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Toxic effects of daily applications of 10% carbamide peroxide on odontoblast-like MDPC-23 cells

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Abstract

Background. Tooth bleaching has been widely studied, mainly due to the possible undesirable effects that can be caused by this esthetic procedure. The cytotoxicity of the bleaching agents and its components to pulp cells has been demonstrated in several researches. The aim of this study was to evaluate the toxic effects of successive applications of 10% carbamide peroxide (CP) gel on odontoblast-like cells. ***Materials and methods.*** Enamel-dentin discs obtained from bovine incisors were adapted to artificial pulp chambers (APCs). The groups were formed as follows: G1: Without treatment (control group); G2: 10% carbamide peroxide, CP (five applications/one per day); G3: 10% CP (one unique application); and G4: 35% hydrogen peroxide, HP (three applications of 15 min each). After treatment, cell metabolism (MTT), alkaline phosphatase (ALP) activity and plasma membrane damage (flow cytometry) were analyzed. ***Results.*** Reductions in cell metabolism and alkaline phosphatase activity along with severe damage of the cytoplasmic membrane were noted in G2. In G3, no damage was observed, compared to the control group. Intermediary values of toxicity were obtained after 35% HP application. ***Conclusion.*** It can be concluded that one application of 10% CP did not cause toxic effects in odontoblast-like cells, but the successive application of this product promoted severe cytotoxic effects. The daily application of the bleaching agents, such as used in the at-home bleaching technique, can increase the damages caused by this treatment to the dental pulp cells.

Key Words: *tooth bleaching, odontoblasts, carbamide peroxide, hydrogen peroxide, toxicity*

Introduction

Tooth bleaching has been widely used as an esthetic procedure, because it is easy, fast, safe and non-invasive. Nevertheless, the terms 'safe and non-invasive' as they relate to bleaching agents are of concern [1], due to some undesirable effects of this procedure, such as a reduction in enamel microhardness [2–5], toxic effects on pulp cells [6–10] and inflammation or pulpal necrosis [11,12].

Different concentrations of hydrogen and carbamide peroxides have been widely used for tooth bleaching according to the therapy selected. Higher concentrations of these peroxides (from 30–35%) have been recommended for in-office tooth bleaching, which is performed by clinicians who are responsible to apply the product on tooth structures for short periods of time (~ 15–45 min) [13]. Lower concentrations of these products (hydrogen peroxide, HP from 3.5–7%; and carbamide peroxide, CP from

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10–22%) have been indicated for at-home or night-guard tooth bleaching therapy. In this kind of esthetic procedure, which is performed without direct professional assistance, the patients maintain the bleaching agent for several hours in contact with enamel using a custom tray [14].

Despite the use of bleaching agents with low concentrations of hydrogen or carbamide peroxide, the night-guard vital bleaching require extended time of contact of the agents with the tooth structure. Current studies demonstrated that long periods of tooth bleaching could reduce the fracture strength in dentin, particularly when the product is applied on this tubular tooth structure [15,16]. Therefore, the bleaching agents should not be applied on exposed dentin, as observed after gingival recession and cement loss.

Regarding the biocompatibility to the pulp cells, when applied to enamel/dentin discs, in-office bleaching agents promote a significant reduction of cell metabolism, as well as important morphologic alterations in odontoblast-like cells in culture, as previously demonstrated [6,7,17,18]. The data obtained in these *in vitro* studies were corroborated by Costa et al. [11], who demonstrated coronal pulp necrosis in human lower incisors bleached with 38% HP.

The damages caused by bleaching agents containing high concentrations of HP (in-office technique) have been well-documented [7,11]; however, the effects of low-concentration bleaching agents [HP or CP, 6–16%] on pulp cells have not been fully elucidated. Fugaro et al. [19] and Anderson et al. [20] previously demonstrated that the night-guard vital bleaching using 10% CP was not capable of producing significant damage to the pulp tissue of premolars. Nevertheless, the main focus of the tooth bleaching treatment is the anterior teeth, like incisors, which present thinner dentin thickness than premolars [11], a fact that may influence the tissue response after the same esthetic therapy.

Previous studies showed that 16% CP promotes a significant reduction in cell metabolism and morphologic alterations in odontoblast-like cells [9,10], when applied to both dentin [9] and enamel-dentin discs [10]. These studies demonstrated that bleaching with 10% CP did not cause significant effects on pulp cells, suggesting that such bleaching probably does not cause relevant alterations in dental pulp. Nevertheless, in these studies, the effect of a single application of 10% CP on cells in culture was evaluated. When used in compliance with manufacturer's instructions, at-home bleaching agents are customarily used for at least 2 weeks to obtain satisfactory esthetic results. Therefore, the objective of this study was to analyze the trans-enamel and trans-dentinal effects of successive applications of 10% CP on odontoblast-like MDPC-23 cells, evaluating the cell metabolism, alkaline phosphatase activity and cell membrane damage.

Materials and methods

Preparation of discs

Two hundred and sixty sound bovine incisors were collected and scaled for the removal of periodontal tissue remnants and other debris. Teeth with enamel cracks, hypoplasia or other morphological alterations were excluded. Enamel/dentin discs were obtained from the buccal surface of each tooth, using a high-speed, water-cooled cylindrical diamond bur (1095; KG Sorensen) with a diameter of 5.2 mm. Dentin surfaces were polished with wet 400- and 600-grit silicon carbide paper (T469-SF- Norton; Saint-Gobain Abrasivos Ltda., Jundiaí, SP, Brazil). The final thickness of the enamel/dentin discs was 3.5 mm (1.5 mm enamel and 2.0 mm dentin), as measured with a digital caliper (Model 500-144B; Mitutoyo Sul America Ltda., São Paulo, SP, Brazil). A 0.5 mol/L ethylenediamine tetraacetic acid (EDTA) solution, pH 7.2, was applied to the dentin surface for 30 s to remove the smear layer and the discs were then rinsed thoroughly with sterile deionized water [21].

Artificial pulp chamber (APC)

Each enamel/dentin disc was adapted individually to an APC with two compartments, which was previously described [18]. The disc, which was positioned in the upper compartment of the APC with the enamel surface facing upward to receive the bleaching treatment, was maintained between two silicone rings (Rodimar Rolamentos Ltda., Araraquara, SP, Brazil) that promoted a tight seal between the upper and lower compartments of the device. In the lower APC compartment, circular perforations permitted free contact of the culture medium with the dentin surface of the disc. A 5-mm stainless steel matrix (Injecta Products Odontológicos, Diadema, SP, Brazil) was inserted between the silicone ring and the lateral wall of the upper compartment of the APC. The APCs with the discs in position were autoclaved at 120°C for 15 min and received an additional seal with autoclaved number 7 wax (Wilson Polidental, Cotia, SP, Brazil) in the region between the enamel/dentin disc and the stainless steel matrix in a laminar flow chamber.

Culture of MDPC-23 cells

The MDPC-23 cell line used in this study was kindly provided by Dr Carl T. Hanks and Dr Jacques E. Nör, from the University of Michigan. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), with 100 IU mL penicillin, 100 μg mL streptomycin and 2 mmol/L glutamine (Gibco) in a humidified incubator with 5% CO_2 and

95% air at 37°C (Isotemp, Fisher Scientific, Pittsburgh, PA). The MDPC-23 cells were sub-cultured every 3 days until an adequate number of cells were obtained. The cells were then seeded (12,500 cells/cm^2) in sterile 24-well plates (Costar Corp., Cambridge, MA), which were maintained in the humidified incubator with 5% CO_2 and 95% air at 37°C for 72 h.

Bleaching procedure and analysis of cell metabolism (MTT assay)

Bleaching gels containing 10% carbamide peroxide (CP-Whiteness Perfect; FGM, Joinville, Brazil) or 35% hydrogen peroxide (HP-Whiteness HP; FGM) were evaluated. For the bleaching procedure, the APCs with the enamel/dentin discs in position were placed individually in the wells of sterile 24-well plates, containing 1 mL of DMEM culture medium without FBS. The enamel surface of each disc (facing upward) was washed with 1 mL of sterile deionized water and dried with absorbent paper. Five microliters of artificial saliva was applied on the enamel surface and then the bleaching treatment was performed to establish the following groups ($n = 26$): G1, control (without treatment); G2, 10% CP (one application/8 h per day during 5 days); G3, 10% CP (one unique application/8 h); and G4, 35% HP (three consecutive applications of 15 min, totaling 45 min of contact with the enamel). For all groups, after the end of the bleaching treatment, the gel was aspirated and the enamel surface was thoroughly rinsed with 1 mL of sterile deionized water with concomitant aspiration. For experimental groups 3 and 4, after the end of the treatment, aliquots of 500 µL of the extract obtained from the bleaching agents diffused by the enamel/dentin were collected and applied to the wells and were maintained in contact with the cells during 1 h. For Group 2, after each 8-h bleaching time in a humidified incubator with 5% CO_2 and 95% air at 37°C, the extracts were collected and applied for 1-h to the wells of 24-well plates containing previously cultured MDPC-23 cells, and this procedure was repeated during the 5 days of the bleaching treatment, simulating the daily applications of home bleaching. After each 8-h bleaching time, a 100-µL quantity of artificial saliva was applied to the enamel surface, which remained in contact with the enamel for 16-h in the incubator, corresponding to the time for re-mineralization of the dentin substrate to occur [22]. In the control group (G1), the enamel surface remained in contact with deionized water (8-h per day) and saliva (16-h per day) for 5 days.

In each group, 10 wells were used for analysis of cell metabolism by the cytochemical demonstration of succinic dehydrogenase (SDH) activity, which is a measure of the mitochondrial respiration of the cells by the methyl tetrazolium (MTT) assay, as described previously by Mosmann [23] and as used in several studies [6,24,25]. The means were calculated for the groups and transformed into percentages, which represented the percentage of cell viability. The negative control (G1) was defined as having 100% cell metabolism.

Alkaline phosphatase (ALP) activity

Ten wells per group were used to evaluate the total protein expression and ALP activity by means of a colorimetric end-point assay (ALP Kit; Labtest Diagnóstico S.A., Lagoa Santa, MG, Brazil) with a thymolphthalein monophosphate substrate. This is a phosphoric acid ester substrate that is hydrolyzed by ALP and releases thymolphthalein, which gives a bluish color to the solution. The intensity of the resulting color is directly proportional to the enzymatic activity and can be analyzed by spectrophotometry.

The culture medium was aspirated, and the cells were washed with sterile PBS at 37°C. A 1-mL quantity of 0.1% sodium lauryl sulfate (Sigma Chemical Co.) was added to each well and maintained for 30 min at room temperature to produce cell lysis. Next, Falcon tubes (test, standard and blank) were properly labeled and a 50-µL quantity of substrate (thymolphthalein monophosphate 22 mmol/L – Kit's reagent #1) and 500 µL of buffer (300 mmol/L, pH 10.1 – Kit's reagent #2) were added to each tube. A 50-µL quantity of the standard solution 45 U/L (Kit's reagent #4) was added only to the standard tube. Thirty minutes after cell lysis, the tubes were placed in a double boiler (Fanem, Guarulhos, SP, Brazil) at 37°C for 2 min. The samples were homogenized, and a 50-µL quantity from each plate was transferred to the test tubes and maintained in the double boiler under gentle agitation. After 10-min incubation, 2 mL of color reagent (sodium carbonate 94 mmol/L and sodium hydroxide 250 mmol/L – Kit's reagent #3) were added.

The absorbance of the test was measured at a 590-nm wavelength with a spectrophotometer (Micronal B382, São Paulo, SP, Brazil). ALP activity was calculated by multiplication of the absorbance values by the calibration factor and the final values were normalized.

Analysis of cell membrane damage

Cell membrane damage was determined with propidium iodide (PI) staining by flow cytometry ($n = 6$). After treatment, a 200-µL quantity of trypsin was added to each well and the cells were centrifuged (2000 rpm/2 min). The supernatant was discarded and a 300-µL quantity of ligation buffer (10 mM of HEPES, pH 7.4, 150 mM of NaCl, 5 mM of KCl, 1 mM of $MgCl_2$ and 1.8 mM of $CaCl_2$) was added. The cells were treated with 1 µg/mL of PI and the intensity of stained cells was determined

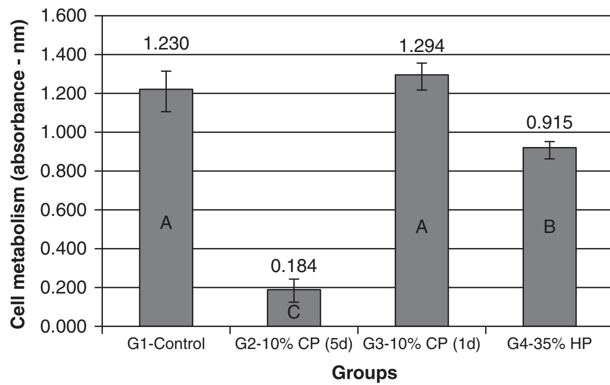

Figure 1. Graphical representation of the cell metabolism (means of absorbance in nm) as a function of the different treatments. Different letters indicate statistical differences (Tukey; $p < 0.05$).

immediately in a FACS Canto System (Becton Dickinson, San Jose, CA).

Three independent experiments (MTT, ALP activity and cell membrane analysis) were undertaken at different times to demonstrate reproducibility.

Statistical analysis

After exploratory data analysis for evaluation of the homogeneity of variances and normality of data, the data obtained from MTT assay and flow cytometry were analyzed by analysis of variance (one-way ANOVA). Multiple comparisons were performed by Tukey's test. Since the results of the alkaline phosphatase activity did not present normal distribution, the data was statistically analyzed by Kruskal-Wallis and Mann-Whitney tests. Both multiple-comparison tests were evaluated at a significance level of 5%.

Results

One application of 10% CP did not cause a significant reduction in the metabolism of MDPC-23 cells, compared with the negative control group (Tukey, $p > 0.05$) (Figure 1). However, in the groups with application of 10% CP on 5 consecutive days, a reduction in cell metabolism was observed (75%), statistically superior to that in the other groups (Tukey, $p < 0.05$). When 35% HP was applied, a reduction in cell viability (45%) was noted, statistically significant compared with that in the negative control group (G1) and the group with one application of 10% CP (G3).

In the evaluation of ALP (Figure 2), the group with five consecutive applications of 10% CP demonstrated a significant reduction in the activity of this protein, compared with the other experimental and control groups (Mann-Whitney, $p < 0.05$). The ALP activity was significantly similar among the other groups (G1, G3 and G4) (Mann-Whitney, $p > 0.05$).

The results of the membrane cell damage, analyzed by flow cytometry, are presented in Figure 3. The daily application of 10% CP caused a high intensity of propidium iodide (~ 67%), suggesting the disruption of the plasma membrane and cell necrosis. The single application of 10% CP and 35% HP caused cellular damage similar to that seen in G2; however, the PI fluorescence intensity was lower than seen with daily application of the at-home agent. All experimental groups were different from the negative control (without treatment) in this evaluation (Tukey; $p \leq 0.05$).

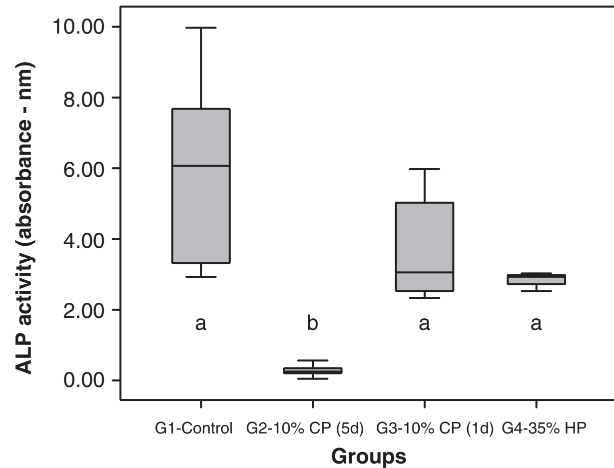

Figure 2. Graphical representation of the ALP activity (medians of absorbance in nm) after different treatments. Different letters indicate statistical differences (Mann-Whitney; $p < 0.05$).

Discussion

The present study analyzed the toxic effect of daily application of the 10% CP to odontoblast-like cells. Odontoblasts were used in this study since these are the first pulp cells to be reached by the products of the materials that diffuse through the tooth structure into the pulp space [6,8,18]. The MDPC-23 cells present an odontoblast phenotype, expressing pulp proteins such as sialoprotein and dentin phosphoprotein [26,27] and have been widely used for evaluation of the biocompatibility of dental materials [6,7,18].

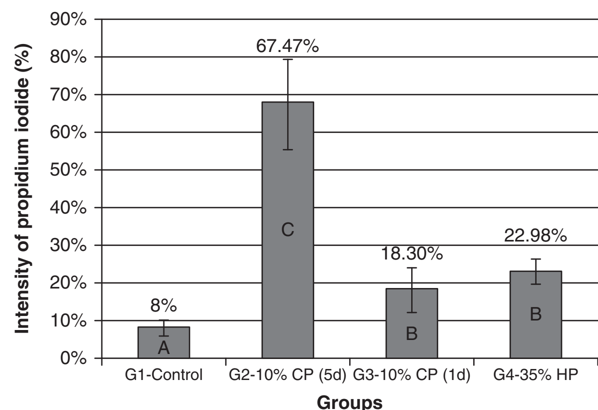

Figure 3. Intensity of propidium iodide (means of percentages) in the analysis of cell membrane damage. Different letters indicate statistical differences (Tukey; $p < 0.05$).

In the present study, significant reductions in cell viability (75%) and ALP activity were noted after daily exposure to the extracts obtained from the bleaching gel. In addition, numerous cells showed damage to the plasma membrane, suggesting cell death by necrosis. The toxic effects observed are probably due to the H_2O_2 released from the bleaching agents, which diffuse through the dental hard tissue to the pulpal space, as demonstrated in previous studies [28–30]. The products diffused by the enamel and dentin reached the culture medium, forming the extract applied to the cells in the different groups. The H_2O_2 and other molecules released from bleaching agents, such as hydroxyl radicals (OH^-), are reactive oxygen species (ROS), which cause oxidative stress when in contact with the cell membrane, characterized by the imbalance between the ROS and antioxidizing agents [31].

The results of the present study showed that one unique application of 10% CP (8-h) does not cause a significant reduction in the metabolism of MDPC-23 cells. These results are corroborated by previous studies of the same cell line [9,10]. The absence of toxicity after a single application of 10% CP to enamel-dentin discs can be related to the minimal concentration of H_2O_2 released from the bleaching agent (3.5%), forming an extract with a very low amount of ROS diffused, incapable of causing significant damage to odontoblast-like cells. However, the cumulative effect caused by daily application was capable of promoting an intense reduction in cell viability, even with low concentrations of H_2O_2 in the extract.

One possible explanation for these results is that the reduced time between the bleaching sessions prevented cell healing, showing that the increased application frequency of 10% CP can cause relevant deleterious effects in the pulp cells. The data of the present study are in contrast with those obtained by Fugaro et al. [19], who demonstrated that the daily application of 10% CP caused only a mild inflammation on dental pulp of premolars, with the damage completely reversed after 2 weeks. It may be suggested that these contradictory data occurred due to the thicker dentin thickness presented by premolars compared to the samples used in this study in which the authors intended to simulate the dentin thickness of human incisors. The thinner dentin barrier probably allows diffusion of higher concentration of peroxides to the pulp space, promoting severe damages to pulp cells, different than observed by Fugaro et al. [19].

In addition, the daily application of 10% CP caused a significant reduction in ALP activity, not observed in the other experimental groups. Part of these results is due to the reduced number of viable cells, resulting in lower ALP activity, a protein that is involved in the mineralization of the dentin matrix [28]. Another observation is that the interaction between the ROS, released from the bleaching agents, and the protein chain can promote a breakdown of the amino acid sequence near the active center of the molecule, causing protein fragmentation [32] and, consequently, reduction of activity.

The reduced cell metabolism and ALP activity were complemented by the results of the flow cytometry, where a higher PI intensity was observed in the group with daily application of 10% CP, compared with the other groups. Propidium iodide is a nucleus marker that exclusively penetrates cells with plasma membrane damage. The damage is caused by the ROS released from the bleaching agents, which break down the double bonds of unsaturated carbons of the membrane, triggering an autocatalytic reaction known as lipid peroxidation [33]. The high PI intensity, indicating cell necrosis after bleaching, combined with the significant reduction in odontoblast-like cells viability and ALP activity, demonstrates that the daily application of low-concentration bleaching agents possibly promotes critical pulp damage *in vivo*.

A significant reduction in cell metabolism (45%) was observed after the bleaching protocol with 35% HP, corroborating the *in vitro* toxicity of this technique, as demonstrated in previous studies [7,17,18]. The toxicity was possibly caused by the high amount of ROS released from the bleaching agent and diffused through the tooth structure, resulting in the cell damage observed. The toxicity presented by this protocol was statistically high compared with that in the group with one application of 10% CP or with the negative control; however, the severity was lower than in the group with five applications of the at-home bleaching agent tested. It should be noted that the daily application of 10% CP promoted a cumulative toxic effect, probably preventing cell healing during the sessions, as previously described, and this can be one factor accounting for the high severity observed in this group.

Another possible reason relates to methodology. Due to the different bleaching protocols, the treatment with daily application of 10% CP began 72 h after the cells were seeded, with a confluence rate lower than that on the day of application of the 35% HP. In light of this fact, fewer cells were available on the last day of application of 10% CP (day 5), when the cell metabolism analysis was performed, promoting intense reduction in the cell viability for group 2, statistically superior to that achieved with 35% HP.

Despite the severe reduction in cell metabolism caused by the products released from the bleaching agents after the daily application of 10% CP, one unique application of the same product during 8 h did not cause significant damage to the odontoblast-like cells. These results demonstrate the lower toxicity of the at-home bleaching agent tested compared with the 35% HP, used for in-office bleaching, when the products are applied for a similar period.

The at-home technique requires daily use of low-concentration bleaching agents and the results of the present study demonstrate that the consecutive application of these products can promote higher toxicity to the pulp cells, indicating that these low-concentration agents should be used with caution. In the present study, the bleaching agent was maintained on enamel for 8 h, simulating a night-guard vital tooth bleaching. Variations in this protocol, such as reduction on enamel bleaching gel-contact time or increasing interval between the bleaching sessions, may be interesting alternatives to reduce the toxic effects of the at-home bleaching agents to pulp cells. Moreover, the presence of intra-pulpal pressure, dentinal fluid, immunological and lymphatic systems, production of antioxidizing agents and other factors can modulate the effects of different treatments on pulp cells for *in vivo* situations [34]. Although the results of *in vitro* studies cannot be directly extrapolated to *in vivo* situations, clinical trials demonstrated increased tooth sensitivity during the course of the studies [35–37]. It has been reported that the tooth sensitivity, that is frequently mild and resolved during or immediately after the tooth bleaching treatment, is directly related to the time/length of the gel application on enamel, which, in turn, is associated with the concentration of peroxide in the pulp chamber [36,37]. Therefore, *in vivo* studies should be performed to evaluate the effects of at-home bleaching techniques with carbamide peroxide for long periods on pulp tissue.

Conclusion

Based on the results obtained in the present study, it can be concluded that a single application of 10% CP agent on enamel does not cause toxic effects on MDPC-23 cells. Nevertheless, the sequential application of this bleaching agent promoted severe toxicity to the cultured cells, which was higher than observed after a single section using 35% HP agent.

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