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ABSTRACT

Hydrogen peroxide is an oxidative agent commonly used for dental bleaching procedures. The structural and biochemical responses of enamel, dentin, and pulp tissues to the *in vivo* bleaching of human (n = 20) premolars were investigated in this study. Atomic force microscopy (AFM) was used to observe enamel nanostructure. The chemical composition of enamel and dentin was analyzed by infrared spectroscopy (FTIR). The enzymatic activities of dental cathepsin B and matrix metalloproteinases (MMPs) were monitored with fluorogenic substrates. The amount of collagen in dentin was measured by emission of collagen autofluorescence with confocal fluorescence microscopy. The presence of Reactive Oxygen Species (ROS) in the pulp was evaluated with a fluorogenic 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) probe. Vital bleaching of teeth significantly altered all tested parameters: AFM images revealed a corrosion of surface enamel nanostructure; FTIR analysis showed a loss of carbonate and proteins from enamel and dentin, along with an increase in the proteolytic activity of cathepsin-B and MMPs; and there was a reduction in the autofluorescence of collagen and an increase in both cathepsin-B activity and ROS in pulp tissues. Together, these results indicate that 35% hydrogen peroxide used in clinical bleaching protocols dramatically alters the structural and biochemical properties of dental hard and soft pulp tissue.

KEY WORDS: tooth bleaching, cysteine proteases, matrix metalloproteinase, collagen, FTIR, confocal microscopy.

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Tooth Bleaching Increases Dentinal Protease Activity

INTRODUCTION

Hydrogen peroxide (H₂O₂) is widely used in different concentrations as a dental bleaching agent. Although it is effective in whitening darkened/colored teeth, the oxidative effect of H₂O₂ has been claimed to be responsible for histochemical (Rotstein *et al.*, 1996; Fattibene *et al.*, 2005; Bistey *et al.*, 2007) and morphological alternations in the structure of dental tissues (Hegedus *et al.*, 1999), which may explain the significant reduction of mechanical properties of these tissues after bleaching treatment (Attin *et al.*, 2005; Forner *et al.*, 2009).

The low molecular mass of H₂O₂ (34 daltons) favors its rapid diffusion into enamel prisms and interprismatic spaces (Park *et al.*, 2004), where it can remain entrapped, exerting a prolonged effect on structures that do not necessarily need to be bleached. Moreover, in certain circumstances, H₂O₂ could reach the pulp chamber through dentinal tubules, causing reduction of cell proliferation, metabolism, and viability (Min *et al.*, 2008) and reduction of pulp-reparative capacity (Goldberg and Smith, 2004) and tissue necrosis (Costa *et al.*, 2010) and inducing pulpal pain (Lu *et al.*, 2001; Kugel *et al.*, 2006). Despite literature indicating that, at low concentrations, bleaching agents are not harmful to dental structures (Goldberg *et al.*, 2010), there are few current data regarding the effects of high concentrations of H₂O₂ (35%) on the integrity of dental tissues.

It was recently reported that bleaching agents could increase matrix metalloproteinase (MMP)-mediated collagen degradation in dentin (Toledano *et al.*, 2011). Our group has recently shown that, in addition to MMPs, the human dentin-pulp complex also contains cysteine proteases, which may, in concert with MMPs, be involved in the remodeling/degradation process of dentin matrix in sound and caries-affected teeth (Tersariol *et al.*, 2010; Nascimento *et al.*, 2011).

The objective of this study was to investigate the potential effect of 35% H₂O₂ on the *in vivo* activity of dentin cysteine proteases and MMPs. The tested hypothesis was that H₂O₂ increases the activity of both proteolytic enzymes, promoting collagen degradation in dentin. A combination of atomic force microscopy analysis (AFM), Fourier transform-infrared spectroscopy (FT-IR), confocal microscopy, and fluorogenic substrate enzyme assays was used to characterize the effect of 35% H₂O₂ on tooth substrates under clinical conditions.

Figure 1. AFM image of enamel after H_2O_2 treatment (A) and before bleaching (B). Values (mean $\pm$ SD) of roughness (nm) before and after bleaching (C) (* p < 0.0001, Student t test, 5 replicates). FTIR spectra of enamel (D) and dentin (E) powders before (thick line) and after (thin line) bleaching. Inserted graphs, FTIR spectra at region between 2,100 and 1,200 cm^{-1} , showing in detail amide I band at 1,673 cm^{-1} and carbonate bands at about 1,540 cm^{-1} and at 1,453 cm^{-1} .

MATERIALS & METHODS

This clinical protocol was approved by the Human Assurance Committee of the University of Mogi das Cruzes, São Paulo, Brazil, after the participants' written informed consent had been obtained. Sixty individuals between the ages of 18 and 25 yrs, with 2 or 4 first premolars to be extracted for orthodontic reasons, were examined. From these, 20 volunteers (10 men and 10 women) who satisfied all of the inclusion criteria for whitening treatment were selected. The criteria included plaque index score of $\leq 20\%$, first or second premolars with no gingival recession, caries, or restorations, and no history of previous

whitening treatment or tobacco use. These 20 volunteers provided a total of 56 teeth (experimental and control) that were used in this study. All bleaching procedures were done without heat or light activation. The 2 or 4 premolars received the following tooth-whitening treatments:

- (1) Bleached Group: application of 35% hydrogen peroxide (H_2O_2) applied as described by the manufacturer (Whiteness HP Maxx, FGM, Joinville, SC, Brazil) in the right maxillary ($n = 14$) or mandibular ($n = 14$) first premolars. Teeth were bleached once a wk for 3 wks. Each bleaching session consisted of 3 applications of 35% H_2O_2 for 15 min, for a cumulative exposure of 45 min. The premolars were extracted one day after the end of these sessions.
- (2) Non-Bleached Group (Control): The left upper ($n = 14$) or lower ($n = 14$) first premolars were extracted without application of the bleaching agent. From these 56 extracted teeth, 20 ($n = 10$ from the bleached group and $n = 10$ from the control group) were used for AFM and confocal microscopy experiments. The remaining teeth ($n = 36$) were used for dentin powder preparation. Just before tooth extraction, gingival crevicular fluid (GCF) was collected from the gingival sulcus of the premolars scheduled for extraction, with the aid of a capillary-tipped micropipette. After extraction, the teeth were sectioned at the cemento-enamel junction, pulpal tissue was removed, and the dental hard tissues were frozen at $-30^\circ C$. Pulp tissues were stored by being placed in transport nutritive medium (Dulbecco's modified Eagle medium [DMEM], Life Technologies, Rockville, MD, USA) containing penicillin-streptomycin (Sigma, St. Louis, MO, USA). These

tissues were directly transported to the laboratory for further processing. We applied one-way analysis of variance (ANOVA) and Tukey tests to the data to determine whether there were significant differences in each quantitative parameter between the groups at a confidence level of 95%.

Atomic Force Microscopy

The enamel surface morphologies of the tooth specimens of the bleached and non-bleached (control) groups were analyzed by AFM in contact mode (Scanning Probe Microscope, Shimadzu

SPM 9600, Kyoto, Japan). Imaging was performed with a standard geometry tip made from silicon nitride (see Appendix for details).

Fourier Transform–Infrared Spectroscopy

Enamel and dentin from control and experimental teeth were frozen at -30°C and then reduced to powder (see Appendix for details). A Spectrum 100 FT-IR Spectrometer (Perkin–Elmer, Waltham, MA, USA) was used to collect IR spectra from the enamel and dentin powders (Taube *et al.*, 2010) of the bleached and non-bleached controls (see Appendix for details).

Proteolytic Activities in Dentin, GCF, and Pulp Tissue

Cathepsin B activities from dentin powder, GCF, and pulp tissue were monitored with the fluorogenic substrate Z-FR-MCA in a Hitachi F-2500 spectrofluorometer (Hitachi Scientific Instruments, Inc., Hialeah, FL, USA) as previously described (Scaffa *et al.*, 2012). The total collagenolytic/gelatinolytic MMP activities from dentin powder, GCF, and pulp tissue were monitored spectrofluorometrically with the synthetic internally quenched fluorescent peptide substrate Abz-GPLGLWARG-EDDnp (gift from Dr. L. Juliano, University of São Paulo, S.P., Brazil), with excitation and emission wavelengths of 320 and 420 nm, respectively (Nascimento *et al.*, 2011) (see Appendix for details).

Confocal Fluorescence Microscopy

The autofluorescence of collagen from 200- μm -thick dentin specimens from bleached and non-bleached (control) groups was analyzed by intravital microscopy with an inverted confocal fluorescence microscope, Zeiss LSM 510 (Lin *et al.*, 2010).

The presence of cathepsin B in dentin slices was detected by immunohistochemical staining. Briefly, dentin sections were incubated for 1 hr at room temperature with polyclonal anti-cathepsin B (1:100) primary antibody (Calbiochem, Merck KGaA, Darmstadt, Germany) diluted in PBS. Secondary antibody, goat anti-rabbit Alexa Fluor 595 (Invitrogen, Carlsbad, CA, USA), was diluted (1:200) in 0.2% Triton X-100/PBS plus 5% albumin. The negative control was performed with pre-immune serum instead of primary antibody (see Appendix for details).

Detection of Reactive Oxygen Species in Pulp

The presence of reactive oxygen species (ROS) in pulp tissue was measured by spectrofluorometry from the dental pulp samples collected *in vivo* from both groups ($n = 5/\text{group}$). The pulp tissues were homogenized in 50 mM sodium phosphate buffer, pH 7.4, in the presence of 0.2% Triton X-100. About 1.0 mL of the pulp tissue homogenate, with 1.0 mg/mL protein, was incubated with 4 mM 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes, Eugene, OR, USA) fluorogenic probe for 30 min. After incubation, the solution was centrifuged, and ROS was quantitated from the supernatant by fluorescence in a Hitachi F-2500 spectrofluorimeter with wavelengths of excitation and emission of 503 to 529 nm, respectively (see Appendix for details).

Figure 2. Proteases from dentin powders (A) and from gingival crevicular fluid (GCF) (B) before (control) and after (H_2O_2) bleaching. The bars on the left represent cathepsin B activity of dentin and cysteine cathepsin activities of GCF; and bars on the right represent MMP activities (degradation unit/ μg of sample; mean with SD in both cases). Bars with an asterisk (*) are significantly different from control ($p < 0.05$, Student's *t* test, 5 replicates).

RESULTS

Impact of Vital Bleaching in Dental Structures

AFM 10 x 10 μm images of non-bleached enamel specimens showed a smooth enamel surface. We can visualize enamel rods with several longitudinal grooves (Fig. 1A). The depths of the grooves varied between 18 and 75 nm, and their widths ranged from 0.25 to 1.0 μm . In contrast, bleached specimens revealed a much more irregular surface, with several spikes of larger size with deep microporosities (Fig. 1B). Statistical analysis of R_z data indicated significantly more roughness ($p < 0.05$) in the enamel after H_2O_2 treatment (Fig. 1C).

The typical FTIR spectra of human enamel and dentin powder are shown in Figs. 1D and 1E, respectively. The peaks in these spectra (4,000–400 cm^{-1}) have been assigned according to the literature (Taube *et al.*, 2010). The phosphate content in enamel and dentin is seen in the bands at about 1,040 and 958 cm^{-1} , corresponding to the ν_3 antisymmetric PO stretching mode and the ν_1 symmetric stretching mode of the PO_4^{3-} group, respectively, but also in the strong phosphate bands at 568 and 603 cm^{-1} ; phosphate bands were also observed at 470 cm^{-1} . Compared with the baseline, the intensities of the peaks related to the phosphate stretching modes did not change after H_2O_2 treatment. However, the amount of carbonate in both enamel and dentin powders observed in stretching mode at about 1,540 cm^{-1} and bands at 1,453 cm^{-1} was decreased after *in vivo* bleaching. The amount of amide I bending vibrational modes from proteins in the organic matrix of the enamel and dentin

Figure 3. Fluorescence confocal microscopy image of dentin before (A-D) and after (E-H) bleaching. Dentin morphology differences could be visualized by DIC (A, E). The autofluorescence of collagen was visualized by its intrinsic fluorescence in the green channel (B, F). Dentin tubules can be clearly seen. Cathepsin B was immunolabeled in dentin with anti-human cathepsin B antibody conjugated with Alexa Fluor® 594 in the red channel (C, G). Co-localization between collagen and cathepsin B can be seen as the yellow color formed by the overlapping channels green and red in merged images (D, H). The intensity of collagen autofluorescence emission (green channel) was monitored before (control) and after (H_2O_2) bleaching (I). The amount of cathepsin B in dentin was monitored by measurement of the intensity of fluorescence emission at the red channel before (control) and after bleaching (J). Bars with an asterisk (*) are significantly different from control ($p < 0.05$, Student's *t* test, 5 replicates).

(Magne *et al.*, 2001), observed at the region of $1,673\text{ cm}^{-1}$, was also decreased after H_2O_2 treatment.

Proteolytic Enzyme Activities in Dentin and GCF after Vital Bleaching Treatment

A statistically significant increase in both cathepsin B and MMP proteolytic activities ($p < 0.05$) was observed in dentin after teeth underwent vital bleaching treatment (Fig. 2A). No significant differences ($p > 0.05$) in cysteine-cathepsin activities before and after bleaching were observed in GCF (Fig. 2B).

Collagen Analysis of Dentin Treated with Vital Bleaching

To better understand the microstructure and the changes in collagen within normal dentin and bleached dentin, we reconstructed the 3-D microscopy confocal images of collagen, cathepsin B, and DIC dentin images in the normal (Figs. 3A-3D) and bleached dentin (Figs. 3E-3H). The gray color-coded areas (Figs. 3A, 3E) show dentin morphology analyzed by DIC, the green color-coded structure is autofluorescent collagen (Figs. 3B, 3F), and red indicates cathepsin B (Figs. 3C, 3G) proteolytic enzyme in dentin. Figs. 3D and 3H show the reconstructed

overlaid 3-D images of collagen, cathepsin B, and DIC. Taken together, these images showed that there were large differences in the morphological structures of collagen and cathepsin B in normal dentin and bleached dentin. When teeth were treated with vital bleaching, the dentin's intrinsic collagen autofluorescence (Figs. 3B, 3F) values significantly decreased compared with those of untreated dentin (Fig. 3I); the decrease ($p < 0.05$) observed in the collagen autofluorescence is accompanied by a significant ($p < 0.05$) increase in the amount of cathepsin B (Figs. 3C, 3G) proteolytic enzyme present in dentin of bleached teeth (Fig. 3J).

Cathepsin B and ROS in Pulp after Vital Bleaching

The effect of hydrogen peroxide on human pulp tissue was evaluated by measurement of the amount of reactive oxygen species and by the level of lysosomal cathepsin B proteolytic enzyme in pulp induced by bleaching. Statistically significant increases ($p < 0.05$) in both cathepsin B (Fig. 4A) and ROS (Fig. 4B) were observed in pulp after teeth underwent vital bleaching.

DISCUSSION

The alterations in enamel in the H_2O_2 -treatment group were similar to those obtained by Hegedüs *et al.* (1999). These alterations are thought to be related to the molecular changes in both organic and inorganic composition (Fattibene *et al.*, 2005; Zimmerman *et al.*, 2010). FTIR analysis revealed that H_2O_2 treatment induces the loss of both carbonate and proteins from enamel (Fig. 1C) and dentin (Fig. 1D). Substitutions in the hydroxyapatite mineral crystal by carbonate increase mineral solubility at low pH (Taube *et al.*, 2010). Indeed, the loss of carbonate in enamel and dentin relates to the acidity of 35% H_2O_2 , whereas changes in enamel and dentin organic content relate to protein loss, especially collagen in dentin, by the strong oxidizing ability of peroxide (Jiang *et al.*, 2007). Analysis of our data clearly showed that the bleaching agent containing 35% H_2O_2 induced a significant *in vivo* alteration in enamel and dentin, which could potentially trigger biological and/or mechanical responses of dental structures.

The loss of collagen is believed to contribute to the reduction of dentin mechanical properties after exposure to bleaching agents (Forner *et al.*, 2009). Toledano *et al.* (2011) demonstrated that home bleaching agents induce the highest collagen degradation by activation of dentinal MMPs. This study is the first to show that, besides dentinal MMPs, H_2O_2 significantly increases cathepsin B activity in dentin (Fig. 2A). We speculate that the remarkable decrease (50%)

of collagen-induced autofluorescence, coupled with the increase of cathepsin B and total MMPs, indicate that H_2O_2 can diffuse through enamel into mineralized dentin (Fig. 3).

Acidity of bleaching agents can trigger the autocatalytic activation of MMPs and stabilization of cysteine proteases (Turk *et al.*, 2012) present in the dentin-pulp complex (Tersariol *et al.*, 2010; Nascimento *et al.*, 2011). Cathepsin B is also able to degrade purified extracellular-matrix proteins under both acidic and neutral pH (Buck *et al.*, 1992). Also, cysteine cathepsins are able to activate MMPs (Murphy *et al.*, 1992; Aisa *et al.*, 2003). Analysis of our data strongly suggests that both classes of proteolytic enzymes, cysteine cathepsins and MMP, are activated in mineralized dentin during degradation during tooth-whitening treatment with 35% H_2O_2 . In peripheral dentin, H_2O_2 is able to diffuse around or etch through apatite crystallites and interact with MMPs, thereby activating them. The literature has reported that HOCl generated by H_2O_2 markedly enhances the proteolytic activity of MMPs (Weiss *et al.*, 1985; Peppin and Weiss, 1986; Fu *et al.*, 2001). Also, an independent cellular mechanism may operate in the pulp and be activated by ROS, NO, or other inflammatory mediators, that cause odontoblasts, fibroblasts, or other pulpal cells to secrete more MMPs and cathepsins.

Tooth-whitening procedures may promote gingival irritation, tooth sensitivity, and pulpal necrosis (Jorgensen and Carroll, 2002). The bleaching did not affect cysteine cathepsin or MMP activities in GCF (Fig. 2B), suggesting that the procedure does not induce gingival damage. H_2O_2 can diffuse through enamel and dentin, reaching dental pulp (Gokay *et al.*, 2005). It has been demonstrated that the coronal pulp cells of the *in vivo* bleached human incisors exhibited coagulation necrosis (Costa *et al.*, 2010).

Analysis of our data showed that, *in vivo*, 3 consecutive 15-minute applications of 35% H_2O_2 induced ROS generation in the pulps of sound teeth (Fig. 4B). The probable inflammation/cell death process triggered by H_2O_2 in pulpal cells may be related to the significant 2.5-fold increase in cathepsin B activity in pulp tissue of bleached teeth (Fig. 4A). According to this scenario, high cysteine cathepsin levels in dentin and pulp tissue can relate to the inflammatory/cell death process triggered by the bleaching agent (Min *et al.*, 2008; Costa *et al.*, 2010).

Despite reports that the use of bleaching agents at low concentrations has been considered absolutely safe, analysis of our data shows that the use of 35% H_2O_2 as a bleaching agent provokes biological responses of dental tissues that can be clinically adverse in the long term and/or after recurring bleaching treatments. Individuals intending to undergo dental bleaching should be warned about the biological hazards potentially caused by the use of extensive and unbridled H_2O_2 .

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Figure 4. Bleaching agent effects in pulp chamber. (A) The bars represent the amount of cathepsin B activity in pulp tissues before (control) and after (H_2O_2) bleaching (degradation unit/μg of sample; mean with SD). (B) The bars represent the amount of reactive oxygen species (ROS) measured by DCFDA in the pulp tissue before (control) and after (H_2O_2) bleaching (ROS fluorescent unit/μg of sample; mean with SD). Bars with an asterisk (*) are significantly different from control ($p < 0.05$, Student's *t* test, 5 replicates).

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