EVALUATION OF FIBRIN CLOT ADHESION AS A PRECURSOR FOR NEW ATTACHMENT FOLLOWING ROOT CONDITIONING

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ABSTRACT

The objective of the present study was to evaluate the influence of various root conditioning modalities on adsorption and adhesion of blood to scaled and planned root surfaces. This research work was done in the Department of Periodontics, DAV Dental College, Yamunanagar, Haryana, India (Year 2006-2009). The SEM analysis was done in …SEM & TEM centre, Department of Radiology, AIIMS, New Delhi (India)

A total of 24 specimens were obtained from fresh extracted human teeth which were divided into 4 groups comprising of 6 specimens in each group. The root conditioning groups included 3 experimental – Citric acid, EDTA (Ethylene diamine tetra acetic acid), Tetracycline hydrochloride and 1 PBS (Phosphate buffered saline) as control group. The root surfaces were planned and specimen blocks (4x4x1mm) were obtained. They were subject to various conditioning agents and then exposed to fresh blood which was allowed to clot. These specimens were then rinsed and subjected to SEM analysis.

The results showed that Citric acid treated planned root specimens presented a dense blood cells and fibrin attachment, while tetracycline treated showed moderate and EDTA treated showed scarce attachment of blood clot elements. In contrast, untreated planned dentin exhibited smear layer. The adhesion of fibrin network and blood cell attachment was best following application of Citric acid followed by moderate attachment seen in Tetracycline.

Key words: *Root-conditioning, Blood, Fibrin, Smear*

INTRODUCTION

The ultimate goal of periodontal therapy is the regeneration of the supporting tissues at the site previously exposed by periodontal disease.[1] One of major hindrance in inhibiting predictable regeneration appears to be the nature of the periodontitis affected root surface. Complex inflammatory, enzymatic and other biological influences which accompany periodontal disease produce physical or chemical alterations which are particularly apparent in root cementum.

The traditional treatment of such pathologically altered root surfaces has relied on mechanical removal of plaque and calculus, root bound toxins, and contaminated cementum. But such mechanical debridement and planning of teeth generate a smear layer. Root conditioning by topical application of acidic solutions has been demonstrated to remove not only this smear layer but also any remaining root surface contaminants. A number of agents have been proposed for the demineralization including Phosphoric acid, EDTA, Citric acid. Tetracycline and Fibronectin.[2] Citric acid and tetracycline exert their etching action through a low pH (1 and 1.3 respectively) while EDTA chelates divalent cations, such as Ca^{2+} at neutral pH (7.5). Surface demineralization of dentin by these agents exposes the collagen matrix, there by providing a substrate that supports the chemotaxis, migration and attachment of cells involved in wound healing

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and formation of new connective tissue attachment.[3,4,5]

In vitro studies have indicated that cells attach better to etched than to non etched dentin surfaces, regardless of choice of etching agent.[6] The dentinal tubules and intra – and peritubular collagen matrix exposed by these acids and chelating agents can support adhesion of fibrin clot.[7] This is important as the first requirement for successful regeneration rests with clot adhesion to the root surface, possibly by virtue of fibrin linkage.[8]

Various experimental studies have suggested that periodontal regeneration is dependent upon the adsorption, uninterrupted adhesion and maturation of the fibrin clot positioned between the gingival flap and a periodontally compromised root.[9] The blood elements imposed must establish an attachment that endures the forces and remain stable until sufficient tensile strength is achieved by the matured tooth gingival interface.[7] This rapid adherence of blood clot to the root surface may thus form a sufficient barrier to apical epithelial cell migration and lead to connective tissue attachment following periodontal regenerative surgery.[10]

METHODOLOGY

Sample preparation

Twenty-four dentin blocks, approximately 4x4x1mm in size were prepared from roots of freshly extracted human teeth. The tooth included in the study were those affected by periodontal disease, characterized by bleeding on gentle probing, grade III mobility, radiographic evidence of proximal bone loss. The exclusion criteria included the presence of caries, history of scaling, root planning in the previous 6 months, teeth with periapical infection or nonvital teeth and patients with history of systemic disease. The samples were obtained after planning of root surfaces and stored in PBS (phosphate buffered saline) pH 7.4 at 4°C until use.[11]

Preparation of chemical agents

a) Tetracycline HCl: 500 mg of tetracycline HCl capsule (Hostacycline,™ Aventis Co) was dissolved in 5 ml of sterile water i.e. distilled water under continuous stirring for 10 minutes to obtain tetracycline HCl solution of pH 1.3 as checked using a pH meter.[12]

b) Citric Acid: 65 grams of anhydrous citric acid crystals (Glaxo Smith Klein Co) were dissolved in 100 ml of distilled water at room temperature till the solution became saturated to obtain citric acid at pH 1 which was checked using pH meter.[13]

c) EDTA: 15% EDTA solution was prepared by combining 25 ml of distilled water with 2.31 ml of 5 N NaOH (Glaxo Smith Klein Co), and then adding 4.25 gm of the disodium salt of EDTA (Glaxo Smith Klein Co). This solution had a pH of 7.5 as checked by using a pH meter.[14]

Each of the four groups analysed in this study contained 6 samples: 1) Immersed in citric acid solution 2) in tetracycline hydrochloride solution 3) in EDTA solution 4) in PBS.

Immersion was carried out for 5 minutes. After conditioning, three-5 minutes washes in PBS were carried out. After this, a healthy female donor was hematologically tested and a drop of her whole peripheral blood applied to external dentin surface on each root block. The blood was allowed to clot for 20 minutes in a humidifier chamber. Block were than rinsed three times for 5 minutes in PBS. Washes and rinses were carried out in small petri dish with gentle swirling motion. All steps were carried out at room temperature.[15]

SEM analysis preparation

Immediately after rinsing, the blocks were fixed in 1% formaldehyde in PBS for 15 minutes followed by three 5 minute PBS rinses. After this the blocks were incubated for 10 minutes in 0.02 M glycine in PBS and rinsed again, as before. The samples were post-fixed in 2.5% glutaraldehyde in PBS for 30 minutes and rinsed again, as above. The samples were then dehydrated through a graded ethanol series: 25%, 50%, 75%, 95% and 3 exchanges of 100%, all steps at room temperature. The teeth were kept for fifteen minutes in all graded series and for half an hour in 100% acetone.[11]

The samples were subsequently dried overnight in a dehydration jar. The dentine blocks were mounted on aluminium stubs with an adhesive tape with the labial

side of the roots facing the beam of the SEM and in such a ways that the root were placed in the center of the stubs. They were vacuum coated with a thin layer of carbon, sputter coated with gold/palladium. The gold coating was done to ensure a proper conducting surface to the non-conducting specimen. The coated specimens were removed from the sputter coating unit and then the stubs were mounted on the specimen stage of the SEM unit. Observations were performed with a scanning electron microscope (Jeol JSM, Tokyo, Japan) at 15.0 KV and observed on the computer screen fitted with SEM. Photomicrographs of representative areas were taken with a 35-mm camera.[11]

SEM examination

All specimens were examined and one photomicrograph obtained from a random area treated with blood tissue at 2000x magnifications using scanning electron microscope operated at an accelerated voltage of 15 KV. After the photomicrographs were obtained, they were identified and analyzed through scores in order to verify the adhesion of blood components and to analyze the morphological characteristics of root surface obtained after treating with various root conditioning agents. Using a single blind method, the photomicrographs obtained from samples that received blood tissue were examined three times by an operator who was previously trained. It was again calibrated with two other operators. Each sample received the score that prevailed among the three readings.[15,16]

Scoring criteria[15]

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

Statistical analysis was done with standard computer software. The non parametric Kruskal Wallis test ($p<0.05$) was employed to compare the rank of the evaluated groups. This procedure was followed by non parametric Mann-Whitney U test when the Kruskal Wallis test suggested a significant difference between the groups ($p<0.05$). The Mann-Whitney U test ($P\leq0.05$)

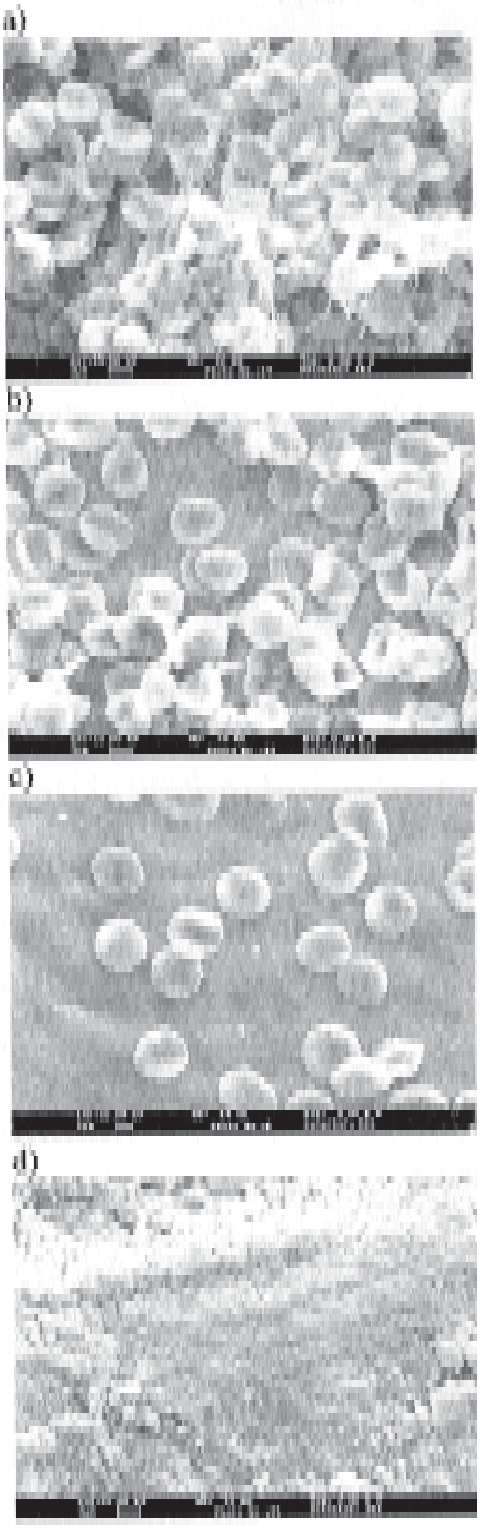

Fig 1: Photomicrographs of the root surfacesshowing a) dense red blood cells and fibrin attachment aftertreatment with CA, b) moderate red blood cells and fibrin attachment after treatment with TTC HCl, c) scarce red blood cells and fibrin attachment after treatment with EDTA, d) smear layer after treatment with PBS (Original magnificaion x2000)

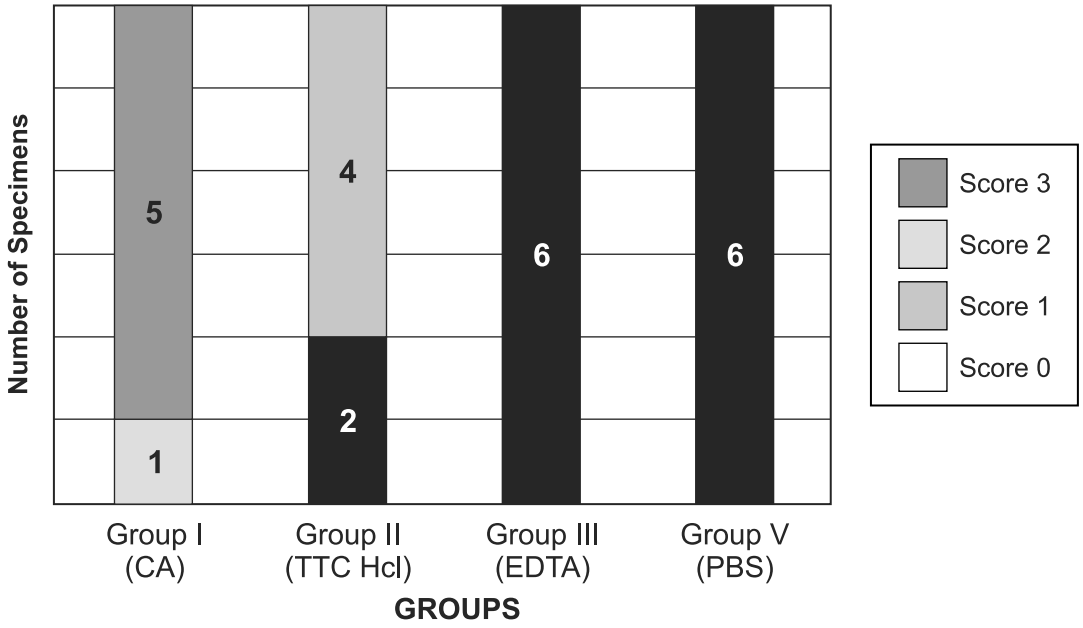

Fig 2: Score distribution according to the different treatments performed

was calculated to determine differences between individual groups.

RESULTS

Scanning electron microscopy analysis

*In group 1 i.e CA treated samples, the*re was dense fibrin network and entrapped blood cells (score 3) in five samples while one sample showed moderate fibrin and blood cells attachment (score 2) (Fig 1a).

In group 2 i.e TTC HCl treated samples, four samples showed moderate fibrin and blood cells attachment (score 2) while only two samples showed scarce fibrin and blood cells attachment (score 1) (Fig 1b).

In group 3 i.e EDTA treated samples, all the 6 sample showed scarce fibrin and blood cells attachment (score 1) (Fig 1c).

In group 4 i.e PBS treated samples (control), none of the 6 samples showed any attachment of fibrin or blood cells (score 0) (Fig 1d).

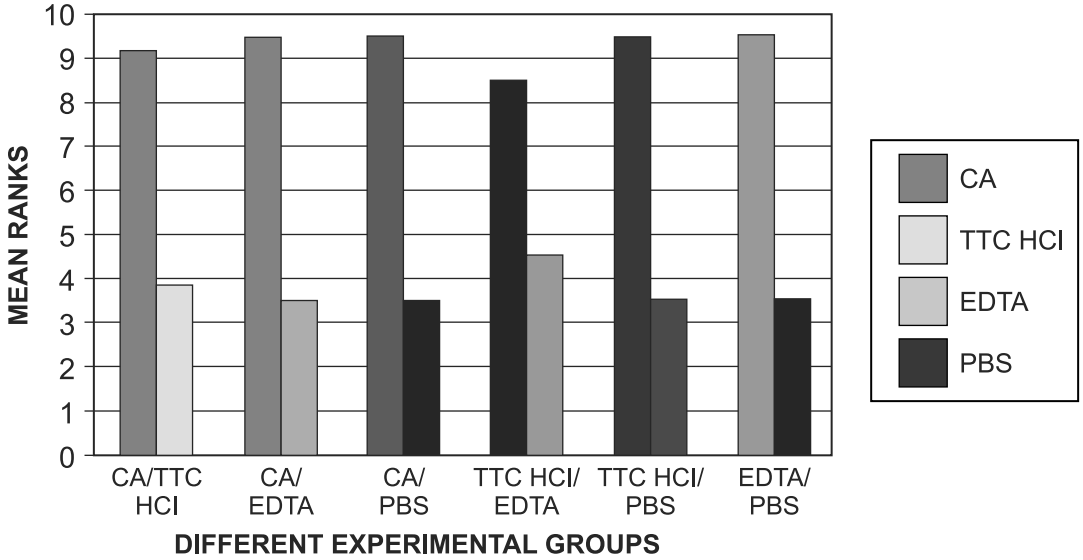

Fig 3: Shows inter group comparison of adhesion of fibrin and blood cells between different groups using mean ranks

TABLE 1: ALLOCATION AND COMPARISON OF ADHESION OF BLOOD COMPONENTS AND FIBRIN NETWORK BETWEEN DIFFERENT GROUPS

Groups	Absence of fibrin network and blood cells (Score 0)	Scarce fibrin network and/ or blood cells (Score 1)	Moderate fibrin network and blood cells (Score 2)	Dense fibrin network and trapped blood cells (Score 3)	Mean Ranks (R)	P value
Group I Citric Acid (CA)	0	0	1	5	21.17	
Group II Tetracycline HCl (TTC HCl)	0	2	4	0	14.83	(p=0.000
Group III EDTA	0	6	0	0	10.50	
Group IV PBS	6	0	0	0	3.50	

TABLE 2: INTER GROUP ANALYSIS

Comparison	Groups	No of specimens	Mean rank	Z value	P value
1	Group I CA	6	9.17	-2.768	0.006
	Group II TTC HCl	6	3.83		
2	Group I CA	6	9.50	-3.207	0.001
	Group III EDTA	6	3.50		
3	Group I CA	6	9.50	-3.207	0.001
	Group IV PBS	6	3.50		
4	Group II TTC HCl	6	8.50	-2.345	0.019
	Group III TTC EDTA	6	4.50		
5	Group II TTC HCl	6	9.50	-3.146	0.002
	Group IV PBS	6	3.50		
6	Group III EDTA	6	9.50	-3.317	0.001
	Group IV PBS	6	3.50		

Statistical Results

Considering groups as an independent variable, the use of non parametric Kruskal Walli test showed a significant difference among the evaluated groups regarding blood components adhesion scores (p=0.000) (Fig 2).

The comparison between the mean ranks of the groups (Mann-Whitney U test) showed a significant differences between group 1 and group 2 (p=0.006), group 1 and group 3 (p=0.001), group 1 and group 4 (p=0.001), group 2 and group 3 (p=0.019), group 2 and group 4 (p=0.002), group 3 and group 4 (p=0.001) (Fig 3).

When group 1 was compared with group 2, 3 and 4, it was observed that the blood components adhesion score was highest in group 1, in the same way, group 2 showed higher scores for blood components attachment when compared to group 3 and 4.

DISCUSSION

Root surface conditioning by topical application of acidic solutions has been demonstrated to remove the smear layer and also any remaining root surface contaminants.[1] It uncovers and widens the orifice of dentinal tubules[12] with unmasking of the intra-and peri-tubular dentin collagen matrix[6,12] due to demineralization. This is indispensable for adhesion of fibrin clot.

The clot adhesion appears vitally dependent on the formation of a resilient unit between the clot (fibrin

network) and the collagen fibers exposed at the root surface.[7,15]

The present study was designed to evaluate the extent of the fibrin clot adhesion on cleansed root conditioned surfaces.

The study sample consisted of 24 single rooted human teeth, affected by periodontitis with grade III mobility extracted from patients with no systemic disease.

Only single rooted teeth were selected for the purpose of standardization. Teeth affected by caries were not included in the study as it could adversely affect the root surface topography.[17] Minimal instrumentation during extraction was considered to avoid chipping off root structure.[18,19] Teeth with immediate past history of scaling and root planning procedures were excluded from the study as these procedures may alter the root surface. Teeth with attrition, abrasion and erosion were excluded as they are shown to produce secondary changes in the tooth structure like alteration in mineral composition and the formation of sclerotic dentin.[20]

The obtained specimens were categorized into 4 groups (one control and three experimental) consisting of 6 specimens in each group. Phosphate buffered saline was used for temporary storage of the teeth. The solution used during this short holding time should not have affected the final surface characteristics.[21]

The root specimens were immersed in root conditioning agents for five minutes. This is in accordance to the study done by Wen CR et al (1992)[13] showing that wide opening of dentinal tubules and tufting of inter tubular dentin fibrils was highest in immersion group as compared to cotton pellet placement or burnishing technique or those treated with camel hair brush. The application of root conditioning agents resulted in root surface demineralization exposing dentinal tubules and collagen of intra-and peritubular dentinal matrix[11] which leads to adhesion of fibrin clot.[7]

The in vitro modeling system[11] used in this study provides a simulation of most of the critical steps during the earliest healing events following periodontal tissue regeneration procedures. This method allows observations of the dentin surface directly following various stages of simulation i.e 1) root instrumentation; 2) dentin conditioning to remove the instrumentation surface smear layer; 3) formation of the fibrin clot. It is conceivable that those conditions that may promote or adversely affect fibrin clot adhesion in this model system may also produce similar effects in vivo.

Instrumentation of the human dentin blocks produced a smear layer as has been shown in previous reports.[22,23] Conditioning of the instrumented dentin with a saturated citric acid solution, tetracycline hydrochloride solution or EDTA solution, but not PBS, at least in part, removed the smear layer exposing dentinal tubules and the intra and inter tubular collagenous matrix. This observation is in harmony with a large number of reports evaluating the effect of acidic and chelating agents on the ultrastructure of dentin surfaces.[6,7,11,23]

Group 1 (Citric acid) exhibited vast three dimensional array of dense inter connected fibrin strands enmeshing the trapped blood cells (seen in 5 samples) to moderate fibrin network and blood cells (seen in 1 sample).

When citric acid was compared with other groups, the attachment of fibrin and blood cells was statistically significant. The mean rank of citric acid was 9.17 while for TTC was 3.83, mean rank of citric acid was 9.50 while for EDTA was 3.50 and mean rank of citric acid was 9.50 while for PBS was 3.50 (Table 2).

The possible explanation of the results seen in the citric acid could be that saturated citric acid successfully removes the smear layer[24,25] and provides greater depth of demineralization as compared to TTC HCl as well as increased tubular diameter, as was seen in citric acid conditioned root surfaces.[12,21] All this increased the wettability of dentin resulting in enhanced attachment of the fibrin clot imposed on to the root surfaces, as continuous adhesion of fibrin clot appears dependent on the wettability of the substrate.[7] It is observed that although citric acid is an anticoagulant, it has shown no adverse effects on early fibrin polymerization.[8]

The results obtained in this study are in accordance to those obtained by Baker PJ et al (2000)[11]; Baker DL et al (2005)[7] in their in vitro studies. Also, Polson AM (1983)[26] have shown the efficacy of citric acid conditioning to support maturation of fibrin clot into a new

connective tissue attachment using a non human primate replantation model.

In contrast, animal studies by Nyman et al (1981), Gottlow et al (1984) and clinical trials by Stahl and Froum (1977); Stahl et al (1983), Smith et al (1987) utilized citric acid for root conditioning have failed to result in new attachment.[5] Speculative explanation for these in consistent findings have included variations in animal models, inconsistent flap adaptation, inadequate demineralization of periodontitis affected root surface[3] and repopulation of the root surface with inappropriate cell types.[27]

Group II (TTC HCl) exhibited the presence of moderate fibrin network and moderate attachment of blood cells (in 4 samples) to scarce fibrin network and blood cells (in 2 samples).

When tetracycline was compared with other groups i.e EDTA and PBS, attachment of fibrin and red cells was statistically significant. The mean rank of TTC was 8.50 while for EDTA was 4.50, the mean ranks for TTC was 9.50 while for PBS was 3.50 (Table 2).

The possible explanation of these results seen in tetracycline hydrochloride could be that tetracycline hydrochloride was successful in removing the smear layer and exposed the opening of dentinal tubules with collagen matrix.[1,28] In the present study TTC HCl fared better than EDTA group as regards to fibrin network and blood cell attachment. It can be due to greater demineralization potential of tetracycline than EDTA. According to Claffey et al 1987 more superficial demineralization obtained with tetracycline is responsible for a more favourable healing response. Several in vivo studies have demonstrated that tetracycline demineralized dentin surfaces showed greater number of attached cells[24], greater connective tissue attachment[28,29], reattachment and new cementum formation.[28]

In contrast, in a study done by Frantz B and Polson A (1988)[24] using a non human primate implantation model, it was shown that enhanced response to cell attachment after demineralization by tetracycline did not result in a connective tissue attachment and tetracycline released from root surface altered the biological cascade of healing by altering PMN functions. Also Delazari FMC et al (1999)[30] found fibrin network formation in situ was not improved by application of TTC-HCl.

The root specimens treated with EDTA (Group III) demonstrated scarce fibrin network and blood cells. When EDTA was compared to PBS, the attachment of fibrin and red cells was found to be statistically significant. The mean rank of EDTA was 9.50 while for PBS was 3.50 (Table 2).

It was observed that most samples in group III inhibited blood element adsorption and adhesion to the root surface. The possible explanation could be that EDTA is a calcium chelator and may have inhibited or retarded coagulation events.[I5,30,31] It was also observed that EDTA did not consistently produce a smear layer free dentin surface.[7]

In contrast, studies conducted by Blomlof and Lindskog (1995)[6] have shown that etching at neutral pH with EDTA have shown to be equally, if not more efficient compared to agents operating at low pH in exposing collagen fibres on dentin surfaces.

The root specimens treated with PBS (group IV) demonstrated no fibrin network or blood cell attachment. This could be due to presence of smear layer on root specimens after treatment with phosphate buffered saline. Specific dentin topographic features such as dentinal tubules, intra-and inter-tubular collagen fibrils were not readily discernible.[7,16] The results obtained in this group are in contrast to the study done by Leite FRM et al (2005)[15] who showed that untreated planed dentin presented the best results with blood cells entrapped in a thick web of fibrin. This could be due to the more repeated 5 min washings with PBS done after individual treatment with Formaldehyde, Glycine, Glutaraldehyde in this study which might have washed the fibrin network [11] as compared to 5 min. washings with PBS done only after Glutaraldehyde treatment by Leite FRM et al in their study.

In perspective, it appears reasonable to suggest that in vitro protocols that sustain fibrin clot adhesion to dentin may support wound maturation into a connective tissue attachment in vivo. However, protocols that are less successful in vitro should not be expected to support fibrin clot adhesion in a clinical scenario.[7]

CONCLUSION

From this study it is concluded that the application of citric acid and tetracycline on instrumented

periodontally diseased roots were better root conditioning agents for the adhesion of the fibrin clot and could prove to be a useful tool in the establishment of a new connective tissue attachment in periodontal regenerative procedures.

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