Demineralization Effects of Phosphoric Acid on Surface and Subsurface Bovine Enamel Bleached with In-office Hydrogen Peroxide

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\textbf{Purpose:} To measure the demineralization capacity of 37% phosphoric acid on surface and subsurface bovine enamel after bleaching with hydrogen peroxide (H$_2$O$_2$).

\textbf{Materials and Methods:} Three equally-sized sections with 16 mm$^2$ of exposed enamel surface were obtained from the enamel of 10 bovine incisors. One specimen sample from each crown was assigned to one of three groups (n = 10): group I, no bleaching agent; group II, bleached with 38% H$_2$O$_2$ for 20 min; or group III, 30% H$_2$O$_2$ for 60 min. After 24 h, the thickness of specimens was measured and they were immersed in 37% phosphoric acid solution, from which 5-mL aliquots were collected at 30 s and 60 s. Specimens were then ground to a depth of 25 μm and again immersed in 37% phosphoric acid solution. This procedure was repeated for enamel depths of 50 and 100 μm. Ca$^{2+}$ concentrations in the phosphoric acid aliquots were measured by atomic absorption spectroscopy.

\textbf{Results:} No significant differences were found in the total amounts of extracted Ca$^{2+}$ between bleached and unbleached specimens (F = 0.142; p = 0.869). The amount of Ca$^{2+}$ extracted was similar among the four depth levels in the unbleached and in the 30% H$_2$O$_2$ bleached specimens. A significantly larger amount of Ca$^{2+}$ was obtained at 25 μm depth (subsurface) from specimens treated with 38% H$_2$O$_2$.

\textbf{Conclusions:} Pre-bleaching with 38% H$_2$O$_2$ significantly increased the decalcifying effect of phosphoric acid on subsurface enamel at a depth of 25 μm compared to 100 μm, whereas pre-bleaching with 30% H$_2$O$_2$ did not modify this effect at any level.

\textbf{Keywords:} hydrogen peroxide, decalcifying, subsurface enamel, phosphoric acid, enamel acid etching, atomic absorption spectroscopy.


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\textbf{Numerous techniques have been used for vital dental bleaching. With the in-office technique, high concentrations of hydrogen or carbamide peroxide are applied during the clinical treatment session. With home tooth bleaching, the same peroxide-based gels are applied at lower concentrations, using custom-made trays\textsuperscript{17} or placing bleaching strips on the buccal surface of the teeth.\textsuperscript{14}}

The mechanisms underlying tooth bleaching have not yet been fully elucidated. However, it has been proposed that the strong oxidative action of the free radicals generated by hydrogen peroxide (H$_2$O$_2$) acts by breaking the polypeptide chain caused by the destruction of amino acids that are part of the composition of the organic substance, and the main agents responsible for tooth bleaching may be hydroxyl radicals.\textsuperscript{21}
There is no general consensus on the negative effects of bleaching agents on enamel and dentin. Most studies have reported that peroxide-containing bleaching agents have no significant deleterious effects on the enamel or dentin surface or subsurface morpholology, chemistry, microhardness, or ultrastructure. However, some researchers have claimed that tooth-whitening procedures irreversibly damage tooth structure and may cause changes in enamel microstructure, notably a demineralization that is similar to initial caries. It was reported that the microhardness of enamel is reduced by in-office and at-home bleaching, and that its nano-hardness is significantly increased by even a short (1 h) bleaching procedure. The organic material around the enamel rods is mobilized by the attack of free oxygen radicals, preferentially damaging the enamel sheath area and increasing the surface roughness.

It has been demonstrated that the peroxide readily diffuses through the enamel and dentin, reaching the pulp tissue. De Lima et al. found that carbamide peroxide (CP) gel extracts exerted dose-dependent cytotoxic effects on cultured pulp cells, even at low concentrations. There is also controversy about whether it can affect subsurface as well as surface enamel. A recent study using confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and Raman spectroscopy plus CLSM technique found no significant ultrastructural or chemical effects on surface or subsurface enamel or dentin. However, other researchers have suggested that bleaching causes microstructural changes to surface and subsurface enamel layers mainly localized in the outer enamel, which have been associated with a loss of calcium and a significant decrease in calcium/phosphate ratios.

Microcomputed tomography studies demonstrated post-bleaching demineralization of the enamel from 50 to 150 μm in depth. It was observed that a larger amount of ions was released at higher H2O2 concentrations, which may be explained by changes in the chemical composition of dental enamel caused by the bleaching agents. Patients often need further interventions after bleaching in order to restore esthetic effects. Acid etching of the enamel is required shortly after bleaching in numerous clinical procedures, including composite restorations, replacement of old restorations, porcelain veneers, composite veneers, and bonding of orthodontic brackets. The acid etching of bleached enamel surfaces has been reported to produce a loss of prismatic shape, giving the surface an over-etched appearance. A spectroscopy study found that a larger amount of Ca2+ was extracted from enamel by phosphoric acid after application of 30% CP and that this greater susceptibility to the action of the acid persisted for at least 1 week after bleaching. In another, similar study, the largest amount of Ca2+ was extracted by 37% phosphoric acid at 24 h after application of 30% peroxide, coinciding with a significant decrease in the shear bond strength of brackets to enamel. After one week, the shear bond strength with bleached enamel progressively recovered to reach the value observed with unbleached enamel, and extracted Ca2+ values stabilized.

Many clinical esthetic procedures require bleaching agents or some type of enamel preparation before acid etching, and subsurface decalcification caused by bleaching agents may influence the effectiveness of the etching. The null hypothesis of the present study was that a bleaching treatment with 30% or 38% H2O2 does not modify the decalcifying ability of phosphoric acid in enamel acid etching at either the surface or subsurface level. Therefore, the objective of this in vitro study was to quantify, using atomic absorption spectroscopy, the amount of calcium extracted from surface and subsurface enamel (depths of 0, 25, 50, and 100 μm) by 37% phosphoric acid after its treatment with 30% and 38% H2O2.

MATERIALS AND METHODS

After careful visual inspection, 10 bovine incisors with no signs of cracks or structural anomalies were selected and stored under refrigeration in solutions of distilled water and saturated thymol until their preparation for testing. All roots were sectioned at the cementoenamel junction using an Accutom-50 diamond cutter (Accutom Hard Tissue Microtome; Struers; Ballerup, Denmark). Crowns were polished with silicon carbide paper disks on a polishing (Exakt Apparatebau; Norderstedt, Germany) to obtain a flat vestibular surface and a uniform substrate for bleaching. Each crown was then fixed with Coltene utility wax (Whaledent; Mahwah, NJ, USA) to an acrylic base and cut into three 4 x 4 mm sections of equal size (sample specimens). Each specimen was then attached, with the enamel surface pointing downwards, using a Coltene utility wax to the central part of a 12 x 3-mm embedding mold that was then filled with Inplex acrylic resin (Bayer Materialscience; Leverkusen, Germany) until the whole specimen was covered. For every 10 ml of resin, 5 drops of accelerator and 10 drops of hardener were used. The exposed enamel surface of specimens was polished with 500-grit then 1200-grit silicon carbide paper disks to obtain a flat surface and remove possible excess resin, so that all specimens had the same surface area (16 mm2) of exposed enamel. The opposite end of the embedded specimen was also polished, obtaining two smooth and parallel surfaces. Specimens were stored in distilled water until the experimental procedure.

One sample specimen from each crown was assigned to one of three groups (n = 10); group I, no bleaching agent (control group); group II, bleached with 38% H2O2 (Opalescence Boost, Ultradent; South Jordan, UT, USA, batch no. 5332) for 20 min; and group III, bleached with 30% H2O2 (Illumine Office, Dentsply de Troy; Konstanz, Germany, batch no. 0708001853) for 60 min, replacing all of the gel at 30 min as per manufacturer’s instructions.

After the whitening treatment, specimens were stored in distilled water for 24 h and then dried with absorbent paper. Next, the thickness at the central part of the exposed enamel area was measured with a micrometer (Mitutoyo 350-MHNl.25-DM), which has a ratchet stop to ensure a constant measuring force, preventing trans-
mission to the sample of the pressure exerted by the micrometer. Subsequently, specimens were immersed in 20 ml of 37% phosphoric acid solution, which was prepared by dissolving 311.1 ml of 85% phosphoric acid (Scharlau Comise; Barcelona, Spain) at a concentration of 1.71 g/ml in 689 ml distilled water to obtain 1000 ml of 37% acid solution with pH 0.14 as measured with a Micro pH 2000 pH-meter (Crisol; Alella, Spain).

A blank 5-ml sample of phosphoric acid solution and another blank with phosphoric acid solution plus embedding material alone were prepared in order to determine Ca$^{2+}$ levels in absence of the specimen. All unbleached and bleached specimens underwent the same experimental procedure, ie, immersion in 20 ml of phosphoric acid solution followed by constant agitation using a magnetic multi-stirrer (SBS A-09 series C; Barcelona, Spain) to uniformly mix the Ca$^{2+}$ extracted into the solution. At 30 s and 60 s, 5-ml aliquots were drawn using a calibrated micropipette with a disposable tip that was replaced after each extraction. Extracts were placed in hermetically sealed, labeled glass tubes. Hence, two extracts were obtained for each sample specimen.

After specimens were removed from the phosphoric acid solution, they were washed with abundant distilled water and dried with absorbent paper. Specimens were then ground with 1200-grit silicon carbide paper disks until the enamel depth was reduced by 25 μm. Specimens were washed again with abundant distilled water, dried with absorbent paper, and immersed in the phosphoric acid solution, followed by constant agitation using a magnetic multi-stirrer, collecting 5-ml aliquots at 30 s and 60 s. The same procedure was repeated after reducing the enamel depth by 50 and 100 microns with respect to the initial dimensions. Hence, two aliquots were obtained for each specimen (at 30 and 60 s) at enamel depths of 0, 25, 50, and 100 μm (Fig 1).

Ca$^{2+}$ concentrations in solutions were determined by atomic absorption spectroscopy (Perkin Elmer mod. 5100ZL; Waltham, MA, USA) using the flame technique. Extract readings were expressed in ppm and subsequently transformed into mg of Ca$^{2+}$ lost per area of specimen by applying the following formula:

$$\text{mg Ca}^{2+} = \left( [\text{ppm Ca}^{2+}] \cdot 10^{-3} \text{ l/ml} \times V \right) / A$$

where ppm Ca$^{2+}$ are ppm of Ca$^{2+}$ in each time period, V is the volume of solution in ml (at 30 s, V1; and at 60 s, V2), and A is the area of the sample in mm$^2$. The volume was 20 ml at 30 s and 15 ml at 60 s (due to the removal of 5 ml from V1 for testing). Therefore, the mg of Ca$^{2+}$ corresponding to 5 ml of V1 solution were added to the mg of Ca$^{2+}$ obtained at 60 s. Likewise, since the volume at 60 s was 15 ml, the mg of Ca$^{2+}$ corresponding to 5 ml of V1 solution plus 5 ml of V2 solution were added to those obtained at 60 s. By this means, we obtained the mg of Ca$^{2+}$ passing into the solution per mm$^2$ of specimen, expressing the values in micrograms due to the low values found.

**Statistical Analysis**

We used a full factorial model of ANOVA for repeated measures, with two within-subject factors (subsurface level of enamel depth [0, 25, 50, or 100 μm] and immersion time in 37% phosphoric acid [30 or 60 s]) and a between-subject factor (treatment [control, 38% H$_2$O$_2$, 30% H$_2$O$_2$]).

The repeated-measures ANOVA revealed that the enamel depth and 37% phosphoric acid application time and their first-order interactions with the enamel treatment were all significant. Next, repeated-measures ANOVA was independently applied for each treatment
Table 1 Mean (SD) of Ca²⁺ extracted by etching time and enamel depth level (µg/ml)

<table>
<thead>
<tr>
<th>Depth</th>
<th>0 µm</th>
<th>25 µm</th>
<th>50 µm</th>
<th>100 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etching time</td>
<td>30 s (2.64)</td>
<td>6.33 (1.69)</td>
<td>5.51 (1.50)</td>
<td>4.66 (1.50)</td>
</tr>
<tr>
<td></td>
<td>60 s (2.63)</td>
<td>7.19 (2.54)</td>
<td>6.55 (2.54)</td>
<td>4.86 (1.62)</td>
</tr>
<tr>
<td>Control</td>
<td>30 s (2.35)</td>
<td>6.05 (3.70)</td>
<td>8.17 (3.70)</td>
<td>6.25 (2.25)</td>
</tr>
<tr>
<td></td>
<td>60 s (3.35)</td>
<td>6.72 (3.35)</td>
<td>8.83 (3.35)</td>
<td>7.65 (3.35)</td>
</tr>
<tr>
<td>38% hydrogen peroxide</td>
<td>30 s (2.80)</td>
<td>6.97 (3.94)</td>
<td>8.32 (3.94)</td>
<td>6.75 (2.74)</td>
</tr>
<tr>
<td></td>
<td>60 s (3.56)</td>
<td>4.39 (0.79)</td>
<td>5.24 (0.79)</td>
<td>4.41 (1.31)</td>
</tr>
<tr>
<td>38% hydrogen peroxide</td>
<td>30 s (4.26)</td>
<td>4.31 (0.92)</td>
<td>5.60 (0.92)</td>
<td>4.63 (1.59)</td>
</tr>
<tr>
<td></td>
<td>60 s (3.03)</td>
<td>4.82 (0.86)</td>
<td>4.28 (0.86)</td>
<td>6.73 (2.12)</td>
</tr>
<tr>
<td>30% hydrogen peroxide</td>
<td>30 s (3.57)</td>
<td>4.82 (0.86)</td>
<td>4.57 (0.86)</td>
<td>6.76 (2.50)</td>
</tr>
<tr>
<td></td>
<td>60 s (3.57)</td>
<td>5.58 (0.89)</td>
<td>5.58 (0.89)</td>
<td>6.76 (2.50)</td>
</tr>
</tbody>
</table>

The same superscripts represent statistically significant differences (p < 0.05) between enamel depth levels in the experimental group treated with 38% H₂O₂. The same superscripts represent statistically significant differences (p < 0.05) between enamel depth levels in the experimental group treated with 38% H₂O₂.

Results

According to the Kolmogorov-Smirnov test, results were not significant for any study group and were normally distributed. Results were between Z = 1.06 (p = 0.25) for the treatment with 30% H₂O₂ at a depth of 25 µm, etching time 60 s, and Z = 0.34 (p = 0.30) in the control group at the superficial level (0 µm depth), etching time 30 s.

Table 1 shows the amounts of Ca²⁺ extracted from the enamel specimens (after subtracting the amount of Ca²⁺ in the specimen-free solution) for each whitening treatment and each enamel depth studied. Significant differences (ANOVA, p < 0.05) were found in the amount of Ca²⁺ present in the solution of phosphoric acid as a function of: enamel depth (F = 5.564; p = 0.026), interaction between enamel depth and bleaching treatment (F = 5.197; p = 0.012), phosphoric acid application time (F = 45.853; p = 0.000) and interaction between application time and bleaching treatment (F = 3.506; p = 0.044).

First-order interactions between phosphoric acid etching time and enamel depth and second-order interactions between etching time, enamel depth and bleaching treatment were not significant. Hence, the effects of enamel depth and phosphoric acid application time are mutually independent, but are not independent of the bleaching treatment applied.

No significant difference was detected in the global amount of Ca²⁺ extracted between bleached and unbleached specimens (F = 0.142; p = 0.869) (between-subject factor). The effects of enamel depth and phosphoric acid immersion time were therefore analyzed for each bleaching treatment. In all treatment groups, Ca²⁺ extraction by 37% phosphoric acid was significantly influenced by the enamel depth and acid etching time, and these variables were independent of each other. In the unbleached control group and the group bleached with 30% H₂O₂, no difference in the amount of Ca²⁺ extracted was observed among the four enamel depths. The Bonferroni test verified that only the use of 38% H₂O₂ produced significant differences in extracted Ca²⁺ among the different enamel depths (Fig. 2). Thus, the amount extracted at 25 µm was significantly greater than at 100 µm and was more than extracted at any depth in the other groups.

The separate analysis of extracted Ca²⁺ in the three treatment groups for each phosphoric acid application time showed similar results, with a larger amount of Ca²⁺ extracted after 30 s of acid etching at 25 µm than at 100 µm in the 38% H₂O₂ group.

Acid etching time significantly influenced the amount of Ca²⁺ extracted for all treatment groups and depth levels. Thus, significantly more Ca²⁺ was obtained after 60 s than after 30 s immersion in phosphoric acid, except at a depth of 25 µm in the 38% H₂O₂ group and at depths of 25 and 50 µm in the 30% H₂O₂ group, where no significant difference in Ca²⁺ extraction was observed between 30 and 60 s of acid etching.

Discussion

Bleaching agents usually contain either H₂O₂ or CP. The concentrations and conditions of vital tooth bleaching systems vary considerably. In the present study, we used 30% H₂O₂ for 60 min and 38% H₂O₂ for 20 min in a single application on the enamel of bovine teeth. Bovine enamel was used in this study because it has been proposed as a reliable substitute for human teeth.
in research. SEM observations revealed the similar morphology of bovine and human teeth and of their enamel after etching with 35% H₃PO₄. Although bovine enamel undergoes faster demineralization in comparison to human enamel, and suffers greater erosion and erosion-abrasion, it can be accepted as a suitable substitute to evaluate enamel demineralization.

The repercussions of bleaching agents on the structure of hard dental tissues remain controversial. It has been reported that even over-bleaching produced no changes in observed subsurface enamel or dentin ultrastructure or architecture. Conversely, it has been reported that continued bleaching caused a progressive demineralization of enamel and the concomitant enamel matrix degradation of a layer that is probably only a few microns deep. Many H₂O₂ bleaching systems are formulated at extremely low pHs (often below 4) to ensure stability. The combination of low pH and high peroxide levels might be expected to affect the surface and subsurface integrity of dental tissue and react with tooth mineral substrates including both surface and subsurface structures. These deleterious effects are not evident when the pH is above 5.5, which can be considered a critical cut-off point. The pH of both agents used is above this point.

In a recent study, a larger amount of Ca²⁺ was extracted from enamel by phosphoric acid after the application of 30% CP for 90 min, contrasting with the present finding of no significant differences in the global amount of Ca²⁺ extracted between bleached and unbleached specimens. However, although we used high concentrations, they were applied on the enamel for a short time. Bistey et al. and Lee et al. reported that bleaching-induced structural changes to surface enamel were proportionally more severe with longer treatment times and higher H₂O₂ concentrations. An increase in the quantity of ions released was also reported with higher H₂O₂ concentrations. It is therefore necessary to explore the amount of Ca²⁺ extracted by phosphoric acid after the prolonged application of H₂O₂.

H₂O₂ has a high capacity for diffusion, and it is known that post-bleaching intervals will influence the amount of Ca²⁺ extracted by phosphoric acid. In a previous study, De Medeiros et al. found no significant difference in Ca²⁺ loss between an unbleached control group and a group etched immediately after bleaching, whereas significantly more Ca²⁺ was extracted from the bleached group at 24 h after bleaching, explaining our choice of this interval for our experiment.

The acid etching time significantly influenced the amount of Ca²⁺ extracted for all treatment groups and depths. Thus, significantly more Ca²⁺ was obtained after 60 s than after 30 s of immersion in phosphoric acid, although this difference was not evident at an enamel depth of 25 μm.

In the unbleached control group and the group bleached with 30% H₂O₂, the amount of Ca²⁺ extracted by phosphoric acid was the same at all four enamel depths studied. It should be borne in mind that the manufacturer’s label describes this product as 30% H₂O₂, but the mixed gel has a concentration of only 15% (manufacturer’s information). We emphasize that Ca²⁺ extraction was higher at 25 and at 100 μm (although without significant differences). This could be explained by the enamel microstructure, where Retzius lines have a regular periodicity (of around 30 μm), alternating light and dark bands and with a lower crystal concentration in the dark bands.

The amount of Ca²⁺ extracted at 25 μm was only significantly higher than that obtained at 100 μm depth when 35% H₂O₂ was applied. It can be speculated that there may be a critical point at which there is a correlation among final H₂O₂ concentration, application time, and delay time (post-bleaching period). Our results confirm
the possible decalcifying effect of H$_2$O$_2$, because a higher Ca$^{2+}$ loss was detected at a depth of 25 µm when a high H$_2$O$_2$ concentration was applied for a short time. Enamel demineralization of 50 µm$^2$1 and 150 µm$^2$2 has been reported with the use of low and high H$_2$O$_2$ concentrations, respectively. In a Raman spectroscopy study, Götze et al.[6] found no alterations of phosphate bands, which were similar to synthetic pure hydroxyapatite after the chemical removal (by acetone or hydrazine extraction) of the organic constituents. Peroxides are known to produce the whitening effect by destroying one or more of the double bonds in the conjugated chain of chromophores. It is possible that peroxides only affect the organic matrix of enamel, facilitating the detachment of calcium phosphates and their extraction by phosphoric acid. Raman spectroscopy and infrared spectroscopy can determine qualitative changes due to modifications in bonds of functional groups but do not quantify mineral losses, whereas Ca$^{2+}$ losses can be measured using spectroscopy. An absence of alterations in hydroxyapatite composition may be compatible with a greater release of Ca$^{2+}$, since more unaltered hydroxyapatite may become detached if peroxides affect the organic matrix. It is therefore of interest to study changes in bands corresponding to amides in order to detect whether alterations are produced in the enamel organic matrix. McCracken et al.[25] used atomic absorption spectroscopy and demonstrated that CP has an intrinsic demineralizing effect. Carboxyl peroxide causes local microstructural and chemical changes in enamel that are similar to those of initial caries. Electron probe microanalysis showed reduced concentrations of Ca and P and a lower Ca/P ratio. Some Ca and P were also reported in the bleaching gel after use.[27] The null hypothesis of our study must be partially accepted. The amount of Ca$^{2+}$ extracted by phosphoric acid from groups bleached with 30% and 38% H$_2$O$_2$ for a short application time is the same as that extracted from unbleached teeth. Previous whitening with 30% H$_2$O$_2$ does not influence the amount of Ca$^{2+}$ extracted by phosphoric acid at the four enamel depth levels studied (from 0 to 100 µm). A significantly higher calcium extraction was only observed at a depth of 25 µm when 38% H$_2$O$_2$ was used.

CONCLUSIONS

Within the limitations of our in vitro study and taking due caution in extrapolating results from bovine to human teeth, we can conclude that pre-bleaching with 38% H$_2$O$_2$ significantly increases the decalcifying effect of phosphoric acid on subsurface enamel at a depth of 25 µm compared to 100 µm, whereas pre-bleaching with 30% H$_2$O$_2$ does not modify this effect at any level.

REFERENCES


Clinical relevance: 38% H2O2 increases the decarboxylating capacity of phosphoric acid at the subsurface level.