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Influence of Restoration Type on the Cytotoxicity of a 35% Hydrogen Peroxide Bleaching Gel

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Clinical Relevance

The application of a 35%-H_2O_2 bleaching gel to a dental surface containing a hydrolytically degraded resin-modified glass ionomer cement restoration results in more intense diffusion of H_2O_2 into the pulp chamber, which increases pulp cell cytotoxicity.

SUMMARY

Objectives: The tooth/restoration interface may act as a pathway for hydrogen peroxide (H_2O_2) diffusion into the pulp chamber. Therefore, the influence of resin-modified glass ionomer cement (RMGIC) and resin composite simulated restorations on the cytotoxicity of an in-office bleaching gel was assessed *in vitro*.

Materials and Methods: Cavities in enamel/dentin discs restored with RMGIC Vitremer (3M ESPE) or Single Bond/Filtek Z350 (3M ESPE) resin composite (RC) were subjected or not subjected to hydrolytic degradation (HD). A 35%-H_2O_2 bleaching gel was applied to simulated restored and nonrestored enamel surfaces, and culture medium in contact with the dentin substrate (extract) was collected and applied to MDPC-23 cells. Nonrestored discs subjected or not subjected to bleaching were used as positive and negative controls, respectively. Cell viability, oxidative stress, interleukin (IL)-1β expression, alkaline phosphatase (ALP) activity, and mineralized nodule deposition were evaluated. The H_2O_2 in the extracts was quantified. Data were subjected to statistical analysis.

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Results: Higher oxidative stress associated with reduced cell viability, ALP activity, and mineralized nodule deposition was observed for all bleached groups compared with the negative control group. The RMGIC/HD group, which presented the highest H_2O_2 diffusion, had the lowest values of cell viability, ALP activity, and mineralized nodule deposition, as well as significantly increased IL-1β expression.

Conclusions: Dental cavities restored with the RMGIC subjected to hydrolytic degradation allowed for more intense diffusion of H_2O_2 into the pulp chamber, intensifying the toxicity of a 35%-H_2O_2 bleaching gel to pulp cells.

INTRODUCTION

Vital tooth bleaching is a cosmetic technique widely used in clinical dental practice. However, this procedure is characterized by a high prevalence of tooth sensitivity, which may cause considerable to intolerable discomfort to patients.[1-5] This side effect has been associated with the rapid transtooth diffusion of hydrogen peroxide (H_2O_2),[6,7] which is toxic to pulp cells.[8-12] An important factor that can influence the diffusion of H_2O_2 into the pulpal space is the presence of cavity restorations on tooth surfaces.[13-15] It has been shown that the tooth/restoration interface may act as a pathway for H_2O_2 diffusion into deep dentin and, consequently, into the pulp chamber, depending on the dental material used to create the restoration.[14,15] Bonafé and others[16] showed that the application of a 35%-H_2O_2 bleaching gel to anterior teeth containing aged resin composite restorations resulted in more intense and prevalent tooth sensitivity. The authors reported that the dental material and technique used to restore dental cavities, as well as the quality of restoration margins, may affect H_2O_2 diffusion. This fact was corroborated by Soares and others,[17] who showed that resin composite (RC) restorations performed with etch-and-rinse adhesives did not influence the transtooth cytotoxicity of a professional bleaching protocol. However, a significantly higher toxic effect was observed when a two-step self-etch adhesive was used to perform such adhesive restorations.[18]

Instead of the limited range of clinical indications, resin-modified glass ionomer cements (RMGICs) have been used in operative dentistry to restore carious or noncarious cervical lesions in permanent teeth, since these areas are not subjected to compressive loads.[19] The main advantage of this kind of dental material in this clinical situation is related to the chemical adhesion to tooth structure, biocompatibility, and release of fluoride.[19] However, the bond strength of RMGICs to enamel and dentin is lower than that observed for RC restorations,[20,21] making the application of bleaching gels to tooth/RMGIC restorative material interfaces a concern.[22] Therefore, the present study evaluated the effects of RMGIC and etch-and-rinse adhesive/RC restorations, subjected or not subjected to hydrolytic degradation, on the indirect toxicity of a 35%-H_2O_2 bleaching gel to odontoblast-like cells. The null hypothesis is that these restorations have no effect on the cytotoxicity of the bleaching protocol.

METHODS AND MATERIALS

Specimen Preparation

Standardized 3.5-mm-thick and 5.6-mm-diameter enamel/dentin discs were obtained from intact bovine incisors, as previously described.[12] Round cavities measuring 1.6 mm in diameter and 2.5 mm deep were prepared on part of each disc by means of a high-speed, water-cooled cylindrical diamond bur (#1095; KG Sorensen, Barueri, SP, Brazil). Therefore, the remaining dentin thickness between the base of the cavity preparation and the simulated pulp chamber wall was set to 1.0 mm.[17] The cavities were restored with a RMGIC or with RC associated with two-step etch-and-rinse adhesive, following the manufacturer's instructions, as described in Table 1. The etch-and-rinse adhesive/RC restoration was used as a control since it was previously demonstrated that this restoration does not impact the transenamel and transdentinal MDPC-23 cell cytotoxicity when bleaching, even when it is subjected to hydrolytic degradation (HD).[17]

In the nonrestored discs, no cavity preparation was performed; however, a resin coating was prepared on the enamel surface, with the same diameter as that of the restoration performed on discs of restored groups. Therefore, the enamel surface subjected to bleaching was standardized in both restored and nonrestored groups. For the nonrestored groups, a round enamel surface with 1.6-mm diameter was etched with 37% phosphoric acid for 30 seconds, followed by the application of two layers of bonding agent and light-curing for 20 seconds. Finally, a layer of composite resin (1.0 mm thick × 1.6 mm diameter) was applied and also light-cured for 20 seconds (Figure 1A).

Table 1: *Application Protocol for Each Restorative Material*

Material	Application Protocol
RMGIC (Vitremer; 3M ESPE, St Paul, MN, USA)	A brush was used to apply the Vitremer primer (HEMA+polycarboxylic acid) for 30 seconds to enamel and dentin, followed by gentle air-drying for 15 seconds. This process was repeated once more, and the primer was then light-cured (450 mW/cm^2 Curing Light XL 300, 3M ESPE) for 20 seconds.
	One level scoop and one drop of liquid of Vitremer were dispensed onto the mixing pad and mixed within 45 seconds.
	The material was applied to the cavity by means of a Centrix applicator and light-cured for 40 seconds.
	Twenty-four hours after cavity restoration, the restoration surface was polished with sequential Soflex discs (3M ESPE) at low speed.
RC (Single Bond + Filtek Z350; 3M ESPE, St Paul, MN, USA)	The 37% phosphoric acid etchant (Scotchbond, 3M ESPE) was applied to enamel for 30 seconds and to dentin for 15 seconds and then rinsed for 30 seconds, and the tooth structure was dried with sterilized cotton.
	The first layer of Single Bond was applied under friction, followed by 30 seconds of resting and 10 seconds of gentle air-drying. A second bonding agent layer was applied, and the product was light-cured for 20 seconds under a halogen lamp (450 mW/cm^2 Curing Light XL 300, 3M ESPE).
	The cavity was restored by the application of two increments of the nanofilled composite resin Filtek Z350, which were individually light-cured for 20 seconds.
	Twenty-four hours after cavity restoration, the resin surface was polished with sequential Soflex discs (3M ESPE) at low speed.

Abbreviations: HEMA, 2-hydroxyethyl methacrylate; RC, resin composite; RMGIC, resin-modified glass ionomer cement.

Half of the restored discs were subjected to HD, which consisted of a thermocycling regimen in a thermal cycler (MSCT-3 plus; Marcelo Nucci-ME, São Carlos, SP, Brazil) for a total of 20,000 cycles at 5°C and 55°C, with a 30-second dwell time in each bath, followed by 12 months of storage at 37°C in 0.1% thymol-buffered solution, which was replaced weekly. The thymol solution was used to prevent microbial contamination during the storage periods. The dentin surfaces of discs were treated with ethylenediaminetetraacetic acid (EDTA), 0.5 N, for 30 seconds for smear layer removal, and discs were then individually adapted to artificial pulp chambers (APCs) (Figure 1B) by means of two silicone O-rings (Rodimar Rolamentos Ltda, Araraquara, SP, Brazil). To avoid any H_2O_2 diffusion through the edges of the discs, a seal was created with wax on the silicone ring area. The APCs with the enamel/dentin discs in position were sterilized in ethylene oxide gas.

Experimental Procedure

The disc/APC sets were positioned in 24-well plates (Costar Corp, Cambridge, MA, USA) containing 1 mL of serum-free Dulbecco Modified Eagle Medium (DMEM; Sigma-Aldrich, St Louis, MO, USA). A 35% H_2O_2 bleaching gel (Whiteness HP; FGM Produtos Odontológicos Ltda, Joinville, SC, Brazil) was applied to simulated restoration and enamel surfaces of the discs three times for 15 minutes each time. The groups are described in Table 2. Two control groups were used in the present investigation. Nonrestored/nonbleached discs were used in the negative control group (NC), which represents normal cell parameters. The nonrestored/bleached discs were used in the positive control group (PC), which represents the cell responses to bleaching. In total, six APC/disc sets were used for each experimental group. The MDPC-23 cells were seeded on 96-well plates (Costar Corp) at 80% confluence (6000 cells/well; 48 hours) in

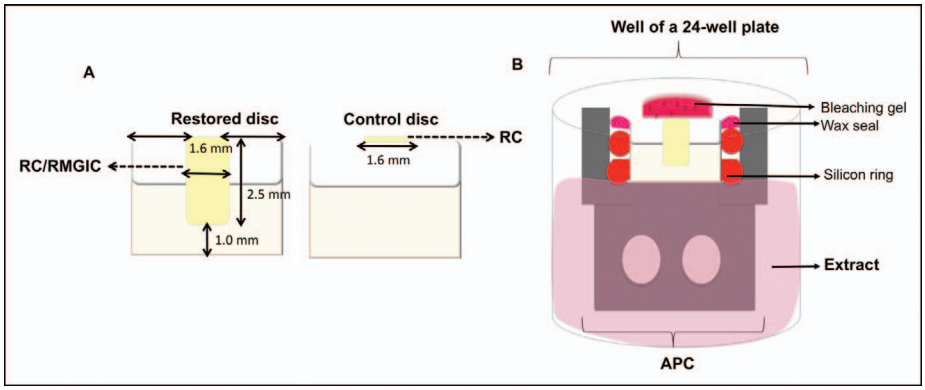

Figure 1. *Representative illustration of experimental protocol. (A) Restored and control discs. (B) APC/disc set positioned in a well of a 24-well plate.*

Table 2: *ACP Disc/Set Distribution into Experimental and Control Groups*

Group	Treatment	Restorative Material	Aging Procedure
NC	Nonbleached	Nonrestored	Water storage – 24 h
PC	Bleached	Nonrestored	Water storage – 24 h
RMGIC	Bleached	RMGIC	Water storage – 24 h
RMGIC/HD	Bleached	RMGIC	Water storage – 12 mo + thermocycling
RC	Bleached	RC	Water storage – 24 h
RC/HD	Bleached	RC	Water storage – 12 mo + thermocycling

Abbreviations: HD, hydrolytic degradation; NC, negative control group; PC, positive control group; RC, resin composite; RMGIC, resin-modified glass ionomer cement.

DMEM plus 10% fetal bovine serum (FBS; Sigma-Aldrich). Immediately after bleaching, the culture medium in contact with the internal dentin surface (extract) of each APC/disc set was collected and distributed into aliquots of 100 μL, which were applied to the cultured cells. The MPDC-23 cells were incubated with the extracts for a period of 1 hour at 37°C and at 5% CO_2, and then the experimental protocols were conducted, as follows.

Cell Viability

For cell viability analysis, the methyl tetrazolium (MTT) assay was performed. Immediately after the one-hour contact time with extracts, the cells were incubated for four hours with MTT solution (5 mg/mL; Sigma-Aldrich) diluted in DMEM without FBS (1:10), and the absorbance of formazan crystals on viable cells was read at a 570-nm wavelength (Synergy H1, Biotek, Winooski, VT, USA). The numeric values obtained from the MTT assay were converted into percentages according to mean absorbance observed in the NC, which was considered as 100% of cell viability.

Interluekin (IL)-1β Protein Expression

The enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA) was used for this analysis. Two 100-μL aliquots of the extracts from each APC/disc set were applied to two wells (100 μL per well) with previously seeded MDPC-23 cells. Immediately after the contact time with extracts, the cells were incubated for 24 hours in DMEM with no FBS (100 μL). The culture media of the two wells of each APC/disc set were then collected (total of 200 μL) and incubated for two hours in ELISA plates previously coated with the primary antibody (overnight at room temperature) and blocked with bovine serum albumin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) solution (one hour). Secondary antibody was then added, followed by reagent and stop solutions. Protein expression was analyzed by spectrophotometry (455 nm) with standard curves containing defined concentrations of the cytokine (Synergy H1, Biotek).

Oxidative Stress Generation

The production of reactive oxygen species (ROS) in cultured cells was quantified by means of a cell-permeant fluorescence probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA; Life Technologies, San Francisco, CA, USA) (n=6). The cells were incubated at 37°C and at 5% CO_2 with 5 μM carboxy-H_2DCFDA for 10 minutes and were then exposed for one hour to the extracts (100 μL) of each experimental group. After this period, the fluorescence intensity was monitored at 592-nm excitation and 517-nm emission by means of a microplate fluorescence reader (Synergy H1, Biotek).

Alkaline Phosphatase (ALP) Activity

Immediately after the contact with the extracts, the cells were cultured in osteogenic medium (DMEM plus 10% FBS, supplemented with 10 nmol/L β-glycerophosphate and 50 μg/mL sodium ascorbate; Sigma Chemical Co, St Louis, MO, USA) for seven days (the medium was changed daily). After the seven-day incubation period, the ALP activity was assessed (n=6). Cell lysis was performed (0.1% sodium lauryl sulfate; Sigma), and an aliquot was transferred to glass tubes containing thymolphthalein monophosphate substrate at 37°C (End point assay; Labtest, Lagoa Santa, MG, Brazil). After a 10-minute incubation period at 37°C, the color reagent (sodium carbonate and sodium hydroxide) was added, and the absorbance was read at a 590-nm wavelength (Synergy H1, Biotek), which was converted into U/L by means of a standard curve. Total protein (TP) dose was performed for normalization of ALP according to the Read and Northcote protocol, as previously described.[10] The absorbance of the test and blank tubes was measured at a 655-nm wavelength (Synergy H1, Biotek) and converted into mg/L by a standard protein curve. The final value of ALP was normalized by TP obtained from each well,

with the value of ALP activity measured as U/mg of protein. The mean ALP activity value of the NC was considered as 100% of ALP activity, and the percentage value for each sample was calculated based on this parameter and used for statistical analysis.

Mineralized Nodule Deposition

For assessment of the quantity of mineralized nodules deposited (n=6), Alizarin Red staining was performed. The cell cultures after the incubation period with extracts were also cultured in osteogenic medium for seven days. The cells were then fixed with cold 70% ethanol for one hour, washed with deionized water, and then stained with Alizarin Red dye (40 mM, pH 4.2; Sigma-Aldrich) for 20 minutes under gentle shaking (VDR Shaker, Biomixer, Ribeirão Preto, SP, Brazil). After aspiration of unincorporated dye, the cells were washed twice with deionized water for the removal of excess stain. The cells were then incubated with 10% cetylpyridinium chloride (Sigma-Aldrich) for 15 minutes under agitation to solubilize the nodules. The absorbance of the resulting solution was measured at 570 nm (Synergy H1, Biotek). The percentage of calcium deposition for each experimental group was calculated based on the mean value of the NC as 100% of staining.

Quantification of H_2O_2 Diffusion

The amount of H_2O_2 present in the extract was also quantified. Aliquots of 100 μL obtained from extracts of each group were transferred to tubes containing 900 μL of acetate buffer solution (2 mol/L, pH 4.5) to stabilize the H_2O_2. After that, an aliquot of 500 μL of the buffer solution plus H_2O_2 from each tube was transferred to test tubes containing 2.2 mL of deionized water and 250 μL of 0.5 mg/mL leucocrystal violet (Sigma-Aldrich Corp). The tubes were agitated, and a 50-μL quantity of horseradish peroxidase enzyme solution (1 mg/mL; Sigma-Aldrich Corp) was added. Then, aliquots of each tube were transferred to 96-well plates, and the optical density of the solutions was measured at 600-nm wavelength (Synergy H1, Biotek). A standard curve of known H_2O_2 concentrations was used for conversion of the optical density obtained in the samples into μg of H_2O_2 per mL of extract.

Statistical Analysis

To verify the reproducibility of data, we performed two independent experiments for all protocols in this study. Thereafter, data were compiled and subjected to the Levene test for the verification of homoscedasticity. Data from cell viability, H_2O_2 diffusion, Alizarin Red, and ALP activity were subjected to one-way analysis of variance and Tukey test. Fluorescence intensity data of carboxy-H_2DCFDA and protein expression of IL-1β were analyzed by the Kruskal-Wallis and Mann-Whitney tests. The variables cell viability and H_2O_2 diffusion were correlated by Pearson linear correlation analysis. All tests were set at a significance level of 5%. SPSS 19.0 software (SPSS Inc, Chicago, IL, USA) was used to run the statistical analyses.

RESULTS

Cell Viability/H_2O_2 Diffusion

Significant reductions in cell viability relative to the NC were observed for all bleached groups. The percentages of cell viability reduction were 38.7%, 38.5%, 61.7%, 39.4%, and 31.5% for the PC, RMGIC, RMGIC/HD, RC, and RC/HD groups, respectively. A significant difference from the PC was observed only for the RMGIC/HD group. No H_2O_2 was detected in the NC, which was discarded from statistical analysis. The highest H_2O_2 value was found for the RMGIC/HD group, which was significantly higher than that of the other experimental groups. Considering the PC as presenting 100% of H_2O_2 diffusion, about 125% of H_2O_2 diffusion was observed in the RMGIC/HD group. These data are demonstrated on Figure 2. The linear Pearson correlation analysis demonstrated a significant negative correlation between the variables cell viability and H_2O_2 diffusion (Pearson coefficient of correlation=−0.87, p=0.0001) (Figure 2). Therefore, the higher the H_2O_2 diffusion, the lower the cell viability.

Oxidative Stress Generation/IL-1β Protein Expression

All bleached groups presented significantly more intense fluorescence values for the carboxy-H_2DCFDA probe than did the NC group, demonstrating that in all bleached groups, the cells were under oxidative stress. For IL-1β protein expression, only RMGIC/HD presented values significantly higher than those of the NC and PC (Figure 3).

ALP Activity/Mineralized Nodule Deposition

Significant reductions in ALP activity and mineralized nodule deposition were observed for all bleached groups relative to those in the NC. A reduction of around 29% to 67% of ALP activity and 66% to 82% of mineralized nodule deposition was observed in

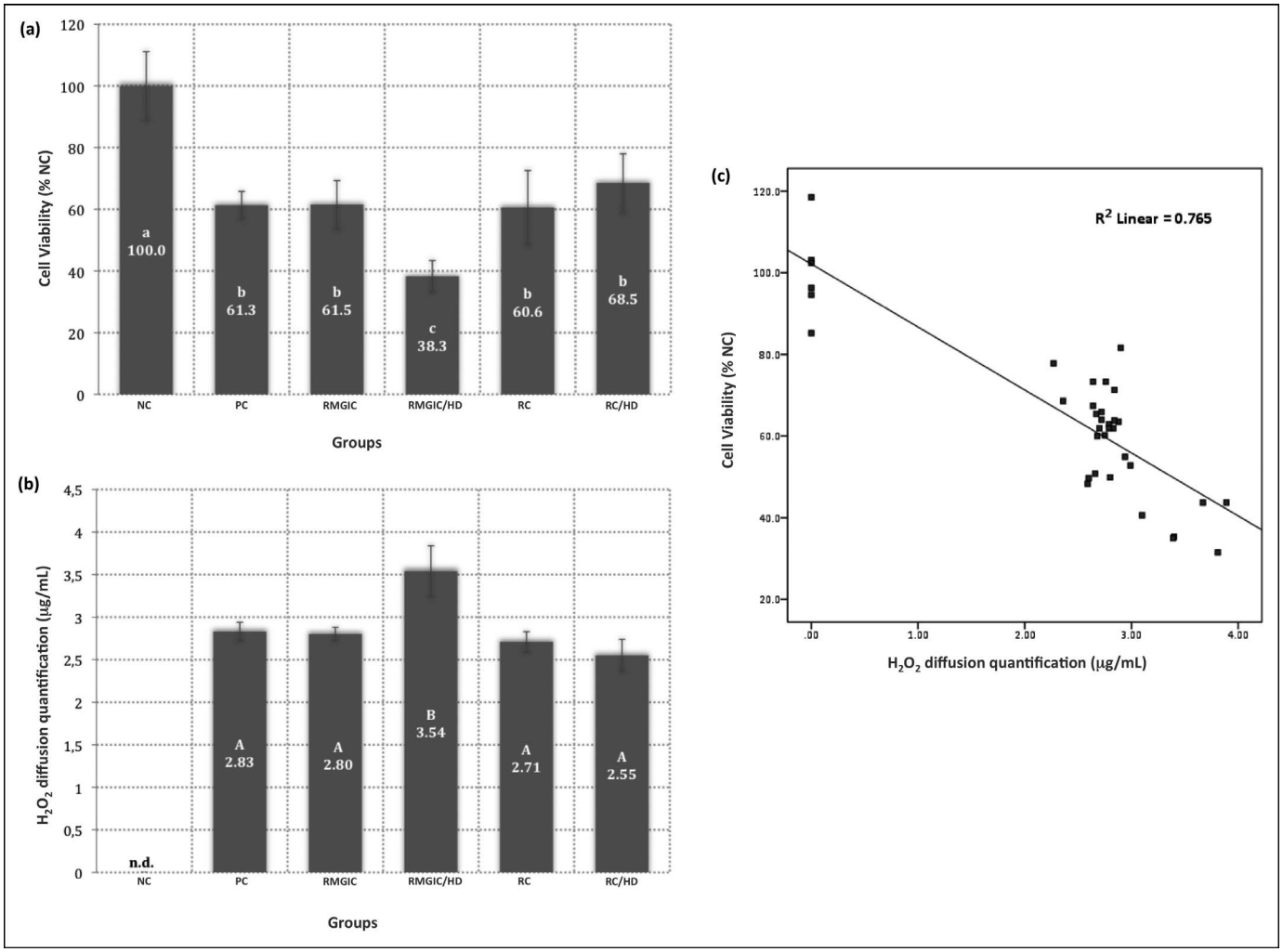

Figure 2. *Bar graph of cell viability (a) and H_2O_2 diffusion (b). Different letters indicate statistically significant differences among groups (Tukey, $p<0.05$). Values are average ± standard deviation ($n=6$). (c) The linear Pearson correlation analysis demonstrating a significant negative correlation between the variables of cell viability and H_2O_2 diffusion (Pearson coefficient of correlation=−0.87, $p=0.0001$).*

bleached groups relative to those in the NC, with RMGIC/HD being the group that featured the highest reduction values (Figure 4).

DISCUSSION

In the present investigation, a traditional in-office bleaching technique was performed on enamel/dentin discs restored with RMGIC or etch-and-rinse adhesive/RC, which were subjected or not subjected to hydrolytic degradation. The amount of H_2O_2 that reached the pulpal space was quantified, and its toxicity to cultured odontoblast-like cells was assessed. The RMGIC used in the present investigation (Vitremer) was previously assessed regarding the tooth/restoration interface quality, such as bond strength to enamel/dentin and nano/microinfiltration. The results of *in vitro* and *in vivo* studies demonstrated that Vitremer featured results similar to or better than those of other brands.[23-28] It was also demonstrated that powder/liquid RMGICs have higher microtensile bond strength to dentin and lower microleakage than do the paste/paste restorations when evaluated immediately after cavity restoration placement and following simulated hydrolytic degradation.[29,30] Therefore, the interface created with the powder/liquid Vitremer in the present study represents a challenging situation for RMGIC restorations exposed to bleaching. Regarding the adhesive system and composite resin used in the RC group, the materials and restoration techniques performed were based upon those described in a previous study[17] in which the tooth/restoration interface had no effect on transenamel and transdentinal H_2O_2 diffusion and cytotoxicity to the MDPC-23 cells, even when subjected to hydrolytic

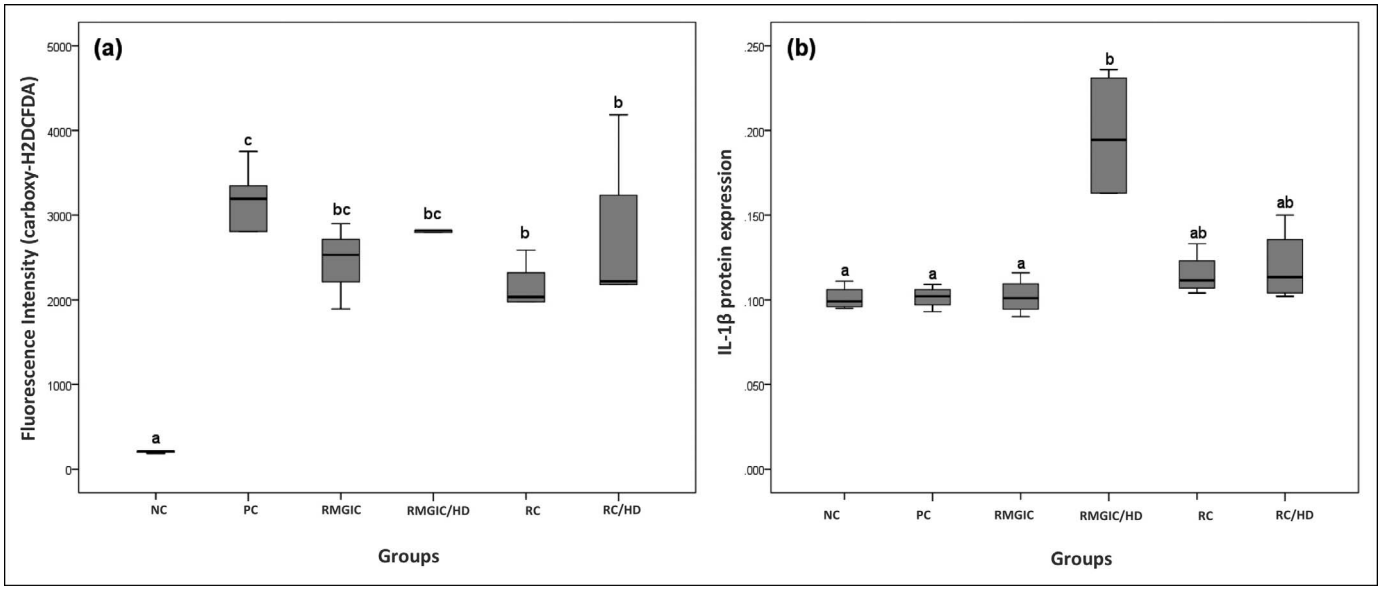

Figure 3. *Box-whisker plot of fluorescence intensity of H_2DCFDA (a), indicating the occurrence or lack of occurrence of oxidative stress and IL-1β expression (b). Different letters indicate statistically significant differences among groups (Mann-Whitney,* $p<0.05$; $n=6$).

degradation and exposed to calcium-containing 20% and 35%-H_2O_2 bleaching gels. Therefore, this group served as a control for comparison of the effect of the RMGIC interface subjected to bleaching. However, materials from only one single brand were tested in the present investigation, which represents a limitation of this study; therefore, the results should be interpreted with caution.

It has already been demonstrated[16] that the in-office bleaching protocol involving three 15-minute applications of a 35% H_2O_2 gel causes intense tooth sensitivity in sound human teeth, which is even higher in adhesive-restored teeth. Therefore, this professional bleaching technique was assessed in the present investigation to determine the role of different tooth/restoration interfaces in transenamel and transdentinal cytotoxicity to pulp cells. Regarding the sound/bleached samples (SD group), the results of the present study are in agreement with those of previous investigations,[10-12,17] in which highly concentrated H_2O_2 bleaching gels applied to sound tooth surfaces for 45 minutes caused moderate cytotoxicity, characterized by decreased cell viability from 21% to 50%.[31,32] These data have been correlated with the intense tooth sensitivity claimed by the patients subjected to traditional in-office bleaching, as observed in clinical trials.[1-5] Using a

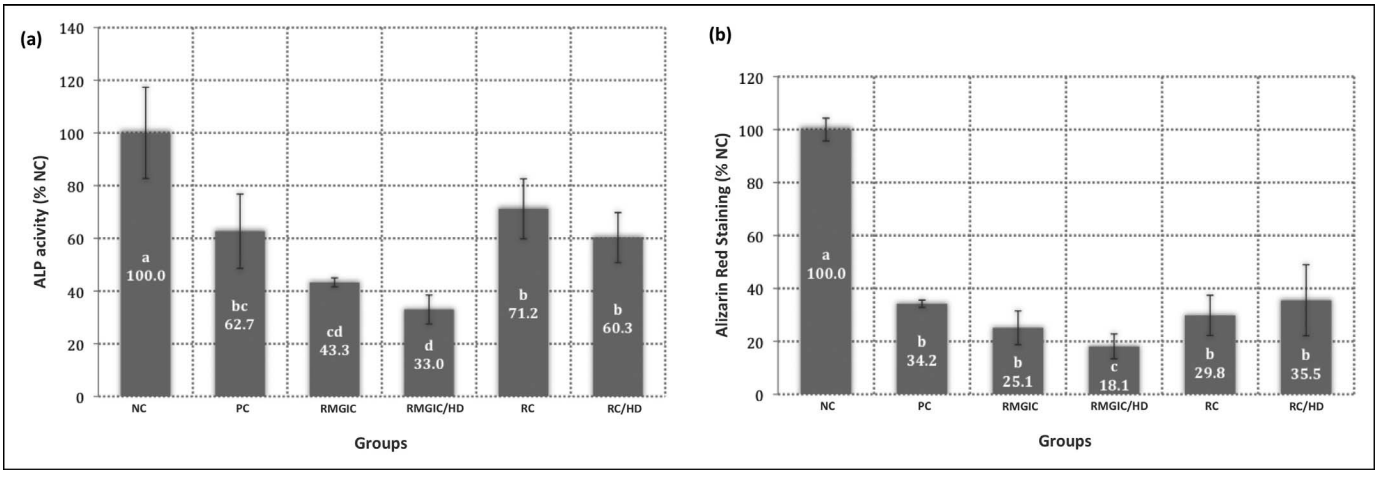

Figure 4. *Bar graph of ALP activity (a) and Alizarin Red staining (b). Different letters indicate statistically significant differences among groups (Tukey,* $p<0.05$*). Values are average ± standard deviation (n=6).*

similar experimental protocol, Soares and others[12] showed that the reduction in MDPC-23 cell viability mediated by traditional in-office protocols is related to intense oxidative stress generation, culminating with cell membrane damage and cell death by necrosis. In clinical situations, oxidative cell damage triggers an inflammatory reaction in pulp tissue,[8,9] during which cell-derived factors are released. This tissue response, associated with increased pulp pressure, may trigger impulses to the intradentinal pulpal nerve fiber endings, causing clinical symptoms of tooth sensitivity.[33] According to the literature, [8-12,17,18] the intensity of these adverse events is directly related to the amount of H_2O_2 that reaches the pulp chamber.

Analysis of the data obtained in the present study also demonstrated that cells exposed to the extracts obtained from bleached enamel/dentin discs were under intense oxidative stress; the fluorescence intensity of carboxy-H_2DCFDA probe increased about 10 to 15 times in bleached groups compared with that in the NC, associated with significant cell viability reduction (from 31.5% to 61.7%). Oxidative stress arises when the balance between the production of ROS and their neutralization by antioxidant systems is disturbed.[34,35] Depending on the intensity of the oxidative stress, the cell components undergo severe oxidative damage, ultimately compromising cell viability.[12] It has been reported[34-36] that ROS accumulated during oxidative stress are transient as a result of their high reactivity, leading to oxidative damage of indispensable biomolecules such as proteins, lipids, and nucleic acids. Therefore, the H_2O_2-mediating reduction in pulp cell viability has been related to two basic mechanisms: 1) induction of oxidative stress conditions due to H_2O_2 diffusion through cell membranes and 2) direct contact of H_2O_2 by-products with cell membranes, causing disruption and cell death.[12,34-36] Associated with these results, significantly decreased ALP activity (from 29% to 57%) and mineralized nodule deposition (from 65% to 82%) were observed at seven days post–bleaching treatment compared with the NC.

The regulation of the odontoblastic phenotype is critical for the homeostasis of the pulp-dentin complex. Under a noxious stimulus of mild intensity, upregulation of odontoblastic secretory activity is observed, which is accomplished by deposition and mineralization of collagen-rich matrix in a process called reactionary dentinogenesis. During this process, several proteins are expressed by odontoblasts, such as DSPP, DMP-1, and ALP.[37] The ALP is considered an initial marker for *in vitro* analysis of the odontoblastic maturation process, since the activity of this protein provides the phosphate needed for the biomineralization process. Nevertheless, mineralization nodule deposition (MND) is considered a late marker, since it demonstrates the capability of mature odontoblasts to deposit and mineralize the organic matrix.[38,39] Recently, Lee and others[40] demonstrated that the toxic concentrations of H_2O_2 in contact with human pulp cells caused intense oxidative stress and cell viability reduction associated with the downregulation of odontoblastic marker expression and the inhibition of odontoblastic differentiation. Also, as determined in the present study, toxic concentrations of H_2O_2 in contact with odontoblast-like cells for a relatively short-term period (one hour) caused intense reductions in ALP activity and MND. Intense reductions in ALP activity by MDPC-23 cells after one-hour exposure to components released from 35%-H_2O_2 gel applied for 45 minutes to enamel/dentin discs were also shown previously.[10] Therefore, traditional in-office bleaching may drastically alter the regenerative capability and homeostasis of pulp tissue.

In the present study, the most intense cell alteration was observed for the RMGIC/HD group, which featured cell viability reduction that was 1.6 times higher, and ALP activity and MND that were around 1.8-1.9 times lower, than in the sound/bleached (SD) group. In addition, in this RMGIC/HD group, higher IL-1β expression was observed compared with that in the other experimental and control groups. One may suggest that the intense cell alterations observed in this study for the RMGIC/HD group were caused by the higher diffusion of H_2O_2 (about 25%) than was observed in the sound/bleached group. Pro-inflammatory cytokines, such as IL-1β, are barely expressed or not expressed in healthy human dental pulp, but their expression level is enhanced in inflamed pulps or after *in vitro* exposure of odontoblasts to lipopolysaccharides.[41-43] The higher the expression of pro-inflammatory cytokines, the higher the intensity of tissue damage in the *in vivo* situation, since pulp cells under inflammatory reaction increase the expression of proteolytic enzymes, promoting disruption of the extracellular matrix.[44,45] This histological event, characterized by inflammatory reactions associated with partial tissue necrosis and the absence of tertiary dentin deposition, was reported by de Souza Costa and others[8] after applying a bleaching gel with a high concentration of H_2O_2 to human sound teeth for 30 minutes. This effect has also been observed in previous *in vitro* studies, in which pulp cells exposed

to pro-inflammatory cytokines at high concentrations for long periods impaired odontoblastic marker expression and the deposition of mineralized matrix.[46-48] Treatment of mesenchymal pulp stem cells from pulp tissue with IL1-β for 48 hours significantly reduced the expression of odontoblastic markers and MND, demonstrating that this cytokine significantly affected odontoblastic differentiation capability.[49]

In groups restored with etch-and-rinse adhesive/RC, no significant difference, compared with the sound/bleached group, was observed for all cell analyses performed, nor was any difference observed for H_2O_2 diffusion. Significant correlation between the amount of H_2O_2 diffusion and cytotoxicity was demonstrated by Pearson correlation analysis; therefore, the higher the H_2O_2 diffusion, the lower the cell viability. One can conclude that the exposure of RMGIC restorations to hydrolytic degradation created an interface pathway for H_2O_2 diffusion. Therefore, the null hypothesis was partially rejected, since the RMGIC restoration, subjected to hydrolytic degradation, had a significant effect on the indirect cytotoxicity of the 35%-H_2O_2 bleaching gel to pulp cells. It has already been demonstrated[20,21] that the shear bond strength of RMGIC to tooth structure is significantly lower than that observed for etch-and-rinse/RC restorations. Consequently, the nano/microleakage of RMGIC is highly affected by hydrolytic degradation.[50,51] A clinical study with cervical restorations of different brands of RMGICs demonstrated that Vitremer scored the highest percentage of intact margins after 18 months relative to other RMGICs; however, this material showed significant reductions in perfect margin adaptation (48%) and microleakage (26%) after six months in the oral cavity. An 18-month period of restoration margin evaluation showed that material fragments crumbled off at the interface, leaving clear local defects.[23] Additionally, Yu and others[22] demonstrated that the tooth-bleaching procedure increased the microleakage at the RMGIC interface subjected to hydrolytic degradation by a factor of 3, whereas no alteration was observed for etch-and-rinse adhesive/RC restorations. According to the authors, the marginal gaps at the RMGIC interface favor H_2O_2 diffusion to cause oxidation on both the restoration[52-54] and the tooth structure,[55,56] as previously demonstrated. In the present study, the RMGIC interface not subjected to hydrolytic degradation did not allow for more intense H_2O_2 diffusion or cytotoxicity to the pulp cells. Khoroushi and Fardashtaki[57] also observed that the microleakage of class V RMGIC restorations (Vitremer), subjected only to 500 cycles of thermocycle regime, was not affected by a 38%-H_2O_2. According to the authors, an appropriate bond in the cavity margins protects restoration from the risk of peroxide penetration during the bleaching procedure, as those promoted by new and nondegraded RMGIC restorations.[57] It is important to mention that the data obtained in this study should be interpreted with caution, since only the conventional RMGIC interface was tested. Previous studies showed that the inclusion of hydroxyapatite (HA) or bioactive glasses to RMGICs may result in the deposition of HA at the restoration interface.[58] Although several investigators have reported that the inclusion of these bioactive substances has little or no effect on bond strength to dentin and microinfiltration,[59-61] one study has reported that these materials have a protective effect when dentin is subjected to demineralization, leading to the deposition of HA at the tooth/restoration interface.[61,62] Therefore, future studies should be performed to assess the effects of bleaching agents on the bioactive RMGICs tooth/restoration interface and pulp cell cytotoxicity.

The more resistant interface created with etch-and-rinse/RC restorations may act as a barrier for H_2O_2 diffusion, and no significant alteration in the tooth/restoration interface takes place after bleaching.[22] Previous studies[63-66] have demonstrated that the quality of the adhesive interface has a significant effect on H_2O_2 susceptibility and that different adhesive systems have variable degrees of susceptibility, as follows: one-step self-etch > two-step self-etch > etch-and-rinse systems. Soares and others[17] did not report differences in H_2O_2 diffusion and its cytotoxicity to MDPC-23 cells when the bleaching gel was applied to sound or etch-and-rinse/RC restored enamel/dentin discs, as observed in the present investigation. However, the authors[18] described the occurrence of higher cytotoxicity when the same bleaching gel was applied to two-step self-etch adhesive/RC restored discs compared with sound/bleached discs.

Bonafé and others[16] observed that teeth containing old adhesive restorations with no clinically perceptible margin degradation and subjected to bleaching presented more intense tooth sensitivity compared with that of sound and bleached teeth. However, the authors had no control over the materials and application protocols used to perform the adhesive restorations. This same situation may be frequently experienced in the clinical dental practice. Some authors[22] have suggested the application of a resin coating around the restoration

margins to avoid the contact of bleaching gels with the tooth/restoration interface. According to Yu and others,[22] the protection of RMGIC restoration margins with resin prevented bleaching-induced microleakage *in vitro*. The authors observed no visually detectable color difference between the coated and uncoated tooth surfaces after bleaching. Therefore, this method seems to be an interesting alternative to be assessed to verify its protective effect on bleaching-induced tooth sensitivity/pulp cell damage in restored teeth.

Finally, according to the results of the present investigation, it is possible to affirm that a degraded tooth/RMGIC interface may act as a pathway for H_2O_2 diffusion through the tooth structure, indirectly influencing pulp cell cytotoxicity. It is known that the association of H_2O_2 and resin monomers results in increased cytotoxicity to pulp cells *in vitro*;[67,68] however, the effects of H_2O_2 and RMGIC components on the increased toxicity observed in the present investigation are unknown. Also, it is important to note that only the in-office bleaching protocol with the three 15-minute applications of 35%-H_2O_2 gel to sound and restored teeth was evaluated in the present investigation. It is well known that H_2O_2 diffusion,[6,7,10-12] pulp cell toxicity,[10-12] and the tooth sensitivity intensity/prevalence[1-5] are directly related to the bleaching protocol. Therefore, analysis of the data obtained in the present study can guide clinicians to an understanding of the risks involved in the application of bleaching gels to restored teeth. However, the toxic effects of at-home and other alternative professional bleaching protocols on pulp cells should be addressed in future studies.

CONCLUSION

According to the results found in this investigation, the presence of a tooth/RMGIC hydrolytically degraded interface favors transtooth H_2O_2 diffusion after three 15-minute applications of a 35%-H_2O_2 gel to its surface, which may increase its toxic effects on pulp cells.

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Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of Departamento de Fisiologia e Patologia Faculdade de Odontologia de Araraquara - UNESP.

Conflict of Interest

The authors 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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