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Influence of Manganese Oxide on the Esthetic Efficacy and Toxicity Caused by Conventional In-office Tooth Bleaching Therapy

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Clinical Relevance

The improved efficiency and reduced toxicity observed after adding manganese oxide to a bleaching gel with 35% H_2O_2, commonly used for professional therapy, may prevent pulp damage and post-bleaching (in-office) dental sensitivity.

SUMMARY

Objective: This study aimed to evaluate the esthetic efficacy, cytotoxicity, and kinetics of decomposition of hydrogen peroxide (H_2O_2) present in a commercial bleaching gel with 35% H_2O_2 (BG35%) chemically activated with manganese oxide (MnO_2).

Methods and Materials: After incorporating 2 mg/mL, 6 mg/mL, and 10 mg/mL of MnO_2 into BG35%, the stability of pH and temperature of

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the products were analyzed. To assess the esthetic efficacy (ΔE and ΔWI), the BG35%s with MnO_2 were applied for 45 minutes on enamel/dentin discs (DiE/D). BG35% or no treatment were used as positive (PC) and negative (NC) controls, respectively. To analyze the cell viability (CV) and oxidative stress (OXS), the same bleaching protocols were performed on DiE/D adapted to artificial pulp chambers. The extracts (culture medium + gel components that diffused through the discs) were applied to pulp cells and submitted to H_2O_2 quantification. BG35% with MnO_2 that showed the best results was evaluated relative to kinetic decomposition of H_2O_2, with consequent generation of free radicals (FR) and hydroxyl radicals ($OH^•$). The data were submitted to the one-way analysis of variance complemented by Tukey post-test (α=0.05). Data on kinetics of H_2O_2 decomposition were submitted to the Student's-*t* test (α=0.05).

Results: All the BG35%s with MnO_2 showed stability of pH and temperature, and the gel with 10 mg/mL of this activator had an esthetic efficacy 31% higher than that of the PC (p<0.05). Reduction in OXS and trans-amelodentinal diffusion of H_2O_2 occurred when all the BG35%s with MnO_2 were used. The addition of 6 and 10 mg/mL of MnO_2 to BG35% increased the CV in comparison with PC, confirmed by the cell morphology analysis. An increase in FR and $OH^•$ formation was observed when 10 mg/mL of MnO_2 was added to BG35%.

Conclusion: Catalysis of BG35% with MnO_2 minimized the trans-amelodentinal diffusion of H_2O_2 and toxicity of the product to pulp cells. BG35% containing 10 mg/mL of MnO_2 potentiated the decomposition of H_2O_2, enhancing the generation of FR and $OH^•$, as well as the efficacy of the in-office tooth therapy.

INTRODUCTION

The perception of tooth color alteration following dental bleaching therapy is believed to be caused by the interaction of hydrogen peroxide (H_2O_2) and its degradation products with the chromophores present in enamel and dentin.[1-4] However, studies have shown that a large quantity of residual H_2O_2 that does not interact with the chromophores, termed as free-H_2O_2, remains in dentin[5-6], where it can rapidly diffuse to reach the pulp chamber.[7,9-11] Once in contact with pulp cells, the H_2O_2 causes cell injury and pulp necrosis associated with tissue inflammation,[7,8] which has frequently been correlated with post-bleaching (in-office) tooth sensitivity.[6-8,12] Therefore, new dental bleaching protocols have been evaluated with the purpose of preventing these negative side effects caused by this professional esthetic therapy.[13-14]

With the aim of improving the efficacy and simultaneously minimizing the biological damage caused by in-office tooth bleaching, some studies have been developed about incorporating catalysts into gels with high concentrations of H_2O_2.[15-18] The purpose of this alternative is to accelerate the process of H_2O_2 decomposition into free radicals with an extremely short half-life (HLT). This strategy leads such molecules to be rapidly eliminated from the dental tissues after reacting with the chromophores.[1-2,18] Researchers have shown that hydroxyl ($OH^•$, HLT=10^{-9} sec), singlet oxygen (O^{-2}, HLT=10^{-5} sec), and peri-hydroxyl ($HO^{2\cdot}$, HLT=7 sec) are the main free radicals derived from the dissociation of H_2O_2.[19-20] Duque and others[18] demonstrated that the incorporation of catalysts into a bleaching gel with 35% H_2O_2 reduces the toxicity of the product by over 60%. Taking into account these data, some enzymes and salts of transition metals, such as Fe, Cu, Cr, and Mn, may be used as catalysts to promote the dissociation of H_2O_2 into free radicals.[15-18] Therefore, the generation of more reactive molecules with reduced half-life appears to be fundamental for the development of innovative bleaching gels, which can improve the efficacy as well as the compatibility of the in-office bleaching therapy with pulp tissue.

Chemical catalyzers derived from manganese (Mn) have been extensively studied[19-23] because, in addition to their effective catalyzer potential, Mn is abundantly available on the planet,[24] making it a low-cost product.[25-30] Furthermore, the ionic compounds arising from Mn act as co-factors of innumerable enzymes that participate in important biological processes related to the production of energy.[26] Another aspect to be considered is the ample variety of possible groupings to be formed with Mn acting as the central chemical element. This makes it possible to obtain different catalyzers for the formulation of chemical activators.[24,27] It is also imperative to emphasize that Mn-based oxides are capable of catalyzing organic substances since their oxidized form has greater reduction potential than their reduced form.[31-32] Another interesting benefit of chemical catalyzation via metallic oxides, when compared with enzymatic catalyzation, lies in the fact that enzymatic activity, in addition to being high cost, is completely influenced by the pH and temperature of the medium.[33]

The main objective of the present study was to assess the esthetic efficacy, cytotoxicity, and kinetics of

the decomposition of H_2O_2 present in a commercial bleaching gel with 35% H_2O_2, which was chemically activated with manganese oxide (MnO_2). The null hypothesis of this study was that the incorporation of MnO_2 into a highly concentrated bleaching gel does not influence the esthetic efficacy, cytocompatibility, and decomposition of the H_2O_2 present in the dental product.

METHODS AND MATERIALS

SAMPLE PREPARATION

Eighty enamel/dentin discs (DiE/D) were obtained from the buccal surface of bovine incisors by using a diamond trephine bur (Dinser Broca Diamantadas LTDA, São Paulo, SP, Brazil) coupled to a bench drill (FSB 16 Pratika, Schultz, Joinville, SC, Brazil). The disc diameters were standardized to measure 5.6 mm, and the total thickness (enamel/dentin) was established at 2.3 ± 0.2 mm, by wear performed on dentin with water abrasive papers grit 400 and 600 (T469-SF- Norton, Saint-Gobam Abrasivos Ltda, Jundiaí, SP, Brazil), as described by de Oliveira Duque and others.[14]

After regularizing the dentin surface, prophylaxis of the enamel surface was performed with pumice stone and water to eliminate superficial pigments. The dentin was treated with EDTA (ethylenediaminetetraacetic acid; Sigma-Aldrich Corp, St Louis, MO, USA) 0.5 N for 30 seconds to remove the smear layer.[14] Afterward, the discs were placed in a white silicone matrix, in a standardized manner, so that only the enamel surface remained exposed. The spectrophotometer (Color Guide 45/0; BYK-Gardner GmbH, Geretsried, BAV, Germany) was positioned over each specimen with the support provided by the appliance. Then, three initial readouts were taken to obtain a mean of the color values of each sample, determined by the coordinates L*a*b* (CIE L*a*b* system). The values of the coordinates L*a*b* (La Commission Internationale de l'Eclairage [CIE]) were measured with a wavelength ranging from 400 nm to 700 nm, standard illuminant D65, and angle of illumination/observation 45/0°.[14]

The DiE/D discs were submitted to an intrinsic staining protocol after initial color readout, by means of infusion in 2 mL of black tea (Matte Leão, Curitiba, PR, Brazil) for 24 hours.[2-14] After staining, the enamel surface was submitted to prophylaxis and the discs underwent another color readout to determine the mean values of the variables L* (48.61±5.82) and b* (7.07±5.67), because this guide is the standard of tooth staining. Subsequently, the discs with similar mean values of L* and b* were distributed into to the following groups: [NC] - without treatment (Negative Control); [PC] - gel with 35% H_2O_2 (Whiteness HP - WHP, FGM, Joinville, SC, Brazil; Positive Control); [WHP+ 2(MnO_2)] - gel with 35% H_2O_2 containing 2 mg/mL MnO_2; [WHP+6(MnO_2)] - gel with 35% H_2O_2 containing 6 mg/mL MnO_2; and [WHP+10(MnO_2)] - gel with 35% H_2O_2 containing 10 mg/mL MnO_2 (Table 1).

Bleaching Procedure

The chemical activator manganese oxide (MnO_2, Santa Cruz Biotechnology, Texas, USA) was incorporated into the thickener of the commercial bleaching gel (WHP) so that the concentration of the chemical activator would remain at 2 mg/mL, 6 mg/mL, and 10 mg/mL. Then, three drops of the liquid phase containing 35% H_2O_2 (thickener/H_2O_2 proportion in accordance with the manufacturer's indication) were added to the thickener

Table 1: *Experimental Groups and Controls Established According to the Incorporation, or Not, of Different Concentrations of MnO_2 into Bleaching Gel with 35% H_2O_2*

Groups	Treatments	MnO_2 Concentrations	Protocol of Application
NC	Without treatment	—	—
PC	Bleaching Gel (WHP) with 35% H_2O_2	—	Three 15-min applications
WHP+2(MnO_2)	Bleaching Gel (WHP) with 35% H_2O_2+ MnO_2	2 mg/mL	Three 15-min applications
WHP+6(MnO_2)	Bleaching Gel (WHP) with 35% H_2O_2+ MnO_2	6 mg/mL	Three 15-min applications
WHP+10(MnO_2)	Bleaching Gel (WHP) with 35% H_2O_2+ MnO_2	10 mg/mL	Three 15-min applications

Abbreviations: NC, negative control; PC, positive control; WHP, whiteness HP (commercial bleaching gel). "—" signifies that no concentrations of MnO_2 were used. In the negative control group (NC), no protocol of bleaching gel application was used

whether or not it contained MnO_2. After this, 20 μL of the bleaching gels and controls were applied to the enamel of the discs for 15 minutes. This procedure was repeated another two times until a total of 45 minutes of bleaching treatment was concluded. After each application of the bleaching gel, the enamel surface of the discs was rinsed with sterilized water or PBS for bleaching efficacy or cytotoxicity analysis, respectively.

Immediately after obtaining the experimental bleaching gels containing 2 mg/mL, 6 mg/mL, and 10 mg/mL of MnO_2, the homogeneity of the products, and possible formation of bubbles or colored residues during the reaction, were visually analyzed. After this, the variation in pH and temperature was verified by means of a bench microelectrode (HI-2221 Calibration Check pH/Bench Meter; Hanna Instruments Ltd, Leighton Buzzard, AD, United Kingdom). These measurements were taken every 15 minutes in all the bleaching gels, whether or not they contained MnO_2.

Evaluation of Bleaching Efficacy (ΔE and ΔWI)

Forty DiE/Ds (n=8), distributed among the study groups, were stored in an environment with 100% humidity, at 37°C, for 72 hours.[14] Immediately after the bleaching procedures, the DiE/D were again stored in an environment with 100% humidity, at 37°C, for an additional 72 hours. Thus, readouts were performed again to obtain the color change data. Complete color change was obtained by means of the formula $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$. The whiteness index for Dentistry was calculated using the L*, a*, b* parameters of transmittance analysis, according to the equation: $\Delta WI = 0.511L - 2.3424a - 1.100b$,[34] in which higher WI values indicate whiter samples and lower WI values indicate darker samples. According to this index, the whiter the material, the higher and more constant the reflectance across the visible wavelength range.[34] The baseline and final (after bleaching) whiteness index was calculated according to: $\Delta\Delta WI = \Delta WI_{\text{after bleaching}} - \Delta WI_{\text{baseline}}$.

Cytotoxicity Evaluation

Cell Culture—Immortalized odontoblast-like MDPC-23 cells, stored in the Experimental Pathology and Biomaterials Laboratory of the Araraquara Dental School - UNESP, SP, Brazil, were used in the study. These cells were seeded in wells of 96- and 24-well plates (KASVI Imp, Curitiba, PR, Brazil) with Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Grand Island, NY, USA) culture medium, containing 10% Bovine Fetal Serum (BFS; GIBCO, Grand Island, NY, USA), 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 2 mmol/L of glutamine (GIBCO, Grand Island, NY, USA), in a humidified atmosphere, at 37°C, with 5% of CO_2 and 95% of air.[2,3-14]

Experimental Procedure—Forty DiE/Ds were adapted to artificial pulp chambers (APCs), using two silicone rings for this purpose. After sealing the edges of the discs with baseplate wax (pink) (7 Wax Pink Wilson, Polidental, Cotia, SP, Brazil), the disc/APC sets were sterilized in ethylene oxide (Acecil, Central de Esterilização Comércio e Indústria LTDA, Campinas, SP, Brazil).[2,13-14] Then, the DiE/D/APC sets were positioned in wells of 24-well plates (KASVI Imp) containing 1 mL of DMEM without BFS so that the solution remained in contact with the dentin and exposed enamel, either to receive the treatments, or not (Table 1).[35] Immediately after concluding the bleaching procedures, the extracts (DMEM + gel components that diffused through the DiE/D) were collected, homogenized, and divided into aliquots of 100 μL for the purpose of performing the tests described below.

Cell Viability (MTT Assay)—One aliquot of 100 μL of the extracts was applied to the MDPC-23 cells (n=8) for 1 hour, which had previously been seeded in wells of 96-well plates. Then, the extracts were aspirated and the cells were incubated for 4 hours in contact with 90 μL of DMEM + 10 μL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich Corp, St Louis, MO, USA) in the concentration of 5 mg/mL of PBS, in the ratio of 10:1. After this period, the formazan crystals were dissolved in 100 μL of an acidified isopropyl alcohol (0.04 N HCl) solution, and the absorbance was measured at 570 nm (Synergy H1, Biotek). The mean absorbance value obtained in the NC Group was considered 100% cell viability, and this parameter was used to calculate the cell viability of the other groups.[2,13-14]

Intracellular Reactive Oxygen Species (Oxidative Stress - H_2DCFDA Probe)—The cells seeded in wells of 96-well plates (KASVI Inp) were pretreated with the fluorescent probe carboxy-H_2DCFDA (Invitrogen, San Francisco, CA, USA) in the concentration of 5 μM at 37°C for 30 minutes, and were then exposed to 100 μL of the extracts (n=8). Immediately afterward, fluorescence was evaluated at 492 nm wavelength excitation and 517 nm wavelength emission (Synergy H1, Biotek), and the values were normalized by the mean values of Group NC.[1,2,35]

Analysis of Cell Morphology by Scanning Electron Microscopy (SEM)—For this analysis, the MDPC-23 cells were seeded on glass slide measuring 13 mm in diameter (KASVI Imp), positioned on the bottom of wells of 24-well plates. After being exposed to 100 μL of the extracts (n=4), the cells were fixed in 2.5% glutaraldehyde (Vetec Química Fina LTDA, Duque de Caxias, RJ,

Brazil) for 24 hours and post-fixed in 1% osmium tetroxide (Sigma-Aldrich). After this, dehydration was performed with ascending changes of ethanol solutions (30%, 50%, 70%, 95%, and 100%) and chemical drying with HMDS (1,1,1,3,3,3-hexamethyldisilazane; Sigma-Aldrich). Then, the samples were positioned on metal stubs, stored in a glass desiccator (Labor Quimi, Poá, SP, Brazil) for seven days, at ambient temperature, and sputtered with gold.[1,2,13-15] Finally, the samples were evaluated by SEM (5kv; JEOL JSM 6610, JEOL Ltd, Akishima, Tokyo, Japan) when photomicrographs were obtained at 1000× and 4000× magnification.

H_2O_2 Quantification

To determine the quantity of H_2O_2 that diffused through the enamel/dentin discs, an aliquot of 100 μL of the extracts (n=8) was placed in wells of 24-well plates containing 900 μL of an acetate buffer solution (2 mol/M, pH 4.5). After this, a volume of 500 μL of this solution was transferred to tubes containing 100 μL of Leuco Crystal Violet (LCV) coloring reagent (0.5 mg/mL; Sigma-Aldrich), 50 μL of a horseradish peroxidase (HRP) enzyme solution (1 mg/ml; Sigma-Aldrich), and 2.750 mL distilled water. The absorbance of the solutions was measured in a spectrophotometer at a wavelength of 596 nm (Synergy H1, Biotek). The optical density values were converted into μg H_2O_2 per mL of extract (standard curve), and these values were transformed into percentages, considering the positive control group (35% 3×15) as being 100% of residual H_2O_2 diffusion.[1,2,14,35]

Kinetics of the Decomposition of H_2O_2

For this analysis, the concentration of the chemical activator MnO_2 with the best esthetic and biological performance was selected. The aim of this stage was to evaluate whether the presence of the catalyzer induced the formation of free radical and $OH^{\bullet}$ radicals in the bleaching gel (n=3) during the time interval of 15 minutes. To determine the production of free radicals, an aliquot of the selected bleaching gel was diluted in buffer solution, in the ratio of 1:10 in each time interval of analysis. After this, 50 μL of this sample was transferred to wells of 96-well plates. To proceed with quantification, the solutions were incubated at 37°C with the carboxy-H_2DCFDA probe (Invitrogen, Eugene, OR, USA) in the concentration of 1 mM (1:1), and the emission of fluorescence was evaluated with excitation of 492 nm and emission of 527 nm (Synergy H1, Biotek) for 15 minutes. To quantify the $OH^{\bullet}$ released by the selected bleaching gel, the fluorescence probe of the OxiSelect Hydroxyl Radical Antioxidant Capacity (HORAC) Activity Assay (Cell Biolabs Inc, San Diego, CA, USA) kit was used, of which the principle is based on oxidation of the probe by $OH^{\bullet}$ via transference of an atom of oxygen. Thus, the fluorescence of the reaction is reduced in the presence of $OH^{\bullet}$. For this analysis, 20 μL of the samples were incubated with 140 μL of the probe, and the fluorescence was monitored at 480 nm excitation and 530 nm emission (Synergy H1, Biotek) throughout the period of 45 minutes of incubation at ambient temperature.[1,2]

Statistical Analysis

The quantitative data were evaluated relative to the normal adherence curve and all showed normality (Shapiro-Wilk, $p>0.05$) and homogeneity of variance (Levene, $p>0.05$). Therefore, these data were applied to the one-way analysis of variance tests that were complemented with the Tukey test for color and cytotoxicity assays. For the analysis of the kinetics of the decomposition of H_2O_2, the Student's-*t* test was applied. All statistical analyses inferences were based on the level of significance of 5%. The statistical power of the analyses was calculated by means of DDS Research (Statistical Power Calculator, α=5%). Visual analyses were performed for the qualitative data.

RESULTS

Analysis of pH and Temperature of MnO_2-Containing Bleaching Gels

Visual analysis of the bleaching gels allowed for the determination of the fact that there was no formation or precipitation of residues after the incorporation of MnO_2. The possible changes in pH and temperature were evaluated in the time intervals of 0.5, 5, 10, and 15 minutes after addition of the chemical activator to the commercial bleaching gel (WHP). The pH of the gels activated with MnO_2 were maintained close to neutral or slightly alkaline (Figure 1A). The temperature also remained stable during all periods, and did not exceed 30°C (Figure 1B).

Bleaching Efficacy (ΔE and ΔWI)

All the bleached groups showed a significant increase in the values of ΔE when compared with NC Group. Significant increases of 15% and 31% in esthetic efficacy occurred after addition of 6 mg/mL and 10 mg/mL of the catalyzer MnO_2, respectively, to the gel, in comparison with PC Group. The bleaching gel with 10 mg/mL of MnO_2 showed the highest ΔE values when compared with the other groups (Figure 2A). All the bleached groups showed a significant increase in the values of ΔWI when compared with the NC Group. No

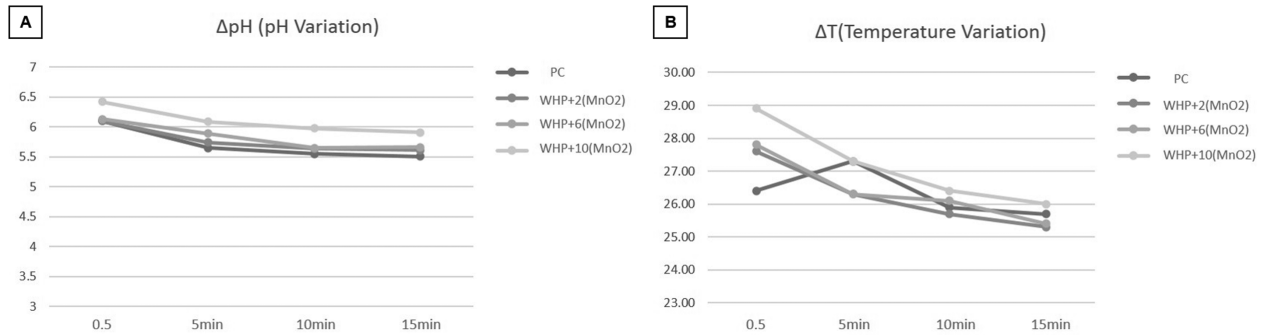

Figure 1. *(A): Analysis of variation in pH. Comparative graph of variation in pH (ΔpH) between WHP and bleaching gels catalyzed with MnO_2, represented by Groups PC, WHP+2(MnO_2), WHP+6(MnO_2), and WHP+10(MnO_2). The analyses were performed in time intervals of 0.5, 5, 10, and 15 minutes. (B): Analysis of variation in temperature. Comparative graph of variation in temperature (ΔT) between WHP and bleaching gels catalyzed with MnO_2, represented by Groups PC, WHP+2(MnO_2), WHP+6(MnO_2), and WHP+10(MnO_2). The analyses were performed in time intervals of 0.5, 5, 10, and 15 minutes.*

differences were found in the whiteness index among groups after the addition of 2 mg/mL and 6 mg/mL when compared with PC Group. The addition of 10 mg/mL showed the highest ΔWI values in comparison with the other groups (Figure 2B).

Cytotoxicity

The NC Group was considered to have a cell viability of 100%. Reduction in cell viability and increase in oxidative stress occurred in all the bleached groups ($p<0.05$). Groups WHP+6(MnO_2) and WHP+10(MnO_2) showed 28% and 45% higher cell viability, respectively, in comparison with PC Group ($p<0.05$; Figure 3A). All the groups in which bleaching was performed with gel containing MnO_2 showed significant reduction in oxidative stress when compared with PC Group (Figure 3B), with outstanding results for Group WHP+10(MnO_2). Changes in number and morphology of cells adhered to the substrate were observed by SEM in PC Group in comparison with NC Group. However, a less deleterious effect on cells occurred in the groups in which 6 mg/mL and 10 mg/mL of MnO_2 were added to the bleaching gel, and the cells of Group WHP+10(MnO_2) exhibited a morphological pattern similar to that of the NC Group (Figure 4).

Quantification of Residual H_2O_2

Significant reduction in the diffusion of residual H_2O_2 occurred in all the groups in which bleaching was performed with gel containing MnO_2, in comparison with the PC Group (Figure 3C). Group WHP+10(MnO_2) had a lower quantity of residual H_2O_2

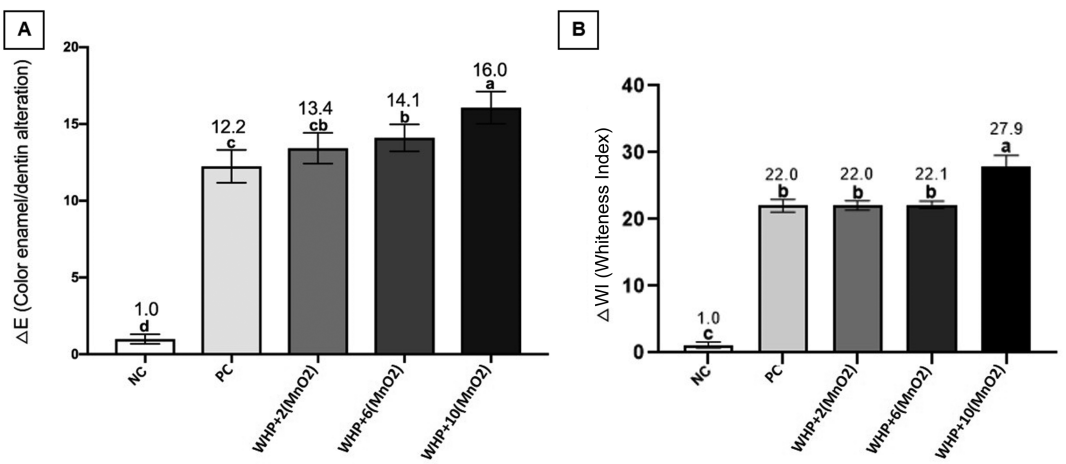

Figure 2. *(A): Analysis of bleaching efficacy (ΔE) of bleaching gels. Bar graph of mean values and standard deviation determined by calculation of total ΔE of bleached groups. (B): Analysis of bleaching efficacy (ΔWI) of bleaching gels. Bar graph of mean values and standard deviation determined by calculation of total ΔE of bleached groups. Different letters demonstrate statistically significant difference among groups (One-way ANOVA; Tukey Test, $\alpha=0.05$ / n=8)*

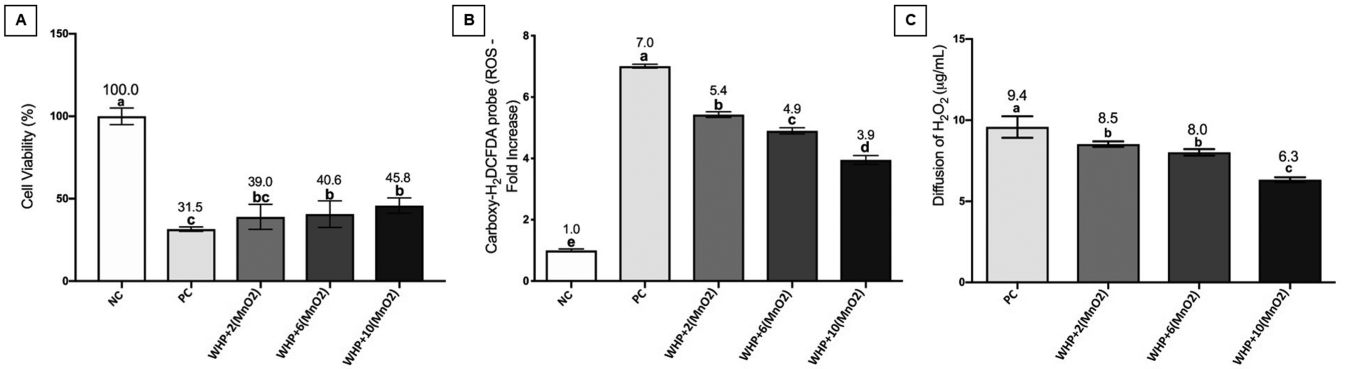

Figure 3. *(A): Analysis of cell viability performed by MTT assay. Bar graph showing mean values and standard deviation of cell viability. (B): Analysis of cell oxidative stress measured by the OXS values present in the culture medium. Bar graph of mean values and standard deviation of emission of fluorescence normalized by control of carboxy-H_2DCFDA probe. (C): Analysis of residual H_2O_2: bar graph of mean values and standard deviation of H_2O_2 concentration in the extracts. Different letters demonstrated statistically significant difference among groups. (One-way ANOVA; Tukey Test, α=0.05 / n=8)*

in the culture medium when compared with the other bleached groups.

Kinetics of the Decomposition of H_2O_2

Because of having shown the best results of esthetic efficacy and reduction in cytotoxicity, only the bleaching gel containing 10 mg/mL of the catalyzer MnO_2 was evaluated. A significant increase in the production of free radicals (Figure 5A) and hydroxyl radicals (Figure 5B) was observed in this Group WHP+10(MnO_2) in comparison with the PC Group, and the formation of free radicals reached its peak in the time interval of 15 minutes.

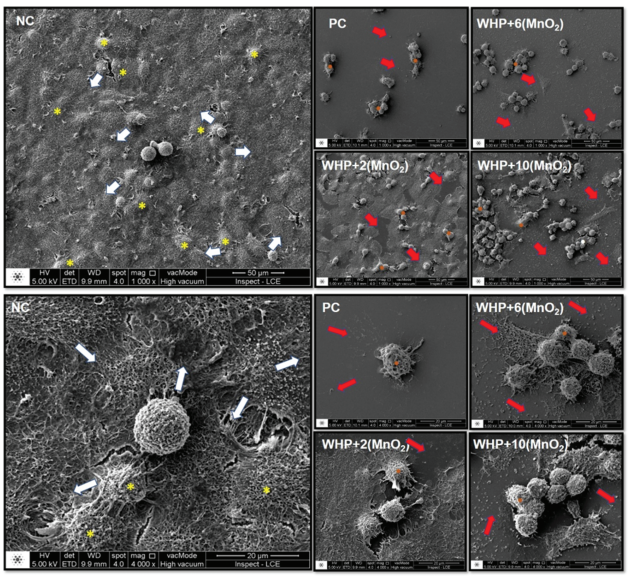

Figure 4. *Representative images obtained by scanning electron microscopy (SEM) for each study group. It was possible to observe a significant reduction in the quantity of cells adhered to the glass slide in the NC Group, which had a well-defined polygonal morphology (*) with cytoplasmic projections (white arrows), whereas in the bleached groups it was possible to observe the bottom of the glass slide onto which the cells exposed to the extract detached themselves (red arrows). Furthermore, it was possible to observe morphological change in the cell surface after bleaching (*). Magnifications of 1000× and 4000× (n=4).*

DISCUSSION

The chemical decomposition of H_2O_2 in an aqueous medium, which may be potentiated by different factors—such as those described by Choudhary and others[36]—appears to be an interesting alternative for making in-office tooth bleaching more compatible with the dentin-pulp complex, without harming the esthetic outcome of the treatment.[1-2,18] Suty and others[19] reported that catalyzers could accelerate the decomposition of H_2O_2, which resulted in the formation of intermediate chemical species. The dissociation of this reactive oxygen species (ROS), which is the main active component of bleaching gels, into other highly reactive molecules with a short half-life has been considered crucial for reducing the toxicity of this modality of esthetic therapy, widely used in dental offices.[2] Therefore, with the aim of increasing the reactivity of bleaching gel with the dental structures and minimizing the trans-amelodentinal diffusion of the toxic components released by the product, in the present study the proposal was to incorporate the chemical activator manganese oxide (MnO_2) into a commercial bleaching gel containing 35% H_2O_2.

Initially, 2 mg/mL, 6 mg/mL, and 10 mg/mL of MnO_2 were incorporated into the thickener, and the new products obtained remained viscous, with a homogeneous pattern, without forming colored residues. These bleaching gels containing MnO_2 showed stability of pH (close to neutral) and temperature during the different time intervals of

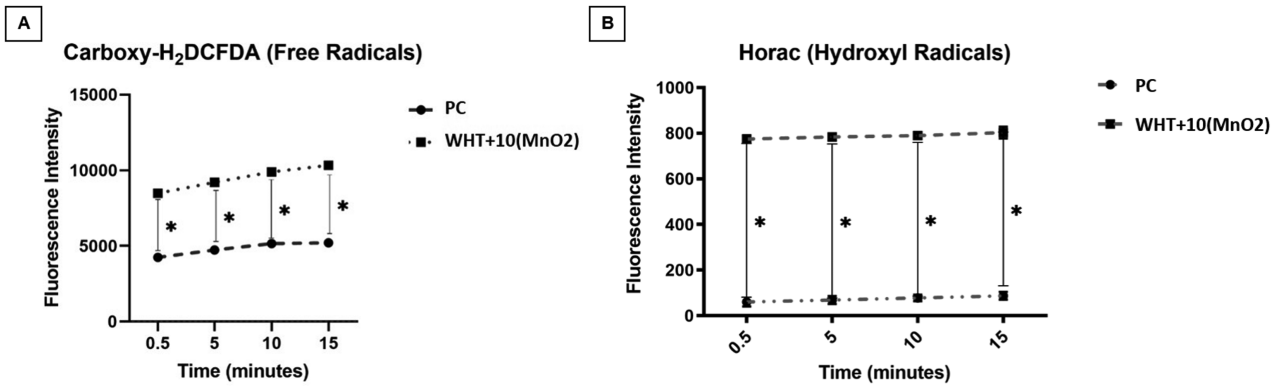

Figure 5. *(A): Analysis of the kinetics of decomposition of H_2O_2 — formation of free radicals (carboxy-H_2DCFDA probe). The values corresponded to the emission of fluorescence in the two groups evaluated and tested in the time interval of 15 minutes (0.5, 5, 10, and 15 minutes). (B): Analysis of the kinetics of decomposition of H_2O_2 — formation of hydroxyl radicals (HORAC probe). The values corresponded to the emission of fluorescence in the two groups evaluated and tested in the time interval of 15 minutes (0.5, 5, 10, and 15 minutes). The symbol * indicated statistical difference between the groups in the formation of hydroxyl radicals within each time interval in the analysis (Student's-t Test; n=3).*

evaluation. In a recent study, Guan and others[37] used amperometry to demonstrate that the catalytic current of chemical activation performed by MnO_2 attains the ideal point at a pH within a range of values between 5.0 and 7.0, at the time when the chemical interaction of this activator with H_2O_2 reaches its maximum point. In addition, bleaching agents with neutral pH do not cause changes in the enamel surface that may favor the inward diffusion of H_2O_2.[38] Therefore, in the present research the stability pH of the bleaching gels containing MnO_2 (close to 7.0) certainly favored the kinetics of decomposition of H_2O_2, potentiating the generation of free radicals (Figure 5A) and hydroxyl radicals (Figure 5B), which was clearly observed in Group WHP + 10 (MnO_2).

This positive behavioral profile of the bleaching gels containing MnO_2 enabled continuity of the subsequent analyses, which consisted of determining the esthetic efficacy and cytotoxic potential of these new products. With regard to the esthetic efficacy, a significant increase in the total ΔE of the DiE/D was observed with the use of the bleaching gel containing 6 mg/mL of MnO_2. Nevertheless, the highest ΔE values were obtained in Group WHP+10(MnO_2), in which the total change in color was 31% higher in comparison with PC Group, which in this study, represented the conventional in-office tooth bleaching protocol. This result could be explained, even partially, by the excellent catalytic activity that transition metals, such as MnO_2, have when they come into contact with substances with oxidative potential (H_2O_2), and this activity is further enhanced when the medium contains a high concentration of H_2O_2.[36] Therefore, one may suggest that the greater availability of MnO_2 in the bleaching gel containing 10 mg/mL of this chemical activator may have accelerated the decomposition of H_2O_2. One of the possible interpretations for the mechanism of this reaction consists of the process of reduction of MnO_2 that occurs in a medium rich in H_2O_2:[20]

$$MnO_2 + H_2O_2 \rightarrow MnO \text{ (or } Mn_2O_3) + O_2 + H_2O$$

$$MnO \text{ (or } Mn_2O_3) \rightarrow MnO_2 + 2e^-$$

Concerning the trans-amelodentinal cytotoxicity of the products evaluated in this study, reduction in intracellular oxidative stress values of approximately 23%, 30%, and 44% occurred when 2 mg/mL, 6 mg/mL, and 10 mg/mL of MnO_2, respectively, were incorporated into the bleaching gel. This datum is directly related to the significant reduction in toxic residual H_2O_2 present in the culture medium, as was demonstrated by means of the leucocrystal violet test. Values of 28% and 45% higher cell viability in comparison with the PC Group were observed in Groups WHP+6(MnO_2) and WHP+10(MnO_2), respectively. These data were corroborated by the qualitative analysis of MDPC-23 cells by SEM, in which it was observed that the higher the concentration of MnO_2 incorporated into the gel, the larger the number of cells that remained adhered to the substrate. Furthermore, the cells of Group WHP+10(MnO_2) were shown to have a morphology similar to that of the cells of the NC Group, which showed evidence of the reduction in the toxic potential of the bleaching gel containing MnO_2. Recently, Ortecho-Zuta and others[2] demonstrated that a bleaching gel with 35% H_2O_2 activated with horseradish

peroxidase (HRP) enzyme resulted in cell viability that was almost two times higher than that observed for the same gel without the addition of the enzyme. However, the authors reported that in spite of the limited trans-amelodentinal toxic effect of the gel with HRP, the cells that remained adhered to the substrate exhibited important morphological changes, characterized by the reduction in their size, rupture of the membrane, and/or loss or contraction of the cytoplasmic processes. This cellular response may have been triggered by oxidative stress, which causes direct cellular lesions or even reduces the repair capacity of cells in the long term.[14] Studies have shown that pathological oxidative stress in pulp cells causes acute tissue inflammation[39-40] with a consequent expression of proteases and degradation of the extracellular matrix,[41] which may result in areas of pulp necrosis.[7,8] In the present investigation, it was observed that the addition of 10 mg/mL MnO_2 to the bleaching gel reduced by 67% the quantity of H_2O_2 that diffused through the enamel and dentin. In a recent study, using an *in vitro* experimental model similar to that used in the present research, the authors demonstrated that concentrations of H_2O_2 ranging from 2.55 μg/mL to 3.54 μg/ mL were sufficient to reduce pulp cell viability by 31.5% to 61.7%.[42] Therefore, in the present study it was to be expected that the concentration of 6.3 μg/mL of H_2O_2 that diffused through the enamel/dentin discs would also cause some toxicity to the MDPC-23 cells in culture, however, with a lower intensity than that observed for the PC Group.

In the present study, the reduction in the concentration of residual H_2O_2 in the culture medium was associated with the chemical catalyzation of H_2O_2 mediated by MnO_2. This result, which was more evident in Group WHP+10(MnO_2), can be justified by greater availability of the catalyst present in the bleaching gel. Based on the fact that manganese is a transition metal, one may suggest that the mineral phase of this oxide could have catalyzed the H_2O_2 by means of a Fenton-like reaction,[43] which explains the high generation of $OH^{\bullet}$. However, it is necessary to emphasize that the degradation rate of organic pigments increases until achieving the ideal concentration of the catalyst. It is known that concentrations beyond this threshold do not enhance the degradation levels of such pigments. This is because the high concentrated free radicals tend to react with each other, then being quickly consumed.[45-46] Another important aspect related to the high catalytic potential of MnO_2 is that the metallic portion of this oxide tends to react quickly with H_2O_2 at neutral/basic pH.[47-48] These properties and characteristics of MnO_2 may have enhanced the degradation of the bleaching gels used in this study.

It is known that high tissue levels of manganese in the central nervous system creates clinical symptoms of cognitive dysfunction, behavioral changes, and movement disorders resembling Parkinson's disease.[49] However, Finley and others[50] followed women who were fed with 0.8 or 20 mg Mn/day for 60 days to determine how the diets containing low or high amounts of Mn affect neuropsychological measures and basic metabolic function. The authors showed that there was no association between Mn intake and performance in neurological tests. They concluded that efficient mechanisms operate to maintain Mn homeostasis over a wide range of Mn intake levels that can be found in the diet and that, for healthy adults, Mn intake from 0.8 to 20 mg for 8 weeks does not result in Mn toxicity. In the present study, concentrations of MnO_2 lower than 10 mg/mL was added to a commercial bleaching gel, which has been recommended to be applied for a short time (45 minutes) on enamel. Therefore, it is not expected that the addition of such oxide into the dental product causes any neurotoxicity risk to humans. So, according to the interesting scientific data achieved in the present research, it is possible to consider MnO_2 a promising catalyzer to act as coadjutant in the chemical catalysis of H_2O_2. The presence of the catalyst was responsible for the reduction of the intracellular oxidative stress and diffusion of H_2O_2. These analyses directly impact on the reduction of toxic effects caused by the gels with 35% H_2O_2. In addition, it was possible to observe a significant increase in the esthetic result achieved with in-office bleaching. This increase is mainly related to free radical production due to high H_2O_2 degradation in the presence of the catalyst. Based on these data, the null hypothesis of this study was rejected.

Despite the motivating results presented, it is important to consider that *in vitro* data, such as these obtained in the present study, cannot be immediately extrapolated to clinical situations.[38] This is because the outward dentin fluid movement, presence of collagen, cytoplasmic processes of the odontoblasts, and other structures within the dentinal tubules of vital teeth may interfere in the trans-amelodentinal diffusion of H_2O_2 and, therefore, in the cytotoxicity of the bleaching gels. Based on the results of the present laboratory research, one may suggest that new *in vitro* studies must be conducted to determine the dynamics of MnO_2 decomposition, such as the ideal concentration of this chemical catalyzer being incorporated into bleaching gels so that even more effective products may be obtained, with potential to be evaluated *in vivo* and perhaps be more safely used clinically, without causing discomfort to patients.

CONCLUSION

According to the methodology used in the present research, it was concluded that the activation of the bleaching gel with 35% H_2O_2, by means of incorporating 10 mg/mL of MnO_2 into the product, accelerated the decomposition of H_2O_2, stimulating the generation of $OH^{\bullet}$ and other free radicals. These effects of MnO_2 increased the esthetic efficacy of the in-office tooth bleaching and led to a smaller quantity of residual H_2O_2 being diffused through enamel and dentin, thereby minimizing the trans-amelodentinal toxic effect caused to pulp cells by this professional therapy.

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Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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