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ORIGINAL ARTICLE

Efficacy and cytotoxicity of a bleaching gel after short application times on dental enamel

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Abstract
Objectives This study aimed to evaluate and correlate the efficacy and cytotoxicity of a 35 % hydrogen peroxide (HP) bleaching gel after different application times on dental enamel.
Materials and methods Enamel/dentin disks in artificial pulp chambers were placed in wells containing culture medium. The following groups were formed: G1, control (no bleaching); G2 and G3, three or one 15-min bleaching applications, respectively; and G4 and G5, three or one 5-min bleaching applications, respectively. Extracts (culture medium with bleaching gel components) were applied for 60 min on cultured odontoblast-like MDPC-23 cells. Cell metabolism (methyl tetrazolium assay) (Kruskal–Wallis/Mann–Whitney; α=5 %) and cell morphology (scanning electron microscopy) were analyzed immediately after the bleaching procedures and the trans-enamel and trans-dentinal HP diffusion quantified (one-way analysis of variance/Tukey's test; α=5 %). The alkaline phosphatase (ALP) activity was evaluated 24 h after the contact time of the extracts with the cells (Kruskal–Wallis/Mann–Whitney; α=5 %). Tooth color was analyzed before and 24 h after bleaching using a spectrophotometer according to the Commission Internationale de l'Eclairage *L*a*b** system (Kruskal–Wallis/Mann–Whitney; α=0.05).
Results Significant difference (p<0.05) in cell metabolism occurred only between G1 (control, 100 %) and G2 (60.6 %). A significant decrease (p<0.05) in ALP activity was observed between G2, G3, and G4 in comparison with G1. Alterations on cell morphology were observed in all bleached groups. The highest values of HP diffusion and color alterations were observed for G2, with significant difference among all experimental groups (p<0.05). G3 and G4 presented intermediate color change and HP diffusion values with no statistically significant differences between them (p>0.05). The lowest amount of HP diffusion was observed in G5 (p<0.05), which also exhibited no significant color alteration compared to the control group (p>0.05).
Conclusions HP diffusion through dental tissues and its cytotoxic effects were proportional to the contact time of the bleaching gel with enamel. However, shorter bleaching times reduced bleaching efficacy.
Clinical relevance Shortening the in-office tooth bleaching time could be an alternative to minimize the cytotoxic effects of this clinical procedure to pulp tissue. However, the reduced time of bleaching agent application on enamel may not provide adequate esthetic outcome.

Keywords Odontoblasts · Tooth bleaching · Cytotoxicity · Tooth color

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Introduction

In-office tooth bleaching is a widely employed esthetic dentistry procedure, especially because it produces fast

positive esthetic outcomes, promoting a great deal of fulfillment of patients' expectations for a bright white smile. This kind of tooth therapy may be performed under the dentist's supervision using agents with high concentrations (30–38 %) of hydrogen peroxide (HP) on enamel surface and require one to three 10- to 15-min applications of the product in each clinical session [1]. The esthetic efficacy of tooth bleaching has been extensively demonstrated both in vivo and in vitro [2–6]. However, postoperative tooth sensitivity is the most common adverse effect associated with bleaching procedures, affecting around two thirds of patients [7].

It has been demonstrated that the HP released from bleaching agents can diffuse through enamel and dentin and penetrate the pulp chamber [8–11]. Therefore, one of the hypotheses to explain the occurrence of post-bleaching sensitivity is that HP and its degradation products could trigger a local pulp inflammatory reaction [12–17]. Previous studies have demonstrated that pulpal response to tooth bleaching could range from a mild inflammatory reaction [14, 17] to an acute inflammation [12, 13] or even partial necrosis of the coronal pulp tissue [16].

Several studies have shown that the indirect cytotoxicity of the active compounds released from bleaching gels is proportional to their concentration in the agents as well as the contact time with enamel [18–22]. However, some authors have demonstrated that exposure of pulp cells to low concentrations of HP promoted overexpression of odontoblast differentiation markers [23–25] and formation of mineralization nodules [23, 25]. This means that a low-intensity stimulus is capable of inducing dentin matrix deposition and mineralization, which are favorable events to the healing of the pulp–dentin complex. Reducing the contact time of the bleaching gels with enamel could be an interesting alterative to minimize their deleterious effects to the pulp tissue and enhance their participation in the process of reactionary dentin matrix synthesis and deposition. On the other hand, shortening the bleaching time could reduce the clinical esthetic outcome of this procedure. Therefore, the purpose of this study was to evaluate and correlate the bleaching efficacy and the trans-enamel and trans-dentinal cytotoxicity of a 35 % HP bleaching gel recommended for in-office therapy, after different application times on dental enamel.

Material and methods

Enamel/dentin disks

Sound bovine central incisors from 24- to 30-month-old bullocks were cross-sectioned with a diamond trephine bur (Dinser brocas diamantadas Ltda., São Paulo, SP, Brazil) coupled to a bench drilling machine (FSB 16 Pratika; Schultz, Joinville, SC, Brazil) to produce 5.6-mm-diameter enamel/dentin disks. The dentin side of the disks was ground wet with 400- and 600-grit silicon carbide paper (T469-SF; Norton, Saint-Gobam Abrasivos Ltda., Jundiaí, SP, Brazil) to obtain a standardized final thickness of 3.5 mm.

Trans-enamel and trans-dentinal cytotoxicity

A 0.5-M ethylenediaminetetraacetic acid solution, pH 7.2, was applied on the dentin surface during 30 s for removal of the smear layer [18–20, 22] and the disks were washed with sterile distilled water. Then, the disks were adapted to artificial pulp chambers (APC) and the APC/disk sets were autoclaved at 121 °C for 15 min in a receptacle with water. The APC/disk sets were placed in sterile 24-well plates (Costar Corp., Cambridge, MA, USA) containing 1 ml of serum-free Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) in such a way that the enamel surface remained exposed for the subsequent bleaching treatments (Fig. 1). A 35 % HP bleaching gel (Whiteness HP; FGM, Joinville, SC, Brazil) was applied on the enamel side of the disks for different times, originating the

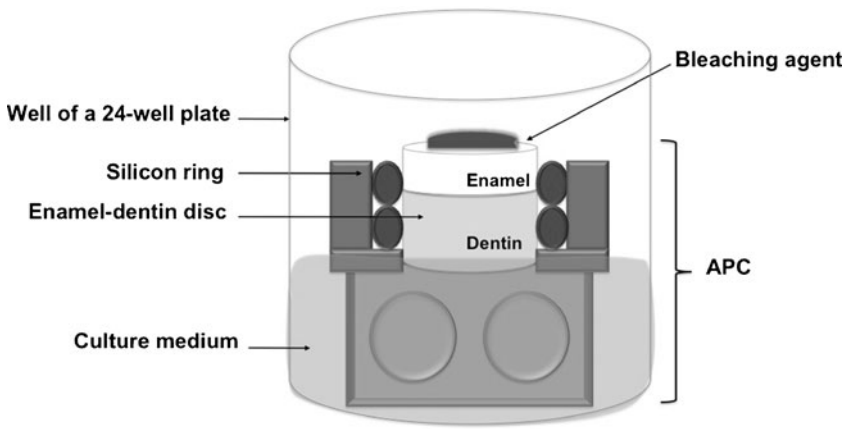

Fig. 1 Schematic presentation of the APC with the enamel and dentin disk in position into a 24-well plate

following groups: G1, no bleaching (control); G2, three consecutive 15-min applications; G3, one 15-min application; G4, three consecutive 5-min applications; and G5, one 5-min application. Eight APC/disk sets were obtained for each experimental group. Six of them were used to evaluate the succinic dehydrogenase (SDH) and alkaline phosphatase (ALP) activities and the other two APC/disk sets were assigned to cell morphology analysis. For each APC/disk sets, an amount of 1 ml of extract was obtained, and two aliquots of 500 μl were used for SDH and ALP evaluation. Two independent experiments were performed for each test (SDH and ALP).

Cell metabolism (SDH activity)

Prior to the bleaching procedure, odontoblast-like cells MDPC-23 (30,000 cells/cm^2) were seeded on wells of 24-well sterile acrylic plates using plain DMEM supplemented with 10 % fetal bovine serum (Gibco, Grand Island, NY, USA). The plates containing the cells were maintained in a humidified incubator (Isotemp; Fisher Scientific, Pittsburgh, PA, USA) with 5 % CO_2 and 95 % air at 37 °C for 72 h.

Immediately after the bleaching procedure, 500 μl of extract, i.e., culture medium containing the products of bleaching gel degradation that diffused through the enamel/dentin disks, was collected from each APC/disk sets (n=6) and applied on the previously cultured MDPC-23 cells for 60 min. Then, six wells of each group were used for analysis of cell metabolism by assessment of the activity of mitochondrial SDH enzyme using the methyl tetrazolium assay, as previously described [18, 20, 22]. Two independent experiments were performed. The data for SDH activity from the two independent experiments (n=6) were compiled and submitted to the Kruskal–Wallis test complemented by the Mann–Whitney test for pairwise comparison of treatments. A significance level of 5 % was set for all analyses.

Alkaline phosphatase activity

Aliquots (500 μl) of the extracts from the same APC/disk sets used for SDH evaluation were assigned for the ALP activity analysis (n=6). Therefore, aliquots from each APC/disk sets were used for analysis of ALP activity using the colorimetric endpoint assay (ALP Kit; Labtest Diagnóstico S.A., Lagoa Santa, MG, Brazil) employed in previous studies [18]. The MDPC-23 cells were maintained in contact with the extracts for 60 min. Then, the extracts were aspirated and 1 ml of serum-free DMEM was applied on the cells. After 24-h incubation, 0.1 % sodium lauryl sulfate (Sigma-Aldrich, St. Louis, MO, USA) was added to the wells for 30 min to produce cell lysis. In a buffered environment (pH 10.1) at 37 °C, thymolphthalein monophosphate (22 mmol/L) was added, which is hydrolyzed in the presence of ALP, releasing thymolphthalein. Next, a color reagent (sodium carbonate, 94 mmol/L; sodium hydroxide, 250 mmol/L) was added, which reacts with the hydrolyzed thymolphthalein, changing the color of the final product. A photometric analysis was performed at 590 nm wavelength with an enzyme-linked immunosorbent assay (ELISA) plate reader (Tp Reader; Thermoplate, Nanshan District, Shenzhen, China). The absorbance value for ALP was converted into units per liter by a standard curve with known amounts of ALP.

Total protein (TP) dosage was performed for normalization of ALP according to the Read and Northcote [26] protocol. Lowry reagent solution (Sigma-Aldrich Corp.) was added to the remaining sample in each well. After 20 min at room temperature, Folin–Ciocalteu's phenol reagent solution (Sigma-Aldrich Corp.) was added to each sample and homogenized. Thirty minutes later, the absorbance of the test and blank tubes was measured at 655 nm wavelength with the ELISA plate reader. The absorbance value obtained was converted into milligrams per liter by a standard curve of protein.

The final value of ALP was normalized by TP obtained from each well, dividing the value of ALP dosage by the value of TP dosage, obtaining the value of ALP activity into units per milligram of protein. Two independent experiments (n=3) were performed and the data compiled were submitted to Kruskal–Wallis test complemented by Mann–Whitney test for pairwise comparison of treatments. A significance level of 5 % was set for all analyses.

Cell morphology (scanning electron microscopy [SEM])

Cell morphology was examined on cells adhered to cover glasses that had been placed on the bottom of the 24-well plates before seeding. Two APC/disk sets from each experimental group were used for this analysis. A 500-μl aliquot obtained from the extracts was applied to the cultured cells. The MDPC-23 cells were incubated in contact with the extracts for 60 min. Then, the extracts were aspirated, and the cells rinsed with phosphate buffer saline solution and fixed with 2.5 % buffered glutaraldehyde at room temperature. After that, the cells were postfixed with 1 % osmium tetroxide, dehydrated in a series of increasing ethanol concentrations (30, 50, 70, 95, and 100 %), and finally immersed in 1,1,1,3,3,3-hexamethyldisilazane. The cover glasses with the cells on them were mounted on metallic stubs, stored in a vacuum desiccator for 72 h at room temperature, and sputter-coated with a gold layer. Cell morphology was examined with a scanning electron microscope (Philips FEG XL 30; Oxford Instruments, Inc., Concord, MA, USA).

Quantification of HP diffusion

In order to quantify HP trans-enamel and trans-dentinal diffusion, APC/disk sets were placed in sterile 24-well plates containing 1 ml of acetate buffer solution (2 mol/L, pH 4.5) in such a way that the enamel surface of the disks was left exposed. The bleaching procedure was performed as described previously (n=8). An aliquot of 100 μl of buffer solution from each well was transferred to test tubes containing 2.6 ml deionized water and 250 μl of 0.5 mg/ml leucocrystal violet (Sigma-Aldrich Corp.). The tubes were agitated and 50 μl of horseradish peroxidase enzyme solution (1 mg/ml; Sigma-Aldrich Corp.) was added. Then, three 100-μl aliquots of each tube were transferred to 96-well plates and the optical density of the solutions was measured at 600 nm wavelength in an ELISA plate reader. A standard curve of known HP concentrations was used for conversion of the optical density obtained in the samples into micrograms of HP and the data were related to micrograms per milliliter of acetate buffer solution. HP quantification data (micrograms per milliliter) were analyzed statistically by analysis of variance and Tukey's test at 5 % significance level.

Bleaching efficacy

To analyze the efficacy of the bleaching protocols, enamel/dentin disks were subjected to staining in a black tea solution (n=8) [27]. The dentin surface of the disks was etched with 37 % phosphoric acid (3M ESPE, St. Paul, MN, USA) during 60 s and rinsed with distilled water for the same amount of time. The disks were placed individually in the wells of 24-well plates with the dentin side turned upward. One milliliter of a standardized solution of black tea (Leão Jr. S.A.; Fazenda Rio Grande, PR, Brazil) produced by filtration of 2 g of tea in 100 ml of boiling water during 5 min. The disks were left in the black tea solution at 37 °C during 6 days. Color assessment was done using a portable UV–Vis spectrophotometer (Spectro Guide 45/0; BYK-Gardner GmbH, Geretsried, Germany). The measurements were done in the visible range of wavelength (400–700 nm) and using the standard illuminant D65.

The specimens were placed in a white silicone matrix, leaving exposed only the enamel surface. The portable spectrophotometer was positioned over the sample and three readings were made for calculation of the average. The analysis of tooth color was performed before and 24 h after the bleaching procedure. The enamel surface of the disks remained in contact with artificial saliva between readings [22]. In the control group, no treatment was performed on the enamel surface.

Color change values of the specimens were determined using the $L^*a^*b^*$ system established by the Commission Internationale de l'Eclairage (CIE), which allows color specification within a three-dimensional space. The L^* axis represents the degree of lightness in a sample and is measured in a scale that ranges from 0 (black) to 100 (white). The a^* plane represents the saturation of green and red in the sample, while the b^* plane represents the saturation of blue and yellow. L^*, a^*, and b^* values were recorded for each specimen before and after bleaching. The difference between L^*, a^*, and b^* at the beginning and end of the experiment was expressed as ΔL^*, Δa^*, and Δb^*. The overall color change of each specimen, expressed as ΔE^*, was calculated using the following equation: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Data were analyzed by Kruskal–Wallis and Mann–Whitney tests for pairwise comparison. All statistical tests were considered at the level of significance of 5 %.

Results

The results for SDH activity (cell metabolism), ALP activity, and TP dosage are presented in Table 1. A significant decrease in SDH activity was observed only between G2 (three 15-min applications) and G1 (control) (Mann–Whitney; $p<0.05$). Considering G1 as having 100 % of cell metabolism, the percentage of cell metabolism in G2, G3,

Table 1 Data from SDH activity (absorbance, 570 nm), TP dosage (in milligrams per liter), and ALP activity (in units per milligram) of MDPC-23 cells exposed to extracts from the different groups

Group	SDH enzyme	TP production	ALP activity
G1 (control)	1.054 (1.005–1.084)a	87.32 (84.54–89.51)a	5.50 (5.38–5.56)a
G2	0.628 (0.699–0.757)b	34.42 (34.46–37.29)c	2.03 (1.87–2.26)c
G3	1.042 (1.023–1.098)a	56.43 (49.95–63.35)bc	2.70 (2.08–2.91)b
G4	1.113 (1.098–1.123)a	72.40 (68.02–75.97)ab	2.25 (2.17–2.35)b
G5	1.254 (1.209–1.301)a	106.45 (103.30–109.97)a	6.25 (6.04–6.46)a

All values are given as median (25th percentile–75th percentile); n=6. Data represent the results obtained from two independent experiments. In columns, medians followed by the same letters do not differ significantly (Mann–Whitney, $p>0.05$)

G4, and G5 was 59.6, 99.0, 105.5, and 119.0 %, respectively. A statistically significant decrease (Mann–Whitney; $p<0.05$) of TP dosage relative to the control group (G1) was observed for G2 and G3, while in G4 and G5, MDPC-23 cells produced similar amounts of TP compared to the control (G1) (Mann–Whitney; $p>0.05$). Similarly, statistically significant decrease of ALP activity was observed between the control group G1 and the experimental groups G2, G3, and G4, with the most accentuated decrease in G2 (Mann–Whitney; $p<0.05$). A discrete increase in TP content (21.9 %) and ALP activity (13.63 %) was observed in G5, though without statistical significance when compared with the control group (G1) (Mann–Whitney; $p>0.05$).

A large number of MDPC-23 cells adhered to the glass substrate was observed in the control group (G1). These cells exhibited a wide cytoplasm covering almost the entire cover glass surface (Fig. 2a, b). Application of the 35 % HP gel on the enamel/dentin disks resulted in alterations in cell morphology, regardless of the experimental group. The intensity of these alterations varied according to the contact time of the bleaching gel with the dental surface. In G2, in which three 15-min applications were performed, there were large cell-free areas, indicating the death of cells with consequent detachment from the glass substrate. A few cells that remained adhered to the cover glass exhibited severe morphological alterations (Fig. 2c). In G3 and G4, there was a slight decrease in the number of cells adhered to the glass substrate, and they presented a round morphology (Fig. 2d, e). In G5, where one 5-min bleaching gel application was performed, no decrease in the number of cells adhered to the glass substrate was observed, but the cells exhibited a shrunken cytoplasm, with consequent reduction of their size (Fig. 2f).

The highest HP value was obtained in G2 (6.94 μg/ml), with a statistically significant difference in comparison with the other experimental groups (Tukey; $p<0.05$). For G3 and G4, HP values were 4.76 and 5.09 μg, respectively, without statistically significant difference between them (Tukey; $p>0.05$). The lowest HP values were observed in G5 (2.66 μg/ml), which differed significantly from the other experimental groups (Tukey; $p<0.05$). HP was not detected in the control group. These results are summarized in Table 2.

The results of ΔL^*, Δa^*, Δb^*, and ΔE^* for the specimens subjected to the bleaching procedure are shown in Table 3. Statistically significant alterations were observed only for ΔL^*, Δb^*, and ΔE^*. In all groups, there was a tendency to increased L^* values, indicating color change towards white, and a decrease of b^* values, which demonstrates reduction

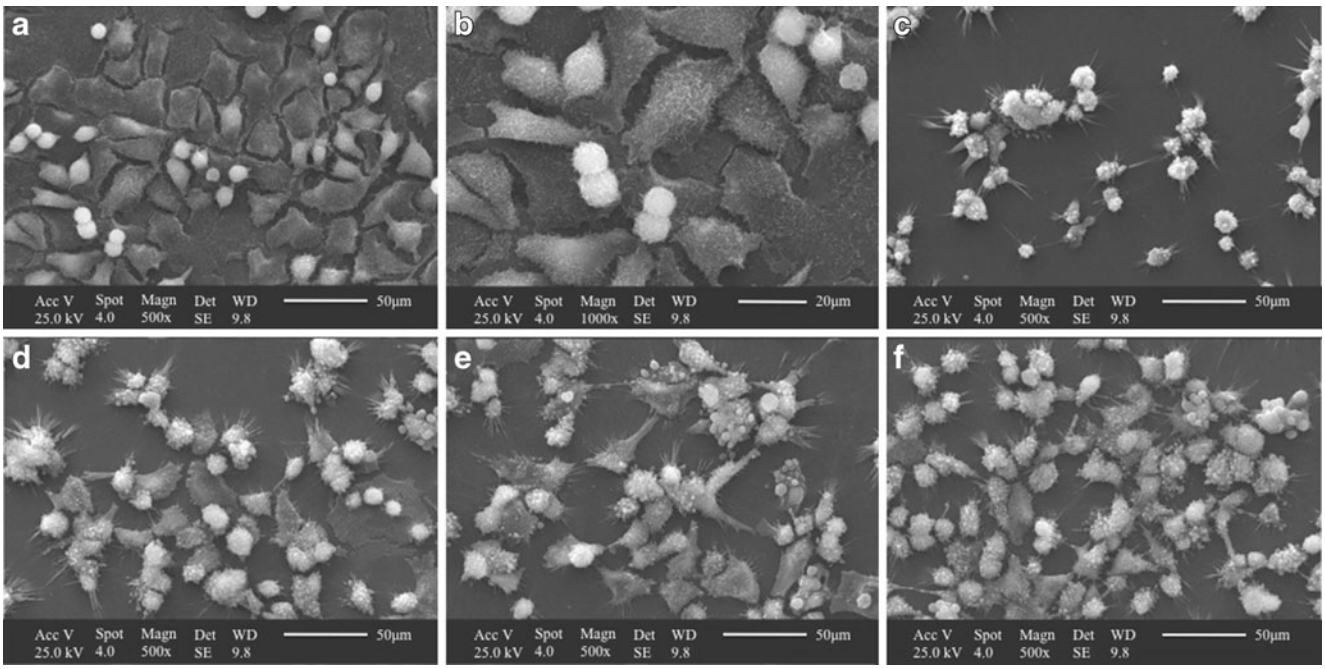

Fig. 2 SEM ×500 and ×1,000. Composite figure of SEM micrographs representative of the control and experimental groups. **a**, **b** G1 (control): A large number of MDPC-23 cells near confluence can be observed on the cover glass where they had been cultured. The cells exhibit a wide cytoplasm, covering almost the entire glass substrate. **c** G2 (three 15-min applications): Several cells were lethally damaged and detached from the cover glass. The few cells that remained adhered to the substrate exhibited remarkable shrinkage of the cytoplasm and some cytoplasmic processes originating from the plasma membrane, which seemed to be adhering them to the substrate. **d** G3 (one 15-min application): A reduction in the number of cells adhered to the glass substrate can be noticed, exhibiting a more rounded morphology and few cytoplasmic processes. **e** G4 (three 5-min applications): In the same way as observed for G3, a smaller number of MDPC-23 cells remained adhered to the cover glass, and these cells exhibited morphological alterations. **f** G5 (one 5-min application): Although a larger number of MDPC-23 cells could be seen on the glass substrate, these cells also exhibited remarkable morphological alterations

Table 2 HP content (in micrograms per milliliter) detected after the application of a 35 % HP bleaching gel on enamel/dentin disks for different times (in minutes)

Group	Hydrogen peroxide (μg/ml)[a]	Statistical comparison[b]
G1 (control)	Not detected	–
G2	6.94 (0.86)	a
G3	4.76 (0.28)	b
G4	5.09 (0.43)	b
G5	2.66 (0.48)	c

[a] Numbers are mean (standard variation), $n=8$

[b] The same letter do not differ statistically (Tukey, $p>0.05$)

of the yellowish hue. G2 exhibited the highest ΔL^*, Δb^*, and ΔE^* values, with a statistically significant difference from all other groups ($p<0.05$). G3 and G4 presented intermediate color change values, with no statistically significant differences between them for all the analyzed variables ($p>0.05$). On the other hand, G5 exhibited the lowest color change values, with no statistically significant differences from the control group, G3, and G4 ($p<0.05$).

Discussion

Traditional in-office vital tooth bleaching therapies applying gels with high HP concentrations on the enamel surface for 30–45 min in a single session is frequently associated with a high incidence of postoperative tooth sensitivity [7]. It has also been reported that severe pulpal damage may occur when these agents are applied on the buccal surface of teeth with small enamel and dentin thicknesses [12, 13, 16]. Despite these shortcomings, tooth bleaching has brought an important contribution to modern esthetic dentistry by offering a noninvasive procedure in which severe tooth darkening can be minimized without removal of sound dental tissue [1]. Overtime, much effort has been directed to the search of bleaching protocols that are at the same time clinically efficient and harmless to the teeth [4–6, 28]. The present study evaluated how shortening the application time of a commercial 35 % HP bleaching gel on enamel would affect the trans-enamel and trans-dentinal HP diffusion as well as its toxic effects to odontoblast-like cell cultures and in vitro bleaching efficacy.

All the bleaching protocols evaluated in this study resulted in trans-enamel and trans-dentinal HP diffusion in an application time-dependent manner; longer application times resulted in higher penetration of HP through the dental tissues. The highest HP diffusion (6.94 μg/ml) was observed in G2, in which the bleaching protocol followed the manufacturer's instructions (three consecutive 15-min applications). This protocol also resulted in a significant decrease (40.42 %) in the metabolism of the MDPC-23 cells, TP dosage, and ALP activity. The SEM analysis showed a significant reduction in the number of viable cells adhered to the cover glasses after this bleaching protocol, and the few cells remaining on the substrate exhibited severe morphological alterations (Fig. 2c).

Camargo et al. [10] found an amount of 3.0 μg/ml of HP in the pulp chamber of extracted bovine lateral incisors after application of a 38 % HP bleaching gel on the enamel surface during 40 min. The authors also showed that 43 μg/ml of HP reached the pulp chamber of human third molars subjected to the same bleaching protocol used in bovine incisors. These data confirmed that enamel and dentin from human teeth presents greater permeability than observed in bovine teeth. Therefore, it can be suggested that the same bleaching protocols evaluated in the present study (bovine teeth) may cause higher toxic effects when evaluated in human teeth. Bowles and Ugwuneri [29] found 25.4 μg/ml of HP in the pulp chamber of extracted human maxillary incisors after the application of a 30 % HP agent on enamel for 15 min. Therefore, both these previous studies demonstrated that the diffusion of HP across enamel and dentin to reach the pulp chamber is directly related to the

Table 3 Color change (ΔL^*, Δa^*, Δb^*, and ΔE^*) of the enamel after the application of a 35 % HP bleaching gel for different times (in minutes)

Group	Enamel color change (CIE $L^*a^*b^*$ color system)[a]			
	ΔL^*	Δa^*	Δb^*	ΔE^*
G1	0.15 (−0.23/0.69)c	0.56 (−0.21/−0.94)a	−0.42 (−1.09/0.05)a	1.37 (0.80/1.60)c
G2	5.09 (3.46/5.47)a	−0.77 (−0.87/−0.11)a	−3.74 (−5.96/−2.50)c	8.59 (7.37/9.13)a
G3	2.27 (1.82/2.67)b	−0.43 (−0.55/−0.35)a	−2.20 (−2.79/−1.86)bc	3.52 (3.11/3.65)b
G4	2.12 (1.94/3.70)b	−0.46 (−0.63/−0.24)a	−1.48 (−2.20/−0.85)ab	3.07 (2.17/3.66)b
G5	0.55 (0.35/0.80)bc	−0.25 (−0.34/−0.08)a	−1.86 (−1.99/−0.89)ab	2.09 (1.27/2.70)bc

[a] The maximum for L^* is 100, which represents a perfect reflecting diffuser. The minimum for L^* is 0, which represents black. The a^* and b^* have no specific numerical limits. Positive a^* is red; negative a^* is green. Positive b^* is yellow; negative b^* is blue. ΔL^*, Δa^*, and Δb^* indicate how much the baseline and after treatment differ from one another in L^*, a^*, and b^*. ΔE^* is the total color difference. Values are presented as the median (25th percentile/75th percentile), $n=8$. Within each column, groups identified by the same letter do not differ statistically (Mann–Whitney, $p>0.05$)

contact time of the bleaching agent with enamel, such as that observed in the present investigation, as well as the concentration of HP in the product. The high inward HP diffusion described in human teeth may explain the data from de Souza Costa et al. [16], who demonstrated the occurrence of coagulation necrosis in the pulp tissue of human mandibular incisors subjected to a 38 % HP bleaching gel application on enamel for 30 min (three applications of 10 min each).

Using a methodology similar to that employed in the present in vitro study, Trindade et al. [20] demonstrated that three 15-min applications of a 35 % HP bleaching gel on enamel/dentin disks obtained from bovine incisors decreased the MDPC-23 cell metabolism by 92 %. However, the authors applied the extracts (culture medium+components released from bleaching agent that diffused across enamel and dentin) on the cells for 24 h, resulting in a higher toxic effect compared to the present investigation in which the extracts were applied on the cultured cells for only 1 h. These data determined that HP diffused through enamel and dentin remains active to cause cell damage for long periods, such as 24 h.

When the 35 % HP bleaching gel was applied for a total time of 15 min (G3 and G4), the values of SDH activity obtained were not statistically different compared to the control group (G1). The SEM analysis for these groups showed a slight reduction in the number of cells adhered to the glass substrate and only a discrete alteration in the cell morphology (Fig. 2d, e). Therefore, in spite of being less aggressive to the cells compared to the traditional bleaching protocol (G2), both therapies using shorter application times still caused in vitro cell aggression. A similar result was found in a recent investigation in which a 10 % carbamide peroxide bleaching gel was applied on enamel/dentin disks for 8 h [22]. In that study, no significant reduction in the cell metabolism was observed; however, the SEM analysis revealed alterations in cell morphology, especially cytoplasm shrinkage.

In the present study, application of the HP bleaching gel for only 5 min on the enamel surface of the disks resulted in an increase in cell metabolism (18.98 %), TP dosage (21.9 %), and ALP activity (13.63 %). Although this increase in cell activity was not statistically significant compared with the control group, it seems to indicate that the low HP content (2.66 μg/ml) that crossed the enamel/dentin barrier caused a low-intensity aggression that might have stimulated the MDPC-23 cells. In the study by Lee et al. [23], low HP concentrations, such as 0.2 and 0.3 mmol/L, applied directly on MDPC-23 cell cultures induced the occurrence of oxidative stress with a significant increase in the ALP activity and formation of mineralization nodules. Pulp cell stimulatory effects were also demonstrated Min et al. [24]. The authors showed that a 0.2-mmol/L concentration of HP applied directly on primary human pulp cell cultures produced an increase in HO-1 protein expression, upregulating the expression of DSPP and DMP-1 proteins which are both intimately related to dentin matrix deposition and mineralization. Matsui et al. [25] obtained similar results after exposure of primary human pulp cell cultures to 100 μmol/L HP for 10 min. According to the authors, the generation of reactive oxygen species as a result of HP treatment led to the formation of calcium phosphate in the cells and increased the expression of osteocalcin and osteopontin proteins. Thus, the authors demonstrated that HP induced the pulp cells to differentiate into specific kinds of cells with the ability to form dentin. Therefore, based on the data obtained in the present in vitro study which corroborated with those previous investigations [23–25], it may be speculated that the presence of low concentrations of HP in the pulp tissue may play an important role in pulp–dentin stimulation and healing. Nevertheless, further studies are needed to determine tooth bleaching therapies capable of causing pulp cell stimulatory effects and bleaching outcome as esthetically effective as required by the patients.

In the analysis of tooth color change, it was observed that the bleaching efficacy was also dependent on the contact time of the product with the enamel surface. It has been suggested that ΔE^* values above 3.3 are clinically perceptible color changes [30, 31]. According to this parameter, in the present study, only G2 and G3 presented a clinically relevant color change after a single bleaching session. As expected, the highest ΔE^* value (8.59) was obtained in the group in which the bleaching protocol recommended by the manufacturer was employed (G2). Several studies have demonstrated that traditional in-office tooth bleaching therapies using agents with high HP concentrations for 30 to 45 min produce an intense tooth color change already in the first clinical session [4–6, 18, 32]. In the present study, when a total bleaching time of 15 min was used (G3 and G4), the ΔE^* values (3.52 and 3.07, respectively) were significantly lower than those obtained in G2. However, both groups presented an increase in ΔL^* values and a decrease in Δb^* values, which demonstrates color change towards white and a decrease of yellowish hue, producing the whitening effect on dental structure. Therefore, it could be suggested that additional bleaching sessions would produce a bleaching outcome as esthetically effective as the one observed in G2. This phenomenon was also observed in G5 where enamel/dentin disks received a single 5-min application of the bleaching gel. Although this group did not differ significantly from the control group (G1), no significant differences were found from G3 and G4 either for any of the analyzed color parameters. So, it is likely that longer sessions when using these bleaching protocols would increase bleaching efficacy, as the saturation of the chromophores of hard dental tissues would occur in a slow and gradual manner.

As far as dental procedures are concerned, esthetic efficacy should always be preceded by low—or ideally no—toxic potential to the pulp–dentin complex. The present study and previous investigations [18–20] have demonstrated that the

traditional tooth bleaching protocol with 35 % HP gels is toxic to the pulp cell cultures. However, the results of laboratory researches cannot be directly extrapolated to the clinical condition. Further studies are required to evaluate whether some of the deleterious effects observed in vitro would also occur in vivo, as it is impossible to replicate in the laboratory all physiological conditions of the pulp–dentin complex. It is known that teeth present a number of factors, such as intrapulpal pressure, local antioxidant activity, immunological pulp system, blood vessels, and a lymphatic system, that could prevent or at least reduce the damage to the pulp cells [33–35]. Therefore, bleaching protocols in which an agent with high HP concentration is used on dental enamel with short application times in different clinical sessions, in spite of increasing the treatment time, would considerably minimize the aggressive effects associated with the traditional bleaching protocol with longer application times. Continuous research will determine the clinical viability of such protocols as well as their possible adverse effects in vivo.

Conclusions

According to the methodology used in this study, it can be concluded that HP diffusion across the dental structure as well as the trans-enamel and trans-dentinal cytotoxicity of the 35 % HP bleaching gel is proportional to the contact time of the product with the tooth surface. Despite the intense tooth color change caused by the 35 % HP bleaching gel applied for 45 min on enamel, this bleaching protocol produced the highest diffusion of HP through the dental tissues which promoted the most severe toxic effects to the cultured pulp cells. On the other hand, the total bleaching times of 5 or 15 min resulted in lesser trans-enamel and trans-dentinal HP diffusion, which minimized the cytopathic effects to the odontoblast-like cell cultures.

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Conflict of interest The authors declare that they have no conflict of interest.

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