Resin Penetration in Artificial Enamel Carious Lesions after Using Sodium Hypochlorite as a Deproteinization Agent

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Objectives: *To study the degree of penetration of an adhesive resin in artificial enamel carious lesions after using sodium hypochlorite as deproteinization agent.* ***Study design:*** *Twenty included human third-molars, extracted for surgical indication, were used. Artificial lesions were created in the buccal and lingual sides of each specimen through a cycle of demineralization-remineralization. Samples were then incubated in human saliva for 7 days at 37 ° C. After surface cleaning, lesions and the peripheral sound enamel were etched with 37% orthophosphoric acid for 20 seconds. One lesion of each specimen was treated with 5.25% sodium hypochlorite (NaOCl) for one minute. The other lesion of each specimen was used as a control. Experimental and control lesions were sealed with a fluid resin marked with Rhodamine B. Lesions were sectioned for microscopic observation by epifluorescence and polarized light. The images obtained were analyzed morphometrically. The micrometer measurements were made with ImageJ ® software. The level of significance was assessed at $p<0.05$.* ***Results:*** *The average sealant depth penetration in the control group was 94.9 ± 28.6 µm versus 122.8 ± 25.3 µm in the experimental group. This represents Δ 20.1% significantly greater penetration when using sodium hypochlorite ($p<0.001$).* ***Conclusion:*** *The results demonstrated a significant penetration of the sealing resin when the conventional technique is complemented with the application of 5.25% sodium hypochlorite for one minute in artificial enamel carious lesions.*

Keywords: *sodium hypochlorite, resin penetration, artificial carious lesions, epifluorescence microscopy.*

INTRODUCTION

Studies performed 35 years ago conceived the idea of sealing noncavitated natural enamel lesions with light cured resins of low viscosity in fissures and smooth surfaces. These studies suggested that sealing could be an effective measure to stop the progress of enamel lesions and thus avoiding their restoration.[1, 2]

However so far, the most effective preventive methods for stopping non-cavitated enamel lesions are based on effective oral hygiene (plaque control) with fluoride dentifrices and the limitation of frequency of sugar intake. Nevertheless, these strategies are dependent on the patient's compliance. Enamel lesion sealing is a professional decision and must be adopted with care when the patient fails to follow the standard preventive protocols previously described.

Conversely, recent evidence indicates that the seal or infiltration of noncavitated lesions in fissures and proximal tooth surfaces may be a routine procedure in the future and considered a minimally invasive alternative to traditional restorative care.[3-8]

The procedure of conventional sealing with fluid resins applied *in vivo* on proximal noncavitated lesion has had promising results in long-term studies. This approach is simple, non-invasive, and conservative with respect to the surrounding intact enamel.[9-12] However, when following a conventional sealant placement protocol, the penetration of a fluid resin to the interior of natural lesions, both *in vitro* and *in vivo*,[4,5,13-14] does not reach the approximate etch depths (27.1 µm) calculated by Legler *et al,*1990.[15]

Considering the previous points, several studies have suggested that the presence of salivary proteins and organic material from the saliva, which normally are included and adsorbed in the superficial zone of the lesion, may interfere both with the conventional etching technique and with the deepest penetration of the resin.[16-19] This has been addressed, both *in vitro* and *in vivo*, by using sodium hypochlorite as a deproteinizing agent prior to etching intact human enamel with 37% orthophosphoric acid.[20-21].

It is unknown whether the deproteinization with sodium hypochlorite, in natural or artificial lesions, before sealing with a fluid

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resin, would optimize resin penetration or diffusion into the superficial zone and / or the body of the lesion, which might result in better retention of the sealant.

The main objective of this study was to confirm if the use of a deproteinizing agent, such as 5.25% sodium hypochlorite for one minute, would improve the sealant resin penetration in human enamel and artificial lesions using a conventional sealant placement technique.

The hypothesis of the study suggests that the use of a deproteinizing agent, prior to the sealing of an artificial lesion with a fluid resin, optimizes its diffusion into the superficial zone of the lesion, including the body of the lesion, without needing to micro-eroding the surface area.

MATERIALS AND METHOD

This randomized *in vitro* study was conducted in the cariology laboratories of Dental Schools of Valparaiso University, Valparaiso and Finis Terrae, Santiago, Chile. The study was approved by the ethics committee and Department of Oral Research of the Faculty of Dentistry, Valparaiso.

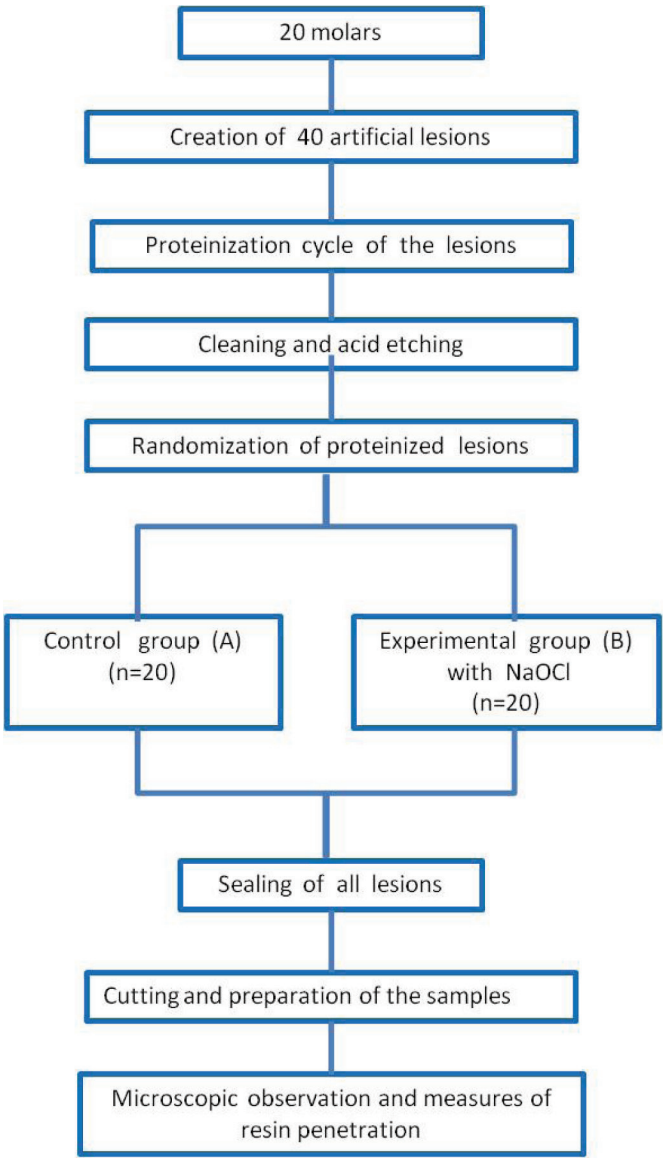
Twenty human third-molars, extracted for orthodontic indication in patients 18-20 years old, were used in this study. The inclusion criteria were a third molar recently extracted with no superficial and structure alteration and not previously exposed to the oral environment. All molars were covered with an acid-resistant nail varnish, leaving 6x4 mm windows in the buccal and lingual surfaces of each tooth. The samples were subjected to demineralization-remineralization cycles as described by Miake et al., 2003.[22] Briefly, the samples were immersed in a 0.01 M acetate buffer solution (pH 4.0) at 50°C for 2 days to form several layers of demineralization. The demineralizing solution (100 ml) used for each sample was replaced with fresh solution daily. The specimens were subsequently washed with distilled water and left to dry. The samples were immersed in a remineralizing solution (Ca2+ 1 mM, PO4 3– 0.6 mM, F– 0.05 mM, pH 7.3) and maintained at 37°C for 2 weeks. The remineralizing solution (100 ml) used for each block was replaced weekly. After the 2-week period the nail varnish was removed with acetone. The lesions were proteinized by placing the teeth in a recipient with a pool of centrifuged human saliva for 7 days at 37°C to simulate oral conditions. The artificial lesions were randomly divided into two groups (n=20 each). After surface cleaning, all lesions and the peripheral sound enamel were etched with 37% orthophosphoric acid (Total etch, Ivoclar Vivadent, AG, Schaan, Liechtenstein) for 20 seconds. After etching of each specimen, the vestibular lesion was treated with 5.25% sodium hypochlorite for one minute and removed profusely with distilled water for another minute serving as the experimental group (Group B). The lingual lesion of the same specimen was used as the control (Group A). In order to control this step, two extra control teeth were stained with 0.1% toluidine blue and assessed with a stereo microscope (Fig 2a and 2b). Experimental and control lesions were sealed with a low viscosity resin (Heliobond, Ivoclar Vivadent AG, Schaan, Liechtenstein) which had been labeled with Rhodamine B (Sciencelab, St. Augustine, Houston, Texas, USA).

Tissue processing and digital image analysis

Each tooth with artificial lesions was embedded in self-curing methacrylate resin (Marche, Feliz Martinez y Cia Ltda, Santiago, lot m641074, Chile blocks (5 x 4 x 3 mm). Once embedded, each tooth was assessed by 3 slices parallel to the occlusal surface of the molar, under running water with a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA), covering both experimental lesions as the control in each cut, thus obtaining a total of 80 samples (four artificial lesions of 500 µm -thick each corresponding 40 lesions to each group). Nevertheless, at the end of the grinding technique only 20 sections of each group were selected for examination because of its homogeneous and integral histological structure. This sample size was statistically calculated to consider $\alpha = 5\%$ and $1\text{-}\beta = 85\%$ (ANOVA one factor and type II error probability). Using this formula, about 10 samples would be examined in each group; however, 20 specimens per group were selected to achieve better results (Fig 1).

After cuts were made the samples were attached to a glass slide using a resin (Eukitt, O.kindler GmbH & Co, Freiburg, GO Lot 55, Deutschland) and left undisturbed for 24 hours prior to the next step. The surfaces of the specimens were ground flat using a diamond

Fig 1 The experimental design flowchart.



disc mounted on a rotating machine (Polishing Machine GMC80, Bilbao, España). The samples were reduced to a coplanar surface of 100 µm thickness using a diamond wheel mounted for fine grain wear and abrasive paper (2500 and 4000 of Al_2O_3).

The sections were examined in a Zeiss Axioscop microscope equipped with an epi-fluorescence attachment (Carl Zeiss, Göttingen, Germany) by one of the authors specializing in enamel histology (AO). Enamel lesions observed under epifluorescent and transmitted light conditions were captured using a Micropublisher 3.3 RTV cooled digital camera (Q Imaging, Surrey, BC, Canada) and Q Capture Pro Image Software for Windows (Q Imaging, Surrey, BC, Canada) and stored as JPEG file formats.

The merged images obtained by Adobe Photoshop CS5.1 software were analyzed with Image J software (http://imagej.en.softonic.com/) to measure the penetration of the resin into the lesion. Eleven measurements of lesion depth were done in each lesion by one of the authors (RM) in a single-blind manner standardizing the distance between each of them to 90 µm. All measurements were made from the surface zone (Sz) to the advanced zone (Az) on the superimposed pictures. Averages were calculated for each lesion resin depth penetration in both the control and the experimental group (Fig 1). To assess the intraexaminer reliability, 12 (30%) images from both the test and control group were measured twice, with an interval of 4 weeks. Measurements were recorded in a Microsoft Excel 2007 database (2007 Microsoft Office System, Microsoft Corporation).

Statistical analysis

The statistical analysis was performed using Minitab® Statistical Software, version 16.0 for Windows (Minitab®; Pennsylvania; USA). The model for the statistical analysis was the T test for paired samples (t student). Normality of data distribution (Kolmogorov-Smirnov) and homogeneity of variances (Bartlett y Levinne) was assessed. The significance level was 0.05 ($\alpha = 0.05$).

RESULTS

The average depth of 40 artificial lesions analyzed was of 129.2 microns ± 22.9 microns. Table 1 shows the average penetration of the resin sealing in both groups, expressed in microns and percentage. No significant differences were observed on the depth of the lesions assigned between the two groups.

By comparing the images obtained from samples treated and untreated with sodium hypochlorite, a difference in penetration of the sealant could be detected, being higher in the experimental group covering almost the entire body of the lesion, whereas in the control group the penetration was smaller. (Fig 3a, 3b, 3c, 3d, 3e, 3f). The experimental group showed a Δ 27.9 microns and Δ 20.1% significantly higher penetration than the control ($p<0.001$). Figure 4 shows the data distribution from both groups. Additionally, in the ground sections, the body of the lesion in the control group were stained with toluidine blue (Fig 2a). In the hypochlorite treated specimens, the lesion body remained unstained (Fig 2b).

Fig 2a In this figure obtained by bright field microscopy under polarized light (10x), it is possible to observe in purple the presence of organic material of salivary origin (Om) stained with toluidine blue, occupying part of the superficial zone (Sz) and body of the lesion (Bl).

Fig 2b This figure obtained by bright field microscopy with polarized light (10x) shows the results after the application of 5.25% NaOCl (1min). It is possible to observe that there was a significant decrease in organic material of salivary origin (Om) in both the surface area (Sz) and the body of the lesion (Bl).

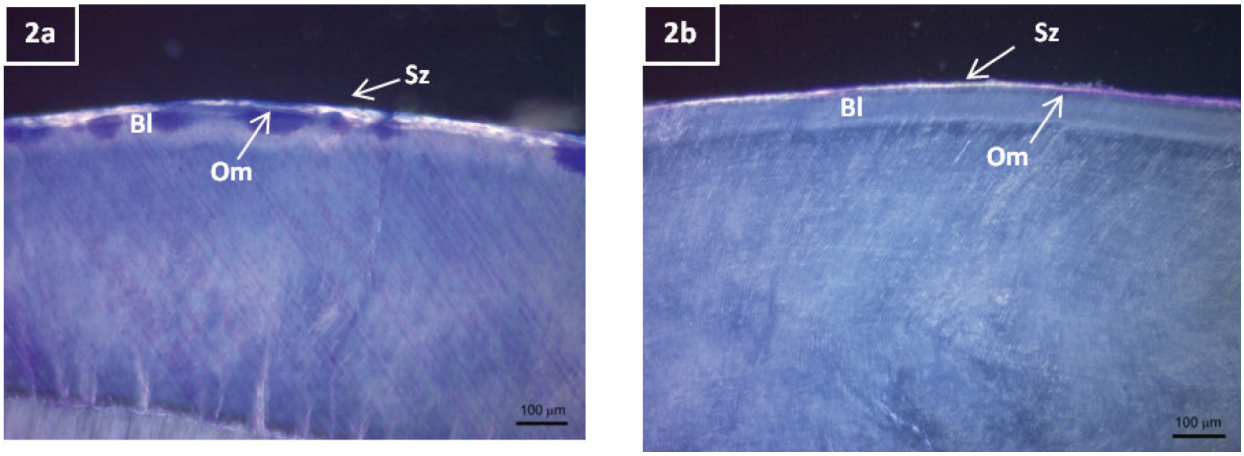


Table 1 Mean penetration of the resin sealing in both groups, expressed in microns and percentage.

Group	n	Mean depth and SD of the lesions (µm)	Mean penetration ± SD (µm)*	Mean standard error	% of penetration into the lesion
A. Conventional sealed treatment *(Control)*	20	128.1 ± 20.6	94.9 ± 28.6	6.41	74.1
B. Conventional sealed treatment + NaOCl *(Experimental)*	20	130.3 ± 25.5	122.8 ± 25.3	5.68	94.2

*Differences between groups are statistically significant, (*t* test) $p < 0.001$

Figs 3a and 3d Images obtained by bright field microscopy (10x), which are observed in different parts of an artificial lesion: (Sz) superficial zone of the lesion, (Bl) body of the lesion and (Az) advanced zone of the lesion. The experimental group (treated with NaOCl) is portrayed in Fig 3a and the control group is portrayed in Fig 3d.

Figs 3b and 3e Images obtained by epifluorescence microscopy (10x) show the penetration of the sealing resin (R) in the artificial lesion of the experimental group with NaOCl (Fig 3b) and in the control group (Fig 3e).

Figs 3c and 3f An overlay of images digitized from bright field microscopy and epifluorescence (10 xs) in Figs 5a and 5b respectively, where they quantified the penetration of the resin, in red line, in the experimental group (Fig 3c), and in the control group (Fig 3f).

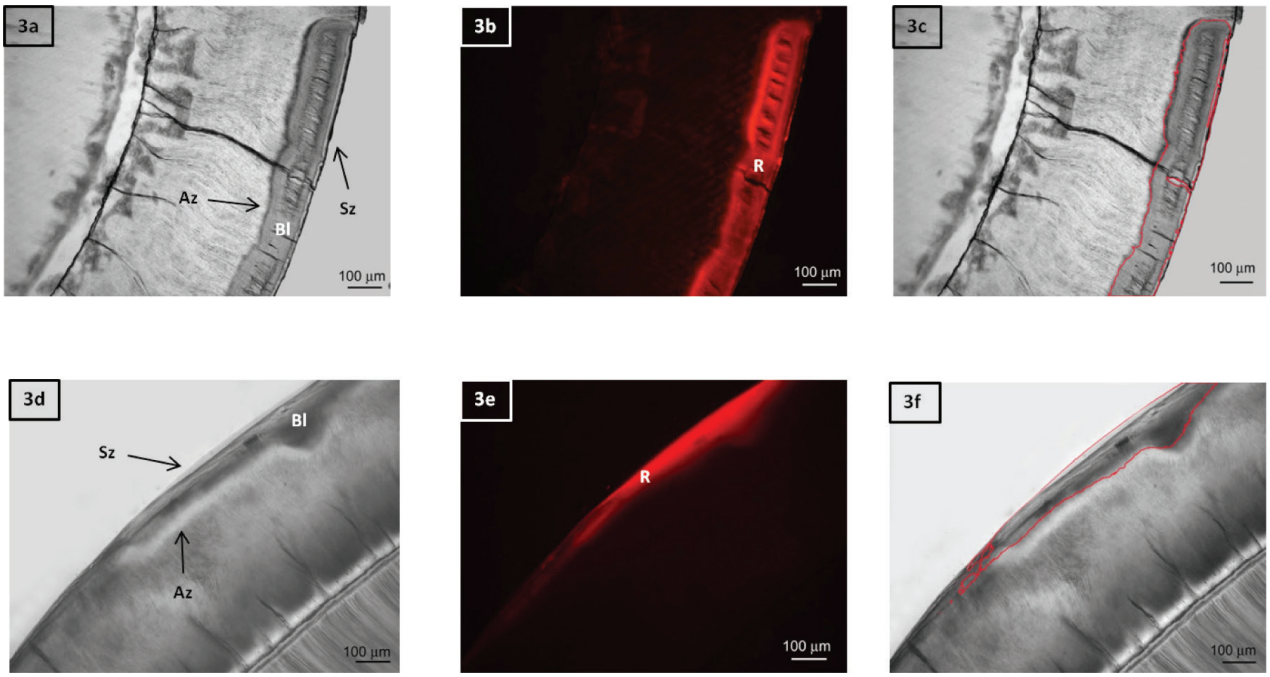
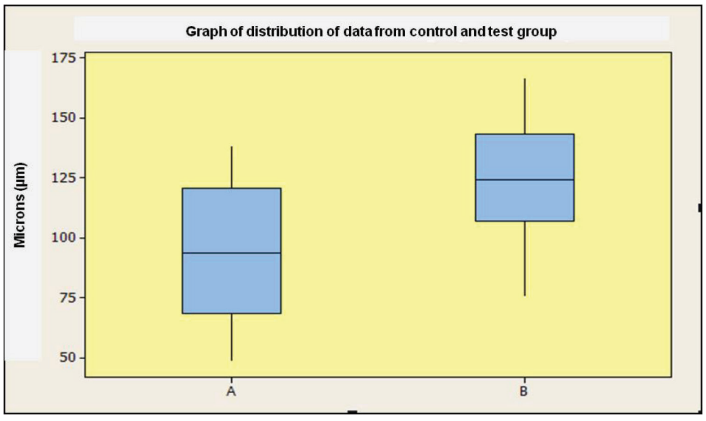


Fig 4 Graph illustrating distribution of data from both groups, where A is the control group and B is the experimental group (with sodium hypochlorite). The greatest concentration of data is contained in the boxes, while the averages are reflected in the center line of each box.



DISCUSSION

In cariology, the noninvasive strategy of sealing proximal lesions is an emergent aspect studied and discussed.[6,8] because it is a viable and safe alternative for caries lesion control.[4,9,11-13,23] Given the success of this procedure, several authors have developed different sealant and infiltration methods using diverse materials and techniques.[24-28] Among them, Paris and Meyer-Lueckel, 2010 [5] have tested a technique using hydrochloric acid and fluid resin, obtaining good results in relation to the infiltration of the body of the lesion. However, this method removes a considerable amount of enamel (about 30 µm), and can be potentially iatrogenic to the tooth mineralized tissues on the cervical areas. Therefore, the purpose of this study was to test if the incorporation of a deproteinizing agent to the conventional sealant technique would increase sealant penetration on in vitro artificial lesions.

The literature has described various methods for creating artificial lesions. The method described by *Miake et al*, 2003 [22] was used in this study. It should be noted that diligence with the instructions and steps outlined for the development of artificial lesions is important. Otherwise, it will result in simple erosions without a well-formed surface zone. Verification of the structure of the developed artificial lesions can be made only by histological analysis, for example, through observation with polarized light microscopy, a technique that was used in this investigation.

After obtaining the artificial lesions by the cyclical process of demineralization and remineralization, we proceeded to the stage of proteinization, with the aim of submitting them to the same conditions seen in the mouth.

The lesions were stained with toluidine blue to check the penetration of the enamel organic material from the saliva. This dye specifically stains protein material from the saliva. We used the toluidine blue metachromatic due to its characteristics, which means that the stain has the ability to take two different shades, violet or blue, depending on the tissue to which it is applied, dyeing violet for proteoglycans and blue for glycoproteins. These proteins have been described in natural lesions by several authors.[16-19] Those studies led us to suggest that their presence may make it difficult to penetrate the sealing resin. Our experience not only demonstrated the presence of organic material from salivary proteins in the superficial zone, but also parts of the body of the lesion.

Moreover, we could note that the lesions, after being immersed in saliva and subsequently treated with sodium hypochlorite for one minute, showed a change in color, staining the sample purple, mixed with some areas more intensely stained than others. This would indicate that, macroscopically, the enamel surface treated with sodium hypochlorite would be free of organic material from saliva, indicating its practical effectiveness as a deproteinizing agent.

The choice of 5.25% sodium hypochlorite as a deproteinizing agent was for its known antioxidant properties produced in dentin when it is used endodontically.[21] However, **there is limited literature to support its use with** enamel. Recently, Espinosa et al., 2010 and Justus et al., 2010 [29-30] demonstrated that the application of 5.25% sodium hypochlorite for one minute on the enamel improves the etching pattern and orthodontic brackets retention, respectively.

Similarly, our study clearly demonstrated that using sodium hypochlorite at the same concentration and time, made it possible to remove the organic material from saliva in the artificial lesion. This was histologically **verified** (Figs 2a and 2b).

With the results of this investigation the hypothesis of this study was accepted by demonstrating that it was possible to quantify the penetration of the resin in a precise way, using digital morphometric analysis of images (digital overlay), with the epifluorescence microscopy. This is an ideal method to observe the penetration limit of the resin.

The percentage of infiltrated areas was 20.1% higher in the experimental group treated with sodium hypochlorite than in the control group. Use of hypochlorite could have an important clinical application in the therapeutic management of noncavitated enamel lesions, as this could enhance the penetration and retention of the resin without requiring a deep erosion of the surface area of the lesion.

In addition, the use of a single tooth to produce two lesions, allowed one of the samples to be used as test and the other as control. Thus, the same tooth was used for experimental and control samples, maintaining both the same chemical, physical and structure properties of the enamel.

Finally, it should be noted that all the steps of this study were conducted in the same procedural conditions with histology as the gold standard assessment strategy. The limitations of this study were the use of artificial enamel carious lesions and the limited number of the section examined per lesion. Therefore, its results cannot be extrapolated to clinical use. For this reason, we suggest the use of a similar procedure on natural carious lesions and increase the number of sections examined for each lesion with the purpose of best validation of the results in upcoming studies. We also suggest that to improve the comparison between the original depths of the lesion with the penetration of the resin, the measurements should be also performed by areas of penetration.

CONCLUSIONS

With the limitations of this experimental protocol, it is possible to suggest that the inclusion of organic material from saliva into the surface zone and part of the body of the lesion could be obstructing the penetration of a sealing resin. Similarly, the results showed a significant difference ($p < 0.001$) in the sealant resin penetration (Δ 27.9 μm) when the conventional technique was complemented with the application of 5.25% sodium hypochlorite for one minute as the deproteinizing agent.

ACKNOWLEDGEMENTS

The authors would like to thank the University of Valparaíso and the University Finis Terrae for their support, and to express their gratitude to Dr. Margherita Fontana DDS, PhD, Associate Professor Cariology, Restorative Sciences and Endodontic School of Dentistry, University of Michigan, USA, for her most valuable contribution to this study.

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