

Bleaching Agents Increase Metalloproteinases-mediated Collagen Degradation in Dentin

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Abstract

Introduction: Tooth bleaching is based on hydrogen peroxide application. The Objective of this study was to determine whether dental bleaching agents affect metalloproteinases-mediated dentin collagen degradation. **Methods:** Human dentin specimens were subjected to different treatments: (1) untreated dentin; (2) demineralization by 37% phosphoric acid (PA); (3) demineralization by 37% PA, followed by application of Single Bond (SB); (4) 2 immersions of 7 days each in a nonvital bleaching agent, followed by PA; (5) 2 immersions of 7 days each in nonvital bleaching, followed by PA and SB application; (6) 3 immersions by using in-office bleaching gel for 20 minutes; (7) 3 immersions by using in-office bleaching gel for 20 minutes plus activation with a light source; and (8) immersion in home bleaching gel for 8 hours per day during 3 weeks. Specimens were stored in artificial saliva. C-terminal telopeptide determinations (radioimmunoassay) were performed after 24 hours, 1 week, and 4 weeks. **Results:** Bleaching agents increased collagen degradation, but C-terminal telopeptide of type I collagen (ICTP) values were higher when dentin was PA-demineralized. Nonvital bleaching plus PA promoted the highest collagenolytic activity, which was reduced after SB infiltration. Halogen light application did not influence ICTP values. At 24 hours, home bleaching exhibited high collagenolytic activity, which decreased up to 4 weeks. After 4 weeks of storage, all bleaching procedures showed similar values of collagen degradation, which were not different from those of PA-demineralized and resin-infiltrated dentin. **Conclusions:** All tested bleaching agents increase matrix metalloproteinases-mediated collagen degradation in dentin. This effect was not completely reverted after 4 weeks. Home bleaching induced the highest collagen degradation. (*J Endod* 2011;37:1668–1672)

Key Words

Adhesives, bleaching, degradation, dentin, matrix metalloproteinases, peroxides

Contemporary tooth whitening processes involve the use of either hydrogen peroxide or carbamide peroxide. Two of the key factors in determining overall tooth whitening efficacy from peroxide-containing products are the concentration of the peroxide and duration and number of times of application (1).

Hydrogen peroxide (H_2O_2) might be applied directly or produced in a chemical reaction from sodium perborate or carbamide peroxide. Hydrogen peroxide acts as a potent biological oxidant (2) of organic and inorganic compounds through the formation of free radicals, reactive oxygen molecules, and hydrogen peroxide anions. Because of its low molecular weight, hydrogen peroxide can penetrate into and through the enamel to reach the enamel-dentin junction (1, 3) and dentin, capable of releasing oxygen that breaks the double bonds of organic and inorganic compounds of dentin structure (2).

Carbamide peroxide [$CO(NH_2)H_2O_2$] is an organic white crystalline compound that is formed by urea and hydrogen peroxide. In a hydrophilic environment it breaks down into approximately 3% hydrogen peroxide and 7% urea (4).

Bleaching of vital teeth includes dentist-supervised night-guard bleaching and in-office bleaching. Night-guard bleaching or home bleaching typically uses a relatively low level of whitening agent (10% carbamide peroxide gel tray application or 5.3% H_2O_2 impregnated strips) (5) applied to the teeth via a custom-fabricated mouth guard and is worn at night for at least 2 weeks. In-office bleaching generally uses relatively high levels of whitening agents, for example, 25%–35% hydrogen peroxide containing products, for shorter time periods (5). Contemporary approaches have focused on accelerating peroxide bleaching, trying to accelerate the chemical redox reactions, with simultaneous illumination of teeth with various sources having a range of wavelengths and spectral power such as lasers, light-emitting diodes, plasma arc lamps, and halogen curing lights (1). In nonvital tooth bleaching, the medicament is placed in the pulp chamber, sealed, left for 3–7 days, and is thereafter replaced regularly until acceptable lightening is achieved (3).

Concerns have been expressed regarding the safety of bleaching agents, especially H_2O_2 . Associated undesirable complications included changes in the surface morphology and structure of dentin, loss of mechanical integrity, increased dentin permeability, and external cervical root resorption (6, 7). Bleaching reduces the microhardness of dentin by the loss of calcium and alterations in the organic substance (6, 7), and these factors might be important to cause decreased dentin bonding efficacy (7).

Dentin metalloproteinases (MMPs) -2, -8, -9, and -20 are structural endopeptidases that contribute to dentin matrix organization and mineralization (8–10). MMPs produce collagen degradation at the dentin-resin bonded interfaces, jeopardizing the efficacy of bonded restorations (11–13). The relation between MMP

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collagenolytic activity in dentin and bleaching agent application has never been elucidated and might be a factor contributing to bond strength reduction in bleached dentin (7).

ICTP is the carboxyterminal telopeptide of type I collagen that is joined via trivalent cross-links and liberated during collagen degradation. The telopeptide is only produced through the action of matrix MMPs, and it is considered an index of MMP-driven collagenolysis (14).

Because an augmented collagenolytic activity of MMPs was described in models in which there is an increased production of reactive oxygen species, we aimed to ascertain the effect of hydrogen peroxide or carbamide peroxide bleaching agents on MMP-mediated dentin collagen degradation. This study tested the null hypothesis that collagenolytic activity of MMPs in dentin is not affected by different dental bleaching procedures.

Materials and Methods

Twenty extracted noncarious human third molars were obtained with informed consent from different donors under a protocol approved by the institution review board. The teeth were stored in 0.1% (w/v) thymol solution at 4°C and used within 1 week after extraction. Dentin disks (0.75 ± 0.08 mm thick) were obtained from the mid-coronal portion of each tooth by using a slow-speed diamond saw (IsoMet; Buehler Ltd, Lake Bluff, IL) under water cooling. Four dentin beams (0.75 × 0.75 × 5.0 mm) were obtained from each dentin disk as previously described by Osorio et al (15, 16). A total of 80 dentin beams were obtained.

The dentin beams were rinsed in deionized water under constant stirring at 4°C for 72 hours. They were then dried over anhydrous calcium sulfate (Sigma-Aldrich, St Louis, MO) for 8 hours. The dry mass of each dentin beam was measured with a digital microbalance (Model HR202; A&D, Japan). Specimens were rehydrated in 0.9% NaCl (Braun Medical SA, Barcelona, Spain) containing 10 U/mL of penicillin G (Sigma-Aldrich) and 300 µg/mL of streptomycin (Sigma-Aldrich) for 24 hours (pH 7.0) (15, 16).

Ten dentin beams were submitted to each of the different treatments:

1. Untreated dentin
2. Demineralization by 37% phosphoric acid (PA 37%, pH 1.0) (Pro-clinic; Dentsply, Bradent, FL) for 15 seconds
3. Demineralization by 37% PA for 15 seconds, followed by application of Single Bond Plus (PA 37% + Single Bond) for 20 seconds and light-cured for 10 seconds with a halogen curing unit (Bluephase; Ivoclar Vivadent AG, Schaan, Principality of Liechtenstein) at 750 mW/cm² of light energy density
4. 2 immersions in nonvital bleaching agent (BNV), each immersion lasted for 7 days, followed by demineralization with 37% PA for 15 seconds (nonvital bleaching + PA 37%)
5. 2 immersions in BNV, each immersion lasted for 7 days, followed by demineralization with 37% PA for 15 seconds and application of Single Bond Plus for 20 seconds, light curing for 10 seconds with a halogen curing unit at 750 mW/cm² of light energy density (nonvital bleaching + PA 37% + Single Bond)
6. 3 immersions in in-office bleaching gel (Opalescence Boost PF; Ultradent Products Inc, South Jordan, UT), immersions were performed for 20 min/day, with 3-day interval between them (in-office-bleaching)
7. 3 immersions in in-office bleaching gel (Opalescence Boost PF) for 20 min/day with 3-day intervals plus activation with a light source (e-bright; Beyond, Stafford, TX) (in-office-bleaching + halogen light)

8. 21 consecutive immersions were performed in home bleaching gel, Opalescence 10%, each immersion lasted 8 hours and was done during 21 consecutive days (home bleaching)

After each bleaching agent immersion, the dentin beams were rinsed with copious water. Bleaching agents, adhesive formulations, and manufacturers are listed in Table 1.

Two dentin beams (from same experimental group and different donor) were incubated in each Eppendorf tube containing 500 µL of artificial saliva: 50 mmol/L HEPES (Applichem GmbH, Darmstadt, Germany), 5 mmol/L CaCl₂·2H₂O (Sigma-Aldrich), 0.001 mmol/L ZnCl₂ (Sigma-Aldrich), 150 mmol/L NaCl, and 100 U/mL penicillin, 1000 µg/mL streptomycin (pH 7.2) at 37°C. After 24 hours, 1 week, and 4 weeks, supernatants (100 µL) of the conditioning medium were withdrawn after agitation and analyzed for the collagen degradation product liberation (C-terminal telopeptide of type I collagen, ICTP determination) by using a radioimmunoassay kit (ICTP-RIA; Orion Diagnostica Oy, Espoo, Finland) (15, 16). A standard curve was constructed with ICTP ranging from 0.01–250 µg/L. Five ICTP measurements were performed in each experimental group for each storage period.

Mean ICTP concentration values were calculated in micrograms per liter and analyzed by analysis of variance and Student-Newman-Keuls multiple comparisons. Differences between storage times were analyzed by Friedman and Wilcoxon pair-wise comparison tests. Significance was considered at $P < .05$.

Results

Mean ICTP values were affected by dentin treatment ($F = 174.28$; $P < .001$) and by storage time ($F = 26.44$; $P < .001$). Interactions were also significant ($F = 12.54$; $P < .001$). The power of the analysis of variance was 0.85. The total amount of ICTP liberated from the dentin beams is displayed in Table 2.

Untreated dentin beams released only negligible amounts of ICTP.

For dentin demineralized with PA 37% only, the ICTP values ranged from 11.03 µg/L after 24 hours to 22.00 µg/L after 4 weeks. The amount of ICTP liberated increased between 24 hours and 1 week of incubation and then remained stable. The application of Single Bond after PA 37% demineralization significantly reduced the ICTP values in all storage periods. No significant differences were found in the amount of ICTP liberated from dentin between 24 hours and 1 week of incubation and between 1 week and 4 weeks in this group. However, from 24 hours to 4 weeks of incubation there was a significant increase in the ICTP values.

Collagen degradation of dentin beams treated with nonvital bleaching + PA 37% was significantly higher compared with untreated dentin in all storage periods. In this group, the amount of ICTP liberated increased between 24 hours and 1 week of incubation and then remained stable. When Single Bond was applied to the dentin treated with nonvital bleaching + PA 37%, the ICTP values significantly decreased for each period of storage compared with the treatment with only nonvital bleaching + PA 37%. The amount of ICTP released from dentin beams treated with nonvital bleaching + PA 37% + Single Bond significantly increased after each storage period.

The ICTP values for in-office bleaching and in-office bleaching + halogen light dentin were similar and were significantly higher than those of untreated dentin at the 3 study time-points. For both groups, the ICTP values increased significantly from 24 hours to 4 weeks.

ICTP values from home bleaching specimens were the highest among all the tested bleaching methods. A significant decrease in ICTP liberation was detected after 4 weeks. Compared with the in-office bleaching groups, the liberation of ICTP from home bleaching dentin

TABLE 1. Materials Used in the Experiment and Respective Manufacturers, Batch Numbers, Basic Formulation, and Mode of Application

Material (manufacturer/batch number)	Basic formulation	Mode of application
Adper Single Bond Plus (3M ESPE, St Paul, MN/8PT)	Bis-phenol A diglycidylmethacrylate, 2- hydroxyethyl methacrylate, dimethacrylates, ethanol, water, a novel photoinitiator system, a methacrylate functional copolymer of polyacrylic, polyitaconic acids.	Etch dentin for 15 seconds and rinse for 30 seconds. Blot excess water by using a cotton pellet or mini-sponge without air drying, leaving a shiny surface. Apply 2–3 consecutive coats of adhesive for 20 seconds with gentle agitation and gently air thin for 20 seconds to evaporate solvent. Light cure for 10 seconds.
BNV Bleaching Nonvital (Futura Medical SA, Spain/1023)	Liquid: hydrogen peroxide. Powder: monohydrated sodium perborate.	Incorporate the powder into the liquid until a homogenous mixture is obtained. Apply the paste on the surface and leave it for 7 days. Rinse with copious water. Repeat the procedure for 1 more time.
Opalescence Boost PF (Ultradent Products Inc, South Jordan, UT/G032).	38% hydrogen peroxide, chemical activator, 1.1% fluoride, 3% potassium nitrate.	In-office bleaching agent. Three applications of 20 minutes. Remove it with a soft pellet; rinse twice with copious water. Repeat the procedure in 2 other applications with 3-day interval between each one.
Opalescence 10% (Ultradent Products Inc/3MPI).	10% carbamide peroxide, Carbopol, glycerin, flavoring.	Home bleaching agent. Apply the bleaching agent on the surface for 8 h/day. Remove it with a soft pellet; rinse twice with copious water. Repeat the procedure for 21 days.

beams was higher at 24 hours and 1 week, but there was no significant difference among the ICTP values of those groups after 4 weeks.

Discussion

Determination of ICTP is one of the most reliable techniques to quantify MMP-driven enzymatic activity on type I collagen (14, 17). Assessment of other collagen fragments such as hydroxyproline or even CTX-epitope of C-telopeptide might not be as sensitive or specific (14). Dentin contains other collagen-degrading enzymes such as cysteine cathepsin (18, 19), but it has been stated that among known collagenolytic proteinases relevant to hard tissue resorption, only MMPs can generate ICTP. This liberation is inhibited by MMP inhibitors but not by cysteine proteinase inhibitors. Thus, determination of ICTP is preferred to other less specific collagen fragments for detecting the activity of MMPs (14). Other enzyme assays used synthetic peptides as substrates, but it is important to confirm the activity of MMP inhibitors against the native substrate and in the presence of endogenous tissue inhibitors (20).

The results of this study do not support the null hypothesis that collagenolytic activity of MMPs in dentin is not altered by different dental bleaching procedures. It has been shown for the first time that bleaching procedures increased MMP-mediated collagen degradation in dentin.

The amount of collagen degradation obtained from the untreated dentin beams confirms that dentin collagen is not degraded if it remains in its mineralized state (15, 16). The collagen-degradation values of PA-demineralized dentin were higher than those of untreated dentin specimens during the 3 periods of study (Table 2). Controversy exists over the interaction of PA with MMPs. It has been reported that PA increased dentin MMP activity (21), whereas Mazzoni et al (22) reported a transient PA-related inactivation of MMPs under different experimental conditions. However, recent studies have demonstrated that MMPs drastically increased their collagenolytic activity after PA demineralisation (13, 21, 23).

The collagen-degradation values of PA-demineralized dentin plus Single Bond Plus infiltration at 24 hours were significantly lower than those of PA-demineralized dentin samples, therefore reducing the degradation of the target proteins. These differences remained during 1 and 4 weeks of the study. The results in the present study are consistent with recent findings in which adsorption of soluble MMPs to biomedical polymers (especially to resins such as polymethylmethacrylate and poly-2-hydroxyethyl methacrylate [HEMA]) came out in reversible blocking of the MMPs (24). It is possible that the resins might molecularly immobilize the catalytic sites of MMPs. HEMA was also shown to inhibit the activity of MMP-2 by zymography (25). Nevertheless, the ICTP values increased significantly after 1 and 4 weeks of

TABLE 2. Mean and Standard Deviation of ICTP Liberated ($\mu\text{g/L}$) from Human Dentin Explants for Each Treatment and Storage Time ($n = 5$)

Dentin treatment	24 Hours	1 Week	4 Weeks
Untreated dentin	0.98 (0.12) ^{1 A}	2.92 (0.78) ^{2 A}	2.96 (0.57) ^{2 A}
PA 37%	11.30 (1.41) ^{1 C}	18.66 (3.47) ^{2 C}	22.00 (3.06) ^{2 C}
PA 37% + Single Bond	1.86 (0.31) ^{1 AB}	2.70 (0.70) ^{1,2 A}	3.76 (0.13) ^{2 AB}
Nonvital bleaching + PA 37%	27.43 (1.22) ^{1 D}	51.84 (8.83) ^{2 D}	71.26 (14.80) ^{2 D}
Nonvital bleaching + PA 37% + Single Bond	0.55 (0.11) ^{1 A}	1.99 (0.22) ^{2 A}	4.14 (0.89) ^{3 B}
In-office-bleaching	3.03 (0.32) ^{1 B}	4.23 (0.86) ^{1,2 A}	4.88 (0.48) ^{2 B}
In-office bleaching + halogen light	3.20 (0.30) ^{1 B}	4.03 (0.75) ^{1,2 A}	5.08 (0.50) ^{2 B}
Home bleaching	9.76 (1.59) ^{1 C}	10.76 (1.90) ^{1 B}	5.13 (0.96) ^{2 B}

For each horizontal row, values with identical superscript numbers indicate no significant difference by using Friedman and Wilcoxon pair-wise comparisons tests ($P > .05$).

For each vertical column, values with identical superscript letters indicate no significant difference by using Student-Newman-Keuls test ($P > .05$).

ICTP, C-terminal telopeptide of type I collagen.

artificial saliva immersion. The hydrolytic degradation process of the resin and the solubilization of the unprotected collagen fibrils within the decalcified dentin (26) could have contributed to these results.

The application of bleaching agents before PA drastically increased the proteolytic action of MMPs in bleached dentin (Table 2). Resin infiltration and polymerization reduced ICTP values, but the durability of this reversible blocking of the dentin matrix MMPs produced by resin (24, 25) remains to be determined (16).

Former theories suggested that application of bleaching agents led to denaturation of dentin proteins by the oxidizing agents (27). In addition, peroxides might also cause a modification in chemistry of dental hard tissues by changing the ratio between organic and inorganic components (2).

The exact role of H₂O₂ in MMP's collagenolytic activity at the dentin substrate has not been elucidated yet, although in other systems, increases in oxidants and oxidative stress have been implicated in many disease processes, including activation of MMPs, cleaving proteins, and compromising the extracellular matrix, thus leading to substrates remodelling under pathologic conditions (28–31). Schulz (30) stated that the consequences of MMP activation are likely to occur in organs and cells subjected to oxidative stress. The proteolytic removal of the propeptide region perturbs the interaction of the thiol moiety of a key cysteine residue from this region to the catalytically active Zn²⁺ site. Disrupting this cysteine-Zn²⁺ bond, either by limited proteolysis or by conformational changes induced by oxidizing agents, is a crucial step in MMP activation. Oxidants have also been shown to inactivate tissue inhibitors of metalloproteases. Conceivably, superoxides induce MMP-2 activity on inactivating the ambient antiprotease TIMP-2, thereby causing an alteration in the protease-antiprotease balance in favor of the protease (31). In addition, Ca²⁺ plays the role of a second messenger in many biochemical and physiological events. In some systems, an increase in free Ca²⁺ level caused by oxidants stimulates activities of different proteases that are known to modulate activities of a variety of signal transduction enzyme processes (31).

ICTP values after 1 and 4 weeks were higher than at 24 hours of study in nonvital bleaching plus PA. These data are consistent with the findings of other studies that reported that when the surface organic components are removed by H₂O₂, the surface mineral components collapse and form a protective layer for the underlying dentin, increasing the crystallinity of dentin, thereby lessening the direct contact of H₂O₂ with underlying dentin and greatly slowing the attack (6). Practically the same ICTP concentration values were observed in untreated dentin as in nonvital bleaching plus Single Bond, except after 4 weeks of immersion, where the bleached specimens originated more collagen degradation. Furthermore, it was shown that after Single Bond infiltration, collagenolytic activity was similar in bleached or not bleached specimens.

All tested external bleaching agents produced an increase in MMP-mediated collagen degradation of untreated dentin. Our study raises the possibility that the mineralized dentin surface might be dissolved in H₂O₂ that diffuses from the dentin substrate (32). This dissolution of mineralized dentin should be mainly attributed to the strong oxidizing ability, more than to the natural acidity, of H₂O₂ (6).

The acidic nature of in-office bleaching agents has been stated (33), undertaking an acid-etch effect and causing superficial structural changes to dentin, which increase its permeability and permit greater diffusion of hydrogen peroxide through dentinal tubules (2). When in-office bleaching protocols were selected, to accelerate the whitening process the bleaching agent can also be heat or light activated by thermocatalysis and photolysis procedure, both releasing hydroxyl radicals from H₂O₂ (5). The fact that the heated and lighted bleaching agents penetrate into dental hard tissue more rapidly might explain the whit-

ening effect of heat and light activated bleaching methods, but it did not affect collagen degradation by dentin matrix MMPs (Table 2). The values of collagen degradation became stable after 1 week of immersion, even when they remained higher than those of untreated and nonbleached dentin.

Home bleaching showed the highest ICTP values among clinically proposed bleaching treatments; it was the most evidenced collagenolytic degradation 1 week after treatment. However, collagen degradation values became similar in all external bleaching systems after 4 weeks. The amount of hydrogen peroxide that diffused through the dentin was most dependent on its original concentration within the bleaching agent and the length of time the agent came into contact with the dentin (32). Home bleaching was performed during 8 hours/day for 21 consecutive days (Table 1). The diffusion of H₂O₂ is complex. It took as little as 15 minutes for H₂O₂ from bleaching agents to diffuse through 0.5 mm of dentin and reach a level capable of causing harmful biological effects, in spite of convection caused by positive pulpal pressure and osmotic pressure of the gels; both worked against the diffusive flux of molecules from the bleaching agents toward the pulp (32). The extended application time that was used in this protocol might account for the highest collagenolytic activity observed at 24 hours and 1 week.

All bleaching methodologies, except home bleaching, that were used in the present study showed higher values of proteolytic activity at 4 weeks than at 24 hours of storage. It is expected that in junction with some other side effects that are unveiled after H₂O₂ treatment as obliteration of the odontoblastic layer, loss of predentin, dense inflammatory infiltrate, and areas of internal resorption (32), the residual oxygen from the bleaching agent might interfere with resin attachment and inhibits resin polymerization, increasing the porosity of the resin material and producing poor uniformed and defined interfaces (7). There are remarkable variations among the recommended post-bleaching time needed to delay bonding procedures (24 hours–4 weeks) (1); some authors thought that a delay of at least 2 weeks is needed after bleaching for the tooth structure to regain its prebleaching adhesive properties. But at least in dentin, the effect of bleaching might be more permanent or take longer to eliminate. It is clear that the encountered high levels of ICTP indicate that created hybrid layers on these substrates will be more susceptible to enzymatic degradation, and it remains to be ascertained whether these changes might be completely reverted. Further studies are needed to investigate these alterations, which might give rise not only to clinical manifestations in the biomechanical properties of teeth but also in adhesion mechanisms of resins to dentin after bleaching treatments.

Reactive oxygen species generated by dental bleaching treatments increased MMP-mediated collagen degradation in bleached dentin. Blocking MMPs though resin infiltration is possible, exerting a protector effect and decreasing the proteolytic activity.

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The authors deny any conflicts of interest related to this study.

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