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Original Article

Fibrin clot adhesion to root surface treated with tetracycline hydrochloride and ethylenediaminetetraacetic acid: A scanning electron microscopic study

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ABSTRACT

Background: Connective tissue attachment following periodontal regenerative surgery is directly related to the attachment of fibrin clot on to the root surface during early wound healing events. The adhesion of fibrin clot to the root surface affected by periodontal disease depends on the biologic acceptance of the root surface which can be accomplished by various root conditioning procedures during periodontal therapy. The present *in vitro* study has been designed to evaluate and compare the degree of fibrin clot adhesion to root surfaces treated with root conditioning agents tetracycline hydrochloride and ethylenediaminetetraacetic acid (EDTA).

Materials and Methods: A total of 30 dentin blocks are divided into three groups and treated with tetracycline hydrochloride, EDTA and phosphate buffered saline and a drop of blood is added to each dentin block. The dentin blocks are then prepared for scanning electron microscopic analysis and examined for the degree of fibrin network frmation and entrapped erythrocytes.

Results: The degree of fibrin clot adhesion was highest with tetracycline hydrochloride group, then with control group and least with EDTA treated group.

Conclusion: According to the results of the present study, root conditioning with tetracycline hydrochloride produces a biologically acceptable root surface with enhanced fibrin clot adhesion, which is a critical step in early wound healing process. EDTA gel appears less effective in producing a root surface necessary for the adhesion of fibrin clot. The control without any root conditioning procedure showed poor fibrin clot adhesion when compared to tetracycline treated group, but when compared to EDTA treated group the fibrin clot adhesion was slightly better.

Key Words: Ethylenediaminetetraacetic acid, fibrin clot, root conditioning, tetracycline hydrochloride, wound healing

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INTRODUCTION

Periodontal diseases are infections of the periodontium producing complex inflammatory, enzymatic and other biologic influences that cause physical and chemical alterations particularly apparent in the root cementum.

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The formation of connective tissue attachment after regenerative therapy is directly related to the adhesion of fibrin clot to root surface during early wound healing events.[1] Fibrin clot mediates initial attachment of the gingival tissues to the root surface and the matrix of fibrin serves as a scaffold for cell migration, attachment and collagen synthesis. The adhesion of fibrin clot to the root surface affected by periodontal disease depends on the biologic acceptance of the root surface and tensile strength of the healing wound.[2,3] Root biomodification with root conditioning agents removes the smear layer and exposes the dentinal tubules and the intra and peri-tubular dentin collagen matrix.[4] *In vitro* studies

have shown improved fibrin clot adhesion to conditioned root surfaces.[5] Evidence shows the formation of a new connective tissue attachment rather than an epithelial attachment when periodontally affected root surfaces are treated by root conditioning after mechanical instrumentation.[1,3] The present *in vitro* study has been designed to evaluate and compare the degree of fibrin clot adhesion to root surfaces treated with root conditioning agents tetracycline hydrochloride and ethylenediaminetetraacetic acid (EDTA).

MATERIALS AND METHODS

Preparation of dentin blocks

Thirty dentin blocks approximately 4 mm × 6 mm × 1 mm in size, were prepared from the cervical third of mesial portion of roots of thirty freshly extracted mandibular second premolars affected by periodontal disease. Two parallel grooves of 0.5 mm depth are made with a cylindrical bur under copious irrigation. The first groove was positioned horizontally at the cementoenamel junction (CEJ) and the second groove parallel and 4 mm apical in relation to the first. The area between the two grooves is then scaled with a sharp universal curette (Hu-Friedy, Chicago, IL). The dental crown above the first groove was removed and a longitudinal cut was performed in the central part of the root portion of the tooth splitting into mesial and distal halves. This is followed by a horizontal cut on the mesial half of the root portion to produce the samples. The dentin blocks obtained are then stored in individual sterile capped tubes containing phosphate buffered saline (PBS) until use.

Fresh human whole blood

Fresh human whole blood of about 0.5 ml from one healthy volunteer after hematologic investigation was used in this study.

Root conditioning agents

Tetracycline hydrochloride solution

Tetracycline hydrochloride solution was prepared by dissolving commercially available 4000 mg capsule of tetracycline hydrochloride (Hostacycline®, Aventis Pasteur) in 80 ml distilled water with constant stirring at 37°C for 10 min to give a solution of 50 mg/ml. A magnetic stirrer was used to mix the solution. This solution gave a pH of 1.11 when checked with a pH meter.

Ethylenediaminetetraacetic acid gel

The EDTA used was commercially available 24% EDTA gel of pH 7.3 (PrefGel, Biora, Malmo, Sweden).

Phosphate buffered saline

PBS of pH 7.4 was used as the control media.

Thirty dentin blocks were randomly divided into 3 groups of 10 each. Group I dentin blocks, which is the control group are treated with PBS, Group II dentin blocks are conditioned with tetracycline hydrochloride solution of concentration 50 mg/ml and pH 1.11 and Group III dentin blocks with 24% EDTA gel (PrefGel) of pH 7.3.The dentin blocks were conditioned for 3 min with a soft brush using one of the three agents and then rinsed 3 times for 5 min in 10 ml PBS. The dentin blocks are then allowed to air dry for about 20 min. After that one drop of fresh human whole blood was added to each of the dentin blocks and allowed to clot for about 20 min. The blocks were then rinsed 3 times for 5 min in 10 ml PBS. All steps were carried out at 36°C degrees (normal body temperature) and rinses were carried out in small Petri dishes with gentle swirling motion.

Scanning electron microscope (SEM) analysis

The dentin blocks were fixed in 3% gluteraldehyde for 12 h at 4°C. After fixation the blocks were washed with PBS. The specimens were dehydrated through a graded series of ethanol (JEBSEN and JESSEN, GmbH [Gesellschaft mit beschränkter Haftung] & Co, Germany) of 30%, 50%, 70%, 90%, and 100% concentration. Then the samples were dried in a critical point dryer using liquid carbon dioxide. The dried specimens were then mounted on metallic stubs and gold coated and desiccated at room temperature. Scanning photomicrographs were obtained at ×5000 magnification at 15 kv with a scanning electron microscope (S-2400, Hitachi). In order to determine the degree of fibrin clot adhesion to the root surface, the following scores were used.[6]

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.

Statistical analysis

In statistical analysis, data were expressed in its frequency and percentage as well as mean, median and standard deviation. To elucidate the associations and comparisons between different parameters, Chi square (χ^2) test was used as nonparametric test in this study.

RESULTS

Scanning electron microscopic examination

Group I

Group I which is the control showed root surfaces covered with sparsely distributed erythrocytes entangled in an organized fibrin network (score 1) obscuring the root planed dentin surfaces [Figure 1]. Three samples were covered with moderate fibrin network and moderate quantity of blood cells with score 2.

Group II

Group II is the tetracycline HCL treated group which demonstrated extensive adhesion of fibrin clot with densely distributed erythrocytes entangled in a thick network of fibrin (score 3) [Figure 2]. The underlying root surface was completely obscured by the extensive surface coverage of the dentin block by the fibrin clot. Only some dentin blocks showed moderate amount of blood cells in moderate amount of fibrin mesh (score 2).

Group III

Group III dentin blocks treated with EDTA showed sparsely distributed erythrocytes in a poorly organized fibrin network with score 1 [Figure 3]. Some samples showed moderate amount of fibrin network but with little or no erythrocytes with score 1.

The Chi-square value for the present study was 20.267 with P value < 0.001 [Table 1] which shows that the results obtained were statistically significant.

DISCUSSION

The periodontium consists of a cell and tissue complex organized spatially into the basic components of cementum, periodontal ligament, and alveolar bone. The main aim of periodontal regeneration is to reorganize this complex onto a root surface which is affected by periodontal disease. In periodontitis the root surface becomes exposed to the periodontal

Table 1: Percentage scores of fibrin clot adhesion in three groups

Score	Control %	Tetracycline HCL %	EDTA %
Group			
Scarce	70.00		80.00
Moderate	30.00	40.00	20.00
Dense		60.00	

χ^2=20.267, P<0.001, EDTA: Ethylenediaminetetraacetic acid; HCL: Hydrochloride

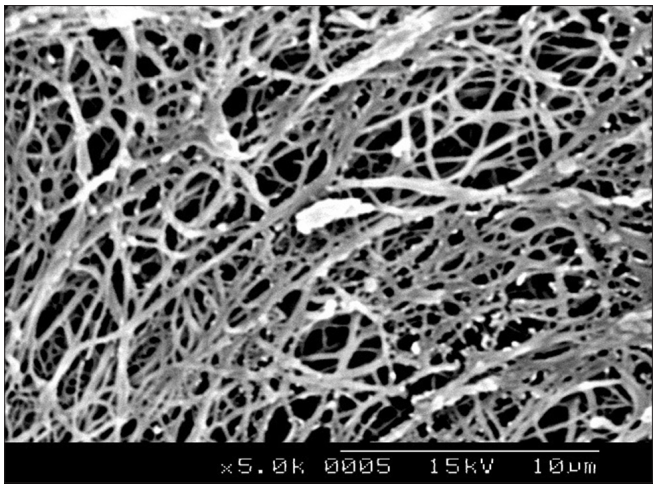

Figure 1: Control showing root surfaces covered with sparsely distributed erythrocytes entangled in an organized fibrin network

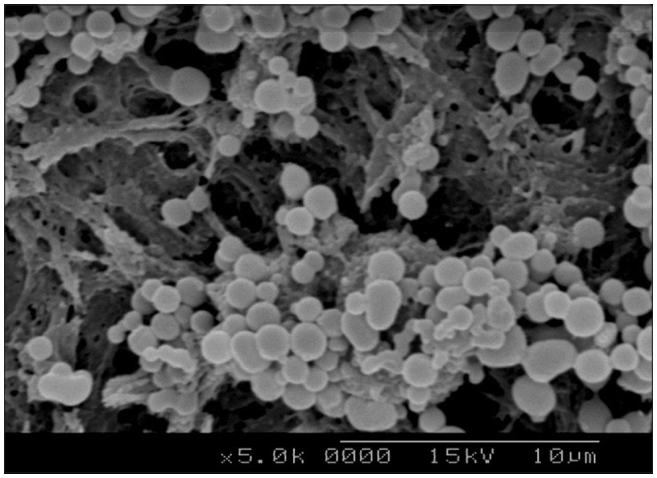

Figure 2: Tetracycline hydrochloride treated group showing extensive adhesion of fibrin clot with densely distributed erythrocytes entangled in a thick network of fibrin

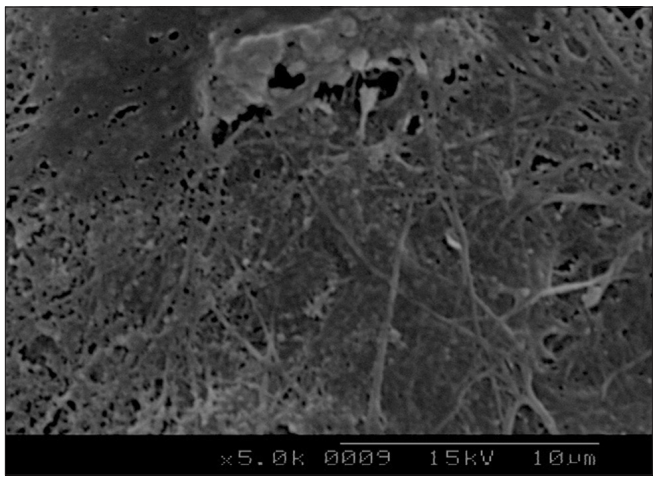

Figure 3: Ethylenediaminetetraacetic acid treated group showing sparsely distributed erythrocytes in a poorly organized fibrin network

pocket with loss of collagen and also there will be cementum bound endotoxin which prevents the *in vitro* growth of fibroblasts.[7] The root surface becomes unsuitable for the new connective tissue attachment necessary for periodontal regeneration.[8]

During wound healing the fibrin clot formed must adhere to the root surface for enough time to allow for proper wound maturation, connective tissue formation and development. Studies have shown that apical migration of the gingival epithelium in periodontal wounds results from the separation or breakdown of the fibrin clot from the root surface. Furthermore, connective tissue attachment following periodontal regenerative surgery is directly related to the adhesion of fibrin clot during wound healing.[1]

Mechanical and chemical means have been used to promote biologically acceptable root surface characteristics. Mechanical means include scaling and root planing, which is effective in removing bacterial deposits along with endotoxins from the root surface; but there will be formation of a smear layer on the root surface and also contamination by bacteria and bacterial products as well as endotoxins. These changes may produce a root surface that is biologically unfit for achieving a stable wound healing interface. *In vitro* studies shows that clot adhesion will be adversely affected in such root surfaces without biomodification.[5] It may also affect the tensile strength of the fibrin clot or may interfere with its formation. This could lead to healing via formation of a long junctional epithelium. Root conditioning agents removes the instrumentation smear layer and also exposes the dentinal tubules and the intra-and peri-tubular dentin collagen matrix. Evidence shows improved adhesion of fibrin clot to conditioned root surfaces.[5] Fibrin clot adhesion to root surface is a crucial step in early healing and whether the root conditioning agents have adverse effects or not on blood clot adhesion or stabilization must be questioned. The objective of the present *in vitro* study was to compare and evaluate the degree of fibrin clot adhesion to root surfaces treated with root conditioning agents tetracycline hydrochloride and EDTA.

Tetracyclines and EDTA are commonly used as root conditioners. They aid in the demineralization of root surfaces, eliminate the smear layer, aid in opening of the dentinal tubules, and expose some components of the matrix like type I collagen.[9] Added advantages of tetracycline on wound healing and regeneration includes, fibrin clot stabilization,[10] increased chemotaxis, adhesion, and growth of fibroblasts on the root surface and inhibition of matrix metalloproteinases.[11] The advantages of using EDTA as a root conditioning agent is that it exposes more intact collagen bundles, there will be less necrosis of periodontal tissues, greater histologic attachment with less long junctional epithelium formation[12] and it does not dissolve root collagen fibers.[13] Furthermore, EDTA etching appears to promote early cell tissue colonization by providing a more biocompatible surface for cell and tissue attachment.[14]

The dentin blocks used in the present study were divided into three groups; first group is the control, second group is treated with tetracycline hydrochloride and third group with EDTA. Tetracycline hydrochloride solution at concentration 50 mg/ml was used. This is according to the study by Wikesjo *et al*. who stated that tetracycline hydrochloride at concentration 50 mg/ml effectively removes the surface smear layer and exposes a partially demineralized dentin surface with open dentin tubules.[15] 24% EDTA gel was used for conditioning dentin blocks because according to Blomlof *et al*. the concentration of EDTA should be somewhere between 15% and 24% in order to obtain an acceptable smear removing and collagen-exposing effect within a clinically acceptable time period.[16] In addition, Babay stated that supersaturated EDTA at 24% enhances the attachment of gingival fibroblasts to the root surface.[17] Furthermore, 24% EDTA gel did not interfere with periodontal tissue repair when used in combination with conventional periodontal treatment.[18]

Bal, *et al*. conducted a similar scanning electron microscopic study on the effects of various root surface treatments on initial clot formation. It was observed that organized clot formation occurred more rapidly in the treatment areas where both root planing and root conditioning with tetracycline hydrochloride had been done than in other groups.[10] This is in accordance with the present study where tetracycline treated samples showed extensive fibrin clot adhesion to root surface. But another study was conducted by Fabio, *et al*. on the effect of tetracycline hydrochloride on smear layer removal and fibrin network formation.[19] The results of this study showed that there were no differences in fibrin network formation in control group with periodontal instrumentation alone and in test group after topical application of tetracycline hydrochloride.

The formation of an organized fibrin network totally enmeshing the dense erythrocytes in tetracycline hydrochloride treated dentin blocks can be due to various reasons. According to Larjava *et al*. and Steinberg and Willey collagen fiber exposure by the use of a root conditioning agent could improve clot organization and the superficial demineralization obtained with tetracycline hydrochloride is sufficient to achieve the necessary exposure of the collagen matrix causing improved clot adhesion.[20,21] Another possible explanation is that collagen exposure favors two steps of the clot formation: The first step is coagulation cascade activation, which originates the fibrin network[22] and the second step is platelet adhesion, aggregation, activation and degranulation of its cytoplasmic granules, resulting in thrombus formation. Root conditioning with tetracycline hydrochloride is enough to achieve the necessary exposure of collagen matrix conducive for enhanced fibrin clot adhesion.

A proposed mechanism to explain the initial mechanism of fibrin-collagen linkage involves the plasma protein fibronectin. Fibronectin causes linking of fibrin and collagen by factor XIIIa. The exposure of collagen fibrils on the root surface after root conditioning with tetracycline promote ideal root surface characteristics for binding of fibronectin causing adhesion of fibrin to collagen. Tetracycline also causes fibroblast chemotaxis and binding leading to a more stable initial clot formation.[23]

The control showed root surfaces covered with sparsely distributed erythrocytes entangled in an organized fibrin network obscuring the root planed dentin surfaces. The fibrin clot adhesion in this group was poor when compared to tetracycline treated group because resilient union between the fibrin clot and root surface elements depends on biologic acceptance of the root surface. In the control only scaling and root planing without any chemical root biomodification resulted in the formation of a smear layer which inhibits fibrin clot adhesion. A similar study was done by Baker *et al*. where planed human dentin surfaces without root conditioning revealed a few sparsely distributed adherent erythrocytes in a fibrin mesh without forming an extensive fibrin network. Whereas, citric acid treated group showed a thick web of fibrin with entrapped erythrocytes.[5] This is in accordance with the present study which showed poor fibrin clot adhesion in control where only scaling and root planing was carried out. Polson and Proye used an extraction and reimplantation in monkeys to study the wound healing events with and without root conditioning. The results of this study, indicate that root conditioning promotes wound healing process by increasing the retention of fibrin clot to the root surface which is in accordance with the present study.[3]

In EDTA treated group there was isolated clumps of erythrocytes distributed in a poorly organized fibrin network. This result may be attributed to the following mechanisms. EDTA is a calcium chelator[16] and its residues may have inhibited coagulation events. Another possible explanation for poor fibrin clot adhesion to EDTA treated root surfaces can be due to the incomplete removal of gel from the root surface.[24] Blood cell attachment to root surfaces treated with EDTA gel was evaluated in a study by Fabio *et al*.[24] In this study different patterns of blood element adsorption and adhesion to root surfaces treated with distilled water, which is the control group and after application of two types of EDTA gels; Santa Paula's EDTA and Biora's EDTA (PrefGel) were evaluated. The results obtained showed that fibrin clot organization with blood cell entrapment was obtained in the control group than in the other groups. Santa Paula's EDTA showed a great variation of results and Biora's EDTA inhibited blood element adsorption and adhesion completely. So the conclusion was that the use of EDTA gel in root biomodification can inhibit clot formation and stabilization on root surface. Root debridement and planing is capable of providing a more stable surface for fibrin clot adhesion when compared to EDTA root conditioning. This supports the findings of the present study where the fibrin clot adhesion in EDTA treated group was poor when compared to that in control group. A similar study by Baker, *et al*. evaluated the fibrin clot adhesion to root surfaces after application of citric acid, EDTA and protein constructs like bovine serum albumin or enamel matrix protein.[25] The results showed that citric acid demineralization removes smear layer to promote adhesion of a fibrin clot. The EDTA gel (PrefGel) appears less effective in retention of fibrin clot. Further conditioning of the dentin surface with protein constructs produces a surface morphology similar to that of the smear layer with poor fibrin clot retention. This is also in accordance with the present study where EDTA treated group showed poor retention of fibrin clot. In a study by Blomlof, *et al*. there was improved healing to EDTA root conditioning

in comparison to controls with no etching and citric acid etching.[26] This study negatively correlates with the present study. The possible explanation was that EDTA with neutral pH results in exposure of the collagen causing improved adhesion of biologically active substances such as growth factors and also produces a biocompatible surface more favourable for cell colonization.

CONCLUSION

According to the results of the present study, root conditioning with tetracycline hydrochloride produces a biologically acceptable root surface as evident by the formation of extensive fibrin network and entrapped erythrocytes, which in turn are an important event in early wound healing process leading to the formation of a connective tissue attachment. EDTA gel appears less effective in producing a root surface necessary for the adhesion of fibrin clot. The control without any root conditioning procedure showed poor fibrin clot adhesion when compared to tetracycline treated group, but when compared to EDTA treated group the fibrin clot adhesion was slightly better.

Fibrin clot adhesion to root surface is a crucial step in early wound healing which inturn is necessary for a successful periodontal treatment outcome. There are only a limited number of studies in the literature evaluating the degree of fibrin clot adhesion after demineralization with various root conditioning agents. Hence more number of studies, both *in vitro* and *in vivo* with large sample size should be carried out to assess fibrin clot adhesion after various root conditioning protocols to support the present study.

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