Original paper

Scanning electron microscopy study to evaluate and compare fibrin clot adhesion over the root surface treated with tetracycline, doxycycline and minocycline

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Abstract

Background. Periodontitis is an inflammatory disease caused by a group of specific microorganisms that provoke the destruction of the periodontal ligament and the alveolar bone, along with pocket formation or recession, or both.

Objectives. The present study aimed to compare the efficacy of tetracycline, doxycycline and minocycline in improving fibrin clot adhesion over manually instrumented periodontally affected root surfaces with the use of scanning electron microscopy (SEM).

Material and methods. A total of 45 single-rooted extracted teeth were sectioned into 45 dentinal blocks and divided into 3 groups: tetracycline (group I); doxycycline (group II); and minocycline (group III). A drop of blood was added over the dentinal blocks, allowed to clot, and later rinsed with phosphate-buffered saline (PBS),1% formaldehyde, and 0.02% glycine. Then, the surfaces were post-fixed in 2.5% glutaraldehyde and dehydrated in a graded ethanol series of 30%, 50%, 75%, 90%, 95%, and 100%. Afterward, the samples were examined under a SEM to assess fibrin clot adhesion and the number of blood cells.

Results. Minocycline demonstrated better fibrin clot adhesion, followed by tetracycline and doxycycline. Statistical significance was observed at ×2,000 magnification ($p = 0.021$), whereas no significance was noted at ×5,000 magnification.

Conclusions. Dentinal blocks treated with minocycline had a better fibrin network and a greater number of entrapped erythrocytes, which is vital in the early wound-healing process leading to the formation of connective tissue attachment.

Keywords: tetracycline, fibrin clot, minocycline, root biomodification

Introduction

Periodontitis is an inflammatory disease caused by a group of specific microorganisms that provoke the destruction of the periodontal ligament and the alveolar bone, along with pocket formation or recession, or both.[1] This inflammatory process changes the balance of the periodontium, observed as a loss of connective tissue attachment and of the alveolar bone, and the altered position of the junctional epithelium.[2] The inflammatory changes cause the exposition of cementum, leading to the accumulation of plaque and calculus on the surface, demineralization, the loss of collagen fibers, and contamination with cytotoxins and endotoxins, which in turn results in a reduction in fibroblast growth and viability, hindering new attachment.[3] Therefore, the goal of periodontal treatment is to preserve tooth functionality by attaining proper periodontal regeneration, which is achieved by stable clot formation that aids in optimal periodontal healing.[2,4]

The bacterial endotoxins present over the root surface are removed with the use of mechanical and chemical methods, as the mechanical methods alone do not ensure complete removal, but produce a compact smear layer that inhibits proper periodontal regeneration.[5] Thus, root biomodifiers are used as an adjunct to help remove the smear layer and restore the biocompatibility of the root surface by enhancing the exposure of the underlying radicular collagen fibrils.[6] The presence of the exposed dentin and cementum collagen fibers helps form proper and stable fibrin attachment in the blood clot, preventing epithelial downgrowth and forming a temporary scaffold required for cell growth and mature collagen adhesion.[7,8]

The application of root biomodifiers helps promote periodontal regeneration, as demonstrated by numerous in vitro and in vivo studies. Hence, various root biomodification agents have been introduced to detoxify and decontaminate the root surface.[2] Materials such as tetracycline, doxycycline and minocycline show bacteriostatic activity and have been proven to be very effective against a wide range of organisms when tested in humans and animals; they also affect the regeneration process by improving attachment levels, promoting gingival fibroblast growth, increasing substantivity, and inhibiting parathyroid hormone bone resorption.[3]

The present study used scanning electron microscopy (SEM) to compare the efficacy of tetracycline, doxycycline and minocycline in improving fibrin clot adhesion on manually instrumented periodontally affected root surfaces.

Material and methods

The present study was conducted between February 2021 and July 2021 after obtaining clearance from the Institutional Ethics Committee at the Sree Sai Dental College and Research Institute, Srikakulam, India (SSDCERI/IRB/IEC/2020-21/408/8/2). Forty-five single-rooted extracted teeth were collected at the Department of Oral and Maxillofacial Surgery of the Sree Sai Dental College and Research Institute, Srikakulam, India. The extracted teeth had to fulfill the following inclusion and exclusion criteria:

– the inclusion criteria – single-rooted teeth with normal morphology, and the absence of restorations or dental caries;
– the exclusion criteria – teeth with dental caries, restorations, malformations, or fractures.

Study design

The teeth extracted due to periodontal disease (grade III mobility) were initially cleaned with distilled water and stored in a saline solution for 2 months before the commencement of the experiment. The extracted teeth were then root-planed with area-specific Gracey curettes (Hu-Friedy, Chicago, USA), using 50 apico-coronal strokes parallel to the long axis of the tooth to remove the contaminants and form a smear layer (Fig. 1A). Using a straight bur, two parallel fissures were made on the proximal surface at the cementoenamel junction (CEJ) and 4 mm apically to the 1st fissure. The teeth were then sectioned between the fissures into 2 parts (Fig. 1B) to form 4 mm × 4 mm × 1 mm dentinal blocks (Fig. 1C), which were stored in phosphate-buffered saline (PBS). The prepared dentinal blocks were then randomly divided into 3 groups containing 15 samples each (n = 15).

Group I dentinal blocks were treated using burnishing cotton pellets soaked in tetracycline with light pressure for 2 min (Fig. 2A), and a similar process was followed using doxycycline (group II) and minocycline (group III).

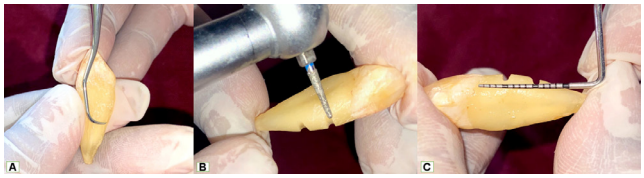

Fig. 1. Scaling and root planing (A), sectioning the tooth (B), and measuring the tooth (4 mm × 4 mm × 1 mm) (C)

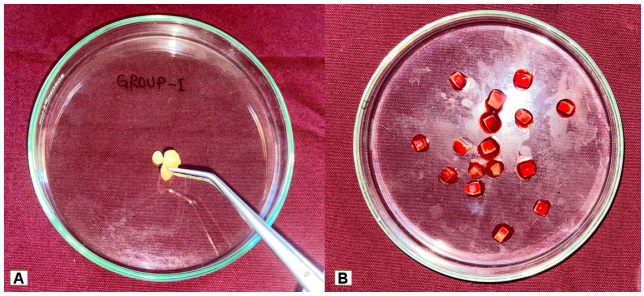

Fig. 2. Application of tetracycline (A) and a blood drop over the dentinal blocks for clot formation (B)

Preparation of tetracycline, doxycycline and minocycline solutions

One 500 mg tetracycline capsule (Resteclin 500; Abbott Healthcare, Mumbai, India), five 100 mg doxycycline capsules (Microdox-LBX; Micro Labs, Bangalore, India) and five 100 mg minocycline capsules (MINOZ™ 100; Sun Pharmaceutical Industries, Bangalore, India) were mixed with a saline solution (50 mL) in 3 separate glass beakers for 10 min by using a stirrer.

After applying tetracycline, doxycycline and minocycline, the samples were washed in 10 mL of saline solution. Blood was drawn from the cubital vein of a healthy volunteer with pristine gingiva and no systemic problems. A drop of blood was placed over the dentinal blocks and allowed to clot for 20 min (Fig. 2B).

Preparation of dentinal blocks for SEM

After clot formation, the samples were rinsed 3 times with PBS for 5 min in a Petri dish. After rinsing, the samples were fixed for 15 min in 1% formaldehyde diluted in PBS, followed by 3 rinses with PBS for 5 min each. The samples were incubated in 0.02 M glycine diluted in PBS, rinsed with PBS, post-fixed in 2.5% glutaraldehyde in PBS for 30 min, and rinsed again. The samples were washed and dehydrated in a graded ethanol series of 30%, 50%, 75%, 90%, and 95% for 10 min each, and then 3 times in 100% ethanol for 10 min. After this, the samples were stored in a desiccator jar until gold sputter coating was carried out. The dried specimens were mounted on the stubs of a scanning electron microscope and coated with gold and palladium in a sputter coating machine (Bal-Tec SCD 050; Bal-Tec, Los Angeles, USA). The samples were again stored in a desiccator jar at room temperature for 3 days,[9] and then examined under the SEM for the presence of a clot. The collected samples were analyzed using the HITACHI S-3700N SEM (Hitachi High-Tech, Hitachinaka, Japan) with a working distance (WD) of 13.3–16.4 mm, 15.0 kV, and magnification of ×2,000 and ×5,000.

The evaluation of fibrin clot adhesion and the number of blood cells was performed according to the blood elements adhesion index (BEAI) by Theodoro et al.[6,10] The scores were as follows: 0 for the absence of a fibrin network and blood cells; 1 for a scarcely distributed fibrin network and/or blood cells; 2 for a moderate number of blood cells and a thin fibrin network with poor interlacing; and 3 for a dense fibrin network with rich interlacing and the presence of blood cells.

Statistical analysis

The data was analyzed using IBM SPSS Statistics for Windows, v. 22.0 (IBM Corp., Armonk, USA). Fisher's test compared the observed results with the expected results.

Results

The assessment of inter-examiner reliability employed Cohen's kappa statistic, and a κ-value of 0.721 indicated substantial agreement among the examiners. Kappa values indicate no agreement (≤0), none to slight (0.01–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), or almost perfect agreement (0.81-1.00).[11] To nullify the variation between examiners, we trained them and checked again for agreement. For the second test, the result produced a value of 0.82, which indicated almost perfect agreement.

Tetracycline group

×2,000 magnification

One sample from group I showed a dense fibrin network and blood cells, while 3 had a moderate fibrin network and blood cells. There was a scarcely distributed fibrin network and/or blood cells in 5 samples, whereas 6 treated samples had no fibrin network under this magnification (Table 1, Fig. 3A).

×5,000 magnification

One sample showed evidence of a dense fibrin network and blood cells, 2 had a moderate fibrin network and blood cells, 7 had a scarcely distributed fibrin network and/or blood cells, and 5 had no fibrin network under this magnification (Table 2, Fig. 3D).

Table 1. Comparison of the 3 groups at ×2,000 magnification (Fisher's exact test)

Group	Score 0	Score 1	Score 2	Score 3	*t*-value	*p*-value
Group I (*n* = 15)	6 (5.0)	5 (6.0)	3 (1.7)	1 (2.3)		
Group II (*n* = 15)	6 (5.0)	9 (6.0)	0 (1.7)	0 (2.3)	13.367	0.021*
Group III (*n* = 15)	3 (5.0)	4 (6.0)	2 (1.7)	6 (2.3)		

Groups: group I – tetracycline; group II – doxycycline; and group III – minocycline. * statistically significant.

Table 2. Comparison of the 3 groups at ×5,000 magnification (Fisher's exact test)

Group	Score 0	Score 1	Score 2	Score 3	*t*-value	*p*-value
Group I (*n* = 15)	5 (5.3)	7 (6.3)	2 (1.7)	1 (1.7)		
Group II (*n* = 15)	6 (5.3)	8 (6.3)	1 (1.7)	0 (1.7)	6.333	0.151
Group III (*n* = 15)	5 (5.3)	4 (6.3)	2 (1.7)	4 (1.7)		

Groups: group I – tetracycline; group II – doxycycline; and group III – minocycline.

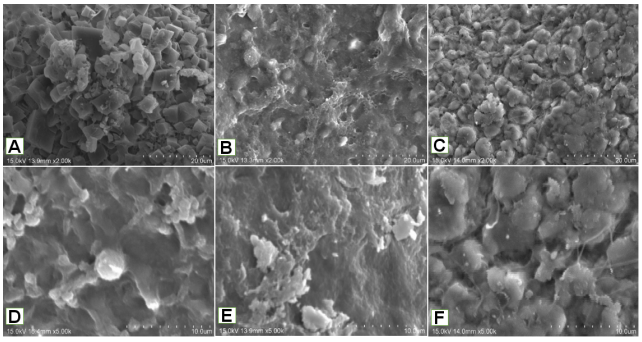

Fig. 3. Scanning electron microscopic (SEM) images

A–C – treated with tetracycline, doxycycline and minocycline, respectively (scores: A – 2; B – 0; C – 3) under ×2,000; D–F – treated with tetracycline, doxycycline and minocycline, respectively (scores: A – 1; B – 0; C – 3) under ×5,000 magnification.

Doxycycline group

×2,000 magnification

Nine samples from group II showed a scarcely distributed fibrin network and/or blood cells, whereas 6 had no evidence of fibrin network formation (Table 1, Fig. 3B).

×5,000 magnification

Eight samples from group II received a score of 1, 1 received a score of 2 and the rest of the root specimens did not appear to have any form of fibrin network (Table 2, Fig. 3E).

Minocycline group

×2,000 magnification

Six dentinal blocks from group III had a BEAI score of 3, and 2 samples received a score of 2. Only 4 samples seemed to have attained a scarcely distributed fibrin network, whereas 3 did not have any fibrin network (Table 1, Fig. 3C).

×5,000 magnification

Four samples in group III received a score of 3, which was attributed to the presence of a dense fibrin network and blood clots. Two samples received a score of 2, and 4 received a score of 1, while the rest of the root specimens did not show the presence of a fibrin network (Table 2, Fig. 3F).

Among the 3 groups, minocycline showed better fibrin clot adhesion and clot stabilization when compared to tetracycline and doxycycline at ×2,000 and ×5,000 magnification. Statistical significance was recorded at ×2,000 magnification (p = 0.021), whereas no significance was found at ×5,000 magnification (p = 0.151).

Discussion

The current study is the first to compare the efficacy of tetracycline, doxycycline and minocycline as root-conditioning agents for enhancing fibrin clot adhesion over the root surface. Hatfield and Baumhammers stated that when the root surface is exposed to mammalian cells and bacterial plaque, the cells show less affinity for cellular attachment unless mechanically debrided or cleaned.[12] Meanwhile, Aleo et al. stated that the primary attachment of connective tissue cells could be prevented by the presence of endotoxins that accumulate and get absorbed over the tooth surface, even after chemical debridement.[13] However, according to Genco and Mergenhagen, the host–microbe interaction is responsible for the destruction of the tooth and its supporting structures when affected with periodontitis.[14] The evaluation of fibrin clot adhesion over the root surface was considered a crucial factor in in vitro studies, as without proper root conditioning, clot formation is affected, leading to alterations in the tensile strength.[15]

In the present study, minocycline produced better results for fibrin clot adhesion among the 3 agents, as it favored periodontal wound healing and promoted periodontal regeneration. Tetracycline demonstrated a moderate effect, and doxycycline was the least effective.

Studies conducted by Somerman et al.[16] and Zhang et al.[17] suggested that treating the root surface with minocycline helped facilitate the adhesion of fibroblasts, as it has the potential to promote appropriate fibrin clot adhesion during periodontal surgery. Indeed, it enhanced periodontal healing, the growth of fibroblasts on the root surface and clinical attachment while promoting periodontal cell attachment. The minocycline ointment used has an optimum viscosity and a low pH, which helped to control the damaging effects at the application areas.[17]

Shetty et al. stated that minocycline had a consistent ability to eliminate the smear layer when compared to tetracycline, and also acts as a calcium chelator; its application resulted in enamel and root surface demineralization and the removal of endotoxins over the untreated root surfaces, which helped remove the smear layer and promoted binding with calcium phosphate, opening the collagen matrix.[18] Atilla and Baylas concluded in 1996 that applying 2.1% minocycline hydrochloride (HCl) ointment to the root surface binded calcium phosphate, and thus opened the collagen matrix, inhibiting the collagenase mechanism, and eliminated the smear layer caused by mechanical instrumentation.[19] Therefore 2.1% minocycline HCl ointment can be regarded as a potential root surface-conditioning agent.[19] Widaryono et al. showed that ethylenediaminetetraacetic acid (EDTA) and minocycline both had equal efficacy with regard to producing fibrin clots over the root surface; EDTA demonstrated the ability to eliminate the smear layer to open the cementum-collagen matrix, and also acted as a pH neutralizer, whereas minocycline removed the smear layer by itself.[20]

Tetracycline inhibits collagenase activity and bone resorption, and has antimicrobial activity during regeneration.[21] In its low-pH form, tetracycline can also increase the binding of fibronectin and other extracellular matrix (ECM) proteins to the root surface to enhance fibroblast attachment and growth, which in turn suppresses the proliferation and growth of epithelial cells.[22] According to studies conducted by Terranova et al.,[23] Bal et al.,[24] Larjava et al.,[25] and Steinberg and Willey,[26] using tetracycline as a root conditioner promoted optimal root surface characteristics for the binding of fibronectin, which causes the adhesion of fibrin to collagen, thus helping with fibroblast chemotaxis and binding, and leading to stable initial clot formation.[23,24] Furthermore, tetracycline improved clot organization and superficial demineralization, which was sufficient to attain the required exposure of collagen matrix, essential for proper clot adhesion.[25,26] In contrast, Delazari et al. stated that tetracycline HCl had no impact on the removal of the smear layer or fibrin network formation in the control and test groups.[27]

In the present study, the samples treated with tetracycline did not show the expected fibrin clot adhesion or presence of blood cells over the root surface, which may be due to the incomplete removal of the smear layer. Shetty et al. made a similar finding when reporting that minocycline had a better ability to remove the smear layer as compared to tetracycline.[18]

Doxycycline is an effective agent against different potentially causative microflora in periodontitis and has enzymatic properties. The topical application of doxycycline had a long-lasting substantivity on periodontally diseased root surfaces. The effect of doxycycline as a root-conditioning agent persists for around 14 days.[19]

A study conducted by Didhra et al. in 2020 used MTAD – a mixture of doxycycline (a tetracycline isomer), citric acid, polysorbate-80 (a detergent), and normal saline – and showed that it promoted better fibrin clot adhesion than saline.[4] Studies have used individual components of MTAD as periodontal conditioners, as low pH helps with the demineralization of the matrix and exposes collagen fibers to promote fibrin clot attachment. However, even with such persistent properties, in the present study, adequate fibrin clot adhesion was not observed for doxycycline, and better results were observed in the minocycline-treated group.

Conclusions

Within the limitations of the study, we concluded that although the 3 materials used belonged to the same group, only minocycline produced a biologically acceptable root surface and functioned as a biomodification agent. Indeed, minocycline helped form an extensive fibrin network with entrapped erythrocytes, which is vital in the early wound-healing process and leads to connective tissue attachment.

Ethics approval and consent to participate

The present study obtainined clearance from the Institutional Ethics Committee at the Sree Sai Dental College and Research Institute, Srikakulam, India (SSDCERI/IRB/IEC/2020-21/408/8/2).

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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