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Trans-enamel and trans-dentinal cytotoxic effects of a 35% H_2O_2 bleaching gel on cultured odontoblast cell lines after consecutive applications

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Abstract

Trindade FZ, Ribeiro APD, Sacono NT, Oliveira CF, Lessa FCR, Hebling J, Costa CAS. Trans-enamel and trans-dentinal cytotoxic effects of a 35% H_2O_2 bleaching gel on cultured odontoblast cell lines after consecutive applications. *International Endodontic Journal*, **42**, 516–524, 2009.

Aim To evaluate the trans-enamel and trans-dentinal cytotoxic effects of a 35% H_2O_2 bleaching gel on an odontoblast-like cell lines (MDPC-23) after consecutive applications.

Methodology Fifteen enamel/dentine discs were obtained from bovine central incisor teeth and placed individually in artificial pulp chambers. Three groups (n = 5 discs) were formed according to the following enamel treatments: G1: 35% H_2O_2 bleaching gel (15 min); G2: 35% H_2O_2 bleaching gel (15 min) + halogen light (20 s); G3: control (no treatment). After repeating the treatments three consecutive times, the extracts (culture medium + gel components that had diffused through enamel/dentine discs) in contact with the dentine were collected and applied to previously cultured MDPC-23 cells (50 000 cells cm^{-2}) for 24 h. Cell metabolism was evaluated by the MTT assay and data were analysed statistically (α = 5%; Kruskal–Wallis and Mann–Whitney *U*-test). Cell morphology was analysed by scanning electron microscopy.

Results Cell metabolism decreased by 92.03% and 82.47% in G1 and G2 respectively. G1 and G2 differed significantly ($P < 0.05$) from G3. Regardless of halogen light activation, the application of the bleaching gel on the cultured odontoblast-like cells caused significantly more severe cytotoxic effects than those observed in the nontreated control group. In addition, significant morphological cell alterations were observed in G1 and G2.

Conclusion After three consecutive applications of a 35% H_2O_2 bleaching agent, the diffusion of the gel components through enamel and dentine caused severe toxic effects to cultured pulp cells.

Keywords: cytotoxicity, hydrogen peroxide, odontoblasts, tooth bleaching.

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Introduction

Professionally applied and home tooth bleaching under supervision are two of the most widely used tooth bleaching therapies (Al-Salehi *et al.* 2007). Professionally applied bleaching procedures are an appropriate alternative to home-bleaching, especially in teeth with severe discoloration or if a rapid treatment is desired. In this technique, a heat or light source is frequently applied on the bleaching gel to accelerate the action of its components, especially hydrogen peroxide (H_2O_2), on the dental structures (Gokay *et al.* 2000a,b) .

H_2O_2 is a type of free radical normally encountered within cell, resulting from a series of intracellular reactions that occur specifically in the mitochondria (Forman & Boveris 1982, Shackelford *et al.* 2000). However, this chemical agent can also reach the cells via exogenous sources (Dizdaroglu *et al.* 1991,

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Martindale & Holbrook 2002). Due to its reactive properties, H_2O_2 is the main active component of most agents used in tooth bleaching therapies (Zantner *et al.* 2007). The potential adverse effects of H_2O_2 on the oral tissues has been widely investigated, especially because it is a thermally unstable chemical agent with a high oxidative power, which may dissociate into water, oxygen and some free radical species, such as hydroxyl radicals (OH^-) (Kawamoto & Tsujimoto 2004). The oxidative reactions and consequently the cell damage caused by free radicals are the main mechanisms responsible for the toxicity of peroxide-containing compounds (Li 1996). The mechanisms by which the teeth are bleached are not yet fully understood, but it is known that the low molecular weight of H_2O_2 permits its trans-enamel and trans-dentinal diffusion, which is responsible for the whitening effect of bleaching agents; on the other hand, it can also cause damage to pulp cells (Benetti *et al.* 2004).

Odontoblasts are typical cells of the pulp tissue and are organized in a monolayer that underlies the dentine (Goldberg & Smith 2004), being therefore the first cells to be damaged by potentially cytotoxic compounds released from dental materials that diffuse through enamel and dentinal tubules. In view of this, several laboratory studies have used different cell types with odontoblast phenotypes to evaluate potential cytotoxic effects of dental materials and/or their individual components (Costa *et al.* 1999, 2003a,b, 2008, He *et al.* 2004, Aranha *et al.* 2006, Souza *et al.* 2006).

Depending on the severity of colour alteration, several bleaching sessions may be required to produce satisfactory whitening (Buchalla & Attin 2007). Therefore, considering that a single application of the bleaching gel on darkened teeth may not be sufficient to reach the desired aesthetic outcome, the purpose of this study was to evaluate the trans-enamel and trans-dentinal cytotoxic effects of a 35% H_2O_2 bleaching gel on cultured odontoblast-like cell MDPC-23 after three consecutive applications of the agent on enamel surface, simulating a professionally applied vital tooth bleaching procedure.

Material and methods

Preparation of discs

Fifteen sound bovine permanent central incisor teeth were collected and scaled for removal of periodontal tissue remnants and other debris. The teeth were mounted in a precision cutting machine (Isomet 1000; Buehler Ltda., Lake Bluff, IL, USA) and 5.2 mm diameter discs containing enamel and dentine were cut transversally from the middle third of the buccal surface of each tooth. Disc refinement was made with a high-speed water-cooled cylindrical diamond bur (size 1095; KG Sorensen, Barueri, SP, Brazil). The discs were examined with a stereomicroscope (SZ2-ILST; Olympus Corporation, Tokyo, Japan) and only those with no enamel defects were selected. Dentine surfaces were polished with wet 400- and 600-grit silicon carbide paper (T469-SF-Norton; Saint-Gobain Abrasivos Ltda., Jundiaí, SP, Brazil) until reaching a standardized dentine thickness of 2.2 mm. The final thickness of the enamel/dentine discs was 3.5 mm, as measured with a digital caliper (Model 500-144B; Mitutoyo Sul América Ltda., São Paulo, SP, Brazil). A 0.5 mol L^{-1} ethylenediaminetetraacetic acid (EDTA) solution, pH 7.2, was applied on the dentine surfaces for 30 s to remove the smear layer and the discs were thoroughly rinsed with sterile deionized water (Jacques & Hebling 2005).

Artificial pulp chambers

Each enamel/dentine disc was adapted individually to the artificial pulp chambers (APCs), which presented an upper and a lower compartment, where circular perforations permitted the free diffusion of the culture medium between the outer and inner portions of the APC (Fig. 1). The discs were positioned in the APC's between two silicon rings (4.47 mm inner diameter; 1.78 mm thick; Rodimar Rolamentos Ltda, Araraquara, SP, Brazil), which promoted a lateral seal between the upper and lower compartments of the device. The APCs with the discs in position were autoclaved at 120 °C for 20 min and then stored at 37 °C with 100% relative humidity for 24 h.

Culture of MDPC-23 cells

Immortalized cells of the MDPC-23 cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA), with 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 2 mmol L^{-1} glutamine (Gibco) in an humidified incubator with 5% CO_2 and 95% air at 37 °C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). The MDPC-23 cells were subcultured at every 3 days until an adequate number of cells were obtained for the study. The cells were then seeded (50 000 cells cm^{-2}) in 30 wells of two sterile 24-well

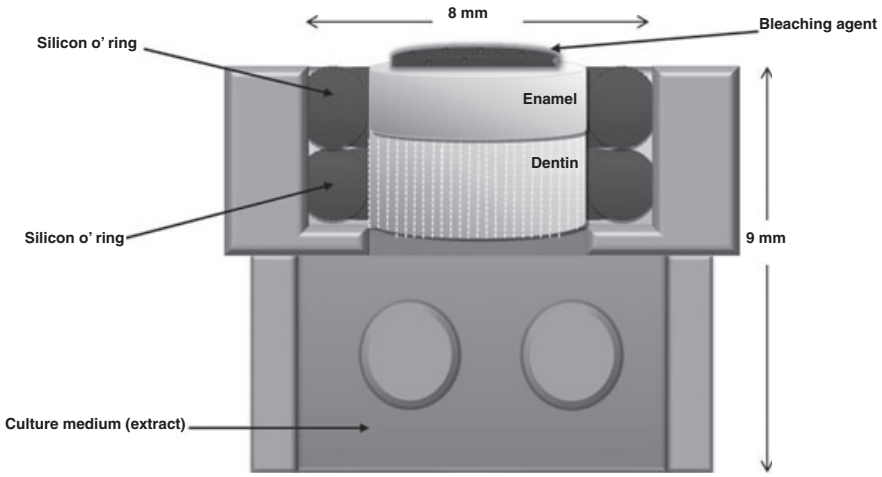

Figure 1 Schematic presentation of the artificial pulp chamber (APC) with the enamel/dentine disc in position.

dishes (Costar Corp., Cambridge, MA, USA), which were maintained in the humidified incubator with 5% CO_2 and 95% air at 37 °C for 24 h.

Bleaching procedure and analysis of cell metabolism (MTT assay)

A 35% H_2O_2 bleaching gel (Whiteness HP, FGM Produtos Odontológicos Ltda, Joinville, SC, Brazil) was used. For the bleaching procedure, the APCs with the dentine/enamel disc in position were placed individually in the wells of sterile 24-well dishes, containing 1 mL of complete culture medium *per* well. The enamel surface of the discs faced upwards in order to receive the bleaching treatments (Fig. 1). The discs were randomly assigned to three groups of five discs each, according to the treatment of enamel surface: G1: 35% H_2O_2 bleaching gel (15 min); G2: 35% H_2O_2 bleaching gel (15 min) + halogen light (20 s); G3: control (no treatment). Ten milligrams of a 35% H_2O_2 bleaching gel (Whiteness HP, FGM Produtos Odontológicos Ltda) was applied to the enamel surface of the discs in the APCs. The halogen light source (Curing Light XL 3000; 3M ESPE, St Paul, MN, USA) used in G2 had an output power of 430 mW cm^{-2}, as measured with a curing radiometer (Demetron, Kerr Corp., Danbury, CT, USA). During light exposure, the light guide tip of the curing unit was held 10 mm from enamel surface. In G1 and G2, the bleaching gel was left in contact with the enamel surface for 15 min and was then aspirated carefully with sterile Pasteur pipettes (Corning Inc., Corning, NY, USA). The bleaching procedure was repeated three consecutive times, to give 45 min of contact between the bleaching agent and the enamel surface (G1 and G2), and 60 s of light activation (G2). After the third and last application, the bleaching gel was aspirated and the enamel surface was rinsed thoroughly with 1 mL of sterile deionized water with concomitant aspiration. The APCs with the discs in position were then incubated for 12 h in 5% CO_2 and 95% air atmosphere at 37 °C. After this period, 1 mL of extract containing components from the bleaching agent that diffused through enamel and dentine was collected from each well**.** In 24-well dishes, aliquots of 500 μL of extract were applied in 30 wells (10 wells per group) containing previously cultured MDPC-23 cells (50 000 cells cm^{-2}). The extract was left in contact with cells in the humidified incubator with 5% CO_2 and 95% air at 37 °C for 24 h.

In each group, seven wells with previously cultured cells were used to evaluate cell metabolic activity by succinic dehydrogenase (SDH) enzyme, which is a measure of the mitochondrial respiration of the cell (Mosmann 1983). For such purpose, the methyltetrazolium (MTT) assay was used. The extracts were aspirated and an amount of 900 μL of culture medium (DMEM; Sigma Chemical Co.) associated to 100 μL of MTT solution [5 mg mL^{-1} sterile phosphate buffered saline (PBS)] was applied to the cells cultured in each well. The viable cells were incubated at 37 °C for 4 h. Thereafter, the culture medium (DMEM; Sigma Chemical Co.) with the MTT solution was aspirated and replaced by 600 μL of acidified isopropanol solution (0.04 N HCl). Next, three 100 μL aliquots of each well were transferred to a 96-well dish (Costar Corp.). Cell viability was determined as being proportional to

the absorbance measured at 570 nm wavelength with an ELISA plate reader (Model 3550-UV, Microplate reader; Bio-Rad, Hercules, CA, USA).

Analysis of cell morphology by scanning electron microscopy

The other 3 specimens were selected for analysis of cell morphology by scanning electron microscopy (SEM). For such purpose, sterile 12 mm diameter cover glasses (Fisher Scientific) were placed on the bottom of the wells of 24-well dishes immediately before seeding of the MDPC-23 cells (50 000 cells cm^{-2}). The extracts were applied to the cells and incubated for 24 h (Mendonça *et al.* 2007). Following this period, the extracts were aspirated and the viable cells that remained adhered to the glass substrate were fixed in 1 mL of buffered 2.5% glutaraldehyde for 120 min. Next, the cells were submitted to three 5-min rinses with 1 mL PBS, post-fixed in 1% osmium tetroxide for 60 min and processed for examination with a scanning electron microscope (DSM 960; Carl. Zeiss Inc., Oberkochen, Germany).

Statistical analysis

The numerical data obtained from the MTT assay, which presented a non-normal distribution, were analysed statistically by the Kruskal–Wallis and Mann–Whitney tests at a level of significance of 5%.

Results

Cell metabolism (MTT assay)

The results of cell viability obtained from the analysis of cell metabolism (MTT assay) after exposure of the MDPC-23 cells to the extracts are presented in Fig. 2.

Considering the control group (G3) as having 100% of cell metabolism, the metabolic activity of the viable MDPC-23 cells that remained adhered to glass substrate in G1 and G2 decreased by 92.03% and 82.47% respectively.

In G1 (bleaching gel), there was a greater tendency for a decrease in cell viability when compared to G2 (bleaching gel + halogen light), but this difference was not significant ($P > 0.05$). This result suggests that the bleaching gel was toxic to the cultured MDPC-23 cells, regardless of halogen light activation. The statistical analysis of the data from the MTT assay revealed a statistically significant difference ($P < 0.05$) in cell viability when comparing G1 and G2 to the control group (G3).

Cell morphology (SEM)

In G1 and G2, in which the MDPC-23 cells were in contact with the extract containing products of the bleaching gel diffusion, a smaller number of viable cells remained adhered to the glass substrate. These cells were small-sized and presented rounded morphology and few or no cytoplasmatic prolongations in their membrane (Figs 3 and 4). In the control group (G3), the MDPC-23 cells were slightly elongated, organized as epithelioid nodules and presented several cytoplasmatic prolongations originating from their membrane, which seemed to be adhering the cells to the glass substrate (Fig. 5).

Discussion

The immortalized cell lines MDPC-23 used in this *in vitro* study were previously described by Hanks *et al.* (1998). These cells were isolated from mouse molar dental papilla present similar characteristics to those of

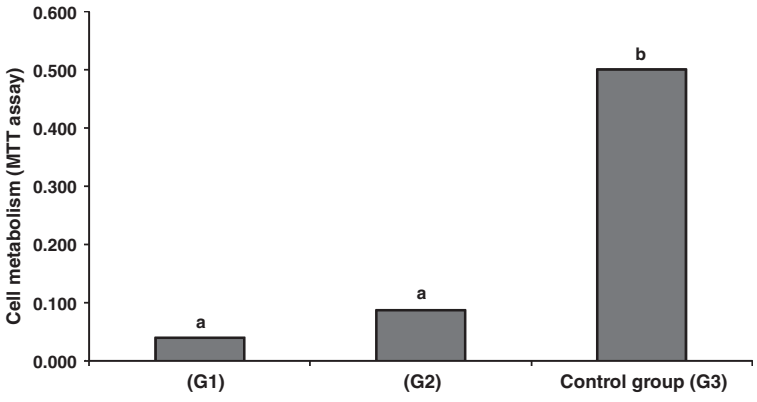

Figure 2 Box-whisker plot [minimum (lower quartile-median-upper quartile) maximum] of the cell metabolism (MTT assay) results for each group ($n = 6$). Groups identified with the same letter do not differ statistically (Mann–Whitney; $P > 0.05$).

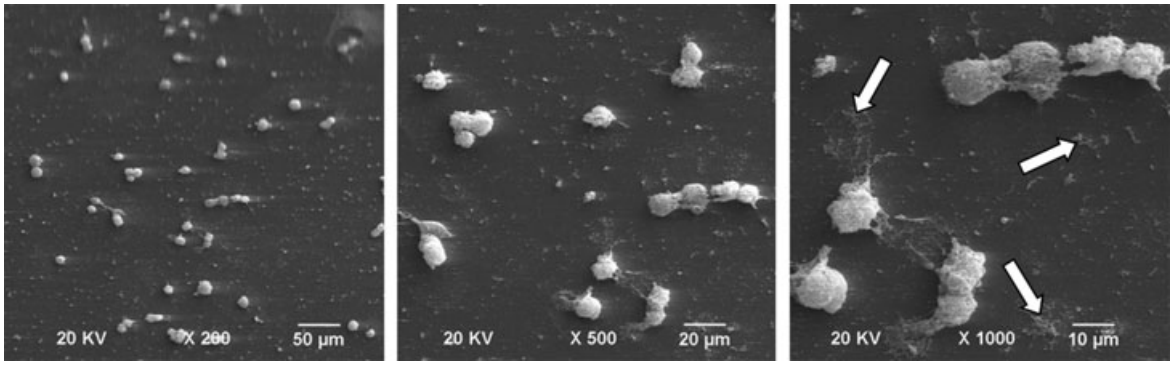

Figure 3 G1 (bleaching gel): The few MDPC-23 cells that remained adhered to the glass substrate exhibit a rounded morphology with few short cytoplasmatic prolongations originating from their membrane. Note that the rests of the cytoplasmatic membrane of lethally damaged cells are clearly observed on the glass substrate (arrows).

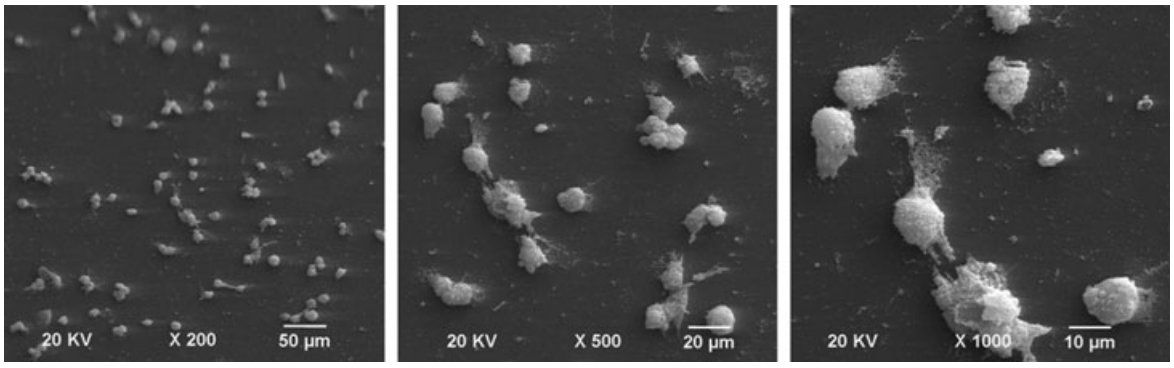

Figure 4 G2 (bleaching gel + halogen light): In the same way as observed in Fig. 3, several MDPC-23 cells detached from the glass substrate, leaving adhered rests of the cytoplasmatic membrane. The cells exhibited a rounded morphology and loss of a large number of cytoplasmatic prolongations.

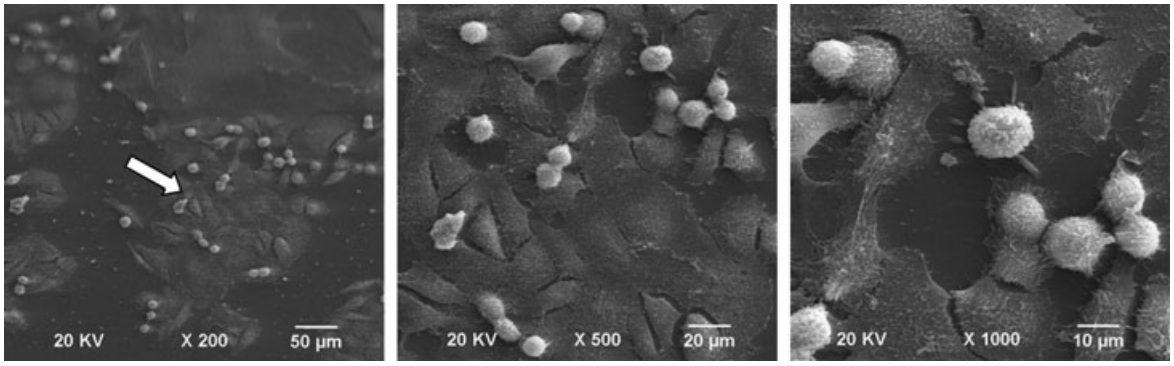

Figure 5 G3 (control group): MDPC-23 cells organized as epithelioid nodules (arrow), exhibiting several cytoplasmatic prolongations originating from their membrane, which seem to be adhering the cells to the glass substrate.

odontoblast-like cells *in vivo*, such as high alkaline phosphatase activity, organization as epithelioid nodules and dentine sialoprotein or dentine phosphoprotein expression (Hanks *et al.* 1998).

Odontoblasts have been described as the most indicated cell type to evaluate the *in vitro* cytopathic effects of dental materials (MacDougall *et al.* 1995) because in mammalian teeth the odontoblasts are

organized in a monolayer that underlies the coronal and root dentine. Therefore, any residual component leached from a dental material that is capable to diffuse through the enamel and dentinal tubules will first interact with these peripheral pulp cells and cause their damage, if cytotoxic (Goldberg & Smith 2004). Therefore, cells with odontoblast phenotype have been widely used to investigate the response of these specific pulp cell types when submitted to different situations of stress or biological stimulus (Costa *et al.* 2003b, 2008, He *et al.* 2004, Aranha *et al.* 2006, Souza *et al.* 2006).

Currently, due to ethical issues, the use of human teeth in *in vivo* studies has become more difficult and alternatives have been proposed, such as the use of discs obtained from sound teeth adapted to APCs, in which it is possible to simulate or not an intrapulpal pressure similar to that of vital teeth (Demirci *et al.* 2008). The products resulting from the diffusion of experimental materials through enamel/dentine discs may be collected in the form of extracts that are applied on pulp cell cultures, producing laboratory conditions close to those observed *in vivo*. Bovine teeth have been widely used in different types of experiments in several fields of dental research (Adibfar *et al.* 1992, Attin *et al.* 1997) because the dental enamel and dentine of the teeth of these animals have similar morphological characteristics to those of the human teeth (Benetti *et al.* 2004). Previous studies have demonstrated structural similarities between human and bovine teeth regarding not only the number and inner diameter of the dentinal tubules, but also the radiodensity of enamel and dentine (Schmalz *et al.* 2001, Fonseca *et al.* 2004, Krifka *et al.* 2008). For these reasons, in the present laboratory study, bovine enamel/dentine discs were obtained and mounted in APCs, and odontoblast-like cell cultures were exposed to extracts containing components from the bleaching gel diffused through enamel and dentine. The enamel/dentine bovine discs prepared for the present study had a final thickness of 3.5 mm, being 1.3 mm of enamel and 2.2 mm of dentine. These enamel and dentine thicknesses are similar to those reported for human teeth (enamel = 0.9–1.05 mm; dentine = 2.33 mm) (Harris & Hicks 1998, Sulieman *et al.* 2005b). However, Camargo *et al.* (2007) reported that the smaller diameter of dentinal tubules presented in bovine teeth when compared with human teeth may explain the greater penetration of components released from the hydrogen peroxide gel in human teeth. In addition, those authors suggested that the interaction of hydrogen peroxide with calcium and phosphate of hydroxyapatite may cause different alterations in human and bovine tooth structures. Consequently, the results observed in the present investigation cannot be directly extrapolated to the clinical situation of dental bleaching applied in human teeth. As previously demonstrated, the higher permeability of human dentine when compared with bovine teeth may result in a more intense diffusion of hydrogen peroxide gel components through human dentine to cause greater toxic effects to pulp cells. Although laboratory studies cannot be directly extrapolated to *in vivo* conditions, the experimental model used in the present investigation attempted to simulate the clinical conditions as closely as possible in such a way that the data could be of interest for researchers and clinicians.

Tooth bleaching procedures are possible due to the permeability of the hard dental tissues and the low molecular weight of some of the active chemical components of bleaching agents, such as H_2O_2 (Arwill *et al.* 1969, Pashley 1988, Hanks *et al.* 1993). Some authors have demonstrated that even at low concentrations, H_2O_2 penetrates easily into enamel porosities and is capable of diffusing deeply through dentine, reaching the pulp tissue (Bowles & Ugwuneri 1987, Cooper *et al.* 1992, Hanks *et al.* 1993, Gokay *et al.* 2000a,b, 2004). Benetti *et al.* (2004) evaluated *ex vivo* the capacity of H_2O_2 to diffuse through bovine hard dental tissues after exposure to bleaching gels containing 10% or 35% H_2O_2 in their composition. Higher concentrations of the bleaching agent produced higher levels of H_2O_2 in the pulp chamber after diffusion through enamel and dentine.

The action of bleaching gels creates porosities on the enamel structure that can permit the diffusion of toxic products to the pulp (Kwon *et al.* 2002). The increase in hard dental tissue permeability might also play a role in postoperative sensitivity following bleaching therapies. It has been reported that postoperative sensitivity or pain is one of the most frequent adverse effects observed in patients submitted to professionally applied vital tooth bleaching (Dahl & Pallesen 2003). The diffusion of H_2O_2 to the pulp tissue may also be involved in postoperative sensitivity or pain due to the toxic effects of this chemical agent to the pulp (Haywood 2000). It is known that H_2O_2 and the products of its degradation, such as hydroxyl (OH^-) ions, are reactive species derived from oxygen, which may have cytopathic effects, like mutagenesis, carcinogenesis, cell membrane damage by peroxidation lipid, and protein fragmentation (Sies 1993, Martindale & Holbrook 2002). Therefore, the elevated exogenous levels of

these highly reactive free radicals (Shackelford *et al.* 2000, Martindale & Holbrook 2002) in contact with cells, as occurs during tooth bleaching, may result in apoptosis and reduced cell proliferation (Slater *et al.* 1995, Allen & Tresini 2000). In the present investigation, both groups in which the cells were exposed to extracts collected after three consecutive enamel bleaching treatments, with or without light activation (G1 and G2), presented a statistically significant reduction in cell viability compared to the control group (G3). In other words, the extracts formed by the culture medium and the products of bleaching gel degradation applied on the cultured odontoblast-like cells caused severe cytopathic effects. These results indicate that toxic components released from the bleaching gel, probably in the form of reactive free radicals, were capable of diffusing through enamel and dentine to reach the culture medium in contact with the pulpal side of the disc. Previous studies have demonstrated the diffusion of bleaching agents through the tooth tissue and the penetration of carbamide/hydrogen peroxide into the pulp chamber (Bowles & Ugwuneri 1987, Cooper *et al.* 1992, Hanks *et al.* 1993, Gokay *et al.* 2000a,b, Gokay *et al.* 2004, Benetti *et al.* 2004).

According to Buchalla & Attin (2007), the use of a light source in tooth bleaching has been recommended in order to speed the bleaching procedure. This effect is due to the fact that the heat generated by light source accelerates the degradation of components of the bleaching gel, especially H_2O_2, resulting in the release a greater amount of highly reactive free radicals, which in turn, will degrade the pigments in the tooth, producing the whitening effect (Buchalla & Attin 2007). From a scientific standpoint, the mechanisms of action and the efficacy of tooth bleaching activated by laser, light or heat are not yet clearly understood. Some authors have stated that halogen light activation of the bleaching agent not only elevates the intrapulpal temperature, but also increases the diffusion of bleaching gel components to the pulp (Bowles & Ugwuneri 1987, Wetter *et al.* 2004). In the present study, the group in which the bleaching gel was irradiated with a halogen light source for 20 s (G2) did not present significantly higher cytotoxicity compared to the use of bleaching agent alone. Therefore, halogen light application on the bleaching gel *per se* seemed not to influence the severe cytotoxic effects observed in both experimental groups (G1 and G2). The aggressive action of the bleaching agent is confirmed by the analysis of the morphological characteristics of the few viable MDPC-23 cells that remained adhered to the glass substrate after the bleaching protocols. MDPC-23 cells that are lethally damaged by toxic dental products loose their cytoplasmatic prolongations, die and detach from the substrate to which they were adhered (Costa *et al.* 2003b, Aranha *et al.* 2006). On the other hand, viable cells that remained adhered to the substrate present few cytoplasmatic prolongations originating from their membrane and exhibit a rounded morphology (Costa *et al.* 2003a, Souza *et al.* 2007).

Although the findings of the present study have clinical relevance, the results of laboratory H_2O_2 diffusion experimental models might not predict the real penetration capacity of this chemical agent *in vivo* (Cooper *et al.* 1992, Hanks *et al.* 1993, Thitinanthapan *et al.* 1999, Gokay *et al.* 2004, Sulieman *et al.* 2005a). Vongsavan & Matthews (1991) evaluated the diffusion of Evans' blue applied on dentine surface and observed that whereas the dye diffused readily into dentine in extracted teeth, a lower dye penetration was observed when it was applied *in vivo*. According to the authors, the presence of a dentinal fluid flow produced by intrapulpal pressure in vital teeth is sufficient to substantially reduce the diffusion of chemicals into the dentinal tubules to reach the pulp. In laboratory experimental models, the absence of this outward intradentinal fluid flow might contribute to the rapid diffusion of bleaching gel components through dentine. Therefore, as stated by Gokay *et al.* (2004), it is possible to assume that the levels of H_2O_2 or the products of its degradation that reach the pulp tissue *in vivo* may actually be lower than those observed *ex vivo*. Further vital tooth bleaching trials as well as laboratory studies using APCs with the application of intrapulpal pressure must be performed to investigate the influence of the outward intradentinal fluid flow on the diffusion of chemicals released by the degradation of bleaching agents, as well as their potential aggressive effects on a more complex and specialized system, like the connective pulp tissue.

Conclusion

Under the tested experimental conditions, the extracts (culture medium plus bleaching gel degradation products that diffused through the enamel and dentine) collected after three consecutive applications of a 35% H_2O_2 bleaching gel caused severe toxic effects on cultured odontoblast-like cell MDPC-23. These cytopathic effects were not increased significantly when the bleaching gel was activated with a halogen light source.

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