UNIVERSIDADE FEDERAL DE UBERLÂNDIA

FACULDADE DE ODONTOLOGIA

MARIA DE LARA ARAÚJO RODRIGUES

USE OF COMPUTERIZED MICROTOMOGRAPHY, ENERGY DISPERSIVE SPECTROSCOPY, SCANNING ELECTRON MICROSCOPY, AND ATOMIC FORCE MICROSCOPY TO MONITOR EFFECTS OF ADDING CALCIUM TO BLEACHING GELS.

USO DE MICROTOMOGRAFIA COMPUTADORIZADA, ESPECTROSCOPIA DISPERSIVA DE ENERGIA, MICROSCOPIA ELETRÔNICA DE VARREDURA E MICROSCOPIA DE FORÇA ATÔMICA PARA MONITORAR OS EFEITOS DE ADIÇÃO DE CÁLCIO À GÉIS CLAREADORES.

UBERLÂNDIA, 2021

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Trabalho de conclusão de curso apresentado à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito parcial para obtenção do título de Graduado em Odontologia

Orientador: Prof. Dr. Carlos José Soares

Co-orientadora: Profª. Msª. Ludmila Cavalcanti de Mendonça

UBERLÂNDIA, 2021

Dedico este trabalho

A Deus,

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À Faculdade de Odontologia da Universidade Federal de Uberlândia

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"Se cheguei até aqui foi porque me apoiei no ombro dos gigantes."

(Isaac Newton)

SUMMARY

RESUMO:

O objetivo deste estudo foi avaliar a perda de conteúdo mineral, expressa por cálcio (Ca) e fosfato (P), no esmalte dental exposto a agentes clareadores por meio de microtomografia computadorizada (micro-CT), microscopia eletrônica de varredura (MEV), espectroscopia de energia dispersiva (EDS) e microscopia de força atômica (AFM). Sessenta espécimes de esmalte dentário bovino foram divididos aleatoriamente em três grupos (n = 20): HP35ca (clareado com peróxido de hidrogênio a 35% com Ca); HP35wca (clareado com peróxido de hidrogênio 35% sem Ca); e controle (sem clareamento). Cinco amostras de cada grupo foram usadas para análises MEV e EDS, 10 amostras foram usadas para análise de AFM e as cinco amostras restantes foram usadas para análise de micro-CT. O pH dos géis foi medido usando um medidor de pH. Os dados de EDS e micro-CT foram analisados usando ANOVA de uma via e teste de correlação de Pearson. Os dados de AFM foram analisados usando ANOVA de uma via ($\alpha = 0,05$). As porcentagens em peso de Ca e P obtidas por EDS foram semelhantes entre os grupos clareado e controle. Pequenas mudanças superficiais foram observadas por MEV no grupo HP35wca. O grupo HP35ca apresentou padrões semelhantes ao grupo controle. Os resultados de AFM não mostraram alterações significativas na rugosidade do esmalte em nenhum dos grupos testados. Nenhuma diferença significativa no volume ou profundidade da perda estrutural de esmalte foi encontrada entre os géis com e sem Ca. Nenhuma perda mineral foi observada no substrato dentinário. Os dados de análise de EDS e micro-CT exibiram uma alta correlação ($P < 0,001$). A adição de Ca ao gel clareador não teve efeito benéfico sobre o esmalte clareado do dente em termos de composição, perda mineral e rugosidade superficial. Os resultados de micro-CT exibiram uma alta correlação com os resultados de EDS.

Palavras-chaves: Clareamento dental; esmalte dentário; peróxido de hidrogênio; perda mineral.

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Use of computerized microtomography, energy dispersive spectroscopy, scanning electron microscopy and atomic force microscopy to monitor effects of adding calcium to bleaching gels.

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Running title: Monitoring the effect of calcium addition to bleaching gels

Keywords: Tooth bleaching; mineral loss; enamel; White Spot Injuries.

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Use of computerized microtomography, energy dispersive spectroscopy, scanning electron microscopy, and atomic force microscopy to monitor effects of adding calcium to bleaching gels.

CLINICAL RELEVANCE

Bleaching teeth with hydrogen peroxide gels containing calcium could not prevent mineral loss at the enamel surface. However, the demineralized regions did not exhibit an increase in surface roughness.

SUMMARY

Objectives: The aim of this study was to evaluate the loss of mineral content, expressed by calcium (Ca) and phosphate (P), in dental enamel exposed to bleaching agents using micro-computed tomography (micro-CT), scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), and atomic force microscopy (AFM).

Methods: Sixty bovine dental enamel specimens were randomly divided into three groups (n=20): HP35ca (bleached with 35% hydrogen peroxide with Ca); HP35wca (bleached with 35% hydrogen peroxide without Ca); and control (without bleaching). Five specimens from each group were used for SEM and EDS analyses, 10 specimens were used for AFM analysis, and the remaining five specimens were used for micro-CT analysis. The pH of the gels was measured using a pH meter. The EDS and micro-CT data were analyzed using one-way ANOVA and Pearson's correlation test. The AFM data were analyzed using one-way ANOVA (α=0.05).

Results: The weight percentages of Ca and P obtained using EDS were similar between the bleached and control groups. Small superficial changes were observed by SEM in the HP35wca group. The HP35ca group showed similar patterns to the control group. AFM results showed no significant changes in the enamel roughness in any of the tested groups. No significant difference in the volume or depth of structural enamel loss was found between gels with and without Ca. No mineral loss was observed in the dentin substrate. The EDS and micro-CT analysis data exhibited a high correlation ($P < 0.001$).

Conclusion: The addition of Ca to the bleaching gel had no beneficial effect on the bleached tooth enamel in terms of composition, mineral loss, and surface roughness. Micro-CT results exhibited a high correlation with the EDS results.

INTRODUCTION

Chromatic alterations in teeth compromise the esthetics of the smile, adversely affecting the social and emotional behavior of patients.[1] Bleaching procedures are the preferred method to treat tooth discoloration because they involve a simple and minimally invasive protocol.[1] Although there have been many studies on bleaching treatments, the performance of bleaching agents has not been fully demonstrated.[1]

Hydrogen peroxide is the most commonly used bleaching agent.[1] Different theories have been proposed to explain its bleaching mechanism, which involves the penetration of hydrogen peroxide and its decomposition into oxygen molecules capable of breaking down pigment macromolecules. However, studies have claimed that free radicals released by hydrogen peroxide are unstable and unspecific and react with the inorganic enamel matrix in addition to the pigment organic molecules.[2-5] Hydrogen peroxide can diffuse through the tooth enamel and dentin, releasing free radicals that oxidize the chromophores of molecules.[6,7] These chromophores, rich in electrons that absorb specific wavelengths of visible light, break down when attacked by free radicals.[8,9] Free radicals attack the double bonds responsible for the color of chromophores, thus making the teeth appear lighter in color. [8,9] Another theory suggested that peroxide causes minor morphological changes in the enamel that increases its opacity due to the dispersion of light and hides the underlying dentin layer.[10-13] Whitening agents can also function by oxidizing the fluorescent components in dentin, such as dentin phosphoproteins. Hydrogen peroxide can whiten the dentin by oxidizing the aromatic amino acids in the dentin phosphoprotein.[14]

Several studies claim that bleaching is a completely safe procedure.[15-17] However, enamel demineralization upon bleaching can cause alterations in the tooth such as an increase in roughness, reduction in microhardness, and changes in the superficial micromorphology.[18-22] To prevent demineralization (especially the loss of calcium and phosphate ions) and reduction of enamel hardness during tooth bleaching, [23] calcium and fluoride are added to the gel composition.[24] A significant increase in enamel permeability and roughness and a decrease in microhardness compared to the untreated control group have been reported after bleaching with 35% hydrogen peroxide gel with Ca or fluoride.[21] Bleaching with 10% carbamide peroxide showed that the enamel was susceptible to

mineral loss during the whitening treatment, but this loss was minimized by the addition of F and Ca to the whitening agents.[24]

Different methods have been used to evaluate the changes that occur after tooth enamel bleaching, including quantitative tests to assess the changes in physical properties and mineral composition via biochemical measurements and qualitative evaluations using different imaging techniques.[19,22,25-28] However, micro-computed tomography is of particular interest to researchers because it can quantify the enamel loss at the surface as well as the subsurface.[29]

The clinical relevance of the undesirable effects of dental bleaching on tooth structures is yet to be addressed.[15-22] The methods used for analyzing enamel mineral loss after bleaching are important to confirm the effect of bleaching, to obtain new information, and to determine the association between different and complementary methods. Therefore, the aim of this study was to evaluate the mineral loss in dental enamel exposed to bleaching agents by micro-computed tomography (micro-CT), scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), and atomic force microscopy (AFM). The following hypotheses were tested: 1) micro-CT can be used as an alternative method to determine the loss of enamel and dentin structures; 2) the presence of calcium in the bleaching gel can reduce enamel mineral loss and retain the surface roughness; 3) the enamel mineral loss occurs primarily at the surface of the enamel structure.

METHODS AND MATERIALS

Preparation of specimens - Bleaching Procedures

Incisor teeth of bovine animals of equal ages were extracted immediately after sacrificing the animals. The teeth were stored in distilled water at -10 °C for a maximum of 30 d. Specimens of dimensions 5 mm × 5 mm × 4 mm (approximately 1.5 mm - enamel and 2.5 mm – dentin) were obtained from the central region of the buccal surface of each tooth using a water-cooled low-speed diamond saw (Buehler Ltd, Lake Bluff, IL, USA). The specimens were randomly divided into three groups (N=20): HP35ca [bleached with 35% hydrogen peroxide with Ca ions (Whiteness HP Blue Calcium - FGM, Joinvile,

Brazil)], HP35wca [bleached with 35% hydrogen peroxide without Ca ions (Whiteness HP Maxx - FGM, Joinvile, Brazil)], and control (without the application of a bleaching gel). Five specimens from each group were used for SEM and EDS analyses, 10 specimens for AFM analysis, and the remaining five specimens for micro-CT analysis. For the micro-CT and AFM analyses, the specimens from the bleached groups were analyzed before and after the bleaching procedures, while the specimens from the control group were analyzed before and after immersion in artificial saliva. The tooth enamel surfaces of the specimens were regularized using 600-, 1000-, 1200-, and 1500-grit abrasive papers (Arotec, Cotia, SP, Brazil) and polished with a polishing cloth and 6-, 3-, 1-, and 0.5-μm diamond pastes (Arotec) in a polishing machine (Arotec) to standardize the surface. The lateral and bottom surfaces were covered with nail polish to isolate the contact of the products only to the sample buccal surface during the treatments (Rísque, SP Brazil). The HP35wca group was treated for two 40 min sessions, with 7 days of interval. The HP35ca group was treated for two 45 min sessions, with three applications every 15 min per session; there was also a 7-days interval between the sessions. All bleaching procedures were performed according to the manufacturer's instructions (Table 1). The specimens were rinsed with distilled water spray after each session and then immersed in artificial saliva at 37 °C until the next application of the whitening gel. After the last session of each group, the specimens were rinsed and stored in distilled water. The samples were ultrasonicated in distilled water for 10 min before all tests. The debris layer produced during specimen preparation was not intentionally removed before analyses.

Micro-CT analysis

Herein, micro-CT, which is a 3D imaging technique that utilizes X-rays to see inside a sample, slice by slice, was conducted (Nrecom software version 1.6.10.1; DataViewer software version 1.5.1.2; CTAn, version 1.13; CTVol, version 2.0; SkyScan Bruker Belgium). In the reconstructed image, the internal structure of the sample can be analyzed. The reconstructed images were then overlaid using the DataViewer software (version 1.5.1.2, SkyScan, Bruker, Belgium). Comprehensive 3D image analysis capability of techniques such as morphometry, densitometry, segmentation, and advanced image processing methods allowed the quantification of mineral loss at the surface and subsurface. [30]

The bleached tooth analysis methodology was based on a previous study that conducted micro-CT to evaluate the cusp deformation produced by resin composite restorations. The images of the prepared tooth (reference) and the image of the restored tooth (target) were overlaid, generating a difference in the volume of the image (Diff). This Diff image represents the volume of cusp deformation caused by the polymerization contraction of the composite resin restoration with high resolution. [30] Similar protocol was used in the present study to determine the structural alterations produced by the bleaching process.

In this study, the samples (n = 5) were scanned using a high-resolution micro-CT instrument with a resolution of 0.35 μ (Bruker, Kontich, Belgium). The device was set to the following configuration: 100 kV and 100 mA, a 0.11 mm Cu filter, an image pixel size of 13 μm, a resolution of 1632 × 1092, and a 0.6° rotation pitch, and generated three image slices over 1850 ms with 20 random movements, resulting in 1692 image slices. NRecon® software (Bruker) was used to reconstruct the images by adjusting to the appropriate parameters for smoothing and beam-hardening artifact correction. The images of each specimen before and after treatment were overlaid using DataViewer software (Bruker) and the difference between them (Diff) was used to characterize the mineral loss in the entire sample, enamel and dentin, on a cubic millimeter scale. These differences were measured by 3D morphometric analysis using CTAn software (Bruker) that also allowed the measurement of mineral loss in millimeters via 2D morphometric analysis. In a 2D longitudinal cross-section view of the specimen, a micrometer was used to measure the enamel and dentin mineral loss, which was represented by the difference in depth in the overlapping images. To guide the overlay process, cavities were made at the base of the specimen with spherical drills and subsequently filled with composite resin.

Prior to the study, pilot tests were performed to confirm the accuracy of the technique by superimposing two different scans of the same specimen without treatment and specimen stored in distilled water, which showed no significant difference in the calculated tissue volume (Figure 1A). However, significant mineral loss was detected in the treated specimens stored in distilled water, as marked by the dark gray line at the specimen surface (Figure 1B).

SEM and EDS analysis

The specimens were vacuum-plated with gold (Balzers, Berlin, Germany) and analyzed at a magnification of 20,000× (Zeiss, Jena, Germany) by SEM. The EDS software, model INCA X-act (Oxford, Abingdon-on-Thames, United Kingdom), was calibrated based on the information that the sample was covered with a 56 nm thick gold layer; therefore, the software was able to perform the adjusted calculation. The content of calcium (Ca) and phosphate (P) ions (wt%) on the enamel surface was measured using EDS (Oxford, Abingdon-on-Thames, United Kingdom). The Ca/P ratio was calculated for each specimen and compared to the stoichiometric ratio of hydroxyapatite (1.67). Five measurements were made per sample in the area corresponding to the 20,000× magnification image. This image was consistently used for all measurements and was defined during the pilot experiment. EDS analyzes were normalized to 100% windowless detector, which determine semi-quantitative evaluation of lighter elements. The acquisition time was 30 s per measurement.

AFM analysis

To determine the alteration of the surface roughness of the specimens, scans (in the positive X-axis direction) were performed on the specimens using an AFM machine in the dynamic force mode (Shimadzu, Chiyoda-ku, Tokyo). The probes (Tap190Al-G - Budget Sensors), which are designed to work at resonance frequencies between 160 and 220 kHz, performed the scans at the frequencies between 160 and 170 kHz without specimen distortion, damage, or removal from the surface. The scan speed (rate) was set to 0.5 Hz, collecting data from an area of 30 × 30 μm (resolution of 512 × 512 pixels) in approximately 18 min with a scan speed of 2 s per line. The equipment settings were as follows: operating point between 0.100 and 0.160, integral gain fixed at 1800, and proportional gain at 0. Using the Gwydion analysis software (version 2.57, open-source software for scanning probe microscopy data processing, (http://gwyddin.net) ten measurements (five vertical and five horizontal line scans) were extracted, and the roughness of the specimen was determined.

Measurement of pH

The pH of the bleaching gels was measured with a pH meter (Adwa, Szeged, Hungary), which monitors the degree of acidity or alkalinity via an electrode coupled to a potentiometer (potential difference meter). The pH measurements were calibrated using

a standard buffered potassium chloride solution. Three measurements were taken for each gel and the average was obtained. The pH electrode was calibrated with standard solutions before each measurement to ensure the sensitivity of the pH meter.

Statistical analysis

After checking the data from micro-CT and EDS for normality (Shapiro-Wilks) and homogeneity (Levene), the volume and depth loss data for enamel and dentin obtained from micro-CT analysis and the volume losses of Ca and P measured using EDS were analyzed by one-way ANOVA. Surface roughness data measured by AFM were analyzed by one-way repeated measures ANOVA. All tests were performed using Sigma Plot (Systat Software Inc., Chicago, IL, USA) at a level of significance (α) of 0.05.

RESULTS

The means and standard deviations of the pH values of the HP35ca and HP35wca groups are shown in Table 1.

The means and standard deviations of the loss of enamel (μm) in terms of depth and total volume loss (mm^3) are shown in Table 2. One-way ANOVA demonstrated no significant difference in the volume ($P = 0.000$) or depth of enamel loss ($P = 0.000$) of HP35wca and HP35ca groups. However, the bleached groups showed significant differences compared to the control group. No mineral loss was detected in the dentin substrate in any of the groups. The losses of the enamel structure in the HP35wca and HP35ca groups were located close to the surface. Micro-CT images showed similar volume losses for HP35wca and HP35ca (Figure 2).

The means and standard deviations of the Ca and P (wt%) compositions are listed in Table 3. The Ca ($P = 0.955$) and P ($P = 0.393$) contents analyzed by EDS were similar between the bleached and control groups. SEM images revealed superficial alterations in HP35wca, such as pores and depressions, and images obtained from the HP35ca group showed slight alterations (Figure 3).

The means and standard deviations of the surface roughness (Ra) are listed in Table 4. The Ra values were similar for all groups before ($P = 0.690$) and after treatment ($P = 0.630$). No significant variation was found before and after bleaching HP35ca ($P = 0.340$), HP35wca ($P = 0.213$), and control ($P = 0.412$) groups. The AFM images showed no significant changes in any of the tested groups (Figure 4). The correlation coefficient for EDS data (Ca and P) and micro-CT data (depth and volume) is shown in Figure 5. Pearson's correlation exhibited high values between the Ca and P percentages measured by EDS and the depth and volume of mineral losses measured by micro-CT ($P < 0.001$) for all combinations.

DISCUSSION

Micro-CT is a nondestructive method that allows the evaluation of the internal structure of the whitened enamel and can be used to evaluate the surface and subsurface enamel layers in 3D. The results of the study demonstrated that micro-CT can be recommended to assess the structural volume and depth loss of enamel and dentin tissues; therefore, the first hypothesis of our study was confirmed. Pilot tests performed prior to the study by overlaying two different scans of each specimen showed no significant differences when the images of specimens with no treatment were overlaid. In contrast, differences were observed between the treated specimens. In another study, the authors suggested that micro-CT was an adequate method to assess the mineral content of tooth enamel after the application of whitening gels.[31] However, the study tested only one group by applying 10% carbamide peroxide for two weeks, which caused enamel demineralization up to a depth of 50 mm below the enamel surface, and did not have a control group.[30] Although the effectiveness of the method in the analysis of the integrity of treated tissues has been proven, studies evaluating structural alterations after bleaching treatment using micro-CT are scarce.[32-35]

This study used overlapping images of the initial and final scans to show the surface alterations of enamel and the loss of enamel structure in terms of depth after using 35% hydrogen peroxide (HP) in-office bleaching gels, regardless of the calcium content in the gels. No mineral loss was observed in the dentin tissue using the resolution of the micro-CT analysis used in this study. New studies using nano-CT may detect mineral loss in the

dentin; however, it is also important to consider the clinical relevance of minor mineral losses in the dentin caused by bleaching gels. Previous studies have shown similar morphological changes in enamel, and no changes in dentin have been reported after treatment with 37,5% HP and 35% HP. Ca and P decreased in the enamel and dentin with no significant differences between them or in relation to the untreated control specimens,[36] while another in vitro study with a 35% HP gel showed no evidence of deleterious effects of bleaching on enamel or dentin and suggested that studies reporting adverse effects on enamel and/or dentin actually reflected the pH of the formulation used.[37]

Ca is used in bleaching gels to achieve Ca ion supersaturation, preventing the dissolution of hydroxyapatite.[38] The optimal Ca concentration required during bleaching protocols is not well defined.[38] The addition of 0.5% calcium gluconate to a 35% HP gel was unable to prevent demineralization of the enamel; [38] therefore, this concentration was insufficient to supersaturate Ca ions relative to the enamel hydroxyapatite crystals.[38] A previous study found that bleaching enamel using a 35% HP gel containing sodium fluoride or calcium gluconate resulted in higher microhardness values than using gels without these compounds and that high concentrations of calcium gluconate in bleaching gels had a positive effect on enamel, but not at low concentrations in gels.[39]

Although studies have reported that the addition of Ca or fluoride to the 35% HP bleaching gel can reduce demineralization of the enamel surface,[40] it cannot fully prevent it or remineralize the subsurface enamel.[25] The increase in the permeability and roughness of the enamel surface and the decrease in the microhardness of the enamel are not prevented when Ca and F ions are added to 35% HP bleaching gels.[21] The results of the present study demonstrate the loss of the enamel structure in terms of volume by micro-CT in the studied groups; however, there was no significant difference in the results obtained by bleaching using HP gels with or without Ca. Therefore, the second hypothesis of this study was rejected because the presence of Ca of a certain concentration in the tested bleaching gel did not inhibit the mineral loss of the enamel structure. It is important to emphasize that the percentages of Ca and P on the enamel surface, which are the main constituents of hydroxyapatite crystals, were similar in both the bleached and control groups. Therefore, the 35% HP gel used for the 80–90 min in-office bleaching technique caused no significant change in the mineral composition of the tooth. Demineralization in some regions promoted the redistribution of minerals, as reported in previous studies,[34]

which may explain the EDS results in our study. This result corroborates the results of those studies that used the same bleaching gels and determined from the EDS results that there was no statistically significant loss of Ca and P during treatment.[41]

Hydroxyapatite is a hydrated calcium phosphate from the mineral group of apatite, whose stoichiometric chemical formula is $Ca_{10}(PO_4)_6(OH)_2$ with a molar Ca/P ratio of 1.67.[42] The Ca/P ratios of the groups were calculated using the compositions of Ca and P obtained by microanalysis in EDS.[43] The calculated Ca/P ratio varied between 1.89 to 2.13 and showed no significant differences between the groups. Therefore, the presence or absence of calcium in the bleaching gel composition caused no significant alteration in the Ca/P molar ratio. In a previous study, where in a whitening treatment with 10% carbamide peroxide (CP) containing calcium (Ca) or amorphous calcium phosphate (ACP) was conducted, concluded that the enamel microhardness decreased after the whitening process, regardless of the presence of Ca or ACP. However, no significant change in the enamel Ca/P ratio was detected, indicating that the bleaching gels have an erosive potential, causing enamel softening without promoting surface loss, irrespective of the presence of calcium or ACP ions.[44]

The concentration and duration of bleaching as well as the pH of bleaching agents can influence the mineral loss of bleached enamel.[22,45] An acidic pH causes changes in the mineral composition of the enamel structure,[4,46] contributing to enamel surface erosion.[33] No morphological or chemical alteration was found in the enamel surface in neutral or alkaline bleaching solutions.[47] The tested HP35ca group had a basic pH (8.3), while the HP35wca group had an acidic pH (6.8), which may justify some of the changes in the enamel observed in the SEM images before and after bleaching. A representative HP35wca SEM image suggests the presence of pores and depressions on the enamel surface. The presence of Ca ions in the HP35ca group may have promoted crystal formation on the enamel surface.[48,49] However, the loss of enamel minerals was concentrated near the surface for groups treated with or without Ca, confirming the third hypothesis of our study. The application of silver nitrate to dental structures after bleaching with 35% HP gels in a previous study demonstrated moderate penetration of the enamel gel through the surface and subsurface prisms to a greater depth through the cracks and microcracks present in the enamel structure.[18] Transverse morphological observations with SEM in another study revealed morphological changes limited to a depth of less than 5 μm (0.005 mm) below the enamel surface with 30% HP gels for 30

or 180 min of immersion.[32] In this study, micro-CT showed significantly higher mineral loss for both bleached groups HP35wca group (0.033 mm) and HP35ca group (0.032 mm) than the control group (0.0064 mm).

The overlapping technique of micro-CT scans has proven to be a promising method to assess the loss of tooth structure caused by tooth whitening. A strong correlation was found between the EDS and micro-CT findings. The percentage of Ca and volume of enamel tissue loss (R = 0.8283), the percentage of Ca and depth of enamel tissue loss (R = 0.8050), the percentage of P and volume of enamel tissue loss (R = 0.896), and the percentage of P and depth of enamel tissue loss (R = 0.8283) are indicators of the efficiency of this method. Pilot tests also confirmed the accuracy of the technique by superimposing two different sweeps of the same specimen stored in distilled water, without treatment. There was no significant difference in the calculated tissue volume (Figure 1A), while significant mineral loss was detected in the treated samples and those stored in distilled water (Figure 1B).

In our study, AFM analysis showed no significant alteration in the surface roughness for all tested groups. Another study evaluated the effects of using an in-office 35–40% HP bleaching gels with or without Ca or F on teeth and found that the 35% HP gel without Ca exhibited a slight increase in Ra, which was statistically different from the control.[50] On the other hand, another study that carried out bleaching using 20–45% CP gels, and 9.5–38% HP gels, reported no effect on the surface roughness.[51] A study carried out using 35% HP gels with and without calcium in the composition showed that the addition of calcium gluconate and the high and stable pH of the calcium-containing gel reduced tooth sensitivity in the study participants.[52] In our study the presence of Ca in the bleaching gel showed no benefit; however, the study was designed using simulated artificial saliva containing Ca and P. For patients having a different saliva composition, the presence of Ca in the bleaching gel may prevent enamel demineralization.

One of the limitations of this study is the use of bovine teeth instead of human teeth. The majority of human teeth available for laboratory studies are extracted from the third molars. Because it is difficult to obtain human anterior incisors, alternatives such as bovine teeth were chosen. In this study, we opted to use bovine teeth enamel due to their histological and structural similarity to human teeth.[53] Bovine enamel exhibits a reproducible surface, especially when its buccal surface is polished; hence, it can be safely used in a study that requires serial measurements.[54] Several related studies have used

specimens of bovine teeth due to the difficulty in controlling the testing parameters with human teeth and the morphological variability of human teeth.[55] Other limitations are related to the use of gels with similar concentrations and classifications as well as different treatment protocols. The non-inclusion of other control groups indicated that saliva effects were not accounted for; therefore, the resolution limits of micro-CT could not be verified. This oversite did not consider the possible accumulation of debris resulting from the regularization and polishing of samples. In the present study, the control group, stored in saliva, did not significantly different micro-CT, SEM, and AFM results. Therefore, the ions present in the formulation of the saliva did not interfere with the results. Artificial saliva was used to simulate the clinical environment and was replaced daily.[27] Studies in situ and in vivo have shown that the presence of saliva promotes remineralization on the enamel surface and does not make it porous.[56] Future studies are needed in order to test different resolutions of micro-CT; different devices with higher resolutions such as nano-CT can investigate different products with greater variability. A previous study evaluated whether there were significant long-term clinical benefits or side effects caused by the addition of ACP to PC16% whitening gel. The effects on tooth color, gingival health, and dentin hypersensitivity were evaluated after 90 and 180 days. After 180 days, the PCA group retained nearly 10% more of the original whitening treatment compared to that of the control. No other significant differences were found between groups. Tooth sensitivity, soft tissue health, and gingival health remained similar to baseline levels, proving the long-term safety of whitening treatment.[57]

Although we performed an in vitro evaluation using bovine teeth with artificial saliva, which does not accurately reproduce the clinical environment, these results have important clinical significance because they indicate that bleaching with a 35% HP gel can cause enamel demineralization regardless of the presence or absence of calcium in the gel. However, this demineralization did not change the surface roughness and the Ca and P levels on the surface of the treated enamel. Treatment with whitening gels is generally safe,[34] as long as it is performed by respecting the particularities of each patient and by a dentist. It is necessary to relativized the amount of mineral loss observed in this study with the clinical performance of bleaching procedures. Any adverse effects associated with use of the bleaching gel are temporary, easily controlled, and often disappear within minutes or hours of treatment.

CONCLUSIONS

Within the limitations of this study design, the following conclusions were drawn:

1. The micro-CT method was able to assess the loss of enamel structure in terms of volume and depth with a high correlation with EDS results.

2. The addition of Ca to the bleaching gel composition was not able to prevent enamel surface demineralization, that was minimal and surficial.

3. The enamel underwent mineral loss primarily near the surface regardless of whether bleaching gel with or without Ca was used; however, no alteration in surface roughness was observed.

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TABLES

Table 1. Products composition, pH values and manufacturer's recommendations for use.

Material	Group	Treatment	Composition	Batch number	pH	Manufacture
Whiteness HP Blue Calcium - HP 35%	HP35ca	2 sessions of 40 min, with an interval of 7 days.	PH 35% (after mixing the phases), Thickeners, Inherent pigment, Neutralizing agents, Calcium gluconate, Glycol and purified water.	010319	8.3 (0.3)	(FGM, Joinville, SC, Brazil)
Whiteness HP Maxx HP 35%	HP35wca	2 sessions of 45 min each (3 applications of 15 min), with an interval of 7 days.	PH 35% (after mixing the phases), Thickeners, Mixture of dyes, glycol, inorganic filler and deionized water.	060619	6.8 (0.1)	(FGM, Joinville, SC, Brazil)
Specimen without contact with bleaching gel	Control	Storage in artificial saliva	1.5mm Ca and 0.9mm P in 0.1mm Tris buffer solution	-	7.0 (0.1)	-

Table 2. Means and standard deviations of depth of mineral loss and total volume between the experimental groups obtained by micro-CT, calculated by One-way ANOVA.

Group	**Depth of Loss (mm)** ($P < 0.001$)	**Total Loss (mm^3)** ($P < 0.001$)
HP35ca	0.0326 (0.0008) ^{B}	0.4691 (0.2090) ^{B}
HP35wca	0.0338 (0.0008) ^{B}	0.4488 (0.2150) ^{B}
Artificial Saliva (control)	0.0064 (0.0008) ^{A}	0.0005 (0.0002) ^{A}

Capital letters establish relationships among columns. Different uppercase letters indicate statistically significant differences ($P > 0.05$). The standard deviations are presented in parentheses.

Table 3. The means and standard deviation of the Ca and P values (wt%) and Ca/P ratio in enamel after application of bleaching gels and in the control group obtained by EDS.

Group	**Calcium (Ca)** (wt%) ($P = 0.955$)	**Phosphate (P)** (wt%) ($P = 0.393$)	**Ca/P ratio** ($P = 0.021$)
HP35ca	36.5 (6.1) ^{A}	17.5 (2.4) ^{A}	2.1 (0.1) ^{A}
HP35wca	37.3 (4.8) ^{A}	19.3 (0.4) ^{A}	2.1 (0.2) ^{A}
Control	36.6 (2.3) ^{A}	17.5 (2.9) ^{A}	1.9 (0.1) ^{A}

The same capital letters indicate that there was no significant difference among the groups analyzed by one-way ANOVA ($P > 0.05$). No significant difference among groups within columns is observed. The standard deviations are presented in parentheses.

Table 4. Means and standard deviations of Ra (nm) between the experimental groups obtained by AFM – one-way repeated measures ANOVA.

Group	**Initial Ra** (nm) (P = 0.690)	**Final Ra** (nm) (P = 0.630)
HP35ca	3.7 (1.3) ^{Aa}	2.7 (0.8) ^{Aa}
HP35wca	3.4 (1.2) ^{Aa}	2.8 (1.1) ^{Aa}
Artificial Saliva (control)	3.1 (0.4) ^{Aa}	2.7 (0.8) ^{Aa}

The same capital letters indicate that the Ra values were similar for all groups before and after treatment. The same lower case letters indicate that no significant variation was found before and after bleaching the HP35ca (P = 0.340), HP35wca (P = 0.213), and control (P = 0.412) groups. The standard deviations are presented in parentheses.

Figures

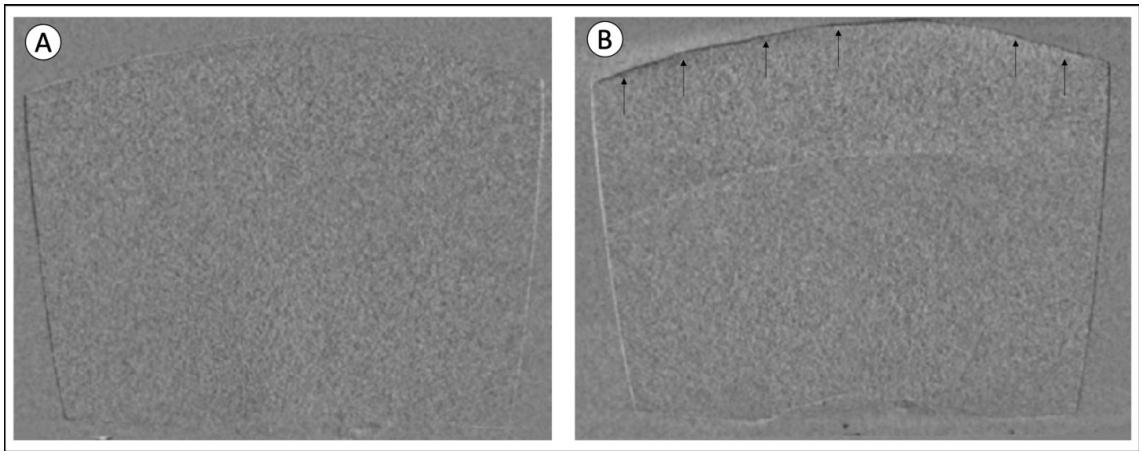

Figure 1. A) Representative image of the superposition of two scans of the same sample without any treatment; B. Representative image of the overlay of the previous scan image with the post-bleach image of the same sample.

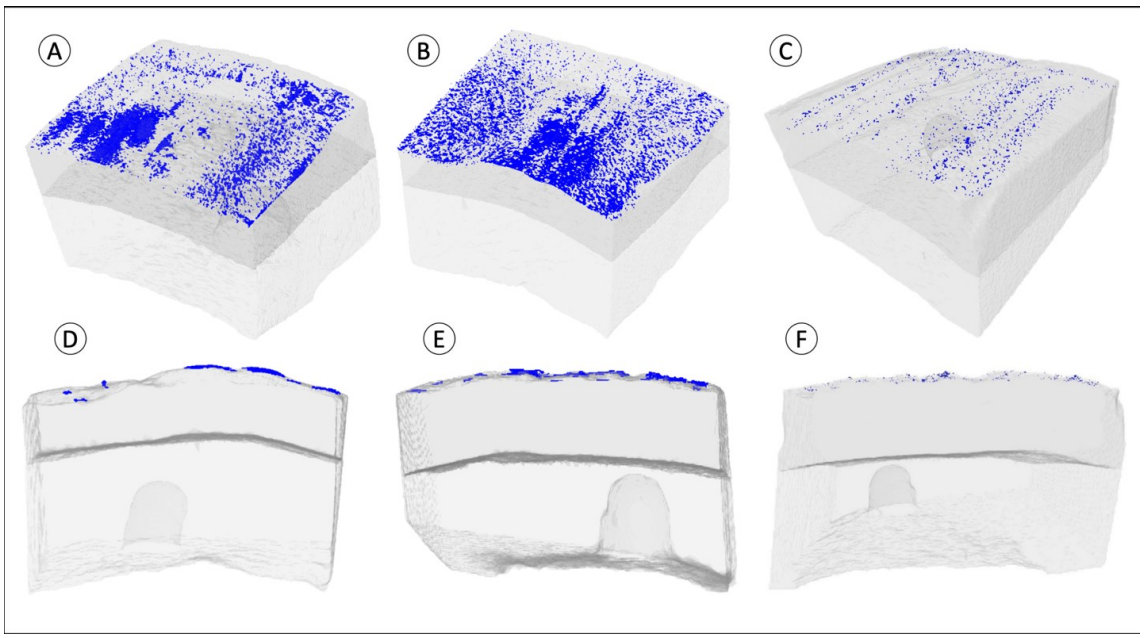

Figure 2. Micro-CT images demonstrate similar volumes and depths of the loss of enamel structure in bleached groups. In the control group, only the difference related to the deviation of precision from the real measurement of the object is measured by the software. Representative image of depth loss for A) HP35wca; B) HP35ca and C) control. Representative image of enamel surface demonstrating volume loss for D) HP35wca; E) HP35ca; F) control.

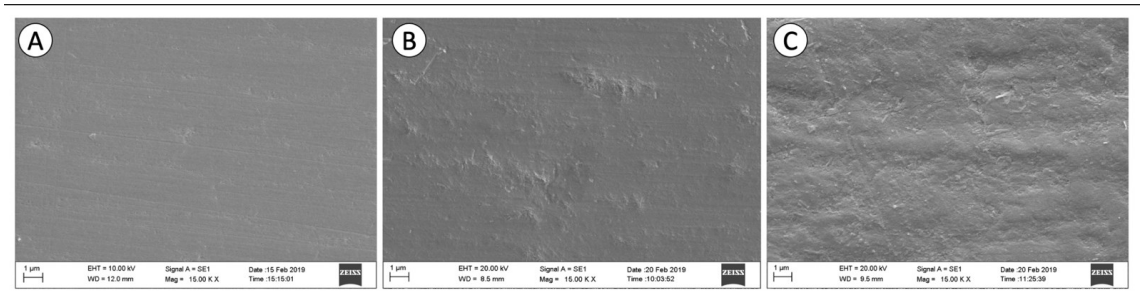

Figure 3. Representative scanning electron microscopy images of the following tested enamels: A) no treatment (control group) showing no changes in the smooth polished surface; B) HP35ca application showing slight surface alterations and areas with calcium deposition; C) HP35wca application showing porosities and depressions.

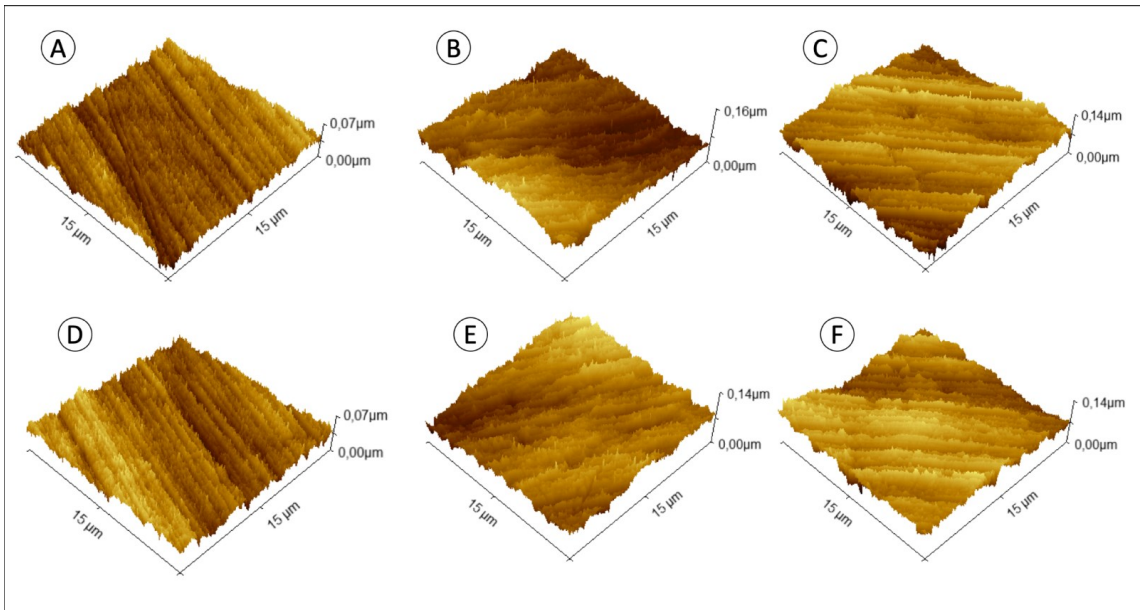

Figure 4. Representative images of AFM of the following tested enamels: A) and D) representative images of the HP35wca group showing no significant changes on the enamel surface; B) and E) representative images of the HP35ca; C) and F) without treatment (control group), showing no significant changes on the enamel surface.

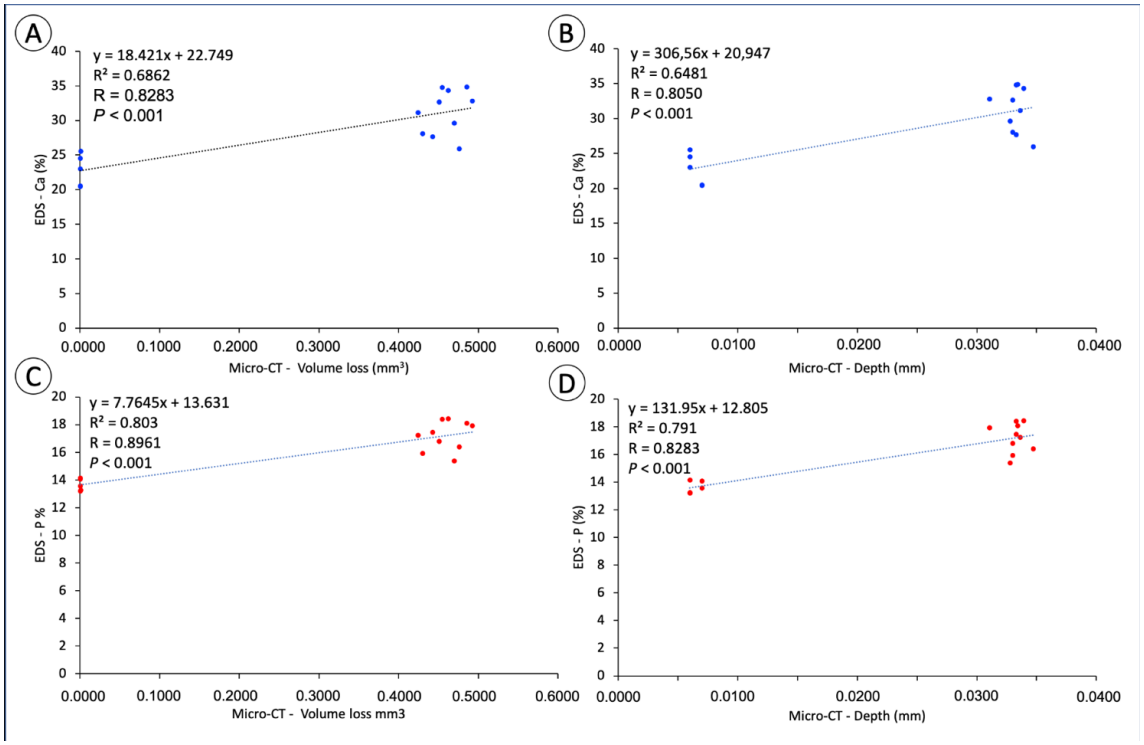

Figure 5. Pearson's correlation between EDS and micro-CT data. Correlation between: A) Ca percentage and volume of enamel tissue loss; B) Ca percentage and depth of enamel tissue loss; C) P percentage and volume of enamel tissue loss; D) P percentage and depth of enamel tissue loss; $P < 0.001$ for all tested correlations.