



Research Article

COMPARATIVE SEM STUDY ON TETRACYCLINE HYDROCHLORIDE ROOT CONDITIONING: THE EFFECTS OF DIFFERENT CONCENTRATIONS AND APPLICATION TIMES

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Article Received on: 25/05/15 Revised on: 30/06/15 Approved for publication: 08/07/15

DOI: 10.7897/2230-8407.06788

ABSTRACT

Smear layer removal and collagen fiber exposure may improve periodontal treatment and regeneration. This *in vitro* study assessed smear layer removal and morphological changes (collagen fiber exposure) after tetracycline hydrochloride (TTC) application on root surfaces using different concentrations and application times by scanning electron microscopy (SEM). 672 samples were divided into eight groups: a control (distilled water) and seven different TTC concentrations were applied at doses of 10, 25, 50, 75, 100, 125 and 150 mg/ml. The TTC application was performed in all groups at three different periods of conditioning (1, 2, and 3 minutes) by burnishing method. A previously trained, calibrated examiner evaluated photomicrographs. Statistical analysis was performed using the Fischer Exact test. Tetracycline hydrochloride concentrations between 50 mg/ml and 150 mg/ml and all application periods used, showed effective root surface changes. The concentrations of 50 mg/ml, 75 mg/ml and 100 mg/ml at 3 and 5 minutes showed most effective smear layer removal. Most effective morphological root surface changes were seen at 100 mg/ml TTC solution at 5 minutes. The concentrations of 50 mg/ml, 75 mg/ml and 100 mg/ml during 5 minutes were the most effective for smear layer removal and 100 mg/ml TTC solution at 5 minutes showed most effective morphological root surface changes.

Keywords: Tetracycline hydrochloride, root conditioning, smear layer, demineralization, periodontal regeneration, collagen fiber exposure, SEM

INTRODUCTION

Roots of periodontally affected teeth become hypermineralized¹ and are contaminated by bacteria and endotoxins.² Periodontal therapy such as scaling and root planing, is aimed to remove the bacterial deposits and pathogenic microbiota from the tooth surface and to achieve a surface, compatible with periodontal health³.

Instrumentation of root surface with mechanical instruments results in formation of smear layer composed of organic and mineralized debris of 2-15 µm in thickness and serves as a physiological barrier between periodontal tissue and root surface, which may inhibit the formation of new connective tissue attachment to root surface.⁴ Root conditioning has been proposed as a means to circumvent this problem.⁵

A number of agents have been used in conjunction with demineralization/new attachment procedures, including phosphoric acid, EDTA, citric acid and tetracycline. Among these, the tetracyclines have been shown to produce a dentin surface that can potentially enhance periodontal regeneration. TTC application on root surface shows exposure of root collagen and opening of the dentinal tubules, removal of smear layer thereby causing demineralization and detoxification of the root surfaces.⁶ It has been indicated that tetracycline is adsorbed and subsequently desorbed from dentin, maintaining its antimicrobial activity.⁷ It aids in fibrin clot stabilization,⁸ adhesion and growth of fibroblasts on the root surface⁹ and inhibition of matrix metalloproteinases.¹⁰

However, there appear to be dose-dependent effects on the root surfaces.¹¹ Conflicting data regarding the efficacy of tetracycline hydrochloride with respect to the optimal concentrations and application times still exist. Previous studies are limited to a small group and there is a need to elucidate this by comparing it with a larger sample size of specimens. In view of this fact, the present *in vitro* study was designed with the main objective to assess, smear layer removal and exposure of collagen fibers after TTC application on root surfaces by scanning electron microscopy (SEM) in order to evaluate the most effective application parameters.

MATERIALS AND METHODS

A total of 336 periodontally affected single rooted teeth were obtained from the patients visiting the Department of Oral and Maxillofacial Surgery, Krishnadevaraya College of Dental Sciences and Hospital. The Ethics Committee, Krishnadevaraya Dental College, Rajiv Gandhi University of Health Sciences, approved the study protocol. Written consent was obtained from the patients whose extracted teeth were included in the study.

The selection criteria were periodontally affected single rooted teeth from patients with chronic periodontitis with an attachment loss of 5 mm or more and evidence of calculus deposits. Teeth that were affected with root surface caries and abrasive lesions, teeth with root restorations, patients who had undergone scaling and root planing within 6 months before the date of examinations and teeth with root fractures or anatomical abnormalities were deemed unfit for the

study. Scaling and root planning of the roots of the teeth was carried out with Gracey curette No. 5-6 (HuFriedy U.S.A.).

Sample Preparation

Samples were obtained from the cervical third of the roots. The crown portion of each extracted tooth was sectioned following the cemento-enamel junction as a guide line using a diamond disk in a slow-speed hand piece under copious water irrigation. Following the separation of crowns from the roots (Figure 1), a longitudinal sectioning was done at the lines marked on the facial and lingual or palatal surfaces of the roots extending apically from the cemento-enamel junction up to the 6 mm marking and the two proximal halves were separated with a cross section at the termination of 6 mm vertical cut (Figure 2). The root blocks measured individually at three dimensions as follows: length: 6 mm, width: 6 mm and depth: 2 mm.

Two samples were produced from each tooth, thus, a total of 672 samples were obtained and stored in sterile and clean labelled bottles containing distilled water until further procedures.

Conditioning Procedure

The 672 samples were conditioned with different TTC concentrations, periods of conditioning by burnishing with a cotton pellet. Specimens were divided into 8 groups for conditioning at tetracycline hydrochloride concentration (weight per volume) of 0 (control group using distilled water), 10, 25, 50, 75, 100, 125 and 150mg/ml and 3 application periods of 1, 3, and 5 minutes (28 samples per group). Fresh tetracycline hydrochloride[†] solutions were prepared at room temperature by weighing pure tetracycline hydrochloride powder and dissolving it in distilled water, to mix the solution to a final volume of 50 ml. With respect to each concentration, the dose of tetracycline hydrochloride (TTC HCl) was calculated to be mixed with 50 ml of distilled water, so that the desired concentrations were obtained per ml of distilled water. The specimens in each group were then conditioned with one of the 8 concentrations and the root surface was burnished for 1, 3 or 5 minutes with a solution- soaked cotton pellet changed every 30 seconds (Figure 3); the area was then flushed for 30 seconds with distilled water.

[†] Karnataka Antibiotics and Pharmaceuticals LTD, Quality assurance department, Bangalore

Preparation for SEM

All specimens of the 8 groups were fixed with 2.5% glutaraldehyde in 0.2M phosphate buffer (pH 7.2) at the room temperature for 24 hours. The specimens were then washed twice with the same buffer and post fixed in phosphate buffer for 1 hour. Following fixation, the specimens were dehydrated using ascending series of grades of aqueous ethanol solutions with the concentrations 50%, 70%, 85%, 95%, & 100% for 10 minutes at each concentrations. The specimens were then air dried overnight.

A conductive layer of carbon was applied on specimens mounted on copper stubs. The mounted specimens were then sputter coated with gold in the JEOL-1100E- Ion sputter coating device. The teeth were

examined by a JSM-840A scanning electron microscope[‡] at 0° tilt angle, operated at 20 KV. The teeth specimens were subjected to Scanning Electron Microscopy and examined at magnification of 2500X. Polaroid photomicrographs were taken and the photomicrographs analysis was carried out.

[‡] JEOL- Japan

Statistical analysis

Descriptive statistical analysis was carried out in the present study. Results on continuous measurements are presented on Mean $\pm$ SD (min-max) and results on categorical measurements are presented in number (%). Significance is assessed at 5 % level of significance. 2x2 Fisher Exact test has been used to find the significance of study parameters on categorical scale between two or more groups.

Significant figures-

** Significant (P value : $P \leq 0.01$)

RESULTS

A comparison of TTC solution concentration groups showed differences between all groups.

Regarding the smear layer removal, no specimens in the control group showed removal of smear layer (Figure 4). Statistically significant results were observed for specimens that were conditioned at 50 mg (100%), 75 mg(100%), 100 mg(100%), 125 mg(94.0%) and 150 mg (92.9%) concentrations of TTC HCl solutions and for 1, 3 and 5 minute duration. However, concentrations of 50 mg/ml, 75 mg/ml, 100 mg/ml showed most effective smear layer removal ($p < 0.01$) (Table 1, Graph 1) (Figure 5, Figure 6, Figure 7)

Evaluation of application times on the removal of smear layer showed statistically significant results with all the three application times, i.e, 1, 3 and 5 minutes. However, 5 minute application periods exhibited the most effective smear layer removal (Graph 2).

Considering the effect of TTC on the morphological changes seen on the root surfaces, TTC treated specimens exhibited significant root surface alterations compared to control. Control specimens exhibited an amorphous, irregular surface (Figure 4). Statistically significant results were observed for specimens that were conditioned at 50 mg, 75 mg, 100 mg, 125 mg and 150 mg concentrations of TTC HCl solutions and for 1, 3 and 5 minute duration. ($p < 0.01$)(Table 2, Graph 3) (Figure 5, Figure 6, Figure 7). All eighty four specimens that were conditioned at 100 mg concentration of TTC HCl solution showed effective cemental surface changes, exposing the collagen fibers. Therefore, the most effective results were obtained with specimens conditioned at 100 mg/ml concentrations of TTC HCl solution.

Evaluation of application times on the morphological changes on the root surfaces (collagen fiber exposure) showed that statistically significant results were obtained with all the three application times, i.e. 1, 3 and 5 minutes. ($p < 0.01$).However, most effective results were observed with specimens treated for 5 minutes duration (Graph 4).

Table 1: Evaluation based on the various concentration of tetracycline hydrochloride and time of application on removal of Smear layer

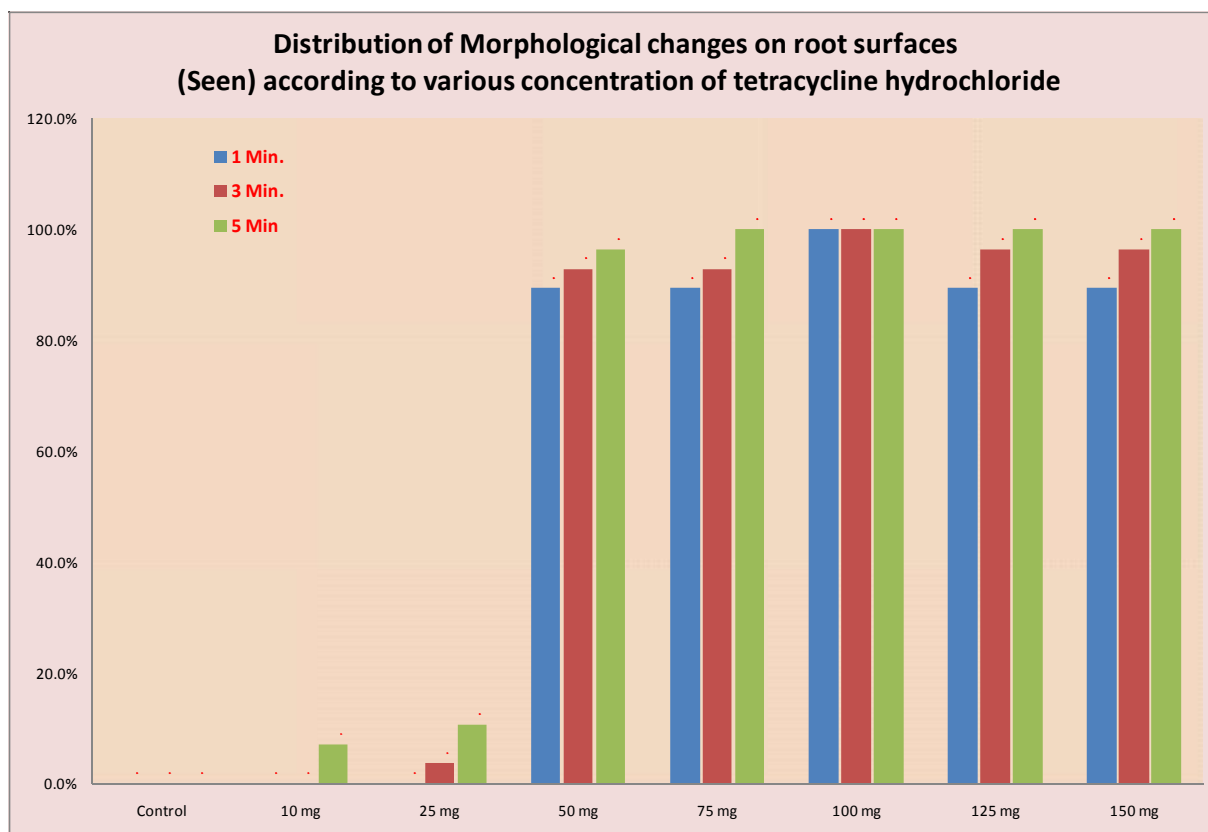
Time of application	Removal of smear layer	Control (n=84)	10 mg (n=84)	25 mg (n=84)	50 mg (n=84)	75 mg (n=84)	100 mg (n=84)	125 mg (n=84)	150 mg (n=84)
Control	No	84 (100.0%)	-	-	-	-	-	-	-
	Yes	0	-	-	-	-	-	-	-
1 minute	No	-	28 (100.0%)	28 (100.0%)	0	0	0	3 (10.7%)	4 (14.3%)
	yes	-	0	0	28 (100.0%)	28 (100.0%)	28 (100.0%)	25 (89.3%)	24 (85.7%)
3 minute	No	-	28 (100.0%)	26 (92.9%)	0	0	0	2 (7.1%)	2 (7.1%)
	Yes	-	0	2 (7.1%)	28 (100.0%)	28 (100.0%)	28 (100.0%)	26 (92.9%)	26 (92.9%)
5 minute	No	-	24 (85.7%)	25 (89.3%)	0	0	0	0	0
	Yes	-	4 (14.3%)	3 (10.7%)	28 (100.0%)	28 (100.0%)	28 (100.0%)	28 (100.0%)	28 (100.0%)
Over all	No	84 (100.0%)	80 (95.2%)	79 (94.0%)	0	0	0	5 (5.9%)	6 (7.1%)
	Yes	0	4 (4.8%)	5 (6.0%)	84 (100.0%)	84 (100.0%)	84 (100.0%)	79 (94.0%)	78 (92.9%)
Significance from control		-	0.121	0.059	<0.001	<0.001	<0.001	0.059+	0.028*

Table 2: Evaluation based on the various concentration of tetracycline hydrochloride and time of application on Morphological changes on root surfaces

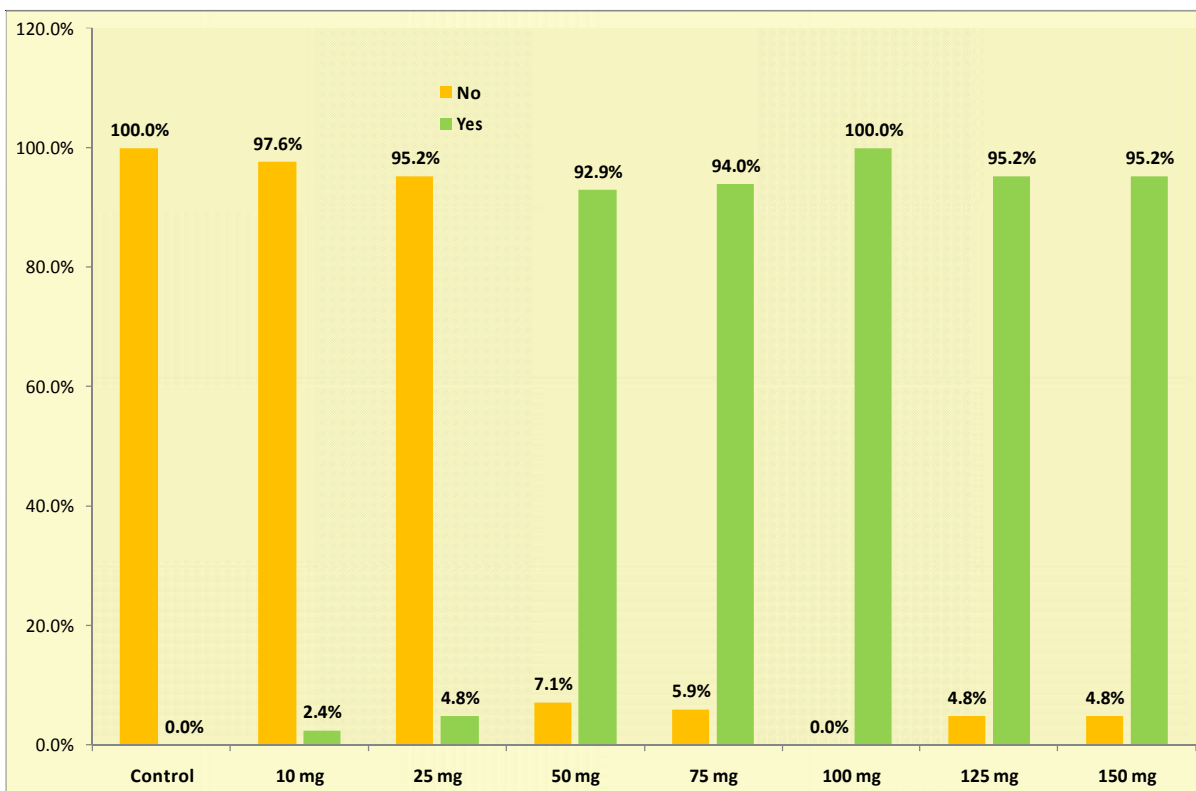
Time of application	Morphological changes	Control	10 mg	25 mg	50 mg	75 mg	100 mg	125 mg	150 mg
Control	Not seen	84 (100.0%)	-	-	-	-	-	-	-
	Seen	0	-	-	-	-	-	-	-
1 minute	Not seen	-	28 (100.0%)	28 (100.0%)	3 (10.7%)	3 (10.7%)	0	3 (10.7%)	3 (10.7%)
	Seen	-	0	0	25 (89.3%)	25 (89.3%)	28 (100.0%)	25 (89.3%)	25 (89.3%)
3 minute	Not seen	-	28 (100.0%)	27 (96.4%)	2 (7.1%)	2 (7.1%)	0	1 (3.6%)	1 (3.6%)
	Seen	-	0	1 (3.6%)	26 (92.9%)	26 (92.9%)	28 (100.0%)	27 (96.4%)	27 (96.4%)
5 minute	Not seen	-	26 (92.9%)	25 (89.3%)	1 (3.6%)	0	0	0	0
	Seen	-	2 (7.1%)	3 (10.7%)	27 (96.4%)	28 (100.0%)	28 (100.0%)	28 (100.0%)	28 (100.0%)
Over all	Not seen	84 (100.0%)	82 (97.6%)	80 (95.2%)	6 (7.1%)	5 (5.9%)	0	4 (4.8%)	4 (4.8%)
	Seen	0	2 (2.4%)	4 (4.8%)	78 (92.9%)	79 (94.0%)	84 (100.0%)	80 (95.2%)	80 (95.2%)
Significance from control		-	0.497	0.121	<0.001	<0.001	<0.001	<0.001	<0.001



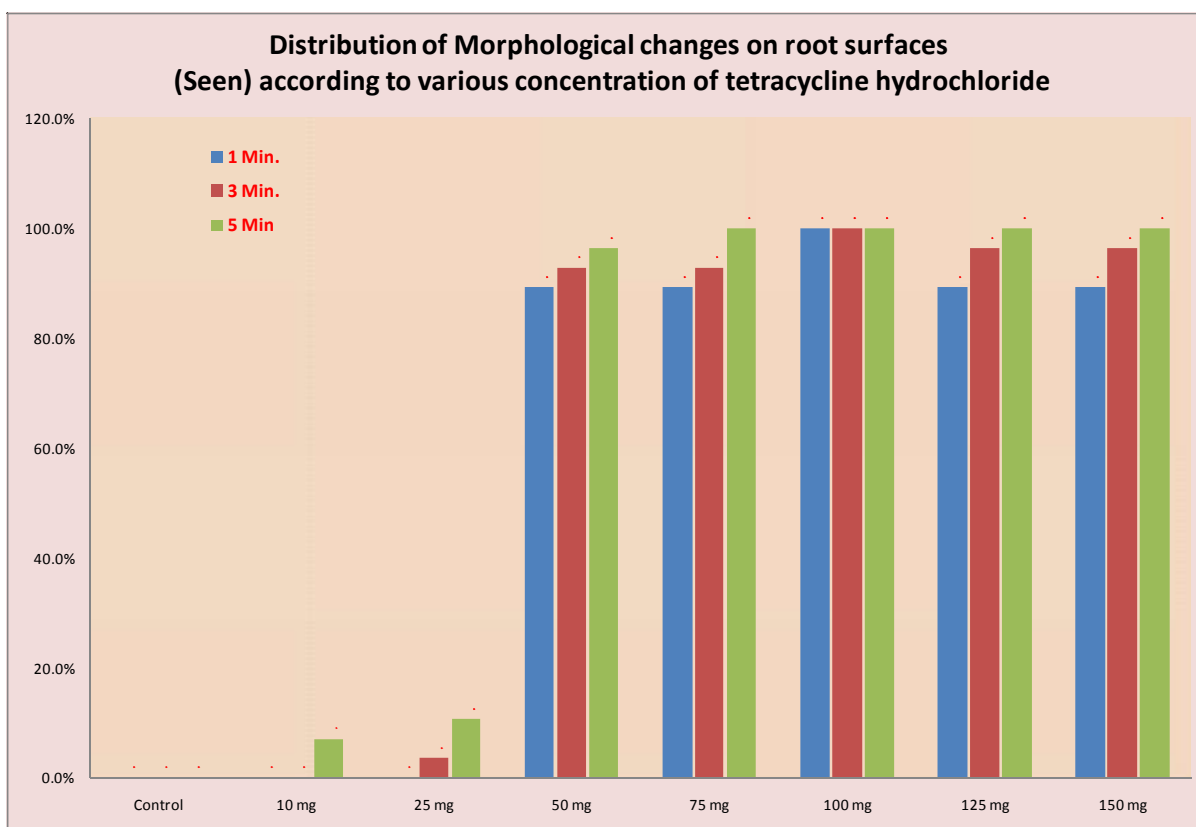
Graph 1



Graph 2



Graph 3



Graph 4

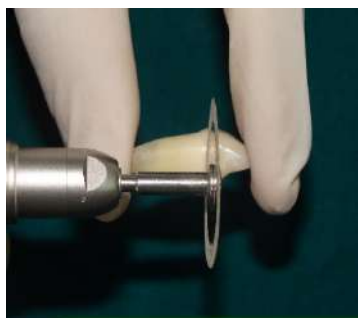


Figure 1: Horizontal sectioning of the tooth specimen



Figure 2: Vertical sectioning of the tooth specimen



Figure 3: Burnishing of the cemental root surface using tetracycline hydrochloride solution

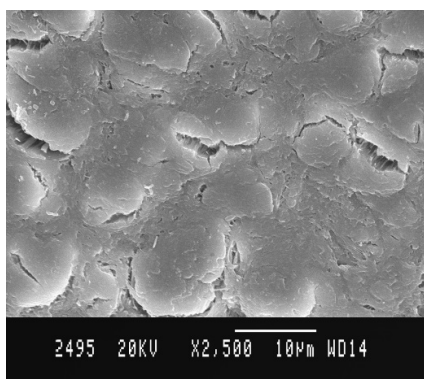


Figure 4: Control specimen

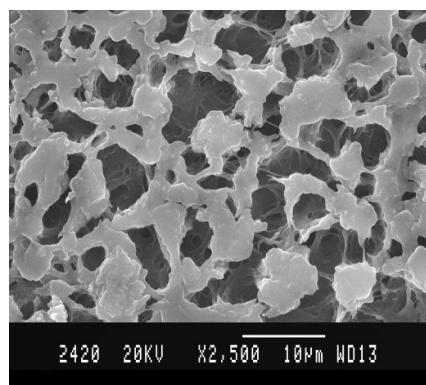


Figure 5: SEM of the specimen conditioned using 50mg/ml for 5 minutes

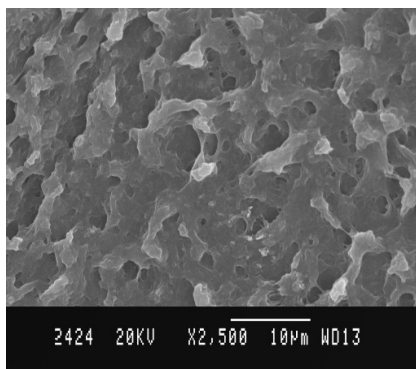


Figure 6: SEM of the specimen conditioned using 75mg/ml for 5 minutes

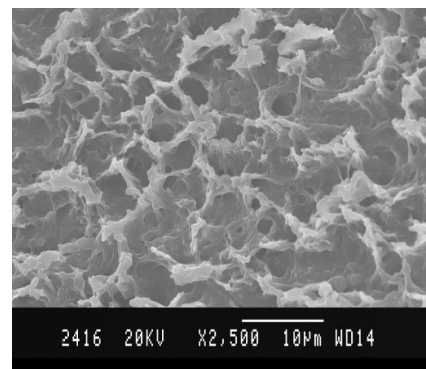


Figure 7: SEM of the specimen conditioned using 100mg/ml for 5 minutes

DISCUSSION

Results of the present study showed that TTC demineralized the root surface and exposed collagen fibers of the cemental matrix. In the present study, observations of the control specimens showed the presence of smear layer in control specimens and none of the control specimens exhibited effective changes on the cemental surface; there was no evidence of demineralization of the root surface. This is consistent with the findings of Trombelliet al¹² in which the control specimens (sterile saline for 1 minute followed by SRP) exhibited an amorphous, irregular surface and did not show any evidence of exposure of collagen fibres.

In the present study, among the specimens conditioned at 10 mg/ml and 25 mg/ml TTC, only 2.4% specimens (2 specimens) and only 4.8% specimens (4 specimens) respectively that were conditioned for

five minutes showed dissolved cores of cementum and exposure of underlying collagen network. All the specimens (100%) conditioned at 100 mg/ml of TTC solution showed effective root surface morphological changes, such as demineralized cores of cementum, complete peeling effect of cementum, with exposure of large and small fibers of cementum. All specimens (100%) conditioned at 50, 75, and 100 mg/ml, showed effective removal of smear layer ($p < 0.001$). This is consistent with the findings of Eduardo et al¹¹ who observed strongly significant smear layer removal with specimens treated at 50 mg/ml and 75 mg/ml concentrations of TTC. Previous *in vitro* studies by Terranova et al⁸ have shown that fibroblast attachment to TTC treated root surfaces at 100 mg/ml concentrations was greatly enhanced.

Evaluation of application times on the removal of smear layer and morphological changes on root surfaces showed that statistically

significant results were obtained with all the three application times, i.e. 1, 3 and 5 minutes. However, for smear layer removal, most effective results were seen with specimens conditioned for 5 minutes. Considering the morphological root surface changes (collagen fiber exposure), statistically significant results were obtained with the specimens conditioned for 1, 3 and 5 minutes. This is similar to the study conducted by Isik et al,¹³ which revealed that TTC solutions used between 50 mg/ml and 150 mg/ml at all application periods used in their study i.e. 1, 3 and 5 minutes exhibited effective smear layer removal and morphological root surface changes.

However, Madison and Hokett⁵ in their *in vitro* study, using TTC at 250 mg/ml, applied for 0.5, 1, 3, 5, and 10 minutes, did not find samples presenting chemical dissolution of dentin, although they reported crystalline deposits on the samples attributed to the high TTC concentration used. Study conducted by Babay¹⁴, suggested that 1 minute immersion with TTC is not enough to remove the smear layer, but 4 minute applications, irrespective of the method of application used and it was adequate to completely expose the dentinal tubules.

It is well known that, smear layer has been characterized as an amorphous structure obscuring the underlying dentin surface. Such a layer has been shown to vary in thickness from one location to the next, and may well comprise different percentages of organic and inorganic material ranging in size from less than 1 µm to more than 15 µm and the detrimental role of such a surface covering in periodontal healing is now recognized.¹⁵ It appears reasonable to assume that a longer exposure time would enhance the smear removing effect. This is in agreement with Sterret et al who found that effectiveness of TCC HCl>75 mg/ml was time dependent and concentrations at 3 and 5 minutes were more effective.¹⁶

It is noteworthy that in the present study, pure form of tetracycline hydrochloride in powder form was used. This was advantageous over the use of tetracycline hydrochloride capsules as capsules introduce a significant amount of fillers into the solution and therefore, the practice of using tetracycline hydrochloride directly from capsules provided from the pharmacy to prepare an acid solution should be avoided.¹⁷

Burnishing method was preferably used for application of tetracycline HCl in the present study as according to the study conducted by Isik et al, it may be desirable to apply tetracycline HCl using a burnishing technique to maximize exposure of intertubular fibrils and tubular openings.¹⁶

From the observations made in the present study, it is clearly evident that concentrations of TTC at and above 50 mg/ml showed statistically significant removal of smear layer and morphological root surface changes at 1, 3 and 5 minutes ($p<0.01$). However, most effective smear layer removal was observed with specimens conditioned at 50 mg/ml, 75 mg/ml and 100 mg/ml (100%) of TTC and most effective morphological root surface changes at 100 mg/ml of TTC at 5 minute application period. One possible explanation for this is the high TTC concentrations (for example, 125mg/ml and 150mg/ml as in the present study) present a greater surface tension which could reduce effectiveness.¹⁰

Results of the present study may potentially contribute to the interpretation of other investigations found in literature, to the design of *in vivo* studies on root conditioning and to the clinical application of TTC root conditioning in periodontal treatment and regenerative procedures. The present study may facilitate clinicians to take advantage of the beneficial properties of TTC while avoiding its shortcomings.

The results of the present study are limited to physical findings of root surface changes and do not present *in vivo* differences that may result from the physiologic effect of these root conditioning agents. Hence, additional studies of these variables, are needed to validate the present findings.

CONCLUSION

Within the limits of the present study, it can be concluded that the concentrations of 50 mg/ml, 75mg/ml and 100mg/ml during 5 minutes were the most effective for smear layer removal and 100 mg/ml TTC HCl solution at 5 minutes showed most effective morphological root surface changes. Therefore, the present study suggests that the most effective root surface changes can be obtained at 100 mg/ml of TTC at 5 minute application period.

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- Cite this article as:**
- Shaeesta Khaleelahmed Bhavikatti, Karthikeyan B V, MLV Prabhuji. Comparative SEM study on tetracycline hydrochloride root conditioning: The effects of different concentrations and application times. *Int. Res. J. Pharm.* 2015; 6(7):423-430 <http://dx.doi.org/10.7897/2230-8407.06788>

Source of support: Nil, Conflict of interest: None Declared

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