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RESEARCH

Co-doped titanium dioxide nanoparticles decrease the cytotoxicity of experimental hydrogen peroxide gels for in-office tooth bleaching

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Abstract

Objective To evaluate the efficacy and cytotoxicity of experimental 6% and 35% hydrogen peroxide gels (HP6 or HP35) incorporated with titanium dioxide nanoparticles (NP) co-doped with nitrogen and fluorine and irradiated with a violet LED light (LT).

Methods Bovine enamel-dentin disks adapted to artificial pulp chambers were randomly assigned to bleaching ($n = 8$/group): NC (negative control), NP, HP6, HP6 + LT, HP6 + NP, HP6 + NP + LT, HP35, HP35 + LT, HP35 + NP, HP35 + NP + LT, and commercial HP35 (COM). Color (ΔE_{00}) and whiteness index (ΔWI_D) changes were measured before and 14 days after bleaching. The extracts (culture medium + diffused gel components) collected after the first session were applied to odontoblast-like MDPC-23 cells, which were assessed concerning their viability, oxidative stress, and morphology. The amount of HP diffused through the disks was determined. Data were analyzed by generalized linear models or Kruskal Wallis Tests ($\alpha = 5\%$).

Results HP6 + NP + LT exhibited ΔE_{00} and ΔWI_D higher than HP6 ($p < 0.05$) and similar to all HP35 groups. HP6 + NP + LT showed the lowest HP diffusion, and the highest cell viability (%) among bleached groups, preserving cell morphology and number of living cells similar to NC and NP. HP6 + LT, HP6 + NP, and HP6 + NP + LT exhibited the lowest cell oxidative stress among bleached groups ($p < 0.05$). HP35, HP35 + LT, and HP35 (COM) displayed the lowest cell viability.

Conclusion HP6 achieved significantly higher color and whiteness index changes when incorporated with nanoparticles and light-irradiated and caused lower cytotoxicity than HP35 gels. The nanoparticles significantly increased cell viability and reduced the hydrogen peroxide diffusion and oxidative stress, regardless of HP concentration.

Clinical Significance Incorporation of co-doped titanium dioxide nanoparticles combined with violet irradiation within the HP6 gel could promote a higher perceivable and acceptable efficacy than HP6 alone, potentially reaching the optimal esthetic outcomes rendered by HP35. This approach also holds the promise of reducing cytotoxic damages and, consequently, tooth sensitivity.

Keywords Tooth bleaching · Hydrogen peroxide · Light irradiation · Nanoparticles · Toxicity

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Introduction

In-office tooth bleaching, a procedure commonly performed in dental practices, offers the advantages of being a low-cost and ultraconservative alternative compared to restorative treatments [1]. This procedure addresses tooth discoloration by applying high-concentration hydrogen peroxide (H_2O_2) gels (25–40%) to the buccal surface of dental enamel [2, 3]. Due to its low molecular weight, H_2O_2 can diffuse through the interprismatic spaces of the enamel into the dentin. In dentin, reactive oxygen species (ROS) generated by the decomposition of H2O2 interact with and break down staining compounds known as chromophores [1, 4].

Despite the optimal aesthetic outcomes achieved with high-concentration H_2O_2 gels [3], several studies have shown that standard in-office protocols can adversely affect enamel surface properties [5–7]. These professional protocols have also been associated with undesirable cytotoxic effects, which are dependent on both application time and concentration [8, 9]. This is due to the trans-amelodentinal diffusion of high concentrations of H_2O_2 reaching the pulp chamber. Clinically, bleaching protocols using such high concentrations of H_2O_2 are expected to increase the risk and intensity of tooth sensitivity [10, 11], and their long-term effects on the pulp structure remain unknown. A previous study indicated that reducing the concentration of H_2O_2 in the bleaching gel and shortening the application time up to three times significantly protected cell viability, although the aesthetic outcome was compromised [8].

In this critical context, recent efforts have focused on incorporating catalysts (e.g., titanium dioxide [TiO_2] and manganese oxide [12, 13]) into low-concentration experimental gels. These efforts aim to balance reduced cytotoxicity with the maintenance of highly effective aesthetic outcomes by accelerating the decomposition of H2O2 into ROS. Additionally, reports have demonstrated a significant increase in the efficacy of 6 to 15% hydrogen peroxide and 37% carbamide peroxide gels when irradiated with a new generation of violet LED light for in-office tooth bleaching [10, 14].

Recently, an in vitro study successfully incorporated TiO2 nanoparticles co-doped with nitrogen and fluorine into experimental 6% and 15% hydrogen peroxide gels used for in-office bleaching [15]. These co-doped TiO_2 nanoparticles were synthesized through robust solvothermal reactions, resulting in a stable anatase phase of TiO_2 and the generation of long-lasting reactive oxygen species (ROS) [16]. The study demonstrated that the combination of co-doped TiO_2 nanoparticles in the 6% gel, when coupled with violet LED irradiation, achieved aesthetic outcomes comparable to those of a 35% hydrogen peroxide gel [15]. Additionally, the experimental gels containing co-doped TiO_2 nanoparticles exhibited a higher pH, maintained enamel surface topography, and preserved carbonate and phosphate levels on the enamel compared to gels without nanoparticles [15]. These promising results suggest potential for clinical success, which was not achieved in a previous clinical trial that used commercially available TiO2 nanoparticles in a 6% hydrogen peroxide gel, failing to match the efficacy of a 35% gel [17].

Because the co-doped TiO_2 nanoparticles have been shown to optimize the dissociation of H_2O_2 dissociation into long-lived ROS [18], it is expected that longer interaction time between ROS and organic chromophores would lead to better esthetic outcomes and lower levels of cytotoxicity that precipitates from unreacted H_2O_2 by-products. Therefore, low-concentrated experimental gels containing these nanoparticles could allow for an improved esthetic and reduced cytotoxicity effects following in-office tooth bleaching. Based on context provided, the present study aimed to evaluate the effects of these novel gels on the bleaching efficacy, on amounts of diffused $H_2O_{2,}$ and on the viability of odontoblast-like cells. The null hypotheses were that the nanoparticle incorporation i) would not improve the efficacy of in-office bleaching gels and ii) would not increase the cytotoxicity of the proposed treatments.

Material and methods

Experimental design

Enamel-dentin disks (n = 8/group) were submitted to bleaching with 6% and 35% hydrogen peroxide experimental gels (HP). These gels were incorporated or not with TiO_2 nanoparticles (NP) co-coped with nitrogen and fluorine and irradiated or not with a violet LED light (LT). A commercial 35% HP gel (HP COM) and an experimental gel only with NP were also used. The negative control (NC) did not receive any bleaching treatment. The color change (ΔE_{00}) and whitening effect (ΔWI_D) were assessed before the bleaching procedures (T_0) and 14 days after the last session (T_4). The trans-amelodentinal hydrogen peroxide diffusion and cytotoxicity (cell viability, oxidative stress, cell morphology, and Live/dead assay) were measured at the first bleaching session (T_0).

Specimens' preparation and group distribution

Intact bovine incisors were extracted, cleaned, and stored at 4 °C for no longer than 30 days. Eighty-eight enamel-dentin disks were obtained from the middle third of the incisor's buccal surface using a diamond bur for glass (⌀8 mm, Di

Table 1 Composition of the experimental materials used in the study

Experimental Material	Composition
Carbomer-based gel	Carbomer 940 NF (Spectrum, Gardena, CA), ultrapure water, and potassium hydroxide
Hydrogen peroxide solution	Hydrogen speroxide solution at either 6% or 35% (Sigma-Aldrich, St. Louis, MI, USA)
Co-doped titanium dioxide nanoparticles	Ti $(OBu)_4$(Aldrich, 97%), C_2H_5OH (200-proof Decon Labs, King of Prussia, PA, USA), $C_{18}H_{35}NH_2$(Aldrich, 70%), $C_{18}H_{34}O_2$(Aldrich, 90%), NH_4F (based on Ti content; crystalline, ACS, Alfa Aesar), and ethanol–water solution

Martino Brocas Diamantadas Ltda, Campinas, SP, Brazil) coupled to a bench drill (Pratika FSB16P, Schultz, Joinville, SC, Brazil). The diameter of the disks was 5.6 mm and the thickness (enamel-dentin) was standardized at 2.3 ± 0.2 mm using 400-grid sandpapers (3 M Brasil, Sumaré, SP, Brazil). Then, the disks were positioned in a white and opaque tile to allow the colorimetric measurement with a hand-held spectrophotometer (Easy Shade,Vita Zahnfabrik, Bad Sackingen, Germany). The coordinate values (L*, a*, b*, h, and C) were used to randomly allocate the specimens into 11 groups. One-way ANOVA test confirmed that no significant differences were found among the groups regarding the color coordinates ($p > 0.05$). The groups were randomly assigned to each proposed bleaching protocol, as follows:

- NC: negative control – without treatment;
- NP: experimental bleaching gel only incorporated with the co-doped nanoparticles;
- HP6: experimental 6% hydrogen peroxide gel;
- HP6 + LT: experimental 6% hydrogen peroxide gel irradiated with violet LED light;
- HP6 + NP: experimental 6% hydrogen peroxide gel incorporated with NP;
- HP6 + NP + LT: experimental 6% hydrogen peroxide gel incorporated with NP and light-irradiated;
- HP35: experimental 35% hydrogen peroxide gel;
- HP35 + LT: experimental 35% hydrogen peroxide gel irradiated with violet LED light;
- HP35 + NP: experimental 35% hydrogen peroxide gel incorporated with NP;
- HP35 + NP + LT: experimental 35% hydrogen peroxide gel incorporated with NP and light-irradiated;
- HP35 (COM): commercial 35% hydrogen peroxide gel (Whiteness HP, FGM, Joinville, SC, Brazil).

Nanoparticles' synthesis

The synthesis of the co-doped titanium dioxide nanoparticles has been reported in previous publications [15, 16]. Briefly, 1.7 g of Ti $(OBu)_4$(Aldrich, 97%), 4.6 g C_2H_5OH (200-proof Decon Labs, King of Prussia, PA, USA), 6.8 g $C_{18}H_{35}NH_2$(Aldrich, 70%), 7.1 g $C_{18}H_{34}O_2$(Aldrich, 90%) and 5% of NH_4F (based on Ti content; crystalline, ACS, Alfa Aesar) were mixed with an ethanol–water solution (4%, 18-Milli-Q; total weight=13.10 g). The final solution was dispensed into a high-pressure reaction vessel (Borosilicate Glass-lined; Paar Series 4593, Bench Top Reactor System, Moline, Il, USA), reacted (180 °C, 24 h, 15 psi), and stirred (280 rpm) during 24 h. Following this cycle, the solution was transferred to a falcon tube with ethanol (200-proof, Decon Labs, King of Prussia, PA, USA) and centrifuged for 15 min at 8,000 rpm. This procedure was repeated two additional times, using 20 mL of ethanol.

Experimental gel's synthesis and incorporation of NPs

The synthesis of the experimental gel was previously described [15]. A commercially available hydrophilic polymer (Carbomer 940 NF, Spectrum, Gardena, CA) was mixed within ultrapure water using a planetary and orbital stand-alone mixer (Speed Mixer, DAC 400.1 FVZ, FlackTek Inc, Laudrum, SC, USA). The pH of the resulting gel was around 6.0. One-mL aliquots of the co-doped nanoparticles (NP, ~ 40 mg/mL) suspended in ethanol were transferred to separated falcon tubes and were centrifuged at 8,000 rpm for 5 min. The ethanol was removed from the tube and the NPs were incorporated into 20 g of the experimental gels, which were mixed at 2,450 rpm for 20 s (Speed Mixer, DAC Iso.1 FVZ, FlackTek Inc, Laudrum, SC, USA). As a result, the gels contained 5% of NP. The composition of the experimental materials used in this study is summarized in Table 1.

Bleaching procedures

The gels (with or without the incorporation of NP) were manually mixed with stock 6% or 35% HP solutions following the previously published protocol [15]. At the first bleaching session (T_1), 20 µL of the commercial or each experimental gel was applied onto the buccal enamel surface using a viscosity pipette. The gels remained in contact with the surface for 30 min without renewal. Light-irradiated groups (LT) received twenty 1-min irradiations with consecutive 30-s intervals from a violet LED light (Bright Max Whitening, MMOptics, São Carlos, SP, Brazil) [19]. The light unit was positioned 8 mm away from the buccal surface

of the specimens. The same procedures were repeated at the second (T_2) and third (T_3) sessions. The specimens were stored in 100% humidity at 37 °C [20] among the sessions and for 14 days (T_4) after the last bleaching session.

Colorimetric evaluation

At T_0 and T_4, the enamel-dentin disks were positioned in the white and opaque tile to allow the measurement of the color coordinates with the spectrophotometer. The color change (ΔE_{00}, Eq. 1) was calculated with the CIEDE2000 system formula, taking into consideration the L*, a*, b*, h, and C values collected at T_0 and T_4. The whiteness change was calculated with the whiteness index for dentistry (WI_D, Eq. 2) [21]. The ΔWI_D was calculated subtracting the WI_D values (T_4—T_0).

$$\Delta E_{00} = \sqrt{\left(\frac{\Delta L'}{K_L S_L}\right)^2 + \left(\frac{\Delta C'}{K_C S_C}\right)^2 + \left(\frac{\Delta H'}{K_H S_H}\right)^2 + RT \bullet \left(\frac{\Delta C'}{K_C S_C}\right) \bullet \left(\frac{\Delta H'}{K_H S_H}\right)} \quad (1)$$

$$WI_D = 0.55L^* - 2.32a^* - 1.1.00b^* \quad (2)$$

ΔE_{00} values adopted for perception (PT) and acceptance (AT) limits (50:50%) were 0.81 (PT) and 1.8 (AT) units, respectively. ΔWI_D adopted for PT and AT limits (50:50%) were 0.7 (PT) and 2.6 (AT) units, respectively [21].

Trans-amelodentinal diffusion

Experimental procedure

The enamel-dentin disks (n = 8/group) were inserted into artificial pulp chambers (APC). The disks were adapted with two silicon rings and the edges of the disks were sealed with utility wax (Cera 7 Rosa Wilson, Polidental, Cotia, SP, Brazil). Following the disk/APC sterilization with ethylene oxide (Acecil, Central de Esterilização Comércio e Indústria, Campinas, SP, Brazil), the sets were positioned in 24-well plates (KASVI, São José dos Pinhais, PR, Brazil) in contact with 1 mL of DMEM without FBS. The bleaching procedures were then executed, and the extracts containing the DMEM and the bleaching gel components that diffused through the disks were collected and homogenized. These extracts were split into 100-µL aliquots to run the next tests described.

Quantification of H_2O_2

An aliquot of 100 µL of the extracts was dispensed in 96-well plates with 900 µL of 2 mol/M acetate buffer (pH = 4.5). This solution (500 µL) was collected and placed in tubes with 100 µL of 0.5 mg/mL leucocrystal violet reagent (Sigma-Aldrich, St. Louis, MI, USA), 50 µL of 1 mg/mL horseradish peroxidase enzyme solution (Sigma-Aldrich) and 2750 mL of distilled water. The plates were then transferred to a spectrophotometer (λ = 596 nm, Synergy H1, Biotek Instruments, Winooski, VT, USA) for measuring the absorbance of the resulting solutions. A standard curve with known concentrations of H_2O_2 was obtained to allow the optical density values conversion into 100 µg of H_2O_2 per mL of extract [13].

Trans-amelodentinal cytotoxicity

Cell cultivation

This study used immortalized odontoblast-like MDPC-23 cells cultivated in 24- and 96-well plates (KASVI, Curitiba, PR, Brazil) with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 2 mmol/L of glutamine (Gibco), 100 IU/mL of penicillin and 100 g/ of streptomycin (Gibco).

Cell viability

For this test, 10% Alamar Blue (Life Technologies; Grand Island, NY, USA) solution was prepared in DMEM without FBS. Then, 500 µL was distributed to each well containing the extracts and the plates were incubated with 5% CO_2 at 37^0C for 4 h. The oxidized form of Alamar Blue presents a blue color, which is converted into its reduced form (a pink color) due to mitochondrial activity. Two hundred µL of each sample (extract) was transferred to a 96-well plate in order to determine the mitochondrial activity by measuring the fluorescence of the reduced salt at a spectrophotometer (excitation at 530–560 nm; emission at 590 nm, Synergy H1) [8]. These fluorescence values were converted into percentages by normalization using the mean of the negative control (NC) group.

Oxidative stress

The intracellular oxygen reactive species from the cell were measured using the carboxy-H_2DCFDA fluorescent probe (Invitrogen, San Francisco, CA, USA). The probe was dispensed at a 5 mM concentration for 30 min (37^0C) in 96-well plates containing the cultivated cells exposed to the extracts. The fluorescence was evaluated immediately after 30 min (429-mm excitation and 518-nm emission, Synergy H1), and the values were also normalized using the mean of NC [8, 13].

Live/dead evaluation

The Live/Dead Cell Viability/Cytotoxicity kit (Invitrogen) was used to qualitatively evaluate the Live/Dead expression of the cells (n = 4/group). In this method, the Ethyl1 homodimer-1 (EthD-1) fluorescence (red signal) probe binds the DNA bands solely to cells whose membranes are ruptured. The second probe (green signal), named Calcein AM (CA), is hydrolyzed with esterases present in the cytoplasm in viable cells. The bottom of the wells was analyzed using fluorescence microscopy (20x; Leica DM 5500B, Nussloch GmbH, Nussloch, Germany) [8, 13, 22].

Cell morphology

The cells were seeded on glass slides sitting at 24-well plates (n = 2/group). The cells were fixed using 2.5% glutaraldehyde (Sigma-Aldrich) and post-fixed with 1% osmium tetroxide (Sigma-Aldrich). Then, the cells were dehydrated with alcohol in decreasing concentrations (30, 50, 70, 90, and 100%) and chemically treated with HMDS (1,1,1,3,3,3-hexamethyldisilazane, Sigma-Aldrich). After being desiccated for at least 72 h, the glass slides were submitted to sputter coating with gold and analyzed under scanning electron microscopy (SEM, JEOL-JSM, 6460LV, Tokyo, Japan) at 200- and 500-times magnification [8].

Statistical analyses

The analyses were performed using the R program (R CORE TEAM, 2024), with a significance level of 5%. Initially, descriptive and exploratory analyses of the data were conducted, from which subsequent analyses were defined. Since the data did not meet the assumptions of a classic ANOVA with a general linear model, generalized linear models were adjusted to analyze the effect of treatment on ΔE_{00}, H_2O_2 diffusion, Cell Viability, and Oxidative Stress. For the variable ΔWI_D, it was not possible to apply a parametric test, so non-parametric tests of Kruskal–Wallis and Dunn were used.

Results

Bleaching efficacy

Table 2 reveals that the bleaching protocols promoted significant differences in terms of color change (ΔE_{00}) and whiteness index change (ΔWI_D) ($p < 0.001$). No differences in ΔE_{00} were detected among NC, NP, and HP6 ($p > 0.05$), while the ΔWI_D of NC and NP were negative and significantly lower than all the bleached groups ($p < 0.05$). No significant differences in ΔE_{00} were observed among groups bleached with 35% hydrogen peroxide, but HP35 + LT, HP35 + NP, and HP35 + NP + LT exhibited higher ΔWI_D than HP6. In addition, no differences were observed among HP6 + LT, HP6 + NP, and HP6 + NP + LT and the HP35 bleaching protocols. HP6 + NP + LT displayed ΔWI_D significantly higher than HP6 ($p < 0.0001$) but no different than all the groups modulated by HP35 gels ($p < 0.05$).

Table 2 ΔE_{00} and ΔWI_D considering the values collected before bleaching (T_0) and 14 days after the last bleaching session (T_4)

Group	ΔE_{00}	ΔWI_D
	Mean (standard deviation)	Median (Interquartile range)
NC	3.4 (1.3) ^{D}	-6.1 (3.1) ^{D}
NP	3.9 (1.5) ^{D}	-4.8 (8.6) ^{D}
HP6	5.9 (3.0) ^{CD}	11.1 (7.4) ^{C}
HP6 + LT	8.6 (4.7) ^{BC}	22.2 (10.5) ^{BC}
HP6 + NP	7.8 (3.3) ^{BC}	14.2 (11.1) ^{BC}
HP6 + NP + LT	10.7 (2.7) ^{AB}	24.1 (12.8) ^{AB}
HP35	9.2 (4.0) ^{ABC}	20.6 (16.6) ^{BC}
HP35 + LT	13.6 (5.7) ^{A}	34.2 (12.7) ^{A}
HP35 + NP	14.4 (6.4) ^{A}	32.7 (14.5) ^{AB}
HP35 + NP + LT	12.3 (4.4) ^{AB}	25.3 (17.6) ^{AB}
HP35(COM)	10.6 (4.3) ^{AB}	33.4 (24.2) ^{AB}
p-value	< 0.0001	< 0.0001

Different letters indicate statistical differences within the same collum, for each variable response ($p \leq 0,05$)

Quantification of H_2O_2

Table 3 displays the trans-amelodentinal diffusion of H_2O_2 (µg/mL) at the first bleaching application. HP35 and HP35 (COM) exhibited the highest H_2O_2 (µg/mL) values ($p < 0.05$) among groups. Within the groups treated with experimental HP35 gels, the quantification of H_2O_2 was ranked by irradiation of LT, presence of NP, and NP + LT combination (HP 35 > HP35 + LT > HP35NP > HP35 + NP + LT; $p < 0.05$). Bleaching with experimental HP6 gels significantly reduced the trans-amelodentinal diffusion of H_2O_2 in comparison to the HP35 gels, and the HP6 + NP + LT protocol further decreased H_2O_2 values ($p < 0.05$).

Cytotoxicity

Table 3 demonstrates that the different bleaching protocols significantly influenced the cell viability and oxidative stress ($p < 0.001$). NC and NP ($p = 0.999$) maintained the MDPC-23 cell viability at 100%. Experimental HP35, HP35 + LT, and HP35 (COM) caused the lowest percentage [12.5 (4.8) to 14.4 (2.6)] of viable cells and the highest oxidative stress [4.8 (0.5) to 5.0 (0.4)] levels ($p < 0.05$). Among the high-concentrated gels, cells in HP35 + NP + LT exhibited higher viability

Table 3 Mean and standard deviation values of the H_2O_2 diffusion (µg/mL), cell viability (%) and oxidative stress (fold increase) obtained at the first bleaching session (T_1)

Group	H_2O_2 (µg/mL)	Cell Viability (%)	Oxidative Stress
NC	–	100.0 (5.3) A	0.3 (0.1) F
NP	–	100.0 (1.7) A	0.3 (0.07) F
HP6	1.1 (0.02) E	48.6 (2.0) D	1.5 (0.4) C
HP6+LT	0.9 (0.05) F	66.5 (8.1) C	1.1 (0.3) D
HP6+NP	0.8 (0.01) G	70.6 (2.7) BC	1.0 (0.3) DE
HP6+NP+LT	0.3 (0.02) H	80.1 (2.2) B	0.8 (0.3) E
HP35	6.4 (0.67) A	13.1 (2.4) F	5.0 (0.5) A
HP35+LT	5.2 (0.80) B	14.4 (2.6) F	4.8 (0.5) A
HP35+NP	3.9 (0.58) C	23.9 (3.2) E	4.1 (0.2) AB
HP35+NP+LT	2.5 (0.01) D	43.7 (3.3) D	3.4 (0.3) B
HP35(COM)	6.7 (0.21) A	12.5 (4.8) F	5.0 (0.4) A
p-value	<0.0001	<0.0001	<0.0001

Different letters indicate statistical differences within the same collum, for each variable response ($p \leq 0,05$)

and lower oxidative stress than cells in HP35 (experimental and commercial), HP+LT, and HP35+NP gels ($p<0.05$). Among bleached groups, the highest percentage of cell viability was observed in HP6+NP+LT ($p<0.05$).

Figures 1 and 2 illustrate the cell morphology under SEM following the bleaching protocols. In NC and NP groups, a number of MDPC-23 cells with wide cytoplasm covered almost the entire glass substrate. In HP6, a small number of cells adhered to the substrate was observed in comparison with NC and NP groups. However, higher number of MDPC-23 remained on the glass substrate when NP was incorporated to the gel, which was submitted to LT. Only in HP6 groups, some of the cells were in mitosis, as indicated by an asterisk (*). In HP35 (experimental and commercial), only a few round-shaped and contracted cells remained attached to the glass in comparison to NC and NP groups. The number of cells exposed to the extracts from experimental HP35 gels seems higher in groups containing NP and irradiated with LT, exhibiting a pattern similar to the HP6 group.

Figure 3 displays the fluorescence microscopy images of the Live/Dead assay. NC and NP exhibited a higher number of viable cells stained with Calcein AM (green color) than observed in commercial and all the experimental HP35 groups. In HP35 (COM), most of cells with disrupted cytoplasm membrane were stained with EthD-1 (red). HP35+NP+LT presents slightly more live cells and a lower number of red staining than the other experimental HP35 groups. All the HP6 groups exhibited a high number of viable cells, such as observed in NC and NP.

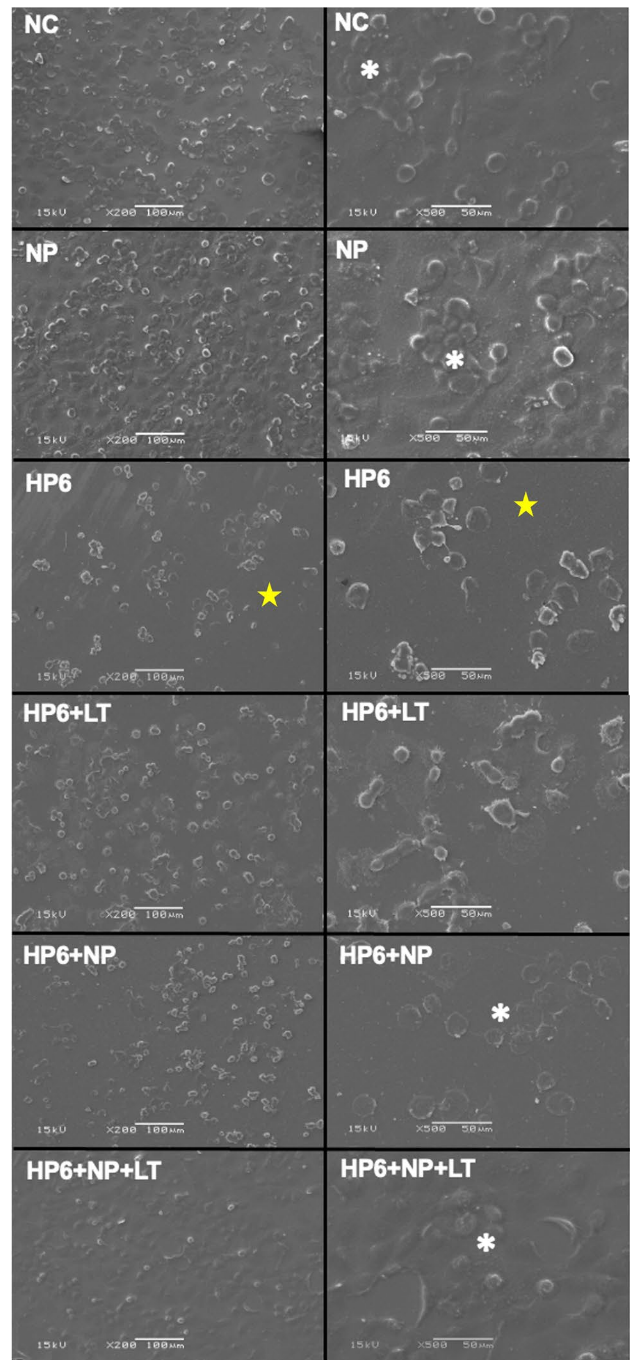

Fig. 1 Representative SEM images (200x [in the left side] and 500x [in the right side]) of glass slides on which MDPC-23 cells were cultured and then exposed to extracts collected from the APCs after the bleaching protocols. In NC and NP groups, a high number of round-shaped cells remained attached to the glass. Some of those cells were in mitosis (*). The number of cells with morphology similar to NC and NP was remarkably reduced in HP6 group. Note that the number of MDPC-23 cells in HP6+LT, HP6+NP, and HP6+NP+LT was higher than in HP6 group

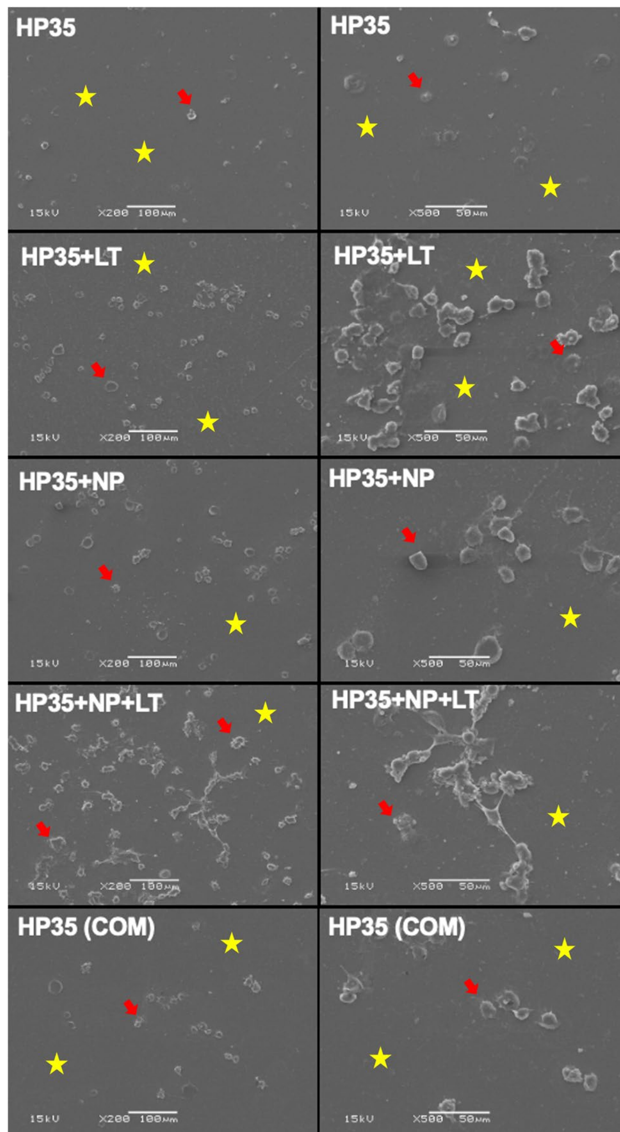

Fig. 2 Representative SEM images (200x [in the left side] and 500x [in the right side]) of glass slides on which MDPC-23 cells were cultured and then exposed to extracts collected from the APCs after the bleaching protocols. In HP35 and HP35 (COM), most of lethally damaged cells detached from the glass substrate, on which only fragments of death cells were seen. The number of cells with morphology similar to NC and NP groups was higher in HP35 + LT, HP35 + NP, and HP35 + NP + LT than in HP35 and HP35 (COM)

Discussion

The combination of violet irradiation and nanoparticles incorporation (HP6 + NP + LT) significantly increased the color and whiteness changes compared to HP6 alone, which was not achieved by groups HP6 + LT or HP6 + NP. Such increase was higher than the PT and AT values described in the literature [21, 32]. Therefore, the first null hypothesis that the NP incorporation would not increase the efficacy of the experimental gels was rejected.

Although no statistical differences were detected among experimental HP6 and HP35 groups in terms of ΔE_{00} and ΔWI_D, it is clear that HP35 reached more perceivable and acceptable mean color and median whiteness changes than HP6, contrarily to that observed between HP6 + LT, HP6 + NP + LT and HP35. According to Paravina et al. (2019) [21], regardless of analytic statistics and research data, results cannot be fully interpreted in terms of real-life relevance without comparison with perceptibility (PT) and acceptance (AT) thresholds. Therefore, it is possible to say that the HP6 + LT and, mostly, HP6 + NP + LT protocol rendered esthetic outcomes similar or even higher to those attained with the light-irradiated HP35 groups containing only one-fifth of the H_2O_2 concentration. These findings corroborate the first report of these experimental bleaching protocols, in which the incorporation of 5% of the co-doped titanium dioxide nanoparticles not only enhanced the esthetic outcomes of HP6 but also upheld the gel's pH and preserved the enamel mineral content following bleaching [15].

The metal oxide nanoparticles synthesized using solvothermal reactions in this study were previously reported to be predominantly in the anatase phase, to have a well-defined pore size distribution, to be electron deficient, and to generate substantial amounts of ROS [16], even under dark conditions [23]. This suggests that the nanoparticles could have spontaneously accelerated the decomposition of H_2O_2. However, it has been well-described in the literature that the fundamental mechanism by which TiO_2 generates ROS depends on light irradiation using appropriate wavelengths (i.e., UV-C, UV-B, or UV-A) [18]. When such an electronic requirement is reached, the electrons from the valence band (fundamental state) are promoted into the conduction band (excited state). As a result, the valence band presents an electron vacancy that is positively charged and likely to recombine with the conduction's free electrons, releasing heat or light [24]. However, if this recombination does not occur, continuous generation of positive holes and excited electrons migrate to the surface of the nanoparticles and may participate in various oxi-reduction reactions in the surface of the photocatalyst, including the generation of longer-lived ROS by follow-on reactions (i.e., hydroperoxyl -·OOH) [18].

In this direction, the TiO_2 nanoparticles used in this study were already proven to render an optical absorption spectrum in the violet light range ($\lambda = 390–420$ nm) twice as much as a commercially available TiO_2 nanoparticle (P25, Degussa), which could be explained by the TiO_2 doping with other chemical elements (nitrogen and fluorine) in the present study [25]. Therefore, the light source ($\lambda = 401.82$ nm) [19] used and the length of the irradiation protocol (30-min total) might have accelerated H_2O_2 dissociation as well as favored follow-on

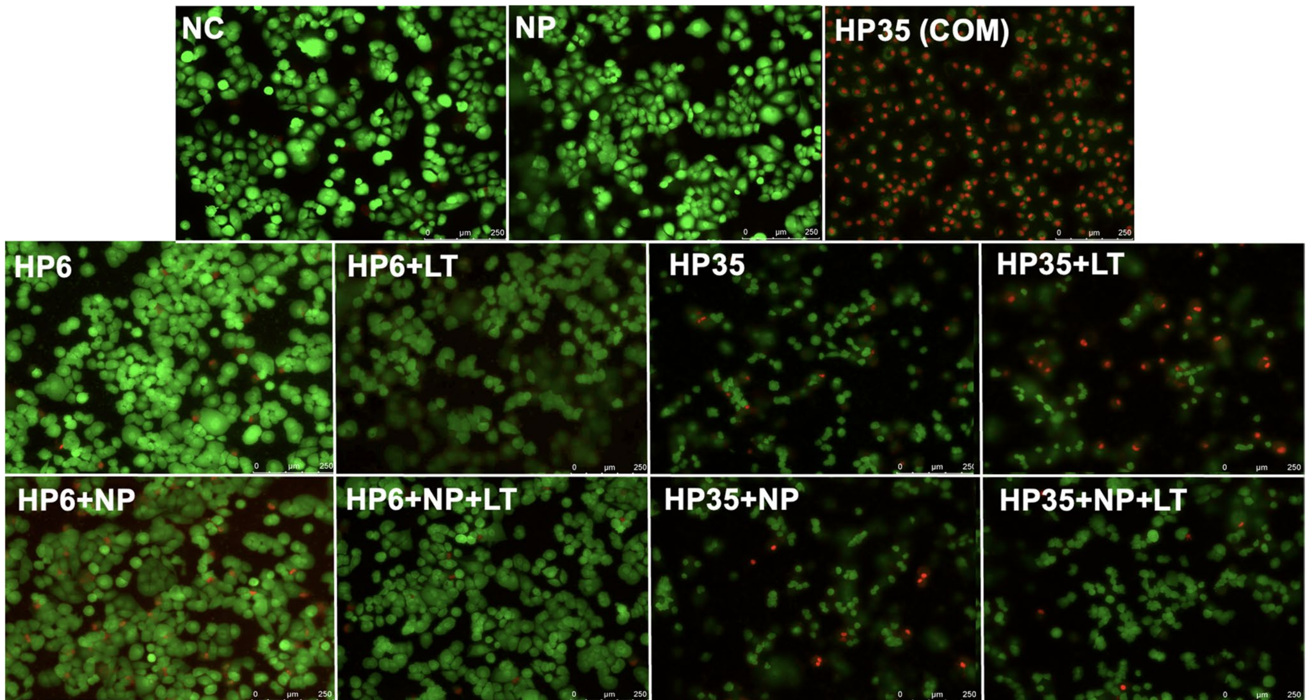

Fig. 3 Representative images of live (green) and dead (red) cells exposed to the extracts collected after the bleaching protocols. NC and NP showed that viable cells were entirely stained with Calcein AM (green). HP35 groups presented a larger number of cells with disrupted cytoplasm membrane (stained with EthD-1 probe – red color) in comparison to all other groups assessed. The incorporation of NP and irradiation with LT seem to have increased the number of live cells and slightly decreased the red staining in the HP35 + NP + LT

reactions in the bulk of the TiO_2, generating longer-lived ROS. Supposedly, these ROS would interact more intensely with the long-chained chromophores that are responsible for the discoloration of dentin[4]. Previous studies showed that the violet irradiation by itself was responsible for increasing the esthetic outcomes of low-concentrated bleaching gels [14, 26, 27], but possibly by increasing the gel's temperature and increasing the speed of the decomposition of HP into short-lived ROS. Based on findings of the present study, it could be inferred that NP and LT worked synergistically to improve the efficacy of experimental gels containing 6% of H_2O_2, which highlights the importance of combining light and nanoparticles to render relevant esthetic outcomes as optimal as possible when using a low-concentrated gel for in-office bleaching.

Regarding HP35, NP and LT combination did not act synergically. An expected higher number of free radicals in HP35 + NP + LT could have interacted with each other and not with the chromophores [28, 29]. Carlos et al. (2022) also showed that the incorporation of TiO_2 nanotubes into 35% HP gel alongside with violet LED irradiation did not enhance the efficacy of in-office tooth bleaching [30]. Likewise, some reports also pointed out that the violet LED light itself did not improve the ΔE_{00} and ΔWI_D of high-concentrated gels (35–40%) [26, 31]. Thus, it could be speculated that the high concentration of H_2O_2 in a gel is already sufficient to promote ΔE_{00} and ΔWI_D values compatible with excellent tooth bleaching efficacy [21, 32], which would refuse the necessity of approaches to improve the efficacy attained by a 35% HP gel.

On the other hand, the incorporation of NP into experimental HP35 gels decreased the trans-amelodentinal diffusion of H_2O_2 that reduced the oxidative stress and consequently the toxic effects of the bleaching protocol to pulp cells. These effects were positively affected by the violet LED irradiation of the gels. Hence, the second null hypothesis that the co-doped nanoparticles would not increase the cytotoxic effects of the experimental bleaching gels was accepted. In view of this fact, even though HP35 + NP + LT did not render better esthetic outcomes compared to both commercial and experimental HP35 groups, this approach might have indeed accelerated the decomposition of HP into ROS, thereby increasing their interaction with each other, and decreasing the number of non-reacted H_2O_2 diffusing towards the culture medium (extracts). Similarly, Martins et al. (2022) recently demonstrated that the application of a nanofiber scaffold and a heme-peroxidase enzyme-based polymeric primer on the enamel surface also protected the cell viability and minimized the oxidative stress and quantification of H_2O_2 against 35% H_2O_2 hydrogen combined with violet LED [33].

However, it is still possible to observe that all HP35-containing protocols in the present study not only dramatically reduced the cell viability (from ≅ 50% [HP35 + NP + LT] to ≅ 90% (HP35 and HP35 [COM]), but also altered the cell morphology, disrupted the cell membranes (EthD-1 red fluorescence signal) and led to the highest levels of oxidative stress. High concentrations of ROS are directly correlated with increases in the oxidative stress due to the accumulation of oxidized-damaged molecules in the cells, impairing the cellular homeostasis [34]. Moreover, ROS can react with lipidic structures from the cell membranes, leading to lipid peroxidation initiation and, consequently, membrane rupture [35]. An in vivo study showed that 35% H_2O_2 significantly increased the number of ROS in human pulp, showing a positive relation with the presence of lysosomal cathepsin B enzymes, inferring that the oxidative stress influences the protein matrix degradation and this relates to the inflammatory and death process of the pulp cells [36]. On the other hand, Duque et al. (2017) reported that lower concentration of H_2O_2 resulted in higher expression of odontoblast differentiation predictors (alkaline phosphatase activity and mineralized nodule deposition) in human pulp cells after 14 and 21 days from bleaching with 10% when compared to 35% H_2O_2 [37]. Therefore, cells exposed to lower concentration of H_2O_2 would be more prone to regulate their oxidative stress and regenerative potential over time.

Despite the similar cell viability between HP35 + NP + LT and HP6 groups, reducing the H_2O_2 concentration to as low as 6% was noticeably more effective in decreasing the diffusion of H_2O_2 and, more importantly, the oxidative stress. In fact, previous researchers revealed that cellular oxidative stress caused by H_2O_2 works in a concentration-dependent manner [22]. In the HP6-containing groups, the use of LT and NP separately was already responsible for upregulating the % of MDPC-23 cells viability and downregulating their oxidative stress. Nonetheless, HP6 + NP + LT reached the most favorable results, indicating, once more, a higher H_2O_2 decomposition and/or ROS that interacted longer with the staining molecules in dentin. The highest percentage of cell viability following this experimental protocol could be related to the significantly lower trans-amelodentinal H_2O_2 diffusion and the similar oxidative stress to the negative control ($p > 0.05$). Besides, representative SEM images revealed that the cell morphology in HP6 + NP + LT maintained the characteristics seen in the NC and NP. Previous studies stated that decreasing the application time and concentration of H_2O_2 in the bleaching gels reduces the cytotoxicity of bleaching protocols, including the preservation of the cell morphology [9, 22]. However, taking into consideration the SEM images and data from other tests performed in the present investigation, one may consider that NP incorporation and LT irradiation would provide an additional protection factor to the cells.

Recently, the incorporation of manganese oxide into 6% and 10% H_2O_2 gels further irradiated with a violet LED light exhibited a similar pattern to HP6 + NP + LT in terms of esthetic outcomes and cytotoxic effects [8, 13], which reinforces that bleaching approaches with photocatalyst-containing gels could protect the viability and minimize the negative impacts of bleaching treatments onto cells homeostasis. Even though these in vitro data should be carefully extrapolated into the clinical settings, experimental materials containing low concentrations of HP and NP were shown to render esthetic results that were comparable to those attained with 35%- containing gels while significantly reducing the adverse effects on pulp-like cell cultures in a validated artificial pulp chamber model. The results of the present study could also indicate that experimental materials and techniques investigated could possibly decrease dentin hypersensitivity and negative effects on enamel microhardness and chemical makeup.

Despite the inherent limitation of an in vitro study, the use of enamel-dentin disks coupled with the APCs is a technique well-established in the literature as an approach to mimic the trans-amelodentinal diffusion of H_2O_2 [9, 22, 37–39]. When reaching the pulp tissue, odontoblasts underlying the dentin would be the first cells exposed to highly toxic components. In this sense, the immortalized odontoblast-like MDPC-23 cell line with a similar phenotype to human odontoblast was used [40]. However, it is important to bear in mind that the vital human pulp holds other organic structures, and it receives exudation pressure from the dentinal fluid, which is likely to influence the internal diffusion of H_2O_2. Therefore, future in vitro and in vivo studies are paramount to confirm these data and to further investigate the clinical efficacy and adverse effects of co-doped TiO_2 nanoparticles incorporation into bleaching gels.

Conclusions

Within the limitation of this study, the following conclusions could be drawn:

- HP6 gel incorporated with nanoparticles and light-irradiated achieved significantly higher bleaching efficacy than HP6 alone, without significant differences to HP35 groups;
- Irradiation of HP35 gel incorporated with NPs did not significantly increase the colorimetric changes for high-concentrated bleaching gels;
- Independently of the peroxide concentration considered, the incorporation of the nanoparticles into the experimental bleaching gels significantly increased the cell viability, reduced the oxidative stress, and hydrogen peroxide diffusion, and this scenario was positively influenced by light irradiation.

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Authors contributions M.K. conducted the methodology, formal analysis an wrote the initial draft. R.A.O.R. and C.A.S.C. conducted the methodology, provided resources, and edited the manuscript draft. F.L.E.F. conceptualized and edited the manuscript draft. V.C. conceptualized, supervised, provided resources, and edited the manuscript draft.

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

Declarations

Ethical approval and informed consent statements Not applicable.

Conflict of interest The authors declare no competing interests.

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