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ORIGINAL ARTICLE

Evaluation of bleaching efficacy, microhardness, and trans-amelodentinal diffusion of a novel bleaching agent for an in-office technique containing hexametaphosphate and fluoride

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Abstract

Objective This study evaluated in vitro the effects of calcium gluconate (CaGlu), sodium fluoride (NaF), sodium hexametaphosphate (HMP), and NaF/TMP added to a 35% hydrogen peroxide (H_2O_2) bleaching gel on the color change, enamel hardness, and trans-amelodentinal diffusion.

Materials and methods Enamel discs/bovine dentin ($n=150$) were divided according to the bleaching gel: 35% H_2O_2 (H_2O_2); 35% H_2O_2 + 0.1% NaF (H_2O_2/NaF); 35% H_2O_2 + 1% HMP (H_2O_2/HMP); 35% H_2O_2 + 0.1% NaF + 1% HMP (H_2O_2/NaF/HMP), and 35% H_2O_2 + 2% CaGlu (H_2O_2/Caglu). The bleaching gels were applied three times (40 min/session) at 7-day intervals between each application. Then, color alteration (ΔE), whitening index (ΔWI_D), percentage of surface hardness loss (% SH), cross-sectional hardness (ΔKHN), and trans-amelodentinal diffusion were determined. Data were submitted for analysis of variance (ANOVA), followed by the Student–Newman–Keuls test ($p<0.05$).

Results All bleaching gels showed significant color changes after treatment ($p<0.001$). ΔE and ΔWI_D were similar among the evaluated gels. Mineral loss (% SH and ΔKHN) and trans-amelodentinal diffusion of hydrogen peroxide were lower for H_2O_2/NaF/HMP; the H_2O_2/CaGlu group presented the highest values about the other groups ($p<0.001$).

Conclusion It is possible to conclude that the addition of NaF/HMP to the in-office bleaching agent did not interfere with the bleaching efficacy and reduced enamel demineralization and H_2O_2 diffusion.

Clinical significance The association of NaF/HMP to the bleaching gel can be used as a novel approach for minimizing the adverse effects of H_2O_2 by-products and with similar clinical efficacy.

Keywords Tooth bleaching agents · Peroxides · Phosphates · Sodium fluoride · Esthetics · Tooth demineralization

Introduction

Beauty is a combination of qualities that arouses admiration through the senses, especially vision. One of the main ways to express it is through a smile [1]. The appearance of a smile is associated with self-confidence and social interactions [2]. So, tooth whitening is very required in dental clinics [3]. In the in-office technique, a bleaching agent with a high concentration (35–38%) of hydrogen peroxide (H_2O_2) is used. It is known that the higher the concentration of H_2O_2 and the longer the exposure time of the dental tissue to the bleaching product [4, 5], the greater the release of reactive oxygen species and their effects on hard tissues and pulp. Some studies report that bleaching can cause demineralization in teeth that undergo the procedure, as well as a decrease in enamel hardness [6–8]. On the other hand, studies show that bleaching does not cause any structural changes to the enamel [5, 9]. However, even with the scientific evidence pointed out, the in-office bleaching esthetic treatment with a high concentration of H_2O_2 gel is still the most used one by clinicians and preferred by patients [3, 10].

Thus, there is a need to seed strategies and adopt new dosages that minimize these undesirable effects resulting

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from the bleaching procedure. Alternatives have been used, such as the use of cyclic phosphates in the composition of the bleaching gel [8, 11, 12]. The addition of sodium trimetaphosphate (TMP) in the bleaching gel led to less enamel demineralization, H_2O_2 diffusion, and cytotoxicity [8, 11]. Sodium hexametaphosphate (HMP) is another cyclophosphate that has 3 more phosphates sites in its structure than TMP and thus may result in a greater reduction of adverse effects resulting from bleaching therapy. In addition, it was observed that HMP reduces the diffusion of H^+ ions in the dental enamel since it retains this ion in its structure, which also minimizes the propagation of acid in the enamel, contributing to reducing the demineralization process of dental tissues [13, 14].

In vitro studies have shown that the effects of the association of HMP and sodium fluoride (NaF) depend on the molar proportion between these compounds [14–16]. Therefore, the ideal concentration of HMP to be used is 1% in toothpaste formulations containing 1100 ppm NaF [14, 15]. In addition, several studies have shown that the association of this salt with NaF helps to inhibit demineralization and favors the process of remineralization of dental substrates [15, 16].

Thus, it is expected that HMP can reduce enamel demineralization and favor the remineralization process [16], as well as reduce H_2O_2 diffusion, in an attempt to minimize the potential adverse effects of bleaching dental. Therefore, the objective of this study was to evaluate, in vitro, the effects of calcium gluconate (CaGlu), sodium fluoride (NaF), sodium hexametaphosphate (HMP), and NaF/TMP added to a 35% hydrogen peroxide (H_2O_2) bleaching gel on the color change, enamel hardness, and trans-amelodentinal diffusion. The null hypothesis of the study was that bleaching gels containing 35% H_2O_2 and NaF/HMP or CaGlu do not show a difference in promoting bleaching effect and in reducing mineral loss and trans-amelodentinal diffusion compared with bleaching gel containing only 35% H_2O_2.

Materials and methods

Formulation of bleaching gels and determination of experimental groups

The bleaching gels were prepared in each application session since there were no stabilizers in their composition. The basic components of the gels were composed of thickener (12% Carbopol), bleaching agent (35% H_2O_2), glycerin and water (qs), and NaOH required to maintain a pH of approximately 7.0. Depending on the experimental group, 0.1% sodium fluoride (NaF) and/or 1% sodium hexametaphosphate (HMP) was added [14, 15]. A commercial (marketplace) bleaching gel containing 35% H_2O_2 and 2% calcium gluconate (CaGlu), neutral pH (Whiteness HP Blue, FGM, Joinville, SC, Brazil) was used as the positive control. Thus, five experimental bleaching gels were defined: (1) 35% hydrogen peroxide (H_2O_2); (2) H_2O_2 + 0.1% NaF (H_2O_2/NaF); (3) H_2O_2 + 1% HMP (H_2O_2/HMP); (4) H_2O_2 + 0.1% NaF + 1% HMP (H_2O_2/NaF/HMP); (5) H_2O_2 + 2% calcium gluconate (H_2O_2/CaGlu). The sample size per group was 10 enamel/dentin discs, which was based on a previous study [8], adopting surface and cross-sectional hardness as primary outcomes, mean difference between groups (9 and 3500, respectively), standard deviation (5 and 2000, respectively), an α error of 5%, and a β error of 10%. Disks were randomly divided (Excel, Microsoft Corporation, USA) among the 5 experimental groups ($n = 10$).

Color alteration analysis

Preparation and pigmentation of enamel/dentin discs

Enamel/dentin discs (5.7 mm diameter × 3.5 mm thick) were obtained from bovine incisor teeth using an 8-mm-diameter grinding wheel under water cooling (4 °C). Then, the discs were cleaned in deionized water and stored in a physiological saline solution containing 0.1% thymol at 4 °C [8].

After the initial reading of the color values, determined according to the Commissione Internationale de l'Eclairage (CIE L* a* b* color system), the discs were stored in microtubes (Kasvi K6-0150, 1.5 mL, São José dos Pinhais, PR, Brazil) containing 1 mL of room temperature black tea infusion for pigmentation. The infusion was made with 1.6 g of black tea (Chá Matte Leão, Curitiba, PR, Brazil) for every 100 mL of deionized water [17]. The pigmentation process was monitored for 6 days, and the infusion was changed daily. Then, a new reading of the color values was determined by the CIE L* a* b* [8, 17].

Bleaching gel treatments and color alteration measurement

After pigmentation, bleaching gels were applied to the enamel surface (0.04 mL) for 40 min, using a dosing syringe and a microbrush (KG Sorensen, Cotia, SP, Brazil). For 14 days, 3 bleaching sessions were performed at 7-day intervals. The gels were removed with gauze, followed by washing with deionized water for 30 s. Between bleaching sessions, the discs were kept in individual plastic containers containing 2 mL of artificial saliva (1.5 mmol/L of $Ca(NO_3)_2.H_2O$; 0.9 mmol/L of $NaH_2PO_4.H_2O$; 150 mmol/L de KCl; in 0.1 mol/L of sodium cacodylate buffer, pH 7.0) and renewed every day [8].

Calculation of total color alteration and whiteness index for dentistry

The enamel/dentin discs were fixed in black silicone supports with a diameter of 5.7 mm and a thickness of 3.5 mm, standardizing the incidence of the light beam in the Visible Ultraviolet Reflection (UV-2450 Model, Shimadzu, Kyoto, Japan), with wavelength ranging from 400 to 700 nm, under standard lighting D65 and an illumination/observation angle of 45/0°. The measurement of colors was performed on the vestibular surface of the enamel after preparation of the discs and their pigmentation, 1st, 2nd, and 3rd bleaching sessions, as well as on the 7th and 14th days after the end of bleaching. Then, the absolute differences (Δ) of the colors coordinates (L*, a*, b*) were calculated between the time analysis (post-staining, post-bleaching, and after 7 and 14 days) and the initial values: ΔL* (positive value indicates more brightness, a negative value indicates darker), Δa* (positive value indicates redder, a negative value indicates more green), and Δb* (positive value indicates more yellow, negative indicates more blue). To determine the total color change between the three coordinates, the following formula was used: $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ [8, 17]. Subsequently, the whiteness index for dentistry (WI_D) was determined according to the following equation: $\Delta WI_D = 0.511L^* - 2.324a^* - 1.100b^*$ [18].

Surface and cross-sectional hardness analysis

The enamel surface of the enamel/dentin discs was flattened and polished, to remove approximately 120 µm of the surface enamel, according to a previous study [8]. After polishing, five impressions, 100 µm equidistant from each other, were made (initial surface hardness: SHi) using a Knoop diamond tip under 25 g for 10 s (Micromet 5114, Buehler, Lake Bluff, IL, USA) [13, 16]. Enamel/dentin discs, with SHi values between 342.0 and 378.0 KH, were randomly distributed (Excel, Microsoft Corporation, USA) in the 5 previously defined experimental groups ($n = 10$). After the 3 bleaching sessions, as described above, the final surface hardness (SHf) was determined to calculate the percentage change in surface hardness (% SH = [[SHf − SHi] / SHi] × 100) [8]. After SHf determination, the enamel/dentin discs were sectioned in half, and one of the halves was embedded in acrylic resin and polished [8]. Knoop hardness in the cross-sectional was determined with a load of 5 g/10 s at 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, 120, 140, 160, and 180 µm from the surface. The integrated area (AI) under the curve of hardness values (KHN × µm) was calculated by the trapezoidal rule using the software GraphPad Prism (Prism 7 for Windows, version 7.00, San Diego, CA, USA) [8].

Determination of trans-amelodentinal diffusion of H2O2

To quantify the amount of H_2O_2 that permeated the dental tissues, enamel/dentin discs ($n = 10$/group) were placed in an artificial pulp chamber (APC) between two silicone rings (5.60-mm internal diameter; 1.78 mm thick; Ref. OR 008—Rodimar Rolamentos Ltda, Araraquara, SP, Brazil) and sealed with melted pink wax nº 7 (Wilson®, Polidental, Cotia, SP, Brazil) restricting the lateral penetration of the bleaching agent, according to a study by Briso et al. [19]. CPAs were placed individually in 24-well cell culture plates (Costar Corp., Cambridge, MA, USA). Each well was filled with 1 mL of sodium acetate buffer solution (2.0 mol/L, pH 4.5 by acetic acid, Sigma Chemical Co, St Louis, MO, USA) and subsequently received the APCs, already containing the disks of enamel/dentin. Thus, the dentin surface remained in contact with the acetate solution during the bleaching protocol, where the bleaching gels were applied once as described above (item 2.2.2) [19]. Then, the amount of 500 µL of buffer solution plus extract was transferred to experimental tubes to react with leuco-crystal violet (0.5 mg/mL; Sigma Chemical Co, St Louis, MO, USA) and horseradish peroxidase enzyme (1 mg/mL; Sigma Chemical Co, St Louis, MO, USA) [19]. The final reaction volume was adjusted to 3 mL with deionized water inserted into a cuvette and the optical density of the solutions was measured at a wavelength of 596 nm (UV-2450, Shimadzu, Kyoto, Japan) [20]. A standard curve (0.5–5.0 µg/mL) was used to convert the optical density obtained in the samples into l g/mL of H_2O_2.

Statistical analysis

The Sigmaplot® for Windows version 12.0 statistical program was used, with a significance level of 5%. For the color alteration data, the different bleaching gels and the time of analysis were considered as variation factors and, as variables, the parameters ΔE and ΔWID. The data were homogeneous and submitted to analysis of variance (ANOVA) of repeated measurements, followed by the Student–Newman–Keuls multiple comparison test. For the analysis of the hardness and diffusion of H_2O_2, the values were considered as variables and the bleaching gels as a factor of variation. The values were homogeneous and were submitted to one-way ANOVA followed by the Student–Newman–Keuls test.

Results

The bleaching agents evaluated promoted a significant change in ΔE after bleaching sessions ($p < 0.001$) in previously pigmented enamel. All treatments showed similar performance, regardless of the period evaluated ($p > 0.05$). The

color change was progressive and continuous from the first bleaching session (Fig. 1A). In ΔWID, all groups promoted gradual and continuous color changes after the bleaching sessions, making the samples whiter ($p < 0.001$). Only the 35% H_2O_2 group showed significant partial regression in whiteness values 14 days after the bleaching procedures ($p < 0.05$) (Fig. 1B).

All bleaching gels led to hardness loss (Table 1), and the greatest hardness loss was approximately between 0 and 40-µm enamel depths for all groups tested (Fig. 2). Compared to the 35% H_2O_2 group, the addition of NaF reduced the loss of surface hardness (SHf: $p < 0.001$; %SH: $p < 0.001$), and CaGlu had the highest losses on the surface and inside the enamel. The bleaching gel containing 35% H_2O_2/NaF/HMP led to the lowest hardness loss values (SHf: $p < 0.001$; %SH: $p < 0.001$; KHN × µm: $p < 0.001$; respectively) followed by H_2O_2/NaF (Table 1). Furthermore, it can be noted that the hardness values, as a function of depth, were higher for the H_2O_2/NaF/HMP group (Fig. 2). The bleaching gels H_2O_2/NaF and H_2O_2/CaGlu showed the highest values of trans-amelodentinal diffusion of H_2O_2, and the 35% H_2O_2/NaF/HMP group had the lowest value ($p < 0.001$).

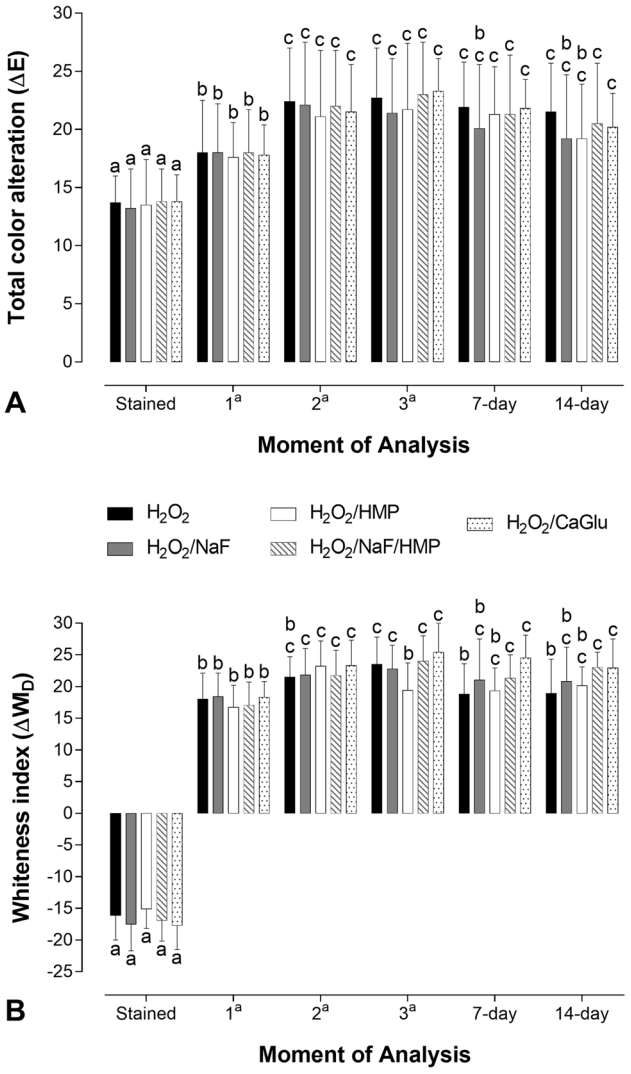

Fig. 1 Mean values (±SD) of total color alteration (ΔE) (**A**) and whitening index in dentistry (ΔWI_D) (**B**) by the International Commission of I'Eclairage (CIE) according to bleaching gels and time of analysis ($n = 10$). Distinct superscript lowercase letters indicate statistical difference among the bleaching gels at each moment of analysis and among the moments of analysis for each bleaching gel (Student–Newman–Keuls; $p < 0.05$)

Table 1 Mean values (±SD) of initial (SHi) and final (SHf) surface hardness, percentage of loss surface hardness (%SH), integrated area (KHN × µm), and trans-amelodentinal penetration of H_2O_2, according to experimental gels ($n = 10$)

Bleaching gels	Variables				
	SHi	SHf	%SH	KHN × µm	H_2O_2 (µg/mL)
H_2O_2	363.2[a] (7.4)	290.1[a] (9.2)	-20.1[a] (1.6)	18,019.1[a] (1,174.8)	6.56[a] (0.33)
H_2O_2/NaF	363.4[a] (7.8)	307.9[b] (7.5)	-15.3[b] (0.8)	18,621.9[a] (633.1)	6.99[b] (0.22)
H_2O_2/HMP	363.4[a] (7.7)	294.8[c] (7.8)	-18.9[c] (1.1)	18,425.6[a] (438.8)	6.54[a] (0.58)
H_2O_2/NaF/HMP	363.3[a] (7.9)	331.6[d] (8.5)	-8.7[d] (1.2)	20,461.8[b] (485.4)	6.03[c] (0.57)
H_2O_2/CaGlu	363.8[a] (7.8)	286.0[a] (7.3)	-21.4[a] (1.5)	17,062.8[c] (784.9)	6.75[a] (0.33)

Distinct superscript lowercase letters indicate statistical difference among bleaching gels in each variable (Student–Newman–Keuls test, $p < 0.001$)

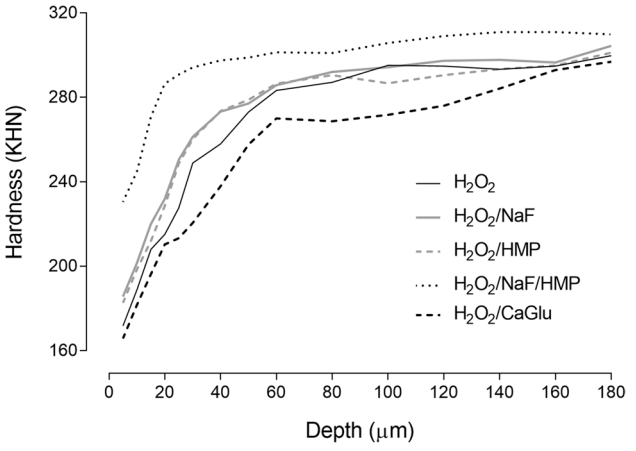

Fig. 2 Cross-sectional hardness profiles at different depths in the enamel ($n = 10$) according to the bleaching gels

Discussion

Currently, new bleaching dosages are being developed to minimize the adverse effects of this therapy without interfering with its clinical effectiveness. In the present study, the addition of remineralizing agents CaGlu, NaF, HMP, and NaF/TMP did not change the esthetic efficacy of the bleaching gel at 35% H_2O_2; thus, the first null hypothesis was accepted. However, the presence of NaF/HMP in the gel reduced mineral loss and trans-amelodentinal diffusion of H_2O_2; therefore, these null hypotheses were rejected.

It has been suggested that ΔE* values above 3.3 show clinically perceptible color changes [21, 22]. ΔE values between 3 and 8 are moderately visible and above 8 are extremely noticeable [23]. According to this parameter, in the present study, all evaluated gels showed clinically relevant color change after the adopted bleaching protocol. Several trials have shown that traditional in-office tooth bleaching therapies using agents with high concentrations of H_2O_2 for 30 to 45 min produce an intense change in tooth color already in the first clinical session and less chromatic changes in subsequent sessions [8, 24]. According to Sun [25], this occurs because the darker molecules react more easily with free radicals, causing a great visual impact in the first application of tooth whitening with a high concentration agent.

Postoperative color stability is as important as the ability to lighten during treatment. Throughout the post-treatment period (7 and 14 days), there was the maintenance of color for all bleaching gels evaluated. According to Bizhang et al. [26], color degradation is minimal and linear over time, showing adequate color stability after the end of treatment at 18 months. The bleaching effectiveness, as well as its maintenance, is derived from a large diffusion of H_2O_2 in the enamel prisms and reaction with the chromophore molecules, resulting in greater reflection and less absorption of light [27]. The dental whitening index (ΔWI_D) indicates that higher values correspond to whiter teeth, and lower ΔWI_D (including negative values) denotes darker teeth. Positive ΔWI_D results for all groups indicated the bleaching ability of the agents [18], and no differences were observed between the experimental groups after 14 days of bleaching completion (chromatic stabilization). Although ΔE is an important parameter to observe color changes based on L*, a *, b * parameters, we must keep in mind that it should not be interpreted individually, but together with other variables, such as ΔWI_D, which correlates color with the proximity of white [18].

These data are important, given the great interest and expectation of the patient when seeking the bleaching procedure [28]. The improvement of the chromatic and esthetic characteristics of the teeth has been required since the introduction of whitening agents in the dental clinic [2, 28] and is associated with beauty, engagement, and social and mental well-being, positively impacting the patient's self-perception and quality of life [2]. Furthermore, it is reported that disadvantages experienced by a person due to cosmetic dental problems may profoundly affect their self-esteem, interactions, environmental adaptations, personal relationships, job opportunities, and other fundamental aspects affecting their quality of life [29]. Thus, it is essentially fundamental to maintain the functional properties and clinical effectiveness of the bleaching gel when adding and/or changing its chemical composition.

Overall, the results of the color analysis suggest that, even with the addition of NaF and/or HMP agents, H_2O_2 was able to diffuse through enamel and dentin and react with the organic and bioorganic matter, or biological waste products from dental tissues, and provide lightning through the oxidation of pigments. It is worth mentioning that, although promoting a similar whitening effect, the addition of NaF and HMP in the bleaching gel reduced the trans-amelodentinal H_2O_2 diffusion (Table 1). Thus, possibly the number of reactive oxygen species released was lower than for the groups without NaF/HMP, which hypothesizes that clinically it could culminate in lower postoperative sensitivity.

On the other hand, all the gels evaluated in the present study negatively affected the enamel microhardness, promoting its decrease. This is mainly justified because the decomposition products of H_2O_2 not only react with the dark-colored chromophore molecules but also with the organic ones (such as proteins, lipids, and substances that stain teeth) and inorganic components of enamel [30]. However, the group exposed to the bleaching gel containing NaF/HMP had the highest KHN values, which were higher than the other gels evaluated. In addition, it was the only group that showed a loss of surface hardness within the acceptable range recommended by the ISO 28399 standard [31]. In addition to this, the presence of NaF/HMP in the bleaching gel can also protect the enamel against deep mineral loss (Fig. 2). This result is mainly attributed to the synergistic effect resulting from the association NaF/HMP incorporated in the bleaching agent.

It is noteworthy that HMP forms strong complexes with metal ions [32, 33] in the oral environment, which adsorbs to the enamel surface and retains the charged ions CaF^+ and Ca^{2+} through Na^+ substitution in the cyclic structure, leading to a reticular formation [34] through the binding of Ca^{2+} to one or more HMP molecules. Through these multiple bonds, the HMP molecules form a layer of condensed phosphates, altering the selective permeability of enamel [35] and, in this case, increasing the selectivity to cations. Furthermore, HMP is a negatively charged cyclic phosphate that leads to a greater amount of electron donor sites on the enamel surface and, consequently, increases the adsorption of ionic species

such as Ca^{2+}, $H_2PO_4^-$, and CaH_2PO_4 [13, 16, 36], which are important for increasing the diffusion of fluoride, calcium, and phosphate in enamel.

Recently, a study showed that HMP adsorption provides more calcium phosphate-binding sites and promotes the supersaturation of these ions near the enamel surface [36], which explains the ability of HMP to reduce demineralization of this substrate (Table 1; Fig. 2). In addition, the results support that the presence of HMP/NaF in the bleaching gel possibly minimizes changes in the organic matrix of enamel, since the removal or degradation of the organic matrix of enamel has been shown to significantly reduce its mechanical properties [37], as products derived from H_2O_2 in tooth whitening give rise to unstable oxygen free radicals that are capable of inducing structural changes in matrix proteins, located between the lenses and in the area of the stem sheath, as well as significant alteration of enamel interrod proteins [30].

Although even the incorporation of NaF/HMP in the gel minimized changes in hardness, it is important to mention that all bleaching gels evaluated promoted a reduction in hardness in depth, especially up to 60 µm from the surface (Fig. 2), a finding that has already been reported in the literature [38]. This data reinforces the demineralizing potential that H_2O_2 exerts on dental enamel, since all the gels were manipulated, and the pH was previously adjusted to neutrality (pH = 7.0) to avoid biases resulting from acidic pH that could raise doubts about whether enamel effects are attributable to peroxide action or acid etching secondary to the product's low pH value [5]. In addition, the adoption of a protocol with a high exposure time and with three sessions of bleaching agent application caused a greater loss of hardness.

Regarding the presence of the other remineralizing agents tested (NaF and CaGlu), there was no decrease in H_2O_2 diffusion, nor changes in enamel hardness (Table 1). The literature has positive antecedents with the addition of these agents in the bleaching gel [7, 31 39]. Possibly, the good results observed in these studies with the incorporation of NaF are related to the use of this ion in a higher concentration (0.2–1.1% NaF), since in our study 0.1% NaF was used. As for CaGlu, Vieira et al. [7], when using a bleaching gel composed of 2% CaGlu, reported a reduction in mineral loss, unlike the data of the present study and other works reported in the literature [8, 31, 39]. According to previous studies [40, 41], CaGlu is incompatible with strong oxidizing agents and cannot be released during bleaching decomposition.

Thus, it is possible to perform an efficient in-office tooth whitening protocol that is less aggressive to mineralized tissues with NaF/HMP supplementation in bleaching gels. However, the results should be extrapolated to clinical practice with caution, as in vitro studies have limitations, since they do not fully reproduce intra-oral conditions, especially regarding the effect of human saliva and the use of fluoride products, such as the daily use of fluoridated toothpaste. Moreover, some differences are observed between the laboratory conditions and the natural atmosphere of the oral cavity. As well as many other interventions, some discoloration and some continual whitening processes can be observed in the natural condition of the oral cavity. Therefore, clinical studies are needed to confirm the efficacy of using NaF/HMP in the bleaching agent, as well as the biological responses to dental and pulp hard tissues are necessary to obtain clinical validation of the use of these agents as an additive to bleaching gel.

Conclusion

Taking into account the data obtained and the limitations of the experimental model, we can conclude that the addition of NaF/HMP to the bleaching gel does not interfere with bleaching efficacy and significantly reduces the loss of surface and cross-sectional hardness in enamel, as well as the trans-amelodentinal diffusion of H_2O_2. Thus, future studies should be performed to assess the esthetic outcome and biocompatibility of the different in-office bleaching gels.

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Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

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