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MARIANGELA IVETTE GUANIPA ORTIZ

Avaliação da incorporação de sub-micropartículas de polifosfato de cálcio em clareadores à base de peróxido de hidrogênio de alta concentração e análise dos seus efeitos nas propriedades físico-químicas do esmalte dental

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Orientadora: Profa. Dra. Débora Alves Nunes Leite Lima
Coorientador: Prof. Dr. Klaus Heinz Rischka

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RESUMO

O presente estudo caracterizou géis clareadores experimentais à base de peróxido de hidrogênio (PH) em alta concentração (35%) contendo sub-micropartículas de polifosfato de cálcio (PPCa) associado ou não a uma enzima fosfatase (ALP), e avaliou *in vitro* o seu efeito em as propriedades físico-químicas do esmalte dental. Para a primeira fase do Estudo o PPCa foi sintetizado por co-precipitação. A morfologia, tamanho e características químicas e a fase do PPCa foram caracterizados por microscopia eletrônica de varredura e transmissão, espectroscopia de raios X por energia dispersiva (EDX), e difração de raios X, respectivamente. Os géis clareadores avaliados foram: Comercial (Whiteness HP Maxx, FGM); Sem-PPCa; 0,5%-PPCa; 1,5%-PPCa. Os valores de pH dos géis e concentração de PH (titulação) foram determinados. A viabilidade de células tipo odontoblasto (MDPC-23) após a exposição do gel foi avaliada pelo ensaio MTT. Para a segunda fase do Estudo blocos de esmalte/dentina foram preparados e aleatorizados em: Controle (Sem tratamento); Comercial; Sem-PPCa; 0,5%-PPCa; 1,5%-PPCa. Os grupos avaliados receberam três sessões de clareamento (três aplicações de 15 min). Antes, durante e depois do clareamento os espécimes (n=12) tiveram as propriedades físicas, mecânicas e químicas avaliadas mediante espectrofotometria de refletância, determinando os índices de alteração de cor e clareamento (ΔE, ΔE00, ΔWID), rugosidade (Ra), microdureza de superfície (SMH), EDX e espectroscopia de Raman (FT-Raman), respectivamente. Para a terceira fase do Estudo a atividade da ALP e as propriedades químicas (concentração de PO_4^{3-}) dos géis contendo ou não ALP foram avaliadas mediante ensaios colorimétricos em espessantes à base de água ou tampão-tris. Também discos de esmalte/dentina foram preparados e alocados, para receber três sessões de clareamento (três aplicações de 15 min), em: Controle (Sem tratamento); Comercial; Exp-H (Sem-PPCa-H_2O); PPCa-H (0,5 wt%); PPCa-H+ALP (ALP); Exp-T (Sem-PPCa-Tris); PPCa-T (0,5 wt%); PPCa-T+ALP (ALP). Antes, durante e depois do clareamento os espécimes (n=10) tiveram as propriedades físico-mecânicas avaliadas: ΔE, ΔE00, ΔWID, SMH, e microdureza transversal (CSMH). Após análise estatística de cada fase observou-se que: as partículas de PPCa eram esféricas, com Ca e P, diâmetro de 135,7±80,95 nm e amorfas. A concentração de PH diminuiu em todos os grupos após a mistura. O grupo 0,5%-PPCa apresentou níveis de pH mais estáveis (neutro) e de viabilidade celular mais altos do que o Sem-PPCa. Os níveis de PO_4^{3-} foram maiores

nos espessantes contendo ALP. Os grupos tratados com géis contendo PPCa e/ou ALP apresentaram eficácia clareadora (ΔE, ΔE00, ΔWID) similar ao Comercial. Não existiu alteração significativa da Ra dos espécimes. O grupo Sem-PPCa apresentou alteração do seu conteúdo de carbonato e menores valores de Ca:P. Enquanto que os valores de SMH e CSMH foram maiores para os grupos contendo PPCa, especialmente quando associado à ALP. De tal forma é possível estabelecer que após a adequada síntese, caracterização e incorporação do PPCa em géis clareadores (PH-PPCa), as propriedades físicas e químicas do esmalte clareado com PH-PPCa, especialmente quando associado à ALP, apresentaram um potencial positivo para reduzir as alterações físico-químicas associadas à terapia clareadora de forma biologicamente inspirada.

Palavras-chave: Polifosfatos. Clareamento dental. Clareadores Dentários. Fosfatase Alcalina.

ABSTRACT

The present study characterized an experimental bleaching gel based on hydrogen peroxide (HP) at high concentration (35%) containing sub-microparticles of calcium polyphosphate (CaPP) associated or not with a phosphatase enzyme (ALP), and evaluated *in vitro* its effect on the physicochemical properties of dental enamel. For the first phase of the study, CaPP sub-microparticles were synthesized by co-precipitation. The morphology, size, and chemical characteristics and the CaPP phase were characterized by scanning and transmission electron microscopy, energy dispersive X-ray spectroscopy (EDX), and X-ray diffraction, respectively. The bleaching gels evaluated were: Commercial (Whiteness HP Maxx, FGM); Experimental (without CaPP); 0.5%-CaPP; 1.5%- CaPP. The pH values of the gels and HP concentration (titration) were determined. The viability of odontoblast-like cells (MDPC-23) after gel exposure was assessed by MTT assay. For the second phase of the study enamel/dentin specimens were prepared and randomized into: Control (No treatment); Commercial; Experimental; 0.5%-CaPP; 1.5%-CaPP. The bleached groups received three bleaching sessions. Before, during, and after bleaching the specimens had their physical, mechanical, and chemical properties evaluated by reflectance spectrophotometry (ΔE, ΔE00, ΔWID), roughness (Ra), surface microhardness (SMH), and EDX and Raman spectroscopy (FT-Raman), respectively. For the third phase of the study, ALP activity and chemical properties (PO_4^{3-} concentration) of gels containing or not containing ALP were evaluated by colorimetric assays in water-based or tris-based thickeners. Also enamel/dentin specimens were allocated to receive three bleaching sessions in: Control (No treatment); Commercial; Exp-H (Experimental in H_2O); CaPP-H (0.5 wt%); CaPP-H+ALP (ALP); Exp-T (Experimental in Tris); CaPP-T (0.5 wt%); CaPP-T+ALP (ALP). Before, during, and after bleaching the specimens had their physical and mechanical properties evaluated by reflectance spectrophotometry (ΔE, ΔE00, ΔWID), SMH, and cross-sectional microhardness (CSMH). After statistical analysis of each phase of the study, it was determined that the: CaPP particles were spherical, with Ca and P, a diameter of 135.7 ± 80.95 nm, and amorphous. The HP concentration decreased in all groups after mixing. The 0.5%-CaPP group showed more stable pH levels and higher viability than the Experimental. PO_4^{3-} levels were higher in the thickeners containing ALP. After bleaching, the groups treated with CaPP-containing gels showed similar bleaching efficacy (ΔE, ΔE00, ΔWID) to the Commercial. There was no significant change in the

specimens' Ra. The Experimental group showed a change in its carbonate content and lower Ca:P values. While SMH and CSMH values were higher for the groups containing CaPP, especially when associated with ALP. Thus, it is possible to establish that the synthesis, characterization, incorporation, and analysis of the physical and chemical properties of the enamel bleached with experimental bleaching gels containing CaPP and especially associated with ALP presents a good potential to reduce the alterations associated with the bleaching procedures with HP in high concentration in a biologically inspired way.

Key words: Polyphosphates. Tooth Bleaching. Tooth Bleaching Agents. Alkaline Phosphatase.

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1 INTRODUÇÃO

A maior causa de insatisfação dos pacientes é o escurecimento dental, o que se reflete no fato de que pelo menos 30% dos pacientes se sentem insatisfeitos com a cor dos dentes naturais (Vilhena et al., 2019). Para atender a demanda destes pacientes, diferentes sistemas clareadores a base de peróxido de hidrogênio (PH) ou peróxido de carbamida (PC) têm sido introduzidos no mercado (Qin et al., 2017; Kutuk et al., 2018).

Independentemente da técnica ou produto utilizado, o mecanismo de ação dos agentes clareadores baseia-se no processo de oxidação gerado pela dissociação do PH em espécies reativas de oxigênio (EROs), que seriam responsáveis pela quebra de moléculas orgânicas e produção de moléculas menores e mais claras, e consequentemente do efeito clareador (Hyland et al., 2015; Kutuk et al., 2018). Porém, além desse efeito, tem sido relatado que a ação oxidante “inespecífica” das EROs agiria também sobre os componentes estruturais orgânicos e inorgânicos do esmalte (Gomes et al., 2018; Vieira et al., 2018; Torres et al., 2019; Vieira et al., 2019; Wijetunga et al., 2021) e da dentina (Jiang et al., 2018) gerando mudanças topográficas e perdas minerais nestas estruturas, mesmo aplicando géis mais neutros ou alcalinos (Elfallah et al., 2015; Vilhena et al., 2019).

É relevante considerar que os potenciais efeitos colaterais associados ao clareamento são: sensibilidade dentária, alterações pulpares, irritação dos tecidos moles, alteração na morfologia e rugosidade do esmalte, e a redução da sua dureza de superfície (Yang et al., 2021). Particularmente, as alterações no esmalte são mais acentuadas quando o tempo de contato da superfície com o agente clareador e a concentração do produto são aumentados (Pini et al., 2022). Ainda que a exposição à saliva humana após o clareamento auxilie no processo de remineralização do esmalte clareado, a extensão deste efeito ainda é discutida, sendo comprovado que a reparação da perda mineral pode ser limitada às áreas superficiais ou desenvolver-se de forma irregular (Franco et al., 2016; Cavalli et al., 2018; Kutuk et al., 2018; Ferraz et al., 2021).

Nesse sentido, na tentativa de reverter o efeito do clareamento na deterioração do esmalte, alguns agentes remineralizantes, como: fosfopeptideo de caseína com fosfato de cálcio amorfo (Kutuk et al., 2018; Vilhena et al., 2019), quitosana (Pini et al.,

2022), gluconato de cálcio e/ou flúor (Borges et al., 2014; Torres et al., 2019; Vieira et al., 2020), sílica de cálcio hidratada (Yang et al., 2021), vidro bioativo (Yang et al., 2022), tetrafluoreto de titânio (Lins et al., 2021), e dióxido de titânio (Monteiro et al., 2020) têm sido associados e/ou incorporados em géis contendo PH em alta concentração apresentando resultados promissores.

Embora alguns desses clareadores com efeito remineralizante aumentaram a microdureza do esmalte e/ou reduziram o dano celular gerado pelo PH sem interferir com a eficácia clareadora (Borges et al., 2011; Hyland et al., 2015; Qin et al., 2017; Kutuk et al., 2018; Vilhena et al., 2019; Torres et al., 2019; Lins et al., 2021), outros estudos não encontraram aumento satisfatório dos níveis de cálcio, nem preenchimento da estrutura mineral do esmalte sub-superficial, ou ainda relataram alterações na estrutura mineral dental e nas propriedades mecânicas do esmalte (Pinto et al., 2017; Cavalli et al., 2018; Orilisi et al., 2021). Além disso, algumas das evidências positivas deste tipo de geís poderiam ser atribuídas não somente ao efeito do composto *per se*, mas às mudanças do pH (Yang et al., 2021; Yang et al., 2022), ou à diminuição da concentração do peróxido (Vilhena et al., 2019).

Considera-se, por tanto, que o tipo e a quantidade do agente remineralizante a ser incorporado no gel clareador são fatores que supõem ainda desafios à odontologia estética (Monteiro et al., 2020; Akabane et al., 2021). Sendo possível inferir que ainda não existe um composto de cálcio/fosfato “padrão ouro” associado ao clareamento dental, o que determina a necessidade do desenvolvimento de sistemas clareadores com aditivos remineralizantes que apresentem adequado efeito clareador e, ao mesmo tempo, reduzam ou eliminem os efeitos deletérios gerados na estrutura dental (Pinto et al., 2017; Gomes et al., 2018; Kwon et al., 2018).

Apesar do esmalte ser um tecido biologicamente inerte, foi demonstrado que depósitos de Ca-P podem ser formados na sua superfície, se os precursores apropriados forem fornecidos (Müller et al., 2016). Entre estes, o polifosfato (PoliP) tem sido descrito como uma fonte promissora de orto-fosfato, necessário para a formação de tecido duro em mamíferos (Müller et al., 2016). Baseando-se na atividade bio- e morfo-genética do PoliP, um grupo de pesquisa produziu sub-micropartículas de polifosfato de cálcio (PPCa), cujo tamanho foi ajustado usando uma razão molar Pi: Ca^{2+} definida de 1: 1 ou 1: 2 (Müller et al., 2015), e que tem mostrado resultados promissores na área de biomineralização ao ser uma fonte amorfa de cálcio e fosfato

que estimula a deposição mineral tanto no esmalte quanto na dentina (Müller et al., 2016; Müller et al., 2017a; Ackermann et al., 2019; Desbord et al., 2022).

A capacidade biomimética do PPCa (Deng et al., 2022), se basearia no deposito mineral induzido por duas vias: 1) fusão direta: onde os grupos PO_4^{3-} expostos da hidroxiapatita se ligariam ionicamente com os Ca^{2+}, e estes por sua vez agiriam como ponte de união para mais grupos fosfatos da cadeia do polifosfato, o que permitiria o acúmulo do polímero na superfície (Müller et al., 2016); 2) durante o processo de cisão da cadeia amorfa do PPCa (por degradação hidrolitica), a precipitação dos íons cálcio/fosfatos formaria uma camada mineral amorfa, que agiria como precursor de uma matriz mineral cristalina e organizada (hidroxiapatita) (Omelon et al., 2014; Ackermann et al., 2019; Ionescu et al., 2022).

Com relação ao processo de cisão do PPCa, deve-se esclarecer que o fornecimento dos íons da cadeia polimérica acontece de forma regulada, o que evita precipitação mineral "fora do local de ação" (Omelon et al., 2014). Esse processo pode então acontecer mediante: 1) um processo de degradação de hidrólise espontâneo, num ritmo lento, ou; 2) pela clivagem da cadeia mediada por alguma enzima fosfatase, como a fosfatase alcalina (ALP), num ritmo mais acelerado (Omelon et al., 2014; Ackermann et al., 2019; Zhang et al., 2021). O papel da ALP como regulador no processo de liberação do Ca^{2+} e PO_4^{3-} é de extrema importância para atingir, mediante a integração iônica na superfície do dente, uma mineralização bioinspirada dos tecidos dentários (Hasselgren et al., 1978; Müller et al., 2016; Ackermann et al., 2019; Guanipa Ortiz et al., 2023b).

Devido à persistência dos efeitos adversos dos géis clareadores convencionais e ao aumento na demanda pela manipulação de novos clareadores experimentais, que possam garantir o máximo benefício com o mínimo de prejuízo (Torres et al., 2019; Monteiro et al., 2020; Vieira et al., 2020; Lins et al., 2021; Pini et al., 2022; Yang et al., 2021; Yang et al., 2022), considera-se que a incorporação do PPCa em géis clareadores experimentais é uma alternativa de tratamento inovadora e promissora associado ou não a enzima ALP. O efeito do PPCa quando incorporado em géis clareadores de alta concentração à base de PH nem a sua associação com a enzima ALP neste tipo de formulações, até onde os autores tem conhecimento, não tem sido previamente descrito.

Assim, o objetivo neste trabalho foi sintetizar e caracterizar as sub-micropartículas de polifosfato de cálcio (PPCa) e incorporá-las em diferentes concentrações (0,5 e 1,5 wt%) na formulação de agentes clareadores à base de PH em alta concentração (PH-PPCa) com ou sem enzima fosfatase alcalina (ALP). Adicionalmente, as propriedades químicas e biológicas destes géis experimentais e seus efeitos nas propriedades físico-químicas dos esmaltes humano ou bovino foram avaliadas.

2 ARTIGOS

2.1 Calcium-polyphosphate sub-microparticles (CaPP) improvement effect of the experimental bleaching gels' chemical and cellular viability properties

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Abstract

The aim of this research was to develop and characterize the chemical and cellular-viability properties of an experimental high-concentration bleaching gel (35 wt%- H_2O_2) containing calcium-polyphosphate particles (CaPP) at two concentrations (0.5 wt% and 1.5 wt%). The CaPP sub-microparticles were synthesized by coprecipitation, keeping a Ca:P ratio of 2:1. The CaPP morphology, size, and chemical and crystal profiles were characterized through scanning and transmission electron microscopy, energy-dispersive X-ray analysis, and X-ray diffraction, respectively. The assessed bleaching gels were experimental (without CaPP); 0.5% CaPP; 1.5% CaPP; and commercial. The gels' pH values and H_2O_2 concentrations (iodometric titration) were determined. The odontoblast-like cell viability after a gel's exposure was assessed by the MTT assay. The pH and H_2O_2 concentration were compared through a repeated-measures analysis of variance (ANOVA) and a Tukey's test and the cell viability through a one-way ANOVA and a Tukey's test using a GraphPad Prism ($\alpha < 0.05$). The CaPP particles were spherical (with Ca and P, 135.7 ± 80.95 nm size) and amorphous. The H_2O_2 concentration decreased in all groups after mixing ($p < 0.001$). The 0.5% CaPP resulted in more-stable pH levels and higher viability levels than the experimental one ($p < 0.05$). The successful incorporation of CaPP had a positive impact on the bleaching gel's chemical and cellular-viability properties when compared to the experimental gel without these particles.

Keywords: hydrogen peroxide; bleaching; cell survival; pH; polyphosphates; sub-microparticles

1. Introduction

Given the high levels of dissatisfaction (30%) with teeth darkening, bleaching gels based on hydrogen peroxide have been extensively introduced into the market [1]. Their chief mechanism of action is the oxidation of the organic molecules, which stain teeth, into smaller and lighter compounds [2]. Along with their bleaching effect, both the H_2O_2 and its reactive oxygen species (ROS) are responsible for the "nonspecific" oxidative action on the organic and inorganic structural components of enamel [3,4,5,6,7] and dentin [8], generating topographic and mineral changes in these structures, even applying more-neutral or -alkaline gels [9,10,11].

Although enamel is a biologically inert biomaterial, remineralizing components that dissolve through a chemical process can generate local precipitation of biosimilar Ca/PO_4^{3-} on the surface. This must be considered for the development of dental materials with remineralizing properties, such as bleaching gels, that can achieve an adequate bleaching effect while reducing the intrinsic adverse effects of the therapy [3,12,13,14].

Therefore, the demand for experimental bleaching agents that can ensure maximum benefit with minimum harm has grown in recent years [5,14,15,16,17]. Those that incorporate a source of Ca/PO_4^{3-} compounds still report changes in the tooth mineral structure [18], and some of the positive evidence could be attributed not only to the compound effect but also to pH changes [14,17] or the decrease in peroxide concentration [1]. For this, it can be inferred that there is still no gold standard Ca/PO_4^{3-} compound for bleaching-gel preparation.

One promising material is polyphosphate (PolyP), a natural compound of living cells and a source of ortho-phosphate (PO_4^{3-}), which is required for hard tissue formation [19,20]. More recently, the synthesis of calcium-polyphosphate (CaPP) sub-microparticles by a defined Ca:P molar ratio of 2:1 has been described [21]. CaPP is an amorphous source of Ca and PO_4^{3-} that stimulates mineral deposition in both enamel and dentin [19,22]. The interaction of CaPP with these tissues can be partially explained by the formation of Ca^{2+} bonds between the mineral matrix of dental hydroxyapatite and CaPP [19,23].

In view of bleaching treatment–induced mineral alterations and given CaPP's possible remineralizing effects, the incorporation of CaPP in experimental bleaching

gels is an innovative and promising alternative for treatment. Thus, the aim of this research was to develop and characterize the chemical and cellular-viability properties of an experimental high-concentration bleaching gel (H_2O_2/HP—35 wt%) containing calcium-polyphosphate (HP-CaPP) particles in two concentrations (0.5 wt% and 1.5 wt%). For this study, the null hypotheses were as follows: (1) the H_2O_2 concentration and pH levels of the HP-CaPP bleaching agent will not differ from those of a commercial bleaching agent and an experimental bleaching gel without CaPP, and (2) the cytotoxic potential of the HP-CaPP bleaching agent will not differ from that of a commercial bleaching agent or that of an experimental bleaching gel without CaPP.

2. Results

2.1. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Analysis (EDX)

SEM analysis showed that the particles had a spherical morphology (Figure 1a). The presence of Ca and P was detected by using the EDX system. Although certain levels of Na and Cl persisted, the amounts were minimal. The particles presented a Ca:P atomic ratio of 1.11, as shown in Figure 1b.

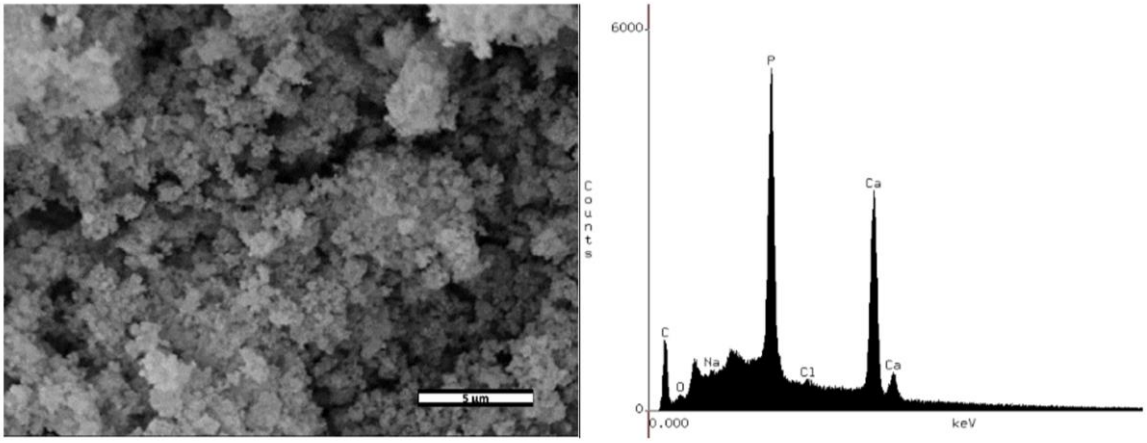

Figure 1. Morphological and chemical characterization of the synthesized calcium-polyphosphate particles: (a) scanning electron microscopy (SEM) images (5000); (b) energy-dispersive X-ray analysis (EDX).

2.2. Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS)

According to the TEM images, the CaPP particles showed a sub-microparticle size (135.7 ± 80.95 nm; Figure 2a). The hydrodynamic radius of the CaPPs showed a distribution of 257.1 nm (± 26.52 nm), with a polydispersity index of 0.43 (±0.05) and a correlation of 0.90 (Figure 2b).

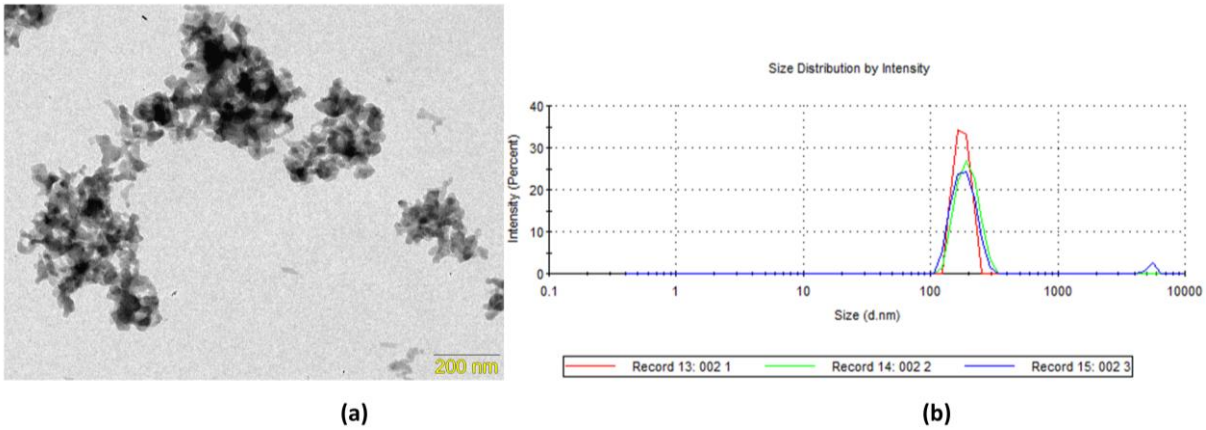

Figure 2. Sized and size distribution of the synthesized calcium polyphosphate particles: (a) Transmission electron microscopy (TEM); (b) Dynamic Light Scattering (DLS).

2.3. X-ray Diffraction (XRD)

XRD analysis demonstrated the amorphous character of the synthesized CaPP particles given that the characteristic peaks of crystalline materials were not observed in the diffractogram (Figure 3).

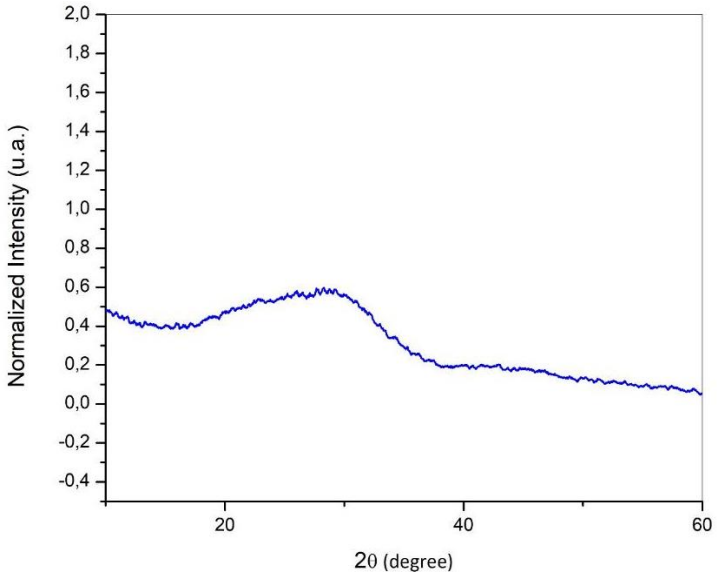

Figure 3. Results of X-ray diffraction analysis (XRD) of the calcium polyphosphate particles.

2.4. Visual and pH Evaluation Results

The visual aspect of the experimental gels (Figure S1) denoted an opaque appearance with increasing CaPP concentrations. The pH values (without dilution) varied among the periods of time (ANOVA; $p < 0.0001$) and among the bleaching gels (ANOVA; $p < 0.0001$). The gel with 0.5% CaPP had the highest pH values when compared to the others. After 15 min of mixing, all gels maintained pH values above 5.1. The 1.5% CaPP and commercial gels (Tukey; $p < 0.05$) had the lowest pH values in this assessment period. After 45 min of mixing, the experimental bleaching gel had the lowest pH value (Tukey; $p \leq 0.007$) (Figure 4).

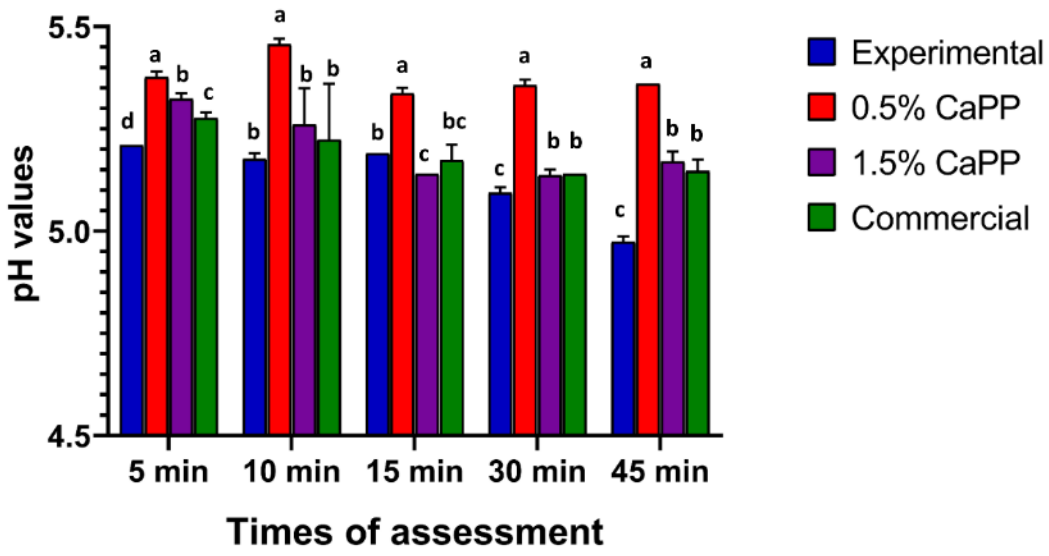

Figure 4. The pH mean values of the used bleaching gels in the different periods of assessment. Different letters in the bars indicate statistically significant differences among the groups ($p \leq 0.05$). The results were obtained through triplicate analyses.

2.5. Hydrogen Peroxide Concentration of the HP-CaPP—Titrimetric Analysis (Iodometric)

The H_2O_2 concentration decreased with time in all groups (ANOVA; $p < 0.0001$). There were no significant differences among the groups for H_2O_2 concentration after 15, 30, and 45 min of gel mixing. After 90 min, the experimental bleaching gel presented the lowest H_2O_2 concentration (Tukey; $p < 0.005$); however, after 150 min, the 0.5% and 1.5% CaPP gels presented the highest H_2O_2 levels (Tukey; $p < 0.005$) (Table 1).

Table 1. Hydrogen peroxide concentration of the bleaching gels determined through the iodometric method.

Gel	15"	30"	45"	90"	150"
	Hydrogen peroxide concentration mean (wt%) *				
Experimental	26.74 **a**	25.28 **a**	21.39 **a**	17.01 **b**	14.10 **c**
0.5% CaPP	25.16 **a**	24.67 **a**	23.19 **a**	22.69 **a**	23.68 **a**
1.5% CaPP	24.91 **a**	24.91 **a**	23.25 **a**	23.50 **a**	21.85 **a**
Commercial	29.86 **a**	23.08 **a**	22.10 **a**	22.10 **a**	18.79 **b**

Different letters in the columns indicate statistically significant differences among the groups ($p \leq 0.05$). * The results were obtained through triplicate analyses.

2.6. Odontoblasts-like Cells (MDPC-23) Viability after Exposure to the HP-CaPP Bleaching Gel

The cell viability results are displayed in Figure 5. For the 10 µg/mL concentration, the experimental gel was significantly different from the other gels ($p < 0.05$; Tukey's post hoc ANOVA test). The IC50 of this experimental gel (9.81) was significantly different from the 0.5% CaPP (35.92), 1.5% CaPP (22.65), and commercial (26) gels. Additionally, the IC50 of the 0.5% CaPP was significantly different from the 1.5% CaPP ($p < 0.05$).

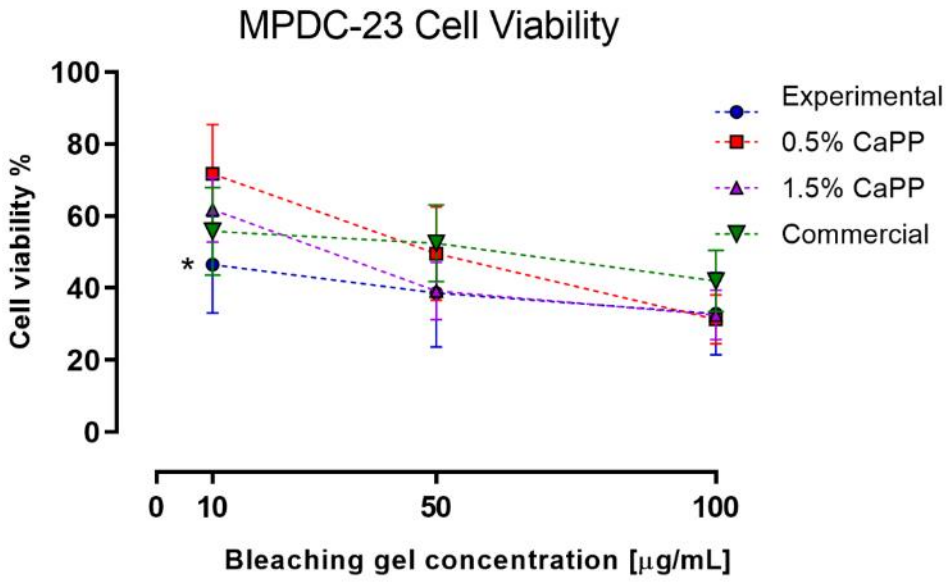

Figure 5. Cell viability (%) of the MDPC-23 cells after exposure to different concentrations of the bleaching gels (10, 50, and 100 µg/mL). * Statistically different from the rest of the groups ($p < 0.05$).

3. Discussion

An experimental bleaching gel based on H_2O_2-35 wt% with calcium-polyphosphate sub-microparticles (HP-CaPP) was developed, and its chemical (pH and H_2O_2 concentration) and biological (cellular-viability) properties were assessed. According to the obtained results, the first null hypothesis was rejected, as the HP-CaPP bleaching gels (CaPP—0.5 and 1.5 wt%) had higher pH levels compared to the experimental gel without CaPP and similar values to the commercial bleaching gel. The H_2O_2 concentration was similar among the bleaching gels. The second hypothesis was also rejected, as the cell viability was higher for the HP-CaPP groups (CaPP—0.5 and 1.5 wt%) than for the experimental gel without CaPP but was similar to the commercial bleaching gel.

Following the coprecipitation method [21], CaPPs were synthetized and characterized for later incorporation into an experimental bleaching gel (HP-CaPP). The SEM images display particles with spherical morphology. Additionally, the EDX spectra display marginal Na levels but significant levels of P and Ca, as previously reported [21,22,24].

Concerning the CaPP size, the TEM analysis indicated submicrometric particles (135.7 ± 80.95 nm). The starting Ca:P molar ratio was 2:1, which allowed the formation of smaller particles [22]. Although the DLS results display a higher hydrodynamic radius (257.1 ± 26.52 nm), this analysis considers the particles' hydrodynamic diameters (257.1 ± 26.52 nm). Another factor that may influence DLS analysis and contribute to a higher hydrodynamic radius reading is the particle agglomeration that usually occurs throughout the reading [25,26]. As determined by XRD analysis, the CaPPs retained their amorphous character. The amorphous state is relevant for increasing the remineralizing potential of the particles. This is related to the fact that when dental tissues are exposed to highly supersaturated phosphate solutions (e.g., CaPP), an amorphous calcium phosphate precipitate can be formed and later transformed into an organized crystal apatite structure [22,23,27].

The initial pH values of the experimental formulations were intended to remain close to those of the undiluted commercial bleaching gel to determine the effect of the CaPP particles within a similar pH range [28]. The smallest CaPP concentration (0.5 wt%) seemed to have a beneficial effect on pH value stability. Initially, the medium

protons, thanks to their high electronegativity, replace the Ca^{2+} ions on the CaPP structure, leading to an increase in pH, as observed after 10 min in the 0.5% CaPP gel. Moreover, phosphoric acid is also produced during the hydrolytic cleavage of the CaPP chain, which decreases the gel's pH [29]. When we tripled the CaPP concentration (1.5 wt%), this latter effect was predominant, as after 15 min, this gel had a lower pH than the experimental and 0.5% CaPP gels. The commercial gel showed a pH decrease 15 min after mixing (5.17), which followed the trend of previous assessments [15,30].

After the longest assessment time, 45 min after the preparation of the experimental gel without CaPP, the lowest pH values were reached. The lower pH values have been related to higher enamel alterations (e.g., lower microhardness, higher superficial roughness) and to reduced bleaching efficacy [5,7]. Thus, the incorporation of CaPP into an experimental bleaching gel could be regarded as positive for the pH of the bleaching gels and therefore positive for the enamel properties in future *in vitro/in vivo* applications.

The H_2O_2 concentration was lower than 35 wt% 15 min after mixing the gels. However, given the H_2O_2 instability, the exact concentration is difficult to determine by titration [31], which can be considered a limitation of the current study. After gel preparation, the H_2O_2 present in the bleaching gel starts to decompose into free radicals and H_2O as time passes [31]. Nevertheless, in the experimental gel containing CaPP, this reduction was not as accentuated as in the experimental gel without CaPP and the commercial gel. This could occur because of the H_2O_2 stabilization promoted by the phosphate molecules released from the CaPP chain [31,32,33,34].

Given that CaPP is a biocompatible compound, its presence in bleaching gels, as expected, did not increase their cytotoxic potential [21,35,36]. Interestingly, lower amounts of CaPP (0.5 wt%) were better at sustaining MDPC-23 cells' viability compared to the experimental gel without CaPP. CaPP chain cleavage not only provides ortho-phosphate units that aid in the remineralization process but also generates "metabolic fuel" by supporting ATP production [21]. This intracellular burst of energy could increase cell proliferation [37,38], which could explain why this group presented the best results for cell viability at a concentration of 10 µg/mL.

However, a higher polyphosphate concentration within the bleaching gel (1.5 wt%) did not result in an increase in cell viability. The hydrolytic cleavage of the CaPP chain led to a more acidic gel, as seen in our pH assessments. Under these conditions, cell growth, proliferation, and differentiation were retarded [39]. It could also be speculated that H_2O_2 decomposition into free radicals at this pH level is reduced; therefore, the free remaining H_2O_2 cytotoxic potential could have affected cell viability [40,41], surpassing the CaPP protective effect displayed at a lower CaPP concentration and a higher pH level (0.5% CaPP).

As previously described, CaPP, as a long-chain bioinorganic polymer, is a promising bioactive compound in bioremineralization [19,23,42]. This characteristic behavior would be useful to reduce the enamel mineral loss associated with high-concentration bleaching gels, while the presence of the ions would have a potential effect on reducing dental sensitivity, which remains the most common clinical symptom of the bleaching treatment [1,5]. Although the successful incorporation of CaPP did not negatively alter the experimental gels' chemical and biological properties compared to commercial and experimental bleaching gels based on 35% H_2O_2 without these particles, further studies should be conducted to determine the effect of CaPP on the bleaching efficacy and the enamel chemical and mechanical properties of this type of experimental bleaching gel when compared to commercially established treatments.

4. Conclusions

In the present study, calcium-polyphosphate fine particles were successfully synthesized through the coprecipitation method [21] and incorporated into bleaching gels based on 35 wt% H_2O_2. The incorporation of CaPP, even in smaller concentrations (0.5% w/t), stabilized the pH, reduced the cytotoxic potential, and retained similar hydrogen peroxide concentration values compared to the experimental bleaching gel without CaPP after 45 min of mixture *in vitro*. Because the CaPP gel's retained similar chemical and biological properties to the commercial bleaching gel without CaPP, their remineralizing potential turns them into a promising clinical alternative to conventional bleaching gels.

5. Materials and Methods

5.1. Synthesis of Calcium-Polyphosphate Particles (CaPP) and Characterization

To obtain CaPP particles, a coprecipitation technique was used [21]. Based on our own pilot synthesis and previous works [19,21], a sodium polyphosphate (NaPP), with an average chain length of ~35 phosphate units (Chemische Fabrik; Budenheim, Germany), was used for CaPP synthesis (Table 2).

Table 2. Main reagents used for the synthesis of calcium polyphosphate (CaPP)

Reagent	Brand	Lot
$CaCl_2 \cdot 2H_2O$ (C3306-1000g)	Sigma	#SLBZ8395
Na-PolyP (DP-PCB/O-2018-001)	Chemische Fabrik Budenheim	MV58/371
NaOH (Pearls)	Labsynth	H2000.01.AH

The pH of the NaPP solution (5 g in 250 mL H_2O) was adjusted to 10 using an aqueous 1 M NaOH solution. Then, the $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, Taufkirchen, Germany) solution (14 g in 250 mL H_2O) was added to the NaPP solution at a controlled rate, 1 mL/min, using a peristaltic pump (P-1; Pharmacia Biotech, Uppsala, Sweden) to obtain a Ca:P ratio of 2:1. During the addition, the pH was maintained (10) at room temperature using 1 M NaOH solution. After 4 h of stirring, the final solution was washed for 10 min and centrifuged (3500 rpm × 20 min) twice with distilled water and at last twice with absolute ethanol. The final slurry was kept in an oven at 60 °C (14 h) [21,22]. The dried material was crushed with a pestle in a mortar to obtain a fine CaPP powder (Figure 6).

Figure 6. Graphical summary of the synthesis procedure of calcium-polyphosphate (CaPP) particles employing the coprecipitation method: (**a**) flow pump; (**b**) controlled addition of $CaCl_2 \cdot 2H_2O$ solution (1 mL/min; pH = 10) into the NaPP solution; (**c**) After 4 h of stirring, distribution of the crude product in falcon tubes; (**d**) washing of CaPP (twice with water and twice with ethanol); (**e**) Centrifugation after every wash; (**f**) CaPP slurry obtained; (**g**) CaPP dried fine powder.

5.1.1. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Analysis (EDX)

A thin gold layer was deposited on the CaPP surface (BAL-TEC SCD 050; Capovani Brothers Inc., NY, USA) to assess CaPP morphology. Images at ×5000 magnification were obtained on an SEM (JSM-5600, JEOL; Tokyo, Japan) with a 15 kV accelerating voltage, 13 mm Z, and 15 mm WD.

The powder was coated with carbon by vapor deposition (Delton vacuum Desk II, Moorestown, NJ, USA) to characterize the chemical elements of the CaPP particles. Elemental analysis was performed using an EDX detector (Vantage, Acquisition Engine Company, Tokyo, Japan) connected to an SEM (JSM-5600, JEOL, Tokyo, Japan). The EDX system was operated at 15 kV with a collection time of 100 s, 30° incidence, Z = 20 mm, and WD = 20 mm. Three areas of approximately 10 μm^2 were evaluated.

5.1.2. Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS)

Morphological and size examinations of CaPP were performed using a transmission electron microscope (Phillips CM 200, Phillips, Amsterdam, the Netherlands) at 200 kV with a LaB6 filament. The sample was prepared, and the

obtained images were analyzed to estimate the particle size on the basis of an average diameter of 60 particles (ImageJ; public domain image software).

To determine the particle hydrodynamic radius, CaPP was diluted in distilled water (1 mg/mL), stirred for 15 min (50 °C), and centrifuged for 10 min (10,000 rpm). The supernatant was analyzed using a laser diffraction particle analyzer (Zetasizer Nano ZS90; Malvern Instruments Ltd., Worcestershire, UK) with the following parameters: 10 runs of 10 s, at 25 °C, with 60 s of stabilization, and a 90° scattering angle.

5.1.3. X-ray Diffraction (XRD)

The CaPP powder crystallinity pattern was recorded on a diffractometer (D8 Advance; Bruker, MA, USA) with Cu-Kα radiation (40 kV, 30 mA) and an equipment geometry of 2θ. Continuous readings were performed with a step of 0.02° and an accumulation time of 0.3 s (interval of 4°/min).

5.2. Experimental Bleaching Gel Containing Calcium-Polyphosphate Particles (HP-CaPP)

Formulations of high-concentration H_2O_2-based bleaching gel (H_2O_2—35 wt%) with two separate components were proposed: A— H_2O_2 (pH ≈ 1.8); B—thickener and calcium-polyphosphate particles (CaPP) (pH ≈ 12) [5]. Three types of experimental bleaching gels were manipulated: without CaPP (experimental) and containing 0.5 and 1.5 wt% CaPP (CaPP 0.5% and CaPP 1.5%). In addition, Whiteness HP Maxx (FGM Dental Products, SC, Brazil) was used as a commercially available control.

The reagents of component A were stirred for 60 min (≈10 °C) in the dark to prevent H_2O_2 decomposition [31,43]. For the gels containing CaPP in component B, CaPP was first diluted in distilled water and stirred in a water bath for 15 min (50 °C).

After cooling down, the remaining reagents of component B were homogenized (Speed Mixer DAC 150.1; FlackTek, Inc., SC, USA) at 2000–2500 rpm for 10 min (Table 3).

For all gel analyses, experimental components A and B were mixed in a 3:1 weight proportion, while the commercial control bleaching gel was prepared according to the manufacturer's instructions. All gels were kept under refrigeration (8 ± 1 °C) before the experiment and were brought to room temperature 30 min before mixing.

Table 3. Composition of the experimental bleaching gels (Component A and B)

Component A	Component B
H_2O_2 (50% sol)	H_2O distilled
Carbopol 940	CaPP (0.5 or 1.5 wt%)
Glycerol	Carbopol 940
Propylene glycol	Glycerol
Citric acid	Propylene glycol
-	NaPP
NaOH to adjust the pH ≈ 1.8	NaOH to adjust the pH ≈ 12

5.2.1. Evaluation of pH

The pH values of 4 g of each bleaching gel were recorded using a benchtop digital pH meter (mPA210; MS TECNOPON, Piracicaba, SP, Brazil), which was previously calibrated with buffered solutions (pH 4, 7, and 10) at 25 °C. The pH values of the gels were recorded in triplicate 5, 10, 15, 30, and 45 min after the mixture of both components. The mean of the three measurements was considered the final value for each assessment time.

5.2.2. Hydrogen Peroxide Concentration of the HP-CaPP—Titrimetric Analysis (Iodometric)

The gels' respective H_2O_2 concentrations were determined by employing iodometric titration with a hydrogen peroxide test kit (Hanna Instruments; Barueri, SP, Brazil) and a standardized sodium thiosulfate solution ($Na_2S_2O_3$; 0.1 N). In brief, iodide ions (I^-) can be oxidized by $H_2O_2$to iodine (I_2), which in the presence of starch forms a blue charge-transfer complex by the imprisonment of polyiodide species within the helix structure of amylose. I_2 can be reduced by titration with a standard $Na_2S_2O_3$ solution to I^-, which is colorless [31,44]. The H_2O_2 concentration can be calculated by the volume of the standard $Na_2S_2O_3$ solution required to change the color of the bleaching solution using Equation (1) [45].

$$C = \frac{[(0.1M \times V)/2] \times 34.0147 g/mol}{Vf} \quad (1)$$

Where C = final concentration; V = volume of the $Na_2S_2O_3$ (0.1 N) solution used; and Vf = final volume of the diluted H_2O_2 solution used to conduct the titration.

All bleaching gels were diluted in distilled water (1 mg/mL of H_2O_2) and remained at room temperature in static and dark conditions. At defined times, samples were collected from the upper area of the samples (15, 30, 45, 90, and 150 min after mixing) and further diluted to obtain 0.2 mg/mL H_2O_2 solutions in three 25 mL glass vials [31]. The titration procedure was performed on the three samples, and the mean was considered the final value of each sample/time assessed.

5.2.3. Odontoblasts-like Cells (MDPC-23) Viability in the HP-CaPP Bleaching Gel

The MTT reduction method was performed to determine the viability of MDPC-23 cells after exposure to the bleaching gels. The MDPC-23 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with antibiotics, 100 IU/mL penicillin and 100 µg/mL streptomycin (Vitrocell Embriolife, Campinas, Brazil), 2 mmol/L glutamine, and 10% fetal bovine serum (FBS—GIBCO; Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in a 5% CO_2 atmosphere [39,46].

After reaching 80% confluence, the MDPC-23 cells were washed with 0.25% trypsin/EDTA (GIBCO; Thermo Fisher Scientific, Waltham, MA, USA) to separate them from the plate. The separated cells were centrifuged at 3000 rpm for 5 min at 4 °C. A Neuberger chamber was used to count the cells. The supernatant was discarded; the cells were passed to a new medium (DMEM), transferred to 96-well cell culture plates (Corning Costar Corp., Cambridge, MA, USA) at a concentration of 5×10^4 cells/mL, and subsequently incubated in a 5% CO_2 atmosphere at 37 °C for 24 h.

After the incubation period, the cells were exposed to diluted bleaching gels (10, 50, and 100 µg/mL) with DMEM for 45 min. The wells were then washed twice with phosphate buffered saline (PBS—pH = 7.4), and 200 µL of MTT diluted in DMEM medium at 0.3 mg/mL (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) was added. After 3 h of incubation in a 5% CO_2 atmosphere at 37 °C, the wells were washed twice with PBS and filled with 200 µL of ethanol. Finally, the absorbance values were obtained using a microspectrophotometer (ASYS UVM340; Biochrome Ltd., Cambridge, England) at 570 nm [39,47].

5.3. Statistical Analyses

Shapiro–Wilk and Levene tests were used to verify the normality and homoscedasticity of variances of the cell viability, pH, and concentration data. The cell-viability data were compared through a one-way analysis of variance (ANOVA) and a post hoc Tukey's test. The IC50 values were determined by logistic regression analysis. The respective pH values and concentrations were assessed through a repeated-measures ANOVA and a post hoc Tukey's test (significance level 5%) using GraphPad Prism 8.0.2 software for Windows (GraphPad Software, San Diego, CA, USA).

Supplementary Materials

The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/gels9010042/s1, Figure S1: Bleaching gels appearance after manipulation from left to right—experimental; 0.5% CaPP; 1.5% CaPP; commercial.

Author Contributions

Conceptualization, M.I.G.O. and D.A.N.L.L.; methodology, M.I.G.O., J.J.d.S. and J.B.S.; investigation, M.I.G.O., J.J.d.S. and J.B.S.; writing—original draft, M.I.G.O.; writing—review and editing, M.I.G.O., J.J.d.S., J.B.S., U.P.R.-F., F.H.B.A., K.R. and D.A.N.L.L.; validation, J.J.d.S.; resources, U.P.R.-F., F.H.B.A., K.R. and D.A.N.L.L.; supervision U.P.R.-F., F.H.B.A., K.R. and D.A.N.L.L.; project administration, K.R. and D.A.N.L.L.; funding acquisition, K.R. and D.A.N.L.L. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Not applicable.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declare no conflict of interest.

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2.2 Maintenance of enamel properties after bleaching with high-concentrated hydrogen-peroxide gel containing calcium polyphosphate sub-microparticles

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ABSTRACT

Objective: To assessed the physical and chemical properties of human-enamel after treatment with an experimental bleaching gel containing 35%-Hydrogen Peroxide (HP) and calcium polyphosphate sub-microparticles (CaPP).

Materials and Methods: Enamel/dentin specimens (4x4x3mm) were obtained (n=120) and allocated to different groups: Control (without bleaching); Experimental (HP35%); Commercial (Whiteness-HP-Maxx); CaPP0.5% (HP35%+CaPP0.5wt%); CaPP1.5% (HP35%+CaPP1.5wt%). Three sessions were performed. The specimens´ color was assessed using a spectrophotometer and the color ($\Delta E/\Delta E_{00}$) and bleaching index (ΔWID) determined. The surface roughness and microhardness were assessed with a roughness tester and Knoop indenter. Raman spectroscopy was performed to obtained the ratios between the areas under the 431, 580, and 1070cm^{-1} and the 960cm^{-1} bands (430:960, 580:960, 1070:960). Kruskal-Wallis and Dunn compared the color, Ra and SMH data. The Raman data was analyzed with Kruskal-Wallis and Dunn (α=5%).

Results: The ΔE, ΔE_{00}, and ΔWID were similar among the bleached groups ($p>0.05$). The roughness was not different between the groups ($p>0.05$). After the 3rd-session, CaPP0.5% had higher microhardness than the Experimental ($p<0.05$). The 1070:960 was higher in the Experimental than in the CaPP1.5% and Control ($p<0.05$).

Conclusions: In human-enamel, CaPP did not alter the bleaching effectiveness or roughness, and additionally, CaPP-containing-gels increased the microhardness and preserved the mineral content when compared to the experimental without CaPP.

Clinical significance: Experimental bleaching gels containing calcium polyphosphate sub-microparticles as a mineral source reduce the mineral content alteration and superficial microhardness reduction, known potential side effects of the in-office bleaching treatments.

Keywords: Dental Enamel. Tooth Bleaching. Polyphosphates. Calcium polyphosphate. Raman Spectroscopy.

INTRODUCTION

Changes in enamel morphology and surface hardness reduction by demineralization of this tissue after exposure to bleaching agents have been related to the "nonspecific" oxidizing action of hydrogen peroxide (HP) and its by-products on organic and inorganic structures of the tooth structure [1-3]. This would lead to significant changes in dental enamel mineral content and topography [4-7] even when applying more neutral or alkaline gels [8-10].

Even though the contact of the treated surface with human saliva promotes a certain remineralization degree of the bleached enamel [11], the extent of this effect is still debated, as it has been proven that mineral loss replacement may be limited to certain surface areas or may develop irregularly [1,12,13]. Considering this and the persistence of bleaching gels' adverse effects [14,15], the demand for experimental bleaching agents, which can ensure maximum benefit with minimum harm, has grown in recent years [16-20].

Likewise, even when some remineralizing compounds, such as fluoride or calcium/phosphate increase enamel microhardness and/or reduce HP-generated pulpal damage when incorporated into the formulation of bleaching gel [1,21,22]. Some of these products still generate changes in the tooth mineral structure [16,23], and it has been related to their inability to satisfactorily increase the enamel calcium levels [23] or produce a mineral filling of the enamel subsurface [12]. Also, some of the positive evidence could be attributed not only to the effect of the compound *per se* but to pH changes [20] or decrease in peroxide concentration [21].

Based on this, it can be inferred that there is still no "gold standard" of calcium/phosphate compound that can be incorporated into tooth bleaching gels to reduce their adverse effects. A promising bioactive compound in the area of bioremineralization is the recently developed long-chain calcium polyphosphate polymer (CaPP) [24-27], which is considered to have more biological activity than hydroxyapatite [28] and, unlike other amorphous calcium phosphates, is stable enough to prevent precipitate crystallization of the compound when in contact with water [29].

Regarding this, an experimental bleaching gel based on HP containing CaPP have been developed and its chemical and biological properties characterized [30],

based on the co-precipitation method [31]. Hence, this study assessed the bleaching effectiveness and physical and chemical properties on human enamel after treatment with an experimental bleaching gel containing 35%-HP and calcium polyphosphate sub-microparticles (HP-CaPP) in two different concentrations (0.5 and 1.5 wt%). The research hypotheses for this study were: H_1 − the color change (ΔE; ΔE_{00}; Δ_{WID}), topography, and surface microhardness of enamel treated with HP-CaPP will be similar or superior to enamel treated with experimental and commercial 35%-HP gels without CaPP; and H_2 – the mineral content (Raman and EDX) of the enamel treated with HP-CaPP will be better preserved than the treated using experimental and commercial 35%-HP gels without CaPP.

MATERIALS AND METHODS

Sample preparation and allocation

Third molars were donated by adults through a consent form (Ethical Committee: CAAE 24313019.9.0000.5418). One hundred and fifty sound third-molars, without cracks, cavities, or enamel defects (Carl Zeiss; Santo Amaro, SP, Brazil) were cleaned of large debris and stored in a 0.1% thymol solution at ± 8 °C for no more than two months [32].

The molars' roots were cut 2 mm apically to the cemento-dentin junction with a double-face diamond disk (KG Sorensen Ind. Com, Cotia, SP, Brazil) on a low-speed dental handpiece under constant water irrigation. From each crown, enamel/dentin specimens (4 x 4 x 3 mm) were obtained using a diamond disk attached to a precision saw (Isomet 1000 Buehler, Lake Buff, IL, USA). To ascertain the heights and regularize their surfaces, the specimens were ground and polished with granulated silicon carbide papers (CarbiMet 2; Buehler, Lake Bluff, IL, USA) of 600, 1200, 2500, and 4000 grit, and with felts and a diamond paste - 1, and ¼ µm - mounted in a polishing unit (Arotec SA Indústria e Comércio Ltda; Cotia, SP, Brazil) under water cooling. Between each abrasive paper and felt, the specimens were cleaned for 10 min in an ultrasonic bath (Marconi; Piracicaba, SP, Brazil) with distilled water to remove any smears. All specimens remained stored in distilled water at ± 8 °C until the treatment started.

Two sets of specimens were prepared, one set for color/roughness and the other for microhardness analyses. Based on a previous pilot study, the ideal sample size for the color (effect size=0.17; α=0.05; β=0.80; Correlation=0.5) and microhardness specimens (effect size=0.20; α=0.05; β=0.80; Correlation=0.3) was 10

samples per group. Considering 20% specimens' loss, a total of 60 specimens were selected for each set of specimens (n=120).

For the allocation process, specimens with similar surface microhardness values (15% deviation with respect to the total mean – 338 KHN) were selected, while the color/roughness specimens with similar *L** values were selected. The specimens were allocated by stratification considering their KHN and *L** initial values into five different groups: Control (without bleaching); Experimental (HP35%); Commercial (Whiteness HP Maxx); CaPP 0.5% (HP35 wt% + CaPP0.5 wt%); CaPP1.5% (HP35 wt% + CaPP1.5 wt%). The experimental gels were formulated and manipulated as previously described [30]. Briefly, solution A (H_2O_2-based) components were mixed in a light-protected device at ± 10°C during 60 min and solution B (thickener-based) components were mixed into a light-protected storage device and homogenized at 2000–2500 rpm for 10 min (Speed mixer DAC 150.1; FlackTek, Inc., SC, USA). **Table 1**.

Table 1. Treatment groups for the study, composition, batch/manipulation details.

Treatment groups	Composition	Batch	Manipulation
Experimental	Glycerol, Propylene glycol, H_2O_2-35 wt%, deionized water, acrylic acid thickener.	21/16100	Mix the Sol A:Sol B in a 3:1 weight proportion for 30s.
CaPP 0.5%	Glycerol, Propylene glycol, H_2O_2-35 wt%, deionized water, acrylic acid thickener, CaPP 0.5 wt%.	21/16101	
CaPP 1.5%	Glycerol, Propylene glycol, H_2O_2-35 wt%, deionized water, acrylic acid thickener, CaPP 1.5 wt%.	21/16102	
Commercial	Glycol, inorganic fillers, H_2O_2-30-35 wt%, mixture of pigments, deionized water, thickener.	140420	Mix the components in a 3:1 proportion according to the manufacturer instructions for 30s.
Control	No bleaching gel	-	-

Bleaching treatment

The artificial saliva used throughout the experiment contained 1.5 mM Ca, 0.9 mM P, 150 mM KCl, 0.05 µg F / mL, and 0.1 M Tris buffer at pH 7. For the bleached

groups, three sessions were performed at weekly intervals. Three applications (15 min each) were performed during each session. Before bleaching, the specimens were cleaned with absorbent paper, and the non-bleached areas were protected with a resin barrier (Top Dam - Blue; FGM, SC, Brazil) and light-cured for 10 s (Bluephase 20i; Ivoclar, Liechtenstein).

Then, approximately 0.03 g of the bleaching agent (according to the assigned group) was applied over the enamel surface, and the specimen was kept in a moist environment at room temperature. After 15 min, the bleaching gel was removed with gauze and washed with distilled water for 5 s. After the last application, the specimen was washed for 10 s, dried with soft paper, and returned to storage in 1.5 mL of artificial saliva at 37 °C in a laboratory oven. The saliva was renewed daily for all the groups and after the last bleaching session for further 14 days.

Color assessment

For each reading the specimens were placed in a Teflon device in a light booth (GTI Mini Matcher MM-1; GTI Graphic Technology, Newburgh, NY, USA), and the color was evaluated using a previously calibrated spectrophotometer (CM 700d; Minolta, Osaka, Japan). Three readings were performed on each specimen at five moments (baseline, 24 h after the 1st, 2nd and, 3rd session, and 14 days after bleaching), and their spectral distribution was quantified using the CIELab color space. The *L** coordinate represents the degree of brightness ranging from 0 (black) to 100 (white), the *a** coordinate determines the presence of red (+ *a**) and green (- *a**) pigments, and the *b** coordinate refers to the yellow (+ *b**) and blue (- *b**) pigments present in the specimens.

The total color change (ΔE / ΔE_{00}) and bleaching indices (ΔWID) [33] were calculated by using the baseline measurement (ΔE1 / $\Delta E_{00}1$ / ΔWID1 = after 1st session; ΔE2 / $\Delta E_{00}2$ / ΔWID2= after 2nd session; ΔE3 / $\Delta E_{00}3$/ ΔWID3 = after 3rd session; ΔE4 / $\Delta E_{00}4$/ ΔWID4 = 14 days' after bleaching) as a reference based on the following formulas:

$$\Delta E = [(\Delta L^*)\,2 + (\Delta a^*)\,2 + (\Delta b^*)\,2]\,½ \quad (1)$$

$$\Delta E_{00} = [(\Delta L'/K_L S_L)^{2} + (\Delta C'/K_C S_C)^{2} + (\Delta H'/K_H S_H)^{2} + R_T\,(\Delta C'/K_C S_C)\,(\Delta H'/K_H S_H)]\,½ \quad (2)$$

$$\Delta WID = 0.511\ \Delta L^* - 2.324\ \Delta a^* - 1.100\ \Delta b^* \quad (3)$$

Where ΔL = time period L^* - baseline L^*, Δa = time period a^* - baseline a*, and Δb = time period b^* - baseline b^*. Using the CIEDE2000 metric, ΔL', ΔC', and ΔH' represent the brightness, chroma, and hue value differences, respectively. S_L, S_C, and S_H are parameters to adjust the coordinate values as a function of the color difference variation. K_L, K_C, and K_H are correction parameters with respect to experimental conditions, and R_T is a parameter that considers the interaction of chroma and hue differences in the blue region [34,35].

Surface roughness assessment

The roughness of the enamel specimens was also read at five moments: baseline, 24 h after the 1st session, 2nd session, and 3rd session, and 14 days after bleaching. Three readings were performed on each specimen using a surface roughness tester (Surftest SV 2100; Mitutoyo, SP, Brazil) with a cutting point of 0.25 mm, a reading length of 1.25 mm, and a 0.1 mm/s speed. The readings mean was considered the enamel roughness value at each assessment period.

Surface microhardness (SMH) assessment

The SMH was assessed at baseline, 24 h after the 3rd session, and 14 days after bleaching, using a microhardness tester with a Knoop indenter (FM 100, Future-Tech; Kawasaki, Japan). Five indentations (50 gf/5 s) were made in the central area of the specimens with a distance of 100 μm between them [2,19]. The mean values of the five measurements were considered the specimen's Knoop hardness number (KHN) for the time period.

Raman

The inorganic enamel concentration of phosphate and carbonate was determined 14 days after the bleaching treatment finalization with a Raman Spectrometer (Raman LabRAM HR Evolution spectrometer - Horiba). The samples were cleaned with an ultrasonic bath in distilled water before the reading, dried with soft paper, and placed on a glass slide. The analysis was performed with the following specifications: laser wavelength 785 nm, edge grid 1800 (500 nm) diffraction, intensity

180 mW, objective lens x100, 64 scans, acquisition time 2 s, and analysis range 200 to 2000 cm^{-1}. After a baseline correction, the Raman spectra were normalized by the 960 cm^{-1} (PO_4^{3-} v1) peak intensity. To determine the vibrational band relative areas, the band deconvolution was performed by Gaussian functions with analytical software (OriginLab Software, Northampton, MA, USA). The ratio between the areas under the 431-449 cm^{-1} − PO_4^{3-} v2; 540-630 cm^{-1} − PO_4^{3-} v4; and 1043-1070 cm^{-1} − CO_3^{2-} v3 bands and the area under the 960 cm^{-1} v1 band were calculated (430:960; 580:960; 1070:960) [32,36,37].

Scanning electron microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDX)

Samples were prepared 14 days after the bleaching by immersion in serial alcohol solutions (n=6 per group; n=3 for SEM, and n=3 for EDS). The samples for SEM were vacuum-coated (SCD 050; Balzers, Germany) with gold and analyzed with a scanning electron microscope—SEM (JSM 5600LV; JEOL, Tokyo, Japan). Representative areas of each group were photographed for comparison (×4000). The EDX samples were covered with a carbon layer (Delton vacuum Desk II, Moorestown, NJ, USA). Their calcium (Ca) and phosphorus (P) levels were acquired with an energy dispersive X-ray spectrometer (Vantage, Aquisition Engine Company, Tokyo, Japan) connected to a SEM (JSM-5600 LV; JEOL, Tokyo, Japan) with the following parameters: accelerating voltage 15 Kv, 10 mA beam current, working distance of 20 mm, and 100 s acquisition time. Per specimen, three areas (230 μm^2) were measured. The Ca and P contents were converted into the Ca:P ratio [1,6].

Statistical analysis

As the color, roughness, and microhardness data did not meet the assumptions of a parametric analysis, after descriptive and exploratory analyses (homogeneity of variance, quantile-quantile plots and the Shapiro-Wilk tests), the non-parametric tests of Kruskal Wallis and Dunn were used for comparisons between groups and Friedman and Nemenyi for comparisons between times using the R program (R Core Team; Computing, Vienna, Austria), with a significance level of 5%. The Raman and EDX spectroscopy data were submitted to Kruskal Wallis and Dunn for comparison between the groups (SigmaPlot 12; Systat Software, San Jose, CA, USA).

RESULTS

Color (ΔE, ΔE_{00}, ΔWID)

The color variation (ΔE, ΔE_{00}) and the bleaching index (ΔWID) at all the assessment times (after the 1st, 2nd, 3rd bleaching sessions, and 14 days after the treatment's end) were significantly lower in the control group than in the other groups ($p<0.0001$). Among the bleached groups there was no significant difference regarding color variation and bleaching index ($p>0.05$). **Table 2**.

Surface roughness (Ra)

Between the groups, the enamel roughness was not statistically different among the assessment periods ($p>0.04$) (after the 1st, 2nd, 3rd bleaching sessions, and 14 days after the treatment's end). However, the commercial group displayed higher Ra 14 days after the treatment's end than after the first bleaching session ($p<0.05$). **Table 3**.

Surface microhardness (SMH)

At baseline, the SMH was not significantly different between the groups ($p=0.1$). After the 3rd session, the CaPP 0.5% group had significantly higher SMH than the Experimental group ($p<0.05$), while 14 days after the treatment's end, the CaPP 1.5% group had significantly higher SMH values than the Experimental and Commercial groups ($p<0.05$). For the intragroup assessment, the Experimental group had higher SMH values 14 days after the treatment's end than after the 3rd session ($p<0.05$), while the CaPP 0.5% group showed a significant increase in SMH after the 3rd session compared to baseline ($p<0.05$). The CaPP 1.5% group showed greater SMH 14 days after the treatment than in the other two assessment periods ($p<0.05$). **Figure 1**.

Table 2. Color variation (ΔE; ΔE_{00}; Δ_{WID}) according to the treatment group and assessment period

	Experimental	CaPP 0.5%	CaPP 1.5%	Commercial	Control	p-value
			Median (minimum and maximum value)			
ΔE1	10.63 (8.06; 12.12) **A**	10.01 (7.83; 11.95) **A**	10.48 (7.62; 13.13) **A**	10.07 (7.49; 11.56) **A**	3.15 (0.60; 5.42) **B**	<0.0001
ΔE2	11.1 (8.67; 13.41) **A**	11.56 (9.13; 12.92) **A**	11.22 (8.72; 13.56) **A**	10.75 (8.05; 13.73) **A**	3.11 (0.7; 5.03) **B**	<0.0001
ΔE3	14.26 (11.02; 16.08) **A**	13.48 (12.36; 15.26) **A**	14.02 (11.92; 17.08) **A**	14.00 (11.71; 17.20) **A**	2.32 (1.17; 3.48) **B**	<0.0001
ΔE4	6.24 (3.89; 8.02) **A**	7.04 (6.06; 8.80) **A**	6.92 (4.78; 10.48) **A**	7.77 (5.68; 9.30) **A**	2.78 (0.60; 5.94) **B**	<0.0001
$\Delta E_{00}1$	5.90 (4.46; 7.17) **A**	5.86 (3.81; 7.01) **A**	6.29 (4.61; 7.23) **A**	5.76 (4.28; 6.69) **A**	1.83 (0.34; 3.26) **B**	<0.0001
$\Delta E_{00}2$	6.39 (4.86; 6.91) **A**	6.63 (5.18; 7.52) **A**	6.42 (5.14; 7.98) **A**	5.91 (4.39; 7.12) **A**	2.18 (0.49; 3.24) **B**	<0.0001
$\Delta E_{00}3$	8.14 (6.18; 9.03) **A**	7.78 (6.72; 9.07) **A**	8.06 (6.46; 10.17) **A**	7.77 (6.7; 9.7) **A**	1.69 (1.01; 2.28) **B**	<0.0001
$\Delta E_{00}4$	3.50 (2.04; 4.34) **A**	3.73 (3.17; 4.54) **A**	3.68 (2.36; 5.43) **A**	4.02 (3.01; 5.59) **A**	1.60 (0.46; 3.60) **B**	<0.0001
ΔWID1	17.11 (10.73; 20.07) **A**	15.79 (12.00; 18.42) **A**	15.99 (11.44; 21.66) **A**	14.54 (12.58; 20.42) **A**	1.53 (-4.74; 2.70) **B**	<0.0001
ΔWID2	15.17 (8.93; 20.45) **A**	15.86 (10.01; 18.95) **A**	15.76 (11.43; 19.29) **A**	14.46 (11.17; 20.57) **A**	-3.06 (-5.52; 0.23) **B**	<0.0001
ΔWID3	19.19 (13.22; 25.08) **A**	19 (15.64; 21.99) **A**	20.53 (16.11; 23.66) **A**	19.09 (16.11; 26.12) **A**	-1.73 (-7.13; 0.43) **B**	<0.0001
ΔWID4	8.60 (2.53; 12.47) **A**	9.24 (5.00; 13.55) **A**	9.74 (5.13; 13.84) **A**	9.13 (5.21; 14.56) **A**	-3.42 (-4.62; -1.02) **B**	<0.0001

Times: ΔE1; $\Delta E_{00}1$; ΔWID1: Baseline – 1st session. ΔE2; $\Delta E_{00}2$; ΔWID2: Baseline – 2nd session. ΔE3; $\Delta E_{00}3$; ΔWID3: Baseline – 3rd session; ΔE4; $\Delta E_{00}4$; ΔWID4: Baseline – 14 days' after. Groups: Experimental (HP35%); CaPP 0.5% (HP35% + CaPP-0.5%); CaPP 1.5% (HP35% + CaPP-1.5%); Commercial (HP35%-Whitenes HP-Maxx); and Control (without bleaching).
Different case letters indicate significant difference between the groups. p values - Kruskal Wallis and Dunn tests.

Table 3. Enamel superficial roughness values (Ra) according to the treatment group and time of assessment

	Experimental	CaPP 0.5%	CaPP 1.5%	Commercial	Control	*p-value*
	Median (minimum and maximum value)					
Baseline	0.024 (0.014; 0.034) **Aa**	0.026 (0.02; 0.033) **Aa**	0.023 (0.019; 0.035) **Aa**	0.022 (0.017; 0.035) **Aab**	0.03 (0.021; 0.047) **Aa**	*0.0420*
1st session	0.024 (0.014; 0.036) **Aa**	0.025 (0.017; 0.031) **Aa**	0.022 (0.017; 0.034) **Aa**	0.022 (0.018; 0.033) **Ab**	0.029 (0.014; 0.043) **Aa**	*0.5160*
2nd session	0.027 (0.014; 0.035) **Aa**	0.023 (0.018; 0.033) **Aa**	0.023 (0.017; 0.032) **Aa**	0.024 (0.018; 0.032) **Aab**	0.027 (0.016; 0.042) **Aa**	*0.6648*
3rd session	0.023 (0.013; 0.053) **Aa**	0.026 (0.018; 0.035) **Aa**	0.024 (0.016; 0.036) **Aa**	0.023 (0.015; 0.040) **Aab**	0.031 (0.018; 0.038) **Aa**	*0.1884*
14 days after	0.026 (0.015; 0.037) **Aa**	0.026 (0.016; 0.038) **Aa**	0.023 (0.018; 0.035) **Aa**	0.025 (0.022; 0.039) **Aa**	0.033 (0.017; 0.045) **Aa**	*0.1623*

Groups: Experimental (HP35%); CaPP 0.5% (HP35% + CaPP-0.5%); CaPP 1.5% (HP35% + CaPP-1.5%); Commercial (HP35%-Whitenes HP-Maxx); and Control (without bleaching).

Different letters indicate significant difference between the groups (upper case letters in the lines) and within the groups at the different assessment times (lower case in the columns). p values - Kruskal Wallis and Dunn tests.

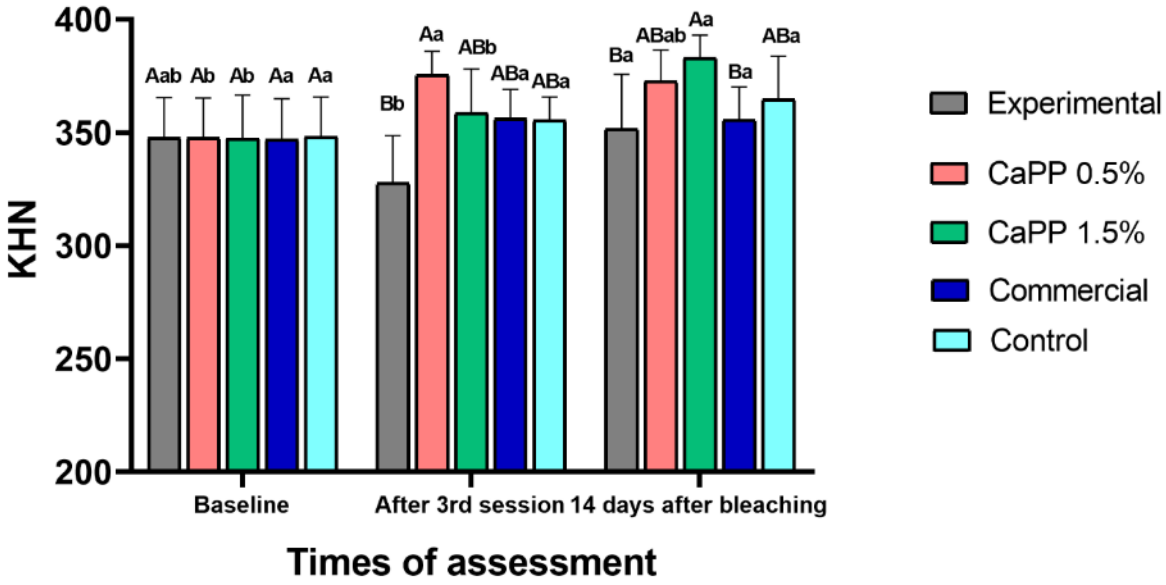

Figure 1. Surface microhardness (SMH) variation according to the treatment group and time of assessment. *Different letters indicate significant difference between the groups (upper case letters) and within the group for each assessment time (lower case).*

Raman

The ratio between the areas under the 431-449 cm^{-1} and the 960 cm^{-1} bands (430:960), and the 540-630 cm^{-1} and the 960 cm^{-1} bands (580:960) did not differ among the groups ($p>0.5$). However, the ratio between the areas under the 1043-1070 cm^{-1} and the 960 cm^{-1} bands (1070:960) was significantly higher in the Experimental group than in the CaPP 1.5% and control groups ($p<0.04$). **Table 4 and Figure 2**.

Table 4. Raman areas ratio between the 431-449 cm^{-1} (PO_4^{3-}), 540-630 cm^{-1} (PO_4^{3-}), 1043-1070 (CO_3^{2-}) and the 960 cm^{-1} (PO_4^{3-}) area according to the treatment group and time of assessment.

	Experimental	**CaPP 0.5%**	**CaPP 1.5%**	**Commercial**	**Control**	***p-value***
Ratios	**Median (minimum and maximum value)**					
430:960	0.309 (0.281; 0.333) **A**	0.307 (0.290; 0.316) **A**	0.304 (0.288; 0.329) **A**	0.299 (0.296; 0.341) **A**	0.332 (0.293; 0.397) **A**	*0.495*
580:960	0.210 (0.156; 0.248) **A**	0.207 (0.190; 0.253) **A**	0.214 (0.179; 0.227) **A**	0.203 (0.187; 0.230) **A**	0.227 (0.180; 0.246) **A**	*0.922*
1070:960	0.118 (0.101; 0.130) **A**	0.109 (0.056; 0.127) **AB**	0.057 (0.032; 0.077) **B**	0.095 (0.027; 0.149) **AB**	0.037 (0.037; 0.083) **B**	*0.009*

Groups: Experimental (HP35%); CaPP 0.5% (HP35% + CaPP-0.5%); CaPP 1.5% (HP35% + CaPP-1.5%); Commercial (HP35%-Whitenes HP-Maxx); and Control (without bleaching). 430:960 (431-449 cm^{-1}:960 cm^{-1}), 580:960 (540-630 cm^{-1}:960 cm^{-1}), 1070:960 (1043-1070 cm^{-1}: 960 cm^{-1})
Different letters indicate significant difference between the groups. p values - Kruskal Wallis and Dunn tests.

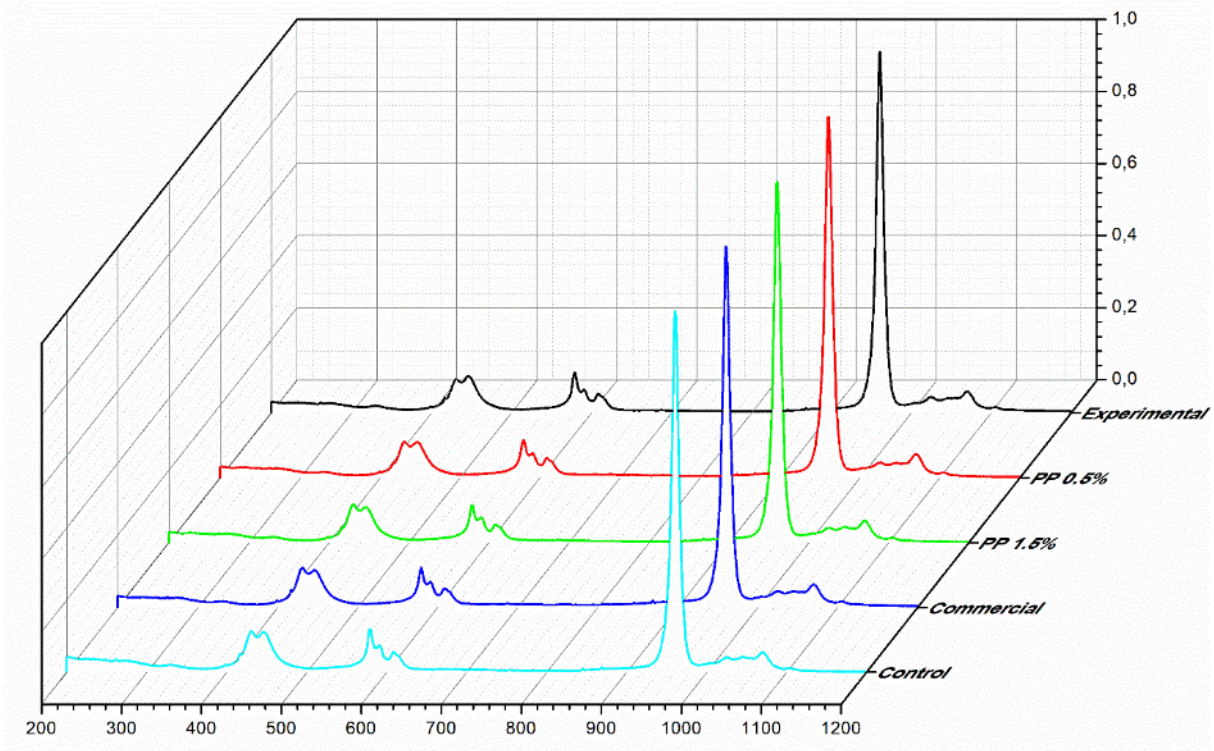

Figure 2. Raman average spectra of the mineral components in the 200-1200 cm^{-1} region of the enamel samples. Showing the phosphate (431-449 cm^{-1} − v2; 540-630 cm^{-1} − v4; 960 cm^{-1}− v1), and carbonate peaks (1043-1070 cm^{-1} − v3) bands intensity.

Scanning electron microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDX)

The SEM images demonstrates the greatest alterations on the enamel surface treated with the Experimental bleaching gel (Figure 3a) when compared with the specimens treated with the other bleaching gels (Figure 3b, c, d) using the control group as reference (Figure 3e), 14 days after the end of the bleaching treatment. **Figure 3**. The EDX results indicated that the Experimental gel presented the lowest Ca:P values and was statistically different from the rest ($p<0.01$). Also, the atomic percentage (at%) of Ca and P were the lowest and highest, respectively, in the Experimental group after 14 days of treatment ($p<0.01$). **Table 5**.

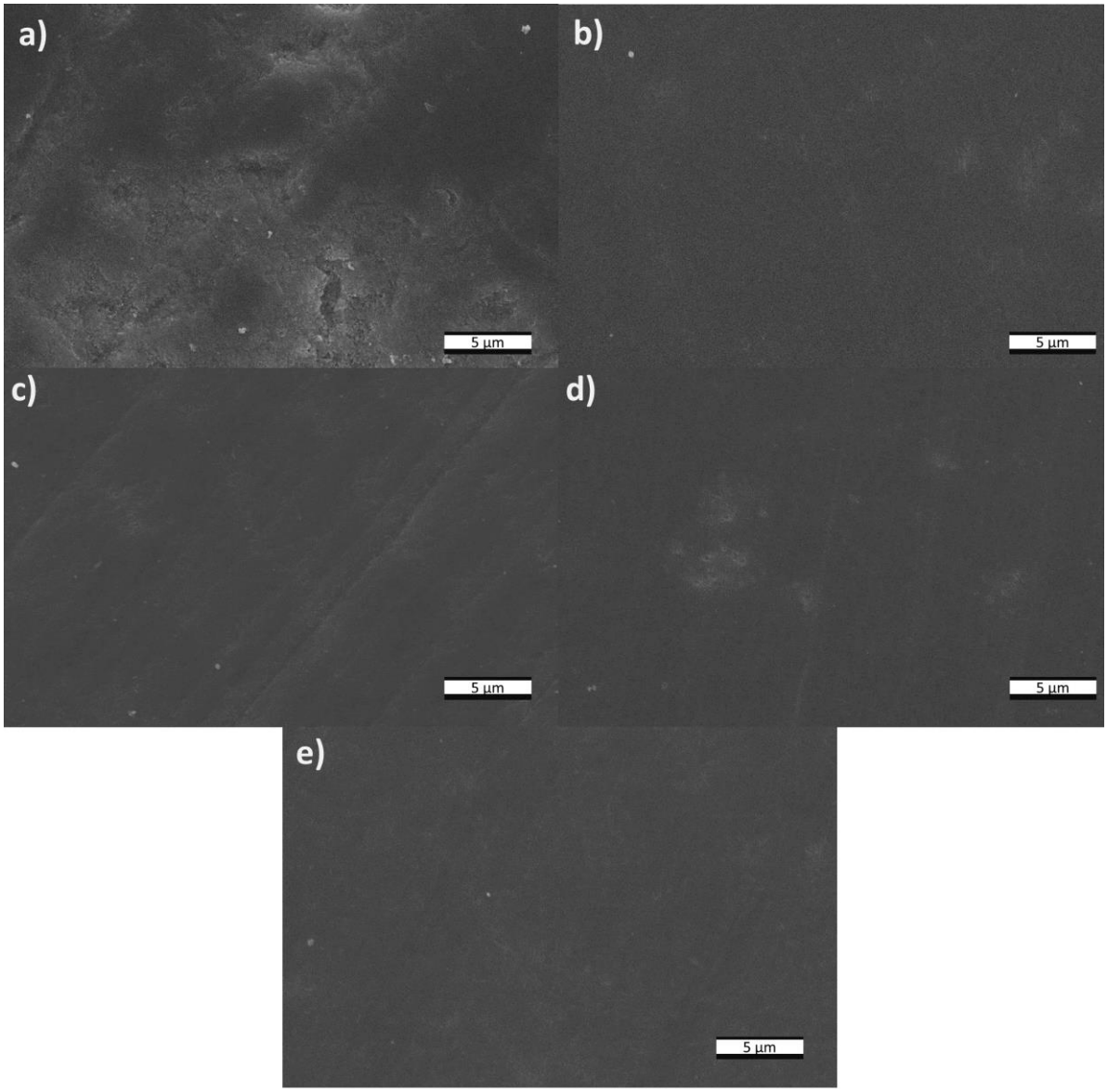

Figure 3. Scanning Electron Microscopy - SEM images (x4000) of the enamel surface 14 days after the bleaching treatment: a) Experimental (HP35%); b) CaPP 0.5% (HP35% + CaPP-0.5%); c) CaPP 1.5% (HP35% + CaPP-1.5%); d) Commercial (HP35%-Whitenes HP-Maxx); e) Control (without bleaching).

Table 5. Ca and P (at%) values and Ca:P ratio as determined by the x-ray diffraction spectroscopy (EDX).

Group	Ca (at%)	P (at%)	Ca:P
	Mean		
Experimental	65.02 **B**	34.98 **A**	1.86 **B**
CaPP 0.5%	65.32 **AB**	34.68 **AB**	1.88 **AB**
CaPP 1.5%	65.60 **A**	34.40 **B**	1.91 **A**
Commercial	65.39 **AB**	34.28 **B**	1.91 **A**
Control	65.71 **A**	34.29 **B**	1.92 **A**

**Different letters in the columns indicate statistically significant differences between the groups (p≤0.05) after Kruskal Wallis and Dunn tests.*

DISCUSSION

This study assessed the color variation, roughness (Ra), surface micro-hardness (SMH), and the mineral content of enamel samples after being treated with experimental bleaching gels containing 35%-HP and CaPP, based on the co-precipitation method [30,31], in two different concentrations (0.5 and 1.5 wt%) that were previously assessed, demonstrating adequate biocompatibility and chemical properties [30]. Based on our results, the first research hypothesis was accepted because the color alteration and Ra were similar among the groups, and the SMH of the samples treated with CaPP 0.5% was higher than the experimental gel without CaPP after the treatments´ end. Similarly, after 14 days of saliva storage, the CaPP 1.5% SMH was higher than the experimental and commercial gels without CaPP. The second research hypothesis was also accepted since the mineral content of the enamel treated with HP-CaPP was better preserved after the treatment's end when compared to the one treated with an experimental gel without CaPP.

The color variation among the bleaching groups was similar during all bleaching sessions. After 14 days of saliva storage, the expected color rebound effect related to the tooth rehydration [34] was observed in all groups. Still, the samples had values above the acceptability level, ΔE (>5.4) and ΔE_{00} (>3.6), for this the bleaching treatments can be considered effective [35] without difference among the treated groups. Also, the bleaching index (ΔWID) highest values on the bleached groups

corroborates the bleaching treatment effectiveness [33] without significant difference between them. Hence, the CaPP incorporation did not interfere with the gel's bleaching effectiveness. Although there is no known report about the effect of CaPP on the HP action, it can be assumed that the small particle size [25,26,30] and the concentrations used could not alter the HP diffusion through the tissues to prevent its bleaching action.

The enamel Ra was similar among all groups at all times. Even though some degree of alteration was expected between the control and bleaching groups due to the HP and its byproducts' effects, the lack of alteration of the surface could be related to the saliva action [14]. There is evidence of the Ra alteration after bleaching treatments; [14,19] however, some studies have found minimum Ra alterations even with high-concentration bleaching gels [7,20]. The discrepancies may be related to the gel formula and/or pH levels and the saliva incorporation in the study design [11]. Furthermore, in this study, only the commercial group had the highest Ra 14 days after the treatment when comparing after the 1st bleaching session; it could be theorized that after the bleaching treatment, the saliva-induced remineralization could have not occurred in a regular manner [13], which may be related to this bleaching gel composition.

The SMH decreased for the experimental group after the 3rd bleaching session and was recovered after 14 days of saliva storage. The decrease could be related to the alterations produced in the inorganic and/or organic content of the enamel due to the oxidative action of the HP and its byproducts [3,5]. However, the experimental and commercial groups had lower SMH when compared to the CaPP-containing groups (CaPP 0.5% and CaPP 1.5%) after 14 days of saliva storage. To illustrate the CaPP remineralizing action, one must consider its biomimetic capacity [31] that would induce the mineral deposition by two routes: 1) direct fusion: where the exposed PO_4^{3-} groups of the hydroxyapatite (HA) would ionically bind with the CaPP Ca^{2+}, and these, in turn, would act as a bonding bridge for other phosphates groups of the polyphosphate chain, allowing the polymer to accumulate on the surface [24]; 2) during the process of scission of the amorphous CaPP chain (by hydrolytic degradation), the precipitation of Ca^{2+}/PO_4^{3-} ions would form an amorphous mineral layer, which would serve as a precursor to a crystalline and more organized mineral matrix (hydroxyapatite) [25,29]. Also, the combination of both routes could occur when some of the amorphous CaPP sub-microparticles fuse with the amorphous mineral layer formed by the scission process [27].

Even if it could have been expected that the CaPP 1.5% group would produce the most positive effects after the 3rd bleaching session, the SMH increase for this group was, unlike the CaPP 0.5% group, more pronounced after 14 days of saliva storage. This could be related to the fact that the transformation of an amorphous precipitated layer is a gradual process, and a higher concentration at first would not have been able to generate a higher remineralizing effect. In a previous study [26], when incorporated into a toothpaste after 5 days of treatment, the CaPP deposit was still amorphous and not crystalline HA; for this, it has been suggested that longer periods of time in a physiological pH, like our artificial saliva, would favor the state transition.

The presence and semiquantitative analysis of inorganic moieties (PO_4^{3-} and CO_3^{2-}) of enamel were calculated after the bleaching treatment's end, using Raman spectroscopy as it allowed us to estimate the tissue composition as well as its quality, indicating the enamel mineralization degree [32,36]. In addition, the areas under the band are an objective measure to define the intensity of the analyzed PO_4^{3-} and CO_3^{2-} groups within the hydroxyapatite crystal phase [32]. For this, we determined the band ratios (430:960; 580:960; 1070:960) [32,36]. As in previous studies, the bleaching treatments used did not alter the PO_4^{3-} content within the enamel hydroxyapatite, which could be related to the sample's saliva storage during and after the bleaching's end [32,37].

Particularly, the Raman peak at 1070 cm^{-1} corresponds to the symmetric stretching mode of CO_3^{2-} that replaces PO_4^{3-} in the apatite lattice (ß-type carbonate) [36]. The Experimental group had an increased carbonate content that signalized a lattice distortion, a tendency to be instable, and thus higher susceptibility to acid attacks [36,38]. The highest ß-type carbonate in this group indicates the PO_4^{3-} substitution by CO_3^{2-} was not upregulated by the saliva action [36], while the CaPP 1.5% group retained similar values to the Control group indicating, in a similar manner to the SMH results, the polyphosphate presence could have diminished the microstructural changes caused by the HP by the remineralizing process previously described.

As in previous studies, the Ca and P levels were determined through EDX which signalized the demineralization and remineralization processes [15]. In our study, the Experimental group had the lowest Ca:P ratio, which would indicate a reduction in mineral content. This was not observed with the manipulated gels that contained CaPP, indicating their positive action in maintaining the mineral balance within the

enamel structure [25]. The Ca content was lowest for the Experimental group as previously related to the HP oxidation-reduction action within the tooth structure [6]. However, the P content was highest for this group, when the opposite trend was expected. This was previously reported and related to the EDX quantification method, where a higher Ca loss may increase the P relative values but does not imply the latter was incorporated into the enamel structure [6].

Although it could have been expected that the CaPP-containing groups would display higher mineral content when compared to the commercial group, this was not the case. This could be related to the fact that the hydrolytic cleavage of the CaPP chain is a time-dependent process [39] and the enamel exposure time to the bleaching gels may not have been sufficient to allow a higher ionic release. This factor could be surpassed by the enzymatic cleavage of the polymer chain promoted by certain phosphatases [40], which should be further studied. Also, the gels compositions determine in great manner their chemical interactions with the substrate [3]. Hence, as the CaPP-containing gels were better than the experimental one, this is a promising indicator of the gel's remineralizing effect. However, relevant factors, such as pulp chamber pressure, HP trans-enamel-dentin diffusion, erosion/abrasion conditions, were not mimicked/assessed in this study, which can be considered limitations of the present study. For this, further investigations should be undertaken to understand more about the CaPP interaction within the gel and tooth tissues.

Both the determination of the best chemical compounds to be incorporated into the bleaching gels as well as their proportion still represent challenges to aesthetic dentistry, which aims to increase the bleaching effect while reducing/eliminating simultaneously its adverse effects [18]. To the authors' knowledge, the CaPP using the co-precipitation method [30,31], and incorporation into highly concentrated bleaching gels for treating human enamel have not been previously described. Within the limitations of the study, it can be stated that the CaPP is a promising polymer to maintain the enamel inorganic structure during and after in-office bleaching treatments, without affecting the bleaching efficacy.

CONCLUSION

Within the limitations of this study, it was concluded that:

1. The addition of CaPP into high-concentration bleaching gels did not affect their effectiveness to produce relevant color changes in human enamel and did not alter the surface roughness.
2. After bleaching, the enamel surface microhardness increased with the CaPP-containing gels, and the mineral content was preserved when compared to an experimental gel without CaPP.

Thus, the bleaching of enamel with a gel containing CaPP was positive for the enamel's physical and chemical properties *in vitro.*

DECLARATIONS SECTION

A. Ethics Approval and Consent to Participate

The biological material used in this study was donated by adults through a written consent form, as previously approved by the university Ethical Committee (CAAE 24313019.9.0000.5418).

B. Funding

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C. Conflict of Interests

The authors declare no conflict of interest

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2.3 Enzymatic-driven mineralization of a calcium-polyphosphate bleaching gel

Artigo submetido ao periódico Journal of Dental Research (Anexo 3)

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ABSTRACT

The remineralizing ability of bleaching gels containing calcium-polyphosphate sub-microparticles (CaPP) could be potentialized through the use of the alkaline phosphatase enzyme (ALP). Therefore, the current study examined ALP activity and the effects of incorporating it in a highly concentrated hydrogen peroxide-based bleaching gel containing CaPP (HP-CaPP-ALP) on orthophosphate (PO_4^{3-}) levels, bleaching effectiveness, and enamel microhardness. ALP activity was assessed at different pH levels, H_2O_2-concentrations, and in H_2O- and Tris-based thickeners. Circular dichroism (CD) was used to examine changes in the secondary structure of ALP in water-, Tris-, or H_2O_2-based mediums. The PO_4^{3-} levels were evaluated in two thickeners with and without ALP. Enamel/dentin specimens were allocated into the following groups: control (without bleaching); commercial (Whiteness-HP-Maxx, FGM); Exp-H (H_2O-based); CaPP-H (H_2O-based, CaPP); ALP-H (CaPP+ALP); Exp-T (Tris-based); CaPP-T (Tris-based, CaPP); and ALP-T (CaPP+ALP). Color changes in the specimens ($\Delta E/\Delta E_{00}$) and the bleaching index (ΔWID) were calculated, and surface (SMH) and cross-sectional microhardness (CSMH) assessed. The two-way ANOVA and Tukey's post-hoc tests were used to compare ALP and PO_4^{3-} levels via pH and H_2O_2 concentration. Generalized linear models were used to examine ΔE, ΔE_{00}, SMH, and CSMH, while the Kruskal–Wallis and Dunn's tests were used to examine ΔWID (α=5%). The findings showed that ALP activity was higher at pH 9, lower in H_2O_2-based mediums, and similar in both thickeners. The changes in CD spectra indicated denaturation of the enzyme upon contact with H_2O_2. The PO_4^{3-} levels were higher after incorporating ALP, and the ΔE, ΔE_{00}, and ΔWID values were comparable among the bleached groups. SMH was lower after bleaching in the Exp-H group, while CSMH was higher in the ALP-T group compared to the remaining groups. Compared to gels containing CaPP only, the higher PO_4^{3-} levels in the HP-CaPP-ALP (ALP-T) group increased microhardness without decreasing bleaching effectiveness, suggesting bioinspired remineralization potential.

Keywords: Biomimetic, Tooth Bleaching, Polyphosphates, Alkaline Phosphatase, Hydrogen Peroxide, Circular Dichroism.

INTRODUCTION

The emergence of new biomimetic materials is accompanied by an increase in their use for the purpose of remineralizing dental hard tissues (Barot et al. 2021; Ionescu et al. 2022). Calcium-polyphosphate sub-microparticles (CaPP), which are amorphous polymers containing ±40 phosphate units linked by phosphoanhydride bonds, have been previously synthetized using co-precipitation (Guanipa et al. 2023a). CaPP act as precursor for the formation of crystalline materials that repair altered dental hard tissues, with the orthophosphates (PO_4^{3-}) releasement from the polymers acting as building blocks for enamel remineralization (Müller et al. 2017; Ackermann et al. 2019).

Upon completion of polymerization, the PO_4^{3-} and calcium divalent cations (Ca^{2+}) in the CaPP become unavailable for mineralization. For this, polyphosphates deliver phosphates to the mineralization sites, preventing “off-site” mineral precipitation (Omelon et al. 2009 & 2014). CaPP can release ions through: 1) spontaneous hydrolytic degradation of the polyphosphate chain at a slower rate, or; 2) cleavage of the chain by a phosphatase enzyme (e.g., alkaline phosphatase [ALP]) at an accelerated rate, especially in mediums with higher pH levels (e.g., buffer solutions) (Omelon et al. 2014; Zhang et al. 2021; Lorenz et al. 2001). The latter method ensures precise apatite saturation at the mineralization site (Omelon et al. 2009).

Much of the evidence on hydrogen peroxide-based (HP) bleaching gels to date have focused on their clinical effectiveness preservation while reducing their main adversary effects such as dental sensitivity and hard tissues surface defects (Balladares et al. 2019; Crastechini et al. 2019; Torres et al. 2019). Although some experimental HP bleaching gels have reported promising results after incorporating chemical activators (Oliveira et al. 2014; Soares et al. 2019) or remineralizing agents (Monteiro et al. 2020; Akabane et al. 2021; Yang et al. 2022; Guanipa et al. 2023b), evidence in this field remains unclear necessitating the development and assessment of new materials (Rauen et al. 2015; Pinto et al. 2017).

CaPP integrates into the tooth surface and releases Ca^{2+} and PO_4^{3-}, facilitating biomineralization of the dental tissues through transformation of the amorphous

precursors into crystalline materials (Müller et al. 2016; Guanipa et al. 2023b). This process is biologically upregulated by the enzymatic activity of ALP (Hasselgren et al. 1978; Sharma et al. 2014; Haarhaus et al. 2017). This boosting of the CaPP degradation is a biological process that within a remineralizing-bleaching system, could can facilitate enamel bleaching and concurrent preservation of its physical properties. However, as ALP activity is pH-dependent (Golub et al. 2007), the properties of such a bleaching system should be assessed in both water and buffer solutions (e.g., Tris-buffer).

The objective of the current study was to assess ALP activity and examine its effects on PO_4^{3-} levels in a highly concentrated hydrogen peroxide-based bleaching gel containing CaPP (HP-CaPP-ALP). Furthermore, its bleaching efficacy and effect on enamel microhardness after *in vitro* treatment were also examined. The research hypotheses were: (1) ALP activity will be higher in the presence of Tris and alkaline mediums but decrease in the presence of H_2O_2; (2) the PO_4^{3-} levels of HP-CaPP-ALP will be higher than that of gels without ALP; and (3) color changes (ΔE; ΔE00; and ΔWID) and the microhardness of enamel treated with HP-CaPP-ALP will be similar to or superior than that of enamel treated with experimental and commercial 35%-HP gels without CaPP and ALP.

MATERIALS AND METHODS

Assessment of ALP activity and its secondary structure by circular dichroism spectroscopy (CD)

The activity of ALP derived from calf intestine (524572; Merck KGaA, Germany) was assessed under different conditions including Tris-HCl buffers with varying pH levels (six, seven, eight, nine, and ten); different concentrations of H_2O_2 (i.e., 7,4, and 0.6 mg/mL); and thickeners prepared with different mediums (i.e., Tris-based and H_2O-based). Assessments were carried out using colorimetric endpoint assays (ALP-Kit; Labtest-Diagnóstico-S.A., Brazil), and all analyses were performed within 10 or 30 minutes of incorporating ALP in the medium of choice. After sample preparation (Akabane et al. 2021), the ALP absorbance values were determined using a microplate reader at 590 nm (Infinite-200-PRO; Tecan, Switzerland) and then converted into units per liter (U/L) using the manufacturer's formula. Structural changes in the ALP by the medium used (i.e., MilliQ water; Tris-buffer; HP-35%-H [water-based]; HP-35%-T [Tris-based]; and Sol-B [Tris-based]) were characterized by circular dichroism (CD) spectra

using an Applied Photophysics Chirascan spectrometer (further details provided in Appendix).

Assessment of PO_4^{3-} levels

Orthophosphate (PO_4^{3-}) levels in the thickener component of bleaching systems containing 0.5 wt% of CaPP in H_2O or Tris-HCl-buffer were assessed, with or without ALP incorporation. A colorimetric Phosphate Assay Kit (MAK-308; Sigma-Aldrich, USA) was used (Rahutomo et al. 2019) to examine: CaPP-H (H_2O-based/CaPP); ALP-H (H_2O-based/CaPP+ALP); CaPP-T (Tris-based/CaPP); and ALP-T (Tris-based/CaPP+ALP). After sample preparation (1.6 mg/mL), the optical densities of the solutions were measured, after 10 or 30min, in triplicate at 620 nm using a microplate reader. The PO_4^{3-} concentration was determined using a calibration curve obtained from aqueous solutions containing known concentrations of PO_4^{3-} (Huang et al. 2018; further details provided in Appendix).

Enamel preparation and bleaching treatment

After assessing the orthophosphate levels, *in vitro* bleaching treatment of bovine enamel specimens was carried out. Power calculations for the color (effect size=0.35; α=0.05; β =0.80; correlation=0.5) and microhardness analyses (effect size=0.35; α=0.05; β=0.80; correlation=0.2) were based on previous evidence, and the findings showed a minimum sample size of 10 per group per analysis (GPower 3.1-software; Heinrich-Heine-Universität, Germany).

A bench drill (FSB16; Schulz, Joinville, Brazil) was used to create 200 enamel-dentin disks (5.7×2.3 mm) from bovine incisors previously disinfected with 0.1% Thymol-solution. The disks were regularized (Freiria et al. 2022) and the prepared specimens were divided into two groups for color and microhardness analyses. The color specimens were stained by immersing them in a black tea solution (more details in Appendix). After initial color and surface microhardness (SMH) assessment (12% deviation considering a 359 KHN mean), the specimens were randomized into eight groups (n=10) including control (without bleaching); commercial (Whiteness-HP-Maxx, FGM); Exp-H (water-based-thickener without CaPP); CaPP-H (0.5wt%-CaPP); ALP-H (0.5wt%-CaPP+ALP); Exp-T (Tris-based-thickener without CaPP); CaPP-T (0.5wt%-CaPP); and ALP-T (0.5wt%-CaPP+ALP; **Appendix Table 1)**.

The specimens were then subjected to three bleaching sessions at weekly intervals, with each session including three applications lasting 15 minutes each. In the ALP-H and ALP-T groups, ALP was first mixed with the thickener solutions and left to react for 10 minutes. Thereafter, similar to the other groups, an H_2O_2-based solution was mixed with the thickener (3:1 weight proportion), and 0.02 g of the bleaching gel was applied to the enamel surface. The specimens were stored in artificial saliva (1.5 mM Ca^{2+}, 0.9 mM P, 150 mM KCl, 0.05 µg F^- /mL, and 0.1M Tris-buffer at pH 7) at 37°C throughout the bleaching process and up to 14 days after completion of treatment (Guanipa et al. 2023b).

Color (ΔE, ΔE00, ΔWID) assessment

Color assessment was carried out using a calibrated spectrophotometer (CM 700d; Minolta, Japan) in a standardized light environment (GTI Mini Matcher MM-1; GTI, USA). Three readings were recorded at baseline; after the 1st, 2nd, and 3rd sessions; and 7 and 14 d after completion of bleaching, and the spectral distribution was quantified using the CIELab color space. The total color change (ΔE/ΔE00) and bleaching indices (ΔWID) (Pérez et al. 2016; Paravina et al. 2019) were calculated using pre-established formulas (Guanipa et al. 2023b) with baseline readings serving as the reference (further details provided in Appendix).

Surface Microhardness and Cross-sectional Microhardness (CSMH) (SMH) assessment

Enamel SMH was assessed using a microdurometer (FM-100; Future-Tech-Corp, Japan) at baseline (prior to 1st bleaching session) and again after completion of the third bleaching session. A Knoop indenter (25 gf/5 s) was used to make five indentations 100 µm apart (Pini et al. 2022). After completion of bleaching treatment, the specimens were prepared and three columns of indentations were created at depths of 10, 20, 40, 60, 80, 100, 120, 140, 160, 180 µm from the enamel surface to determine the specimens' CSMH (Vieira et al. 2019; Freiria et al. 2022; further details provided in the Appendix).

Statistical analyses

After descriptive and exploratory analyses, the two-way ANOVA and Tukey's post-hoc tests were used to compare ALP and PO_4^{3-} levels by pH and H_2O_2

concentration. A t-test (GraphPad-Software-Prism-8; USA) was used to compare ALP levels by thickener type. Generalized linear models (GLM) were used to examine the ΔE and ΔE00, while ΔWID was analyzed using the Kruskal–Wallis and Dunn's tests. SMH was analyzed using a mixed GLM model adjusted for repeated measures in time, while CSMH was analyzed using a GLM model taking the effects of bleaching treatment with subdivided plots in the depths (R Core Team; Computing, Austria) into consideration. All analyses were carried out at a significance level of 5%.

RESULTS

Activity and secondary structure of ALP

The lowest and highest ALP activities were observed at pH levels of 6 ($p < 0.04$) and 9, respectively, after 10 and 30 minutes of incubation ($p<0.009$; **Figure 1 (a)**). In H_2O_2-containing solutions, the highest ALP activity was observed at a concentration of 0.6 mg/mL ($p<0.01$), independent of the time [**Figure 1 (b)]**. Both thickeners exhibited similar ALP activity after 10 minutes; however, the Tris-based medium was seen to exhibit higher ALP activity after 30 minutes ($p=0.0006$; **Figure 1 (c)**).

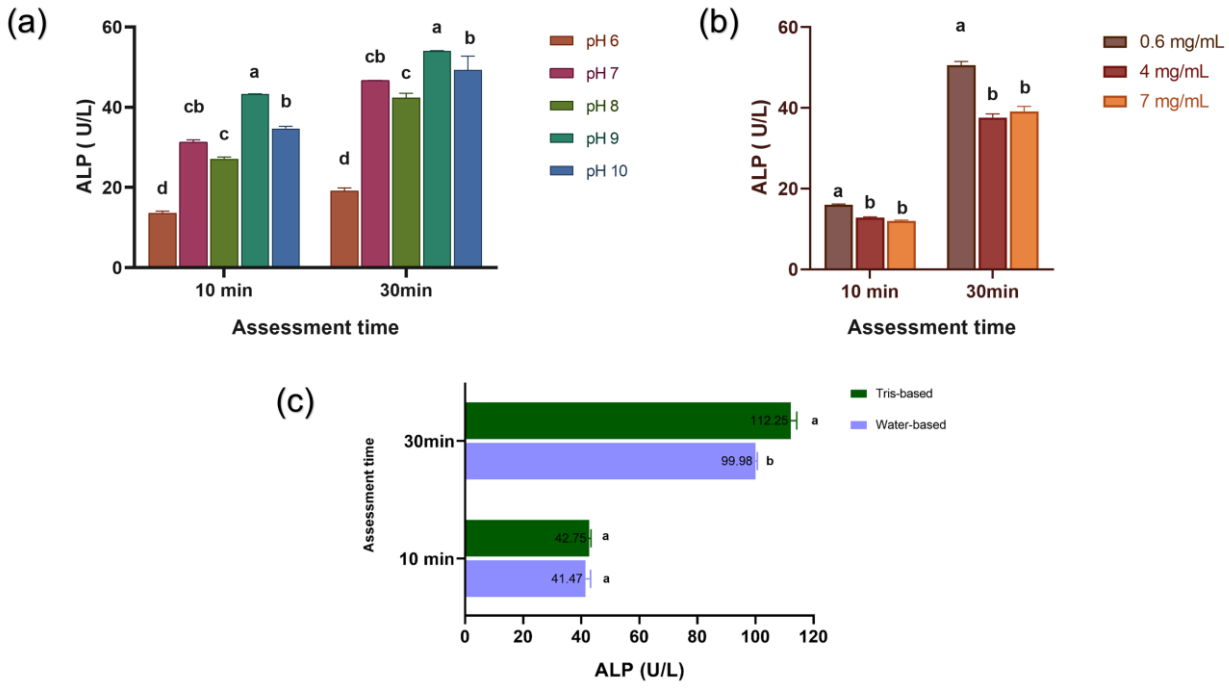

Figure 1. Alkaline phosphatase (ALP) activity as determined by the spectrophotometric technique (590 nm) after 10 and 30 minutes of assessment in: (a) different pH levels; (b) different concentrations of hydrogen peroxide; and (c) thickeners based on water or Tris. *The lowercase letter indicates a statistically significant difference between the groups.*

Significant changes in the CD spectra were observed in the HP-35%-H and HP-35%-T groups, with the negative centered at 225 nm decreasing. Furthermore, a single negative band at 205 nm was present in the HP-35%-H group (**Figure 2)**, and a decrease in the α-Helix (%) of samples diluted with peroxides was observed (**Appendix Table 2**).

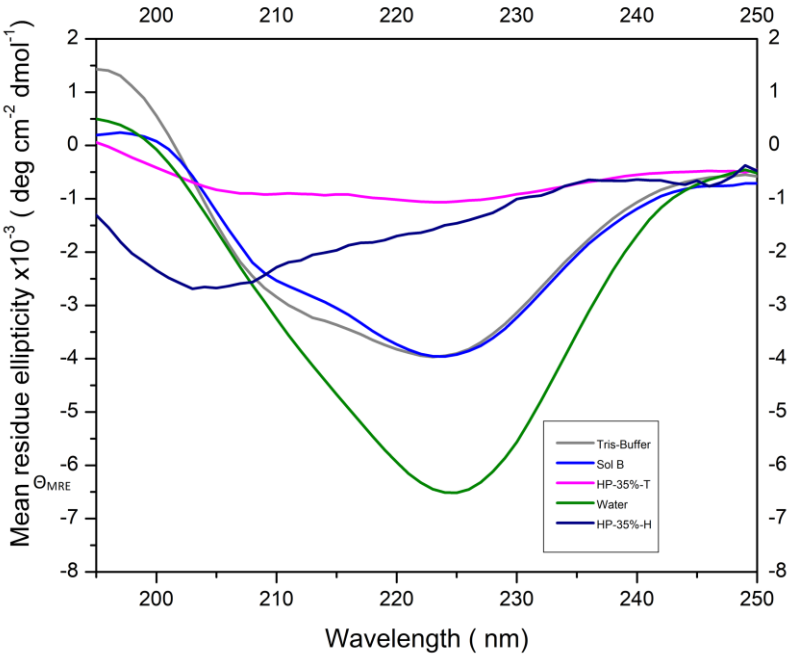

Figure 2. CD spectra of the alkaline phosphatase enzyme (ALP) dispersed in the different solutions.

PO_4^{3-} levels

Incorporation of ALP significantly increased free PO_4^{3-} levels (p<0.001) in both water-based (ALP-H) and Tris-based (ALP-T) thickener solutions. However, PO_4^{3-} levels did not increase significantly upon prolonging incubation time from 10 to 30 minutes (p>0.32; **Figure 3**).

Color (ΔE, ΔE00, ΔWID)

The color difference parameters (ΔE and ΔE00) were higher in the bleached groups compared to the control group (p<0.05), and no statistically significant differences were observed between the commercial and experimental bleaching gels (p>0.05). Similarly, the ΔWID values were significantly higher in the bleached groups

compared to the control group ($p<0.05$), although no inter-group differences were observed in the former ($p>0.05$; **Appendix Table 3**).

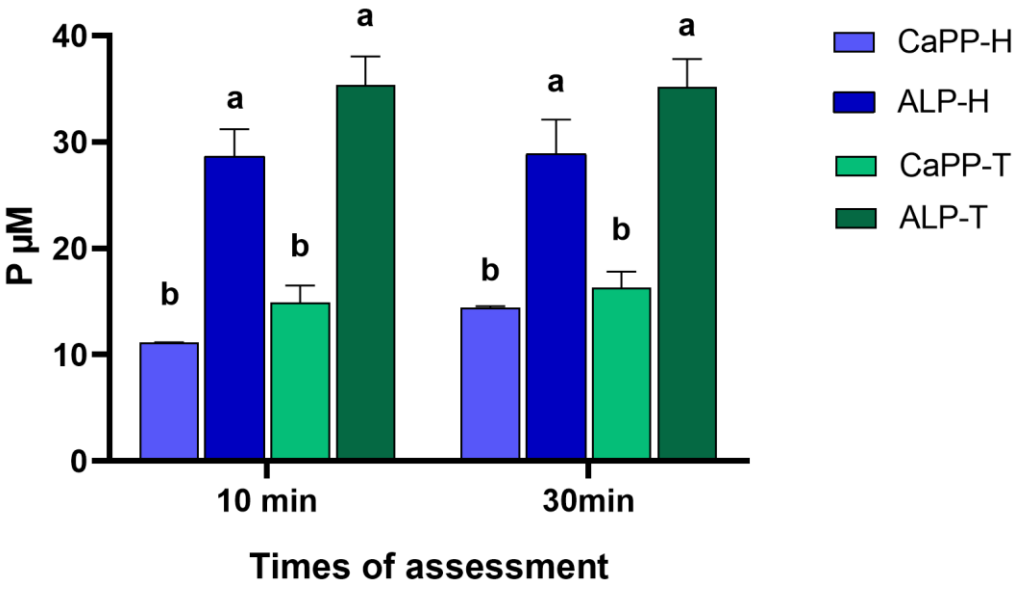

Figure 3. PO_4^{3-} concentrations (P µM) in the thickeners after 10 or 30 minutes of the ALP incorporation (15 U): CaPP-H (H_2O-based with 0.5 wt% CaPP); ALP-H (H_2O-based with 0.5 wt% CaPP + ALP); CaPP-T (Tris-based with 0.5 wt% CaPP); ALP-T (Tris-based with 0.5 wt% CaPP + ALP). *The lowercase letter indicates a statistically significant difference between the groups.*

Surface microhardness (SMH)

All groups exhibited similar SMH values at baseline ($p > 0.05$), although these were seen to be significantly decreased in the Exp-H and commercial groups after the third bleaching session ($p<0.05$). The ALP-T group exhibited the highest SMH values, and this was significantly different from that of the commercial and control groups (p-value<0.05). In contrast, the Exp-H group exhibited the lowest SMH values, and these were significantly different from that of the CaPP-H, ALP-H, Exp-T, CaPP-T, ALP-T, and control groups ($p<0.05$; **Figure 4**).

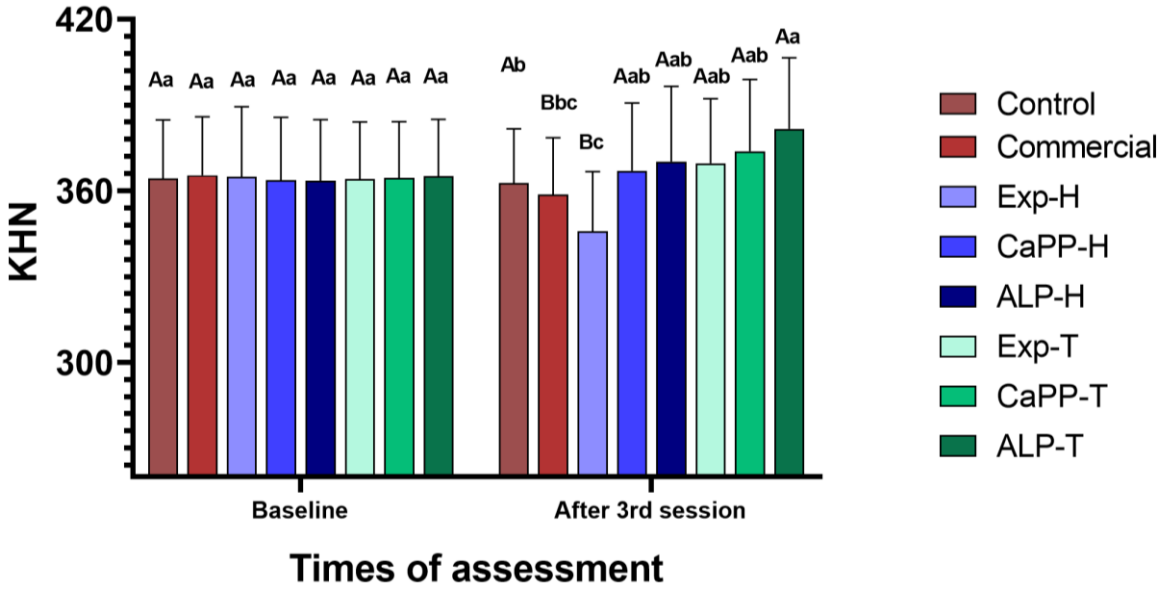

Figure 4. Surface microhardness (SMH) variation according to the treatment group and time of assessment. *Different letters indicate significant difference between the assessment times (upper case letters) and the treatment groups (lower case).*

Cross-sectional microhardness (CSMH)

The CSMH values after treatment were significantly higher in the ALP-T group when compared to the other groups ($p<0.05$). In contrast, the CSMH values were significantly lower in the Exp-H and commercial groups when compared to the control, CaPP-H, CaPP-T and ALP-T groups ($p<0.05$). Moreover, CSMH was seen to increase up to a depth of 100 µm ($p<0.05$) and then stabilize in all groups ($p>0.05$; **Table 1**).

Table 1. Cross-sectional microhardness (CSMH) values according to the treatment group and distance from the enamel surface.

Distance (µm)/ Multiple comparisons (distances)	[1]Group							
	Control	**Commercial**	**Exp-H**	**CaPP-H**	**ALP-H**	**Exp-T**	**CaPP-T**	**ALP-T**
	Mean (Standard deviation)							
10/d	253.80 (46.09)	229.27 (48.36)	225.79 (33.32)	261.52 (44.47)	257.23 (63.62)	252.63 (41.45)	268.00 (41.14)	282.98 (44.07)
20/c	315.00 (38.43)	299.19 (42.05)	290.14 (39.99)	327.24 (29.84)	325.07 (56.57)	308.15 (46.48)	338.72 (44.76)	354.99 (18.63)
40/b	357.19 (29.88)	343.43 (25.72)	309.19 (35.97)	352.00 (26.87)	354.71 (47.32)	346.58 (28.99)	354.93 (36.11)	381.47 (20.81)
60/b	356.66 (26.13)	352.95 (26.42)	317.96 (47.54)	359.04 (23.27)	358.61 (58.31)	340.80 (42.03)	359.27 (37.05)	382.85 (19.23)
80/b	363.74 (28.75)	345.53 (29.77)	322.34 (41.43)	352.46 (18.62)	352.47 (57.49)	339.12 (31.32)	362.22 (36.78)	386.80 (19.36)
100/a	370.85 (39.25)	346.59 (30.10)	329.56 (42.04)	359.77 (18.33)	360.73 (62.74)	349.64 (29.56)	367.34 (40.88)	390.13 (15.44)
120/a	370.64 (32.70)	354.94 (25.09)	329.87 (42.09)	369.51 (19.73)	357.56 (58.48)	348.79 (30.21)	367.02 (39.38)	384.07 (15.88)
140/a	371.80 (36.63)	342.79 (31.93)	330.85 (45.03)	370.35 (15.59)	360.98 (61.72)	343.10 (29.79)	368.86 (48.97)	394.55 (18.15)
160/a	374.00 (36.79)	350.06 (39.45)	335.78 (46.73)	364.65 (18.90)	360.43 (63.80)	344.84 (27.62)	370.25 (36.30)	393.73 (12.76)
180/a	371.32 (36.31)	348.36 (38.10)	330.31 (44.89)	369.91 (28.38)	365.69 (61.55)	348.78 (26.32)	365.95 (36.70)	402.54 (16.18)
Multiple comparisons (groups)	**B**	**C**	**C**	**B**	**BC**	**BC**	**B**	**A**

[1] Control (without bleaching); Commercial (HP35%-Whitenes HP-Maxx); Exp-H (Water based); CaPP-H (0.5 wt% CaPP); ALP-H (0.5 wt% CaPP + ALP); Exp-T (Tris based); CaPP-T (0.5 wt% CaPP); ALP-T (0.5 wt% CaPP + ALP). p(group) = 0.0025; p(distance) < 0.0001; p(interaction) = 0.2899. Different letters (uppercase in the groups and lowercase in the distance) indicate statistically significant differences ($p \leq 0.05$) – pooled mean.

DISCUSSION

Examination of the effects of ALP on experimental bleaching gels containing 35%-HP and 0.5 wt% CaPP (HP-CaPP-ALP) showed increased activity in Tris-based and alkaline mediums (pH 9) and decreased activity in the presence of H_2O_2. Therefore, the first research hypothesis was accepted. The findings of this study also showed that PO_4^{3-} levels increased upon incorporation of ALP; similar color changes (ΔE; ΔE00; ΔWID) were observed in all groups; and the SMH and CSMH values were higher after treatment using gels containing ALP in a Tris-based medium (ALP-T) when compared to the experimental and commercial gels without CaPP. Therefore, the second and third research hypotheses were also accepted.

ALP is a metalloenzyme that catalyzes the hydrolysis of phosphomonoesters such as polyphosphates. It plays a vital role in hard tissue formation due to its increased expression in mineralized tissue cells (Hasselgren et al. 1978; Golub et al. 2007). The current study measured ALP activity in different mediums using a test based on thymolphthalein monophosphate hydrolysis to enable identification of the most appropriate method of incorporating it in bleaching gels containing CaPP (Akabane et al. 2021).

H_2O_2 was seen to downregulate ALP activity. This was in agreement with the findings of a previous cell analysis (Oliveira et al. 2017). Alkaline mediums with pH nine were considered to be ideal (Sharma et al. 2014) as the ALP catalytic mechanism is favor by the formation of serine phosphate at the enzyme active site which then reacts with water to form inorganic phosphates (Golub et al. 2007). The thickener solutions did not decrease ALP activity and, as a result, both formulas were used for ALP incorporation in the subsequent phases of the study. Since the enzyme conformational integrity influences its activity (Zhang et al. 2000), the CD spectra support the ALP activity findings. Exposure to H_2O_2 induced changes in the secondary structure of the enzyme, as evidenced by observation of decreased ALP activity in H_2O_2-containing solutions.

Although encouraging results have been observed upon incorporating CaPP in bleaching gels containing hydrogen peroxide (Guanipa et al. 2023b), addition of ALP can increase the free orthophosphate levels (PO_4^{3-}) and facilitate its subsequent precipitation during bleaching treatment. In this study, the enzymatic effects on PO_4^{3-}

were assessed using a method based on the complexation of triarylmethane dye malachite green with phosphatemolybdate ions as it had greater sensitivity and simplicity (Rahutomo et al. 2019). The two-fold-increase in PO_4^{3-} levels observed in both thickener solutions within 10 minutes of ALP incorporation suggests scission of the polyphosphate chain mediated by the exopolyphosphatase. The maintenance of CaPP in an amorphous state can aid this process as it is more susceptible to enzymatic cleavage by ALP (Müller et al. 2016).

Although the majority of bleaching gels are water-based, previous evidence suggests that Tris-HCl buffers are more suitable when requiring greater ALP activity (Hethey et al. 2002). Therefore, two types of thickeners were formulated and the ALP activity, PO_4^{3-} levels, bleaching efficacy, and enamel microhardness after treatment were assessed using both. The findings showed that both water- and Tris-based thickeners exhibited similar PO_4^{3-} levels, even 30 minutes after incorporation. This could likely be attributed to the fact that higher Ca^{2+} concentrations during CaPP hydrolysis lowered ALP activity up to a certain point, regardless of the medium used (Huang et al. 2018).

The color changes observed in the specimens confirmed the efficacy of all treatments examined in this study, with the observed values being above the acceptability level (ΔE >5.4 and ΔE00 >3.6) (Paravina et al. 2019). Similarly, the bleaching index (ΔWID) exhibited the highest values when compared to the control group, confirming that CaPP (Guanipa et al. 2023b) and ALP incorporation did not alter bleaching effectiveness. Examination after 7 and 14 d showed maintenance of the bleaching effect even after color stabilization. To the best of our knowledge, this is the first study to provide a scientific description of the association between phosphatase and bleaching gel formulations. Based on the initial ALP analysis, it can be inferred that due to its activity reduction in the presence of H_2O_2, the last one mechanism of action was not altered.

A reduction in SMH was observed after bleaching in the commercial and Exp-H groups, with the latter exhibiting the greatest overall reduction, potentially due to the oxidizing action of H_2O_2 (Vieira et al. 2019). In agreement with previous results (Guanipa et al. 2023b), incorporation of CaPP in a bleaching gel was seen to exert a positive effect on enamel properties after treatment, as observed in the CaPP-H and CaPP-T groups. With regards to ALP incorporation, the highest SMH values were

observed in the ALP-T and ALP-H groups, suggesting mineral deposition on the surface and/or maintenance of the enamel mineral content potentiated by ALP (Ackermann et al. 2019). Previous studies examining other re-mineralizing agents such as trimethaphosthate with fluoride or hydrated calcium silicate suggest that mineral deposition can be effective in preventing surface mineral loss after bleaching (Akabane et al. 2021; Yang et al. 2022).

The CSMH analysis examined the efficacy of the experimental gels in preventing in-depth mineral loss in the enamel (Júnior et al. 2022), and the findings showed that remineralization was more effective after incorporating CaPP and ALP in Tris-based thickeners (ALP-T) compared to water-based thickeners (ALP-H) or CaPP alone (CaPP-H and CaPP-T). This suggests that ALP in the water-based thickener was not able to translate the greater ions releasement, reported in the PO_4^{3-} levels, into the CSMH values. This potentially indicate that the lower pH values of this formula (≈5) induced greater demineralization than that generated by Tris-based thickeners with a more neutral pH (≈6). Therefore, the former induced mineral loss that could not be compensated by the ionic burst, unlike the latter, which exhibited CSMH values that surpassed those observed in the control group (without bleaching).

In summary, the hardness values indicate successful mineral gain and/or preservation induced by the effect of ALP on CaPP. Upregulation of apatite biomineralization by ALP is initiated by enzymatic cleavage of one terminal hydrogen-phosphate and one additional proton per anhydride linkage of CaPP (Ackermann et al. 2019). This process releases PO_4^{3-} and Ca^{2+} from the polyphosphate chain, making them locally available. Precipitation of the ions on the dental surface can result in formation of apatite derived from the amorphous precursors (Müller et al. 2019). The main limitations of the current study were that simulations of other oral environments were not considered and more extensive analyses of the chemical properties of the enamel were not undertaken, and future studies should aim to address these.

Previous studies examining enzymatic action within bleaching gels incorporated peroxidase and/or catalase enzymes in HP-based gels and reported promising outcomes with regard to bleaching effectiveness and trans-tissue diffusion (Soares et al. 2019; Zuta et al. 2019). However, to the best of our knowledge, this is the first study to demonstrate that a phosphatase enzyme increases ionic availability in a bioinspired way. Moreover, this was also found to be beneficial for the physical properties of the

enamel *in vitro*, likely due to the bioinspired remineralization potential. Elucidation of the biomimetic principles can impulse the development of modified bleaching gels, such as those used in the current study (i.e., containing CaPP and ALP), and facilitate examination of their effects on undesirable clinical symptoms associated with dental bleaching treatments.

Following examination of the effects of incorporating ALP in highly concentrated hydrogen peroxide-based bleaching gels containing CaPP (HP-CaPP-ALP) its concluded:

- ALP activity was higher in mediums with pH 9, reduced in H_2O_2-mediums, and remained similar in Tris- or water-based thickeners.
- The PO_4^{3-} levels were higher following incorporation of ALP in gel thickener solutions, indicating high polymer scission.
- Both HP-CaPP-ALP (ALP-H and ALP-T) solutions demonstrated adequate bleaching effectiveness.
- The HP-CaPP-ALP gels exhibited increased enamel surface microhardness after treatment when compared to the commercial or experimental gels without CaPP. Moreover, the ALP-T group exhibited the highest microhardness values after treatment.

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Appendix

Materials and methods

Assessment of alkaline phosphatase activity

All assessments were carried out using a colorimetric endpoint assay (ALP-Kit; Labtest Diagnóstico S.A., Brazil) wherein 250 µL of a buffer solution was added to 25 µL of a thymolphthalein monophosphate solution and kept under 700 rpm agitation (Thermomixer Comfort; Eppendorf-SE, Germany) at 37°C (2 minutes). Thereafter, 25 µL of the sample was mixed with the solution, stored under the previously mentioned conditions for 10 minutes, and then 1,000 µL of a color reagent was added to react with the hydrolyzed thymolphthalein and modify the final color of the product (Akabane et al. 2021). The ALP absorbance values were determined using a microplate reader at 590 nm (Infinite-200-PRO; Tecan, Switzerland), and then converted into units per liter (U/L) according to the manufacturer's formula as follows:

$$ALP = (Asamp / Astand) \times 45 \quad (1)$$

Where Asamp = absorbance of the sample and Astand = absorbance of the standard.

Analysis of the secondary structure of alkaline phosphatase using circular dichroism spectroscopy (CD)

CD spectra were recorded using an Applied Photophysics Chirascan spectrometer running the Pro-Data Chirascan software (v4.2.22). At least three scans were recorded for each sample under the following conditions: temperature=25 °C; wavelength range of 190–250 nm using intervals of 1 nm, in Suprasil quartz cells (Hellma U.K. Ltd.) with a path length of 0.02 mm. The mean values of the scans were then calculated and the respective baseline values subtracted. The resulting net spectra were smoothed using a Savitzky–Golay filter with smoothing windows of 5–10 data points.

The mean residue ellipticity (Θ_{MRE}) was defined as $\Theta_{MRE} = \Theta/(c*n*l)$

where Θ is the raw CD ellipticity (mdeg); n is the number of amino acids in the solvated peptide; l is the path length of the quartz cuvette used (mm); and c is the molar concentration of the peptides. The CD spectra were analyzed using the BeStSel web server to allow estimation of the relative amount of specific secondary conformational elements in the samples.

Intestinal-type alkaline phosphatase solution in 6 mM Tris/HCl, 6 mM magnesium chloride, 0.12 mM zinc chloride, and 40% glycerol pH 7.6 (Calbiochem, Germany; activity: 30,100.0 U/mL; molecular weight: 140,000 Da) were used. To 1 µL of the enzyme (concentration 17.8 mg/mL) 60 µL of each solution was added and left to react for 15 minutes before carrying out measurement. The solutions used included MilliQ water; Tris-buffer; HP-35% (water-based); HP-35% (Tris-based); and Sol-B (Tris-based).

Examination of orthophosphate (PO_4^{3-}) levels

A colorimetric Phosphate Assay Kit (MAK-308; Sigma-Aldrich; Missouri, USA) was used to analyze PO_4^{3-} levels (Rahutomo et al. 2019). The thickener was weighed (25 mg) and 15 U of exogenous ALP from calf intestine (524572; Merck KGaA, Darmstadt, Germany) was added and kept in an incubator (700 rpm) at 37°C (Thermomixer Comfort 5355; Eppendorf SE, Hamburg, Germany). Thereafter, the gels were further diluted to a final concentration of 1.6 mg/mL so that they remained within the assay kit detection range (0.4–50 µM PO_4^{3-}).

The prepared samples were mixed with malachite green in a 96-well-plate and kept in an incubator (KS260; IKA®, Staufen, Germany) under agitation (250 rpm) at 37°C. The optical densities of the solutions were read in triplicate at 620 nm using a microplate reader (Infinite 200 PRO; Tecan Trading, AG, Switzerland). The PO_4^{3-} concentration was determined by employing a calibration curve obtained from a series of aqueous solutions containing known concentrations of PO_4^{3-} (40, 32, 24, 16, 12, 8, 4, 0 µM) as per the manufacturer's instructions (Huang et al. 2018). Moreover, thickeners without CaPP or ALP were used as blank controls and subtracted from the group's final values.

Enamel specimen preparation and bleaching treatment

The specimen surfaces were regularized using granulated silicon carbide paper (600, 1,200, 2,500, and 4,000 grid) and polished using felt and diamond pastes (1 and 1/4 µm), mounted in a polishing unit (Arotec S.A. Ltda; Brazil) (Freiria et al. 2022). The color specimens were stained by immersing them in a black tea solution (1.6 g of black tea in 100 mL of boiled distilled water for 5 minutes) that was renewed daily for 6 days, followed by one-week of immersion in artificial saliva.

Color assessment

For the five assessment points (T1=after 1st session; T2=after 2nd session; T3=after 3rd session; T4=7 days after bleaching; T5=14 days after bleaching), the following formulas were used to determine total color change (ΔE/ΔE00) and bleaching indices (ΔWID) (Pérez et al. 2016):

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]\ ½ \quad (1)$$

$$\Delta E00 = [(\Delta L'/KLSL)^2 + (\Delta C'/ KCSC)^2 + (\Delta H'/KHSH)^2 + RT\ (\Delta C'/KCSC)\ (\Delta H'/ KHSH)]^{½} \quad (2)$$

$$\Delta WID = 0.511\Delta L^* - 2.324\Delta a^* - 1.100\Delta b^* \quad (3)$$

Where ΔL = assessment time L* - baseline L*; Δa = assessment time a* - baseline a*; and Δb = assessment time b* - baseline b*. As per the CIEDE2000 metric, ΔL', ΔC', and ΔH' represent changes in brightness, chrome, and hue values, respectively. SL, SC, and SH are parameters that regulate the values of the coordinates; KL, KC, and KH are correction parameters for the experimental conditions; and RT takes into account the interaction between differences in chroma and hue in the blue region (Paravina et al. 2019).

Assessment of cross-sectional microhardness

The specimens were first cut longitudinally using a double-faced diamond disk, and one of the sections was immersed in epoxy resin (2001; Redelease, SP, Brazil). Grinding and polishing was carried out as described previously to expose the specimen surface. A Knoop indenter (25 gf / 5 s) was used to create three columns (spaced at least 100 µm apart) of indentations at depths of 10, 20, 40, 60, 80, 100, 120, 140, 160, 180 µm from the enamel surface to allow measurement of CSMH using a microdurometer (FM 100, Future-Tech Corp; Kawasaki, Japan).

Appendix tables

Appendix Table 1. Treatment groups for the study, composition, batch/manipulation details.

Treatment groups	Composition	Batch	Manipulation
Control	No bleaching gel	-	-
Commercial	Glycol, inorganic fillers, H_2O_2-30-35 wt%, mixture of pigments, deionized water, thickener.	061222	Mix the components in a 3:1 proportion for 30s.
Exp-H / Exp-T	Glycerol, Propylene glycol, H_2O_2-35 wt%, deionized water or Tris Buffer solution, acrylic acid thickener.	23/16101	
CaPP-H / CaPP-T	Glycerol, Propylene glycol, H_2O_2-35 wt%, deionized water or Tris Buffer solution , acrylic acid thickener, CaPP 0.5 wt%.	23/16102	
ALP-H / ALP-T	Glycerol, Propylene glycol, H_2O_2-35 wt%, deionized water or Tris Buffer solution , acrylic acid thickener, CaPP 0.5 wt%, ALP (thickener).	23/16103	

Appendix Table 2. Secondary structure of the alkaline phosphatase (ALP) dispersed in the different solutions

Solution	α-Helix (%)	β-sheets (%)	Turn and coil (%)	NRMSD
Water	11.5	52.7	35.4	0.06
Tris buffer	7.5	28.1	64.4	0.04
Sol B	9.1	26.1	64.8	0.05
HP-35%-T	3.7	27.3	69.1	0.11
HP-35%-H	2.9	28.8	67.4	0.08

Water (MilliQ water); Sol B (Tris-based); HP-35%-T (tris-based); HP-35%-H (water-based); NRMSD: normalized root mean square error deviation

Appendix Table 3. Color variation (ΔE; ΔE_{00}; Δ_{WID}) according to the treatment group and assessment period

	Control	Commercial	Exp-H	CaPP-H	ALP-H	Exp-T	CaPP-T	ALP-T	p-value
					Median (minimum and maximum value)				
ΔE1	3.62 **B** (2.69; 4.70)	11.44 **A** (9.64; 15.11)	12.45 **A** (7.30; 20.33)	11.69 **A** (8.94; 21.38)	11.30 **A** (8.24; 18.71)	12.51 **A** (10.34; 15.18)	13.11 **A** (8.66; 23.20)	12.25 **A** (9.96; 14.16)	<0.0001
ΔE2	3.59 **B** (2.34; 4.18)	14.05 **A** (11.16; 17.67)	14.36 **A** (7.66; 24.82)	13.19 **A** (9.79; 31.17)	14.31 **A** (9.14; 26.15)	14.64 **A** (12.21; 19.16)	16.20 **A** (10.20; 26.95)	14.65 **A** (11.55; 19.78)	<0.0001
ΔE3	2.46 **B** (2.18; 3.09)	11.61 **A** (9.38; 15.68)	12.28 **A** (5.64; 23.33)	11.18 **A** (5.57; 30.39)	11.70 **A** (7.15; 27.20)	11.92 **A** (9.53; 15.16)	14.15 **A** (7.60; 25.38)	12.44 **A** (9.62; 17.57)	<0.0001
ΔE4	4.10 **B** (2.92; 5.61)	8.03 **A** (6.02; 12.53)	9.04 **A** (2.99; 19.52)	8.00 **A** (3.30; 26.35)	8.94 **A** (4.95; 24.29)	9.68 **A** (6.71; 11.93)	10.91 **A** (4.77; 21.86)	9.03 **A** (6.42; 14.44)	<0.0001
ΔE5	4.00 **B** (3.41; 5.30)	8.93 **A** (6.61; 12.52)	9.80 **A** (3.53; 19.84)	8.43 **A** (3.66; 26.35)	9.22 **A** (5.17; 23.48)	10.20 **A** (7.00; 12.49)	11.64 **A** (5.21; 22.23)	9.25 **A** (6.63; 14.89)	<0.0001
$\Delta E_{00}1$	2.43 **B** (1.82; 3.50)	8.08 **A** (6.91; 9.98)	8.35 **A** (5.62; 13.45)	8.14 **A** (6.87; 14.70)	7.90 **A** (5.63; 13.02)	8.65 **A** (7.37; 10.57)	9.28 **A** (6.50; 15.92)	8.39 **A** (7.20; 10.13)	<0.0001
$\Delta E_{00}2$	2.77 **B** (2.00; 3.19)	9.30 **A** (7.27; 11.40)	9.89 **A** (5.34; 16.06)	9.02 **A** (6.80; 20.25)	9.28 **A** (6.08; 17.12)	9.88 **A** (8.67; 12.67)	11.02 **A** (7.38; 17.88)	9.55 **A** (8.03; 13.61)	<0.0001
$\Delta E_{00}3$	3.25 **B** (2.56; 3.56)	7.99 **A** (6.81; 10.58)	8.99 **A** (4.04; 14.61)	7.90 **A** (4.68; 19.78)	7.83 **A** (5.03; 17.34)	8.46 **A** (6.99; 10.38)	10.11 **A** (5.83; 16.53)	8.45 **A** (6.56; 12.65)	<0.0001
$\Delta E_{00}4$	4.31 **B** (3.62; 5.04)	6.03 **A** (4.95; 8.51)	6.97 **A** (2.84; 12.06)	5.82 **A** (3.92; 17.08)	6.25 **A** (4.07; 15.40)	7.06 **A** (5.31; 8.07)	7.76 **A** (4.31; 14.08)	6.47 **A** (5.09; 10.34)	0.0016
$\Delta E_{00}5$	4.27 **B** (3.80; 5.00)	6.43 **A** (5.37; 8.50)	7.46 **A** (3.11; 12.28)	6.04 **A** (4.22; 17.10)	6.49 **A** (4.27; 14.86)	7.49 **A** (5.58; 8.51)	8.31 **A** (4.50; 14.33)	6.68 **A** (5.31; 10.94)	0.0004

	Control	Commercial	Exp-H	CaPP-H	ALP-H	Exp-T	CaPP-T	ALP-T	p-value
	Median (minimum and maximum value)								
ΔWID1	4.49 **B** (2.29; 5.94)	16.03 **A** (12.38; 20.03)	15.55 **A** (10.94; 31.82)	15.86 **A** (10.19; 34.37)	15.10 **A** (10.24; 26.82)	15.51 **A** (12.11; 20.94)	17.52 **A** (9.71; 36.98)	15.73 **A** (11.99; 20.24)	0.0003
ΔWID2	0.56 **B** (-1.25; 1.32)	14.69 **A** (11.10; 19.61)	15.07 **A** (8.47; 35.52)	14.22 **A** (7.80; 46.18)	15.57 **A** (7.94; 37.92)	16.13 **A** (10.63; 23.17)	18.45 **A** (8.52; 39.41)	16.28 **A** (11.18; 23.89)	0.0003
ΔWID3	-4.89 **B** (-7.78; -4.04)	11.08 **A** (7.43; 17.60)	11.12 **A** (3.55; 30.82)	9.79 **A** (1.10; 47.45)	10.80 **A** (3.81; 38.65)	11.17 **A** (5.70; 18.32)	13.33 **A** (4.96; 35.49)	10.96 **A** (4.77; 19.84)	0.0003
ΔWID4	-8.98 **B** (-12.24; -7.61)	5.43 **A** (1.84; 12.83)	6.30 **A** (-1.38; 25.13)	4.27 **A** (-3.35; 41.87)	6.34 **A** (-0.12; 34.02)	6.52 **A** (0.19; 13.45)	7.89 **A** (-0.13; 30.11)	6.06 **A** (-0.60; 14.91)	0.0003
ΔWID5	-8.45 **B** (-11.30; -7.19)	6.58 **A** (2.77; 12.71)	7.58 **A** (-0.63; 25.57)	4.97 **A** (-2.72; 42.05)	6.84 **A** (0.35; 33.26)	7.30 **A** (0.72; 14.20)	8.79 **A** (1.62; 30.49)	6.20 **A** (0.23; 15.52)	0.0003

Times: ΔE1; $\Delta E_{00}1$; ΔWID1: 1st session. ΔE2; $\Delta E_{00}2$; ΔWID2: 2nd session. ΔE3; $\Delta E_{00}3$; ΔWID3: 3rd session. ΔE4; $\Delta E_{00}4$; ΔWID4: 7 days' after. ΔE5; $\Delta E_{00}5$; ΔWID5: 14 days' after. Groups: Control (without bleaching gel); Commercial (HP35%-Whitenes HP-Maxx); Exp-H (Water based); CaPP-H (0.5 wt% CaPP); ALP-H (0.5 wt% CaPP + ALP); Exp-T (Tris based); CaPP-T (0.5 wt% CaPP); ALP-T (0.5 wt% CaPP + ALP). *Different case letters indicate significant difference between the groups.*

3 DISCUSSÃO

O desenvolvimento e análise de géis clareadores experimentais à base de peróxido de hidrogênio (PH) em alta concentração com adição de sub-micropartículas de polifosfato de cálcio (PPCa) associado ou não a enzima fosfatase alcalina (ALP) foi descrito no presente estudo, assim como seu efeito nas propriedades físicas e químicas do esmalte dental humano ou bovino.

Após ter sintetizado o PPCa, usando o método da co-precipitação, foram obtidas partículas compostas principalmente por Ca e P, esféricas, com tamanho sub-micrométrico (135,7 ± 80,95 nm) e de caráter amorfo. O efeito da incorporação do PPCa nas propriedades químicas de géis clareadores experimentais foi avaliado. Observando-se que a menor concentração de PPCa empregada (0,5 wt%) conseguiu estabilizar o valor de pH da formulação (≈5,5). Isto poderia ser justificado pela substituição dos íons Ca^{2+} na estrutura do PPCa pelos prótons eletronegativos presentes no meio, levando ao aumento no pH. Já, nas concentrações maiores de PPCa (1,5 wt%), a geração de ácido fosfórico associado a clivagem hidrolítica do PPCa parece ter sido o efeito predominante reduzindo o pH do gel (Wang et al., 2008). Uma vez que após 45 min de manipulação o gel Sem-PPCa apresentou os menores valores de pH e, portanto, maior potencial desmineralizante, considera-se que a incorporação do PPCa em gel clareador poderia ser considerada positiva para o pH do mesmo.

Embora o PPCa seja um composto biocompativel (Müller et al., 2017a), é relevante determinar as reações biológicas diante de novos produtos odontológicos e/ou seus componentes solúveis por médio de modelos de cultivo celular *in vitro*, verificando desta forma seus efeitos na viabilidade celular (De Lima et al., 2009; Llena et al., 2019). Como esperado, a presença do PPCa na formulação de géis clareadores experimentais à base de PH em alta concentração não alterou a compatibilidade biológica deste tipo de material quando comparada a de géis comerciais e experimentais sem PPCa. Deve-se considerar que neste estudo diluições dos géis clareadores foram preparadas previamente à análise de viabilidade celular, pelo que fatores como difusão trans-amelodentinaria não foram simulados na avaliação biológica o que pode ser considerado uma limitação do presente estudo.

Quantidades menores de PPCa (0,5 wt%) nos géis clareadores permitiram manter maiores níveis de viabilidade das células MDPC-23 em comparação com o gel Sem-PPCa, o que poderia ser explicado pelo fato de que a clivagem da cadeia de PPCa não só fornece unidades de ortofosfato que ajudam no processo de remineralização, mas também gerariam "combustível metabólico" que facilitaria a produção de ATP (Müller et al., 2017a). Este aumento intracelular de energia poderia aumentar à proliferação celular (Dhivya et al., 2018; Darvishnia et al., 2022), o que poderia explicar porque este grupo apresentou os melhores resultados para a viabilidade celular na concentração de 10 µg/mL.

Com base nos resultados positivos obtidos na primeira fase deste estudo com a síntese e incorporação do PPCa em géis clareadores experimentais à base de PH, foi considerado que o seu conteúdo íonico (Ca^{2+} e PO_4^{3-}) viabilizaria seu efeito remineralizante no tratamento clareador do esmalte dental humano.

A maior concentração de PH, segundo a técnica titrimetrica, nos géis clareadores contendo PPCa poderia se associar com uma redução na degradação do PH e menor quantidade de outros radicais livres (Vandersmissen et al., 2008). Porém, os resultados da segunda fase deste estudo demostraram que esta diferença não afetou a eficácia clareadora destes tipos de géis, pois os valores de ΔE, ΔE00, ΔWID foram similares nos grupos independentemente da presença ou não do PPCa. Isto poderia ser explicado pelo fato de que os polifosfastos podem se ligar às estruturas dentais e, por um processo de dessorção, remover suas proteínas pigmentadas (Alshara et al., 2014).

Considera-se que menores valores de pH nos géis clareadores, poderiam reduzir a microdureza de superfície (SMH) do esmalte (Acuña et al., 2019), o que poderia explicar que o gel com a maior estabilidade de pH após manipulação (PPCa 0,5 wt%) na primeira fase foi também o que teve os melhores resultados de SMH após a última sessão de clareamento. Porém, contrário ao esperado, maiores concentrações do polímero (PPCa 1,5 wt%) no gel clareador não aumentaram os valores de KHN, expressivamente quando comparado ao Sem-PPCa, de forma imediata e sim de forma tardia. Após 14 dias do final da terapia clareadora, com armazenamento na saliva artificial, poderia ter acontecido a transformação gradual da camada amorfa precipitada em uma camada mais mineralizada em maior proporção no grupo PPCa 1,5 wt%. Previamente, esta transformação teria sido relatada quando,

ao incorporar o PPCa em um dentifrício, o seu depósito na superfície dental após 5 dias de tratamento ainda era amorfo (Müller et al., 2017b). É plausível que em meio com pH fisiológico como a saliva artificial e em um período de duas semanas de armazenamento a transição do estado amorfo para cristalino teria sido favorecida.

Com respeito à rugosidade do esmalte (Ra), embora fosse esperado certo grau de alteração deste parâmetro entre os grupos clareados e o controle devido à presença e efeito do PH e os seus subprodutos, a falta de alteração da superfície no presente estudo poderia estar relacionada à ação da saliva (de Carvalho et al., 2020) e a falta da replicação de outros tipos de desafios (abrasivos e/ou erosivos). Existem, entretanto, evidências da alteração na Ra após tratamentos clareadores (de Carvalho et al., 2020; Lins et al., 2021), enquanto que alguns estudos encontraram alterações mínimas na Ra mesmo com géis clareadores de alta concentração (Cadenaro et al., 2010; Yang et al., 2022). Tais discrepâncias relacionam-se principalmente à fórmula do gel e/ou níveis de pH (Costa et al., 2022).

Para complementar as análises físicas realizou-se uma análise das moléculas inorgânicas (PO_4^{3-} e CO_3^{2-}) presentes no esmalte, empregando a espectroscopia Raman após a conclusão do tratamento clareador. Esta análise permitiu estimar a composição do tecido, bem como sua qualidade, sendo um indicador do grau de mineralização do esmalte (Lima et al., 2015; Akgun et al., 2021). Além disso, as *"areas under the curve"* (do inglês: áreas debaixo da curva) são uma medida objetiva para definir a intensidade dos grupos PO_4^{3-} e CO_3^{2-} dentro da fase de cristal de hidroxiapatita (Lima et al., 2015; Vargas et al., 2017). No presente estudo, o clareamento não alterou o conteúdo de PO_4^{3-} dentro da hidroxiapatita do esmalte, o que poderia estar relacionado ao armazenamento em saliva da amostra durante e após o término do clareamento como reportado previamente (Lima et al., 2015; Kury et al., 2020).

Na análise do Raman o pico em 1070 cm^{-1} corresponde ao modo de estiramento simétrico do CO_3^{2-} que substitui o PO_4^{3-} na malha apatita (carbonato tipo ß) (Akgun et al., 2021). O grupo Sem-PPCa apresentou aumento do carbonato que sinalizou uma distorção da *"lattice"* (do inglês: malha) uma tendência a ser instável e, portanto, maior suscetibilidade a ataques ácidos (Robinson et al., 2009; Spizzirri et al., 2021; Akgun et al. 2021). A elevação no conteúdo de carbonato do tipo ß neste grupo indicaria que a substituição PO_4^{3-} pelo CO_3^{2-} não conseguiu ser regulada pela ação

da saliva (Robinson et al., 2009; Akgun et al., 2021), enquanto o grupo PPCa 1,5% reteve valores similares ao grupo Controle corroborando o que os resultados do SMH indicaram: a presença do PPCa poderia ter reduzido as mudanças microestruturais induzidas pelo PH no esmalte dental.

É relevante mencionar que os materiais odontológicos com propriedades remineralizantes baseiam sua bioatividade em componentes que se dissolvam num processo químico e gerem a precipitação local de fosfatos de cálcio biossimilares quando em contato com os tecidos dentais (Torres et al., 2019). Sendo assim, a incorporação do PPCa como agente remineralizante em agentes clareadores de alta concentração é relatado de forma inédita neste estudo. Este composto demonstra ser um polímero bioativo promissor pela: sua maior atividade biológica quando comparado à hidroxiapatita (Zhang et al., 2021); sua maior estabilidade que, diferente de outros fosfatos de cálcio amorfos, evitaria a sua cristalização precipitada quando em contato com a água (Omelon et al., 2014); e a sua capacidade remineralizante que permitiria, na hora da sua degradação, a formação de uma camada de fosfato de cálcio amorfa que serviria como precursor para a formação de estruturas cristalinas (Ackermann et al., 2019).

Ainda com resultados promissores no tratamento do esmalte humano, o fato de não ter obtido efeito remineralizante significativo quando comparado a um gel comercial, na segunda fase, poderia ser explicado pelo fato de que depois que ocorre a polimerização, os ortofosfatos (Pi) e o cálcio do PPCa ficam indisponíveis para a mineralização. A liberação destes íons ocorreria por meio de: 1) degradação hidrolítica espontânea da cadeia do polifosfato, numa taxa mais reduzida, ou; 2) pela clivagem da cadeia por uma enzima fosfatase, como a fosfatase alcalina (ALP), numa taxa acelerada (Omelon et al., 2014; Zhang et al., 2021).

Assim, na terceira fase deste estudo, as propriedades químicas de uma ALP e dos géis clareadores após a sua a incorporação foram avaliadas (HP-PPCa-ALP). Subsequentemente, estes géis modificados tiveram a efetividade clareadora e o efeito nas propriedades mecânicas do esmalte bovino analisados. Outros estudos na área de clareamento têm avaliado a incorporação de enzimas (peroxidase e/ou catalase) em géis à base de PH e determinado o seu efeito na eficácia do clareamento e na difusão trans-amelodentinaria com resultados promissores (Zuta et al., 2019; Soares et al., 2019). No entanto, até onde os autores têm conhecimento, neste estudo se

descreveu pela primeira vez a associação de uma enzima fosfatase para aumentar a disponibilidade iônica durante o tratamento clareador de forma bioinspirada.

A metaloenzima ALP é responsável por catalisar a hidrólise de fosfomonoésteres, como os polifosfatos (Müller et al., 2019). Após avaliar a sua atividade em diferentes meios, conseguiu-se determinar que a ALP devia ser incorporada no espessante do gel clareador. Já que a ALP apresentava redução na sua atividade em pH < 7 ou na presença de H_2O_2 conforme relatado anteriormente (Oliveira Duque et al., 2017). Seguidamente, o efeito de cisão da cadeia do PPCa induzido pela ALP foi demostrado pelo aumento expressivo dos níveis de ortofosfato livre (PO_4^{3-}) nos espessantes analisados após 10 min da incorporação da ALP. É importante considerar que a manutenção do estado amorfo do PPCa teria facilitado esse processo, ao torná-lo mais suscetível à clivagem enzimática pela ALP (Müller et al., 2016; Guanipa Ortiz et al., 2023a).

Embora grande parte dos géis clareadores seja à base de água, foi relatado anteriormente que um tampão Tris-HCl é ideal para obter maior atividade de ALP (Hethey et al., 2002). Assim, ambos os meios foram empregados para manipular os espessantes e as suas propriedades químicas, eficácia clareadora e a microdureza do esmalte bovino durante e após o clareamento foram avaliadas. Os géis contendo a ALP apresentaram índices de clareamento (ΔE, ΔE00, ΔWID) similares ao comercial e aos manipulados sem a ALP, indicando de forma similar aos resultados da segunda fase que o PPCa nas concentrações empregadas não altera o efeito clareador do gel (Guanipa Ortiz et al., 2023b).

Os valores da microdureza de superfície e transversal apontaram ganho mineral bem-sucedido e/ou preservação induzida pela ação da ALP no PPCa no meio de Tris (ALP-Tris). Deve-se considerar que essa biomineralização da apatita regulada pela ALP inicia-se com a clivagem enzimática de um hidrogênio-fosfato terminal e um próton adicional por ligação de anidrido do PPCa (Lorenz et al., 2001; Ackermann et al., 2019). Esse processo liberaria PO_4^{3-} e Ca^{2+} da cadeia de polifosfato, tornando-os localmente disponíveis. A precipitação iônica poderia então cobrir a superfície dentária, resultando na formação de apatita derivada de precursores amorfos (Omelon et al., 2014; Haarhaus et al., 2017; Müller et al., 2019; Ionescu et al., 2022).

No entanto, durante o tratamento, condições ainda mais próximas ao meio bucal, como: desafios erosivos, abrasivos e de citotoxicidade trans-amelodentinária não foram realizadas. Pelo que essas análises, assim como estudos *in vivo*, devem ser realizados no futuro para poder analisar com maior profundidade como acontece o deposito do PPCa na superfície no esmalte dental clareado, a sua resistência aos desafios orais, assim como seu efeito na sensibilidade dental associado às terapias clareadoras, para poder assim considerar num futuro estender o uso deste tipo de géis clareadores remineralizantes no nível comercial.

Os resultados deste trabalho como um todo mostraram que a incorporação do PPCa em géis clareadores experimentais à base de PH em alta concentração é promissora por não alterar as propriedades químicas do mesmo e não comprometer a sua eficácia clareadora. Enquanto tem a capacidade de manter a microdureza de superfície e evitar alterações na composição química do esmalte dental. Além disso, a associação deste tipo de gel com uma fosfatase como a ALP aponta à plausibilidade de induzir efeito remineralizante biologicamente inspirado, o que é inovador na pesquisa com géis clareadores remineralizantes.

4 CONCLUSÃO

As conclusões são:

- A sínteses de partículas de polifosfato de cálcio (PPCa) pelo método de co-precipitação gerou sub-microparticulas esféricas, compostas por Ca e P, de caráter amorfo.
- A incorporação do PPCa em géis clareadores a base de peróxido de hidrogénio em alta concentração - 35 wt% (PH-PPCa) não alterou negativamente as propriedades químicas e biológicas dos géis quando comparados a géis clareadores sem PPCa.
- Os géis clareadores contendo PPCa mantiveram a mesma eficácia clareadora, não alteraram a rugosidade, enquanto que mantiveram a microdureza de superfície do esmalte (1,5 wt%) 14 dias após o clareamento quando comparado a géis experimentais e comerciais sem PPCa.
- O gel com 1,5 wt% de PPCa preservou um conteúdo de carbonato similar ao do esmalte que não foi clareado, enquanto que o gel Sem-PPCa apresentou menores valores de Ca:P quando comparado aos demais grupos.
- A atividade de ALP foi maior em meio pH 9, reduzida em meio H_2O_2 e semelhante em espessantes à base de tris ou água. Os níveis de PO_4^{3-} foram maiores nos espessantes contendo ALP.
- Os géis com a enzima (PH-PPCa-ALP) tiveram eficácia clareadora adequada. Enquanto aumentaram a microdureza da superfície do esmalte (ALP-Tris) após o tratamento quando comparados a um gel comercial ou experimental sem PPCa. Ainda, o gel PH-PPCa-ALP à base de tris apresentou os maiores valores de microdureza em profundidade após o tratamento.

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APÊNDICES

Apêndice 1 – Síntese do PPCa, manipulação e análise do gel clareador

Metodologia detalhada

1. **Delineamento experimental**

Fatores em estudo:

- Gel clareador (4 níveis)

Peróxido de Hidrogênio 35% – FGM – Whiteness HP Maxx ®

Peróxido de Hidrogênio 35% – Laboratorial – FOP-UNICAMP

Peróxido de Hidrogênio 35% + PPCa 0,5 wt% – Laboratorial – FOP-UNICAMP

Peróxido de Hidrogênio 35% + PPCa 1,5 wt% – Laboratorial – FOP-UNICAMP

Variáveis de resposta:

- Análise de pH;

- Concentração de peróxido de hidrogênio (PH), pela analise titrimetrica (% - massa/massa);

- Viabilidade celular, pelo ensaio de redução do MTT.

1.1. Síntese do PPCa

A síntese do polifosfato foi realizada seguindo a metodologia de Müller et al., 2015:

- Preparo de solução de PPNa e ajuste do seu pH (10) usando uma solução 1M NaOH
- Preparo de solução de $CaCl_2.2H_2O$.
- Adição controlada por bomba de fluxo (Pump P-1, Pharmacia Biotech) da solução de $CaCl_2.2H_2O$ na solução de PPNa (1 mL/min), durante a adição o pH era mantido em 10 usando a solução 1M NaOH.
- A suspensão formada foi mantida sob agitação magnética durante 4 h.
- Seguidamente, a suspensão foi distribuída em tubos Falcon de 50 mL, passando por lavagem em ultrassom durante 10 min e por centrifugação (3500 rpm x 20 min).
- As amostras foram lavadas em água (duas vezes) e em álcool etílico absoluto (duas vezes)
- O slurry final foi coletado e secado em estufa a 60 °C (14 horas)
- O material seco foi homogeneizado com um pistilo num almofariz, para obter o pó de PPCa. **Figura 1**.

Figura 1. Resumo gráfico do processo de síntese do polifosfato de cálcio (PPCa).

1.2. Caracterização do PPCa

O PPCa sintetizado foi caracterizado mediante diversas analises, sendo as principais delas:

1.2.1. Microscopia eletrônica de transmissão (MET): O exame morfológico e de tamanho foi realizado usando um microscópio eletrônico de transmissão (Phillips CM 200, Phillps, Amsterdam, Holanda) a 200 kV com filamento de LaB6. As amostras foram preparadas em grades de cobre (TED PELLA, ultrathin C type A, 400 mesh). A amostra foi dispersa em isopropanol e foi mantida em banho de ultrassom por 10 min para sua re-dispersão. Gotejou-se 1 µL na grade de cobre, que foi seca em temperatura ambiente para a realização da análise. As imagens obtidas foram analisadas no software ImageJ, a média do cumprimento de 60 partículas por amostra foi calculada para estimar o tamanho das partículas.

1.2.2. Dispersão dinâmica de luz (DLS): Para calcular a distribuição dos tamanhos das partículas, foi utilizado um analisador de partículas por difração de laser (Zetasizer Nano ZS90; Malvern Instruments Ltd. Worcestershire, Reino Unido) realizando 10 corridas de 10 s, a 25 °C, com 60 s de estabilização e ângulo de espalhamento de 90°. Para o preparo das amostras: uma diluição contendo 1 mg/mL de PPCa foi agitada durante 15 min (50 °C), seguindo isto a solução foi centrifugada durante 10 min (10,000 rpm) e o sobrenadante foi levado numa cuba de vidro para a análise.

1.2.3. Difração de raios X (DRX): Para determinar a fase cálcio-fosfato das amostras, foi analisado o padrão de difração de raios-X (DRX) do pó seco de PPCa, usando radiação Cu-Kα de níquel a 40 kV e 30 mA (Difratômetro – D8 Advance, Bruker, Massachusets, USA). Com uma geometria de equipamento de θ /2θ. Leituras continuas foram realizadas com passo de 0,02° e um tempo de acumulação de 0,3 s (intervalo de 4° / min).

1.3. Manipulação do gel clareador

Após análises pilotos, a fórmula apresentada na **Tabela 1** foi empregada para a manipulação laboratorial dos géis clareadores. O componente A era preparado misturando os reagentes em um dispositivo protegido contra a luz que era mantido sob agitação magnética a ± 10 °C durante 60 min. O componente B era preparado pesando e acrescentado os reagentes em um dispositivo protegido da luz e agitado 1800 rpm por 1,5 min (Speed Mixer DAC 150.1; FlackTek, Inc., SC, USA).

Tabela 1. Composição dos géis manipulados laboratorialmente (Componente A - H_2O_2 e Componente B – Espessante)

Componente A	Componente B
H_2O_2 (50% sol)	H_2O
Glicerina	Glicerina
Propilenoglicol	Propilenoglicol
Carbopol 940	Carbopol 940
Ácido cítrico	Polifosfato de sodio
NaOH (50% sol)	PPCa
	NaOH

Figura 3. Preparo dos diferentes tipos de componentes B (espessantes): sem ou com 0,5 ou 1,5 wt% de PPCa.

1.4. Análise de pH

Os géis clareadores tiveram seus componentes (A:B) manipulados numa proporção 3:1 (peso:peso), sem diluir. Seguidamente, o seu pH era mensurado introduzindo o eletrodo de um pH-metro de bancada no gel (**Figura 4**). Leituras em triplicatas eram realizadas em diferentes tempos (após 5, 15, 30, 45 e 60 min) e a média das leituras para cada tempo era calculada.

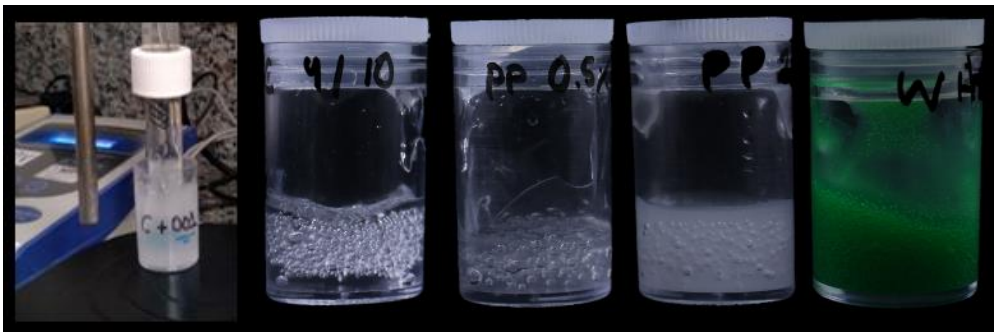


Figura 4. Leitura do pH dos géis clareadores após mistura dos seus componentes empregando um pH-metro de bancada.

1.5. Análise titrimétrica

Para a titulação iodométrica, foi empregado o kit de teste de peróxido de hidrogênio (Instrumentos Hanna; Barueri, SP, Brasil). Para isto, após serem misturados os dois componentes dos géis, os mesmos eram diluídos em água destilada até obter uma amostra com 200 ppm de H_2O_2. Seguidamente, 0,5 mL do reagente do kit HI3844A-0 (ácido) e uma colher do reagente HI3844B-0 (iodeto de potássio) eram acrescentados na amostra. A amostra era agitada durante 30 s e armazenada no escuro durante 15 min. Posteriormente, uma solução de NaS_2O_3 previamente padronizada (0,1N) era gotejada na amostra com uma bureta até obter uma cor amarela clara. Uma gota do reagente HI 3844D-0 (solução de amido) era acrescentada, para facilitar a observação do ponto de virada. Novamente era gotejada a solução de NaS_2O_3 até a amostra ficar transparente (**Figura 5**). A quantidade gotejada da solução de NaS_2O_3 foi usada para calcular a concentração do H_2O_2 nos diferentes tempos de avaliação. Todas as reações foram realizadas em triplicata.

Figura 5. Titulação iodométrica: Onde as mudanças de cor na amostra durante a titulação com uma solução de NaS_2O_3 indicam a redução do iodo em iodeto.

1.6. Análise citotoxicidade

Para determinar a viabilidade de células odontoblastoides, derivadas da papila de camundongo (MDPC-23) o método de redução de MTT foi realizado. As MDPC-23 foram cultivadas em Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) suplementado com antibióticos 100 IU/mL de penicilina e 100 µg / mL de estreptomicina (Vitrocell Embriolife, Brasil), 2 mmol/L de glutamina, e 10% de soro fetal bovino a 37 °C, atmosfera de 5% CO_2. Após atingir uma confluência de 80%, as MDPC-23 foram transferidas para placas de cultura celular de 96 poços na concentração de $5x10^4$ células/mL e posteriormente incubados em atmosfera de 5% de CO_2 a 37 °C por 24 h.

Após o período de incubação, as células foram expostas às diluições dos géis clareadores (1 mg/mL; 0,5 mg/mL; 0,1 mg/mL) com DMEM, durante 45 min. Posteriormente, os poços foram lavados duas vezes com solução salina tamponada com fosfato e neles foram adicionados 200 µl de solução de MTT. Após 3 h de incubação em atmosfera de 5% de CO_2 a 37 °C, os poços foram lavados duas vezes com PBS e preenchidos com 200 µL de etanol PA. Finalmente, as absorbâncias foram lidas num micro-espectrofotômetro (ASYS UVM340, Biochrome Ltd, Cambridge, England) a 570 nm.

Apêndice 2 – Tratamento clareador no esmalte humano

2. Delineamento experimental

Unidades experimentais: 120 blocos de dente humano, sendo 60 blocos utilizados para as análises de cor, rugosidade e de conteúdo mineral e os outros 60 para as análises de microdureza de superfície.

Fatores em estudo:

- Tratamento (5 níveis)

Controle (Sem tratamento)
Peróxido de Hidrogênio 35% – FGM – Whiteness HP Maxx ®
Peróxido de Hidrogênio 35% – Laboratorial – FOP-UNICAMP
Peróxido de Hidrogênio 35% + PPCa 0,5 wt% – Laboratorial – FOP-UNICAMP
Peróxido de Hidrogênio 35% + PPCa 1,5 wt% – Laboratorial – FOP-UNICAMP

- Tempo de análise para cor e rugosidade (5 níveis):

Inicial;
Após primeira sessão;
Após segunda sessão;
Após terceira sessão;
Após 14 dias.

- Tempo de análise para microdureza (3 níveis):

Inicial;
Após terceira sessão;
Após 14 dias.

- Tempo de análise para espectroscopia de raman (1 nível):

Após 14 dias.

Variáveis de resposta:

- Determinação da cor (ΔE, ΔE00, ΔWID);
- Rugosidade do esmalte (Ra);
- Microdureza de superfície (KHN);
- Análise de conteúdo mineral – Espectroscopia de Raman (CO_3^{2-} e PO_4^{3-}).

2.1. Coleta e preparo amostras esmalte/dentina

Terceiros molares hígidos (previamente armezados em solução de Timol 0,1% para desinfeção) foram limpos e tiveram as suas raízes seccionadas usando um disco diamantado dupla face sobre irrigação com água, 2 mm abaixo da junção cemento esmalte. Após isto, cada coroa era cortada usando um disco diamantado montado numa cortadeira metalográfica (Isomet 1000 Buehler, Lake Buff, IL, USA) até obter blocos de esmalte/dentina de 4x4 mm. **Figura 1.**

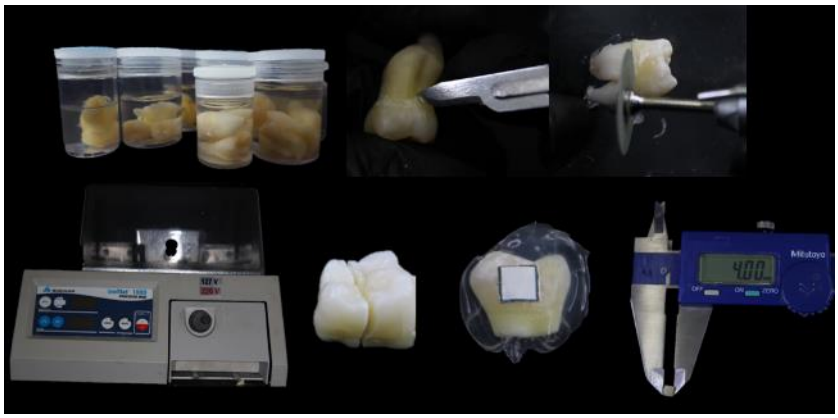


Figura 1. Os terceiros molares eram armazenados, limpos e cortados para obter blocos de esmalte/dentina de 4x4 mm.

Com o fim de retificar e padronizar a altura dos blocos em 3 mm, foram empregadas lixas de 600, 1200, 2500, 4000 grit, assim como panos de polimento com pastas diamantadas (1 e ¼ µm) montados numa politriz (Arotec SA Indústria e Comércio Ltda; Cotia, SP, Brasil) sob irrigação com água. Entre cada lixa e pano de polimento os espécimes eram lavados num ultrassom (Marconi; Piracicaba, SP, Brasil) com água destilada durante 10 min. **Figura 2.**

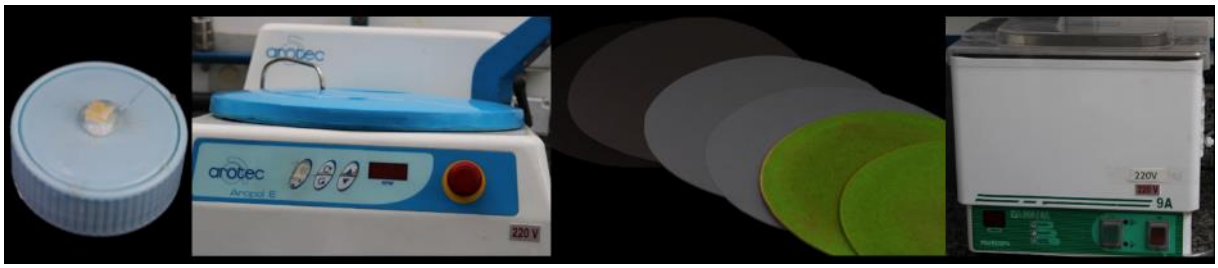


Figura 2. Padronização da altura dos blocos de esmalte/dentina (3 mm) e lavagem num ultrassom.

2.2. Terapia clareadora

Antes de cada sessão de clareamento, os espécimes eram retirados do seu armazenamento na saliva artificial. Seguidamente, eram colados em uma placa acrílica com fita dupla face para estabilizar, e a dentina das laterais era vedada, de tal forma que só o esmalte ficasse exposto ao gel clareador, usando uma barreira resinosa fotopolimerizavel (Top Dam – Blue; FGM, SC, Brasil) que era fotopolimerizada por 10 s (Bluephase Ivoclar). **Figura 3.**

No total foram realizadas três aplicações de 15 min cada uma (para um total de 45 min de clareamento). Em cada aplicação, aproximadamente 0,03 g do gel clareador (misturado antes da aplicação) era aplicado em cada amostra e, ao final da mesma, o gel era retirado com gaze e a amostra lavada com água destilada. Após a última lavagem, a amostra era seca com papel suave e voltava ao armazenamento no eppendorf contendo saliva artificial.

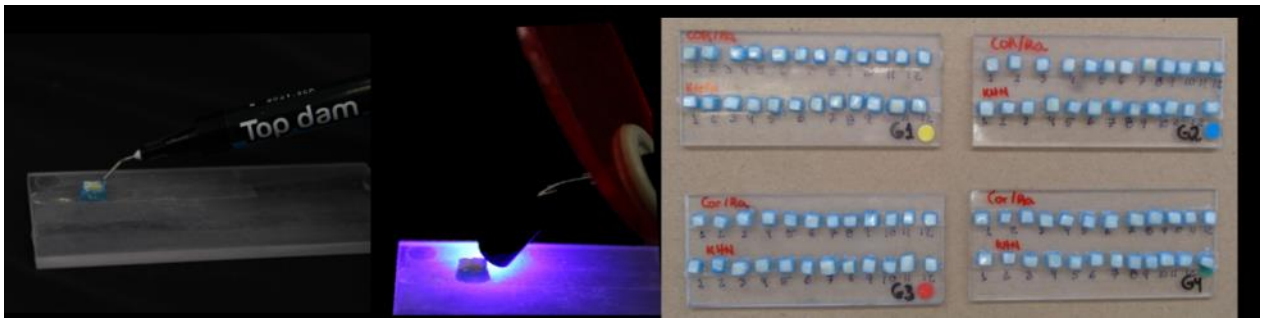


Figura 3. Preparo dos espécimes para iniciar a terapia clareadora, vedando as áreas laterais, para garantir que só a superfície do esmalte fosse a que recebesse o gel clareador.

2.3. Análise de cor, rugosidade e microdureza de superfície

Para análise de cor, os espécimes foram marcados na lateral com uma ponta diamantada 1012 montada em uma peça de alta rotação mantendo irrigação constante com água. A cor foi avaliada usando um espectrofotômetro (CM 700d; Minolta, Osaka, Japão) num ambiente com luz padronizada (GTI Mini Matcher MM-1; GTI Graphic Technology Inc., Newburgh, NY, USA). O espécime era removido do meio de armazenamento, seco com papel suave, posicionado em um dispositivo cerâmico branco e três leituras eram realizadas por espécime, sendo a média calculada para determinar a cor no espectro CIELAB.

Após fixar o espécime em uma placa acrílica a sua rugosidade era avaliada com um rugosimetro (Surftest SV 2100; Mitutoyo, SP, Brasil): 0,25 mm de corte,

1,25 mm de comprimento e uma velocidade de 0,1 mm/s. Três leituras que passavam pelo centro da amostra eram realizadas na superfície do esmalte de cada espécime e a média das mesmas era calculada.

Enquanto que para a avaliação a microdureza de superfície, os espécimes eram fixados em uma placa e mediante um microdurômetro (FM 100, Future-Tech Corp; Kawasaki, Japão) com um indentador Knoop uma carga de 50 gf/5 s era aplicada. No total, cinco endentações com 100 µm de espaçamento entre elas foram realizadas e a média das mesmas calculada. **Figura 4.**

Figura 4. Dispositivos empregados (de esquerda para direita) para a avaliação de cor, rugosidade e microdureza dos espécimes antes, durante e após o clareamento.

Apêndice 3 – Avaliação da enzima ALP e tratamento clareador no esmalte

3. Delineamento experimental

Unidades experimentais: Géis clareadores manipulados com ou sem: o polifosfato de cálcio (PPCa) e a enzima fosfatase alcalina (ALP). 120 blocos de dente bovino, sendo 60 blocos utilizados para as análises de cor e os outros 60 para as análises de microdureza Knoop de superfície e transversal.

Fatores em estudo:

- Soluções ALP (3 níveis)
  Diferentes pH
  Diferentes concentrações de H_2O_2
  Diferentes espessantes
- Géis clareadores (6 níveis)

PH 35% (H_2O) – Laboratorial – FOP-UNICAMP
PH 35% + PPCa 0,5 wt% (H_2O) – Laboratorial – FOP-UNICAMP
PH 35% + PPCa 0,5 wt% + ALP (H_2O) – Laboratorial – FOP-UNICAMP
PH 35% (Tris) – Laboratorial – FOP-UNICAMP
PH 35% + PPCa 0,5 wt% (Tris) – Laboratorial – FOP-UNICAMP
PH 35% + PPCa 0,5 wt% + ALP (Tris) – Laboratorial – FOP-UNICAMP

- Tratamento dos blocos (8 níveis)

Controle (Sem tratamento)
PH 35% – FGM – Whiteness HP Maxx ®
PH 35% (H_2O) – Laboratorial – FOP-UNICAMP
PH 35% + PPCa 0,5 wt% (H_2O) – Laboratorial – FOP-UNICAMP
PH 35% + PPCa 0,5 wt% + ALP (H_2O) – Laboratorial – FOP-UNICAMP
PH 35% (Tris) – Laboratorial – FOP-UNICAMP
PH 35% + PPCa 0,5 wt% (Tris) – Laboratorial – FOP-UNICAMP
PH 35% + PPCa 0,5 wt% + ALP (Tris) – Laboratorial – FOP-UNICAMP

- Tempos para avaliação química do gel (2 níveis):

10 min;
30 min.

- Tempos de análise para cor (5 níveis):

Após primeira sessão;

Após segunda sessão;
Após terceira sessão;
Após 7 dias;
Após 14 dias.

- Tempo de análise para microdureza de superfície (2 níveis):

Inicial;
Após terceira sessão;

- Tempo de análise para microdureza transversal (1 nível):

Após fim do tratamento clareador.

Variáveis de resposta:

- Atividade da ALP (U/L);
- Concentração de PO_4^{3-} (µM);
- Determinação da cor (ΔE, ΔE00, ΔWID);
- Microdureza de superfície – SMH (KHN);
- Microdureza transversal - CSMH (KHN);

3.1. Análise química da ALP

Os níveis de atividade da ALP foram determinados em diferentes condições de pH, concentrações de H_2O_2 e tipo de meio no espessante para determinar a maneira mais adequada de incorporá-la ao gel clareador contendo PPCa. Para todas as análises da atividade da ALP, o ensaio colorimétrico de ponto final (Labtest Diagnóstico S.A., Lagoa Santa, MG, Brasil) foi usado. **Figura 1**.

Para cada análise 25 µL de uma solução de monofosfato de timolftaleína (22 mmol/L, pH 10,1) eram adicionados a 250 µL de uma solução tampão (<330 mmol/L) e mantidos sob agitação de 700 rpm (Thermomixer Comfort 5355; Eppendorf SE, Hamburgo, Alemanha) a 37 °C durante 2 min. Em seguida, foram adicionados 25 µL das amostras e a mistura era mantida nas condições anteriores por 10 min. Depois que o monofosfato de timolftaleína estava hidrolisado pela ALP presente nas amostras, 1000 µL de um reagente de cor (94 mmol/L de carbonato de sódio em 250 mmol/L de NaOH) eram adicionados para reagir com a timolftaleína hidrolisada, modificando a cor do produto final. Os valores de absorbância da ALP eram determinados em um leitor de placas de microplacas a 590 nm (Infinite 200 PRO;

Tecan Trading, AG, Suíça), sendo convertidos em unidades por litro (U/L) de acordo com a fórmula do fabricante.

Figura 1. Ensaio colorimétrico de ponto final empregado para analisar a atividade da ALP.

3.2. Análise química dos espessantes com ALP

Para determinar a concentração do ortofosfato livre (PO_4^{3-}), foi realizado um ensaio colorimétrico com um kit de ensaio de fosfato (MAK-308 Lote 308CB09A22 - Exp Jan/24; Sigma-Aldrich; Missouri, EUA). Para isto, o espessante correspondente (a base de agua ou tampão tris) e 15 U da ALP eram misturadoss e mantidos em uma incubadora (Thermomixer Comfort 5355; Eppendorf SE, Hamburgo, Alemanha) a 700 rpm a 37 °C.

Em seguida, os géis eram diluídos para permanecer dentro da faixa de detecção do ensaio (0,4-50 µM PO_4^{3-}) até uma concentração final de 1,6 mg/mL. Em uma placa de 96 poços, 50 µL da amostra eram misturados com 100 µL de verde malaquita e mantidos em uma incubadora (KS260; IKA®, Staufen, Alemanha) sob agitação a 250 rpm a 37 °C para cada tempo de leitura (10, 30 min). **Figura 2.**

A densidade óptica das soluções foi lida após 30 min de incubação em triplicata em um leitor de placas de microplacas (Infinite 200 PRO; Tecan Trading, AG, Suíça) a 620 nm. A concentração de PO_4^{3-} foi determinada por meio de uma curva de calibração obtida de uma série de soluções aquosas contendo concentrações conhecidas de PO_4^{3-} (40, 32, 24, 16, 12, 8, 4, 0 µM). Além disso, os espessantes sem PPCa ou ALP eram usados como controles em branco e suas densidades ópticas eram subtraídas dos valores finais do grupo Sem-PPCa.

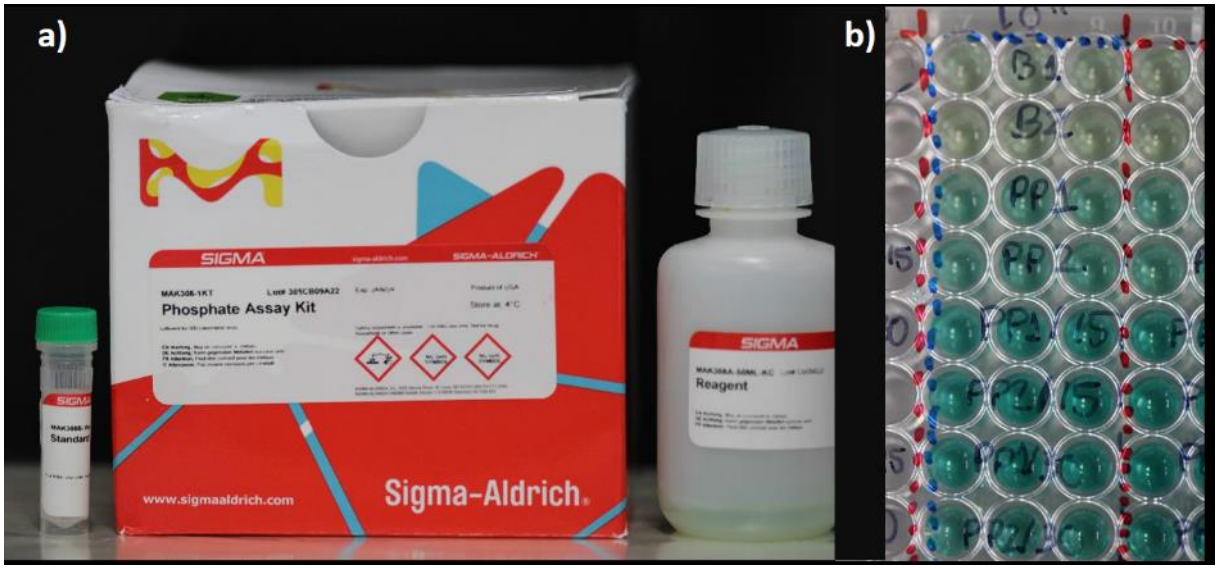


Figura 2. Kit empregado para avaliação do ortofosfato com a técnica colorimétrica (a) e amostras para leitura num espectrofotômetro (b).

3.3. Obtenção de amostras e protocolo de manchamento

Dentes bovinos foram extraídos e mantidos em solução de Timol 0,1 % durante 14 dias. Após limpar as suas raízes e realizar profilaxia com pedra pomes, os dentes foram montados numa furadeira de bancada (FSB16; Schulz, Joinville, SC, Brasil) para cortar a sua coroa e obter espécimes de esmalte/dentina de 5,7 mm de diâmetro e 2,3 mm de altura. A altura dos espécimes foi padronizada empregando lixas de 600, 1200, 2500 e 4000 grit e a também foram polidos com discos de feltro e pasta de polimento de 1 e ¼ µm montados numa politriz (Arotec SA Indústria e Comércio Ltda; Cotia, SP, Brasil) sob irrigação com água. Entre cada lixa/disco os espécimes eram lavados em um ultrassom (Marconi; Piracicaba, SP, Brasil) com água destilada durante 10 min. **Figura 3.**

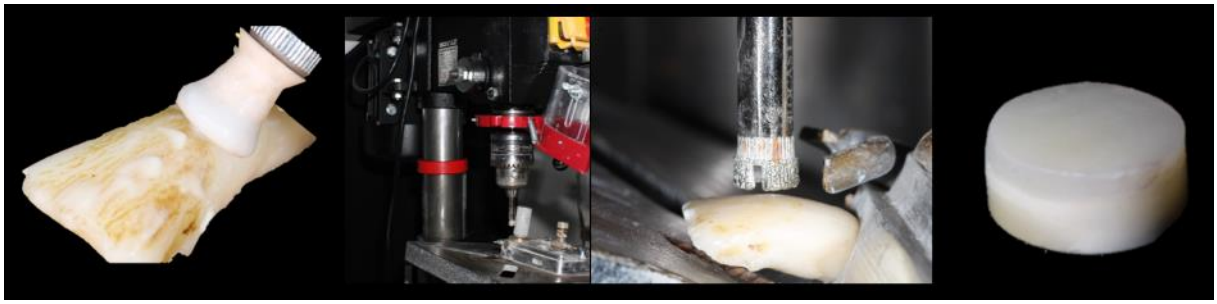


Figura 3. Profilaxia do dente bovino, furadeira de bancada, dente montado na furadeira de bancada, espécime de esmalte/dentina de após regularização e polimento.

Após o preparo dos espécimes, aqueles que teriam a sua cor analisada foram marcados na área lateral com uma ponta diamantada #1012 (KG Sorensen; Cotia, SP, Brasil) e tiveram a sua dentina isolada com verniz ácido resistente incolor (Risqué). Para realizar o manchamento, os mesmos permaneceram imersos em solução de chá de preto a 37 °C durante 6 dias. A solução era preparada diariamente com 1,6 g de chá preto (Dr. Oetker, Curitiba, PR, Brasil) em 100 mL de água destilada fervida. **Figura 4.**

Os resíduos de chá formados nas superfícies de esmalte e dentina foram removidos usando uma taça de borracha com uma mistura de pedra-pomes e água (proporção 2:1) em baixa velocidade por 30 s. Também, para obter estabilização de cor, as amostras foram armazenadas em saliva artificial durante uma semana, a qual era diariamente renovada, com pH neutro a 37 °C.

Figura 4. Espécimes submersos em solução de chá preto e armazenados posteriormente em estufa (37 °C).

3.4. Terapia clareadora

De forma similar ao relatado no **Apêndice 2**, após retirar os espécimes do armazenamento na saliva artificial, estes eram secos com papel suave e colados em placas acrílicas com fita dupla face; as áreas não clareadas eram vedadas com resinosa fotopolimerizavel (Top Dam – Blue; FGM, SC,Brasil). A resina era aplicada em volta do bloco esmalte/dentina, e fotopolimerizada por 10s (Bluephase Ivoclar). Todos os grupos clareados receberam três aplicações de 15 min cada uma.

Com o diferencial de que para os géis com ALP o sistema era manipulado empregando três componentes. A mistura da ALP e o componente B (espessante) era realizada dentro de um eppendorf o qual tinha seu conteúdo agitado empregando um vortex. Seguidamente, a mistura era mantida em repouso durante 10 min para depois

ser misturada como o resto dos géis na proporção 3:1 em peso (componentes A e B). **Figura 5.**

Figura 5. Mistura da ALP com o componente B do clareador laboratorial (espessante), agitação num vortex. Após o tempo de ação (10 min) os espécimes (previamente protegidos) receberiam o gel clareador manipulado (componente A e B).

3.5. Análise de microdureza transversal

Os espécimes foram fixados em uma placa de acrílico e seccionados longitudinalmente com um disco diamantado dupla face. Uma das suas metades foi submersa em resina epóxi (2001; Redelease, SP, Brasil). Após a polimerização completa da resina epóxi (24 h) a superfície transversal do espécime foi exposta e polida gradualmente como descrito para o preparo dos espécimes. Para a leitura, três colunas de 10 endentações foram realizadas na área central de cada espécime com um microdurômetro (FM 100, Future-Tech Corp; Kawasaki, Japão), carga de 25 gf, durante 5 s, medindo a 10, 20, 40, 60, 80, 100, 120, 140, 160, 180 µm da superfície do esmalte. As médias das três colunas a cada distância foram calculadas e posteriormente analisadas.

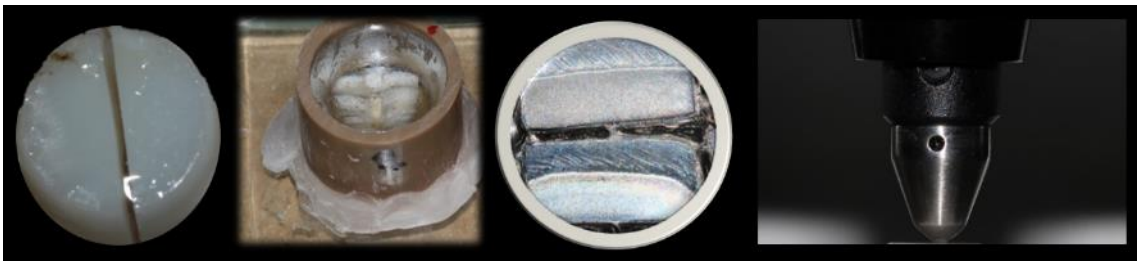
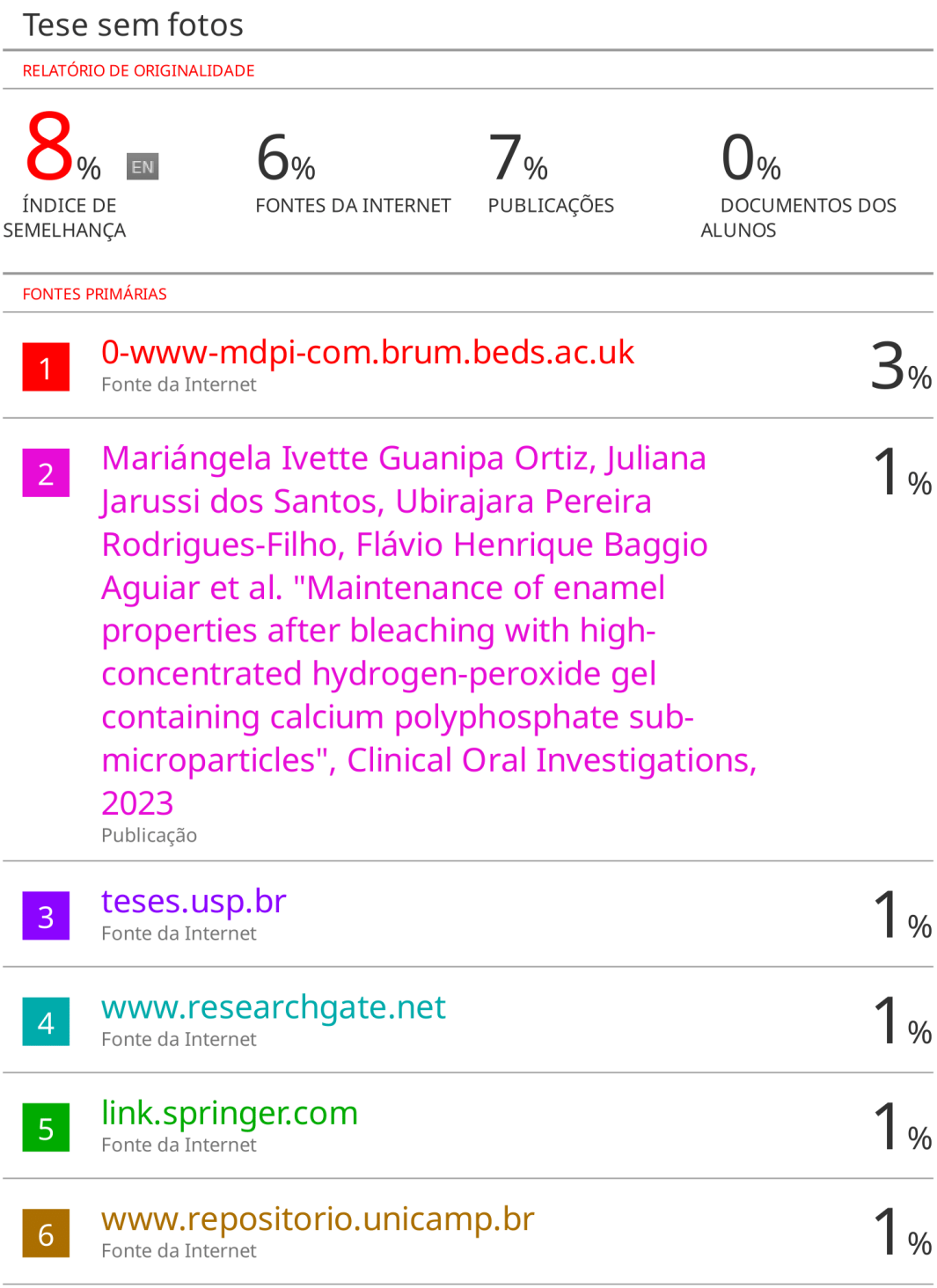


Figura 6. Preparo do espécime para leitura de microdureza transversal empregando um microdurômetro com indentador Knoop.

ANEXOS

Anexo 1 – Verificação de originalidade e prevenção de plágio



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02-Oct-2023

Dear Miss Guanipa Ortiz:

Your manuscript entitled "Enzymatical-driven mineralization of a calcium-polyphosphate bleaching gel" has been successfully submitted online and is presently being given full consideration for publication in Journal of Dental Research.

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PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Desenvolvimento de um agente clareador a base de peróxido de hidrogênio com adição de nanopartículas de polifosfato de cálcio e determinação do seu efeito no clareamento e mineralização do esmalte dental

Pesquisador: Mariangela Ivette Guanipa Ortiz

Área Temática:

Versão: 4

CAAE: 24313019.9.0000.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.854.885

Apresentação do Projeto:

O parecer inicial é elaborado com base na transcrição editada do conteúdo do registro do protocolo na Plataforma Brasil e dos arquivos anexados à Plataforma Brasil. Os pareceres de retorno, emendas e notificações são elaborados a partir dos dados e arquivos da última versão apresentada.

Trata-se de SOLICITAÇÃO DE EMENDA (E1) ao protocolo originalmente aprovado em 12/12/2019 para inclusão de novo método de coleta de amostras biológicas. O texto do parecer foi atualizado conforme a documentação apresentada. A solicitação está detalhadamente descrita ao final do parecer.

Pendência 1 (atendida em 22/11/19)-A LISTA DE PESQUISADORES citada na capa do projeto de pesquisa inclui MARIANGELA IVETTE GUANIPA ORTIZ (Cirurgiã Dentista, Doutoranda no PPG em Clínica Odontológica da FOP-UNICAMP, Pesquisadora responsável), DÉBORA ALVES NUNES LEITE LIMA (Cirurgiã Dentista, Docente da área de Dentística da FOP-UNICAMP) e KLAUS RISCHKA (Químico, Pesquisador colaborador na FOP-UNICAMP), o que é confirmado na declaração dos pesquisadores e na PB.

Pendência 2 (atendida em 22/11/19)- Em sua resposta os pesquisadores informaram que “A

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