentinal tubules is seen in the specimens treated with normal saline (Figure 1a), while patent dentinal tubules were clearly visible in the specimens treated with citric acid (Figure 2a), EDTA (Figure 3a), and tetracycline HCl (Figure 4a). The Mean number of patent dentinal tubules in all the specimens is shown in Table 1. The mean number of patent dentinal tubules in the group treated with normal saline (control/1a) was found to be 2.10 (ranges being 0 - 6). In contrast, the mean number of patent dentinal tubules was found to be 16.00 (ranges being 10 - 25), 14.00 (ranges being 9 - 19), 19.30 (ranges being 6 - 26) in the specimens treated with citric acid (group 2a), EDTA (group 3a) and tetracycline HCl (group 4a), respectively. It is depicted in Figure 2.

The number of patent dentinal tubules in different groups was compared using parametric test (analysis of variance) and the difference was found to be statistically highly significant ($p<0.01$). To confirm this further, mean rank was obtained using non-parametric Kruskal-Wallis test. The mean rank in the group 1a was 5.55, while the mean rank was found to be 24.80, 21.40 and 30.25 in the group 2a, 3a and 4a, respectively. The number of patent dentinal tubules was found to be significantly very higher in the specimens treated with citric acid, EDTA and tetracycline HCl compared to that of the control group ($p=0.000$). However, differences in the patent dentinal tubules among the test groups were found to be not significant statistically ($p> 0.05$).

Table 1. Mean ± SEM of patent dentinal tubules in control and test groups (ranges are shown in bracket)

Group (number)	Mean ± SD (range)
1a (n = 10)	2.10 ± 2.02 (0 - 6)
2a (n = 10)	16.00 ± 4.81 (10-25)
3a (n = 10)	14.00 ± 3.46 (9-19)
4a (n = 10)	19.00 ± 6.43 (6-26)

Clot adhesion: The specimens in group 'b' (which received blood following biomodification) were evaluated for blood clot adhesion using SEM at the magnification of 3000x and photomicrographs were taken (Figure 3). Distribution of the clot adhesion score in all four groups is shown in Table 2. In control group (1b) (n=10), no specimens showed attachment of fibrin or blood cells (score - 0), while in citric acid-treated group (n=10), 70% of the specimens showed moderate fibrin network and blood cell attachment (score 2), while rest of the 30% showed scarce fibrin network and blood cell attachment (score 1). In EDTA-treated group (n=10), 50% of the specimens showed dense fibrin network and entrapped blood cells (score 3), while 40% and 10% of the specimens showed blood clot adhesion score of 2 and 1, respectively. Similarly, tetracycline HCl-treated group (n=10), 60% of the specimens showed blood clot adhesion score of 2 and rest of the 40% showed the score 3. Notable that none of the specimens in test groups showed 0 score of the blood clot adhesion. Graphical representation of the clot adherence scores percentage wise of all the groups is shown in Figure 4. The adhesion of fibrin network and blood cells was found to be significantly higher in the citric acid (subgroup 2b), EDTA- (sugroup 3b), and tetracycline HCl- (subgroup 4b), treated groups compared to that of the control group (subgroup 4b) ($p<0.01$).

On intergroup comparison among the three test groups, the adhesion of fibrin network and blood cells was found to be

Table 2. Distribution of clot adhesion scores

Group	Clot adhesion score			
	0	1	2	3
1b (n= 10)	10 (100%)			
2b (n= 10)		3 (30%)	7 (70%)	
3b (n= 10)		1 (10%)	4 (40%)	5 (50%)
4b (n= 10)			6 (60%)	4 (40%)
Total	10	4	17	9

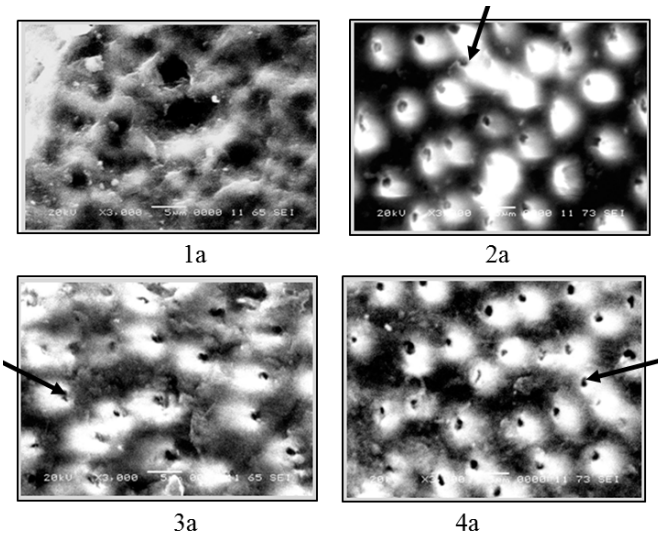


Figure 1. *Surface Morphology*. Note the absence of patent dentinal tubules in 1a, while dentinal tubules are patent in all other specimens indicated by arrows (2a = citric acid-treated; 3a = EDTA-treated and 4a = Tetracycline HCl-treated). (bar = 5 m; magnification = 3000 x)

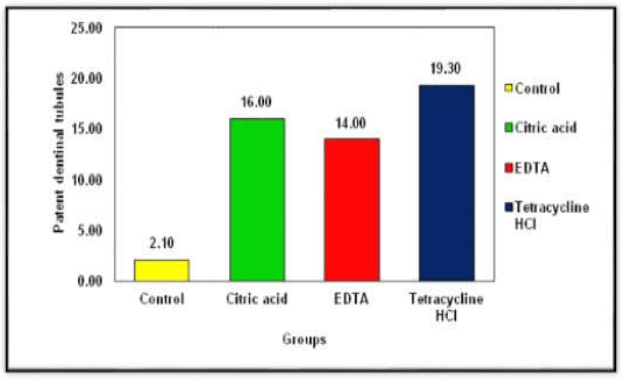


Figure 2. *Number of patent dentinal tubules* Note the lowest number of patent dentinal tubules in control specimens than that of the treated specimens

higher in EDTA- and tetracycline- treated group in comparison to the citric acid-treated group, which was statistically significant ($p<0.05$). Though the adhesion of fibrin network and blood cells was found to be higher in EDTA-treated (subgroup 3b) (10.70) than that of tetracycline HCl-treated (subgroup 4b) (10.30), the difference was not found to be significant statistically ($p>0.05$).

DISCUSSION

Historically, the use of acids in lieu of scaling and root planing was first reported in the New York Dental Record in 1846 and later by Younger (1893, 1897) and Stewart (1899) described

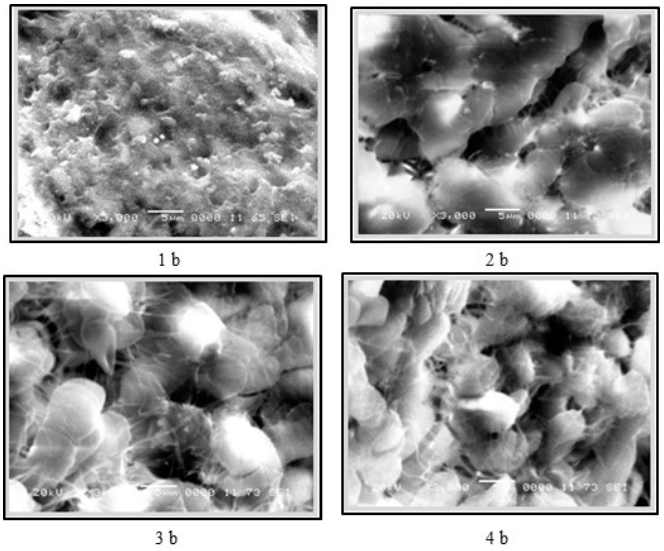
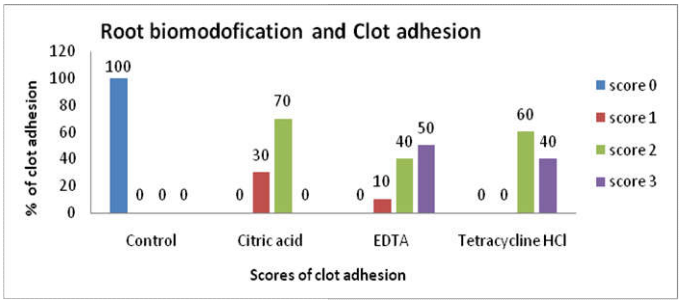


Figure 3. *Clot Adhesion*. Note the absence of fibrin network and blood cells (scroe 0) in 1b, while more fibrin network and blood cells adhere to the other specimens (2b = citric acid-treated; 3b = EDTA-treated and 4b = Tetracycline HCl-treated) (bar = 5 m; magnification = 3000 x)



Note that score '0' in the control group (100%), while none of the specimens in test groups showed 0 score of the blood clot adhesion

Figure 4. Percentage distribution of the clot adhesion scores

the use of acids in conjunction with the mechanical removal of calculus and cementum considering the microscopic evidence of hypermineralization of the roots with obliteration of lacunae of cellular cementum by calcific deposits (Register and Burdick, 1976). Since then various *in vitro* as well as *in vivo* studies have been carried out to evaluate the use of root biomodification as an effective intermediate step in enhancing periodontal regeneration which has been the relentless goal of the periodontal therapy. Several studies have indicated that root biomodification enhances the outcome of periodontal regenerative procedures (Maruyama *et al*., 2008; Lee *et al*., 2010). At the same time, some studies have pointed out that root biomodification have no added advantage in periodontal regeneration and may negatively influence on regenerative therapies (Aukhil and Pettersson, 1987, Dilsiz *et al.,* 2010). Thus, the effectiveness of root biomodification on periodontal regeneration still remains uncertain. So, this study was conducted with an objective to evaluate the effects and compare the efficacy of various root surface biomodification agents on periodontally-compromised human anterior teeth (n= 40). The root biomodification agents selected for the study were citric acid, EDTA and tetracycline HCl.

Normal saline was used as control. Periodontally compromised extracted teeth were considered in this study, on notion that effects of citric acid, EDTA and tetracycline HCl may be directly predictable *in vivo* situation. Again, only single rooted teeth were used in order to avoid bias on

prediction of findings in between single and multirooted teeth. Teeth affected by caries were not included as it could adversely affect the root surface topography (Levespere and Yukana, 1996). Teeth with immediate past history of scaling and root planing procedures were excluded as these procedures may alter the root surface topography. Teeth with attrition, abrasion and erosion were excluded, since secondary changes in the tooth structure, such as in mineral composition and formation of sclerotic dentin occurs in these situations (Ashwini and Mehta, 2001). The specimens were collected according to the protocol suggested by Sterrett *et al*., (1995). The cervical half of the root was attained by preparing two horizontal grooves at 5 mm apart, which was sectioned longitudinally through the root canal with a double-sided diamond disc in a slow-speed hand piece under copious water irrigation. This methodology of specimen preparation was suggested by Grisi *et al*., (2006) and the methodology used for adhesion of blood components was according to Thedoro *et al.*, (2006). Though various agents have been tried for root biomodification, few agents, namely EDTA, tetracycline HCl and citric acid are found to be safe to the surrounding tissues and they have gained popularity (Polson *et al*., 1984; Isik *et al.,* 1997). Tetracycline HCl is preferred over doxycyline and minocycline, as it removes the smear layer fully that forms after scaling and root planing. Various methods of application of root conditioning agents have been tried. These include an active burnishing, passive application and immersion technique. In the present study, passive application technique was used. Because the studies suggested that removal of the smear layer and opening of dentinal tubules varies with the agents irrespective of the methods used (Babay, 1995; Babay and Mokeem, 2005). Moreover, active burnishing may itself form a smear layer and thus obliterates the dentinal tubule (Shetty *et al*., 2008). In comparison to the conventional light microscope, SEM employs electrons rather than light to form an image. Combination of higher magnification, large depth of focus and greater resolution has made SEM an important investigative tool. Considering this fact, SEM was used in the present study to evaluate the patency of dentinal tubules and blood clot adhesion (Babay and Mokeem, 2005, Grisi *et al*., 2006, Galli *et al.*, 2009; Ishi *et al*., 2009; Lee *et al*., 2010). Since tooth is non-conductive in nature, electrons cannot be emitted from the surface and they cannot be directly viewed under SEM, therefore samples were made conductive using an ion-sputtering device under high vacuum.

Evaluation of the photomicrographs revealed that control specimens (group 1a) presented an amorphous irregular surface with no evidence of patent dentinal tubules. This is probably due to the presence of the surface smear layer, consisting of organic and inorganic materials, that appears following the root surface instrumentation (Polson *et al*., 1984). Debris was observed in some areas which could be the fragments of enamel, cementum, or dentin (chipped off during instrumentation), contaminated foreign materials entrapped during preparation of the specimen or a combination of these, as noted by Lasho *et al*., (1983). In contrast, the surfaces treated with citric acid (group 2a), EDTA (group 3a) and tetracycline HCl (group 4a) showed a significant number of patent dentinal tubules, which is probably related to the removal of the smear layers that results in exposure of the dentinal tubules. This observation is consistent with the findings of Lafferty *et al*., (1993), Trombelli *et al*., (1994) and Babay (2000). Blood clot adhesion to the biomodified root specimens was evaluated in the photomicrographs obtained using SEM technique following the criteria given by Theodoro *et al*., (2003). In the present study, clot adhesion to the control specimens (group 1b) was found to be negative (score 0), while clot adhesion on biomodified root specimens varies from a scarce fibrin network along with blood cell attachment (score 1) to a dense fibrin network along with entrapment of blood cells (score 3). The difference in clot adhesion between the treated and control root specimens was highly significant statistically ($p<0.01$). The reason of absence of the blood clot on the untreated control specimens may be related to the presence of smear layer that impedes the dentinal tubules (Polson *et al.,* 1984). Similar observation of greater number of fibroblast attachment to the biomodified root surfaces compared to that of the saline was observed by Babay (2001), Babay and Mokeem (2005) and Silverio *et al*., (2007). However, our finding is in contradiction of Leite *et al*., (2005) who noted maximum capture of blood cell in a thick network of fibrin on teeth treated by root planing alone. A greater variation in blood clot adherence was noted on the root specimens treated with biomodification agents, from score 1 to 3. However, none of the specimens treated with saturated citric acid, EDTA and tetracycline HCl showed the blood clot adherence score of 0. Blood clot adherence score was noted maximum on EDTA-treated roots (50%), which may be related to its demineralizing effect through the chelation of bivalent cations at neutral pH without impairing the vitality of periodontal tissue. However, the findings of the present study contradict the observations of Delazari *et al*., (1999), Blomlof and Lindskog, (1995) and Leite *et al*., (2005) who suggested that since EDTA is a calcium chelator, it inhibits or retards the coagulation events. In comparison to the root specimens treated with citric acid, the root specimens treated with tetracycline HCl showed better clot adherence. This is again in support of the observation of Frantz and Polson, (1988), Wikesjo *et al*., (1988) and Alger *et al.*, (1990) and may be related to the superficial demineralization obtained with tetracycline HCl (Claffey *et al*., 1978). Differences between the findings of the present study and those of other studies may be related to the disease status of the root surfaces used for evaluation, time and mode of application, and concentrations of the biomodification agents or a combination of these variables.

Glossary of Abbreviations:

CO_2: Carbon dioxide

EDTA: Ethylene diamine tetraacetic acid

Er:YAG: Erbium: Yttrium Aluminum Garnet

HCl: Hydrochloric acid

NaCl: Sodium chloride

Nd:YAG: Neodymium-doped: Yttrium Aluminum Garnet

pH: Pourvoir hydrogen

SEM: Scanning Electron Microscope w/v:Weight/volume

Conclusion

In the light of the present study carried out to evaluate the effects of various root surface biomodification agents on

periodontitis-affected teeth, the agents used namely citric acid, EDTA and tetracycline HCl unwraps the dentinal tubules and makes the root surface more conducive for blood clot adhesion. These findings thus, suggest that these agents may play a significant role in attainment of periodontal new attachment.

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