Smear Layer Removal for Collagen Fiber Exposure after Citric Acid Conditionings

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Abstract

Aim: The aim of the present study was to compare the removal of the smear layer and exposure of collagen fibers of the root surface following the application of five citric acid solution concentrations.

Methods and Materials: Two hundred seventy (270) samples were equally divided into six groups (n=45) for treatment with saline solution (control) and five different concentrations of citric acid (0.5, 1, 2, 15, and 25 percent). Three acid application methods were used (passive, brushing, and burnishing) as well as three application periods (1, 2, and 3 minutes). A previously trained, calibrated (kappa score = 0.93), and blind examiner subsequently scored scanning electron micrographs (SEMs) of the samples. Statistical analyses were performed by using Kruskal-Wallis and Dunn’s post-hoc tests.

Results: According to the results obtained and within the limitations of the methodology used, the citric acid applications were more effective than the control treatment of applying saline solution (p<0.05). However, no statistically significant differences were observed among the three application methods and three application periods. Descriptive analyses showed that best results for exposure of collagen fibers were obtained with the application of citric acid at 25 percent by brushing for 1 or 3 minutes, even though there were no statistically significant differences among the groups.

Clinical Significance: The best results for exposure of collagen fibers on root surfaces noted in this study were obtained with application of citric acid at 25 percent by brushing for 1 or 3 minutes.

Keywords: Acid conditioning collagen fibers, citric acid, laboratory study, root surfaces, smear layer

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Methods and Materials

This study was approved by the Research Ethics Committee of Araraquara Dentistry School—UNESP—Brazil (protocol # 31/06). A total of 135 teeth were obtained from the Human Tooth Bank of the institution. These teeth had no evidence of dental caries, restorations below the cementoenamel junction (CEJ), or any other cervical root lesions.

Sample Preparation

The cervical third of roots was chosen for the sample preparation. Samples were prepared by making two parallel grooves, approximately 0.5 mm wide, on the buccal and lingual root surfaces of each tooth using a high-speed cylindrical diamond bur (KG Sorensen, Barueri, SP, Brazil) under copious water irrigation. The first groove was made horizontally at the cementoenamel junction, and the second groove was made approximately 3 mm from the first in the apical direction. The same bur was used to remove the surface layer of the root between the two grooves (Figure 1). The area between the two grooves was then scaled with 50 apical-cervical strokes using a sharp # 5-6 Gracey curette (Hu-Friedy, Chicago, IL, USA) (Figure 2).

Two samples were obtained from each tooth and all of them were stored in containers with saline solution.
Two hundred seventy dentin samples, approximately 3 × 4 × 1 mm in size, were obtained and randomly divided into five experimental groups (n=45 for each group) and one control group (Figure 3).

The control group was conditioned with saline solution and the experimental groups were conditioned with different citric acid concentrations (0.5, 1, 2, 15, or 25 percent). These six groups were further divided into three subgroups (15 samples each) according to the application method of the solutions used. There were also three application methods used: (1) passive application with a small, cotton pellet; (2) brushing application with a soft brush; and (3) burnishing application with a small, cotton pellet. Each of these three subgroups was further divided into three application periods of 1, 2, or 3 minutes with 5 samples in each subgroup.

During each application period, solutions were renewed on the cotton pellet or brush every 30 seconds. A new pellet or brush was used for each solution. Thereafter each sample was rinsed with 10 ml of saline solution and identified with a code for blind scoring of the SEMs.

All samples were then dehydrated in an increasingly graded series of alcohols (30, 50, 70, 80, 95, and 100 percent) for 1 hour each. After immersion in the 100 percent ethanol concentration, samples were placed in a 50 percent (v/v) solution of 100 percent ethanol and

![Figure 2. Fifty apical-cervical scraping strokes made on the area between the two grooves with the curette.](image)

![Figure 3. Sample preparation procedure. (A) The dental crown above the first groove was removed. (B) Then a longitudinal cut was made in the central part of the tooth. (C) Finally, a horizontal cut was made to produce the samples.](image)
hexamethyldisilazane (HMDS) (Sigma, Sigma-Aldrich Inc., St. Louis, MO, USA) for 30 minutes with a final immersion in 100 percent HMDS for 10 minutes. Finally, samples were dried overnight in a dehydration jar (Corning, Sao Paulo, SP, Brazil), mounted on metallic stubs (Senai, Sao Paulo, SP, Brazil), and sputter-coated with a thin 25 nm layer of 99.99 percent pure gold.

**SEM Analysis**
Two photomicrographs were obtained from the center area of each sample under 1,500X and 3,500X magnifications, using a scanning electron microscope operated at an accelerating voltage of 20 kV (Jeol T330 A, Jeol Ltd., Peabody, MA, USA). The photomicrographs were scored according to a root surface modification index (Figure 4) adapted for this study. Three evaluations at 15-day intervals were performed by one previously calibrated and experienced examiner. The examiner was blind to the experimental groups. The final score actually recorded for each sample was the most prevalent score among the three evaluations. The scoring criteria for the adapted root modification index used for this study consisted of eight scores, ranging from 1 to 8, as shown in Figure 4. Good reproducibility was achieved for use of the index with a weighted kappa score of 0.93.

**Statistical Analysis**
The nonparametric analysis of variance (Kruskal-Wallis test) was applied to independently evaluate the effect of the three dependent variables, solution concentration, application mode, and application period, at a 5 percent significance level. If a p value was ≤ 0.05, Dunn’s Multiple Comparison post-hoc test was applied to detect statistically significant differences among the groups. The statistical analyses were performed with the GraphPad Prism 5.00 software (San Diego, CA, USA).

**Results**
Citric acid demineralization produced a variety of surface topographies. The surface appearance ranged from a rough amorphous surface with no evidence of fiber material to a surface made up of collagen fibers, best described as a “shag carpet” appearance of tufted fibers. Samples that exhibited exposure of collagen fibers and opening of dentinal tubules corresponded to concentrations of 1, 15, and 25 percent (Figure 5).

Partial opening of dentinal tubules were observed in all citric acid concentrations and in the control group. Traces of debris were observed in the openings of dentinal tubules at concentrations of 0.5, 1, and 2 percent, and in two samples of the control group. A high frequency of chemical dissolution was observed for all citric acid concentrations evaluated as well as for samples in the control group. Kruskall-Wallis test analysis of variance showed significant differences among groups (p<0.0001). Dunn’s post-hoc test showed that significant differences appeared when comparing the control group to all other groups (p<0.05). However, no statistically significant differences were found among the five citric acid solutions of the experimental groups.

There also were no statistically significant differences observed among the three application periods (p=0.0793). Nevertheless, high frequencies of collagen fiber exposure (Score 1) were observed in the application period of three minutes (eight samples) followed by the application period of one minute (seven samples) of citric acid solutions (Figure 6).

Analysis of the effect of the application methods did not find any significant differences among groups (p=0.8525); however, the highest frequency of collagen fibers exposure was observed for the brushing application method (14 samples) (Figure 7).

**Discussion**
The results of this study showed that scaling and root planing produced a smear layer that was removed from the root surface by a citric acid application. The smear layer itself is comprised of very small particles of organic and inorganic material. These particles vary in size from less than 1 µm to more than 15 µm, and the layer is in intimate contact with the tooth surface and only removed by applying a demineralizing solution. Studies have suggested that this smear layer, interposed between the root surface and adjacent connective tissue, may act as a physical barrier for the development of a connective tissue attachment to the root surface. On the other
Figure 4. Evaluation criteria used in the adapted root modification index. (A) Score 1. Complete smear layer removal with dentin collagen fibers exposure. Complete opened dentin tubules, without a trace of the smear layer on the root surface. (B) Score 2. Complete smear layer removal but no collagen fibers exposure. Complete opened dentin tubules. (C) Score 3. Traces of smear layer remaining in the openings of dentinal tubules. (D) Score 4. Partial opening of the dentinal tubules. (E) Score 5. Smear layer formed by chemical dissolution of the dentin surface covering the root surface. (F) Score 6. Uniform smear layer covering the dentin surface with some signs of tubule openings. (G) Score 7. Dentin surface covered by a uniform smear layer with no signs of dentinal tubule opening. (H) Score 8. Rough smear layer covering the dentinal surface.
Figure 5. Frequency of concentrations 25, 15, 2, 1, 0.5 percent, and the controls in the scores of root surface modification.

Figure 6. Frequency of 1-, 2-, and 3-minute application periods in the scores of root surface modification.

Figure 7. Frequency of passive, brushing, and burnishing applications in the scores of root surface modification.
hand, the collagenous matrix, exposed by the use of demineralizing substances, appears to offer a more “hospitable” environment for cell attachment and may predispose the root to a new connective tissue attachment.

After evaluating the effects of citric acid concentrations and the parameters of the three application methods and three application periods, variability was noted in the scoring results obtained. All five of the experimental groups exhibited significant differences from the control group. However, there were no significant differences in scores recorded for the five citric acid concentrations.

Despite the lack of significant differences, exposure of collagen fibers was obtained with concentrations of 1, 15, and 25 percent. Thus, the discussion of the results focuses on these three specific concentrations (Figure 5).

It was evident that 25 percent citric acid tended to produce more samples with collagen fiber exposure than either the 1 percent or 15 percent group (Table 1). These results are consistent with those from other studies that reported the optimum dentin demineralization was obtained with citric acid solution concentration between 25 and 30 percent.

Although statistical analysis of the application period effects indicated that there were no significant differences among groups, application for one minute as well as three minutes promoted demineralization of dentin and exposure of collagen fibers in a higher frequency than the two-minute application (Figure 6). These results also are in agreement with Codelli et al., who showed that complete demineralization could be achieved in three minutes with a burnishing action, compared to partial demineralization after three minutes with nonburnished (passive) action. Furthermore, five minutes of burnishing actually tended to result in excessive demineralization.

Excessive demineralization seems to occur as a result of chemical and mechanical dissolution of the inorganic hydroxyapatite. The results of this study suggest that excessive demineralization is associated with the burnishing technique, as illustrated in Figure 7. Excessive demineralization of the dentin surface also raises the question of the biological acceptability of the resulting surface. Studies have suggested that a collagenous root surface, devoid of surface debris, offers a higher number of biological mechanisms by which new connective tissue attachment formation can be accelerated. These mechanisms include collagen splicing, mesenchymal cell induction, fibronectin adhesion, and fibroblast attachment. Studies have shown that demineralized dentin surfaces have a greater capacity to bind fibronectin than surfaces that did not undergo demineralization.

Table 1. Sample distribution for the citric acid concentrations that produced a score of 1 according to the root surface modification index.
in binding is due to exposure of more fibronectin
binding sites on the collagen matrix. This change
may increase the total number of binding sites
available for proteins such as fibronectin, which
in turn may help stabilize a mucoperiosteal flap
during the initial stages of wound healing.32–34

One aspect of this study, which differs from
studies, is the use of hexamethyldisilazane. While
other researchers17,11–13,21 used the critical-point
drying with CO2 to prepare samples for SEM,
here hexamethyldisilazane was used to dehydrate
the samples. This method allows visualization
of the collagen matrix in the SEM by preserving
the microporosity of the dentin collagen fibers
and actually preventing the exposed fibers from
collapsing.35–38

The use of citric acid as a conditioning agent is
supported by the literature and its advantages
compared to other conditioning agents is
supported by evaluation of blood cell adhesion20
and by human fibroblast adherence and
proliferation to dentin surfaces.39

Certainly, other methods can be used to evaluate
the effect of the citric acid conditioning and to
verify how the results of this study can help the
periodontal treatment. These include evaluation
of fibroblast behavior on chemically conditioned
dentin,20 evaluation of blood cell adhesion to
dentin-conditioned surfaces,20 quantification of
the parts per million (ppm) of calcium removed
after conditioning,29 and immunohistochemical/
immunocytochemical analysis of the exposed
collagen after conditioning.32–34

Considerable variability in results may be seen
among the various studies. Several factors, such
as scaling details (hand scaling vs. piezoelectric
scaling), could negatively interfere with the amount
of residual calculus and root surface roughness.40–42

There are other considerations when selecting
a substance for root conditioning because the
outcome also may be influenced by the

• application period, application method,
  concentration, and pH of the substance used;43
• dilution and inactivation of substances when in
  contact with blood during surgical procedures;44
• preparation of samples and the storage
  medium, as well as a high degree of
  mineralization of the teeth; and
• pressure applied during the application
  process itself.

In addition, characteristics of the conditioning
substance and a lack of a universal root
modification index also may contribute to the
reporting of varying results in the literature. For
these reasons, we believe that exposure of
collagen fibers by means of root conditioning is
difficult to obtain as explained by the extensive
variability and conflicting results found in the
dental literature.

Conclusion

According to the results obtained in this study and
within the limitations of the methodology used, it
can be inferred that despite the lack of statistical
significance, the best results for exposure of
collagen fibers were obtained with application of
citric acid at 25 percent by brushing for one or
three minutes.

These results may contribute to the interpretation
of other investigations found in the literature, for
the design of in vivo studies on root conditioning,
and for the clinical application of citric acid root
conditioning in both periodontal treatment and
regenerative procedures in order to optimize
results. However, additional studies are
recommended to support or refute these findings.

Clinical Significance

The best results for exposure of collagen fibers
on root surfaces noted in this study were obtained
with application of citric acid at 25 percent by
brushing for one or three minutes.

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