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ORIGINAL RESEARCH

Blood Cells Attachment after Root Conditioning and PRP Application: An *in vitro* Study

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ABSTRACT

Aim: Root conditioning is aimed at smear layer removal and at dental matrix collagen exposure, which may promote periodontal regeneration. This *in vitro* study assessed smear layer removal, collagen fiber exposure and the influence of PRP (platelet-rich plasma) application on adhesion of blood cells to the root surface using scanning electron microscopy (SEM).

Materials and methods: Scaled root samples (n = 160) were set in five groups and conditioned with: group I – control group (saline solution); group II (EDTA 24%); group III (citric acid 25%); group IV (tetracycline hydrochloride 50 mg/ml); group V (sodium citrate 30%). Eighty samples were assessed using the root surface modification index (RSMI). The other eighty samples were set in two groups. The first group (n = 40) received PRP gel application with a soft brush and the second group (n = 40) received PRP application and then a blood drop. The fibrin clot formation was assessed in the first group and the blood cells adhesion was assessed in the second group using the BEAI (blood elements adhesion index). A previously trained, calibrated, and blind examiner evaluated photomicrographs. Statistical analysis was performed using the Kruskal-Wallis's and Dunn's tests.

Results: Group III attained the best results for RSMI and BEAI. Moreover, it was the only group showing fibrin clot formation.

Conclusion: Citric acid was the most efficient conditioner for smear layer removal, collagen fiber exposure and blood cell adhesion. Moreover, it was the only group showing fibrin clot formation after PRP application.

Clinical significance: This study demonstrated that root conditioning followed by PRP application may favor blood cell adhesion on root surface which may optimize periodontal healing.

Keywords: Root conditioning, Blood, Platelet-rich plasma, Smear layer, Edetic acid, Citric acid, Tetracycline, Laboratory research.

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Conflict of interest: None declared

INTRODUCTION

Microorganisms associated toxins adsorbed to dental surface may promote periodontal breakdown trough inflammatory response. Periodontal therapy involves scaling and root planning which is aimed at removing bacterial deposits and byproducts from dental surface. Nevertheless, this procedure produces a smear layer formed by residues of calculus, biofilm, contaminated cement and dentin and bacterial byproducts.[1]

Root conditioning agents may remove smear layer and expose dental matrix associated collagen. Root dental modification may favor formation of new cement, periodontal ligament and bone by facilitating periodontal ligament cells to attach to the root surface.[2-4] Many root conditioning agents have been studied *in vitro* to assess their ability to remove smear layer and expose dental matrix collagen. For instance, chelating agents as EDTA[5] and sodium citrate,[6] low pH agents as citric acid[7] and tetracycline hydrochloride.[8,9] There are also studies assessing the adhesion of blood elements to dental surfaces modificated with this agents.[6,10] Blood clot formation and stabilization is important to periodontal regeneration because it would prevent apical epithelial migration during healing process.[11,12]

Platelets have central participation in coagulation process and produce signaling molecules that modulate inflammation and tecidual healing.[13] Platelets have their hole in hemostasia, coagulation and healing, which led PRP to be assessed to favor tissue regeneration.[14,15] Nevertheless, the authors were unable to find in the literature any study assessing the effect of root surface modification and PRP

application on root surface on the adhesion of blood elements to it. So the objectives of this study were to evaluate using SEM:

1. Root surface modification produced by EDTA 24%, citric acid 25%, tetracycline hydrochloride 50 mg/ml and sodium citrate 30%.
2. The influence of root modification on stabilization of PRP on the root surface.
3. The influence of root modification and PRP application on the stabilization of blood elements on the root surface.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of the School of Dentistry at Araraquara, São Paulo State University – UNESP (protocol # 10/04). A total of 80 teeth were obtained from the undergraduate oral surgery clinic after patients conveyed written consent to use their extracted teeth. Inclusion criteria for teeth were advanced periodontal breakdown (attachment loss in more than 50% of root length), caries-free cementoenamel junction (CEJ) and donator must be never smoker due to higher degree of mineralization of their teeth.[16,17]

Extrated teeth were stored in tubes (Eppendorf, New England, USA) containing filtered water at room temperature. Water has been renewed once a week to avoid medium acidification, microorganism growth and sample dehydration.

Sample Preparation

Samples were obtained from the cervical third of the roots by making two parallel grooves of 0.5 mm depth with a cylindrical bur (KG Sorensen, Barueri, SP, Brazil) under copious irrigation. The first groove was positioned horizontally at the CEJ and the second groove was made parallel and 4 mm apical in relation to the first. The area between the two grooves was then scaled with 50 apico-cervical strokes using a sharp #5-6 Gracey curette (Hu-Friedy, Chicago, IL, USA).

Two samples were produced from each tooth, thus, a total of 160 samples were prepared and stored in the same tubes containing filtered water.

Conditioning Procedure

The 160 samples were conditioned with different substances. Eighty samples were used to assess root surface modification and the other 80 samples were fixed into containers using utility wax to keep them stable during PRP (n = 8) or PRP+blood (n = 8) application.

Samples were divided in five groups containing 32 samples each:

- *Group I (control):* Irrigation with 10 ml of saline solution
- *Group II:* EDTA 24% gel (Farmácia Santa Paula, São Paulo, Brazil) application with a soft brush
- *Group III:* Citric acid 25% solution (Farmácia Santa Paula, São Paulo, Brazil) application using a cotton pellet embebed in the solution
- *Group IV:* Tetracycline hydrochloride 50 mg/ml solution (Farmácia Santa Paula, São Paulo, Brazil) application using vigorous burnishing of a cotton pellet embebed in the solution
- *Group V:* Sodium citrate 30% solution (Farmácia Santa Paula, São Paulo, Brazil) application using a soft brush.

Samples in the groups II, III, IV and V were conditioned during 3 minutes and solutions were renewed every 30 seconds.[18-20] After the conditioning procedure samples were washed with 10 ml of saline solution.

Preparation for SEM

Sixteen samples of each group were prepared for SEM analysis. Samples were dehydrated by the critical point method which consisted of immersion in ethanol concentrations of 30, 50, 70, 80, 95 and 100% for 1 hour each. After the final immersion in 100% ethanol concentration, samples were immersed in a 50% (v/v) solution of 100% ethanol and hexamethyldisilazane (HMDS) (Sigma, Sigma-Aldrich Inc, St Louis, MO, USA) for 30 minutes and a final immersion in HMDS only for 10 minutes. HMDS allows visualization of the collagen matrix in SEM photomicrographs. Finally, samples were dried for 48 hours in a dehydration jar (Corning, Sao Paulo, Brazil) and mounted on metallic holders (Senai, Sao Paulo, Brazil) for sputter coating with 99.99% pure gold.

Blood Elements Adhesion Evaluation

Sixteen remainig samples in each group received PRP (n = 8) or PRP+blood (n = 8) application.

PRP Application

A blood sample of 4.5 ml was retrieved from a male healthy volunteer. Blood was stored in a tube (Vacutainer, BD Becton, Dickinson and Company; Franklin Lakes, NJ, USA) containing sodium citrate 3.2% in a proportion of 10% of the total volume. The tube was centrifuged at 1.100 rpm during 10 minutes (Centrifuga Eppendorf 5702, Becton Dickinson, Germany). After this procedure tube showed two distinct layers: The inferior layer composed of red cells and the upper layer composed of white cells. The PRP was aspirated with a pipette (Biohit Proline Pipettor 100 µl, Helsinki, Finland) and subsequentely activated by calcium clorer 10% by mixing 1ml of PRP with 50 µl of $CaCl_2$. Twenty minutes later PRP gel was ready to use. The PRP gel was applied to samples (n = 16) using a soft brush leaving a uniform layer on the sample.

Blood Application

Imeadiately after PRP application, eigth samples received a drop of fresh blood on it[10] which was left to rest for 20 minutes. After this period, samples were washed three times for 5 minutes in PBS (Merck, Darmstadt, Germany), under soft agitation (60 rpm) (Mini-Rocker Shaker, Boeco, Hamburg, Germany).

Preparation for SEM

Samples were fixed in formaldehyde 1% (Merck, Darmstadt, Germany) solution in PBS for 15 minutes and washed three times for 5 minutes in PBS. After this, samples were incubated for 10 minutes in 0.02 M glycine (Merck, Darmstadt, Germany) solution in PBS and washed as described earlier. Subsequentely, samples were fixed in glutaraldehyde 2.5% solution in PBS for 30 minutes and washed as described earlier. Then they were dehydrated by immersion in ethanol concentrations of 25, 50, 75 and 95% for 10 minutes each and washed three times for 10 minutes each in 100% ethanol. The dehydration process was finished in a critical point device (Baltec CPD 030, Flórida, USA). Finally, samples were mounted on metallic holders for sputter coating with 99.99% pure gold. Table 1 illustrates sample distribution on the experimental groups.

SEM Examination

Photomicrographs were made (JEOL JSM-T330A adjusted to 20kV) of the center area of each sample. Samples that received blood application were evaluated under 500x and 1000x magnifications and all other samples were evaluated under 2000x magnification. Evaluation was performed by a trained and calibrated examiner who was unaware of the experimental groups.

The root surface modification index (RSMI)[6] was used to evaluate photomicrographs of samples that received root conditioning procedures only (Table 2). Photomicrographs of samples that received PRP application were evaluated considering presence (Fig. 1) or absence (Fig. 2) of fibrin network. Finally, photomicrographs of samples that received blood application were evaluated by the blood elements adhesion index (BEAI)[21] presented on Table 3.

Each photomicrograph was evaluated three times in intervals of at least 7 days. The score attributed to each sample was the most prevalent score of the three evaluations. Good reproducibility was achieved in the use of the indexes. Kappa, k = 0.810 (BEAI) and k = 0.949 (RSMI).

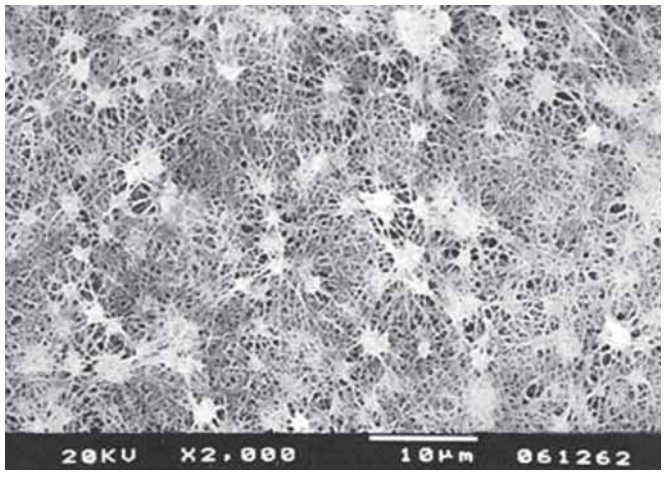
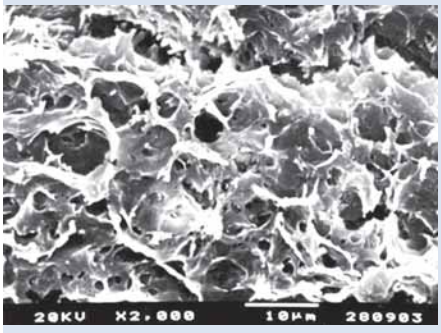


Fig. 1: Presence of fibrin network

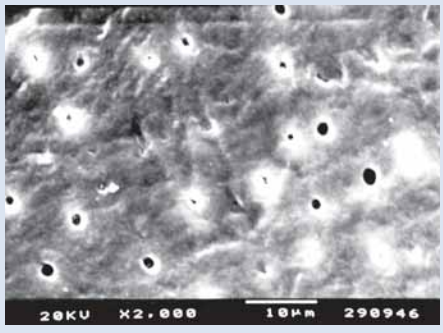
Table 1: Distribution of samples in the treatment groups.

Group treatment	*Control (I)*	*EDTA (II)*	*Citric acid (III)*	*TTCHCl (IV)*	*Sodium citrate (V)*	*Total*
HMDS	16	16	16	15	16	79
PRP	8	8	8	8	8	40
PRP + blood	8	8	8	8	8	40
Total	32	32	32	31	32	159

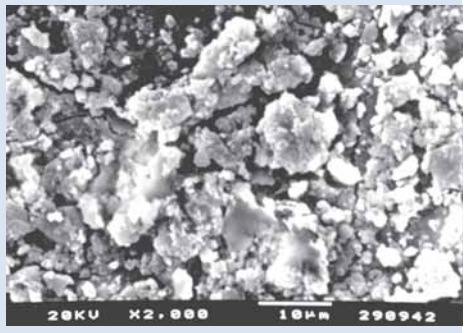
Table 2: Root suface modification index (RSMI)



Score 1: Observation of cementum/dentin and exposure of collagen of dental matrix



Score 2: Observation of cementum/dentin with no exposure of collagen of dental matrix



Score 3: Presence of smear layer

STATISTICAL ANALYSIS

Groups were compared on ordinal (RSMI and BEAI) and dichotomous variables by nonparametric analysis of variance (Kruskal-Wallis and Dunn tests) using a significance of $p \leq 0.05$. These analyses were performed using the Bioestat 3.0 statistical software package (CNPq and Sociedade Civil Mamirauá, Belém PA, Brazil).

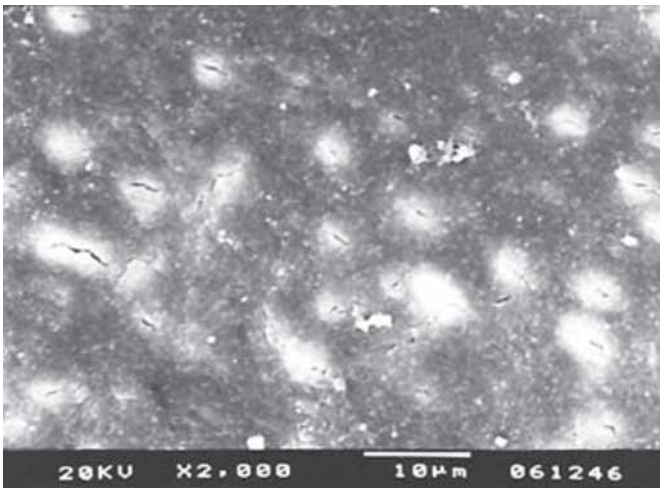


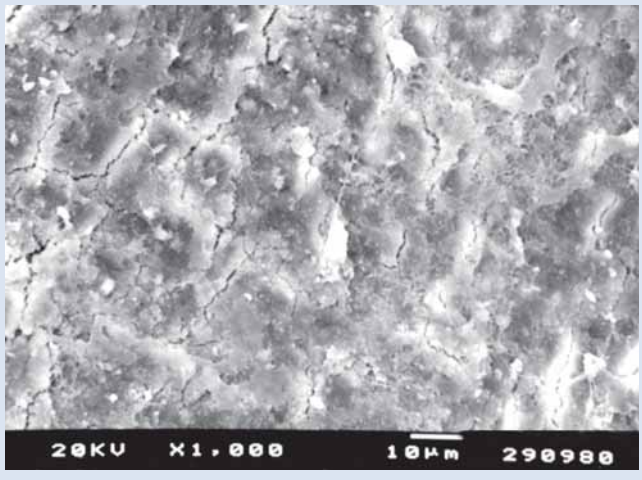
Fig. 2: Absence of fibrin network

RESULTS

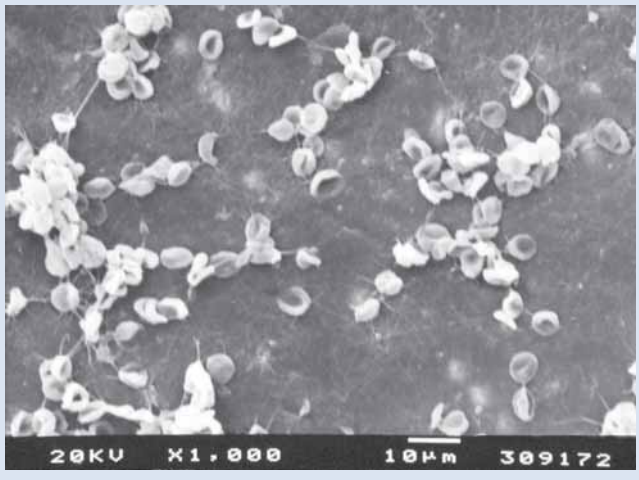
Among five groups compared using the RSMI, difference was detected ($p = 0.0001$) between control group and all other groups. Nevertheless, group II presented score 1 frequency of 25% and score 2 of 75%; the group III presented score 1 frequency of 56.25% and score 2 of 43.75%, the best results for RSMI. Less favorable results were observed in the control group with 6.25% of score 2 and 93.75% of score 3 (Table 4). Groups IV and V presented samples with 100% of score 2 (Fig. 3).

Regarding samples which received PRP application, only those previously conditioned with citric acid 25% showed well defined fibrin network (Table 5).

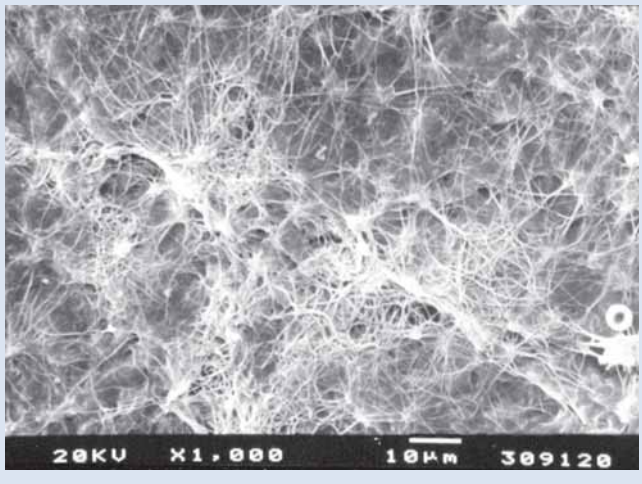
Table 3: Blood elements adhesion index (BEAI)



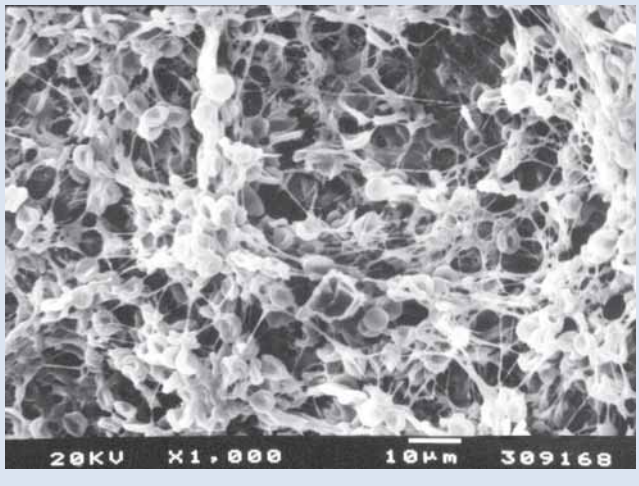
Score 0: Absence of fibrin network and blood cells



Score 1: Scarcely distributed fibrin network and/or blood cells



Score 2: Moderate number of blood cells and thin fibrin network with poor interlacing



Score 3: Dense fibrin network with rich interlacing and presence of blood cells

Table 4: Frequency distribution of scores according to the RSMI

	Control (I) n(%)	*EDTA (II) n(%)*	*Citric acid (III) n(%)*	*TTC HCl (IV) n(%)*	*Sodium citrate (V) n(%)*
score 1	0	4 (25.0)	9 (56.2)	0	0
score 2	1 (6.3)	12 (75.0)	7 (43.4)	15 (100.0)	16 (100.0)
score 3	15 (93.7)	0	0	0	0
Total	16	16	16	15	16

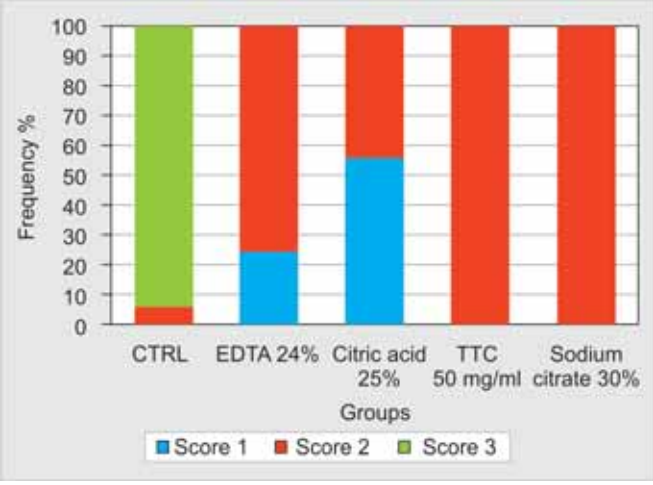


Fig. 3: Frequency of scores 1, 2 and 3 in different groups of demineralizing agents, according to the root surface modification index (RSMI)

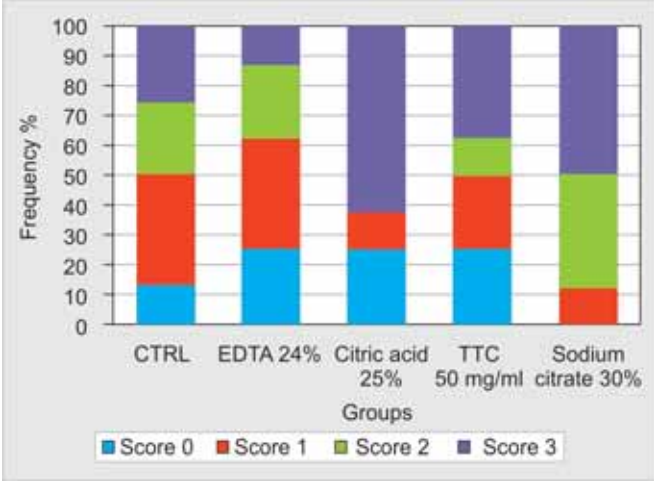


Fig. 4: Frequency of scores 0, 1, 2 and 3 according to the blood elements adhesion index (BEAI), in the samples of groups I (control), II (EDTA 24%), III (citric acid 25%), IV (tetracycline hidrochloride 50 mg/ml) and V (sodium citrate 30%) that received application of PRP and blood

There was no statistical difference among sample who received blood application (p = 0.3380). Nevertheless, groups III and V presented higher frequency of score 3 (62.5 and 50%, respectively) (Fig. 4). Group IV presented 37.5% of score 3, followed by groups I and II (25 and 12.5% respectively) (Table 6).

Table 5: Frequency distribution of samples according to the presence of fibrin network

Groups	*P*	*A*
I-Control n(%)	0 (0)	8 (100)
II-EDTA 24% n(%)	0 (0)	8 (100)
III-Citric acid 25% n(%)	7 (87.5)	1 (12.5)
IV-Tetracycline hydrochloride 50 mg/ml n (%)	0 (0)	8 (100)
V-Sodium citrate 30% n(%)	0 (0)	8 (100)

Presence (P); Absence (A)

DISCUSSION

To achieve periodontal regeneration, it is necessary to remove plaque, calculus and bacterial byproducts from root surface.[7] Scaling and root planning are considered the best method to do it.[7,8] Nevertheless, this procedure leads to smear layer formation[22] which can impair periodontal healing and regeneration. Thus, attention has been given to root surface modification through demineralizing agents, which promotes additional root decontamination and dental matrix collagen exposure aiming new periodontal attachment formation.[10,23]

In the present study, all demineralizing agents were able to remove smear layer, but only some of them exposed the collagen from the dental matrix (Fig. 3). In accordance with

Table 6: Frequency distribution of scores according to BEAI

	Control (I) n (%)	*EDTA (II) n (%)*	*Citric acid (III) n (%)*	*TTC HCl (IV) n (%)*	*Sodium citrate (V) n (%)*
Escore 0	1 (12.5)	2 (25.0)	2 (25.0)	2 (25.0)	0
Escore 1	3 (37.5)	3 (37.5)	1 (12.5)	2 (25.0)	1 (12.5)
Escore 2	2 (25.0)	2 (25.0)	0	1 (12.5)	3 (37.5)
Escore 3	2 (25.0)	1 (12.5)	5 (62.5)	3 (37.5)	4 (50.0)
Total	8	8	8	8	8

previous studies,[24] citric acid 25% and EDTA 24% presented high capacity of root surface modification. Tetracycline hidrochloride 50 mg/ml presented high smear layer removal capacity, which is in accordance with previous studies[22] and showed no capacity of dental matrix collagen exposure, what does no corroborate previous data.[9,25] This unexpected observation may be attributed to the fast deterioration of this chemical after its preparation, once concentration, period of application and mode of application were standardized. Sodium citrate 30% was not able to expose collagen fibers from dental matrix, so new *in vitro* studies are necessary to establish the parameters for its use as dental root modification agent.

It was observed fibrin network and blood cells attached to the dental surface in all groups that received blood application. Nevertheless, there was variability in this phenomenon among groups (Fig. 4). Adhesion of blood elements to dental surfaces in the control group is in discordance with previous studies[6,10] and may be explained by the sample roughness which created a mechanical retention of blood clot.

Although EDTA 24% has a high capacity of smear layer removal and dental matrix collagen exposure working in neutral pH (more biocompatible), it does not favor blood elements adhesion to the root surface,[6] which is in accordance with the results of the present study. This may be explained due to its strong chelation character, which may inhibit formation and adhesion of blood clot to the dental surface and may promote early platelet fragmentation.[26]

Absence of blood elements on the samples conditioned with tetracycline hydrochloride 50 mg/ml may be explained by the weak capacity this chemical showed to expose dental matrix collagen (Table 6).

In vivo studies have shown the ability of citric acid to provide blood clot stabilization on dental surface[27] (corroborating data of the present study) and to provide linkage between collagen from dental matrix and from periodontal connective tissue.[28,29]

The goods results observed in the present study on the adhesion of blood elements over sodium citrate conditioned samples determine the need of additional studies to verify this phenomenon.

Regarding samples that received PRP application, only those previously conditioned with citric acid 25% presented fibrin network (Table 5). The authors attribute this finding to increased dental matrix collagen exposure attained with citric acid 25% conditioning which may favor platelets adhesion to dental surface. The fact that platelets have affinity for collagen and that citric acid is easily washed off from dental surface after conditioning procedure are factors that may be contributed to the citric acid conditioning performance. Chelant agents (EDTA and sodium citrate) and tetracycline hydrochloride (which presents high substantivity)[30] are more difficult to be removed from the dental surface and remaints of these agents may interfere on PRP adhesion.

The encouraging results observed in the present study on the adhesion of blood elements over sodium citrate conditioned samples determine the need of additional studies to verify this phenomenon.

CONCLUSION

According to the results and considering the limitations of the present methodology, the authors conclude that:

Citric acid 25% was the most efficient conditioning agent to remove smear layer, to promote dental collagen exposure and to promote fibrin clot and blood elements adhesion to the dental surface.

CLINICAL SIGNIFICANCE

This study demonstrated that root conditioning followed by PRP application may favor blood cell adhesion on root surface which may optimize periodontal healing.

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