Universidade Federal de Uberlândia
Faculdade de Odontologia
Programa de Pós-Graduação em Odontologia

Ludmila Cavalcanti de Mendonça

Alterações morfológicas e do conteúdo mineral no esmalte dentário induzidas por tratamentos clareadores.

Changes in morphology and mineral content in tooth enamel induced by bleaching *treatments.*

Tese apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito parcial para obtenção do Título de Doutor em Odontologia na Área de Concentração de Clínica Odontológica Integrada.

Uberlândia, 14 de dezembro de 2021

Ludmila Cavalcanti de Mendonça

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“Quem olha para fora sonha,
quem olha para dentro desperta.”
(Carl Jung)

SUMÁRIO

Resumo

Dentre os fatores que provocam comprometimento estético do sorriso estão as alterações cromáticas, tratadas principalmente por protocolos clareadores. O objetivo deste estudo foi avaliar alterações morfológicas e do conteúdo mineral após tratamento clareador com peróxido de hidrogênio, em dentes íntegros e com lesões iniciais de cárie, por meio da microtomografia computadorizada (micro-CT), cromatografia de íons (CI), microscopia eletrônica de varredura (MEV), energia dispersiva (EDS), rugosidade de superfície, microscopia de força atômica (AFM) e microdureza . **Objetivo específico 1:** Avaliar a perda de conteúdo mineral, expressa por cálcio (Ca) e fosfato (P), no esmalte dental exposto a agentes clareadores a base de peroxido de hidrogênio 35% (PH35%) com cálcio e sem cálcio na composição, por meio de micro-CT, MEV, EDS e AFM. **Objetivo específico 2:** Avaliar por meio de micro-CT a perda da estrutura do esmalte, em volume e profundidade frente ao clareamento com géis a base de Peroxido de Hidrogênio 37,5% (PH37,5%) e 7,5% (PH7,5%) e quantificar a perda de cálcio (Ca) e fósforo (P), pós tratamento, por meio de CI. **Objetivo específico 3:** Avaliar à perda mineral em esmalte dental integro (IE) e com mancha branca (WS) pós-tratamentos clareadores com PH37,5% e PH7,5%, e posteriormente submetido à remineralização com flúor (F). Em conclusão**,** o método micro-CT foi eficaz para avaliar perda de estrutura do esmalte em volume e profundidade com alta correlação com resultados de EDS. A adição de Ca na composição do gel clareador não foi capaz de prevenir a desmineralização da superfície do esmalte. O esmalte sofreu perda mínima de mineral próximo à superfície, independentemente do gel clareador com ou sem a presença de Ca; entretanto, nenhuma alteração na rugosidade da superfície foi observada. O tratamento clareador com PH37,5% e PH7,5%, independente da técnica, provoca alterações minerais do esmalte dentário; no entanto, as mudanças foram mais evidentes com PH7,5%. A cromatografia iônica mostrou-se eficaz para detectar no gel clareador, após o contato com o esmalte dentário, a perda de íons causada pelo tratamento clareador. O clareamento não aumenta a perda mineral no WSE. Os protocolos de clareamento utilizando PH7,5% e PH37,5% e diminuíram os valores de VH para o substrato IE, porém não foi verificada

influência sobre Ra e VH para o substrato WSE. Severas mudanças topográficas de superfície após o clareamento PH7,5% foram observadas apenas para substrato IE. A aplicação de F não foi capaz de alterar os valores de percentual de Ca e P, mas aumenta HV d substrato WSE clareado e reduz valor de Ra quando PH7,5% foi utilizado.

Palavras – Chave: clareamento dental, perda mineral, peroxido de hidrogênio, esmalte dental, esmalte dental desmineralizado, mancha branca.

ABSTRACT

Among the factors that cause smile esthetic alteration are the chromatic modifications, which are mainly treated by bleaching protocols. The aim of this study was to evaluate the morphological and mineral content changes after bleaching treatment with hydrogen peroxide, in intact teeth and with initial caries lesions, involving 3 specific objectives: **Specific objective 1**: To evaluate the loss of mineral content, expressed by Calcium (Ca) and Phosphorus (P), in dental enamel exposed to bleaching agents based on 35% hydrogen peroxide (HP35%) with and without calcium in the composition, by through micro-CT, SEM, EDS and AFM; **Specific Objective 2:** To evaluate by using micro-CT the loss of enamel structure, in volume and depth, compared to the bleaching with hydrogen peroxide 37.5% (HP37.5%) and 7.5% (HP7.5%) based gels and also quantifying the loss of Calcium (Ca) and Phosphorus (P), after treatment, by using CI; **Specific objective 3:** To evaluate the mineral loss in intact (IE) and white spot (WSE) dental enamel after bleaching treatments with HP37.5% and HP7.5%, and subsequently submitted to remineralization with fluoride (F). In conclusion, the micro-CT method was effective to assess loss of enamel structure in volume and depth with high correlation with EDS results. The addition of Ca in the whitening gel composition was not able to prevent the enamel demineralization. The enamel suffered minimal mineral loss located near the surface, regardless of bleaching gel or Ca presence. No change in surface roughness was observed. The bleaching treatment with HP37.5% and HP7.5 caused mineral changes in the tooth enamel; however, the changes were more evident for HP7.5. Ion chromatography proved to be effective for detecting the enamel mineral loss transferred to the whitening gel. The bleaching does not increase mineral loss on WSE. The bleaching protocols using HP7,5% and HP37,5% decreased the VH values for IE substrate, however no influence was verified on Ra and VH of WSE substrate. Severe surface topographic changes were observed only after HP7.5% bleaching for IE substrate. The F application was not able to alter the Ca and P values but it increases the HV of bleached WSE and reduces Ra if HP7.5% were used.

Key Words: tooth bleaching, mineral loss, hydrogen peroxide, tooth enamel, demineralized tooth enamel, white spot

1. INTRODUÇÃO E REFERENCIAL TEÓRICO

1. INTRODUÇÃO E REFERENCIAL TEÓRICO

Dentre os fatores que provocam comprometimento estético do sorriso estão as alterações cromáticas, tratadas principalmente por protocolos clareadores. A cor do dente é altamente baseada nas características de reflexão e absorção de luz do esmalte e dentina, bem como na composição química do esmate (Joiner, 2004). Os pigmentos são cadeias moleculares longas de alto peso molecular (Lynch *et al*., 1995), devido a presença dessas cadeias moleculares complexas no interior da estrutura, dentes escurecidos absorvem maior quantidade de luz. A remoção dos pigmentos da estrutura dental promove clareamento e consequentemente aumenta a reflexão (Kwon *et al*., 2002).

O clareamento de dentes vitalizados ocorre devido à interação físico-química entre os tecidos dentais e agente clareador (Efeoglu *et al.,* 2005). Diferentes teorias têm sido propostas para explicar seu mecanismo de clareamento. O peroxido de hidrogênio (PH) se decompõe em moléculas de oxigênio que promovem quebra das macromoléculas dos pigmentos por meio de reações de oxirredução. Isso faz com que as cadeias longas dos cromóforos das moléculas sejam fracionadas em moleculares menores e também mais claras que são total ou parcialmente eliminadas da estrutura dental por meio de difusão (Lynch *et al*., 1995; Sun 2000; Dannacher 2006; Borges *et al*., 2015). No entanto, os radicais livres liberados pelo peróxido de hidrogênio são instáveis e inespecíficos e podem reagir tanto com as moléculas orgânicas pigmentadas quanto com matriz inorgânica do esmalte (Park *et al*., 2004; Jiang *et al*., 2008; Borges *et al*., 2012). Com isso provoca alterações morfológicas no esmalte capazes de aumentar a opacidade devido a dispersão da luz o que oculta a camada dentinária subjacente (Pinto *et al.,* 2004*;* Cavalli *et al*., 2004; Markovic *et al*., 2007) ou ainda pela oxidação dos aminoácidos aromáticos na fosfoproteína dentinária (Fu *et al*., 2007).

Embora estudos afirmarem que há segurança de procedimentos clareadores (Cadenaro *et al.,* 2008; Tanaka *et al*., 2010, De *Carvalho et al.,* 2020), ação nos tecidos dentais e seus possíveis efeitos adversos tem sido relatado (Ferreira *et al.,* 2016, Vieira *et al*, 2020). O clareamento dental pode

resultar em perda mineral do esmalte dental e consequentemente alteração na rugosidade, microdureza e micromorfologia superficial (Attia *et al*., 2015; Rauen *et al*., 2015, Ferreira *et al.,* 2016, Vieira *et al*, 2020).

Perda de íons cálcio (Ca) e fósforo (P) do dente para o gel clareador (tem sido relatada (Lee *et al.,* 2006). Para reduzir a perda de minerais durante o clareamento dental, agentes remineralizantes como flúor, cálcio, fosfato de cálcio amorfo (ACP) e hidroxiapatita podem ser usados por contribuírem com aumento a resistência de desmineralização do esmalte (Cavalli *et al*., 2010; Sasaki *et al*., 2015; Coceska *et al*., 2016;). Esses agentes podem ser aplicados no dentes, bem como adicionados aos géis (Cavalli *et al*., 2011; Vieira-Junior *et al*., 2016; Bilge & Kılıç 2021).

O Ca atua remineralizando lesões incipientes, de modo, a aumentar a microdureza superficial do esmalte (Schemehorn & Novak, 2007). A associação entre PH e hidroxiapatita, pós clareamento dentário devido ao mecanismo químico da hidroxiapatita que é um sal alcalino, pode aumentar o pH do gel. Os cristais de hidroxiapatita reduzem o contato direto do PH com a superfície do esmalte, dessa forma, os efeitos do pH ácido podem ser diminuídos com controle do valor de pH da solução (Jiang, 2008). O pH ácido causa modificações na estrutura dentária em relação a composição mineral (Ushigome *et a.,* 2009; Ferreira *et al.,* 2016; Balladares e*t al*., 2019).

A desmineralização do esmalte dental ocorre com pH inferior a 5,5 (Fujji *et al*, 2011). Para minimizar danos as estruturas dentais, o gel clareador deve apresentar pH superior ou aproximado da neutralidade (Prince *et al*, 2000). Caso o pH da solução seja maior que o pH crítico, a solução é supersaturada em relação ao mineral e maior quantidade do mineral tenderá a se precipitar (Dawes, 2003). A erosão, dano causado ao esmalte por soluções ácidas, é influenciada pela interação do pH, concentração de ácido e presença de Ca (Hughes *et al.,* 2000). Em tratamento com PH a 30%, Ca do esmalte humano é perdido com um pH de 4,7 a 5,3 (Lee *et al.,* 2006).

Lesões de manchas brancas no esmalte, caracterizando a desmineralização é sinal clínico inicial da cárie dental, que quando detectadas precocemente podem ser revertidas com procedimento não invasivo, como o uso

de agentes remineralizantes (El-Sauad *et al*., 2009; Peters *et al*., 2010). No entanto, as lesões não desaparecem e o comprometimento estético persiste. O clareamento dental visando tornar lesões brancas menos perceptíveis constitui alternativa interessante quando comparada à técnica restauradora ou à microabrasão (Owda & Sancakli, 2021). Do ponto de vista estético, o tratamento clareador de dentes contendo lesões de mancha branca é clinicamente relevante para promover efeito de camuflagem óptica (Kim *et al.,* 2016).

Minerais adicionais são unidos à camada superficial quando lesões desmineralizadas são expostas a mecanismos remineralizadores (Jones & Fried, 2006). Embora o mecanismo de ação do clareamento dental ainda não esteja totalmente esclarecido, fenômenos de desmineralização e remineralização são processos contínuos na cavidade bucal. No entanto, para segurança clínica faz se necessários estudos dos efeitos de géis clareadores nos tecidos dentais desmineralizados para que possíveis efeitos deletérios possam ser minimizados.

Diante dos resultados controversos encontrados na literatura, obtidos por metodologias consolidadas na investigação dos protocolos clareadores, observa-se a importância de estudos que envolvam metodologias pouca exploradas para análise da alteração do esmalte dental exposto aos agentes clareadores e os possíveis efeitos a estrutura dental. A validação de novas metodologias, assim como a associação com metodologias já reconhecidas, torna-se importante ferramenta na confirmação de respostas e obtenção de informações por meio da correlação de resultados tendo por objetivo final a associação a estudos clínicos que possibilitem maior evidência científica. O uso de métodos não destrutivos como a microtomografia computadorizada (micro-CT) permite avaliação interna da estrutura dentária clareada (Gomes *et al.,* 2018; Oliveira *et al*., 2018), servindo como ferramenta na análise da integridade dos tecidos tratados. Ganho e perda mineral do esmalte dental pós tratamento clareador, pode ser mensurado pela análise do gel por meio de metodologia de cromatografia iônica (CI). Esse método consiste em processo físico-químico de separação dos componentes de uma mistura, sendo portanto, indicada para

determinação de ânions e cátions inorgânicos (Frankenberger *et al*., 1990; Lee *et al*., 2006; Haddad *et al.,* 2008).

Embora exista diversos estudos envolvendo protocolos clareadores, o efeito dos géis clareadores nas estruturas dentais ainda permanece sistematicamente em análise o que torna oportuno estudos para que efeitos deletérios na prática clínica. Assim este estudo foi realizado com o intuito de agregar novas evidências a cerca das alterações morfológicas e de conteúdo mineral após clareamento em dentes íntegros e desmineralizados, associando métodos pouco explorados ainda na investigação dos efeitos deletérios do tratamento clareador na estrutura do esmalte com métodos já assentidos.

2. OBJETIVOS

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar alterações morfológicas e de conteúdo mineral pós tratamento clareador com peróxidos, em dentes íntegros e com lesões brancas causadas por desmineralização, por meio de métodos pouco explorados ainda na investigação dos efeitos deletérios do tratamento clareador na estrutura do esmalte com métodos já assentidos.

2.2 Objetivos Específicos

Objetivo específico 1

Capítulo 1 – *Use of computerized microtomography, energy dispersive spectroscopy, scanning electron microscopy, and atomic force microscopy to monitor effects of adding calcium to bleaching gels.*

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 a base de peroxido de hidrogênio 35% (PH35%) com cálcio e sem cálcio na composição, por meio de micro-CT, MEV, EDS e AFM.

Objetivo específico 2

Capítulo 2 – *Analysis of the exchange of Ca and P ions between bleaching gel and dental enamel using ion chromatography.*

O objetivo deste estudo foi avaliar por meio de micro-Ct a perda da estrutura do esmalte, em volume e profundidade frente ao clareamento com géis a base de peroxido de hidrogênio 37,5% (PH37,5%) e 7,5% (PH7,5%) e quantificar a perda de Cálcio (Ca) e Fosforo (P), pós tratamento, por meio de CI.

Objetivo específico 3

Capítulo 3 - *Mineral loss and mechanical change in intact and demineralized dental enamel after bleaching and remineralization.*

O objetivo deste estudo foi avaliar à perda mineral em esmalte dental integro (IE) e com mancha branca (WSE) pós-tratamentos clareadores com PH37,5% e PH7,5%, e posteriormente submetido à aplicação de flúor (F).

3. Capítulos

3.1 Capítulo 1

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

Artigo aceito para publicação no periódico **Operative Dentistry.**

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.

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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 Phosphorus (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 effect that any debris layer produced during specimen preparation was not intentionally removed before analyses were conducted.

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 Phosphorus (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 performed without standards, with the XPP correction method, results normalized to 100% and with a window, which allows for 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.[31] 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 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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REFERENCES

1. McEvoy SA (1989) Chemical agents for removing intrinsic stains from vital teeth. II. Current techniques and their clinical application *Quintessence International* **20(6)** 379-384.
2. Sun G (2000) The role of lasers in cosmetic dentistry *Dental Clinics of North America* **44(4)** 831-850.
3. Zalking M, Arwas JR, Goldman A & Roststein I (1996) Surface morphology changes in human enamel, dentin and cementum, following bleaching: a acanning electron microscopy study *Endodontics and Dental Traumatology* **12(2)** 82-88.
4. Hegedüs C, Bistey T, Flóra-Nagy E, Keszthelyi G & Jenei A (1999) An atomic force microscopy study on the effect on the bleaching agents on enamel surface *Journal of Dentistry* **27(7)** 509-515.
5. Park HJ, Kwon TY, Nam SH, Kim HJ, Kim KH & Kim YJ (2004) Changes in bovine enamel after treatment with 30% Hydrogen Peroxide bleaching agents *Dental Materials Journal* **23(4)** 517-521.
6. Borges AB, Torres CR, de Souza PA, Caneppele TM, Santos LF & Magalhaes AC (2012) Bleaching gels containing calcium and fluoride: Effect on enamel erosion susceptibility *International Journal of Dentistry* 347848.
7. Torres CR, Crastechini E, Feitosa FA, Pucci CR & Borges AB (2014) Influence of pH on the effectiveness of hydrogen peroxide whitening *Operative Dentistry* **39(6)** E261-E268.
8. Borges AB, Zanatta RF, Barros AC, Silva LC, Pucci CR & Torres CR (2015) Effect of hydrogen peroxide concentration on enamel color and microhardness *Operative Dentistry* **40(1)** 96-101.
9. Dannacher JJ (2006) Catalytic bleach: Most valuable applications for smart oxidation chemistry *Journal of Molecular Catalysis A: Chemical* 251,1/2 159-176.
10. Markovic L, Jordan RA, Lakota N & Gaengler P (2007) Micromorphology of enamel surface after vital tooth bleaching *Journal of Endodontics* **33(5)** 607-610.

11. Pinto CF, Oliveira R, Cavalli V & Giannini M (2004) Peroxide bleaching agent effects on enamel surface microhardness, roughness and morphology *Brazilian Oral Research* **18(4)** 306-311.
12. Cavalli V, Arrais CA, Giannini M & Ambrosano GM (2004) High-concentrated carbamide peroxide bleaching agent effects on enamel surface. *Journal of Oral Rehabilitation* **31(2)** 155-159.
13. Fu B, Hoth-Hannig W & Hannig M (2007) Effects of dental bleaching on micro- and nano-morphological alterations of the enamel surface American *Journal of Dentistry* **20(1)** 35-40.
14. Jiang T, Guo YR, Feng XW, Sa Y, Yang X, Wang M, Li P & Wang YN (2018) Hydrogen Peroxide Might Bleach Natural Dentin by Oxidizing Phosphoprotein *Journal of Dental Research* **97(12)** 1339-1345.
15. Cadenaro M, Breschi L, Nucci C, Antoniolli F, Visintini E, Prati C, Matis BA & Di Lenarda R (2008) Effect of two in-office whitening agents on the enamel surface in vivo: a morphological and non-contact profilometric study *Operative Dentistry* **33(2)** 127-134.
16. Spalding M, Taveira LA & De Assis GF (2003) Scanning electron microscopy study of dental enamel surface exposed to 35% hydrogen peroxide: alone, with saliva, and with 10% carbamide peroxide *Journal of Esthetic and Restorative Dentistry* **15(3)** 154-164.
17. Unlü N, Cobankara FK, Altinöz C & Ozer F (2004) Effect of home bleaching agents on the microhardness of human enamel and dentin *Journal of Oral Rehabilitation* **31(1)** 57-61.
18. Mendonça LC, Naves LZ, Garcia LFR, Correr-Sobrinho L & Soares CJ, Quagliatto PS (2011) Permeability, roughness and topography of enamel after bleaching: tracking channels of penetration with silver nitrate *Brazilian Journal of Oral Sciences* **10** 1-6.
19. Soares DG, Ribeiro AP, Sacono NT, Loguércio AD, Hebling J & Costa CA (2013) Mineral loss and morphological changes in dental enamel induced by a 16% carbamide peroxide bleaching gel *Brazilian Dental Journal* **24(5)** 517-521.

20. Attia ML, Cavalli V, do Espírito Santo AM, Martin AA, D'arce MB, Aguiar FH, Lovadino JR, do Rego MA, Cavalcanti AN & Liporoni PC (2015) Effects of Bleaching Agents Combined with Regular and Whitening Toothpastes on Surface Roughness and Mineral Content of Enamel *Photomedicine and Laser Surgery* **33(7)** 378-383.
21. Rauen CA, Filho JCC, Bittecourt BF, Gomes GM, Gomes JC & Gomes OMM (2015) Effect of bleaching agents containing fluoride or calcium on enamel microhardness, roughness and permeability *Journal of Oral Science* **14(4)** 262-266.
22. Ferreira AF, Perez FM, Limeira Júnior F de A, de Moura M de F & de Sousa FB (2016) Graded changes in enamel component volumes resulted from a short tooth bleaching procedure *Archives of Oral Biology* **65** 52-58.
23. Lee KH, Kim HI, Kim KH & Kwon YH (2006) Mineral loss from bovine enamel by a 30% hydrogen peroxide solution *Journal of Oral Rehabilitation* **33(3)** 229-233.
24. Cavalli V, Rodrigues LK, Paes-Leme AF, Brancalion ML, Arruda MA, Berger SB & Giannini M (2010) Effects of bleaching agents containing fluoride and calcium on human enamel *Quintessence International* **41(8)** e157-165.
25. Cavalli V, Rosa DAD, Silva DPD, Kury M, Liporoni PCS, Soares LES & Martins AA (2018) Effects of experimental bleaching agents on the mineral content of sound and demineralized enamels *Journal of Applied Oral Science* **4;26** e20170589.
26. Sasaki RT, Catelan A, Bertoldo E, Venâncio PC, Groppo FC, Ambrosano GM, Marchi GM, Lima DA & Aguiar FH (2015) Effect of 7.5% hydrogen peroxide containing remineralizing agents on hardness, color change, roughness and micromorphology of human enamel *American journal of dentistry* **28(5)** 261–267.
27. Silveira J, Coutinho S, Marques D, Castro J, Mata A, Carvalho ML & Pessanha S (2018) Raman spectroscopy analysis of dental enamel treated with whitening product - Influence of saliva in the remineralization *Spectrochimica acta Part A, Molecular and biomolecular spectroscopy* **198** 145–149.

28. Coceska E, Gjorgievska E, Coleman NJ, Gabric D, Slipper IJ, Stevanovic M & Nicholson JW (2016) Enamel alteration following tooth bleaching and remineralization *Journal of microscopy* **262(3)** 232–244.
29. Gomes MN, Rodrigues FP, Silikas N, Francci CE (2018) Micro-CT and FE-SEM enamel analyses of calcium-based agent application after bleaching *Clinical Oral Investigations* **22(2)** 961-970.
30. Oliveira LRS, Braga SSL, Bicalho AA, Ribeiro MTH, Price RB, Soares CJ. (2018) Molar cusp deformation evaluated by micro-CT and enamel crack formation to compare incremental and bulk-filling techniques *Journal of Dentistry* **(74)** 71-78.
31. Efeoglu N, Wood D & Efeoglu C (2005) Microcomputerised tomography evaluation of 10% carbamide peroxide applied to enamel *Journal of Dentistry* **33(7)** 561-567.
32. Efeoglu N, Wood DJ & Efeoglu C (2007) Thirty-five percent carbamide peroxide application causes in vitro demineralization of enamel *Dental Materials* **23(7)** 900-904.
33. Ushigome T, Takemoto S, Hattori M, Yoshinari M, Kawada E & Oda Y (2009) Influence of peroxide treatment on bovine enamel surface--cross-sectional analysis *Dental Materials Journal* **28(3)** 315-323.
34. Tanaka R, Shibata Y, Manabe A & Miyazaki T (2010) Micro-structural integrity of dental enamel subjected to two tooth whitening regimes *Archives of Oral Biology* **55(4)** 300-308.
35. Oldoini G, Bruno A, Genovesi AM, Parisi L (2018) Effects of Amorphous Calcium Phosphate Administration on Dental Sensitivity during In-Office and At-Home Interventions *Dentistry Journal* (Basel) **6(4)** 52.
36. Llena C, Esteve I, Forner L (2018) Effects of in-office bleaching on human enamel and dentin. Morphological and mineral changes *Annals of Anatomy* **217** 97-102.
37. Sulieman M, Addy M, Macdonald E, Rees JS (2004) A safety study in vitro for the effects of an in-office bleaching system on the integrity of enamel and dentine *Journal of Dentistry* **32(7)** 581-90.

38. Torres C, Zanatta RF, Silva TJ & Borges AB (2019) Effect of calcium and fluoride addition to hydrogen peroxide bleaching gel on tooth diffusion, color, and Microhardness *Operative Dentistry* **44(4)** 424-432.
39. Furlan I, Bridi E, Amaral F, França F, Turssi C, Alinhavo (2007) Efeito de agentes clareadores de alta ou baixa concentração contendo cálcio e / ou flúor na microdureza do esmalte *General Dentistry* **65 (3)** 66-70.
40. Alexandrino L, Gomes Y, Alves E, Costi H, Rogez H & Silva C (2014) Effects of a bleaching agent with calcium on bovine enamel *European Journal of Dentistry* **8(3)** 320-325.
41. Moreira RF, Santos FP, Santos EA, Dos Santos RS, Dos Anjos MJ, de Miranda MS (2017) Analysis of the Chemical Modification of Dental Enamel Submitted to 35% Hydrogen Peroxide "In-Office" Whitening, with or without Calcium *International Journal of Dentistry* **2017** 4646789.
42. SRM 2910b; Hydroxyapatite; National Institute of Standards and Technology, U.S. Department of Commerce: Gaithersburg, MD, USA, 2018.
43. Miculescu F, Luță C, Constantinescu AE, Maidaniuc A, Mocanu AC, Miculescu M, Voicu ȘI, Ciocan LT (2020) Considerations and Influencing Parameters in EDS Microanalysis of Biogenic Hydroxyapatite *Journal of Functional Biomaterials* 15 **11(4)** 82.
44. Moura CW, Catelan A, Zanatta RF, Cavalcanti AN, Soares LE, Martins KV, Liporoni PC (2019) Effects of bleaching using 10% carbamide peroxide with calcium or amorphous calcium phosphate on enamel mineral content and hardness *Acta odontológica latinoamericana* **32(3)** 126-132.
45. Balladares L, Alegría-Acevedo LF, Montenegro-Arana A, Arana-Gordillo LA, Pulido C, Salazar-Gracez MT, Reis A, Loguercio AD (2019) Effects of pH and Application Technique of In-office Bleaching Gels on Hydrogen Peroxide Penetration into the Pulp Chamber *Operative Dentistry* **44(6)** 659-667.
46. Pinto CF, Oliveira R, Cavalli V & Giannini M (2004) Peroxide bleaching agents effects surface microhardness, roughness and morphology *Brazilian Oral Research* **18(4)** 306-311.

47. Xu B, Li Q & Wang Y (2011) Effects of pH values of hydrogen peroxide bleaching agents on enamel surface properties *Operative Dentistry* **36(5)** 554-562.
48. Chen HP, Chang CH, Liu JK, Chuang SF & Yang JY (2008) Effect of fluoride containing bleaching agents on enamel surface properties *Journal of Dentistry* 36(9) 718-725.
49. Borges AB, Dantas RL, Caneppele TM, Borges AL & Rocha Gomes Torres C (2013) Effect of remineralizing agents on the bleaching efficacy of gels *General Dentistry* **61(7)** 67-71.
50. Vieira I, Vieira-Junior WF, Pauli MC, Theobaldo JD, Aguiar FH, Lima DA, Leonardi GR (2020) Effect of in-office bleaching gels with calcium or fluoride on color, roughness, and enamel microhardness *Journal of Clinical and Experimental Dentistry* **12(2)** e116-e122.
51. De Carvalho AC, de Souza TF, Liporoni PC, Pizi EC, Matuda LA, Catelan A (2020) Effect of bleaching agents on hardness, surface roughness and color parameters of dental enamel *Journal of Clinical and Experimental Dentistry* **12(7)** e670-e675.
52. Kossatz S, Martins G, Loguercio AD, Reis A (2020) Tooth sensitivity and bleaching effectiveness associated with use of a calcium-containing in-office bleaching gel *Journal American Dental Association* **143(12)** e81-87.
53. Kwon YH, Huo MS, Kim KH, Kim SK, Kim YJ (2002) Effects of hydrogen peroxide on the light reflectance and morphology of bovine enamel *Journal of Oral Rehabilitation* **29(5)** 473-477.
54. Manning MS, Edgar WM (1992) Intra-oral models for studying de- and remineralization in man: methodology and measurement *Journal of Dental Ressearch* **71 (Spec Nº)** 895-900.
55. Lenherr P, Allgayer N, Weiger R, Filippi A, Attin T, Krastl G. (2012) Tooth discoloration induced by endodontic materials: a laboratory study *International Endodontic Journal* **45(10)** 942-949.
56. Attin T, Schmidlin PR, Wegehaupt F, Wiegand A (2009) Influence of study design on the impact of bleaching agents on dental enamel microhardness: a review *Dental Materials* **25(2)** 143-157.

57. Giniger M, Spaid M, MacDonald J, Felix H (2005) A 180-day clinical investigation of the tooth whitening efficacy of a bleaching gel with added amorphous calcium phosphate *Journal of Clinical Dentistry* **16(1**

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 (0.209) ^{B}
HP35wca	0.0338 (0.0008) ^{B}	0.4488 (0.215) ^{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)	**Phosphorus (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.

Figure legends

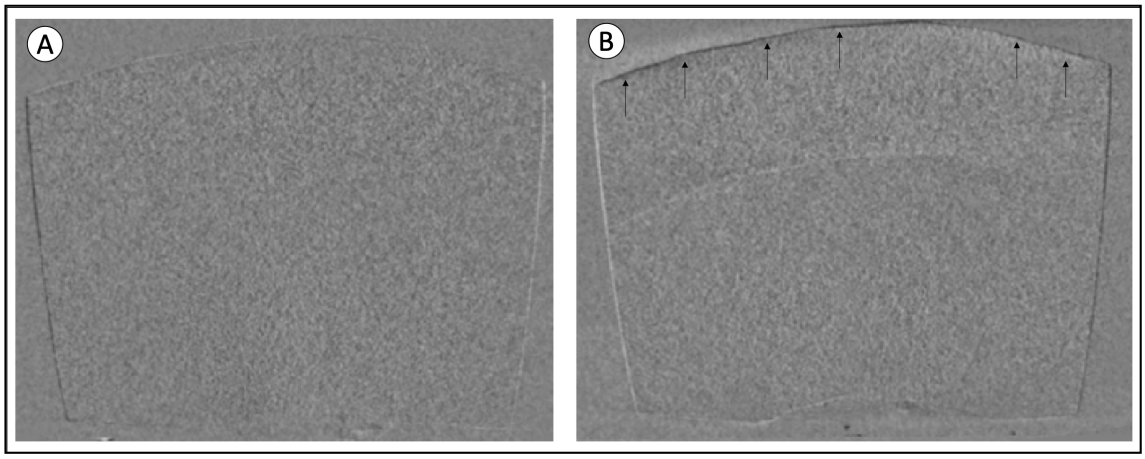

Figure 1. A) Representative image of the superposition of two scans of the same sample without any treatment; B. Representative image of the overlay of the previous scan image with the post-bleach image of the same sample.

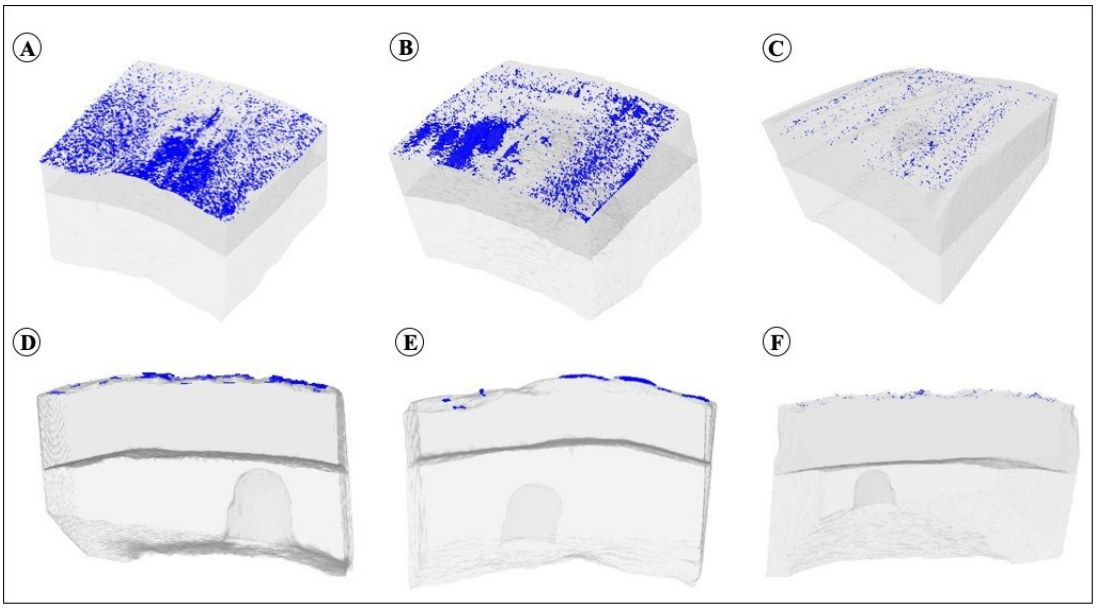

Figure 2. Micro-CT images demonstrate similar volumes and depths of the loss of enamel structure in bleached groups. In the control group, only the difference related to the deviation of precision from the real measurement of the object is measured by the software. Representative image of depth loss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

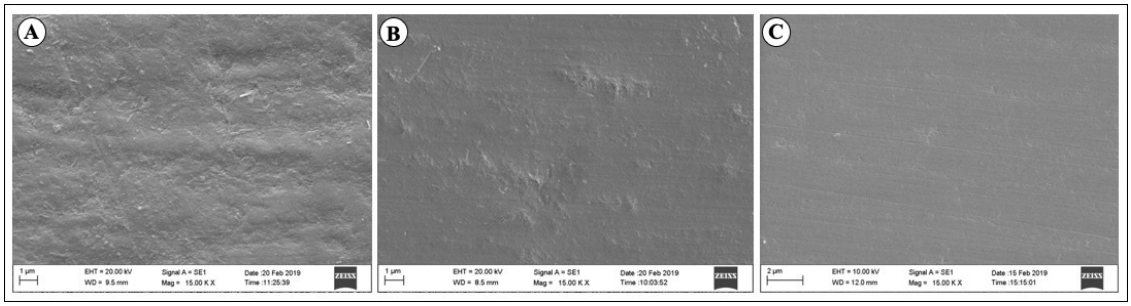

Figure 3. Representative scanning electron microscopy images of the following tested enamels: A) HP35wca application showing porosities and depressions; B) HP35ca application showing slight surface alterations and areas with calcium deposition; C) no treatment (control group) showing no changes in the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

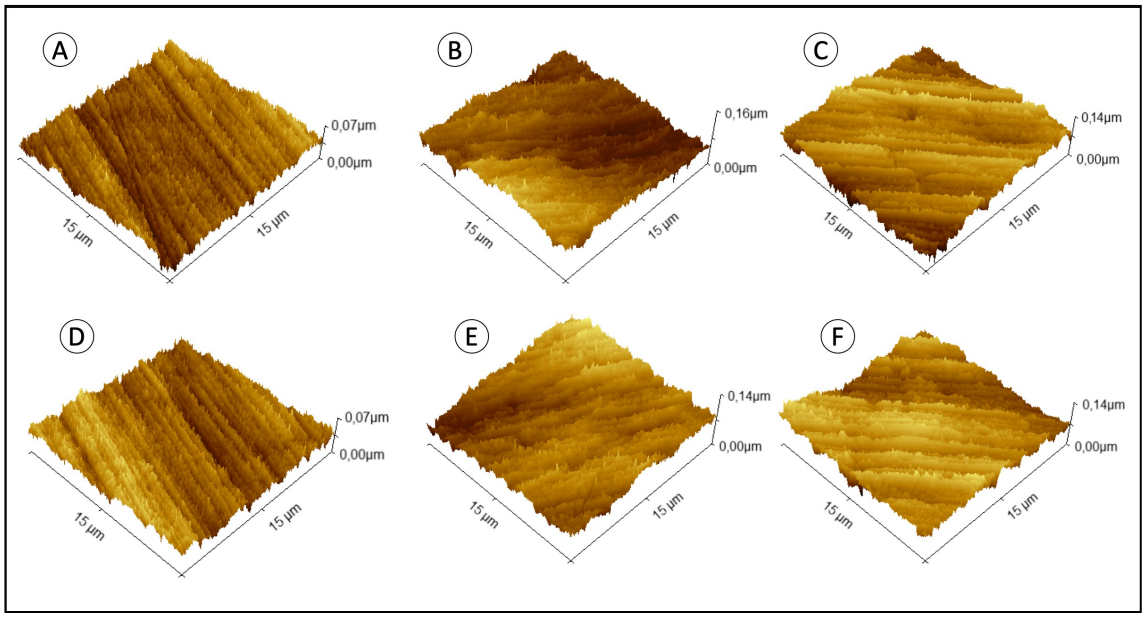

Figure 4. Representative images of AFM of the following tested enamels: A) and D) representative images of the HP35wca group showing no significant changes on the enamel surface; B) and E) representative images of the HP35ca; C) and F) without treatment (control group), showing no significant changes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

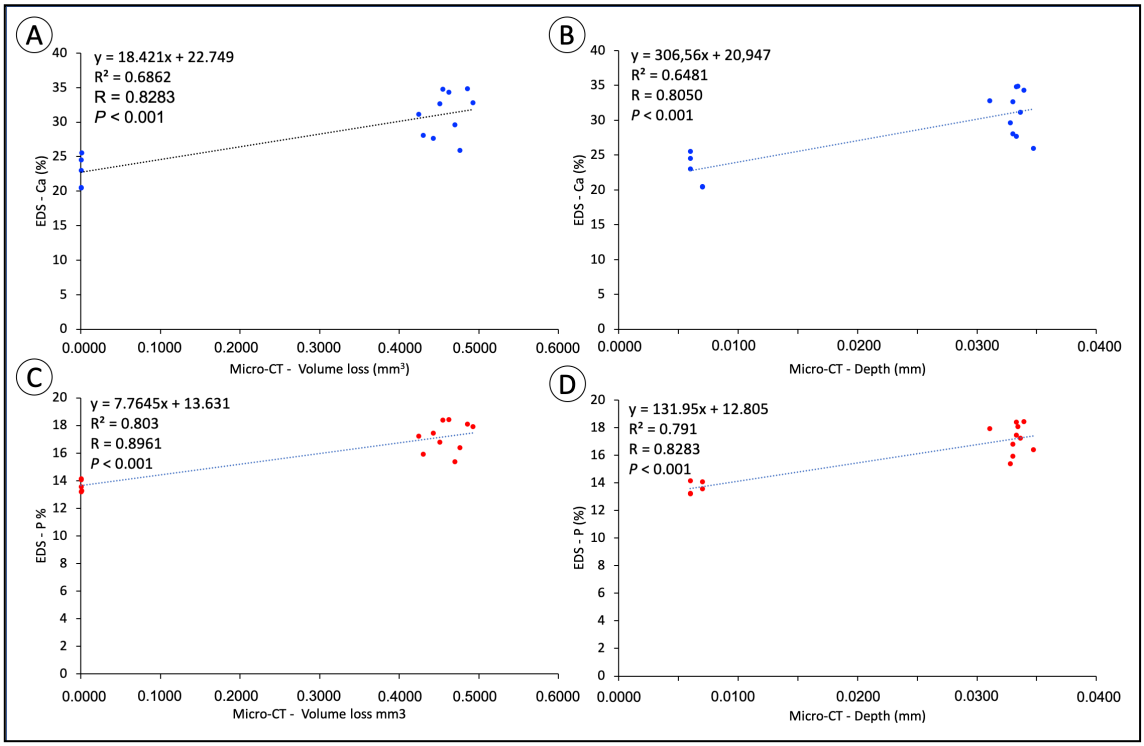

Figure 5. Pearson's correlation between EDS and micro-CT data. Correlation between: A) Ca percentage and volume of enamel tissue loss; B) Ca percentage and depth of enamel tissue loss; C) P percentage and volume of enamel tissue loss; D) P percentage and depth of enamel tissue loss; $P < 0.001$ for all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

3.2. Capítulo 2

Analysis of the exchange of Ca and P ions between bleaching gel and dental enamel using ion chromatogram

Artigo a ser submetido no periódico Operative Dentistry.

Analysis of the exchange of Ca and P ions between bleaching gel and dental enamel using ion chromatography.

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

Keywords: Tooth bleaching; mineral loss; enamel.

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CLINICAL RELEVANCE

Hydrogen peroxide indicated at-home or in-office bleaching techniques promote changes in the enamel structure. Bleaching Protocols with a longer time of contact in the dental enamel, presented the highest levels of alteration in the surface, depth, volume, and enamel loss of minerals.

SUMMARY

Objectives: This study aimed to evaluate the structural loss, in volume and depth of bleached enamel with gels based on 37.5% hydrogen peroxide (HP37.5%) and 7.5 % (HP7.5%). And also quantify the loss of Calcium (Ca) and Phosphate (P), after bleaching with both gels.

Methods: Sixty bovine enamel specimens were randomly divided into three groups (n = 20): HP37.5%, HP7.5%, and Control - without application of the bleaching gel. Five specimens were used for scanning electron microscope (SEM) to surface analysis and dispersive energy system (EDS) to Calcio and Phosphorus dosage. 5 specimens for micro-CT (enamel loss analysis) and 10 specimens for ion chromatography (IC) to analyze Ca and P amount in the bleaching gels. The micro-CT and EDS data were analyzed by 1-way ANOVA followed by Tukey and Dunnett's tests (α = 0.05) and IC data by Two-way repeated measure ANOVA followed by the Tukey's test (α = 0.05).

Results: There was a significant difference in the volume and depth of structural enamel loss in the bleached groups, HP7.5% gel showed more change. EDS analysis showed no significant difference on Ca and P between the experimental and control groups. Pores and depressions on the enamel were observed in SEM after HP37.5% and HP7.5% use when compared to control. The IC demonstrated a significant increase of Ca in the bleached groups, however only HP7.5% showed a increasing to P amount. The HP7.5% showed higher ion exchange than HP 37.5%, for both Ca and P amount ($P<0.001$).

Conclusion: The tested bleaching gels promote changes in the enamel surface. The HP7.5% bleaching gel showed the highest levels of change in surface, depth, volume and loss of Ca and P minerals.

INTRODUCTION

Bleaching products for in-office and at-home treatment contain hydrogen peroxide or carbamide peroxide as an active agent, carbopol as a thickener agent, glycerin as a carrier, and flavoring agents.[1] Among at-home bleaching protocols the concentrations of carbamide peroxide ranging between 10 and 22%, and hydrogen peroxide between 3 and 10%.[2] However, the in-office bleaching uses bleaching gels containing high concentrations of carbamide peroxide at 35 - 37% or hydrogen peroxide at 30 - 40%.[2]

There is no consensus on the negative effects of bleaching gels on tooth enamel,[3-8] although the successful effectiveness of gels has been reported.[9,10] Some studies report morphological changes, suggesting that bleaching is an erosive process.[3] Moreover, effects on organic protein and also the changes in the mineral components, resulting in visible morphological changes to the tooth surface have been shown.[4] These changes were characterized by increased surface porosity, degradation of the organic matrix and loss of calcium and phosphate, and reduced surface microhardness.[5-8] Other studies affirm not only the effectiveness of the tooth bleaching protocols but also the absence of deleterious changes to the enamel.[9,10] These conflicting results can be attributed to different study designs, methodologies, exposure time, storage medium, pH of the solution, and composition of the bleaching agents.[11]

Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) have been used to assess morphological changes on the enamel surface.[12-14] However, they are destructive methods, not allowing the analysis before and after treatment using the same specimen. Micro-CT is a non-destructive method capable of evaluating the superficial and subsurface layers of the whitened enamel in 3D, a tool in the analysis of the integrity of the treated tissues.[15-19] However, the mineral gain or loss of tooth enamel, as a result of demineralization and remineralization after bleaching treatment, can also be measured by analyzing the gel using ion chromatography (IC).[20] This methodology consists on a physical chemical method of separation of the

components of a mixture, been indicated to determine the anions and cations presence in a solution.[21-22]

The use of methodologies for analyzing the bleaching gel after contact with the surface is scarce, although it is an important tool to confirm the results of the dental structure analyses. Therefore, this study aimed to evaluate by using micro-CT, the loss of the enamel structure, in volume and depth after treatment with hydrogen peroxide 37.5% (HP37.5%) and 7.5 % (HP7.5%) bleaching products based. Moreover, the loss of Calcium (Ca) and Phosphate (P) was quantified using ion chromatography. The tested hypotheses were as follows: 1) The bleaching treatment, regardless of the technique, causes mineral changes of the tooth enamel; 2) Ion chromatography is effective for determining the loss of ions in the enamel structure.

METHODS AND MATERIALS

Preparation of specimens - Bleaching Procedures

Sixty bovine incisor teeth, of animals that had all the teeth already erupted and of the same age range, were extracted and stored in distilled water before and during the preparation of the specimens in a refrigerated environment at -10 °C for no more than 30 days.

Thirty specimens for analysis in micro-CT, MEV, and EDS, were obtained per tooth in the region of the middle 1/3 of the buccal surface with dimensions of 5mm x 5mm x 4 mm using a water-cooled, low-rotation diamond saw (Buehler Ltd, Lake Bluff, IL, USA). For specimen preparation, the dental enamel surface was regularized and finished with abrasive sandpaper of decreasing grit size (# 600, #1000, #1200 and #1500, Arotec, Cotia, SP, Brazil) followed by polishing with a polishing cloth and diamond pastes (6 µm; 3 µm; 1 µm; 0.25 µm; Arotec, São Paulo, SP, Brazil) in a polisher (Arotec, Cotia, SP, Brazil). 0.300g of bleaching gel was the quantity established in a pilot test so that analysis in IC was possible, a quantitythat had to be applied to the entire vestibular surface of thirty intact teeth. The sample was randomly divided into three groups (n = 20): HP37.5, 37.5% hydrogen peroxide gel (Pola office + SDI, Victoria, Australia); HP7.5, 7.5% hydrogen peroxide

gel (Pola Day SDI, Victoria, Australia); and Control, without application of bleaching gel. For the micro-CT (n = 5), SEM, and EDS (n=5) methodologies the specimens were analyzed before and after the bleaching procedures and the specimens from the control group before and after immersion in artificial saliva. For IC analysis (n = 10), the bleaching gels were analyzed before and after contact with the enamel. All bleaching procedures were performed according to the manufacturer's instructions (Table 1). The specimens were kept immersed in artificial saliva and changed daily, stored at 37°C until the next application. After the last session of each group, the specimens were rinsed with distilled water and stored in distilled water.

Micro-CT analysis

A methodology of overlapping reference and target image was used to generate a volume difference in the image (Diff).[23] Pilot tests were carried out to confirm the accuracy of the overlaying scans of the same specimen, untreated and after bleaching treatment and storage in distilled water.

The specimens were scanned using a high-resolution micro-CT instrument (Bruker, Kontich, Belgium), resolution of 0.35μ. The device was programmed to scan with a beam at 100 kV and 100 mA, a Cu filter 0.11 mm, an image pixel size of 13 μm, a resolution of 1632 x 1092 and a rotation step of 0.6°, generating 3 image slices over 1850 ms with 20 random movements and resulting in 1692 image slices. The NRecon® software (Bruker, Kontich, Belgium) was applied for the parameters of smoothing and correction of the beam hardening artifact; thus, the images were reconstructed. To characterize the mineral loss of the enamel surface in mm^3, the difference between the images of each specimen before and after treatment were superimposed in the DataViewer software (Bruker, Kontich, Belgium). To guide this overlap, cavities were made with spherical drills at the base of the specimens and then filled with composite resin. These differences were analyzed in enamel and measured using 3D morphometric analysis in the CTAn software (Bruker, Kontich, Belgium). Through 2D morphometric analysis, mineral loss was measured in mm. With a micrometric ruler in a 2D longitudinal

section of half the specimen, the vertical enamel loss was measured in depth, represented by the difference in the overlapping images.

SEM and EDS Analysis

For SEM analysis, the specimens were vacuum-plated with gold (Balzers, Berlin, Germany) and an enlargement of 20.000 X (Zeiss, Jena, Germany) was chosen for surface analysis by obtaining SEM images in greater detail. The percentage of Calcium (Ca) and Phosphorus (P) by weight (wt%) on the enamel surface was measured using EDS (Oxford, Abingdon-on-Thames, United Kingdom) with the same magnification. To perform calculation adjustments, the EDS software, model INCA X-act (Oxford, Abingdon-on-Thames, United Kingdom), has been calibrated to 56nm thick gold layer. The EDS analyses were semi-quantitative, performed with a window, with XPP correction method, without standards and the results were normalized to 100%. Five measurements were made per specimens in the area corresponding to the 20.000x magnification image. The acquisition time was 30s per measurement.

Chromatograph analysis

A 0.300g of bleaching gel was measured on a precision scale (Scientific Marte, São Paulo, Brazil) and was placed on the buccal surface of the enamel per application of each specimens. The specimen was individually washed in a Becker with ultrapure water until all bleaching gel was removed. Three drops of acid Hydrochloric (Dinâmica, Indaiatuba, São Paulo, Brazil) was added to facilitate gel dilution, then ultrapure water was added until reaches 100ml. The solution was then filtered with a 0.45 µm IC filter (Macherey-Nagel, Duren, Germany). The ion chromatography model 883 basic IC Plus (Metrohn Brazil, São Paulo, Brazil,) had the parameters established as follow: Flow rate of 0.7 mL/min; Injection volume of 50 µL; Maximum pressure: 15Mpa; Detection time: 32 min and column temperature at 25°C. Determination of cations with direct conductivity detection, with column 6.1010.230 Metrosep C 2-250, having as eluent 4 mmol/L of tartaric acid and 0.75 mmol/L of dipicolinic acid at a flow rate of 1mL / min, an injection of 10µL. Determination of anions with Metrosep A Supp 5 - 250/4.0 column using subsequent sequential suppression followed by conductivity detection. Having as eluent 3.2 mmol/L of sodium carbonate, 1.0 mmol/L of sodium bicarbonate and suppressor 100 mmol L of sulfuric acid.

Statistical Analysis

Data were tested by normal distribution (Shapiro-Wilk test) and homoscedasticity (Levine test). The micro-CT and EDS data were analyzed by 1-way ANOVA followed by Tukey and Dunnett's tests (α = 0.05) and IC data by 2-way repeated measure ANOVA followed by the Tukey's test (α = 0.05), while the factors evaluated were "bleaching gel" and "assessment time" (repetition factor).

RESULTS

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 demonstrate significant effect for the depth of enamel loss ($P < 0.001$) and volume ($P < 0.001$). Dunnett "s test showed that both bleached groups had significant differences compared to the control group *(*$P < 0.001$*).* The loss of structure of the vertical enamel, in depth, was statistically greater in the 7.5% HP group. Micro-CT images showed distinct volume losses for HP37.5% and HP7.5% groups. (Figure 1).

The means and standard deviations of the (Ca) and (P) (wt%) compositions in dental enamel are listed in Table 3. The (Ca) ($P = 0.213$) and (P) ($P = 0.336$) contents analyzed by EDS were similar between the bleached and control groups (Table 3). SEM images revealed changes such as pores and depressions in the treated groups HP37.5% and HP7.5% when compared to the control. (Figure 2).

The means and standard deviations of the (Ca) and (P) **(**mg/l) compositions in the bleaching gel are listed in Table 4. 2-way repeated measure ANOVA demonstrated that there is a statistically significant interaction between "bleaching gel" and "assessment time" ($P = < 0.001$) to (Ca) and P amount. The IC demonstrated that the HP37.5% ($P = 0.004$) and HP7.5% ($P < 0.001$) gels analyzed after application on the enamel surface showed significant increase for the amount of Ca, however, only HP7.5% showed a significant increase for the amount of P (*P < 0.001)*. The HP7.5% showed higher ion exchange than HP 37.5%, for both Ca and P amount ($P < 0.001$).

DISCUSSION

The present study investigated two bleaching gels containing different concentrations of hydrogen peroxide, one indicated for at-home treatment and the other for in-office use. The first hypothesis tested was confirmed, since tooth enamel changes (volume and in-depth) were observed after bleaching protocols. Moreover, significant changes in the composition of the bleaching gels after contact with the enamel were found, mainly about an increasing amount of (Ca), although EDS analysis has not shown the concentration in % of this mineral on the surface has not been changed significantly.

This study used overlapping images of the initial (reference) and final (target) scans of each specimen performed in micro ct. The representative images of each group showed that both bleaching gels promoted structural alterations when compared to the control, however, the alterations in the 7.5% HP group were much more evident. To investigate the internal structural integrity of human enamel with the application of 35% HP and 10% PC, a previous study quantified and visualized enamel mineral density by micro-CT.[18] The microstructural differences between the bleached enamel specimen were distinct, although the changes in color parameters were equivalent. The color change in the bleached specimens with CP 10% was affected by demineralization, while the bleached specimens with 35% HP depended on redistribution and subsequent remineralization of the enamel.[18]

The inconsistency in the results of studies evaluating the effect of tooth bleaching on the enamel surface is observed among different studies.[3-10] Studies that used SEM have shown different results since no change in surface morphology,[24] even localized or generalized surface changes such as porosity, depressions, and corrosion.[25] In this study, SEM images revealed changes such as pores and depressions in the enamel of the treated HP37.5% and HP7.5% groups when compared to the control group. Although in the present study there were significant changes in volume, depth, and topography of the bleached enamel, no significant quantitative changes of (Ca) and P on surface were

observed in EDS. Which can be explained by a rearrangement of minerals on the surface[18].

The reason why the changes in the present study were more significant in the low concentration bleaching gel can be explained by the long contact time, since, according to the manufacturer, the gels have a neutral pH. The gels were applied according to the manufacturer's orientations, the 7.5% HP product was applied daily for 45 minutes for 14 days, totaling 630 minutes of treatment, while the 37.5% HP product was applied in 2 sessions of 24 minutes each, with 3 applications of 8 minutes per session, totaling 48 minutes of treatment. These results corroborate a previous study that proved that protocols with gels with relatively high concentrations of carbamide peroxide or hydrogen peroxide and shorter application time may be less harmful to the enamel surface regarding hardness, roughness, and modulus of elasticity.[26]

Ion chromatography is a versatile, selective, and sensitive method for determining a variety of anions and cations at trace and ultra-trace levels and has applications in different fields involving ionic analysis, such as clinical specimens, food, pharmaceutical, industrial and environmental.[21-22] This methodology in this study made it possible to observe the ionic profiles of bleaching gels before and after contact with tooth enamel. The bleached groups demonstrated the ion exchange between bleaching gel and tooth enamel. HP7.5% showed higher ion exchange than HP 37.5%, for both (Ca) and P amount. After treatment, this bleaching gel presented an amount of (Ca) (15.65 mg/l) much higher than the initial (0.30 mg/l). Only 7.5% HP a showed statistically significant increase for the amount of P in IC (Figure 3). Therefore, the second hypothesis was accepted, since ion chromatography is effective in determining the loss of ions in the enamel structure through the bleaching gel. (Ca) and P are the elements in the largest amount in the tooth, the decrease of these elements may suggest that there was a change in the mineral phase or significant substitution of these ions.[27] However, possible changes in these elements can be offset by the protective effect of saliva, supersaturated with (Ca) and P, which are re-incorporated into the enamel.[28,29]

A previous study that evaluated the loss of (Ca) in the gel *in situ* and i*n vitro* (mg of Ca/ml) on the 1st, 7th and, 14th day using an atomic absorption spectrophotometer revealed another loss profile.[30] The greatest loss of calcium occurred during the 1st day in both conditions and continued to decrease in the other measurements. Calcium loss was always significantly greater *in vitro* than *in situ* (2.5x greater in vitro).[30] Study that analyzed the amount of (F), (Ca), and (P) ions present in the enamel, ground and diluted for analysis in IC, showed that after bleaching with HP37.5%, the enamel presented lower concentrations of (F) and (Ca) ions when compared to the control group, no treatment.[31]

On the other hand, a previous study with HP30% evaluated the amounts of elements concentrated in the bleaching agent using an atomic emission spectrometer inductively coupled plasma and ion chromatography.[21] And the content of mineral elements in the teeth was through an electron probe microanalyzer. The total mineral element content of unbleached enamel was slightly higher than that of bleached enamel. The amount of (Ca) loss from bleached enamels after 120 h was similar to the amount of (Ca) lose from teeth exposed to a soft drink or juice for a few minutes. Therefore, the authors concluded mineral loss caused by the bleaching process may not be a threatening factor for teeth.[21]In the present study, specimens were stored in artificial saliva throughout the bleaching protocol to simulate real-life. Bovine teeth were used since, from a structural and mechanical point of view, bovine enamel is a suitable alternative to human enamel for in vitro testing of dental products.[32]

Although the results of this study are promising, further *in vivo* studies, are needed to analyze other gels and bleaching protocols. Even being an in vitro study, results are of clinical importance for guiding professionals when choosing the best indication for the bleaching protocols studied. Bleachers with low concentration HP and consequently longer daily contact time with the tooth structure and for a prolonged period, on average 14 days, promote structural changes in the surface and subsurface of the enamel with greater intensity than high concentration HP bleaching gels for periods smaller. So, its indication should be done with caution, observing the clinical conditions of the individual patients,

such as enamel cracks, non-carious cervical lesions, xerostomia and history of sensitivity.

CONCLUSIONS

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

1. The bleaching treatment with HP37.5% and HP7.5%, regardless of the technique, causes mineral changes of the dental enamel; however, the changes were more severe with 7.5%
2. Ion chromatography is effective for detecting in the bleaching gel, after contact with the dental enamel, the loss of ions caused by the bleaching treatment.

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REFERENCES

1. ADA (2002) *Statement on the Safety and Effectiveness of Tooth Whitening Products*, p. 2008.
2. Sulieman M (2005) An overview of bleaching techniques: 2. Night Guard Vital Bleaching and non-vital bleaching *Dental Update* **32(1)** 39-46.
3. Miranda CB, Pagani C, Benetti AR & Matuda FS (2005) Evaluation of bleached enamel by scanning electron microscopy *Journal of Applied Oral Science* **13 (2)** 204–211.
4. Bitter NC (1992) A scanning electron microscopy study of the effect of bleaching agents on enamel: a preliminary report *Journal of Prosthetic Dentistry* **67(6)** 852–855.
5. de Moraes IQ, Silva LN, Porto IC, de Lima Neto CF, Dos Santos NB, & Fragoso LS (2015) Effect of in-office bleaching with 35% hydrogen peroxide with and without addition of calcium on the enamel surface *Microscopy Research and Technique* **78(11)** 975-981.

6. Al-Salehi SK, Wood DJ, & Hatton PV (2007) The effect of 24h non-stop hydrogen peroxide concentration on bovine enamel and dentine mineral content and microhardness *Journal of Dentistry* **35(11)** 845-850.
7. Souza RO, Lombardo GH, Pereira SM, Zamboni SC, Valera MC, Araujo MA, & Ozcan M (2010) Analysis of tooth enamel after excessive bleaching: A study using scanning electron microscopy and energy dispersive x-ray spectroscopy *International Journal of Prosthodontics* **23(1)** 29-32.
8. Cakir FY, Korkmaz Y, Firat E, Oztas SS, & Gurgan S (2011) Chemical analysis of enamel and dentin following the application of three different at-home bleaching systems *Operative Dentistry* **36(5)** 529-536.
9. Borges AB, Zanatta RF, Barros AC, Silva LC, Pucci CR &Torres CR (2015) Effect of hydrogen peroxide concentration on enamel color and microhardness. *Operative Dentistry* **40(1)** 96-101.
10. Cadenaro M, Breschi L, Nucci C, Antoniolli F, Visintini E, Prati C, Matis BA & Di Lenarda R (2008) Effect of two in-office whitening agents on the enamel surface in vivo: a morphological and non-contact profilometric study *Operative Dentistry* **33(2)** 127-134.
11. Lopes GC, Bonissoni L, Baratieri LN, Vieira LCC & Monteiro S (2002) Effect of bleaching agents on the hardness and morphology of enamel *Journal of Esthetic and Restorative Dentistry* **14(1)** 24-30.
12. Coceska E, Gjorgievska E, Coleman NJ, Gabric D, Slipper IJ, Stevanovic M & Nicholson JW (2016) Enamel alteration following tooth bleaching and remineralization *Journal of Microscopy* **262(3)** 232- 244.
13. Pimenta-Dutra AC, Albuquerque RC, Morgan LS, Pereira GM, Nunes E, Horta MC & Silveira FF (2017) Effect of bleaching agents on enamel surface of bovine teeth: A SEM study *Journal of Clinical and Experimental Dentistry* **19(1)** e46-e50.
14. Llena C, Esteve I &Forner L (2018) Effects of in-office bleaching on human enamel and dentin. Morphological and mineral changes *Annals of* Anatomy **217** 97-102.

15. Efeoglu N, Wood D & Efeoglu C (2005) Microcomputerised tomography evaluation of 10% carbamide peroxide applied to enamel *Journal of Dentistry* **33(7)** 561-567.
16. Efeoglu N, Wood DJ & Efeoglu C (2007) Thirty-five percent carbamide peroxide application causes in vitro demineralization of enamel *Dental Materials* **23(7)** 900-904.
17. Ushigome T, Takemoto S, Hattori M, Yoshinari M, Kawada E & Oda Y (2009) Influence of peroxide treatment on bovine enamel surface--cross-sectional analysis *Dental Materials Journal* **28(3)** 315-323.
18. Tanaka R, Shibata Y, Manabe A & Miyazaki T (2010) Micro-structural integrity of dental enamel subjected to two tooth whitening regimes *Archives of Oral Biology* **55(4)** 300-308.
19. Surmelioglu D, Yalcin ED & Orhan K (2020) Analysis of enamel structure and mineral density after different bleaching protocols using micro-computed tomography *Acta Odontologica Scandinavica* **78(8)** 618-625.
20. Lee KH, Kim HI, Kim KH & Kwon YH (2006) Mineral loss from bovine enamel by a 30% hydrogen peroxide solution *Journal of Oral Rehabilitation* **33(3)** 229-33.
21. Frankenberger WT, Mehra HC & Gjerde DT (1990) Environmental applications of ion chromatography *Journal of Chromatography A* **504** 211-245.
22. Haddad PR, Nesterenko PN & Buchberger W (2008) Recent developments and emerging directions in ion chromatography *Journal of Chromatography* **14 1184(1-2)** 456-473.
23. Oliveira LRS, Braga SSL, Bicalho AA, Ribeiro MTH, Price RB & Soares CJ. (2018) Molar cusp deformation evaluated by micro-CT and enamel crack formation to compare incremental and bulk-filling techniques *Journal of Dentistry* **(74)** 71-78.
24. Kwon SR, Kurti SR, Oyoyo U & Li Y (2015) Effect of various tooth whitening modalities on microhardness, surface roughness and surface morphology of the enamel *Odontology* **103(3)** 274-279.

25. Mendonça LC, Naves LZ, Garcia LFR, Correr-Sobrinho L & Soares CJ, Quagliatto PS (2011) Permeability, roughness and topography of enamel after bleaching: tracking channels of penetration with silver nitrate *Brazilian Journal of Oral Sciences* **10** 1-6.
26. Cvikl B, Lussi A, Moritz A & Flury S (2016) Enamel Surface Changes After Exposure to Bleaching Gels Containing Carbamide Peroxide or Hydrogen Peroxide. *Operative Dentistry* **41(1)** E39-47.
27. Mahoney EK, Rohanizadeh R, Ismail FS, Kilpatrick NM & Swain MV (2004) Mechanical properties and microstructure of hypomineralised enamel of permanent teeth *Biomaterials* **25(20)** 5091-5100.
28. Diaz-Arnold AM & Marek CA (2002) The impact of saliva on patient care: A literature review *Journal of Prosthetic Dentistry* **88(3**) 337-343.
29. Hicks J, Garcia-Godoy F & Flaitz C (2004) Biological factors in dental caries: role of remineralization and fluoride in the dynamic process of demineralization and remineralization (part 3) *Journal of Clinical Pediatric Dentistry* Spring **28(3)** 203-214.
30. Justino LM, Tames DR & Demarco FF (2004) In situ and in vitro effects of bleaching with carbamide peroxide on human enamel *Operative Dentistry* **29(2)** 219-225.
31. Petta TM, Gomes YSBL, Esteves RA, Faial KCF, Couto RSD & Silva CM (2017) Chemical Composition and Microhardness of Human Enamel Treated with Fluoridated Whintening Agents. A Study *in Situ Operative Dentistry* **31(11)** 34-40.
32. Arango-Santander S, Montoya C, Pelaez-Vargas A & Ossa EA (2020) Chemical, structural and mechanical characterization of bovine enamel *Archives of Oral Biology* **109** 104573.

Tables

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

Material	Group	Treatment	Batch number	Manufacture
Pola office + hydrogen peroxide 37.5%	HP 37.5%	2 sessions of 3 applications of 8 minutes each	190624132836	SDI, Victoria, Australia
Pola Day + hydrogen peroxide 7.5%	HP 7.5%	45 minutes/day application for 14 days	190719081529	SDI, Victoria, Australia
Specimen without contact with bleaching gel	Control	Storage in artificial saliva (1.5mm Ca and 0.9mm P in 0.1mm Tris buffer solution)	-	-

Table 2. Means and standard deviations of depth of mineral (μm) and total volume (mm^3) enamel loss between groups, obtained by micro-ct analysis.

Group	**Depth of Loss (μm)** *(P < 0.001)*	**Total Loss (mm^3)** *(P < 0.001)*
HP 37.5%	0.042 (0.002) ^{C}	0.341 (0.035) ^{B}
HP 7.5%	0.072 (0.004) ^{B}	0.721 (0.036) ^{C}
Artificial Saliva (control)	0.006 (0.001) ^{A}	0.001 (0.001) ^{A}

Capital letters establish the relationship between columns. Different uppercase letters indicate statistically significant differences at Tukey`s test (P > 0.05). Standard deviations are in parentheses.

Table 3. The means and standard deviation of the Calcium (Ca) and Phosphorus (P) values (wt%) after application of bleaching gels and in the control group, obtained by EDS analysis.

Group	Calcium (Ca) (wt%)	Phosphorus (P) (wt%)
	(P = 0.213)	*(P = 0.336)*
HP 37.5%	31.1 (3.7) **A**	16.2 (1.7) **A**
HP 7.5%	31.7 (2.3) **A**	16.1 (0.8) **A**
Artificial Saliva (control)	28.8 (0.9) **A**	15.2 (0.5) **A**

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

Table 4. Ionic chromatography (mg/l) to Calcium (Ca) and Phosphate (P) values in bleaching gel before and after contact with the enamel surface.

Bleaching agents	**Ionic chromatography (mg/l)**					
	Calcium – (Ca)		***P* value**	**Phosphate (P)**		***P* value**
	Before	***After***		***Before***	***After***	
HP 37.5%	0.46 (0.13) ^{Bb}	2.31 (1.55) ^{Ab}	*P* = 0.044	0.12 (0.01) ^{Aa}	0.11 (0.08) ^{Ab}	*P = 0.414*
HP 7.5%	0.30 (0.05) ^{Bb}	15.65 (3.48) ^{Aa}	*P < 0.001*	0.10 (0.02) ^{Ba}	0.74 (0.38) ^{Aa}	*P < 0.001*
P value	P = 0.852	*P < 0.001*		P = 0.336	*P < 0.001*	

Different letters (uppercase for comparing the assessment times - in lines; lowercase for comparing bleaching gels – in columns) indicate significant difference at Tukey`s test (P<0.05). Standard deviations are in parentheses.

Figure legends

Figure 1. Micro-CT images demonstrate different 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) HP37.5%; B) HP7.5% and C) control. Representative image of enamel surface demonstrating volume loss for D) HP37.5%; E) HP7.5%; F) control.

Figure 2. Representative scanning electron microscopy images of the following tested enamels: A) HP37.5% and B) HP7.5% application showing porosities and depressions; B) no treatment (control group) showing no changes in the smooth polished surface.

Figure 3. Representative graphs of the presence of (Ca) and (P) in the bleaching gel after each application on the enamel surface. A) HP7.5%. B) HP37.5%.

Figures

Figure 1.

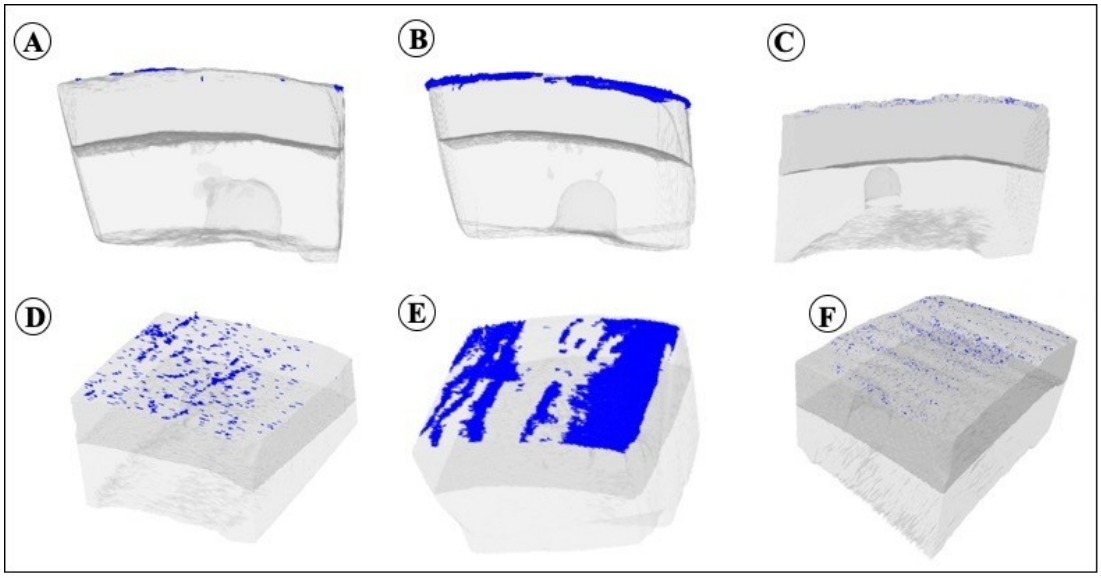


Figure 2.

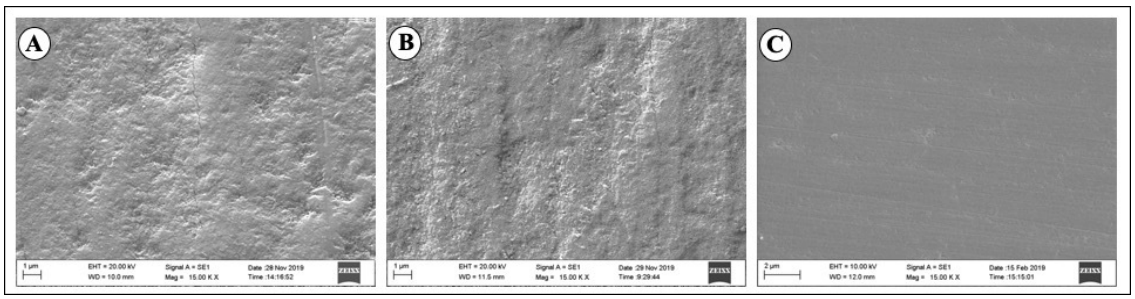
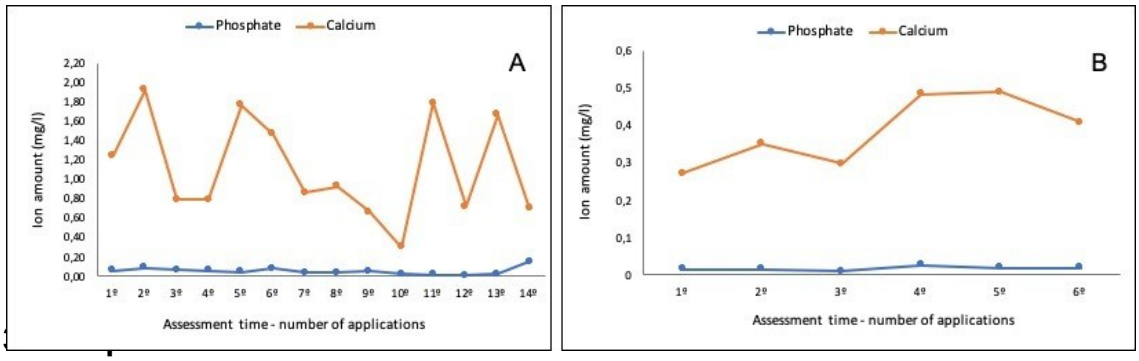


Figure 3.



3.3. Capítulo 3

Mineral loss and mechanical change in intact and white spot dental enamel after bleaching and remineralization

Artigo a ser submetido no periódico Operative Dentistry.

Mineral loss and mechanical change in intact and white spot dental enamel after bleaching and remineralization

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Running title: Change in intact and demineralized dental enamel after bleaching.

Keywords: Tooth bleaching; mineral loss; enamel; white spot injuries.

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CLINICAL RELEVANCE

Hydrogen peroxide applied to white spot enamel does not aggravate possible deleterious effects caused by the gels at 37.5% and 7.5%. The indication of bleaching to camouflage white spots by demineralization tend to be safe.

SUMMARY

Objectives: The aim of this study was to evaluate mineral loss of the intact (IE) and white spot (WSE) dental enamel after bleaching treatments with 37.5% and 7.5% hydrogen peroxide and after fluoride (F) application.

Methods: One hundred and fifty bovine enamel specimens were prepared and half were subjected to dynamic demineralization/remineralization cycling to generate white spot lesion. The specimens were randomly divided into 6 groups (n = 25): expressed by 2 “substrates”: IE and WSE; and 3 “treatment”: submitted to HP37.5% and HP7.5% and control (no treatment). The enamel topography analysis was analyzed by using scanning electron microscope (SEM) and the percentage by weight of Ca and P presented in the enamel substrate was calculated using energy dispersive spectroscopy (EDS). The enamel roughness average (Ra) was obtained by perfilometer and the Vickers hardness (VH) by using. All analyzes were performed before, after the bleaching protocol and after application of F (“assessment time”). Data were analyzed by three-way ANOVA with repeated measurement and Tukey Test (a = 0.05).

Results: Significant effect for the interaction “enamel” and “assessment time” (Ca: P=0.003/ P:P=0.005) for the presence of Ca and P The interaction between “enamel”, “assessment time” and “treatment” was significant to VH (P = 0.04) and Ra (P = 0.016). Bleaching did not influence the VH of the WSE enamel. IE VH has been reduced. The topographic changes were more evident on IE after HP 7.5% treatment. The fluoride application was not able to alter Ca and P contents in WSE but reduced Ra HP7.5%.

Conclusion: Conclusion: The bleaching protocols tested did not change mineral loss, VH and Ra in white spot enamel. The application of fluoride reduced Ra post bleaching with HP7.5%.

INTRODUCTION

The bleaching treatment has become attractive because it is a conservative procedure developed to harmonize the color of teeth.[1] Despite the development of materials and techniques, the concern about the effects on the surface of tooth enamel still exists.[2] Commonly products based on 10% to 22% carbamide peroxide and 10 to 40% hydrogen peroxide are used alone or in combination, in at home bleaching technique or in-office technique.[3,4]

Hydrogen peroxide, the main active agent in bleaching gels, acts as an oxidizing agent.[5,6] Free radicals and reactive oxygen molecules are generated that penetrate into the tooth structure making bonds with pigment molecules or chromophores to stabilize.[7] The breaking of the double bonds of these macromolecules resulting in smaller molecules that absorb less light, become lighter or diffuse away from the tooth.[7]

These free radicals are capable of degrading the organic and inorganic matrix of the dental substrate.[8,9] The degradation of proteins that make up the organic matrix interferes on the enamel mechanical properties,[8,10] and can lead to the microhardness reduction and increase the roughness.[11] Changes in the enamel mineral content contribute to the formation of depressions, porosity and erosion in the enamel.[12] The enamel demineralization caused by the bleaching procedure is also reported due to the loss of Ca and P from the enamel substrate to the saliva or to the bleaching gels.[13] However, when correctly applied, tooth bleaching is considered an effective and safe treatment.[14-17]

White spot lesions may be caused due the fluorosis, enamel hypoplasia, low calcium diet, and poor oral hygiene resulting in initial caries lesions. These lesions located on the buccal surface of the anterior teeth cause aesthetic discomfort and are frequently presented after orthodontic treatment.[18] Fluoride remineralization has been indicated for treating these lesions.[18] However, white spots do not always disappear and, consequently, the aesthetic compromise remains. In order to preserve tooth structure, tooth bleaching can be indicated to make white lesions less noticeable, as an alternative to the invasive techniques.[18,19]

The phenomena of demineralization and remineralization are continuous processes in the oral cavity, when white spot lesions are exposed to the remineralization process, additional minerals are incorporated into the surface layer.[20] There are different theories about the mechanism of action of tooth whitening, [21-29] and the possible interactions between different whitening gels and demineralized tooth enamel are not well established, as the deleterious effects can be reduced by the remineralization of saliva and fluoride.

Considering the knowledge gap regarding the action of bleaching agents on enamel white spot lesions, this study aimed to test the hypotheses that: 1- the white spots bleached with 7.5% or 37.5% hydrogen peroxide are more susceptible to Ca and P mineral loss, 2- fluoride remineralization is able to reduce the sides effects on white spots enamel.

METHODS AND MATERIALS

Preparation of specimens - Bleaching Procedures

One hundred and fifty bovine incisor teeth, of animals that had all the teeth already erupted and of the same age range, were extracted and stored in distilled water before and during the preparation of the specimens in a refrigerated environment at -10 °C for no more than 30 days.

Specimens were obtained per tooth in the region of the middle 1/3 of the buccal surface with dimensions of 5.0 mm x 5.0 mm x 4.0 mm using a water-cooled, low-rotation diamond saw (Buehler Ltd, Lake Bluff, IL, USA). For specimen preparation, the dental enamel surface was regularized and finished with abrasive sandpaper of decreasing grit size (# 600, #1000, #1200 and #1500, Arotec, Cotia, SP, Brazil) followed by polishing with a polishing cloth and diamond pastes (6 µm; 3 µm; 1 µm; 0.25 µm; Arotec, São Paulo, SP, Brazil) in a polisher (Arotec, Cotia, SP, Brazil). The sample was randomly divided into six groups (n = 25), being 3 groups with intact enamel (IE) and 3 with white spot (WSE) after pH cycling submitted to 37.5% hydrogen peroxide gel (HP37,5%, Pola office + SDI, Victoria, Australia), to 7.5% hydrogen peroxide gel (HP7,5%, Pola Day SDI, Victoria, Australia); and control group (C) without application of bleaching gel. In all methodologies the specimens were analyzed before, after the bleaching

procedures and after remineralization. The remineralization was simulated after the last session of each group. The specimens were rinsed in distilled water and two topical applications of 2% neutral fluorine phosphate gel (DFL, Rio de Janeiro, RJ, Brazil) of 60 s.[30] The specimens from the control group were analyzed before, after immersion in artificial saliva and after remineralization. All bleaching procedures were performed according to the manufacturer's instructions (Table 1). The specimens were kept immersed in artificial saliva and changed daily, stored at 37 ^{o}C until the next application.

pH cycling - Formation of artificial caries lesion:

Seventy-five samples underwent a dynamic cycling model of demineralization and remineralization. The demineralizing solution was composed of: 0.1 M acetate buffer, 1.28 mM Ca, 0.74 mM P and 0.03 µg F / mL, pH 5.0, at 37 ° C. Remineralizing solution composed of: buffer 20 mM Tris, 1.5 mM Ca, 0.9 mM P, 150 mM KCl, 0.05 µg F/ml; pH 7.0).[31] The samples were initially immersed in the demineralizing solution, remaining for 72 hours, then rinsed in running deionized water for 10 seconds, and carefully dried with air jets, to later be placed in the remineralizing solution where they remained for 24 hours, thus configuring a pH cycle. 3 consecutive cycles were performed. During the entire process, the samples remained in an oven at 37°C, except for washing intervals and alternating solutions.

SEM and EDS Analysis

For SEM analysis, the specimens were vacuum-plated with gold (Balzers, Berlin, Germany) and an enlargement of 20.000 X (Zeiss, Jena, Germany) was chosen for surface analysis by obtaining SEM images in greater detail. The percentage of Calcium (Ca) and Phosphorus (P) by weight (wt %) on the enamel surface was measured using EDS (Oxford, Abingdon-on-Thames, United Kingdom) with the same magnification. Five measurements were made per specimens in the area corresponding to the 20.000 x magnification image. The acquisition time was 30 s per measurement. To perform calculation adjustments, the EDS software, model INCA X-act (Oxford, Abingdon-on-Thames, United

Kingdom), has been calibrated to 56 nm thick gold layer. The Ca/P ratio was calculated for each specimen. The EDS analyses were semi-quantitative, performed with a window, with XPP correction method, without standards and the results were normalized to 100%.

Surface roughness

The surface roughness was measured using a surface profilometer (SJ-301 Surface Roughness Tester - Mitutoyo, Tokyo, Japan). The parameter used to obtain the surface roughness was roughness average (Ra), defined as the absolute deviation of the mean over the sample length and the most universally used roughness parameter for general quality control / analysis.[32] Five readings were taken to analyze each time (initial, post-bleaching and post-remineralization) for each sample. The extension of each reading was 2.8 mm, using a cutoff of 0.8 mm.

Microhardness

The samples were submitted to the microhardness test (FM700; Future Tech Corp., Kawasaki, Japan) using a Vickers diamond indenter under a load of 200 g for 15 s. Five indentations were performed on the enamel surface for analysis at each time (initial, post- bleaching and post-remineralization) in each sample, with interval of 1 mm between them to obtain an average value.

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 performing a normality and homogeneity test (Shapiro-Willks and Levene) the framing of data that meet the assumptions of parametric analysis was verified. 3-way ANOVA with repeated measurement and Tukey Test (a = 0.05) were applied in all methodologies.

RESULTS

The means and standard deviations of the pH values of the HP37.5% and HP7.5% groups are shown in Table 1. The means and standard deviations of calcium (Ca) and phosphorus (P) values on the enamel surface are showed in Table 2 and Table 3 respectively. The means and standard deviation of Ca/P ratio values are showed in Table 4. The means and standard deviations of microhardness and roughness are shown in Figure 3 and 4. Three-way repeated measures ANOVA revealed a significant effect for the interaction between “substrate”” and “assessment time” in EDS analyzes (Ca: P = 0.003/ P: P = 0.005). The intact enamel showed the increased Ca and P content after bleaching and decreased returning to baseline values after F application. The percentage of Ca and P in the WSE were not affected by regardless the “assessment time” or “treatment”. There was no change in the Ca/P ratio in the WSE lesions.

SEM images revealed changes after bleaching on the surface for both enamel substrates. Greater topographic changes were observed on IE after HP7.5% treatment (Figure 1 and 2). F application slowed topographical changes mainly in IE37.5%.

Three-way repeated measures ANOVA showed that the interaction between “substrate” “assessment time” and “treatment” was significant for microhardness, data are presented in Figure 3 (VH: P = 0.04) Bleaching protocols using HP7.5% and HP37.5% caused decreasing on VH values of the intact enamel. The F application was not able to recover the enamel at the VH baseline value. There was no significant difference in VH in white spot lesions after bleaching protocols or F application. ANOVA showed that the interaction between “substrate” “assessment time” and “treatment” was significant for

roughness data are presented in Figure 4 (Ra: P = 0.016). Ra made no significant changes to intact enamel. In white spot lesions, the application of F was able to reduce the roughness of 7.5% HP.

DISCUSSION

This study evaluated mineral loss of IE and WSE dental enamel after bleaching protocols using HP37.5% and HP7.5% gels indicated for in-office and at-home techniques, respectively, and also the effects of application F after bleaching.

Qualitative analyzes revealed topographic difference with superficial porosities in IE and WSE substrates. The greater topographic changes were observed after HP 7.5% bleaching treatment on IE group. The gels used in this study, HP37.5% and HP7.5% had basic pH (7.3) and acidic pH (5.5) respectively. Enamel exposed to bleaching products with acidic pH can presented the increased risk of superficial changes in the enamel,[33-35] which may explain the severe changes found with HP7,5%. However, remineralization using F solution generated smoothing surface for both IE and WSE substrates, accepting the second hypothesis.

Remineralizing agents such as F, Ca, amorphous calcium phosphate (ACP) and hydroxyapatite can be applied to prevent, minimize or treat potential adverse effects on the enamel surface after tooth bleaching.[36,37] Pre- and post-bleaching treatment with these agents has been recommended to recover the structural integrity of bleached enamel.[38,39] These agents can be applied after bleaching treatments as well as added to gels.[40] Although experimental bleaching agents with Ca or F reduce mineral loss for both intact and demineralized enamel surfaces, these agents do not were able to reverse the subsurface demineralization of the enamel.[41] In the present study, no difference in Ca and P content was observed for WSE after bleaching. The first hypothesis that WSE bleached with HP7.5% or HP37.5% are more susceptible to Ca and P loss was rejected. However, there were an increasing of Ca and P on intact enamel after both bleaching protocols due to hydroxyapatite breaking, resulting on leaving Ca

and P free on the surface leading to aa rearrangement of minerals on the surface.[42] In intact enamel, there is a higher content of Ca and P than in enamel that underwent demineralization and, therefore, the change in the percentage of these ions behaved differently in the two substrates. Thus, fluoride remineralization proved to be important as it manages to return the percentage of Ca and P in intact enamel to baseline values.

The F used induced a chemical reaction with the mineralized structure of the teeth, which leads to the formation of calcium fluoride (CaF2) deposits on enamel surfaces that induce the formation of fluorapatite, a structure with greater resistance.[43] The formation of CaF2 depends on pH, and reducing the pH of the medium increases the amount of CaF2 in the enamel, when it forms, the tooth surface is covered by calcium, phosphate and saliva proteins.

Ca and P in the specimens from the control group, which were not bleached and was kept in the artificial saliva demonstrated similar behavior to the groups bleached with peroxide. Possibly, the high concentrations of Ca and P in the artificial saliva used may have promoted a rapid precipitation of calcium phosphate mineral phases and this action causes the closure of porosities on the enamel surface.[44] The EDS analysis identifies the calcium phosphate compounds evaluating the atomic percentages of the Ca/P ratio.[45] In this study, the WSE, independent of “assessment time” and “treatment”, had a higher Ca/P ratio.

VH was the methodology chosen because it represents the mechanical properties of dental tissues and indirectly expressed their mineral content.[46] In this study, IE submitted to both bleached protocols suffered significant reduction on the VH values when compared to the control groups, even with the application of F. This reduction can be justified by the oxidation suffered by the organic and inorganic components of the enamel when bleached, which leads to changes in the enamel morphology through the development of porosities and microcracks.[47,48] In a previous study, samples bleached with HP7.5% demonstrated significant reduction of more than two-thirds in enamel hardness.[26] Bleaching agents with acidic pH caused greater reductions in VH values when compared to gels with neutral or slightly alkaline pH.[49]

In this study, the bleached WSE groups had no statistically significant differences in VH when analyzed before, after bleaching and after F application. The use of artificial saliva for storing specimens during the bleaching protocol may have promoted mineral deposition and, consequently, can explain the increasing on the VH values of the bleached WSE substrate. It is important to highlight that the WSE and remineralized enamel showed higher VH values than the IE. It is possible that there was calcium fluoride deposition on the surface[43] irregularities leading to an increase in hardness and a reduction in roughness as can be seen.

The bleaching treatments in the WSE showed greater porosity, probably due to the exposure of enamel prisms [50], especially with the use of HP7.5%. HP reacts in a non-selective way with the organic structures of dental tissues, causing porosity in the tooth. Studies comparing bleaching gels with different pHs, revealed an increase in Ra in the bleached enamel as the pH decreases. The pH of the HP7.5% gel tested in this study can be considered acidic (5.5) and resulted in greater damage to the surface of the white spot. However, F application was able to decrease the Ra values of WSE treated with HP7.5% gel, reaching level similar to IE in the same condition. The calcium phosphate mineral precipitation formed may have filled the valleys formed by the bleaching agent [44], leaving the surface smoother.

The limitations of this in vitro study are related to the characteristics of enamel and the structural differences between natural lesions and artificially created by pH cycling protocols. Another limitation refers to the fact that *in vivo* studies, the repair mechanism would more actively neutralize mineral loss than under *in vitro* conditions. Overall, bleaching with HP7.5% and HP37.5% are safe on WSE. Therefore, bleaching treatment for teeth with spot lesions can be recommended as a non-invasive aesthetic treatment with supplementary treatment after remineralization protocols.

CONCLUSION

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

1. The bleaching does not increase mineral loss on WSE.

2. The bleaching protocols using HP7,5% and HP37,5% decreased the VH values of IE, however no influence was verified on Ra and VH of WSE.
3. Severe surface topographic changes after HP7.5% bleaching was observed for only IE substrate.
4. The F application was not able to alter the Ca and P values but it increases the HV of bleached WSE and reduces Ra if HP 7.5% were used.

REFERENCES

1. Dahl JE & Pallesen U (2003) Tooth bleaching—a critical review of the biological aspects *Critical Reviews in Oral Biology and Medicine* **14** 292–304.

2. Trentino AC, Soares AF, Duarte MAH, Ishikiriama SK, Mondelli RFL (2015) Evaluation of ph levels and surface roughness after bleaching and abrasion tests of eight commercial products *Photomedicine and Laser Surgery* **33(7)** 372-377.

3. Sulieman M (2005) An overview of bleaching techniques: 2. Night Guard Vital Bleaching and non-vital bleaching *Dental Update* **32(1)** 39-46.

4. Buchalla A & Attin T (2007) External bleaching therapy with activation by heat, light or laser— A systematic review *Dental Materials* **23(5)** 586–596.

5. Bowles WH & Ugwuneri Z (1987) Pulp chamber penetration by hydrogen perox- ide following vital bleaching procedures *Journal of Endodontics* **13(8)** 375–377.

6. Feinman RA, Madray G, Yarborough D. 1991. Chemical, optical, and physi-ologic mechanisms of bleaching products: a review *Practical periodontics and aesthetic dentistry* **3(2)** 32–36.

7. Sulieman MAM (2008) An overview of tooth-bleaching techniques: chemistry, safety andefficacy *Periodontology 2000* **48** 148–169.

8. Borges BCD, Pinheiro MHM, Feitosa DAS, Correia TC, Braz R, Montes MAJR & Pinheiro IVA (2012) Preliminary study of a novel in-office bleaching therapy modified with a casein phosphopeptide-amorphous calcium phosphate *Microscopy Research and Technique* November **75(11)** 1571-1575.

9. Jiang T, Ma X, Wang Y, Tong H, Shen X, Hu Y & Hu J(2008) Investigation of the effects of 30% hydrogen peroxide on human tooth enamel by Raman

scattering and laser-induced fluorescence *Journal of Biomedical Optics* **13(1)** 014019.

10. He LH, Fujisawa N & Swain MV (2006) Elastic modulus and stress-strain response of human enamel by nano-indentation *Biomaterials* **27(24)** 4388-4398.

11. Jiang T, Ma X, Wang Z, Tong H, Hu J & Wang Y (2008) Beneficial effects of hydroxyapatite on enamel subjected 34 to 30% hydrogen peroxide *Journal of Dentistry*

36 (11) 907-914.

12. Bistey T, Nagy IP, Simó A & Hegedus C (2007) In vitro FT-IR study of the effects of hydrogen peroxide on superficial tooth enamel *Journal of Dentistry* **35(4)** 325–330.

13. LEE KH, KIM HI, KIM KH, & KWON YH (2006) Mineral loss from bovine enamel by a 30% hydrogen peroxide solution *Journal of Oral Rehabilitation* **33(3)** 229-233.

14. Cadenaro M, Breschi L, Nucci C, Antoniolli F, Visintini E, Prati C, Matis BA & Di Lenarda R (2008) Effect of two in-office whitening agents on the enamel surface in vivo: a morphological and non-contact profilometric study *Operative Dentistry* **33(2)** 127-134.

15. Spalding M, Taveira LA & de Assis GF (2003) Scanning electron microscopy study of dental enamel surface exposed to 35% hydrogen peroxide: alone, with saliva, and with 10% carbamide peroxide *Journal of Esthetic and Restorative Dentistry* **15(3)** 154-164.

16. Unlü N, Cobankara FK, Altinöz C & Ozer F (2004) Effect of home bleaching agents on the microhardness of human enamel and dentin *Journal of Oral Rehabilitation* **31(1)** 57-61.

17. Tanaka R, Shibata Y, Manabe A & Miyazaki T (2010) Micro-structural integrity of dental enamel subjected to two tooth whitening regimes *Archives of Oral Biology* **55(4)** 300-308.

18. Owda R & Sancakli HS (2021) Effects of Different Bleaching Agents on the Surface Topography and the Microhardness of Artificial Carious Lesions *European Journal of Dentistry* Aug **24**.

19. Kim Y, Son HH, Yi K, Ahn JS & Chang J (2016) Bleaching Effects on Color, Chemical, and Mechanical Properties of White Spot Lesions *Operative Dentistry* **41(3)** 318-326.
20. Jones RS & Fried D (2006) Remineralization of enamel caries can decrease optical reflectivity *Journal of Dental Research* **85(9)** 804-808.
21. Borges AB, Torres CR, de Souza PA, Caneppele TM, Santos LF & Magalhaes AC (2012) Bleaching gels containing calcium and fluoride: Effect on enamel erosion susceptibility *International Journal of Dentistry* 347848.
22. Torres CR, Crastechini E, Feitosa FA, Pucci CR & Borges AB (2014) Influence of pH on the effectiveness of hydrogen peroxide whitening *Operative Dentistry* **39(6)** E261-E268.
23. Borges AB, Zanatta RF, Barros AC, Silva LC, Pucci CR & Torres CR (2015) Effect of hydrogen peroxide concentration on enamel color and microhardness *Operative Dentistry* **40(1)** 96-101.
24. Dannacher JJ (2006) Catalytic bleach: Most valuable applications for smart oxidation chemistry *Journal of Molecular Catalysis A Chemical* **251 (1)** 159-176.
25.Markovic L, Jordan RA, Lakota N & Gaengler P (2007) Micromorphology of enamel surface after vital tooth bleaching *Journal of Endodontics* **33(5)** 607-610.
26. Pinto CF, Oliveira R, Cavalli V & Giannini M (2004) Peroxide bleaching agent effects on enamel surface microhardness, roughness and morphology *Brazilian Oral Research* **18(4)** 306-311.
27. Cavalli V, Arrais CA, Giannini M & Ambrosano GM (2004) High-concentrated carbamide peroxide bleaching agent effects on enamel surface. *Journal of Oral Rehabilitation* **31(2)** 155-159.
28. Fu B, Hoth-Hannig W & Hannig M (2007) Effects of dental bleaching on micro- and nano-morphological alterations of the enamel surface American *Journal of Dentistry* **20(1)** 35-40.
29. Jiang T, Guo YR, Feng XW, Sa Y, Yang X, Wang M, Li P & Wang YN (2018) Hydrogen Peroxide Might Bleach Natural Dentin by Oxidizing Phosphoprotein *Journal of Dental Research* **97(12)** 1339-1345.

30. Calvo AF, Tabchoury CP, Del Bel Cury AA, Tenuta LM, da Silva WJ & Cury JA (2012) Effect of acidulated phosphate fluoride gel application time on enamel demineralization of deciduous and permanent teeth *Caries Research* **46(1)** 31-7.
31. Queiroz CS Hara AT, Paes Leme AF & Cury JA (2008) pH-Cycling Models to Evaluate the Effect of Low Fluoride Dentifrice on Enamel De- and Remineralization *Brazilian Dental Journal* **19(1)** 21-27.
32. Gadelmawla ES (2002) Roughness parameters *The Journal of Materials Processing Technology* **123** 133-145.
33. Ushigome T, Takemoto S, Hattori M, Yoshinari M, Kawada E & Oda Y (2009) Influence of peroxide treatment on bovine enamel surface--cross-sectional analysis *Dental Materials Journal* **28(3)** 315-323.
34. Ferreira AF, Perez FM, Limeira Júnior F de A, de Moura M de F & de Sousa FB (2016) Graded changes in enamel component volumes resulted from a short tooth bleaching procedure Archives of Oral Biology **65,** 52-58.
35. Balladares L, Alegría-Acevedo LF, Montenegro-Arana A, Arana-Gordillo LA, Pulido C, Salazar-Gracez MT, Reis A, Loguercio AD (2019) Effects of pH and Application Technique of In-office Bleaching Gels on Hydrogen Peroxide Penetration into the Pulp Chamber *Operative Dentistry* **44(6)** 659-667.
36. Coceska E, Gjorgievska E, Coleman NJ, Gabric D, Slipper IJ, Stevanovic M & Nicholson, JW (2016) Enamel alteration following tooth bleaching and remineralization *Journal of Microscopy* **262(3)** 232–244.
37. Sasaki RT, Catelan A, Bertoldo Edos S, Venâncio PC, Groppo FC, Ambrosano GM, Marchi GM, Lima DA & Aguiar FH (2015) Effect of 7.5% hydrogen peroxide containing remineralizing agents on hardness, color change, roughness and micromorphology of human enamel *American Journal of Dentistry* **28(5)** 261–267**.**
38. Vieira-Junior WF, Lima DA, Tabchoury CP, Ambrosano GM, Aguiar FH & Lovadino JR (2016) Effect of Toothpaste Application Prior to Dental Bleaching on Whitening Effectiveness and Enamel Properties *Operative Dentistry* **41(1)** E29-38.

39. Bilge K & Kılıç V (2021) Effects of different remineralizing agents on color stability and surface characteristics of the teeth following vital bleaching *Microscopy Research and Technique* **84(10)** 2206-2218.
40. Cavalli V, Rodrigues LKA, Paes-Leme AF, Soares LES, Martin AA, Berger SB & Giannini, M (2011) Effects of the addition of fluoride and calcium to low-concentrated carbamide peroxide agents on the enamel surface and subsurface *Photomedicine and Laser Surgery* **29(5)** 319-325.
41. Cavalli V, Rosa DAD, Silva DPD, Kury M, Liporoni PCS, Soares LES & Martins AA (2018) Effects of experimental bleaching agents on the mineral content of sound and demineralized enamels *Journal of Applied Oral Science* **26** e20170589.
42. Tanaka R, Shibata Y, Manabe A & Miyazaki T (2010) Micro-structural integrity of dental enamel subjected to two tooth whitening regimes *Archives of Oral Biology* **55(4)** 300-308.
43. Kanzow P, Wegehaupt FJ & Attin T (2016) Wiegand A. Etiology and pathogenesis of dental erosion *Quintessence International* **47(4)** 275-8.
44. García-Godoy F, & Hicks MJ (2008) Maintaining the integrity of the enamel surface: the role of dental biofilm, saliva and preventive agents in enamel demineralization and remineralization. *Journal of the American Dental Association **139** Suppl*, 25S–34S.
45. Miculescu F, Luță C, Constantinescu AE, Maidaniuc A, Mocanu AC, Miculescu M, Voicu ȘI, Ciocan LT (2020) Considerations and Influencing Parameters in EDS Microanalysis of Biogenic Hydroxyapatite *Journal of Functional Biomaterials* 15 **11(4)** 82.
46. Pinto CF, Paes LemeAF, Cavalli V & Giannini M (2009) Effect of bleaching with 10% carbamide peroxide in carious lesions of healthy and artificial enamel *Brazilian Dental Journal* **20(01)** 48-53
47. Klarić E, Marcius M, Ristić M, Sever I, Prskalo K &Tarle Z (2013) Surface Changes of Enamel and Dentin after Two Different Bleaching Procedures *Acta Clinica Croatica* **52(4)** 419-429.

48. Markovic L, Jordan RA, Lakota N & Gaengler P (2007) Micromorphology of Enamel Surface after Vital Tooth Bleaching *Journal of Endodontics* **33(5)** 607-610.

49. Furlan IS, Bridi EC, Amaral FLBD, França FMG, Turssi CP & Basting RT (2017) Effect of High-or Low-Concentration Bleaching Agents Containing Calcium and/or Fluoride on Enamel Microhardness *General dentistry* **65(3)** 66-70.

55. Polydorou O, Scheitza S, Spraul M, Vach K, Hellwig E. (2018) The effect of long-term use of tooth bleaching products on the human enamel surface *Odontology* **106** 64-72.

Tables

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

Material	Group	Treatment	pH	Batch number	Manufactures
Pola office + PH a 37.5%	HP37.5%	2 sessions of 3 applications of 8 minutes each	7.33 (0.1)	190624132836	SDI, Victoria, Australia
Pola Day + PH a 7.5%	HP7.5%	45 minutes/day application for 14 days	5.52 (0.1)	190719081529	SDI, Victoria, Australia
Specimen without contact with bleaching gel	Control	Storage in artificial saliva (1.5mm Ca and 0.9mm P in 0.1mm Tris buffer solution)		-	-

Table 2. Means and standard deviation of Ca values (%) on the enamel surface after application of bleaching gels and control group on intact and white spot enamel.

Groups	Intact Enamel (EI)			Demineralized Enamel (DE)		
	Initial	Treated	Remineralized	Initial	Treated	Remineralized
Control	33.9 (2.1) Bb	35.2 (1.9) Aa	35.8 (1.0) ABb	38.3 (1.3) Aa*	37.9 (2.6) Aa	37.5 (1.2) Aa
HP37.5%	34.0 (1.8) Bb	38.1 (0.9) Aa	34.5 (1.5) Ab	38.0 (2.2) Aa*	36.2 (1.7) Aa	38.9 (3.1) Aa
HP7.5%	33.9 (2.3) Bb	37.4 (1.4) Aa	34.8 (2.2) Ab	38.2 (1.0) Aa*	38.0 (0.6) Aa	36.1 (2.9) Aa

Capital letter to compare assessment time (Initial; bleached; remineralized) for each treatment; Lowercase to compare treatment (Control, HP37.5%; HP7.5%) for each assessment time; *Compares substrate (Intact and Demineralized) for each assessment time and treatment. 3-way RM ANOVA and Tukey Test, $p < 0.05$).

Table 3. Means and standard deviation of P values (%) on the enamel surface after application of bleaching gels and control group on intact and demineralized enamel.

Groups	Intact Enamel (EI)			Demineralized Enamel (DE)		
	Initial	Treated	Remineralized	Initial	Treated	Remineralized
Control	18.0 (0.4) Ba	18.7 (0.7) Aa	18.8 (0.5) Ba	19.1 (0.5) Aa*	19.0 (0.7) Aa	18.6 (0.9) Aa
HP37.5%	18.1 (0.9) Ba	19.1 (0.7) Aa	18.0 (0.2) Ba	18.9 (0.8) Aa*	18.5 (0.9) Aa	19.1 (0.8) Aa
HP7.5%	17.9 (0.9) Ba	19.4 (0.6) Aa	17.6 (0.7)^{Ab} Ba	19.1 (0.2) Aa*	18.9 (0.5) Aa	18.3 (0.6) Aa

Capital letter to compare assessment time (Initial; Bleached; Remineralized) for each treatment; Lowercase to compare treatment (Control, HP37.5%; HP7.5%) for each assessment time; *Compares substrate (Intact and Demineralized) for each assessment time and treatment. 3-way RM ANOVA and Tukey Test, $p < 0.05$).

Table 4. Means and standard deviation of Ca/P ratio on the enamel surface after application of bleaching gels and control group on intact and demineralized enamel.

Groups	Intact Enamel (IE)			White spot (WE)		
	Initial	Treated	Remineralized	Initial	Treated	Remineralized
Control	1.89(0.12) Aa	1,88(0.05) Aa	1,9(0.03) Aa	2,01(0.04) Aa*	1,99(0.09) Aa*	2,02(0.07) Aa*
HP37.5%	1.88(0.07) Aa	1.99(0.04) Aa	1.92(0.06) Aa	2.01(0.06) Aa*	1.96(0.03) Aa*	2,03(0.07) Aa*
HP7.5%	1.89(0.09) Aa	1.93(0.03) Aa	1.97(0.07) Aa	1.99(0.05) Aa*	2.01(0.04) Aa*	1.97(0,12) Aa*

Capital letter to compare assessment time (Initial; Bleached; Remineralized) for each treatment; Lowercase to compare treatment (Control, HP37.5%; HP7.5%) for each assessment time; *Compares substrate (Intact and Demineralized) for each assessment time and treatment. 3-way RM ANOVA and Tukey Test, $p < 0.01$

Figure legends

Figure 1. Representative SEM image of the demineralized enamel surface (DE) after application of bleaching gels and control group: A) HP7.5% and B) HP37.5% application showing porosities; C) no treatment (control group) showing no changes in the surface. F application slowed topographical changes in all groups: D) HP7.5%, E) HP37.5% and F) control group.

Figure 2. Representative SEM image of the intact enamel (IE) surface after application of bleaching gels and in the control group: A) HP7.5% and B) HP37.5% application showing porosities and depressions; B) no treatment (control group) showing no changes in the smooth polished surface. . F application slowed topographical changes in all groups: D) HP7.5%, E) HP37.5% and F) control group.

Figure 3. Graphic image representing the three way ANOVA and Tukey analyzes for roughness (Ra) on the enamel surface after application of bleaching gels and in the control group. (uppercase letters to compare conditions and lowercase letters compare substrate for each solution).

Figure 4. Mean and standard deviation of Vickers Hardness (VH) on the enamel surface after application of bleaching gels and control group (capital letters to compare conditions and lower case letters compare substrate for each solution).

Figures

Figure 1.

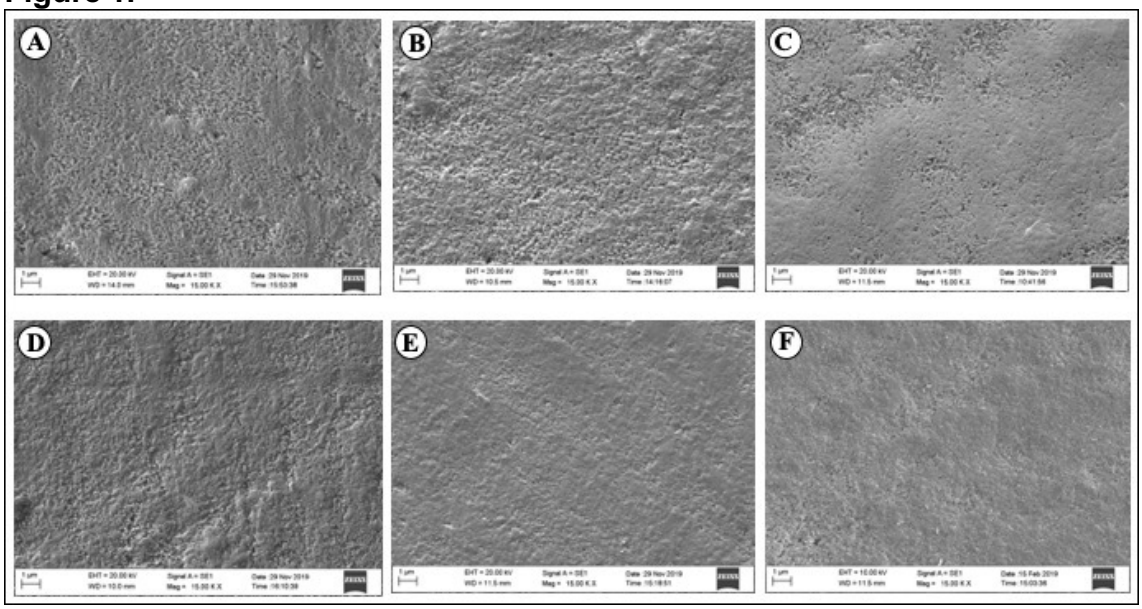


Figure 2.

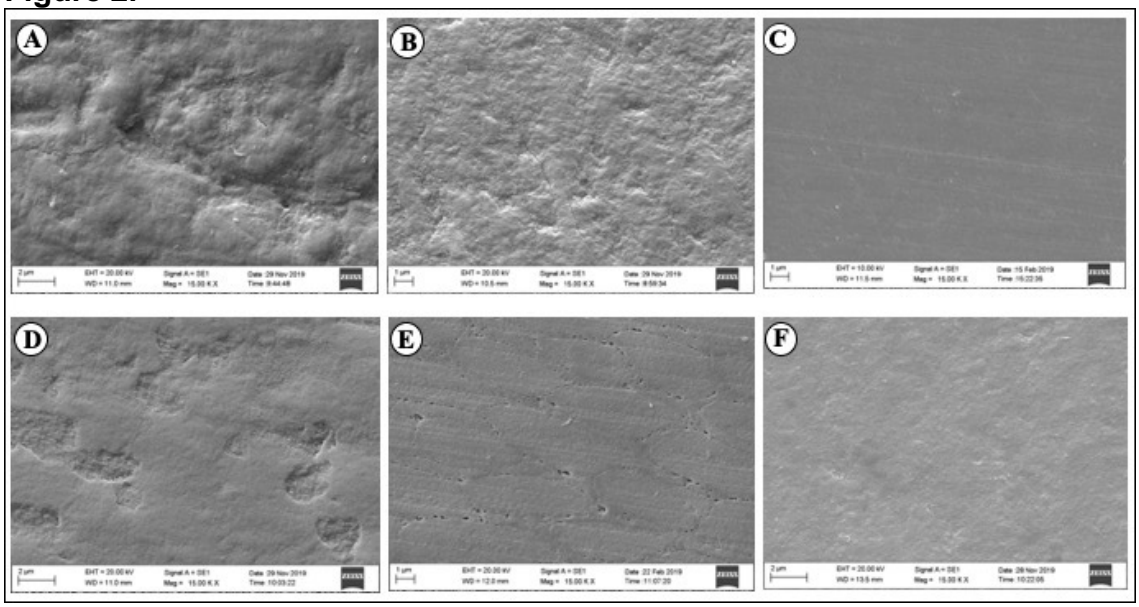
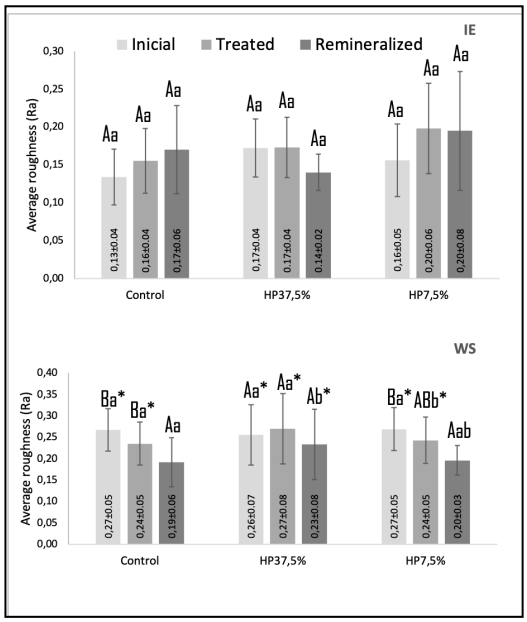
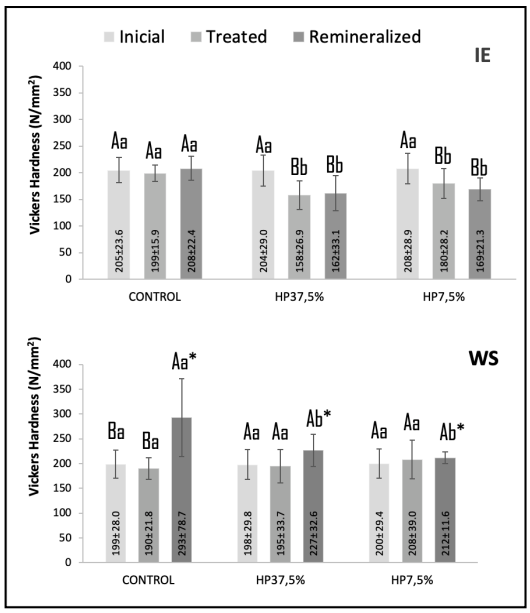


Figure 3



Capital letter to compare assessment time (Initial; Bleached; Remineralized) for each treatment; Lowercase to compare treatment (Control, HP37.5%; HP7.5%) for each assessment time; *Compares substrate (Intact and Demineralized) for each assessment time and treatment.

Figure 4.



Capital letter to compare assessment time (Initial; Bleached; Remineralized) for each treatment; Lowercase to compare treatment (Control, HP37.5%; HP7.5%) for each assessment time; *Compares substrate (Intact and Demineralized) for each assessment time and treatment.

4. Conclusão

4. CONCLUSÃO

Dentro das limitações metodológicas dos três estudos laboratoriais, pode-se concluir que:

- O método micro-CT foi capaz de avaliar a perda de estrutura do esmalte em termos de volume e profundidade com alta correlação com os resultados de EDS;
- A adição de Ca na composição do gel clareador não foi capaz de prevenir a desmineralização da superfície do esmalte, que foi mínima e superficial;
- O esmalte sofreu perda mineral principalmente próximo à superfície, independentemente do uso de gel clareador PH 35% com ou sem Ca mas nenhuma alteração na rugosidade da superfície foi observada;
- O tratamento clareador com PH37,5% e PH7,5%, independente da técnica, provoca alterações minerais do esmalte dentário; no entanto, as mudanças foram mais graves com 7,5%;
- A cromatografia iônica é eficaz para detectar no gel clareador, após o contato com o esmalte dentário, a perda de íons causada pelo tratamento clareador.
- A presença de mancha branca em esmalte causada por desmineralização não reduz a presença de Ca e P;
- A presença de desmineralização no esmalte não potencializa o efeito deletério do clareamento dental com géis de peróxido de hidrogênio 37,5% e 7,5%;

5. Referências

5. REFERÊNCIAS

Attia ML, Cavalli V, do Espírito Santo AM, Martin AA, D'Arce MB, Aguiar FH, et al. Effects of Bleaching Agents Combined with Regular and Whitening Toothpastes on Surface Roughness and Mineral Content of Enamel. **Photomed Laser Surg.** 2015;33(7):378-83. doi: 10.1089/pho.2014.3835. PMID: 26154726.

Balladares L, Alegría-Acevedo LF, Montenegro-Arana A, Arana-Gordillo LA, Pulido C, Salazar-Gracez MT, et al . Effects of pH and Application Technique of In-office Bleaching Gels on Hydrogen Peroxide Penetration into the Pulp Chamber. **Oper Dent.** 2019;44(6):659-667. doi: 10.2341/18-148-L. Epub 2019 Apr 22. PMID: 31009314.

Bilge K, Kılıç V. Effects of different remineralizing agents on color stability and surface characteristics of the teeth following vital bleaching. **Microsc Res Tech.** 2021;84(10):2206-2218. doi:10.1002/jemt.23774.

Borges BC, Pinheiro MH, Feitosa DA, Correia TC, Braz R, Montes MA, et al. Preliminary study of a novel in-office bleaching therapy modified with a casein phosphopeptide-amorphous calcium phosphate. **Microsc Res Tech.** 2012;75(11):1571-1575. doi:10.1002/jemt.22102.

Borges AB, Zanatta RF, Barros AC, Silva LC, Pucci CR, Torres CR. Effect of hydrogen peroxide concentration on enamel color and microhardness. **Oper Dent.** 2015;40(1):96-101. doi:10.2341/13-371-L.

Cadenaro M, Breschi L, Nucci C, Antoniolli F, Visintini E, Prati C, et al. Effect of two in-office whitening agents on the enamel surface in vivo: a morphological and non-contact profilometric study. ***Oper Dent.*** 2008;33(2):127-134. doi:10.2341/07-89.

Cavalli V, Arrais CA, Giannini M, Ambrosano GM. High-concentrated carbamide peroxide bleaching agents effects on enamel surface. **J Oral Rehabil.** 2004;31(2):155-9. doi: 10.1111/j.1365-2842.2004.01138.x. PMID: 15009600.

Cavalli V, Rodrigues LK, Paes-Leme AF, Brancalion ML, Arruda MAZ, Bittencourt Berger S, et al. Effects of bleaching agents containing fluoride and calcium on human enamel. **Quintessence Int.** 2010;41(8):703.

Cavalli V, Rodrigues LKA, Paes-Leme AF, Soares LES, Martin AA, Berger SB, et al. Effects of the addition of fluoride and calcium to low-concentrated

carbamide peroxide agents on the enamel surface and subsurface. **Photomed Laser Surg.** 2011;29(5):319-325. doi:10.1089/pho.2010.2797.

Coceska E, Gjorgievska E, Coleman NJ, Gabric D, Slipper IJ, Stevanovic M, et al. Enamel alteration following tooth bleaching and remineralization. **J Microsc.** 2016;262(3):232-244. doi:10.1111/jmi.12357.

Dawes C. What is the critical pH and why does a tooth dissolve in acid? **J Can Dent Assoc.** 2003;69(11):722-724.

Dannacher JJ. Catalytic bleach: Most valuable applications for smart oxidation chemistry. **Molecular Catalysis**. 2006;251(1):159-176. doi: 10.1016/j.molcata.2006.02.031.

De Carvalho AC, de Souza TF, Liporoni PC, Pizi EC, Matuda LA, Catelan A (2020) Effect of bleaching agents on hardness, surface roughness and color parameters of dental enamel. **J Clin Exp Dent.** 12(7) e670-e675.

Efeoglu N, Wood D, Efeoglu C. Microcomputerised tomography evaluation of 10% carbamide peroxide applied to enamel. **J Dent.** 2005;33(7):561-567. doi:10.1016/j.jdent.2004.12.001

Elsayad I, Sakr A, Badr Y. Combining casein phosphopeptide-amorphous calcium phosphate with fluoride: synergistic remineralization potential of artificially demineralized enamel or not?. **J Biomed Opt.** 2009;14(4):044039. doi:10.1117/1.3210780.

Ferreira AF, Perez FM, Limeira Júnior Fde A, de Moura Mde F, de Sousa FB. Graded changes in enamel component volumes resulted from a short tooth bleaching procedure. **Arch Oral Biol.** 2016;65:52-58. doi:10.1016/j.archoralbio.2016.01.016.

Frankenberger WT, Mehra HC, Gjerde DT. Environmental applications of ion chromatography. **J Chromatogr A.** 1990; 504**:**211-245.

Fu B, Hoth-Hannig W, Hannig M. Effects of dental bleaching on micro- and nano-morphological alterations of the enamel surface. **Am J Dent.** 2007;20(1):35-40.

Fujii M, Kitasako Y, Sadr A, Tagami J. Roughness and pH changes of enamel surface induced by soft drinks in vitro-applications of stylus profilometry, focus variation 3D scanning microscopy and micro pH sensor. **Dent Mater J.** 2011;30(3):404-410. doi:10.4012/dmj.2010-204.

Gomes MN, Rodrigues FP, Silikas N, Francci CE. Micro-CT and FE-SEM enamel analyses of calcium-based agent application after bleaching. **Clin Oral Investig.** 2018;22(2):961-970. doi:10.1007/s00784-017-2175-2.

Haddad PR, Nesterenko PN, Buchberger W. Recent developments and emerging directions in ion chromatography. **J Chromatogr A.** 2008;1184(1-2):456-473. doi:10.1016/j.chroma.2007.10.022.

Hughes JA, West NX, Parker DM, van den Braak MH, Addy M. Effects of pH and concentration of citric, malic and lactic acids on enamel, in vitro. **J Dent.** 2000;28(2):147-152. doi:10.1016/s0300-5712(99)00060-3.

Jiang T, Ma X, Wang Z, Tong H, Hu J, Wang Y. Beneficial effects of hydroxyapatite on enamel subjected to 30% hydrogen peroxide. **J Dent.** 2008;36(11):907-914. doi:10.1016/j.jdent.2008.07.005.

Jiang T, Ma X, Wang Y, et al. Investigation of the effects of 30% hydrogen peroxide on human tooth enamel by Raman scattering and laser-induced fluorescence. **J Biomed Opt.** 2008;13(1):014019. doi:10.1117/1.2870114.

Joiner A. Tooth colour: a review of the literature. **J Dent.** 2004;32 Suppl 1:3-12. doi:10.1016/j.jdent.2003.10.013.

Jones RS, Fried D. Remineralization of enamel caries can decrease optical reflectivity. **J Dent Res.** 2006;85(9):804-808. doi:10.1177/154405910608500905

Kwon YH, Huo MS, Kim KH, Kim SK, Kim YJ. Effects of hydrogen peroxide on the light reflectance and morphology of bovine enamel. **J Oral Rehabil.** 2002;29(5):473-477. doi:10.1046/j.1365-2842.2002.00856.x

Kim Y, Son HH, Yi K, Ahn JS, Chang J. Bleaching Effects on Color, Chemical, and Mechanical Properties of White Spot Lesions. **Oper Dent.** 2016;41(3):318-326. doi:10.2341/15-015-L.

Lee KH, Kim HI, Kim KH, Kwon YH. Mineral loss from bovine enamel by a 30% hydrogen peroxide solution. **J Oral Rehabil.** 2006;33(3):229-233. doi:10.1111/j.1365-2842.2004.01360.x

Lynch E, Sheerin A, Samarawickrama DY, Atherton MA, Claxson AW, Hawkes J, et al. Molecular mechanisms of the bleaching actions associated with commercially-available whitening oral health care products. J Ir Dent Assoc. 1995;41(4):94-102.

Llena C, Esteve I, Forner L. Effects of in-office bleaching on human enamel and dentin. Morphological and mineral changes. **Ann Anat.** 2018;217:97-102. doi:10.1016/j.aanat.2018.01.003

Markovic L, Jordan RA, Lakota N, Gaengler P. Micromorphology of enamel surface after vital tooth bleaching. **J Endod.** 2007;33(5):607-610. doi:10.1016/j.joen.2007.01.011.

Oliveira LRS, Braga SSL, Bicalho AA, Ribeiro MTH, Price RB, Soares CJ. Molar cusp deformation evaluated by micro-CT and enamel crack formation to compare incremental and bulk-filling techniques. **J Dent.** 2018;74:71-78. doi:10.1016/j.jdent.2018.04.015.

Park HJ, Kwon TY, Nam SH, Kim HJ, Kim KH, Kim YJ. Changes in bovine enamel after treatment with a 30% hydrogen peroxide bleaching agent. **Dent Mater J.** 2004;23(4):517-521. doi:10.4012/dmj.23.517.

Peters MC, Bresciani E, Barata TJ, Fagundes TC, Navarro RL, Navarro MF, et al. In vivo dentin remineralization by calcium-phosphate cement. **J Dent Res.** 2010;89(3):286-291. doi:10.1177/0022034509360155.

Price RB, Sedarous M, Hiltz GS. The pH of tooth-whitening products. **J Can Dent Assoc.** 2000;66(8):421-426.

Rauen CA, Filho JCC, Bittecourt BF, Gomes GM, Gomes JC, Gomes OMM. Effect of bleaching agents containing fluoride or calcium on enamel microhardness, roughness and permeability. **J Oral Sci.** 2015;14(4): 262-6.

Sasaki RT, Catelan A, Bertoldo Edos S, Venâncio PC, Groppo FC, Ambrosano GM, et al. Effect of 7.5% hydrogen peroxide containing remineralizing agents on hardness, color change, roughness and micromorphology of human enamel. **Am J Dent.** 2015;28(5):261-267.

Schemehorn BR & Novak ED. Use of a calcium peroxide whitening agent for remineralization and recalcification of incipient lesions. **J Clin Dent.** 2007;18(4):126-30.

Sun, G. The role of lasers in cosmetic dentistry. **Cent Clin Nor Am.** 2000; 44(4): 831-50.

Tanaka R, Shibata Y, Manabe A, Miyazaki T. Micro-structural integrity of dental enamel subjected to two tooth whitening regimes. **Arch Oral Biol.** 2010;55(4):300-308. doi:10.1016/j.archoralbio.2010.02.009.

Pinto CF, Oliveira Rd, Cavalli V, Giannini M. Peroxide bleaching agent effects on enamel surface microhardness, roughness and morphology. **Braz Oral Res.** 2004;18(4):306-311. doi:10.1590/s1806-83242004000400006.

Ushigome T, Takemoto S, Hattori M, Yoshinari M, Kawada E, Oda Y. Influence of peroxide treatment on bovine enamel surface--cross-sectional analysis. **Dent Mater J.** 2009;28(3):315-323. doi:10.4012/dmj.28.315

Vieira-Junior WF, Lima DA, Tabchoury CP, Ambrosano GM, Aguiar FH, Lovadino JR. Effect of Toothpaste Application Prior to Dental Bleaching on Whitening Effectiveness and Enamel Properties. **Oper Dent.** 2016;41(1):E29-E38. doi:10.2341/15-042-L

Vieira I, Vieira-Junior WF, Pauli MC, Theobaldo JD, Aguiar FH, Lima DA, Leonardi GR. (Effect of in-office bleaching gels with calcium or fluoride on color, roughness, and enamel microhardness. **J. Clin. Exp. Dent.** 2020; 12(2) e116-e122.

6. Anexos

6. ANEXOS

6.1 Normas periódico

1, 2 e 3. Operative Dentistry

https://jopdent.com/author-review-for-journal/instructions-to-authors/