

# In vitro evaluation of calcium and phosphorus concentrations in enamel submitted to an in-office bleaching gel treatment containing calcium

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The aim of this in vitro study was to evaluate the calcium and phosphorus concentrations in enamel surfaces before, during, and after treatment with in-office 35% hydrogen peroxide bleaching agents with 2% calcium gluconate (WCa) or without calcium gluconate (W). Twenty sound human third molars were divided into 2 groups of 10. The bleaching agents were applied to the tooth surfaces in accordance with the manufacturer's instructions: WCa, 40 minutes per day at 3 sessions with 7-day intervals; W, 3 × 15 minutes per day at 3 sessions with 7-day intervals. Enamel microbiopsies were performed prior to the bleaching treatment, immediately after each bleaching session (first, second, and third applications), and 7 and 14 days following the last bleaching treatment. The concentration levels of calcium and phosphorus in the microbiopsy specimens were recorded spectrophotometrically. There was a statistically significant decrease in the calcium concentration 7 days after the last bleaching treatment, but there was a recovery to baseline values at

14 days, regardless of the bleaching agent used (WCa and W). When W was used, there was no difference in the phosphorus concentration over time. The phosphorus concentration in the WCa group decreased after the third application, showing a significant difference from the W group at this time. However, an increase in the phosphorus concentration was observed in the posttreatment period, and no significant differences were observed between values at baseline and those at 14 days posttreatment. The in-office bleaching gel containing 2% calcium gluconate did not affect the calcium and phosphorus concentrations in enamel as compared to a calcium-free bleaching agent.

Received: September 5, 2014

Accepted: December 22, 2014

Key words: calcium, enamel, hydrogen peroxide, phosphorus, tooth bleaching

Products containing hydrogen peroxide (HP) in concentrations of 35% to 40% have been used in in-office dental bleaching techniques to gain faster initial results and compliance of patients who do not tolerate the use of trays.<sup>1</sup> This technique is effective and allows total control of product application on the teeth by the professional.<sup>2-4</sup> It also has long-lasting results.<sup>5,6</sup>

Some bleaching products have low pH, leading to mineral loss or a decrease in enamel microhardness over time.<sup>7-13</sup> The addition of calcium and/or fluoride to bleaching gels could be beneficial as compensation for the increased susceptibility of enamel to demineralization or loss of mineral content after use of agents with high concentrations of HP.<sup>8,14</sup> Borges et al observed that the addition of 2% calcium gluconate to hydrogen peroxide gel reduced the susceptibility of enamel to erosion.<sup>15</sup> Moreover, a significant increase in enamel microhardness was found after extracted molars were bleached with a 35% hydrogen peroxide agent containing calcium and fluoride.<sup>16</sup> In contrast, de Oliveira et al showed no significant increase in the microhardness of bleached enamel when using a 10% home-use

carbamide peroxide gel to which calcium and fluoride were added.<sup>17</sup> Berger et al observed that the addition of amorphous calcium phosphate or calcium to bleaching agents did not offset the demineralizing effects caused by bleaching treatments on enamel.<sup>12</sup> According to da Costa Soares et al, in-office bleaching treatments, whether with or without calcium, caused a decrease in enamel microhardness.<sup>14</sup> However, none of the aforementioned studies quantified the calcium and phosphorus content in enamel before, during, or after bleaching with in-office calcium-containing bleaching agents.

Brudevold et al proposed a methodology to monitor calcium and phosphorus in enamel, the enamel microbiopsy.<sup>18</sup> This method was recently used by do Amaral et al to determine in vivo the concentration of calcium and phosphorus in enamel during and after treatment with home-use (10% and 20% carbamide peroxide gels) and in-office (35% and 38% HP) dental bleaching agents, showing that these bleaching gels did not alter the inorganic composition of the enamel.<sup>19</sup> According to the researchers, this method may be more precise than the indirect evaluation of mineral content by

microhardness evaluation and, because there is no need for specimen preparation, could be useful for experiments in which the teeth are constantly submitted to remineralizing-demineralizing conditions and bleaching treatment cycles.<sup>19</sup>

Therefore, the objective of this in vitro study was to evaluate the calcium and phosphorus concentration levels in enamel before, during, and after treatment with a 35% HP in-office bleaching gel with or without the addition of calcium. The null hypothesis tested was that a 35% HP in-office bleaching gel treatment containing calcium would not change the calcium and phosphorus concentration levels in enamel.

## Materials and methods

### Experimental design

The factors under study were *treatment agents* (35% HP agent without calcium [W] and 35% HP with a calcium agent [WCa]); and *microbiopsy evaluation periods* (before treatment agent application [baseline], after the first, second, and third bleaching gel applications, and following the bleaching treatment at 7 and 14 days). The sample consisted of 20 sound human third molars randomly

**Table 1. Bleaching agents and artificial saliva used in the experiment.**

Treatment group	Treatment agent	Composition	Protocol for use
W	35% HP bleaching agent (Whiteness HP Maxx, FGM Produtos Odontologicos)	35% HP, thickening agents, dyes, glycol, inorganic filler, and deionized water	The HP and thickening agent-containing liquids were mixed in a plastic container before use in a ratio of 3 drops of HP to 1 drop of thickening agent to obtain gel consistency. The gel was applied to the tooth surface. The gel was removed with gauze after 15 minutes. This procedure was performed 3 times at each session.
WCa	35% HP bleaching agent with calcium (Whiteness HP Blue Calcium, FGM Produtos Odontologicos)	35% HP, thickening agents, violet dye, neutralizing agents, 2% calcium gluconate, glycol, and deionized water	The HP and the chemical activator-containing syringes were attached and shaken back and forth 8 times to mix the solution thoroughly. The gel was applied to the tooth surface. The gel was removed with gauze after 40 minutes.
	Artificial saliva <sup>20,21</sup>	1.5 mmol/L calcium, 50 mmol/L potassium chloride, 0.9 mmol/L phosphate, 20 mmol/L Tris buffer (pH = 7)	The teeth were immersed in the solution.

Abbreviations: HP, hydrogen peroxide; W, without calcium gluconate; WCa, with calcium gluconate.

**Table 2. pH values of the bleaching agents at different time periods.**

Bleaching agent	Baseline	7 Min	15 Min	30 Min	40 Min
W	5.3	4.5	4.5	NA	NA
WCa	8.0	7.9	7.9	7.8	7.8

Abbreviations: NA, not applicable; W, without calcium gluconate; WCa, with calcium gluconate.

assigned to 2 groups (n = 10) according to the treatment agent under study. A spectrophotometer was used to determine the calcium and phosphorus concentration levels in milligrams per milliliter after the microbiopsy technique was performed.

The research ethics committee of the Sao Leopoldo Mandic Institute Dental School and Research Center, Campinas, Brazil, approved the study protocol (No. 2012/0364).

### **Tooth selection and preparation**

Twenty recently extracted human impacted third molars were selected and stored in an aqueous solution of 0.1% thymol. The teeth were examined under a stereomicroscope (EK3ST, Eikonol do Brazil) at 40× magnification, and any teeth presenting cracks or structural anomalies were discarded. Calculus and soft tissue deposits were removed with a hand scaler. The teeth were cleaned with a rubber cup and fine pumice water slurry.

Each tooth was fixed individually in a cylinder so that its roots were embedded in acrylic resin (VipiFlash, VIPI Produtos Odontologicos) up to 2 mm from the cemento-enamel junction. Each tooth was positioned so that its long axis was parallel to the horizontal plane.

After curing of the resin, the teeth were randomly separated into 2 groups (n = 10). The teeth were kept immersed individually in artificial saliva (20 mL) in an oven (Odontobras) at 37°C for 7 days before the bleaching treatment began. The artificial saliva solution was changed every 2 days.

### **Bleaching treatment**

Table 1 describes the bleaching agents and artificial saliva used in the study and their protocols for use. After the W and WCa agents were prepared according to the manufacturer's instructions, a thin layer of the gel (approximately 1 mm) was applied to the buccal, lingual, and

proximal surfaces of the teeth. The gel was agitated periodically to remove the air bubbles that formed during the procedure. No heat or special lamps were involved in completing the process.

After removal of the gel, each surface was washed with distilled and deionized water for 10 seconds. Between the enamel microbiopsies (described in the next section), the teeth were kept in containers individually with artificial saliva and stored in an oven at 37°C. This protocol was performed in 1 session per week for 3 weeks (corresponding to 21 days of treatment).

The pH values of the bleaching materials were measured at different time periods immediately after the gel was prepared (Table 2). A pH meter (MPA 210, MS Tecnopon Equipamentos Especiais Ltda.) was used after calibration of the equipment with buffer solutions of pH 7.0 and 4.0.

Following the 21-day treatment period, the teeth from both groups were stored in individual containers and immersed in artificial saliva in an oven at 37°C. The artificial saliva solution was changed every 2 days during and after all the treatment agent applications.

### **Enamel microbiopsies**

To determine the concentration of calcium and phosphorus, a specimen of dental enamel was obtained via a technique called *enamel microbiopsy*.<sup>18</sup> The enamel

microbiopsies were performed at baseline; after the first, second, and third bleaching gel applications; and 7 and 14 days after the bleaching treatment.

To obtain the specimen, first a piece of adhesive tape (Scotch Premium Electrical Tape, 3M ESPE) with a circular perforation (1.6 mm in diameter) was placed firmly on the surface of the tooth to demarcate the biopsy site. A different site of the enamel surface was used to obtain the specimen for the microbiopsy at each test time. Then a pipette tip was used to apply 5 µL of 1.6 mol/L of hydrochloric acid in 70% glycerol (vol/vol) to this area for 20 seconds. The specimen was transferred to a tube (Safe-Lock Eppendorf Tubes, Eppendorf North America) already containing 200 µL of ultrapurified water. The surface was rinsed once with 5 µL of 70% glycerol for 10 seconds, and the specimen was again transferred to the same centrifuge tube. Last, the tape was removed from the tooth, and the tooth was washed with water for 30 seconds, dried with an air spray, and returned to storage in artificial saliva.

**Chemical analysis**

Since the chemical analyses were not scheduled to be performed immediately, the specimens were frozen until use to avoid evaporation and loss of specimen volume. At the time of analysis, the specimens were defrosted and vortexed, and half of the specimen volume was used for calcium analysis and the other half for phosphorus analysis.<sup>19</sup>

Phosphorus concentration levels were measured according to the method proposed by Fiske & Subbarow.<sup>22</sup> Each specimen was assayed in triplicate, and the loaded reactions were read in a spectrophotometer plate (ELX800UV, Universal Microplate Reader, BioTek Instruments, Inc.). Reaction mixtures consisted of 30 µL of the specimen, 200 µL of ultrapurified water, and 50 µL of molybdic acid solution (ammonium molybdate at 2.5% [wt/vol] in 4 N sulfuric acid), which were vortexed thoroughly. After 10 minutes, 20 µL of reducing agent was added and the mixture was vortexed again. After 20 minutes, the absorbance was measured at 630 nm. The plate reader was calibrated with standards specifying the known phosphorus concentrations (1, 2, 4, and 8 mg/mL).<sup>19</sup>

**Table 3. Mean (SD) calcium concentrations (µg/mL) for enamel specimens following treatment with bleaching agents.**

Timepoint	Bleaching agent	
	W	WCa
Baseline	7.74 (3.68) <sup>Aa</sup>	7.29 (3.90) <sup>Aa</sup>
After first application	7.50 (2.00) <sup>Aa</sup>	7.84 (1.95) <sup>Aa</sup>
After second application	7.85 (7.25) <sup>Aa</sup>	8.26 (4.94) <sup>Aa</sup>
After third application	7.27 (1.95) <sup>Aa</sup>	6.95 (1.27) <sup>Aa</sup>
7 D posttreatment	2.75 (1.00) <sup>Ab</sup>	2.73 (1.71) <sup>Ab</sup>
14 D posttreatment	5.04 (2.28) <sup>Abb</sup>	5.65 (1.97) <sup>Abb</sup>

Abbreviations: W, without calcium gluconate; WCa, with calcium gluconate. Means followed by the same uppercase letter are not significantly different from each other within the same row. Means followed by the same lowercase letter are not significantly different from each other within the same column.

Calcium concentrations were measured by colorimetric reagent arsenazo end point analysis, in which arsenazo, in the presence of calcium ions in an acidic pH environment, yields a colored complex whose color intensity is directly proportional to the calcium concentration in the tested specimen. Reaction mixtures consisted of 10 µL of the specimen and 200 µL of Calcium-Arsenazo III reagent (K051, Bioclin/Quibasa), which were vortexed and incubated at 37°C for 2 minutes. Next, the absorbance was measured at 630 nm in a spectrophotometer.<sup>19</sup>

**Statistical analysis**

After exploratory analysis was performed, the data were analyzed with a mixed model procedure for repeated measurements and with the Tukey-Kramer test, with a 5% level of significance (SAS software version 8.2, SAS Institute, Inc.).

**Results**

Tables 3 and 4 show the mean calcium and phosphorus concentration levels for each treatment agent at each timepoint. There was a significant decrease in the calcium concentration of the enamel specimens 7 days after the last bleaching treatment, but there was a recovery to the baseline values 14 days after treatment. No differences were found between the two bleaching agents throughout the entire period.

The enamel treated with W showed no differences in phosphorus concentration at any time. However, the phosphorus

concentration for WCa specimens decreased after the third application, showing a statistically significant difference from the W specimens at this time. However, an increase in the phosphorus concentration was observed in the post-treatment period, and no statistically significant differences were observed for WCa between the baseline values and those found 14 days after treatment.

**Discussion**

Adverse effects of in-office bleaching agents on enamel are mainly attributed to the contact time of the gels with the dental surface, the pH of the bleaching gel, and the peroxide concentrations.<sup>8,9,23,24</sup> Bleaching agents with low pH help keep HP stable and facilitate the bleaching process.<sup>25</sup> Therefore, it is common for some bleaching products containing a high concentration of HP (ranging from 35%-40%), used as in-office agents, to have a low pH.<sup>9</sup>

In this study, the bleaching agents presented different protocols for use (different durations of application at each session and different manipulation of the gels) and did or did not contain 2% calcium gluconate. The agents applied in the present study also presented different pH values. The agent W showed lower pH values (ranging from 5.3-4.5) than did WCa (ranging from 8.0-7.8). Nevertheless, no differences were observed in the enamel calcium concentration levels of the two agents during the bleaching treatments. Other studies have

**Table 4. Mean (SD) phosphorus concentrations ( $\mu\text{g/mL}$ ) for enamel specimens following treatment with bleaching agents.**

Timepoint	Bleaching agent	
	W	WCa
Baseline	1.66 (1.05) <sup>Aa</sup>	1.58 (0.49) <sup>Aab</sup>
After first application	2.06 (0.40) <sup>Aa</sup>	1.81 (0.35) <sup>Aab</sup>
After second application	2.01 (0.42) <sup>Aa</sup>	2.34 (0.70) <sup>Aa</sup>
After third application	1.74 (0.31) <sup>Aa</sup>	0.58 (0.78) <sup>Bc</sup>
7 D posttreatment	1.53 (0.42) <sup>Aa</sup>	1.53 (0.41) <sup>Ab</sup>
14 D posttreatment	1.84 (0.32) <sup>Aa</sup>	1.82 (0.27) <sup>Aab</sup>

Abbreviations: W, without calcium gluconate; WCa, with calcium gluconate. Means followed by the same uppercase letter are not significantly different from each other within the same row. Means followed by the same lowercase letter are not significantly different from each other within the same column.

reported surface alterations even with low-concentration and/or nonacidic bleaching agents.<sup>9,26</sup> However, in this study, the calcium concentration was not affected by the bleaching treatment, regardless of the application time, presence of calcium gluconate, or pH value of the gels.

The role of saliva in preventing demineralization caused by bleaching gels was shown by Justino et al, who reported that the amount of calcium on the tooth was highest on the first day of bleaching, in vitro and in situ.<sup>27</sup> In the present study, microbiopsies were taken immediately after the gel application, precluding any effect of saliva on enamel; nevertheless, there were no effects of the acquired pellicle on the enamel surface. However, during posttreatment evaluations, a decrease in the calcium concentration was observed 7 days posttreatment in both groups, and a decrease in phosphorus concentration was observed after the third application of WCa on enamel, despite the remineralizing effect of the artificial saliva.<sup>13,20,21</sup> Nevertheless, the calcium and phosphorus concentration levels 14 days posttreatment showed no difference from the baseline values in either group.

It is possible that bleaching gels containing remineralizing components could act as remineralizing agents, minimizing deleterious effects on enamel.<sup>16,28,29</sup> When a bleaching gel containing 2% calcium gluconate was studied, the remineralizing agent did not interfere with the whitening effect and offered similar bleaching outcomes.<sup>30</sup> It would be expected that

the saturation of calcium in this bleaching agent would allow the calcium to be deposited on the enamel surface or incorporated in the enamel apatite, thus increasing resistance to demineralization, as demonstrated by Borges et al and Cavalli et al, who evaluated bleaching agents containing calcium chloride.<sup>16,28</sup> Their results corroborated those of da Costa Soares et al, who used scanning electron microscopic analysis to show deposits of calcium on the surface of enamel bleached with WCa.<sup>14</sup> Borovskii & Agafonov also showed that a 10% calcium gluconate solution promoted maturation of the enamel sooner than would have occurred naturally and improved the caries resistance of the enamel.<sup>31</sup> However, an in situ study performed by Schirrmeister et al showed that chewing gums with and without calcium—in the form of dicalcium phosphate, calcium gluconate, calcium lactate, or casein phosphopeptides—amorphous calcium phosphate—have no significant effect on the remineralization of initial carious lesions.<sup>32</sup>

The manufacturer of Whiteness HP Blue Calcium has added calcium gluconate in powder form to the thickening phase of the WCa gel, in a concentration of 2%, which does not impair the gel thickness.<sup>15</sup> However, no increase in the enamel calcium concentration was observed in the present study, insofar as no difference in calcium concentrations between specimens treated with WCa and those treated with W (calcium-free) bleaching gel was

recorded. Moreover, a decrease in the phosphorus concentration level of WCa-treated specimens was observed after the third bleaching application.

Calcium gluconate is soluble in water and insoluble in alcohol and organic solvents.<sup>33,34</sup> Since the solubility of a substance fundamentally depends on the physical and chemical properties of the solute and solvent, as well as on the temperature, pressure, and pH of the solution, calcium gluconate was probably not released or did not dissolve in the thickener. In addition, calcium gluconate is incompatible with strong oxidizing agents, and its addition to a bleaching gel may make calcium gluconate incompatible with oxidizing by-products, such as free oxygen ( $\text{O}^\cdot$ ) and hydroperoxyl ( $\text{HO}_2^\cdot$ ), that are released during hydrogen peroxide degradation.<sup>35-37</sup> Therefore, calcium gluconate may not be released during bleaching decomposition and, in the present study, showed no beneficial or expected effects on the enamel mineral content. Further studies are needed to evaluate any potential advantages of adding calcium gluconate to bleaching agents.

## Conclusion

Calcium and phosphorus concentration levels in the enamel surfaces of extracted teeth subjected to bleaching treatments with 35% HP containing 2% calcium gluconate were not significantly different from concentrations in teeth exposed to 35% HP without calcium gluconate. Therefore, the null hypothesis of this study was accepted. Although a statistically significant decrease in the phosphorus concentration in the WCa group was observed after the third application, the baseline values were recovered after storage in artificial saliva during the postbleaching period.

## Disclaimer

The authors have no financial, economic, commercial, or professional interests related to topics presented in this article.

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## Manufacturers

Bioclin/Quibasa, Belo Horizonte, Brazil  
55.31.3439.5454. [www.bioclin.com.br](http://www.bioclin.com.br)

BioTek Instruments, Inc., Winooski, VT  
888.451.5171, [www.biotek.com](http://www.biotek.com)

Eikonal do Brazil, Sao Paulo, Brazil  
55.11.3023.1000, [www.eikonal.ind.br](http://www.eikonal.ind.br)

Eppendorf North America, Hauppauge, NY  
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FGM Produtos Odontologicos, Joinville, Brazil  
55.47.3441.6100, [www.fgm.ind.br](http://www.fgm.ind.br)

MS TecnoPON Equipamentos Especiais Ltda., Piracicaba, Brazil  
55.19.3434.1418, [www.tecnoPON.com.br](http://www.tecnoPON.com.br)

Odontobras, Ribeirao Preto, Brazil  
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SAS Institute, Inc., Cary, NC  
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