

Demineralization and Hydrogen Peroxide Penetration in Teeth with Incipient Lesions

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The aim of this study was to evaluate the demineralization and hydrogen peroxide (HP) penetration in teeth with incipient lesions submitted to bleaching treatment. For analysis of HP penetration, sound and demineralized enamel/dentin discs were placed in artificial pulp chambers containing acetate buffer solution. After bleaching treatment, this solution was subjected for analysis of optical density by spectrophotometry and the disc surfaces were analyzed with scanning electron microscopy (SEM) and polarized light microscopy (PLM). The remaining discs were subjected for cross-sectional hardness analysis at different depths. Data were analyzed by repeated measures ANOVA and PLSD Fisher test ($\alpha=0.05$). It was observed that previously demineralized teeth showed greater HP penetration ($p<0.05$). The bleaching treatment caused changes to a depth of 20 μm in sound enamel and up to 90 μm in demineralized enamel. SEM and PLM images revealed that the bleaching treatment caused superficial changes that were considerably more accentuated in previously demineralized teeth. It may be concluded that the enamel mineralization level influences HP penetration and the bleaching agent contributed to increase the demineralization depth.

Key Words: bleaching, hydrogen peroxide, tooth demineralization, caries.

Introduction

Tooth bleaching is one of the most popular treatments to achieve esthetic whitening and is considered a conservative, easily performed and relatively low-cost procedure (1). At-home bleaching is the most widely used bleaching technique for vital teeth, with recognized efficacy and biosafety (2). The patient uses carbamide peroxide gel at low concentrations under the supervision of a dentist. (3).

Another alternative for vital tooth bleaching is the in-office technique, characterized by the use of products based on hydrogen peroxide (HP) in concentrations ranging from 20% to 38%. Obtaining fast results with immediate lightening effects through observation is the main marketing appeal that anchors the success of this technique (4). It is known that whatever the used bleaching agent, the active ingredient that will act in tooth structure is hydrogen peroxide, which should penetrate into the enamel and reach the dentin to produce the desired effect.

Despite the success of bleaching techniques, it has been demonstrated that application of HP gel on the enamel can result in its partial dissolution, deproteinization, and increased permeability of the tissue (5-7). This fact becomes worrisome because the presence of incipient lesions, often at sub-clinical stage, may suffer interference of bleaching treatment and progress to more advanced lesions, allowing more penetration of peroxides during the treatment.

Several studies *in vitro* show that the HP present in bleaching agents, even at low concentrations, readily crosses enamel and dentin, reaching the pulp tissue (8), which may respond with lipid peroxidation, fragmentation of proteins and result in cell membrane injury (9). In this way, it is believed that the bleaching treatment performed in teeth with incipient caries lesions could result in higher post-treatment sensitivity. Thus, the aim of this study was to evaluate the trans-enamel and trans-dentinal penetrations of hydrogen peroxide in a previously demineralized enamel and, at the same time, evaluate the HP influence in the progress of the lesion.

Material and Methods

Collection and Standardization of Specimens

One hundred sound permanent bovine incisors obtained from steers aged between 24 and 30 months were selected and the teeth were mounted in a cutting machine using a 8-mm-diameter cylindrical diamond tip (Dinser Ferramentas Diamantadas; São Paulo, SP, Brazil). From the middle third of buccal surface of each tooth were cut 5.7-mm diameter discs containing enamel and dentin. The dentin surface was polished with wet 400- and 600-grit aluminum oxide paper (Saint-Gobain Abrasivos, Jundiaí, SP, Brazil) until the specimens reached a thickness of 3.5 mm, approximately 1.3 mm and 2.2 mm (± 0.1 mm) for enamel

and dentin, respectively, measured by a digital caliper (Mitutoyo Sul Americana Ltda, São Paulo, SP, Brazil). A 0.5 mol/L EDTA solution, pH 7.2, was applied on dentin surface for 30 s for removal of the smear layer, and subsequently rinsed with sterile deionized water.

Sample Selection

The remaining tooth structure was used for microhardness analysis to standardize the initial enamel conditions. For this, new fragments were obtained from the region adjacent to the one employed for obtaining the discs. The fragments were fixed on an acrylic base and their surface was polished in a polishing machine (Arotec SA Ind. e Com., Cotia, SP, Brazil), using aluminum oxide sandpaper (Buehler Ltd., Lake Bluff, IL, USA) with water cooling at low speed (90 rpm) - 600- and 800-grit for 2 min and 1200-grit for 4 min. The final polishing was performed with felt disks moistened with diamond paste to 1 µm (Extec Corp., Enfield, CT, USA) for 5 min.

The enamel surface hardness was evaluated in a microhardness tester (HMV-2000; Shimadzu Corp, Tokyo, Japan) equipped with a Knoop indenter type that worked with a static load of 25 g for 5 s. This was performed at 3 indentations in the center of buccal enamel surface fragment, with a distance between them of 100 µm. A total of 60 enamel/dentin discs were selected, with hardness values closer to the mean value (KHN 351).

Experimental Groups

The selected 60 discs were divided into two groups: discs in Group I presented sound enamel (control); discs in Group II were exposed to a demineralizing solution, pH=5.0 for 24 h (11,12) followed by thorough washing with deionized water to produce white spot lesions on enamel.

Preparation of Artificial Pulp Chamber (APC)

The discs were adapted into a stainless steel device developed at the Laboratory of Experimental Pathology and Biomaterials of Araraquara Dental School/UNESP, Brazil (13). The discs were positioned in the APC between silicone rings, which cooperated to stabilize them and create a perfect seal for an APC.

Whitening Procedure

The bleaching treatment was performed on 15 discs from each group with the product based on hydrogen peroxide at 35% (Whiteness HP Maxx; FGM Produtos Odontológicos, Joinville, SC, Brazil) according to the manufacturer's recommendations. After mixing, the final product was inserted into a 1 mL graduated, disposable syringe with 0.04 mL applied to each specimen, and remaining in contact with the dental tissue for 15 min.

Subsequently, the product was removed by sucking and two further applications were made, totalizing 45 min of exposure to bleach. The procedure was performed 3 times with one-week intervals between sessions.

Quantification of HP Penetration

For quantification of the HP that penetrated the enamel/dentin discs, the APCs were individually placed in wells of acrylic plates for cell culture. Each well was filled with 1 mL of acetate buffer solution and subsequently received APCs, already containing the dental fragments. Thus, the dentin surface remained in contact with the acetate solution during all bleaching procedures and the diffused hydrogen peroxide became part of it.

After the whitening procedure had been completed, 25 µL of acetate buffer solution was removed. It was then mixed with 2.750 µL of distilled water, 100 mL of leucocrystal violet (0.5 mg/mL) and 50 µL of peroxidase (1 mg/mL) (Sigma Chemical Co., St. Louis, MO, USA) and the solution was diluted to a final volume of 3 mL with distilled water (1,13,14).

Readings were done using ultraviolet visible reflectance spectrophotometer equipment UV-2450 (Shimadzu), 30 min after each bleaching session. To obtain the CF (calibration factor) equivalent to the ratio of the concentration of the standard solution of hydrogen peroxide to its absorbance, the following equation was used:

$$CF = \frac{\text{[Sample Solution]}}{\text{Absorbance}}$$

Parametric tests were performed using ANOVA repeated measure and PLSD Fisher, using the statistical program StatView (StatView Inc., Nesbit, MS, USA), at a significance level of 0.05.

Cross-Sectional Hardness

After hydrogen peroxide quantification, 10 specimens of each group, including those that were not bleached, were longitudinally sectioned, using a double-sided floppy diamond disk Ø 22 mm (KG Sorensen Ind. and Com., Cotia, SP, Brazil) at low speed and under intense water cooling. Each disc was embedded in acrylic resin using an embedding machine Pre 30 (Arotec). The inner surface was polished with 320-, 600- and 1200-grit aluminum oxide sandpaper and 1 µm diamond paste. The cross-sectional hardness was determined by a microhardness tester with Knoop indenter type that worked with a static load of 5 g for 10 s. The indentations were made at the center of the block at distances of 10, 20, 30, 60, 90 and 120 µm from the enamel surface. Other two indentations rows were made; 200 µm on the right and left from the indentations made at the center of the discs.

Scanning Electron Microscopy

Three specimens from each group, including those not submitted to bleaching treatments, were prepared for surface analysis by scanning electron microscopy - EVO HD LS-15 (Carl Zeiss, Oberkochen, Germany). Therefore, the specimens were cleaned, dehydrated, sputter-coated with gold and enamel SEM micrographs in different surface conditions were obtained at 3,000x magnification.

Polarized Light Microscopy Analysis

Two specimens of each group, including those that did not undergo the bleaching procedure, were intended for analysis by PLM. The specimens were embedded and serially cut longitudinally in the metallographic cutter Isomet 2000 (Buehler Ltd.). The obtained slices were polished with aluminum oxide sandpaper 600- and 1200-grit until the samples presented a thickness of 100 μm .

The analysis was performed using a polarized light microscope AxioPhot Zeiss - DSM 940 A (Carl Zeiss Meditec, Dublin, CA, USA) at 25x magnification. For analysis, the software AxioVision 4 (Carl Zeiss Meditec) was used.

Statistical Analysis

Data were analyzed statistically using repeated-measures ANOVA and PLSD Fisher's test at 5% significance

Table 1. Mean (SD) peroxide concentrations ($\mu\text{g}/\text{mL}$) recovered from artificial pulp chambers after tooth whitening in sound and demineralized enamel, according to the bleaching sessions

Substrate	Bleaching sessions		
	1 st	2 nd	3 rd
Demineralized	5.27 (0.22) Aa	5.10 (0.18) Aa	4.90 (0.42) Ab
Sound	4.68 (0.52) Ba	4.60 (0.33) Ba	4.31 (0.36) Bb

Different uppercase letters in columns and lowercase letters in rows indicate statistically significant difference ($p < 0.05$).

Table 2. Mean (SD) cross-sectional hardness before and after tooth whitening in sound and demineralized enamel, according to different depths

Depth	Sound enamel		Demineralized enamel	
	Not Bleached	Bleached	Not Bleached	Bleached
10 μm	78.9 (20.45) Ea	253.0 (9.96) Eb	94.3 (9.68) Fc	37.8 (13.84) Fd
20 μm	303.8 (5.77) Da	269.1 (8.54) Db	139.0 (32.97) Ec	74.8 (18.26) Ed
30 μm	325.1 (9.77) Ca	313.5 (14.63) Ca	170.5 (52.35) Db	124.5 (39.69) Dc
60 μm	343.2 (10.11) Ba	343.0 (9.55) Ba	249.1 (35.53) Cb	239.7 (22.67) Cb
90 μm	351.1 (7.77) ABa	352.7 (4.57) Aa	313.9 (17.72) Bb	268.9 (9.51) Bc
120 μm	354.6 (8.77) Aa	353.5 (4.55) Aa	350.2 (5.55) Aa	350.3 (6.95) Aa

Different uppercase letters in columns and lowercase letters in rows indicate statistically significant difference ($p < 0.05$).

level

Results

The application of repeated-measures ANOVA showed that HP penetration, as far as cross-sectional hardness is concerned, was different in groups with sound and demineralized enamel ($p < 0.0001$). The application of the PLSD Fisher test at 5% significance level showed that the condition of enamel influenced the intensity of the hydrogen peroxide, presenting the highest values in specimens subjected to cariogenic challenge, regardless of bleaching session (Table 1).

The application of the PLSD Fisher test for cross-sectional hardness analysis data showed that, in general, the cross-sectional hardness (CSH) values were increased in all the experimental conditions, since the depth was 10 μm to 120 μm . Sound enamel showed higher microhardness values to a depth of 90 μm , equated with demineralized specimens at 120 μm in depth (Table 2).

Only the effect of the bleaching agents on the sound enamel led to changes in the microhardness to a depth of 20 μm . Moreover, the action of bleaching in demineralized enamel influenced the microhardness values, which showed more pronounced changes than the group that was not bleached to a depth of 90 μm (Table 2).

Microscopic Observations

The images obtained from SEM (scanning electron microscopy) showed that both the demineralized and sound surfaces exhibit superficial changes (Figs. 1A and 1B), which are considerably more pronounced in teeth that were previously demineralized (Fig. 1B) when compared with Group I, which were only bleached (Fig. 1A).

The PLM analyses showed that the specimens of Group I (Fig. 2A - arrow) as well as those of Group II (Fig. 2B - arrow) presented negative birefringence areas on the enamel surface, which are considerably more pronounced and thicker in previously demineralized teeth (Fig. 2B).

Discussion

Bovine teeth have been widely used for *in vitro* research (15). Specifically in the used methodology, its use allows obtaining experimental units with suitable dimensions for creation of APCs, as it is possible to simulate clinical application of bleaching agents on the enamel. Furthermore, it is a chemically uniform substrate, and allows less variation in response to cariogenic challenges (15), being an interesting

option for this study.

The results showed that the HP penetration in demineralized enamel was significantly greater than in sound enamel. It is believed that increasing diffusion has significant correlation with the histomorphological changes produced by cariogenic challenge, since the control group possibly offered the greatest challenge for the penetration of peroxide. In this context, the microscopic images showed that exposure of enamel to the demineralizing solution modified the surface characteristics of enamel and the bleaching treatment accentuated these changes.

The bleaching effects modifying the characteristics of the enamel surface are well documented (5-7,16-18). In this study was observed that the dental tissues degree of mineralization may have important influence on the HP penetration. This is alarming because it is known that uncontrolled penetration of HP in the pulp tissue has been associated with varying degrees of cell aggression (9,18-21). Thus, it is important that the dentists perform a thorough clinical examination and proper exams before starting

the bleaching treatment, since inadvertent application of whitening gel on incipient lesions may compromise the safety of the procedure (6).

Regarding the CSH analysis, the results showed that bleaching agents were able to increase the depth of incipient lesions demineralization. The CSH data together with images of microscopic observations, makes it possible to consider that under the used experimental conditions, tooth whitening can influence the demineralization progress, also rejecting the second hypothesis of the study. In addition, other studies provide information that sound enamel is more resistant to demineralization than carious enamel when submitted to a bleaching procedure (20).

It was found that in sound enamel, the changes caused by the bleaching treatment were not limited to the surface of the tissue, producing deleterious effects up to 20 µm in depth. These data agree with previous studies (16) where microcomputerized tomography evaluation was used, proving the existence of mineral loss in the subsurface tooth enamel submitted to bleaching procedures.

A.L.F. Briso et al.

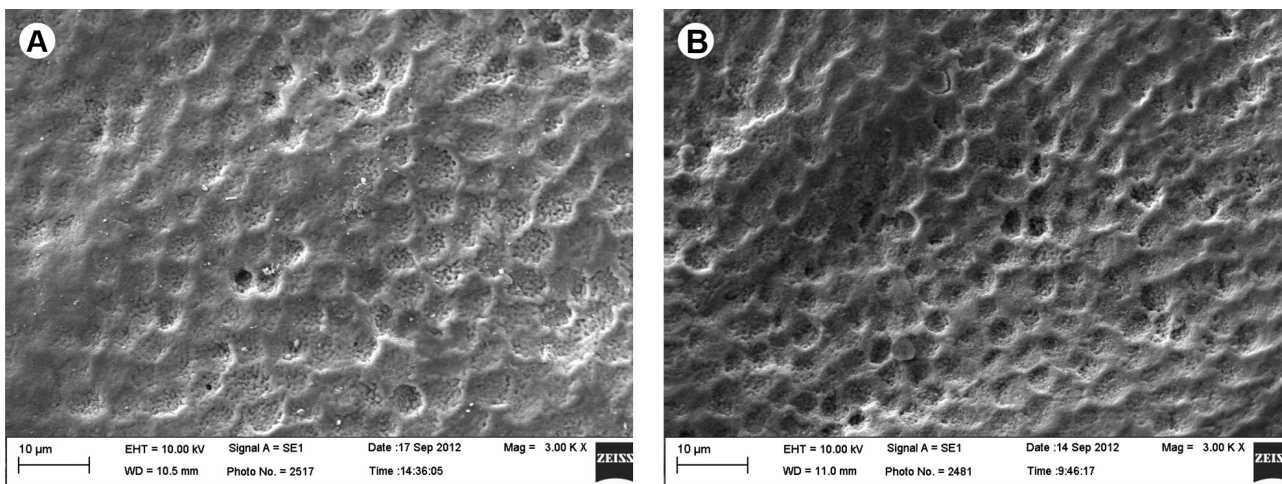


Figure 1. SEM analysis. A: Specimen subjected to cariogenic challenge. The enamel porous surface aspect presents partial dissolution of the aprismatic layer (arrow) (3,000×). B: Specimen subjected to cariogenic challenge and bleaching procedure. The substantial dissolution of the aprismatic layer and increased porosity can be observed, allowing visualization of the enamel prism mouths, forming new channels for dissemination (3,000×).

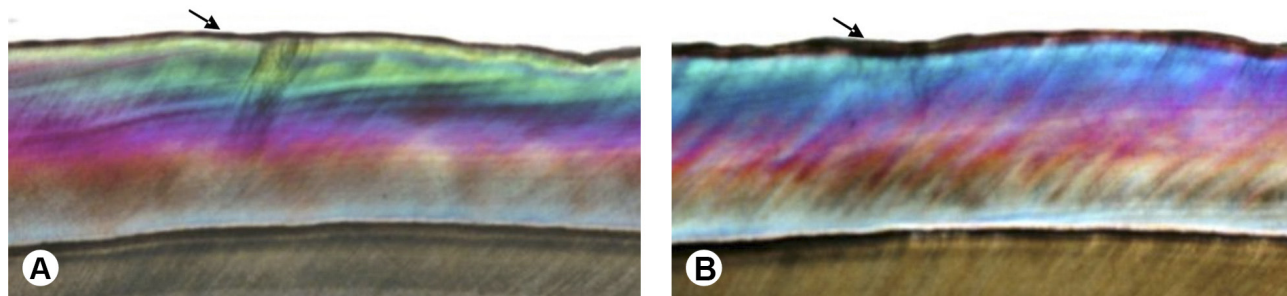


Figure 2. Polarized light microscopy analysis. Image obtained by polarized light microscopy (25×). A: Specimen subjected to cariogenic challenge. Uniform demineralization of the enamel surface (arrow) may be observed. B: Group II specimen subjected to cariogenic challenge and bleaching procedure, arrow indicates a thicker area of negative birefringence, to an average depth of up to 83 µm, without apparent cavitation.

When the bleaching was performed on previously demineralized enamel, the CSH results showed that the bleaching agents produced changes to a depth of 90 μm . These observations are opposed to some studies (6), where it was found that the action of the bleaching is most evident on the surface of this tissue. However, it should be emphasized that the researchers conducted an *in situ* study and possibly the oral environment conditions, especially the action of saliva (21), may explain the difference between the studies.

It is worth noting that from a depth of 120 μm , the bleaching agent, as well as the demineralizing solution, had no influence on CSH, possibly because of the distance to the outer surface (22). Another important factor to be observed is the change of the product pH. During its application, the whitening gel had a pH change from 7 to 5, which might have caused significant histomorphological changes in the enamel surface (23) and influenced the HP diffusion through the tooth structure.

Since this is an *in vitro* study, the results cannot be extrapolated directly to clinical situations, as the presence of intrapulpal pressure, the presence of saliva and odontoblast cytoplasmic processes may decrease the peroxide diffusion (24). In addition, fluoride dilution by saliva, the availability of the product and clearance from oral cavity cannot be taken into consideration in *in vitro* models too. However, *in vitro* models can offer important initial information to provide the investigators with confidence to appropriately design clinical trials (25). Otherwise it offers valuable information on the importance of the enamel integrity on the influence of HP penetration during the bleaching treatment, because bleaching is a cosmetic treatment and therefore should be performed in patients without any pathology in the hard and soft oral tissues.

It may be concluded that the application of bleaching agents in previously demineralized enamel favors HP penetration through the tooth structure and the bleaching treatment can increase the demineralization depth of incipient lesions.

Resumo

O objetivo deste estudo foi avaliar a desmineralização e a penetração do peróxido de hidrogênio (HP) em dentes com lesões incipientes submetidos ao tratamento clareador. Para analisar a penetração do peróxido de hidrogênio, discos de esmalte/dentina hígidos e desmineralizados foram posicionados em câmaras pulpares artificiais contendo solução tampão de acetato. Após o tratamento clareador, esta solução foi submetida à análise da densidade óptica no espectrofotômetro e as superfícies dos discos foram analisadas por meio de microscopia eletrônica de varredura (MEV) e microscopia de luz polarizada (MLP). Os discos restantes foram submetidos à análise de microdureza transversal em diferentes profundidades. Os dados foram submetidos aos testes ANOVA e teste PLSD Fisher ($\alpha=5\%$). Observou-se que os dentes previamente desmineralizados mostraram maior penetração de HP ($p<0,05$). O tratamento clareador causou alterações em uma profundidade de 20 μm em esmalte hígido e até 90 μm em esmalte

desmineralizado. As imagens obtidas em PLM e MEV mostraram que o tratamento clareador promove alterações superficiais no esmalte, sendo mais pronunciadas em dentes previamente desmineralizados. Foi concluído que o nível de mineralização do esmalte influencia a penetração do PH e que o agente clareador contribuiu para o aumento da profundidade de desmineralização.

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