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RESEARCH

Characterization and effects on enamel of low-concentration bleaching gels containing hyaluronic acid, NF_TiO_2 nanoparticles and irradiated with violet LED light

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Abstract

Objective To characterize and evaluate experimental in-office bleaching gels containing hyaluronic acid (HA) or carbomer 940 (CAR), enriched with NF_TiO_2 nanoparticles and irradiated with a violet LED, assessing their effects on the physicochemical properties of enamel.

Materials and methods Bovine enamel-dentin discs were treated according to the parameters: thickener (HA or CAR), HP concentration (1.5% or 6%), and irradiation (with or without LED), resulting in 9 experimental groups ($n=10$/group). An additional control group (35%HP-commercial) was adopted, and three sessions were conducted (30 min each, with a 7-day interval). Gels were evaluated for pH, particle size, polydispersity index, zeta potential and rheological behavior. Samples were assessed for color change (ΔE_{00}), whiteness index (ΔWI_D), Ca/P ratio (EDS), surface microhardness (KHN), roughness (ΔRa), and surface morphology (SEM). Data were analyzed using ANOVA three-way and Tukey/Bonferroni ($\alpha=5\%$).

Results pH remained stable above 6.0. Hyaluronic-based gels exhibited higher particle size and polydispersity, but lower zeta potential and less viscous rheological behavior compared to the carbomer-based ones ($p<0.05$). LED light significantly increased ΔE_{00} and ΔWI_D for all gels, with HA-1.5%HP and HA-6%HP+LED achieving comparable ΔWI_D to 35%HP-commercial ($p>0.05$). Hyaluronic-based gels groups irradiated with LED increased Knoop microhardness ($p<0.05$). No significant changes were found in ΔRa, Ca/P ratio, or enamel morphology ($p>0.05$).

Conclusion Experimental hyaluronic or carbomer bleaching gels incorporated with NF_TiO_2 nanoparticles and irradiated with violet LED showed minimal variations in physicochemical properties, effective bleaching even at low HP concentrations, and no enamel damage.

Clinical relevance Innovative bleaching gel formulations incorporating hyaluronic acid, NF_TiO_2 nanoparticles, and violet LED light irradiation exhibit high efficacy even at low hydrogen peroxide concentrations, thereby minimizing enamel damage and potentially mitigating post-operative sensitivity.

Keywords Dental bleaching · Hydrogen peroxide · Nanotechnology · Hyaluronic acid

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Introduction

Dental bleaching, widely adopted in dental practice, has become a prevalent aesthetic demand [1] and is recognized for its conservative and minimally invasive approach [2, 3]. In-office bleaching gels contain hydrogen peroxide (HP) as the main active ingredient in concentrations ranging from 25 to 40% [4, 5]. These gels generate reactive oxygen species (ROS) that can diffuse through enamel and oxidize the conjugated double bonds of dentin chromophores [6]. Despite the efficacy observed at high HP concentrations [7, 8], studies indicate potential adverse effects, including changes in mineral composition (Ca/P ratio) and substrate morphology [9, 10], decreased microhardness [11], and increased enamel surface roughness [12].

Post-bleaching sensitivity, a clinically reported adverse effect, occurs due to the diffusion of hydrogen peroxide into the dentinal tubules, causing a cytotoxic effect on pulp cells [13]. HP can diffuse through enamel and dentin, reaching the dental pulp and inducing oxidative stress. This stress results from generating ROS, which can damage cellular components such as membranes, proteins, and DNA. Studies indicate that high concentrations of bleaching agents exacerbate pulp cell cytotoxicity, intensifying post-bleaching sensitivity. Additionally, the inflammatory response triggered by ROS contributes to the discomfort [13–15]. Previous studies have reported that the intensity of the bleaching procedure's toxic effect on pulp cells is directly proportional to the contact time with the enamel and the concentration of hydrogen peroxide in the product. Therefore, performing in-office dental bleaching using low-concentration gels is a promising strategy to provide patients with a safer and less painful aesthetic treatment [14, 15].

To accelerate the decomposition of low concentrations of hydrogen peroxide into ROS, titanium dioxide (TiO_2) nanoparticles (NPs) have become a research focus. TiO_2 is a chemically stable, biocompatible compound known for its antimicrobial properties, high oxidative power, and ability to absorb ultraviolet (UV) radiation [16–18]. Literature indicates that the light absorption capacity of TiO_2 in the visible spectrum can be enhanced, thereby intensifying its antibacterial and photocatalytic properties, by introducing dopants. This process involves incorporating metallic ions such as copper (Cu), nickel (Ni), cobalt (Co), chromium (Cr), manganese (Mn), molybdenum (Mo), niobium (Nb), and iron (Fe), or non-metallic elements like nitrogen (N), phosphorus (P), carbon (C), sulfur (S), boron (B), fluorine (F), and iodine (I). These dopants create nanoscale defects within the crystalline structure of TiO_2, improving its functional performance [19, 20]. Moreover, studies show that combining this technology with low concentrations of hydrogen peroxide or carbamide and irradiating with violet LED light enhances the bleaching effect and may reduce the likelihood of sensitivity compared to 35% hydrogen peroxide [21, 22].

Most bleaching agents on the market contain thickeners derived from synthetic polymers, such as carbomer 940 (CAR). This is due to their ability to form gels, providing appropriate viscosity that ensures prolonged contact of the product with the dental surface [23]. However, the combination of this thickener with hydrogen peroxide is susceptible to pH fluctuations, with records indicating changes in the morphology, composition, and structure of dental enamel caused by this substance [24–26]. Considering these circumstances, it is believed that designing a hydrogel with superior hydrophilic properties and biocompatibility compared to CAR could enhance the bleaching effects of the experimental formulation.

Hyaluronic acid (HA) is a biopolymer produced by living organisms, composed of glucuronic acid and N-acetylglucosamine. Its application in health care is widely recognized due to its excellent viscoelastic properties, physiological activity, biocompatibility, biodegradability, and bioactivity [27–29]. HA hydrogel exhibits several advantages, including high transparency and considerable water content [27, 29]. In this context, HA-hydrogel emerges as a promising alternative, demonstrating superior properties compared to synthetic polymers such as CAR. Although CAR is widely recognized for its cost-effectiveness and versatility, hyaluronic acid offers distinct advantages, including excellent biocompatibility, superior hydration capacity, and inherent biological activity. These characteristics position HA as a preferred option in advanced clinical and cosmetic formulations, where enhanced efficacy and safety are essential, such as dental bleaching [30].

In light of the above, the objective of this study was to develop experimental HA-gels containing nitrogen and fluorine co-doped titanium dioxide nanoparticles (NF_TiO_2) (5 wt%) associated with low concentrations of HP (1.5% and 6%), which have not been previously used for in-office bleaching procedures, and to evaluate the efficacy and mineral content of dental enamel bleached with these gels, either irradiated or not with violet LED light. The null hypotheses tested were that the experimental gels (1) would not present suitable physicochemical properties, (2) would not promote the same bleaching efficacy as commercial gels containing high concentrations of hydrogen peroxide (35% HP), and (3) would not cause changes in the mineral content and morphology of the bleached enamel.

Table 1 Experimental design

Experimental units	Bovine enamel	
Parameters under study	1 - Thickeners	• Hyaluronic acid (HA); • Carbomer 940 (CAR).
	2 - Hydrogen peroxide concentration	• HP 1.5%; • HP 6%.
	3 - Irradiated or not with LED	• With LED; • Without LED.
	4 - Time (for surface microhardness analysis)	• T_0 (before bleaching); • T_1 (24 h after the last bleaching session); • T_2 (14 days after the last bleaching session).

Materials and methods

Experimental design

The experimental gels were formulated with HA or CAR and characterized for pH, particle size (P), polydispersity index (PDI), zeta potential (ZP), and rheological behavior (RB). After synthesizing and characterizing the experimental gels, bovine enamel-dentin discs were cut and stained with black tea. The selected samples were subjected to bleaching with experimental gels combined with the following parameters ($n = 10$), as represented in Table 1.

Table 2 presents the composition and specification of each material used in the formulation of the experimental bleaching gels. It describes the components employed, their respective concentrations, and specific characteristics, providing a detailed understanding of the formulations tested in the study.

The control group (35%HP-commercial) was treated with a commercial bleaching gel (Whiteness HP, FGM, Joinville, SC, Brazil) and was not irradiated, following the manufacturer's recommendations (Composition: 35% hydrogen peroxide, glycerol, inert filler and deionized water; pH reported by the manufacturer: 7.0.)

The variable responses from the four study phases are displayed in Fig. 1.

Phase 1: characterization of experimental bleaching gels

Synthesis of NF_TiO_2 NPs

The NF_TiO_2 were synthesized via solvothermal processes [19]. The NPs were fabricated in two stages: the first stage was the solvothermal synthesis of pure TiO_2, and the second stage was the doping of TiO_2 with nitrogen. A solution of 1.7 g of Ti [Titanium (IV) butoxide, Sigma Aldrich, 97%], 4.6 g of ethanol (Decon Labs), 6.8 g of oleylamine (Sigma Aldrich, 70%), and 7.1 g of oleic acid (Sigma Aldrich, 90%) was mixed with 20 mL of 4% hydrated alcohol (18-MΩ Milli-Q, Decon Labs). The solution was divided into two aliquots of 20 mL, and each aliquot was placed in a high-pressure reaction vessel (Paar Series 5000 Multiple Reactor System) at 180 °C for 24 h. The vessels were agitated with an external magnetic field using stirring bars. After cooling, the solutions were decanted and washed three times with anhydrous ethanol to remove surfactants. The pure TiO_2 NPs were immediately stored in 20 mL of ethanol. For the second stage, an aliquot of pure TiO_2 NPs reacted with an equal volume of triethylamine (Sigma Aldrich, 99.5%) using the high-pressure reactor at 140 °C for 12 h. After cooling, the doped NPs were washed three times with anhydrous ethanol. The final solution of NPs in ethanol was determined gravimetrically, with an approximate concentration of 40 mg/mL. Co-doped nanoparticles (NF_TiO_2) were obtained in a single reaction based on stage 1 with the inclusion of 5% (weight/weight; based on Ti content) of fluorine using crystalline ammonium fluoride (ACS, 98%, Alfa Aesar) as the doping source. The particles were washed with anhydrous ethanol and remained suspended in ethanol [19].

Preparation of experimental gels

HA (Sigma Aldrich Chemical, St. Louis, MO, USA) or CAR (Sigma Aldrich Chemical, St. Louis, MO, USA), the polymers used as the matrix for the bleaching gels, were

Table 2 Composition of the experimental gels used in the study

Experimental gels	Materials	Specification/Composition
HA-gels	Hyaluronic acid-based hydrogel used as a thickening agent	Hyaluronic acid (Sigma-Aldrich, St. Louis, MI, USA), ultrapure water and potassium hydroxide.
	Co-doped titanium dioxide nanoparticles (NF_TiO_2) (5%)	Ti $(OBu)_4$ (Aldrich, 97%), C_2H_5OH (200-proof Decon Labs, King of Prussia, PA, EUA), $C_{18}H_{35}NH_2$ (Aldrich, 70%), $C_{18}H_{34}O_2$ (Aldrich, 90%), NH_4F (based on Ti content; crystalline, ACS, Alfa Aesar), and ethanol-water solution.
	Hydrogen peroxide solution	1.5 or 6% hydrogen peroxide solution (Sigma-Aldrich, St. Louis, MI, USA).
CAR-gels	Carbomer 940-based hydrogel used as a thickening agent	Carbomer 940 (Sigma Aldrich Chemical, St. Louis, MO, USA), ultrapure water and potassium hydroxide.
	Co-doped titanium dioxide nanoparticles (NF_TiO_2) (5%)	Ti $(OBu)_4$ (Aldrich, 97%), C_2H_5OH (200-proof Decon Labs, King of Prussia, PA, EUA), $C_{18}H_{35}NH_2$ (Aldrich, 70%), $C_{18}H_{34}O_2$ (Aldrich, 90%), NH_4F (based on Ti content; crystalline, ACS, Alfa Aesar), and ethanol-water solution.
	Hydrogen peroxide solution	1.5 or 6% hydrogen peroxide solution (Sigma-Aldrich, St. Louis, MI, USA).

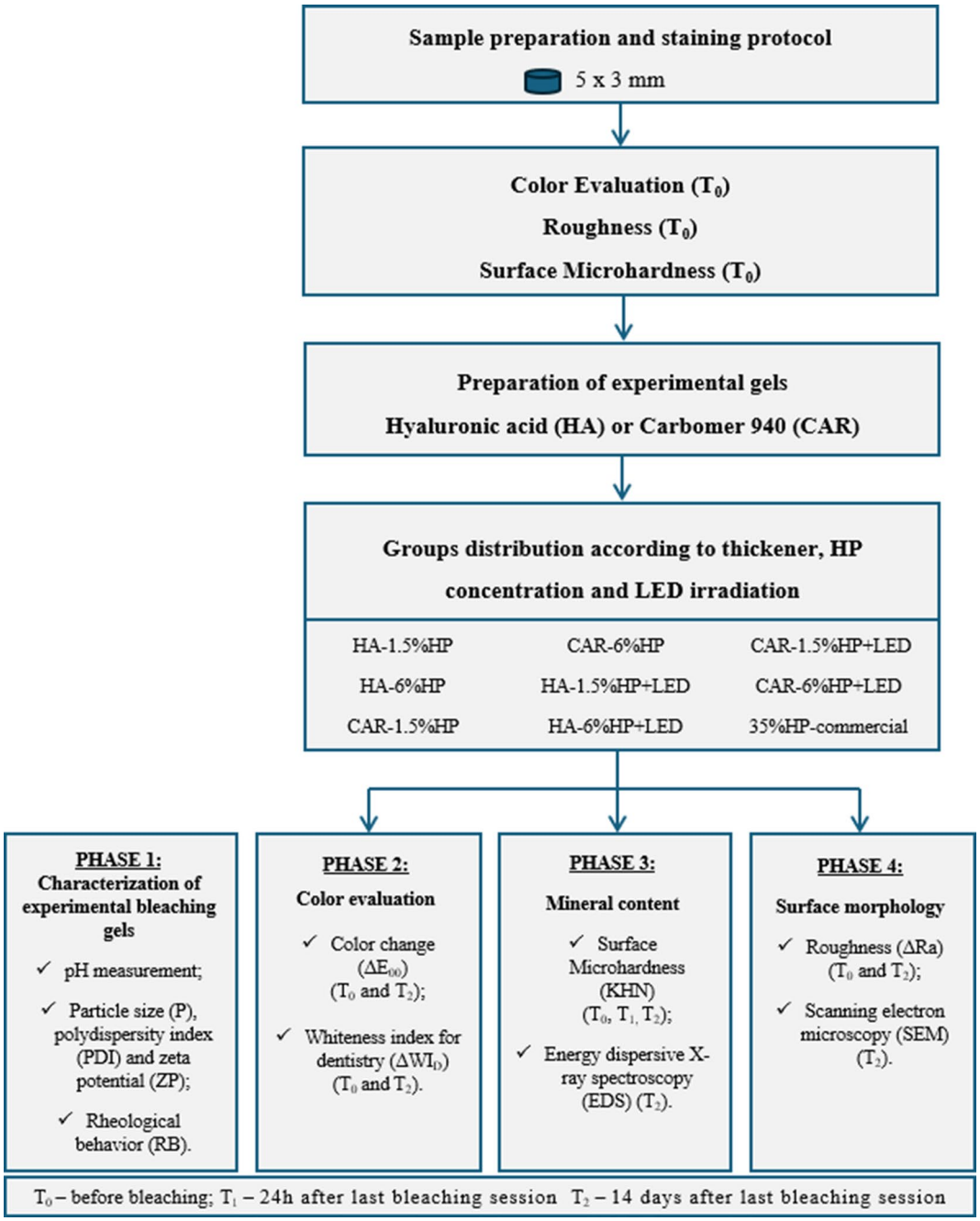

Fig. 1 Flowchart representing the phases of the study

diluted in distilled water and homogenized in a mixer (Speed mixer, Landrum, SC, USA) until the desired viscosity was achieved. The NF_TiO_2 NPs in alcohol were mixed for 30 s, and aliquots corresponding to the intended concentrations (5%) were centrifuged. The ethanol was almost completely removed, and the NPs were added to the hydrogel and homogenized. After, a potassium hydroxide solution (KOH) at a concentration of 60% was added to hydrogel to neutralize the mixture. The HP (35% Merk) was diluted to concentrations of 1.5% and 6% and added to the HA or CAR hydrogel in a 2:1 ratio.

pH measurement

The pH of the bleaching gels was measured using a pH meter (Equilam, Diadema, SP, Brazil) connected to a potentiometer (Orion Research Incorporated, Boston, MA), previously calibrated with pH standards, which analyzed the pH of the gel directly on the dental enamel of the samples.

The first reading was performed 30 s after the bleaching gel contacted the bovine enamel structure (initial), and the second reading was performed 30 s after the end of the session (final), with the analysis performed in triplicate [31].

Particle size (P), polydispersity index (PDI), and zeta potential (ZP)

The P, PDI, and ZP of the bleaching gels were evaluated by light scattering using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) [32]. ZP was assessed to measure colloidal stability using the Helmholtz-Smoluchowski model [33] by measuring the electrophoretic mobility of the particles dispersed in an applied electric field, and the analysis was performed in triplicate. The ZP analysis was conducted via laser electrophoresis with 30 runs at 25 °C. ZP was automatically calculated from the electrophoretic mobility using the Smoluchowski approximation: $UE = 2 * \varepsilon * z * f(ka)/3 * \eta \rightarrow z \approx UE * \eta/\varepsilon$, where UE is the electrophoretic mobility, ε is the dielectric constant, z is the ZP, f(ka) is the Henry function, and η is the viscosity. The particle size and ZP analysis were performed immediately after handling the gels.

Rheological behavior (RB)

The RB of the samples was determined by flow curves at 25 °C, using a controlled stress rheometer (Physica MCR 301 Rheometer, Anton Paar, Ostfildern, Germany), equipped with a stainless-steel cone-plate geometry of 50 mm diameter, 2° angle, and fixed gap of 208 µm. The shear rate was applied between 0 and 150 s-1, measuring 100 points for each curve. The viscosity curves of the samples were obtained from the relationship between shear stress and shear rate, and analyses were performed in duplicate.

Phase 2: color evaluation

Sample Preparation and selection

Bovine teeth with intact enamel surfaces, free of fractures and cracks, were selected, cleaned, and disinfected in a 0.5% thymol solution (Labsynth, Diadema, SP, Brazil). Discs of enamel-dentin with a diameter of 5 mm and a thickness of 3 mm were obtained using a bench drill (FSB 16, Pratika, Shulz). The samples were polished (Arotec, São Paulo, Brazil), and the dentin surface of the discs was initially flattened (#600) to ensure parallelism. Subsequently, the enamel was abraded with silicon carbide sandpapers (#600, 800, 1200, 3 M ESPE 411Q, Sumaré, SP, Brazil) and polished with a felt disk and diamond suspension (with abrasive particles of 1 and ½ µm) for 1 min. The staining protocol was based on the study by Matos et al. (2023) [21]. The samples were isolated with a nail polish base, leaving only the vestibular enamel exposed, and were immersed in a black tea solution (2 g of black tea in 100 mL of boiled distilled water for 4 h). After staining, the samples were cleaned with pumice stone (SS White, São Paulo, Brazil) and a Robson brush to remove non-adhered particles and were stored in artificial saliva (1.5 mM Ca; 0.9 mM PO4 and 150 mM KCl in a 20 mM tris buffer solution, pH 7.0) in an incubator at 37ºC for 7 days to stabilize the color before the treatments, with the artificial saliva being replaced every 2 days, according to a protocol based on Viana et al. (2021) [34].

Group distribution and bleaching protocol

Before bleaching, the samples underwent Knoop microhardness testing with a load of 50.0 g for 5 s and 3 indentations/samples with a 100 µm distance (Future Tech FM-ARS, Tokyo, Japan) (Kury et al., 2022). Samples ($N = 90$; $n = 10$) with standardized microhardness (303.97 kgf/mm² ± 20.47) were randomly distributed and subjected to bleaching with hydrogen peroxide (HP 1.5% and 6%) containing NF_TiO_2 nanoparticles (5%) and violet LED light irradiation (irradiation or not with LED), as represented in Table 3.

The violet LED light was applied immediately after the gel application. The LED device used was the Bright Max Bleaching– BMW (MMOptics, São Carlos, SP, Brazil) with a wavelength of 405 ± 15 nm (violet) and a power of 1.2 W/cm² for all groups. Three applications were performed with 7-day intervals between sessions, and the samples were placed in artificial saliva and stored in an oven at 37ºC.

Color change (ΔE_{00})

The enamel color was assessed at two time points: before bleaching (T_0) and 14 days after the treatments were completed (T_2), with the teeth stored in artificial saliva throughout the interim period. A digital spectrophotometer (EasyShade, Vita Zahnfabrik, Bad Säckingen, Germany) was used to determine the color parameters L* (black-white axis), a* (green-red axis), and b* (blue-yellow axis), as well as h (hue) and C (chroma). Color change was assessed using the CIEDE2000 formula $(\Delta E_{00}) = [(\Delta L'/KLSL)^2 + (\Delta C'/KCSC)^2 + (\Delta H'/KHSH)^2 + RT*(\Delta C'/KCSC)*(\Delta H'/KHSH)]^{1/2}$. In this formula, H represents hue and C represents chroma. The color change was assessed after bleaching at 14 days post-bleaching about the initial time ($T_2 - T_0$). The values for color change (ΔE_{00}) about perception thresholds (PT) and acceptance thresholds (AT) (50:50%) were set at 0.81 and 1.8 units, respectively [35].

Table 3 Groups, gel composition, and application protocols

Groups	Treatments	Protocols
35%HP-commercial	Commercial hydrogen peroxide gel 35% (Whiteness HP, FGM, Joinville, SC, Brazil)	30 min with a single application during the session
HA-1.5%HP	HA-gel as a thickener and 1.5% hydrogen peroxide.	30 min with a single application during the session.
HA-6%HP	HA-gel as a thickener and 6% hydrogen peroxide.	
CAR-1.5%HP	CAR-gel as a thickener and 1.5% hydrogen peroxide.	
CAR-6%HP	CAR-gel as a thickener and 6% hydrogen peroxide	
HA-1.5%HP+LED	HA-gel as a thickener, 1.5% hydrogen peroxide, and irradiation with LED	30 min with a single application during the session and violet LED irradiation (20 cycles of 1 min, with a 30-second interval between each irradiation).
HA-6%HP+LED	HA-gel as a thickener, 6% hydrogen peroxide, and irradiation with LED	
CAR-1.5%HP+LED	CAR-gel as a thickener, 1.5% hydrogen peroxide, and irradiation with LED	
CAR-6%HP+LED	CAR-gel as a thickener, 6% hydrogen peroxide, and irradiation with LED	

Whiteness index for dentistry (ΔWI_D)

The enamel color was assessed at two time points: before bleaching (T_0) and 14 days after the treatments were completed (T_2), with the teeth stored in artificial saliva throughout the interim period. A digital spectrophotometer (EasyShade, Vita Zahnfabrik, Bad Säckingen, Germany) was used to determine the color parameters L* (black-white axis), a* (green-red axis), and b* (blue-yellow axis), as well as h (hue) and C (chroma). The whiteness index for dentistry (WI_D) and the difference in the whiteness index (ΔWI_D) were calculated according to the equations: $WI_D = 0.511\ L^* - 2.324a^* - 1.100b^*$ and $\Delta WI_D = WI_D\ T_2 - WI_D\ T_0$. The values for the difference in ΔWI_D about PT and AT (50:50%) were set at 0.7 (PT) and 2.6 (AT). The 50:50% thresholds for PT and AT refer to the perception and acceptance of professional and non-professional volunteers evaluated in multicentric prospective studies [36].

Phase 3: mineral content

Energy dispersive X-ray spectroscopy (EDS) (T_2)

The samples were selected and analyzed for mineral content 14 days after the treatments were completed (T_2), with the teeth stored in artificial saliva throughout the interim period. After the treatments, the samples ($n=3$) were washed in an ultrasonic bath (Ultra Cleaner, Unique, Indaiatuba, SP, Brazil) for 10 min and dried for 24 h in a drying oven. After drying, the samples were carbon-coated and analyzed using automated imaging scanning electron microscope (SEM–JEOL-JSM, 6460LV, Tokyo, Japan), operating at 15 kV in vacuum mode (45 Pa). This process was carried out concurrently with acquiring scanning electron microscope (SEM) images, using the energy dispersive X-ray spectroscope software (EDS, VANTAGE System– Easymicro Noran Instruments, Middleton, Wisconsin, USA), with measurements taken from three regions per sample to calculate the calcium to phosphorus ratio (Ca/P) [37].

Surface microhardness (KHN) (T_0, T_1, T_2)

The samples were selected at T_0 by performing 3 indentations in the enamel at the central region of each fragment, using a Knoop-type penetrator (Future Tech-FM-1e, Tokyo, Japan), with a load of 50 g for 5 s and a spacing of 100 µm between indentations. Enamel-dentin discs with an average value of approximately 303.97 ± 20.47 kgF/mm² and a variation of up to 10% from the overall mean were selected. Microhardness was analyzed at T_0, T_1 and T_2. The loss of surface microhardness was expressed as a percentage (%SHL) and calculated using the following formula: %SHL = (KHN T_0– KHN T_2) / (KHN T_0) * 100 [38].

Phase 4: surface morphology

Roughness (ΔRa) (T_0 and T_2)

To perform the two-dimensional surface roughness tests, a roughness meter (Surfcorder SE 1700, Kosalab) was used to determine the average roughness value (Ra, µm). Three readings were taken for each sample in three directions at a 45° difference between them, and the average value for each group was calculated. The device was calibrated with a cut-off of 0.25 mm and a speed of 0.2 mm/s, with readings taken before bleaching (T_0) and 14 days after the last bleaching session (T_2), and the difference in ΔRa at the two times was determined ($\Delta Ra = Ra_2 - Ra_0$) [38].

Scanning electron microscopy (SEM) (T_2)

Three samples ($n=3$) from each group were randomly selected 14 days after the treatments (T_2), and representative images of the enamel surface were evaluated using SEM. The samples were washed in an ultrasonic bath (Ultra

Table 4 Mean and standard deviation pH values of the gels at each time point separately

	Initial Time			
	HP 1.5%		HP 6%	
	Without LED	With LED	Without LED	With LED
HA	6.47 (0.06) Ba*♠	6.22 (0.05) Bb♠	6.19 (0.07) Ba♠	6.19 (0.05) Ba♠
CAR	7.03 (0.06) Ab	7.28 (0.17) Aa*	7.02 (0.06) Aa	6.85 (0.13) Ab
Final Time				
		HP 1.5%	HP 6%	
	Without LED	With LED	Without LED	With LED
HA	6.40 (0.18) Ba*♠	6.07 (0.11) Bb♠	6.06 (0.07) Ba♠	6.02 (0.07) Ba♠
CAR	6.98 (0.1) Aa	6.97 (0.11) Aa	7.00 (0.06) Aa	6.92 (0.04) Aa

35%HP-commercial - Initial: 7.11 (0.10); **Final**: 6.85 (0.07)

Distinct uppercase letters indicate statistical differences between CAR and HA and within the same HP concentration and LED condition. Distinct lowercase letters indicate statistical differences within the same gel with or without LED. Asterisks indicate statistical differences between 1.5% and 6% HP and within the same thickener and LED condition. Spades symbols indicate statistical differences with 35%HP-commercial within each time point

Cleaner, Unique, Indaiatuba, SP, Brazil) for 10 min and dried for 24 h in a drying oven. After drying, the samples were coated with carbon and subjected to automated image analysis (SEM– JEOL-JSM, 6460LV, Tokyo, Japan), operating at 15 kV in vacuum mode (45 Pa). The most representative areas of each group were photographed at 1500× magnification and qualitatively evaluated for surface homogeneity and smoothness, as well as regions showing porosity, signs of demineralization, exposure of prisms, or areas with mineral deposits from the artificial saliva [24].

Statistical analysis

The data were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests, respectively. One-way ANOVA and Tukey post-hoc test analyzed P, PDI, and ZP data. A three-factor repeated measures ANOVA and Bonferroni post-hoc test were used for microhardness and pH analysis. A subsequent Dunnet test was performed for each time point among the group bleached with a commercial 35% HP gel and all the experimental groups for both variable responses. A three-factor ANOVA with Tukey post-hoc test analyzed the ΔWI_D data. The non-parametric Kruskal-Wallis test was used for ΔRa data, and a generalized linear model was used for ΔE_{00} data.

Table 5 Means and standard deviations P, PDI, and ZP according to the thickener and irradiation with Violet LED light

Experimental groups	Particle size (em nm)	Polydispersity index (PDI)	Zeta potential (em Mv)
CAR+LED	1199.7 (247.3) C	0.9 (0.1) A	-73.5 (1.8) AB
CAR	1090 (524.1) C	0.8 (0.2) A	-69.5 (1.7) B
HA+LED	2850.7 (116.1) B	1 (0.0) A	-57.4 (2.0) C
HA	4838.3 (477.9) A	1(0.0) A	-78.4 (1.9) A

Capital letters indicate statistical differences between groups, in each analysis (columns) ($p<0.05$). **Legend**: CAR=Carbomer-based gel; CAR+LED=Carbomer-based gel with LED irradiation; HA=hyaluronic acid-based gel; HA+LED=hialuronic acid-based gel with LED irradiation

Results

Phase 1: characterization of experimental bleaching gels

pH analysis

In Table 4, it can be observed that for both concentrations of hydrogen peroxide (1.5% and 6%), the pH of the gels ranged from 6.02 to 7.28, with the CAR-gels being closer to neutrality (pH ~ 7.0). The HA-gels showed a lower pH than CAR-based groups at the initial time point, but after 30 min of treatment, most groups demonstrated a slight decrease in pH values. According to the statistical test, a triple interaction was detected among the parameters thickener, HP concentration and LED irradiation ($p=0.001$), but the time factor (initial x final) did not display any significant interactions with all the other ones. In view of this, Table 4 depicts the significant differences among the groups at the different time points. HA-based groups displayed significant differences with the 35%HP-commercial, regardless of the time.

Particle size (P), polydispersity index (PDI), and zeta potential (ZP)

Table 5 presents the values for average P, PDI, and ZP of the experimental gels immediately after their formulation. The HA (hyaluronic acid) group exhibited the largest average particle size, while the CAR (carbomer-based gel) and CAR+LED (carbomer-based gel with LED irradiation) groups showed smaller average sizes ($p<0.05$).

The HA groups had the highest PDI values for the nanoparticles in the gel, but there were no statistically significant differences compared to the CAR groups, regardless of LED light irradiation ($p>0.05$). The ZP of the experimental bleaching gels varied between −57.4 mV and −78.4 mV, with the lowest value attributed to the HA (hyaluronic acid-based gel) group and the highest value to the HA+LED

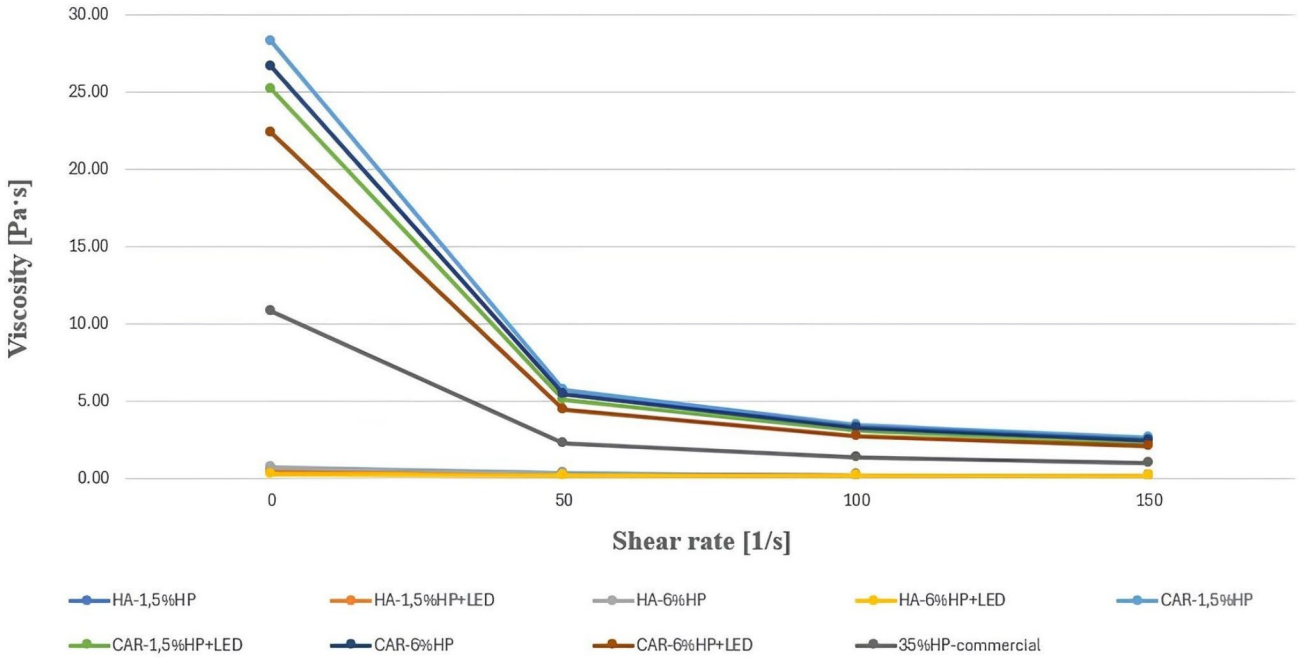

Fig. 2 Rheological behavior analysis based on viscosity changes under different shear rates across experimental groups

Table 6 Means and standard deviations of ΔE_{00} after 14 days of the bleaching protocol

	HP 1.5%		HP 6%	
	Without LED	With LED	Without LED	With LED
HA	4.4 (0.7) Ab*	5.5 (2.7) Aa	4.2 (0.6) Ab*	7.1 (1.9) Aa
CAR	4.4 (1.1) Ab*	6.8 (4.4) Aa	5.5 (1.5) Ab*	6.7 (1.0) Aa

35%HP-commercial: 12.2 (7.7)

Distinct letters indicate statistically significant differences ($p \leq 0.05$) according to generalized linear models. Uppercase letters compare the thickeners (columns), which did not show significant differences among themselves ($p > 0.05$). Lowercase letters compare groups with and without LED light irradiation (rows). Asterisks indicate differences from the control group (35%HP-commercial)

Table 7 Means and standard deviations of ΔWI_D calculated after 14 days of the bleaching protocol

	HP 1.5%		HP 6%	
	Without LED	With LED	Without LED	With LED
HA	17.5 (5.5) Ab*	26.1 (10.6) Aa	17.5 (8.6) Ab*	25.2 (6.1) Aa
CAR	17.5 (5.7) Ab*	21.2 (11.4) Aa*	16.3 (8.1) Ab*	23.4 (4.0) Aa*

35%HP-commercial: 34.5 (12.3)

Distinct letters indicate statistically significant differences ($p \leq 0.05$) according to generalized linear models. Uppercase letters compare the thickeners (columns), which did not show significant differences among themselves ($p > 0.05$). Lowercase letters compare groups with and without LED light irradiation (rows). Asterisks indicate differences from the control group (35%HP-commercial)

(hyaluronic acid-based gel with LED irradiation) group, respectively.

Rheological behavior (RB)

The values presented in Fig. 2 refer to the rheological behavior of the experimental bleaching gels after formulation and manipulation at 25 ºC. The groups containing HA exhibited significantly less viscous rheological behavior values compared to the CAR groups, regardless of the concentration of hydrogen peroxide (1.5% or 6%) and light irradiation. The control group treated with 35% commercial hydrogen peroxide showed intermediate values, positioned between the HA-gels and the CAR-gels.

Phase 2: color evaluation

Table 6 presents the results of ΔE_{00}. There was no significant interaction among the three factors studied ($p > 0.05$). It is noteworthy that, regardless of the hydrogen peroxide concentration and thickener type, the ΔE_{00} was significantly greater when light was used ($p < 0.05$). Additionally, all groups without light showed ΔE_{00} values significantly lower than the control group ($p < 0.05$).

Table 7 displays the results of ΔWI_D, revealing no significant differences between hydrogen peroxide concentrations ($p = 0.974$) and thickener types ($p = 0.339$). However, there was a significant change in the light factor ($p < 0.001$), as demonstrated by the statistically higher values for the HP 6% groups irradiated with light. The HA 1.5% and 6%

groups, both with light, showed no differences compared to the 35%HP-commercial.

Phase 3: mineral content

Energy dispersive X-ray spectroscopy (EDS) (T_2)

Table 8 Presents the values for the Ca/P ratio of the bleached enamel, as determined by EDS analysis. Qualitatively, a similar Ca/P ratio was observed across all evaluated groups, with means ranging from 2.46 to 2.55

Surface microhardness (KHN) (T_0, T_1, T_2)

Table 9 shows the results of KHN values and %SHL values. There were no significant differences in the initial microhardness values between groups ($p > 0.05$). The microhardness of all groups treated with the experimental bleaching gels showed a microhardness loss 24 h after the last bleaching session (T_1). After 14 days (T_2), the groups HA-1,5%HP, HA-6%HP, HA-1,5%HP+LED, HA-6%HP+LED and CAR-6%HP exhibited an increase in microhardness. The groups CAR-1,5%HP, CAR-1,5%HP+LED and CAR-6%HP+LED showed lower microhardness than the 35%HP-commercial control group, whereas the groups HA-1,5%HP+LED and HA-6%HP+LED had higher microhardness than the CAR-6%HP. According to the %SHL, all experimental HA groups showed mineral gain 14 days after the last bleaching session and storage in artificial saliva, while the CAR groups exhibited mineral loss, except for the CAR-6%HP+LED, which showed a slight increase under the same conditions.

Roughness (ΔRa) (T_0 and T_2)

Table 10 presents the ΔRa after 14 days of the bleaching treatment. The initial values showed no statistical difference between the groups. After 14 days of bleaching treatment, no statistical differences were observed among the groups treated with experimental bleaching gels, regardless of irradiation with violet LED light. There was also no difference between the experimental groups and the 35%HP-commercial.

Table 8 Means and standard deviations of the Ca/P ratio (% by weight) based on EDS analysis of bleached enamel according to each group

	HP 1.5%		HP 6%	
	Without LED	With LED	Without LED	With LED
HA	2.48 (0.02)	2.46 (0.04)	2.52 (0.04)	2.49 (0.04)
CAR	2.55 (0.06)	2.49 (0.03)	2.50 (0.01)	2.46 (0.01)

35%HP-commercial: 2.51 (0.01)

Scanning electron microscopy (SEM)

Figure 3 shows representative SEM images of enamel after 14 days of the bleaching treatment with the experimental agents. No areas of porosity or demineralization indicative of mineral loss from the enamel were observed. There was also no evidence of exposed prisms or mineral deposits that could originate from artificial saliva. Even when irradiated with the violet LED light, the enamel surface remained smooth and homogeneous, with no changes when compared to the non-irradiated groups and the 35%HP-commercial.

Discussion

The characterization analyses of the experimental bleaching gels supported the rejection of the first null hypothesis, confirming their minimal variations in rheological properties suitable for clinical application. The pH of the bleaching gels plays a critical role in maintaining the mineral content balance of the enamel [39, 40]. The thickeners utilized in this research (HA and CAR), which were incorporated with NF_TiO_2 nanoparticles, exhibited an acidic pH immediately after production and were neutralized with potassium hydroxide (KOH) solution until reaching a pH of 6, which is comparable to commercial gels [17, 21]. After the initial pH adjustment, the gels showed an average pH ranging from 6.19 to 7.28. There was a slight decrease in pH after 30 min, but the values remained close to the initial readings. This trend was consistent regardless of the thickener used or irradiation with violet LED light, which was expected since H_2O_2 is more stable in a slightly acidic environment [41].

Although a reduction in pH was observed, it is important to note that these values remained above the critical levels for the onset enamel demineralization (pH < 5.5). Furthermore, these pH values were considered safe for application to the enamel structure, as they remained slightly acidic throughout the bleaching protocol which consisted of three 30-minute sessions, with intervals of 7 days. Additionally, enamel-dentin discs were stored in artificial saliva between sessions, facilitating enamel remineralization. Matos et al. (2023) [21] developed a bleaching gel containing NF_TiO_2 nanoparticles, with or without violet light irradiation, and observed a similar pH pattern, noting a slight decrease after 30 min of bleaching.

The average particle size was 1199.7 nm for the CAR group and 4838.3 nm for the HA group, both of which are significantly larger than the typical size range of TiO_2 nanoparticles reported in the literature, typically ranging from 6 to 10 nm [19]. These elevated values can be attributed to the PDI, a dimensionless parameter where values approaching 0 indicate highly monodisperse systems, while

Table 9 Means and standard deviations of initial microhardness(T_0), after 24 h of the last bleaching session (T_1) and after 14 days of the bleaching protocol (T_2)

	HP 1.5%							HP 6%						
	Without LED				With LED			Without LED				With LED		
	T_0	T_1	T_2	%SHL	T_0	T_1	T_2	%SHL	T_0	T_1	T_2	%SHL	T_0	T_1	T_2	%SHL
HA	303.6 (17.6) Ab	323.9 (19.0) Aa	316.2 (11.6) Aab	**4.52%**	303.7 (16.7) Ab	323.0 (26.1) Aa	328.4 (25.3) Aa	**8.24%**	303.0 (17.6) Aa	315.0 (21.73) Aab	316.3 (19.7) Aa	**-4.50%**	302.7 (16.0) Ab	317.0 (28.1) Aa	323.2 (20.1) Aa	**6.82%**
CAR	304.9 (16.7) Aa	260.1 (18.2) Bc*#	291.6 (29.0) Bb*#	**4.38%**	303.5 (16.4) Aa	264.1 (28.2) Bb#	278.2 (17.6) Ab#	**8.38%**	303.4 (17.7) Aa	303.6 (13.8) Aa	314.5 (23.6) Aa*	**-3.84%**	302.4 (16.6) Aa	283.0 (30.6) Bb#	294.2 (29.2) Bab#	**2.62%**

35%HP-commercial - T_0: 303.9 (16.1); **T_1**: 329.0 (38.3); **T_2**: 322.0 (10.7); **%SHL**: −6.11

Uppercase letters compare thickeners at the same time (columns). Lowercase letters compare time within the same thickener (rows). Asterisks compare concentrations of HP within the same thickener, time, and light. Hashtags compare experimental groups with the control group (35%HP-commercial)

Table 10 Means and standard deviations of ΔRa calculated after 14 days of the bleaching protocol

	HP 1.5%		HP 6%	
	With LED	Without LED	With LED	Without LED
HA	0.003 (0.007)	0.001 (0.004)	0.003 (0.006)	0.001 (0.005)
CAR	-0.001 (0.011)	0.004 (0.006)	0.000 (0.004)	0.005 (0.008)
35%HP-commercial: -0.001 (0.006)				

No statistical differences were found among the groups after 14 days of the bleaching protocol, regardless of the thickener, LED, and HP concentration ($p>0.05$)

those closer to 1 suggest a polydisperse sample with a greater tendency for agglomeration [42]. Consequently, the aggregation of NF_TiO_2 nanoparticles may have contributed to the observed particle size values. A high zeta potential (ZP) is crucial for maintaining the stability of sufficiently small particles, preventing gel aggregation. ZP values exceeding (+/-) 40 mV indicate good suspension stability, as the surface charge prevents particle agglomeration. Conversely, low zeta potential values reduce electrostatic repulsion, allowing attractive forces to dominate, which can lead to gel destabilization and flocculation [32].

The negative zeta potential (ZP) values observed in the experimental bleaching gels containing hyaluronic acid or carbomer indicated greater agglomeration of NF_TiO_2 nanoparticles compared to the findings of Carlos et al. (2023) [32]. In their study, TiO_2 nanotubes, irradiated or not with violet LED light, exhibited average particle size and polydispersity index values comparable to the baseline analysis. The higher zeta potential values observed in their study may be attributed to differences in particle morphology, which influence the aggregation potential of TiO_2 particles [42, 43]. Additionally, while Carlos et al. [32] incorporated 1% TiO_2 nanotubes, the present study incorporated 5% NF_TiO_2 in the bleaching gels, which may have further contributed to the observed differences.

Rheological properties are critical in the manufacturing, storage, and application of topical products, as each formulation must exhibit rheological behavior suited to its intended use [44, 45]. Therefore, understanding deformation rates during processing is essential for characterizing the viscosity of bleaching gels and determining whether they exhibit Newtonian or non-Newtonian behavior, as classified in rheological studies [46, 47]. Regarding the viscosity of the experimental gels, Fig. 2 displays values corresponding to the shear rate during analysis. The initial viscosity values are particularly relevant for materials that remain static on the dental surface, such as bleaching gels, as they best represent real clinical application conditions.

In Newtonian fluids, the rate of deformation is directly proportional to the applied shear stress, meaning viscosity remains constant regardless of the shear rate or the duration of force application [46, 47]. This behavior was observed

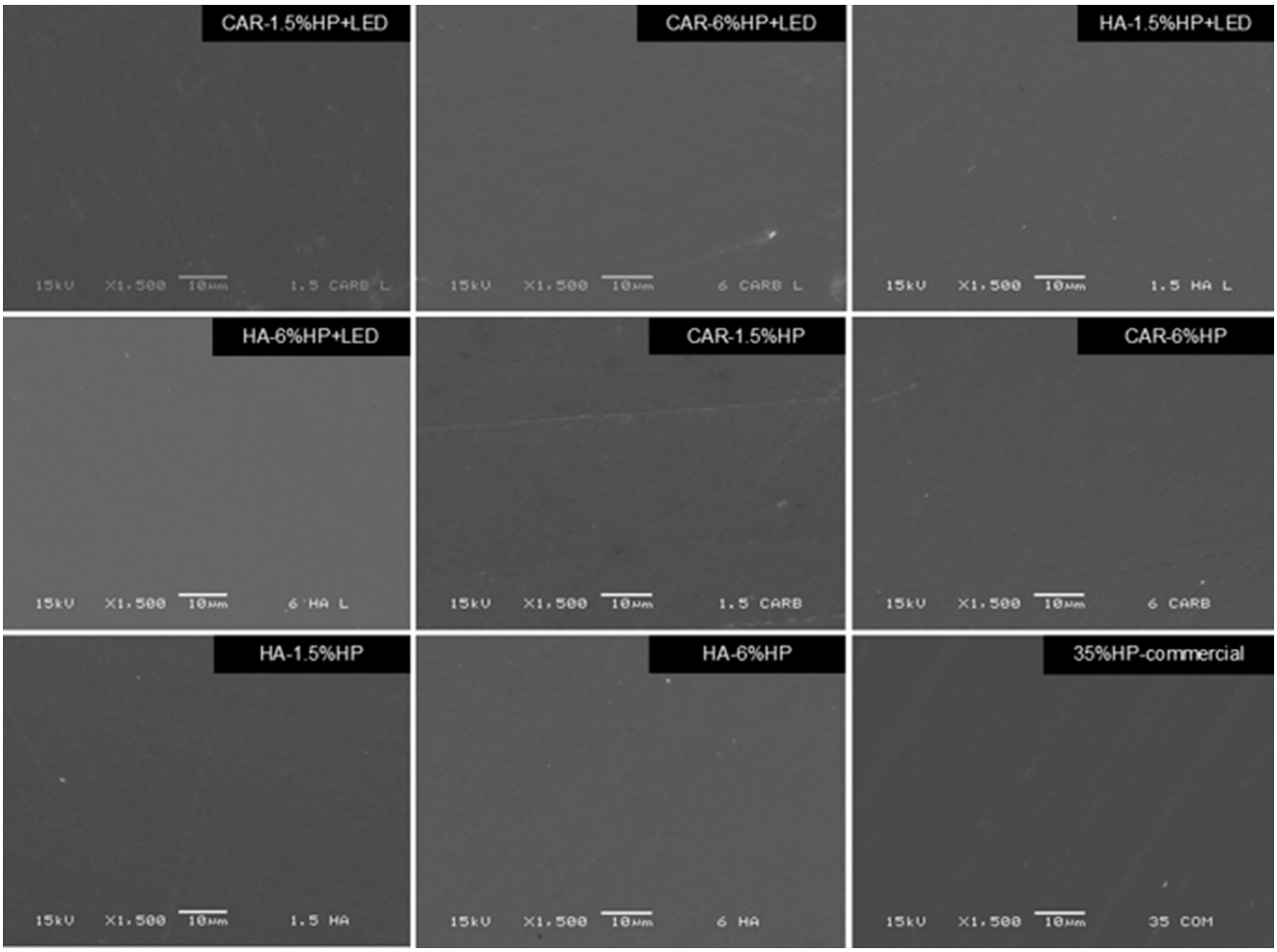

Fig. 3 Representative images were obtained via scanning electron microscopy (SEM). No evidence of demineralized areas or porosities indicates mineral loss of the enamel. Additionally, no areas exhibiting prism exposure or mineral deposits are observed

in the bleaching gels containing hyaluronic acid, regardless of violet LED irradiation. These gels initially exhibited lower viscosity values (0.8 Pa·s not irradiated and 0.5 Pa·s irradiated) with minimal variation throughout the analysis duration and across different shear rates. Currently, no studies have investigated bleaching gels containing hyaluronic acid combined with hydrogen peroxide, making direct comparisons with existing literature challenging. Although its composition is similar to that of hyaluronic acid gels used in facial fillers, its intended application and functional behavior are distinct.

In non-Newtonian fluids, the relationship between shear stress and deformation rate is non-linear, and viscosity may change depending on the deformation rate or the duration of force application [46, 47]. Among these, fluids exhibiting thixotropic behavior are characterized by a time-dependent decrease in viscosity under shear stress. Over time, their internal structure breaks down, leading to reduced viscosity [47, 48]. This behavior was observed in the CAR groups and the commercial control group (35% HP-commercial), which initially exhibited high viscosity values but demonstrated a progressive decline over time.

Incorporation of nanoparticles, such as TiO_2, enables the formulation of products with reduced concentrations of bleaching agents, minimizing potential alterations to the dental enamel structure that are often associated with traditional treatments, without compromising bleaching efficacy [21, 49]. Thus, the second null hypothesis was rejected, as the combination of hyaluronic acid (1.5% and 6% HP) associated with NF_TiO_2 nanoparticles and irradiated with violet LED light showed no statistical difference compared to the 35%HP-commercial control group.

A study by Kury et al. (2022) [20] investigated the application of experimental bleaching gels containing reduced concentrations of hydrogen peroxide (6% and 15%) irradiated with violet LED light. The authors reported comparable aesthetic results in terms of ΔE_{00} and ΔWI_D, to those obtained with high concentration bleaching gels (35% HP). These results are in line with the findings of this study and suggest that the combination of violet LED irradiation and

the experimental gel formulations may have the potential to reduce dental discolorations using lower amounts of hydrogen peroxide.

The results for ΔE_{00} are promising, as the bleaching gels containing NF_TiO_2 nanoparticles (5%, wt%) combined with low concentrations of hydrogen peroxide (1.5% and 6%) achieved results comparable to the commercial 35% HP gel, but only when irradiated with violet LED (405 nm ± 15 nm). Groups treated with the same experimental gels without LED irradiation showed significantly lower ΔE_{00} values, consistent with findings by Kury et al. (2022) [20]. Nonetheless, all experimental groups achieved ΔE_{00} values exceeding the perceptibility (PT) and acceptability (AT) thresholds of 0.8 and 1.8, respectively [35].

Irradiation with violet LED significantly influenced the ΔE_{00} results in the experimental groups. Previous studies indicate that violet LED irradiation can induce color changes, especially when used in combination with gels containing reduced peroxide concentrations [50–52]. Additionally, there are numerous reports in the literature discussing the photocatalytic activity of TiO_2, particularly when doped with metal oxides [21, 49, 53, 54]. Although this study did not directly assess the photocatalytic effect of the nanoparticles, previous studies have shown that incorporating NF_TiO_2 into 6% HP gels irradiated with violet LED light further increased ΔE_{00} and ΔWI_D values [20, 21, 53]. However, since the incorporation of nanoparticles were not designed as a study factor, it is only possible to confirm that violet LED significantly enhanced the color and whiteness changes rendered by HA and CAR-based gels (1.5% and 6% HP).

Similar to the results of ΔE_{00}, the groups irradiated with violet LED exhibited the highest mean ΔWI_D, surpassing the perceptibility (PT) and acceptability (AT) thresholds of 0.72 and 2.60, respectively [36]. However, the influence of the thickening agent on the observed results is notable. The groups containing 1.5% and 6% HP, when irradiated with a violet LED light, were statistically similar to the control group (35%HP-commercial) only when associated with the HA thickening agent. Groups with CAR thickening agents and irradiated with LED displayed significantly higher ΔWI_D averages compared to non-irradiated groups. However, they did not achieve the bleaching efficacy of the control group (35% HP-commercial). Other studies in the literature have reported the influence of thickening agents on color alteration in both in-office bleaching techniques using hydrogen peroxide and at-home techniques using carbamide peroxide [55, 56]. Considering the use of NF_TiO_2 nanoparticles in the experimental gels, it is suggested that hyaluronic acid may act as a nanocarrier, potentially promoting a more favorable environment for molecular movement and decomposition of hydrogen peroxide. However, further investigation is needed to confirm this hypothesis.

Another explanation for the ΔWI_D results is that the type of polymer used in the bleaching gel formulation may influence its rheological behavior. One example, as reported in the literature, is the viscosity of the thickening agent used in the bleaching gel. A study by Torres et al. (2022) [55] investigated the influence of viscosity and thickening agent type in hydrogen peroxide gels at 35% concentration. The authors observed that gels with higher viscosity resulted in reduced color change (ΔEab) in the treated samples. In contrast, Kwon et al. (2018) [57] evaluated the effect of the thickening agent on the penetration of hydrogen peroxide into the pulp chamber. Although no significant difference in color alteration was found, gels with lower viscosity showed greater levels of pulpal penetration.

All groups treated with the experimental bleaching gels demonstrated a loss of microhardness 24 h after the final bleaching session (T_1), a phenomenon commonly associated with the interaction of bleaching agents and the dental structure. Dental enamel, composed of hydroxyapatite, forms a crystalline solid containing calcium, phosphate, and hydroxyl ions. When the bleaching gel contacts the enamel surface, an interface is formed where ionic exchanges occur due to the decomposition of hydrogen peroxide. This results in the dissolution of enamel minerals until ionic equilibrium is reached between the dental surface and the bleaching gel [58]. However, after 14 days in artificial saliva (T_2), an increase in microhardness values was observed, indicating enamel mineral recovery and the restoration of microhardness to levels similar to those observed before the bleaching procedure (T_0).

The hyaluronic acid (HA) thickening agent offers advantages such as biodegradability and hydrophilicity, the latter being attributed to its high-water retention capacity [27, 28]. A slight increase in microhardness was observed in the HA groups, which may be linked to the material's interaction with the enamel surface and its potential to modulate ionic transport within the medium. However, the hypothesis that HA directly influences the release or transport of ROS requires further investigation, as its role in ROS dynamics is not yet fully understood. Additionally, vehicles with lower viscosity, such as HA-based gels, exhibit enhanced wettability, promoting increased interaction between contacting surfaces [59]. Most CAR groups demonstrated minimal reductions in microhardness, consistent with patterns observed in the study by Kury et al. (2024) [25], where NF_TiO_2 nanoparticles combined with low concentrations of hydrogen peroxide (6% and 15%) were incorporated into a carbomer thickening agent. Based on these findings, the third null hypothesis was accepted. The combination of the HA thickening agent (1.5% and 6% HP) with NF_TiO_2

nanoparticles, when irradiated with violet LED, did not alter the mineral content of bleached enamel compared to the commercially bleached group with 35% hydrogen peroxide.

Currently, it is known that treating the enamel surface before and after the bleaching procedure, along with applying fluorides, remineralizing solutions, and adjusting the composition of the bleaching gel, significantly reduces the bleached enamel roughness [60–62]. None of the tested bleaching treatments exhibited an increase in roughness after the bleaching sessions. This indicates that reduced concentrations of hydrogen peroxide (1.5% and 6%) do not alter the enamel surface, corroborating findings from other studies in the literature [21, 63].

Despite the roughness meter's ability to detect minimal variations in superficial topography, the recorded values are minimal and do not exert a substantial impact on the optical characteristics of the enamel, nor do they increase susceptibility to biofilm formation, a phenomenon that could occur on surfaces with roughness exceeding 0.2 µm [64]. The results of the roughness test can be confirmed with images obtained from scanning electron microscopy (SEM), which show no evidence of demineralized areas or porosities indicating enamel mineral loss, corroborating previous studies [21, 65].

Some limitations are inherent to in vitro studies. In the color analyses, the spectrophotometer and sample were kept in standardized positions to ensure the correct alignment of the device's tip, noting that color variations may occur if the tip is placed at a different angle. Measurements were also carried out in a color-matched lightbox, ensuring consistent lighting control. The artificial pigmentation of the samples also presents a limitation and is a recurrent topic of discussion among researchers. Regarding the black tea used in this research, the pigmenting substance was neutralized with KOH before the pigmentation process, as the literature indicates that the acidic pH of black tea can cause enamel demineralization [66]. Despite the ongoing discussion surrounding the issue and the diversity of artificial pigmentation protocols, all are well-established and utilized in other studies [21, 38, 40]. Furthermore, the agglomeration of nanoparticles is a negative factor for photocatalytic performance because it reduces the available surface area for interaction with light and interferes with the production of ROS. Therefore, controlling nanoparticle dispersion is crucial for optimizing the photocatalytic activity in future formulations as well as isolating the NF_TiO_2 nanoparticles as a study factor, especially evaluating HA-type gels.

The experimental bleaching HA or CAR-gels containing NF_TiO_2 nanoparticles and irradiated with violet LED light, demonstrated satisfactory properties in characterization analyses and high bleaching efficacy even with low concentrations of HP. Moreover, the experimental gels maintained the enamel's mineral content and did not cause morphological surface alterations. Thus, it would be beneficial for future studies to evaluate the cytotoxic potential of these experimental gels and the interaction of nanoparticles in different thickening agents. Additionally, conducting specific analyses to evaluate the photocatalytic potential of the nanoparticles is crucial, as this could help to optimize the molecular environment for their photocatalytic action.

Conclusion

Within the limitations of this in vitro study, it is concluded that the experimental bleaching HA or CAR-gels containing nanoparticles showed high bleaching efficacy even with low concentrations of hydrogen peroxide, being favored by violet LED light irradiation. However, the HA groups exhibited better results regarding ΔWI_D. Additionally, they demonstrated minimal variations in physicochemical properties and did not cause significant alterations in mineral content, roughness, surface morphology, and Ca/P ratio in the bleached enamel.

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Data availability No datasets were generated or analysed during the current study.

Declarations

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