UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

ADRIANO FONSECA DE LIMA

CIRURGIÃO-DENTISTA

AVALIAÇÃO DA CITOTOXICIDADE DO PERÓXIDO DE CARBAMIDA E DO EFEITO PROTETOR DO ASCORBATO DE SÓDIO SOBRE CÉLULAS ODONTOBLÁSTICAS MDPC-23

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Mestre em Clínica Odontológica – Área de Concentração em Dentística

Orientadora: Prof. Dra. Giselle Maria Marchi

Co-Orientador: Prof. Dr. Carlos Alberto de Souza Costa

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"Um passo à frente,

e você já não está mais no mesmo lugar."

Chico Science

RESUMO

Os objetivos do presente trabalho foram: a) avaliar os efeitos citotóxicos diretos e transdentinário de diferentes concentrações de peróxido de carbamida (PC) sobre as células de linhagem odontoblástica MDPC-23; b) avaliar o efeito protetor (antioxidante) do ascorbato de sódio (AS) sobre estas células expostas a agentes clareadores, na forma direta e transdentinária; c) avaliar o montante de peróxido de hidrogênio (H_2O_2) liberado por agentes clareadores a base de PC 10% e 16% que se difunde através de discos de dentina com 0,5mm de espessura. No Experimento 1, células odontoblásticas foram cultivadas em *wells* e incubadas por 48 horas. O gel clareador foi solubilizado em meio de cultura (DMEM) originando diferentes extratos, e a quantidade (µg/mL) de H_2O_2 liberado em cada extrato foi mensurada através da técnica de leucocristais violeta/enzima *horseradish peroxidase* (LCV/HRP). Os seguintes grupos foram estabelecidos (n=10): G1-DMEM sem gel clareador (controle); G2-0,0001% PC (0,025µg/ml de H_2O_2); G3-0,001% PC (0,43µg/ml de H_2O_2); G4-0,01% PC (2,21µg/ml de H_2O_2); e G5-0,1% PC (29.74µg/ml de H_2O_2). As células foram expostas por 60 minutos aos diferentes extratos, e então realizada a análise da viabilidade celular (Teste de MTT). Somente os grupos 2 e 3 não apresentaram diferença estatística quando comparados ao controle (G1) ($p>0,05$). Os maiores efeitos citotóxicos foram observados para G4 e G5, sendo que G5 foi estatisticamente diferente que G4, apresentando-se mais tóxico às células. No experimento 2, células MDPC-23 foram cultivadas e incubadas por 48h. O PC e o AS foram solubilizados em meio de cultura (DMEM), para obtenção dos extratos experimentais. Os seguintes grupos foram estabelecidos: G1-DMEM sem gel clareador (controle); G2-0,25mM de AS; G3-0,5mM de AS; G4–0,25mM de AS + 0,01% de PC; e G5–0,5mM de AS + 0,01% PC e G6–0,01% de PC. As células foram expostas por 60 minutos aos diferentes extratos, e depois foi realizado o teste de MTT. O grupo 6 apresentou a maior citotoxicidade quando comparado com os demais grupos, enquanto que o AS produziu uma diminuição dos efeitos citotóxicos do agente clareador, demonstrando uma proteção

frente aos componentes deste produto. No Experimento 3, discos de dentina (0,5mm de espessura) obtidos de terceiros molares humanos foram fixados em uma câmara pulpar artificial (CPA). As células odontoblásticas foram semeadas na superfície pulpar dos discos, e os seguintes grupos foram estabelecidos: Grupo 1 - Sem tratamento (Controle); Grupo 2 - Antioxidante 10% (AS)/6h; Grupo 3- Peróxido de Carbamida (PC) 10% /6h; Grupo 4- AS10%/6h+PC10%/6h; Grupo 5- PC16% /6h; Grupo 6- AS10%/6h+PC16%/6hs. Após os tratamentos, foi realizado o teste de MTT. A difusão de H_2O_2 somente para os grupos 3 e 5 foi mensurada através da técnica de LCV/HRP. Todos os grupos foram estatisticamente semelhantes, exceto o G6. O PC 16% apresentou a maior difusão transdentinária. Pode-se concluir que o PC apresenta efeitos citopáticos para as células odontoblásticas MDPC-23, na forma direta ou transdentinária e que esta citotoxicidade é dose-dependente. O ascorbato de sódio possui a capacidade de reduzir os efeitos citotóxicos do peróxido de carbamida, sobre estas mesmas células em cultura.

Palavras-chave: biocompatibilidade, peróxido de hidrogênio

ABSTRACT

The aims of this present study were: a) to evaluate the direct and transdentinal cytotoxic effects of carbamide peroxide (CP) bleaching gel at different concentrations on odontoblast-like cells MDPC-23; b) to evaluate the protective effect (antioxidizing) of sodium ascorbate (SA) on these cells expose to bleaching agents, on direct and transdentinal mode; c) to evaluate the amount of hydrogen peroxide (H_2O_2) released by bleaching agents based to CP 10% and 16%, that diffuses through dentin discs with 0.5mm thickness. In Experiment 1, odontoblastic cells were seeded in wells and incubated for 48 hours. The bleaching gel was diluted in DMEM culture medium originating different extracts, and the amount (μg/mL) of H_2O_2 released from each extract was measured by the leukocrystal violet/horseradish peroxidase enzyme (LCV/HRP) assay. The following groups were established (n=10): G1-DMEM without bleaching gel (control); G2-0.0001% CP (0.025 μg/mL H_2O_2); G3-0.001% CP (0.43 μg/mL H_2O_2); G4-0.01% CP (2.21 μg/mL H_2O_2); and G5-0.1% CP (29.74 μg/mL H_2O_2). MDPC-23 cells were exposed to the bleaching gel extracts for 60 minutes and then performed the cell viability analysis (MTT assay). Only G2 and G3 were not significantly different from control group (G1) ($p>0.05$). The most severe cytotoxic effects were observed in G4 and G5, and G5 was statistically different to G4, presenting more toxic to the cells. In Experiment 2, MDPC-23 cells were seeded in wells and incubated for 48 hours. CP and SA were dissolved in culture medium (DMEM) in order to obtain the experimental extracts. The following groups were established: G1-no treatment (control); G2-0,25mM SA; G3-0,5mM SA; G4–0,25mM SA + 0,01% CP; e G5–0,5mM SA + 0,01% CP e G6–0,01% CP. The cells were expose to different extracts for 60 min, and then was performed the MTT assay and. Group 6 presented higher cytotoxicity than the other groups, while the SA decreased the cytotoxic effects caused by CP, demonstrating its protective effect against the toxic components of this dental product. In Experiment 3, dentin discs (0.5mm thick) obtained from human third molars were fixed in an artificial

pulp chamber (APC). The odontoblastic cells were seeded on pulp surface of the discs, and the following groups were established: Group 1 – No treatment (Control); Group 2 - Antioxidizing 10% (SA)/6h; Group 3- Carbamide Peroxide (CP) 10%/6h; Group 4- SA10%/6h+CP10%/6h; Group 5- CP16%/6h; Group 6- SA10%/6h+CP16%/6hs. After the treatments, MTT assay was performed. The H_2O_2 diffusion only to the groups 3 and 5 was measured by the LCV/HRP assay. All groups were statistically similar, except G6. The CP 16% presented the higher transdentinal diffusion. It can be conclude that CP presents citotoxic effects to the odontoblastic-like cells MDPC-23, in direct and transdentinal mode and this citotoxicity is dose-dependent. The sodium ascorbate was able to reduce the cytotoxic effects the concentration of 0.1% of PC caused the most intense cytopathic effects of carbamide peroxide on the same cells in culture.

Keywords: biocompatibility, hydrogen peroxide

SUMÁRIO

INTRODUÇÃO

O clareamento dental tem sido muito procurado por pacientes que desejam dentes mais brancos em busca de um sorriso mais atraente (Zantner *et al.*, 2007). Este tratamento, apesar da recente popularização, data de mais de cem anos (Kirk, 1889), sendo relatada a utilização do peróxido de hidrogênio para o clareamento de dentes vitais desde então (Prinz, 1924; Ames, 1937).

Dois tratamentos são os mais utilizados em se tratando do procedimento clareador, são estes o clareamento caseiro ou noturno (Haywood *et al.*, 1989) e o clareamento de consultório. No clareamento caseiro, baixas concentrações de peróxido de hidrogênio (3% a 7%) ou carbamida (10% a 22%) são utilizadas, com o auxílio de uma moldeira, podendo o tratamento ser realizado por semanas, sendo ele realizado sob a supervisão de um cirurgião-dentista (Haywood *et al.*, 1989). O clareamento de consultório utiliza os mesmos peróxidos, todavia, em concentrações mais elevadas, sendo estes produtos aplicados sobre a estrutura dental por curtos períodos de tempo, sendo realizado pelo cirurgião dentista (Joiner, 2006). Para o clareamento de dentes vitais, há também a alternativa da utilização de tiras clareadoras, denominado de "over the counter" (Joiner, 2007), realizado sem a supervisão de um cirurgião-dentista, sendo uma técnica pouco difundida no Brasil.

O peróxido de hidrogênio, principal composto dos géis clareadores, apresenta-se como uma espécie reativa de oxigênio (ERO), e juntamente com outros radicais formados a partir dele, como a OH, se difunde pelos tecidos duros dentais, reagindo com as longas moléculas de pigmento presentes nesses tecidos, deixando-as menores, promovendo a ação clareadora (Cavalli *et al.*, 2004b). O peróxido de carbamida se dissocia em peróxido de hidrogênio e uréia (Cavalli *et al.*, 2004b). Após a dissociação, aquele apresenta o mesmo mecanismo de ação dos géis que contém o peróxido de hidrogênio em sua composição inicial (Haywood *et al.*, 1991).

Todavia, o procedimento clareador apresenta alguns efeitos colaterais, como alterações na estrutura dental (Cavalli *et al.*, 2004a; Kawamoto *et al.*, 2004; Rodrigues *et al.*, 2005); diminuição da resistência de união dos compósitos resinosos ao substrato clareado (Cavalli *et al.*, 2001; Lai *et al.*, 2002; Kaya *et al.*, 2003; Cavalli *et al.*, 2004b; Turkun *et al.*, 2004); e hipersensibilidade dentinária (Robertson *et al.*, 1980; Browning, 2007).

Quanto às alterações na estrutura dental, estas podem ser desde mudanças na microdureza do esmalte ou dentina recém clareados (Basting *et al.*, 2003; Rodrigues *et al.*, 2005; Sasaki *et al.*, 2007), até a uma dissolução de dentina intra e peritubular (Kawamoto *et al.*, 2004).

A diminuição da resistência de união de compósitos resinosos à estrutura dental recém clareada foi investigada por diversos estudos (Cavalli *et al.*, 2001; Basting *et al.*, 2004; Cavalli *et al.*, 2005). Para que esta união seja efetiva, faz-se necessária a espera de 14 a 21 dias, a fim de que os valores de união retornem a um patamar confiável (Cavalli *et al.*, 2001).

Para a reversão do efeito dos agentes clareadores sobre a resistência adesiva dos materiais resinosos à estrutura dental clareada, foi também proposta a utilização de uma solução antioxidante, o acorbato de sódio 10% (Lai *et al.*, 2001). Com a utilização deste agente, o tratamento restaurador pode ser realizado imediatamente após o término do procedimento clareador, obtendo-se uma resistência de união semelhante àquela conseguida em estrutura dental cujo clareamento não foi realizado. Diversos estudos comprovaram a eficiência deste agente antioxidante no restabelecimento da resistência de união à estrutura dental clareada (Lai *et al.*, 2002; Kaya *et al.*, 2003; Turkun *et al.*, 2004).

O ascorbato de sódio é um potente antioxidante hidrossolúvel encontrado em fluidos biológicos, apresenta notável capacidade de promover redução das espécies reativas derivadas do oxigênio e nitrogênio, podendo assim, prevenir danos oxidativos

importantes de macromoléculas biológicas, tais como DNA, lipídios e proteínas (Meister, 1992; Soheili Majd *et al.*, 2003). Tanto o peróxido de hidrogênio como as EROs formadas a partir deste podem se difundir pelos tecidos duros dentais, e atingir o espaço pulpar (Gokay *et al.*, 2000a; Benetti *et al.*, 2004; Gokay *et al.*, 2004; Gokay *et al.*, 2005). Essa difusão pode causar danos às células, através do estresse oxidativo gerado, podendo causar inflamação (Robertson *et al.*, 1980) com conseqüente aumento da pressão intra-pulpar, sendo que estes efeitos podem resultar em hipersensibilidade dental.

A utilização de um agente antioxidante pode ser importante para que os efeitos deletérios causados pelo clareamento dental sejam reduzidos ou até mesmo evitados. Desta forma, o conhecimento dos efeitos nocivos dos agentes clareadores sobre as células odontoblásticas, assim como a avaliação do efeito antioxidante do ascorbato de sódio na proteção destas células contra os agentes clareadores faz-se necessário, para que o conhecimento destas variáveis possa nos auxiliar na obtenção de métodos que tornem o clareamento, além de efetivo, um procedimento mais seguro.

Sendo assim, os objetivos do presente estudo são: a) avaliar os efeitos citotóxicos de diferentes concentrações de peróxido de carbamida sobre as células de linhagem odontoblástica MDPC-23; b) avaliar o efeito protetor (antioxidante) do ascorbato de sódio sobre estas células expostas a uma solução de peróxido de carbamida 0,01%.

CAPÍTULO 1

Cytotoxic Effects of Different Concentrations of a Carbamide Peroxide Bleaching Gel on Odontoblast-Like Cells MDPC-23

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ABSTRACT

This study evaluated the cytotoxic effects of a carbamide peroxide (CP) bleaching gel at different concentrations on odontoblast-like cells. Immortalized cells of the MDPC-23 cell line (30,000 cells/cm^2) were incubated for 48 hours. The bleaching gel was diluted in DMEM culture medium originating extracts with different CP concentrations. The amount (μg/mL) of hydrogen peroxide (H_2O_2) released from each extract was measured by the leukocrystal violet/horseradish peroxidase enzyme assay. Five groups (n=10) were formed according to the CP concentration in the extracts: G1-DMEM (control); G2-0.0001% CP (0.025 μg/mL H_2O_2); G3-0.001% CP (0.43 μg/mL H_2O_2); G4-0.01% CP (2.21 μg/mL H_2O_2); and G5-0.1% CP (29.74 μg/mL H_2O_2). MDPC-23 cells were exposed to the bleaching gel extracts for 60 minutes and cell metabolism was evaluated by the MTT assay. Data were analyzed statistically by one-way ANOVA and Tukey's test (α=0.05). Cell morphology was examined by scanning electron microscopy. The percentages of viable cells were: G1-100%, G2-89.41%, G3-82.4%, G4-61.5% and G5-23.0%. G2 and G3 did not differ significantly ($p>0.05$) from G1. The most severe cytotoxic effects were observed in G3 and G4. In conclusion, even at low concentrations, the CP gel extracts presented cytotoxic effects. This cytotoxicity was dose-dependent, and the 0.1% CP concentration caused the most intense cytopathic effects to the MDPC-23 cells.

Key Words: Tooth bleaching, carbamide peroxide, odontoblasts, cytotoxicity, cell culture

INTRODUCTION

Dentistry has experienced remarkable advances over the last years, especially regarding esthetic materials and techniques [1]. Traditionally, two techniques are used for vital tooth bleaching therapies [2]. In the in-office technique, a gel based on high concentrations of hydrogen peroxide (H_2O_2) or carbamide peroxide (CP) is applied by the dentist during a clinical treatment session, being either activated or not by a light source. Home tooth bleaching involves having the patient wear custom-made trays filled with the same peroxide-based gels at lower concentrations, under the dentist's supervision [3]. Another treatment option is the use of bleaching strips that are applied directly on the buccal surface of the teeth [4].

Some authors have demonstrated that even at low concentrations, H_2O_2 penetrates easily into enamel porosities and is capable to diffuse deeply through dentin, reaching the pulp tissue. In addition, the trans-dentinal diffusion of H_2O_2 may be increased quantitatively by the contact of the bleaching agent with dentin exposed in areas of gingival recessions, abrasions, erosions, areas of wear or enamel defects, and gaps at the cementoenamel junction or tooth/restoration interface [5-7] The effects of CP are similar to those of H_2O_2 because CP is readily dissociated into hydrogen peroxide and urea on contact with water [8]. The ionization and degradation of these chemical compounds of bleaching agents may cause tissue damage with the release of free radicals, which are highly instable and have a great capacity to react with other organic substances [9].

Immortalized odontoblast cell lines have been used to investigate the potential trans-dentinal cytotoxic effects of dental materials and their soluble components [10-12] because in mammalian teeth, following odontogenesis, these cells are organized in a monolayer that underlies the coronal and root dentin, being responsible for the maintenance of tooth integrity due to its capacity to produce new dentin layers [13]. Therefore, any residual cytotoxic component leached from a dental material that is capable to diffuse through the dentinal tubules will first interact with these peripheral pulp cells and cause damage. For these reasons, cell types with odontoblast phenotype have been considered as the most indicated to evaluate potential cytotoxic effects of dental materials and/or their individual components [14]. To evaluate the cytotoxicity of bleaching gels on odontoblast-like cells is important not only to establish safe indications for the clinical application of CP-based vital bleaching therapies, but also to contribute to the search of mechanisms that can eliminate or minimize the deleterious effects caused by residual cytopathic components these agents. The purpose of this study was to evaluate the cytotoxic effects of a CP bleaching gel at different concentrations on odontoblast-like cells.

MATERIAL AND METHODS

Culture of MDPC-23 Cells

Immortalized cells of the MDPC-23 cell line [15,16] 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 penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine (Gibco) in an humidified incubator with 5% CO_2 and 95% air at 37^{o}C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). The MDPC-23 cells were sub-cultured at every 3 days until an adequate number of cells were obtained for the study. The cells were then seeded (30,000 cells/cm2) in 60 wells of three sterile 24-well dishes (Costar Corp., Cambridge, MA, USA), which were maintained in the humidified incubator with 5% CO_2 and 95% air at 37^{o}C for 48 h.

Analysis of H_2O_2 concentration and Collection of the CP extracts

A 10% CP bleaching gel (Whiteness, FGM, Joinvile, SC, Brazil) was used in this experiment. The amount (µg/mL) of H_2O_2 released from the CP gel was measured by the leukocrystal violet/enzyme horseradish peroxidase (LCV/HRP) assay. For such purpose, acetate buffer solutions were prepared with different CP concentrations from the commercial 10% CP bleaching gel. One hundred microliters of 0.5 mg/mlL leukocrystal violet (Sigma Chemical Co., St. Louis, MO, USA), and 50 µL of 1 mg/mL enzyme horseradish peroxidase (Sigma Chemical Co.) were added to the solutions, according to the method described by [17]. The optical density of the resultant blue color in the tubes was measured by a UV spectrophotometer (UV-Vis Spectrophotometer UV-1203; Shimadzu, Kyoto, Japan) at the wavelength of 596 nm. The optical density values obtained from the samples were converted into micrograms equivalent to the H_2O_2 concentration in each solution. The results are presented in Table 1.

After measuring the amount of H_2O_2 released from CP gel in acetate buffer, the same CP concentrations were diluted in pure culture medium (DMEM–2% fetal calf serum - FCS) in order to obtain the extracts to be analyzed. The following groups were formed: G1 (control): DMEM-FCS; G2: 0.0001% CP extract (0.025 μg/mL H_2O_2); G3: 0.001% CP extract (0.43 μg/mL H_2O_2); G4: 0.01% CP extract (2.21 μg/mL H_2O_2); and G5: 0.1% CP extract (29.74 μg/mL H_2O_2).

Analysis of Cell Metabolism (MTT assay)

The control extracts (G1) and those obtained from the dilution of the CP gel in DMEM (G2, G3, G4, and G5) were applied on the cultured MDPC-23 cells. The cells in contact com with the extracts were maintained in a humidified incubator at 37^{o}C with 5% CO_2 and 95% air for 60 minutes and then rinsed carefully in phosphate buffered saline (PBS).

Eight specimens of each group were used to evaluate cell metabolic activity by the methyltetrazolium (MTT) assay [18], which determines the activity of the succinate dehydrogenase (SDH) enzyme produced by the mitochondria of viable cells. In a vertical laminar flow hood, the culture media in contact with cells was aspirated and 900 μL of complete DMEM plus 100 μL of the MTT stock solution (5 mg methyltetrazolium salt in 1 mL PBS) were applied to the cells in each well. The cells in contact with the DMEM/MTT solution were incubated at 37^{o}C for additional 4 h. After this period, the DMEM/MTT solution was aspirated and replaced by 600 μL of acidified isopropanol solution (0.04 N

HCl) to dissolve the blue crystals of formazan present in the cells resulting from the cleavage methyltetrazolium salt by the SDH enzyme produced in mitochondria of the viable cells. After agitation and confirmation of the homogeneity of the solutions, three 100 µL aliquots of each well were transferred to a 96-well dish (Costar Corp., Cambridge, MA, USA). Cell viability was determined as being proportional to the absorbance measured at 570 nm wavelength with an ELISA plate reader (Multiskan, Ascent 354, Labsystems CE, Lês Ulis, France).

The data obtained from the MTT assay were analyzed statistically by one-way analysis of variance and Tukey's test at a level of significance of 5%. The mean value of the production of SDH calculated for each group was transformed into percentage of cellular viability reduction by considering the control group (G1) as 100% of cellular viability. The percentages represent the inhibitory effect of the mitochondrial activity of the cell for the tested CP concentrations.

Analysis of Cell Morphology by Scanning Electron Microscopy

The other two specimens were selected for analysis of cell morphology by SEM. For such purpose, sterile 12-mm-diameter cover glasses (Fisher Scientific, Pittsburgh, PA, USA) were placed on the bottom of the wells of 24-well dishes immediately before seeding of the MDPC-23 cells (30,000 cells/cm^2) [12]. The extracts were applied on the cells according to each group, in a similar way as performed for the analysis of cell viability. Thereafter, 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-minute rinses with 1 mL PBS, post-fixed in 1% osmium tetroxide for 60 min. Afterward, the cover glasses with cells were dehydrated in increasing concentrations of ethanol solutions (30%, 50%, 70%, 90%, 100%). Finally, the cells on the discs were subjected to drying by low surface tension solvent 1, 1, 1, 3, 3, 3,-hexamethyldisilazane 98% (HMDS)-Acros Organics, New Jersey, USA) and kept in desiccators for 12 hours. Then, the cover glasses were fixed on metal stubs and being gold sputtered, being that, these procedures allowed the cell morphology analysis in SEM. (JEOL-JMS-T33A Scanning Microscope, JEOL-USA Inc., Peabody, MA, USA).

RESULTS

The means of SDH enzyme activity in the control and experimental groups are presented Table 2.

The 0.1% CP concentration (G5) presented the most severe toxic effect to the MDPC-23 cells, reducing their metabolism by 78%. For the CP concentrations of 0.001% (G3) and 0.01% (G4), the metabolic activity of the viable cells decreased by 17.6% and 38.5%, respectively, with significantly difference between these groups ($p < 0.05$). For the 0.0001% concentration (G2), a decrease of only 10.5% in cell metabolism was observed. There was no statistically significant difference ($p > 0.05$) in the reduction of cell metabolism between G2 and G3 or between these groups and the control group (G1). The results of cell viability obtained from the analysis of cell metabolism (MTT assay) after

exposure of the MDPC-23 cells to different CP concentrations may be summarized in a decreasing order as follows: 0.1%CP > 0.01%CP > 0.001%CP = 0.0001%CP = control. Considering that the control group (G1) had 0% of death cells, the percentage of decrease of metabolic activity of the MDPC-23 cells was calculated for the different experimental groups, as shown in Figure 1.

Cell Morphology (SEM)

In the control group (G1), it was possible to observe a large number of MPDC-23 cells organized as epithelioid nodules and adhered to the glass substrate. The cells were near confluence and exhibited a large cytoplasm with numerous short cytoplasmatic prolongations originating from the cell membrane (Figure 2). In G2 and G3, the MDPC-23 cells presented morphology similar to that of the control group. In G3, however, cytoplasmatic membrane debris was observed, probably from lethally damaged cells that detached from the glass substrate (Figures 3 and 4). G4 presented a smaller number of cells adhered to the glass substrate. Part of these cells presented a round-shaped morphology and a large amount of cell membrane debris (Figure 5). In G5, the number of MDPC-23 cells that remained adhered to the glass substrate decreased significantly, and a large amount of death cell debris was observed in this group. The metabolic activity of the few MDPC-23 cells that remained viable after incubation with the extracts had a marked decreased of 78%. These cells lost part of their cytoplasm and exhibited a round-shaped morphology with few or no cytoplasmatic prolongations originating from the cell membrane (Figure 6).

DISCUSSION

PC-based products are employed for tooth bleaching therapies at different peroxide concentrations, depending on the treatment modality (in-office or at-home bleaching). Home bleaching techniques use gels that contain CP at low concentrations (10%), and the bleaching action is achieved by the dissociation of CP in 3.5% H_2O_2 (active agent) and urea [19].

Dental enamel is the hardest and most compact tissue of the human body and has a lower permeability compared to dentin [6]. Nevertheless, it has been demonstrated that when applied on enamel, bleaching agents and/or the products of their degradation may diffuse through enamel and dentin, reaching the pulp tissue. This diffusion may be increased in areas of exposed dentin and at the tooth/restoration interface [5-7]. Therefore, under clinical conditions, cytotoxic components released from bleaching agents may cause tissue damage and a local pulpal inflammatory reaction [20]. Hanks et al. (1993) have demonstrated the strong cytotoxicity of H2O2 to fibroblast cultures. The authors evaluated different molarities of H2O2 applied directly on the cells, and several concentrations of products containing H2O2 and CP were applied in vitro on dentin discs, being determined the trans-dentinal diffusion of these chemicals (indirect assay) as well as their direct cytopathic effects to the cell cultures. In both method of evaluation (direct and indirect), the products released from the bleaching agents were highly toxic to the fibroblasts. Therefore, based on the fact that components of the bleaching gels may diffuse through the hard dental tissues and considering that some of these chemical, such

as H_2O_2, are toxic to MDPC-23 cells, it has been suggested that the reversible initial pulp inflammation would be responsible, at least in part, for the occurrence of postoperative sensitivity [20]. The role of odontoblasts on dentinal hypersensitivity has been demonstrated [13]. Therefore, it is important to evaluate the toxic effects of different dental materials, such as bleaching agents and the products of their degradation, on the odontoblast-like cell line (MDPC-23), which are organized in a layer that underlies the dentin and are thus the first cells to be damaged by potentially cytotoxic compounds released by dental materials that diffuse through enamel and dentin and reach the pulp space. In the present study, MDPC-23 cells were exposed to different concentrations of a bleaching gel based on CP. The low concentrations of CP were tested to simulate the concentrations of this chemical that have been reported to reach the pulp tissue after application on enamel and dentin [5,6,21]. Therefore, considering the control group (G1) as having 100% of cell metabolism, no statistically significant differences were observed from G2 (0.0001% CP) and G3 (0.001% CP). However, when the CP concentration increased to 0.01% (G4) and 0.1% (G5), there was a decrease of 38.5% and 78% in the metabolic activity of the MDPC-23 cells, respectively. This important toxic effect observed in G4 and G5 can be attributed to the action of the hydroxyl radicals (OH^-) resulting from the degradation of the bleaching gel [9]. It has been reported that oxidative damage to the cells may be caused by a direct attack of oxygen-derived reactive species, such as H_2O_2 and OH^-, during the process of cellular stress oxidative [22], which is characterized by a severe unbalance between the production of reactive oxygen species (ROS) and the presence of endogenous and

exogenous antioxidants [23]. The OH^- groups may attack double bonds of unsaturated fatty acids from cell membrane lipids, causing an immediate oxidative lesion [24]. The lipid-radical interactions produce peroxides that are instable and reactive, triggering an autocatalytic reaction denominated as lipid peroxidation, which may cause extensive damage to the cell membrane and even cell death [24].

In the present study, SEM analysis of the morphology of the cells exposed to extracts containing different concentrations of CP revealed that the components of the bleaching gel caused direct cell damage. It was characterized especially in G5, in which a significant detachment of cells from the glass substrate was observed, as well as the presence of fragments of the membrane of dead cells. Therefore, it may be suggested that components of the bleaching gel solubilized in culture medium caused a direct damage on the cell membrane, acting as true free radicals [24].

G4 and G5 presented a marked reduction in cell metabolism, and differed significantly from the control group (G1). The amount of H_2O_2 (2.21 μg/mL) released from the 0.001% CP extract (G4) was consistent the values reported in previous studies to diffuse through enamel and dentin, reaching the pulp tissue [5,6,21]. In this group, a 38.5% reduction in cell metabolism was observed. The greater amount of H_2O_2 (29.74 μg/mL) released from the 0.1% CP extract (G5) has been detected in the pulp tissue after bleaching treatment in some situations [6].

The cell metabolic activity of G2 and G3, in which extracts with low CP concentrations were used (0.0001% and 0.001%, respectively), did not differed significantly from that of the control group (G1). However, in spite of the release of smaller amounts of H_2O_2 (G2 = 0.025 μg/mL and G3 = 0.43 μg/mL) from the extracts, these results should not be disregarded because there was a decrease in cell production of the SDH enzyme, characterized by a reductions of 10.5% (G2) and 17.6% (G3) in cell metabolism.

The intensity of the adverse effects of tooth bleaching vary from tooth to tooth and from patient to patient [6,7]. A number of variables should be taken into account: 1) whether the dentinal tubules are directly exposed to the action of the bleaching gel; 2) the thickness and area of dentin exposure; 3) localization of dentin exposure and; 4) previous inflammatory and microcirculatory conditions of the pulp tissue [7]. The findings of the present study showed that, even at low concentrations, PC was toxic to odontoblast-like cells MDPC-23 culture. These results are important to add information about the potential undesirable collateral effects of bleaching systems, especially on the pulpodentinal complex. Further research should be performed to elucidate the effects and mechanisms of action of the components of bleaching agents on MDPC-23 cells in order to establish manners to eliminate or at least minimize the damage caused to the teeth by vital bleaching therapies.

CONCLUSIONS

Under the tested conditions, it may be concluded that even low concentrations of a CP gel presented significant cytotoxic effects on odontoblast-like cells MDPC-23. A CP concentration of 0.1% CP was highly cytopathic, casing a decrease of almost 80% in cell metabolism.

ACKNOWLEDGEMENTS

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Table 1. Amount (μg/mL) of hydrogen peroxide released by the different carbamide peroxide concentrations.

CP concentrations (%)	**H_2O_2 (μg/mL)**
0.0001	0.0257
0.001	0.2547
0.01	2.2207
0.1	29.8143

Table 2. Means (±standard deviation) of the succinate dehydrogenase (SDH) enzyme activity in the control and experimental groups.

Groups (n=10)	SHD
G1 (Control)	0.6251 ± 0.14179a
G2 (0.0001% CP)	0.5589 ± 0.08870a
G3 (0.001% CP)	0.5151 ± 0.11528a
G4 (0.01% CP)	0.3844 ± 0.06657b
G5 (0.1% CP)	0.1437 ± 0.07892c

Different letters indicate statistically significant difference (Tukey's test, $p<0.05$).

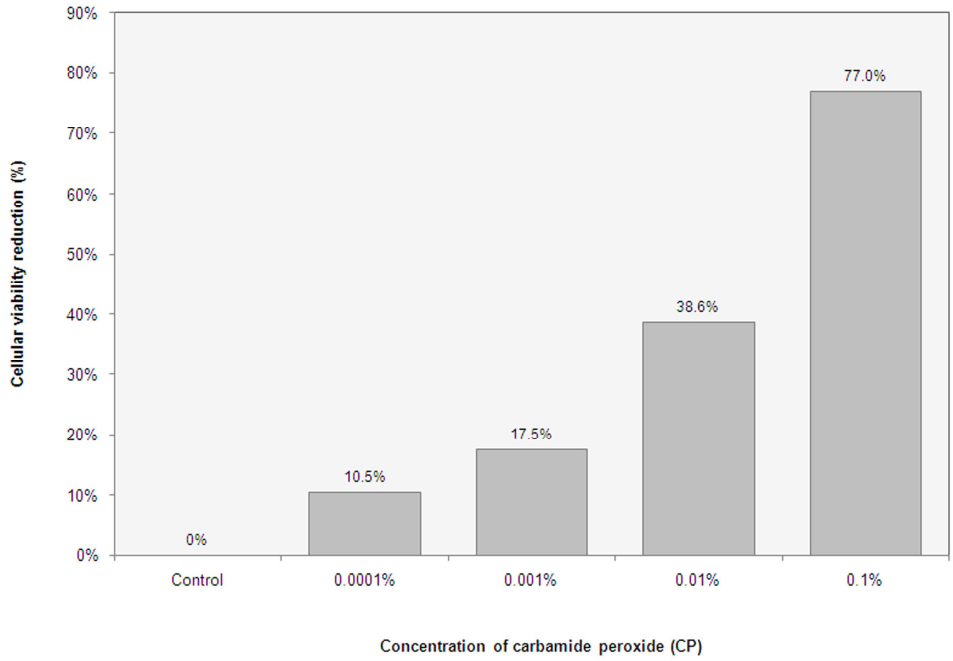

Figure 1. Graphic presentation of the percent reduction of cell viability as a function of the different concentrations of carbamide peroxide.

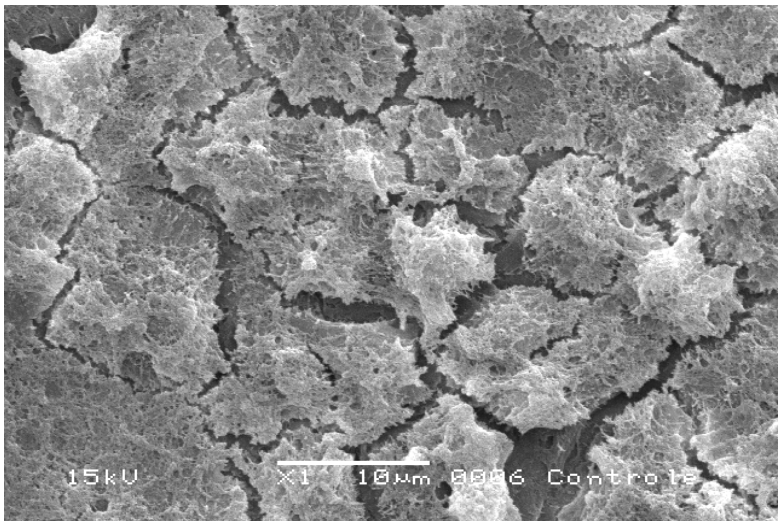

Figure 2: Group 1 (Control). It can be observed that numerous MDPC-23 cells with a large cytoplasm are near confluence on the glass substrate (x1000).

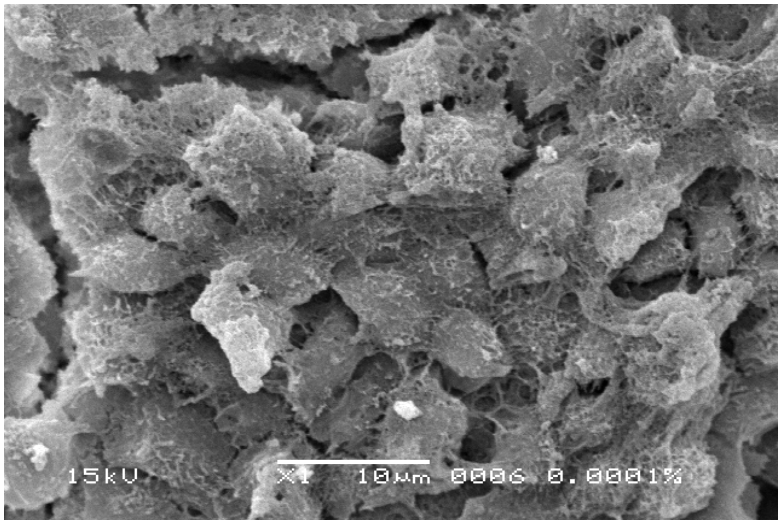

Figure 3. Group 2 (0.0001% PC). Large number of cells with normal morphology adhered to the glass substrate. Note the presence of numerous thin cytoplasmatic prolongations originating from the cell membrane (x1000).

Figure 4. Group 3 (PC 0.001%): MDPC-23 cells organized as epithelioid nodules on the glass substrate, with some cell membrane fragments of covering them (x1000).

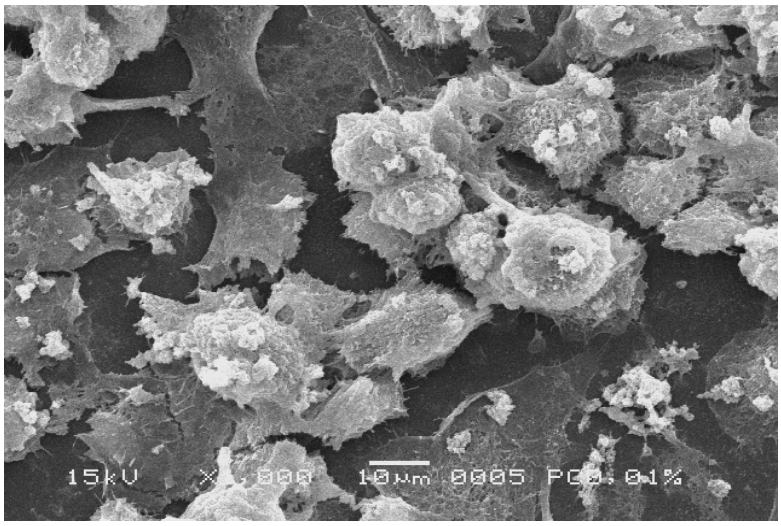

Figure 5. Group 4 (0.01% PC): Discrete reduction in the number of cells on the substrate and a larger amount of cell membrane debris (x1000).

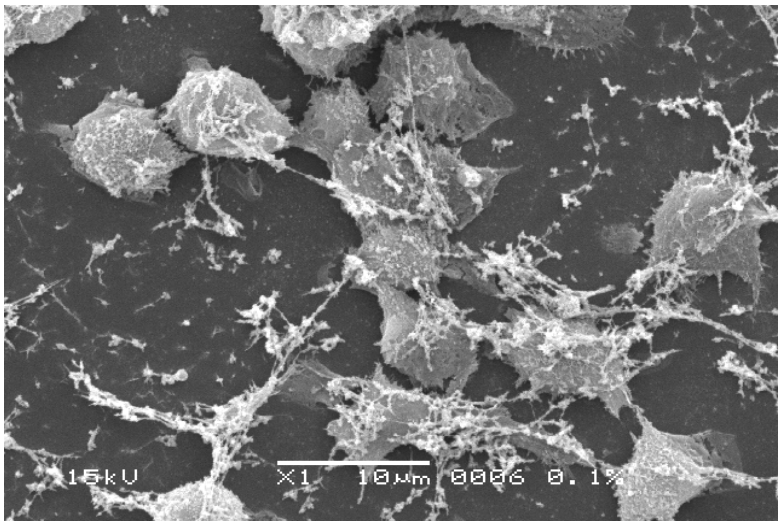

Figure 6. Group 5 (0.1% PC): Few MDPC-23 cells are observed, exhibiting rounded morphology and loss of the cytoplasmatic prolongations. Fragments of the cell membrane can be observed in the spaces previously occupied by cells (arrows) (x1000).

CAPÍTULO 2

Protective effect of sodium ascorbate on odontoblast-like cells MDPC-23 exposed to a bleaching agent

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Running Title: Protection of odontoblasts

Keywords: bleaching agent, carbamide peroxide, odontoblasts, sodium ascorbate

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ABSTRACT

The aim of this study was to evaluate the cytotoxic effects of a bleaching agent composed of 0.01% carbamide peroxide (CP) on MDPC-23 odontoblastic cell line, and to determine if sodium ascorbate (SA) is capable of reducing the toxic effects caused by the bleaching agent. The cells were seeded in wells, incubated for 48 hours, and exposed to different extracts: G1–no treatment (control); G2–0.25mM SA/60min; G3–0.5mM SA/60min; G4–0.25mM SA+0.01% CP/60min; G5–0.5mM SA+0.01% CP/60min and G6–0.01% CP/60 min. G6 showed metabolism statistically lower than the other groups, and the cells that remained on the substrate exhibited changes in their morphology. SA decreased the cytotoxic effects caused by CP. It was concluded that CP gel causes cytopathic effects to the MDPC-23 cells, even at low concentration, such as 0.01%. SA at 0.25mM and 0.5mM is capable to protect the cultured cells against the CP cytotoxic effects.

1 INTRODUCTION

The bleaching treatment, characterized by a procedure that attenuates or remove dyes from the tooth, has been widely used recently, mainly by patients looking for an attractive and apparently healthy smile (Zantner *et al.*, 2007). However, such aesthetic procedure may cause some side effects, such as morphological changes in the hard dental tissues (Kawamoto *et al.*, 2004; Rodrigues *et al.*, 2005; Zantner *et al.*, 2007) and decrease in the bond strength of resin composites to the bleached dental surface (Cavalli *et al.*, 2001; Turkun *et al.*, 2004). Dentin hypersensitivity (Robertson *et al.*, 1980; Browning *et al.*, 2007) is another side effect caused by the diffusion of bleaching agents through the tooth structure to reach the pulp tissue (Hanks *et al.*, 1993; Gokay *et al.*, 2000a; Gokay *et al.*, 2005), resulting in pulp inflammation (Robertson *et al.*, 1980). Such side effects are attributed to reactive oxygen species (ROS), which play an important role in the tooth bleaching therapy, but also may cause deleterious effects on cells due to the lipid peroxidation process(Halliwell, 2006).

In order to reverse the effects of bleaching agents on composite bond strength to the bleached tooth surface, the use of 10 % sodium ascorbate (SA) has been proposed (Lai *et al.*, 2001). SA is considered a powerful hydro soluble antioxidant capable of deoxidizing the reactions of oxygen and nitrogen free radical species. Therefore, SA is able to prevent important deleterious oxidative effects on biological macromolecules, such as DNA, lipids, and proteins (Meister, 1992; Soheili Majd *et al.*, 2003).

Dental materials or their components capable of trans-dentin diffusion can cause irreversible pulp injuries or even induce a death process and tissue necrosis (Costa *et al.*, 2000b). Consequently, the use of materials that can reduce or even eliminate the injuries caused by toxic components diffusing through the dentin tubules to the pulp would be of great value, once the restorative procedures may become not only effective, but also safe. Therefore, the aims of the current study were to evaluate: a) the cytotoxicity of a bleaching agent when applied to immortalized MDPC-23 odontoblastic cell line; and b) if SA can reduce or eliminate the toxic effects caused by the bleaching agent on such cells.

2 MATERIALS AND METHODS

Cell Culture

Immortalized cells of the MDPC-23 cell line were cultured (30.000 cells/cm^2) on sterilized 24-well acrylic dishes (Costar Corp., Cambridge, MA, USA) and were then incubated for 48 hours in a humidified incubator with 5% CO_2 and 95% air at 37^oC. Dulbecco´s Modified Eagle´s Médium (DMEM, SIGMA Chemical Co., St. Louis, MO, USA) with 10% fetal calf serum (FBS, Cultilab, Campinas, SP, Brasil) supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine (GIBCO, Grand Island, NY, USA).

Preparation of the solutions used in the study

One bleaching agent composed of 10% CP (Whiteness, FGM, Joinvile, SC, Brasil) was used in the present in vitro study. The bleaching agent was diluted in culture medium

with no serum fetal bovine (DMEM- SFB) until reaching the extracts with the final concentration of 0.01% (2.21µg/ml of H_2O_2). In order to prepare the antioxidant solution, sodium ascorbate (Sigma Chemical Co. St. Louis, MO, USA) was dissolved in DMEM-SFB to obtain the extracts at 0.25mM/mL and 0.5mM/mL (Soheili Majd *et al.*, 2003). Therefore, the following control and experimental groups (n=10) were created: G1: no treatment (control); G2: SA 0.25mM/mL; G3: SA 0.5mM/mL; G4: SA 0.25mM/mL + 0.01% PC; G5: SA 0.5mM/mL + 0.01% CP; G6: 0.01% CP. The experimental extracts were applied on the cultured MDPC-23 cells and maintained in incubator at 37^{o}C for 60 minutes.

Cytotoxicity Test (MTT assay)

The control extracts (G1) and those obtained from both CP and SA dilution in DMEM (G2-G6) were applied to MDPC-23 cells. The cells in contact with the extracts were maintained in a humidified incubator at 37^{o}C with 5% CO_2 and 95% for 60 minutes. Afterwards, the cells were rinsed carefully in phosphate buffered saline (PBS) and were submitted to cell metabolic activity analysis by metiltetrazolium assay (MTT Assay) (Mosmann, 1983). Such test determines the activity of succinate dehydrogenase enzyme (SDH) produced by the mitochondria of viable cells. In order to prepare the MTT stock solution, 5 mg of methyltetrazolium salt (Sigma Chemical Co. St. Louis, MO, USA) was diluted into 1 mL of PBS. The experimental and control cultures in contact with all cells were aspirated in a vertical laminar flow cabinet and were replaced by a solution composed of 900 µL of DMEM and 100µL of MTT stock solution. The cells in contact with such solution were then incubated for 4 hours. After this period, the DMEM + MTT

solution was aspirated and was replaced by 600 µL of acidified isopropanol solution (0.04 N HCl) to dissolve the blue crystals of formazan present in the cells resulting from the cleavage methyltetrazolium salt by the SDH enzyme produced in mitochondria of the viable cells. After agitation and confirmation of the homogeneity of the solutions, three 100-µL aliquots were transferred from each well to the wells of a 96-well dish (Costar Corp., Cambridge, MA, USA). Cell viability was determined as being proportional to the absorbance measured at 570 nm wavelength with an ELISA plate reader (Multiskan, Ascent 354, Labsystems CE, Lês Ulis, France).

The data obtained from MTT assay were analyzed statistically by 2-way ANOVA (SA and CP) and statistical differences among groups were detected by Tukey's post-hoc test at a pre-set alpha of 5%. The group means were used to determine cell viability (%) in comparison to the control group (G1), which was considered as 100%.

Analysis of Cell Morphology by Scanning Electron Microscopy

The other two specimens were selected for analysis of cell morphology by SEM. For such purpose, sterile 12-mm-diameter cover glasses (Fisher Scientific, Pittsburgh, PA, USA) were placed on the bottom of the wells of 24-well dishes immediately before seeding of the MDPC-23 cells (30,000 cells/cm^2) (de Mendonca *et al.*, 2007). The extracts were applied on the cells according to each group, in a similar way as performed for the analysis of cell metabolism. Thereafter, the extracts were aspirated and the viable cells that remained attached 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-minute rinses with 1

mL PBS, post-fixed in 1% osmium tetroxide for 60 min. Afterward, the cover glasses with cells were dehydrated in increasing concentrations of ethanol solutions (30%, 50%, 70%, 90%, 100%). Finally, the cells on the discs were subjected to drying by low surface tension solvent 1, 1, 1, 3, 3, 3,-hexamethyldisilazane 98% (HMDS)-Acros Organics, New Jersey, USA) and kept in desiccators for 12 hours. Then, the cover glasses were fixed on metal stubs and being gold sputtered, being that, these procedures allowed the cell morphology analysis in SEM. (JEOL-JMS-T33A Scanning Microscope, JEOL-USA Inc., Peabody, MA, USA).

3 RESULTS

The values of SDH enzyme activity determined by MTT assay according to the either the presence or not of bleaching agent and the SA concentration are presented in Table 1. In those groups G2 and G3, in which SA was added to the culture medium a discrete increase in cell metabolism was observed. As a consequence, cell viability values higher than 100 % were recorded in these experimental groups (Figure 1). However, this higher cell metabolism determined in groups G2 and G3 was not statistically different when compared to the control group (G1). When SA was associated with CP, a significant decrease in the cytotoxic effects of CP with higher SDH production was observed ($p<0.05$). The lowest metabolic values were observed in groups in which only the experimental bleaching agent was added to the culture medium. Considering the control group as 100% cell metabolism, the values obtained by the MTT assay for the groups 2, 3, 4, 5, and 6 regarding SDH production were 110.06%; 108.57%; 90.35%; 97.63%; and 66.88%, respectively. These data are demonstrated in Figure 1.

Scanning Electron Microscopy (SEM) analysis of Cell Morphology

In control group (G1) and in groups G2 and G3, a considerable amount of MDPC-23 cell organized in epithelioid nodules remained attached to the glass substrate. Such cells presented large cytoplasm and a number of cytoplasmatic processes originated from its membrane (Figure 2A-C). Similar amount of cells with the same morphological features were observed in group G4 (Figure 2D). In group G5, most of MDPC-23 that remained on the substrate exhibited a few and short cytoplasmatic processes. These cells which were also organized in epithelioid nodules presented smooth round shape (Figure 2E). In group G6, a great number of cells detached from the glass substrate. Therefore, wide areas with granular structures similar to residual membrane from dead cells were seen on the glass disk. However, this small number of cells that remained attached to the substrate still kept organized in epithelioid nodules (Figure 2F).

4 DISCUSSION

The present in vitro study demonstrated that a solution of 0.01% of carbamide peroxide causes cytotoxic effects on MDPC-23 odontoblastic cell line. It has been reported that bleaching agents placed in aqueous medium, decomposes and originates some sub-products, such as hydrogen peroxide and urea (Haywood *et al.*, 1989).

Hydrogen peroxide is classified as one of the many ROS, which consist of highly reactive molecules capable of causing injuries in many cell components, such as plasmatic membrane, organelles, and DNA (Shackelford *et al.*, 2000). The misbalance between

endogen cellular antioxidant agents and ROS results in oxidative stress (Halliwell, 2007), which may cause several injuries that vary from reversible lesions to cell death (Halliwell, 2006). Therefore, the deleterious effects observed when the bleaching agent was applied to the cells (G6) may be attributed to the known cytotoxic effect of hydrogen peroxide, which was at 2.21µg/mL of culture medium. Such toxic effects caused by the CP dissolved in aqueous medium (DMEM) were confirmed not only by the decrease in SDH production by odontoblast-like cells detected by MTT assay, but also by the decrease in number and morphological changes of cells that remained adhered to the glass substrate after bleaching agent application (Figure 2F).

It has been demonstrated that hydrogen peroxide diffuses though enamel and dentin and reaches the pulp tissue when the bleaching agent is applied to the tooth (Gokay *et al.*, 2000a; Gokay *et al.*, 2000b; Camargo *et al.*, 2007). Therefore, the current in vitro study used 0.01% CP dissolved in culture medium (DMEM) in an attempt to simulate the concentration that reching the pulp after clinical procedure of bleaching therapy (Gokay *et al.*, 2000a; Gokay *et al.*, 2000b; Camargo *et al.*, 2007). Several investigations have demonstrated that hydrogen peroxide is released from the bleaching agents applied on enamel, and a defined concentration (2.21µg/ml of H_2O_2) of this oxygen-derived free radical may reach the pulpal chamber after diffusion through enamel and dentin (Gokay *et al.*, 2000a; Gokay *et al.*, 2000b; Camargo *et al.*, 2007) Therefore, it was important in the present study to evaluate the cytotoxic effects of hydrogen peroxide at such concentration on cells with odontoblast phenotype, such as MDPC-23. As the

outer pulp tissue layer is composed of odontoblasts, which are organized in monolayer to underlie the tubular dentin (Arana-Chavez *et al.*, 2004), this kind of cells are the first ones to contact toxic compounds released from dental materials capable to diffuse through the hard tissues, such as enamel and dentin. Consequently, odontoblast cell lines, such as the immortalized MDPC-23 cells, are appropriate to evaluate the toxic effects of dental products applied on tooth structures (MacDougall *et al.*, 1998).

Sodium ascorbate, when applied along with CP (G4 e G5), was capable of protecting the pulp cells against the toxic effects of sub-products released from the bleaching gel dissolved in aqueous medium. The cell protection is attributed to the ROS inactivation by antioxidants agents (Figure 3), which convert the highly reactive radicals into stable molecules (Chaudiere *et al.*, 1999). For this reason, as confirmed by MTT assay and SEM analysis of cell morphology, it is possible to suggest that SA at 0.25mM (G4) or 0.5mM (G5) really prevented cell damages when applied to cells which were later exposed to 0.01% CP. Based on MTT assay, the metabolism of MDPC-23 cell decreased by 33.12% when no SA was used previously to the application of the extract with CP. On the other hand, when SA at 0.25mM (G4) or 0.5mM (G5) was added to the culture medium, the metabolism decreased only 9.65% and 2.37%, respectively. Such SA protector effect at both concentrations was also demonstrated by the SEM analysis of cell morphology. In the experimental groups with no SA (G6) a noticeable decrease in the amount of cells attached to the glass substrate was observed. On that glass surface previously occupied by MDPC-23 cells, only residual fragments of membrane from dead cells were noted.

These results confirm that the misbalance between the presence of antioxidants and ROS on the cells can cause direct cell death (Shackelford *et al.*, 2000). Based upon these results and according to the statistical analysis applied to numerical data obtained from the MTT assay, the protective effectiveness of SA at both 0.25mM (G4) and 0.5mM (G5) concentrations was confirmed for MDPC-23 cells, with no significant difference between them. These favorable results regarding SA antioxidant effects of SA were also observed when resinous materials were applied to cell cultures (Soheili Majd *et al.*, 2003). It is important to emphasize that in groups G2 and G3, in which culture medium with only SA was applied to the cultured MDPC-23 cells, a discrete increase in cell metabolism occurred. However, the results of cell metabolism determined for G2 and G3 were not statistically different of that for control group (G1). Despite the lack of statistical significance, it is possible to speculate that the smooth increase in cell metabolism observed in Groups G2 and G3 might be attributed to antioxidant effect of SA, which may have neutralized the endogenous free radicals produced by the cells during normal mitochondrial oxidative phosphorylation (Shackelford *et al.*, 2000). Therefore, it may be speculated that cell damage or death caused by the endogenous free radicals, which may have occurred in group G1 (control), did not takes place in groups G2 and G3 in which the cell death was prevented and consequently the SDH production increased.

Despite the important scientific data presented in the present investigation, not only regarding the cytopathic effects of CP at low concentration, but also the protective

effect of SA acting as an antioxidant agent, it has been reported that results of in vitro studies cannot be directly extrapolated to clinical situations (Costa *et al.*, 2000a). Therefore, further in vivo studies are required to evaluate the effects of bleaching agents with carbamide peroxide in their composition to the pulp tissue. In addition, it is needed to assess if the in vitro pulp cell protection caused by the sodium ascorbate, such as observed in the present study, may also occur in clinical situations of tooth bleaching treatment.

5 CONCLUSIONS

Based on the methodology used in the current in vitro study, it was possible to conclude that 0.01% CP promoted cytopathic effects to the immortalized odontoblast cell line MDPC-23. It was also concluded that SA at 0.25 mM and 0.5 mM concentrations was capable of reducing the CP cytotoxicity to the cultured cells.

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Table 1. Production of SHD enzyme (means ± standard deviation) detected by MTT assay, according to SA concentration and the presence of the Bleaching agent.

SA	Bleaching agent	
	Absent	Present
0	0.309 ± 0.029ab	0.207 ± 0.019 c
0.25 mM	0.340 ± 0.050 a	0.279 ± 0.027 b
0.5 mM	0.323 ± 0.025 a	0.302 ± 0.036 ab

Significant differences detected by post-hoc Tukey's test are represented by different lower case letters at a pre-set alpha of 5% (n=10).

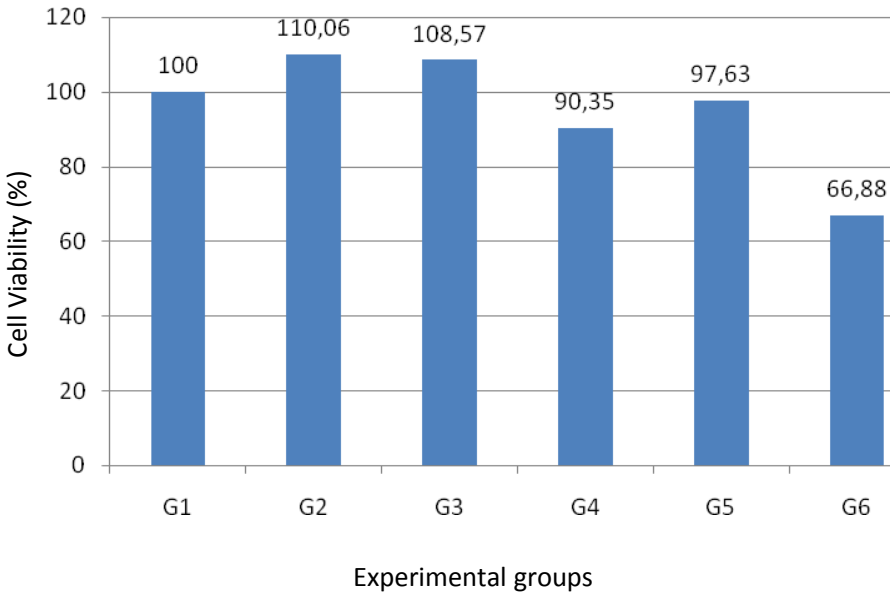

Figure 1 – Bar graph exhibiting cell viability for the experimental groups.

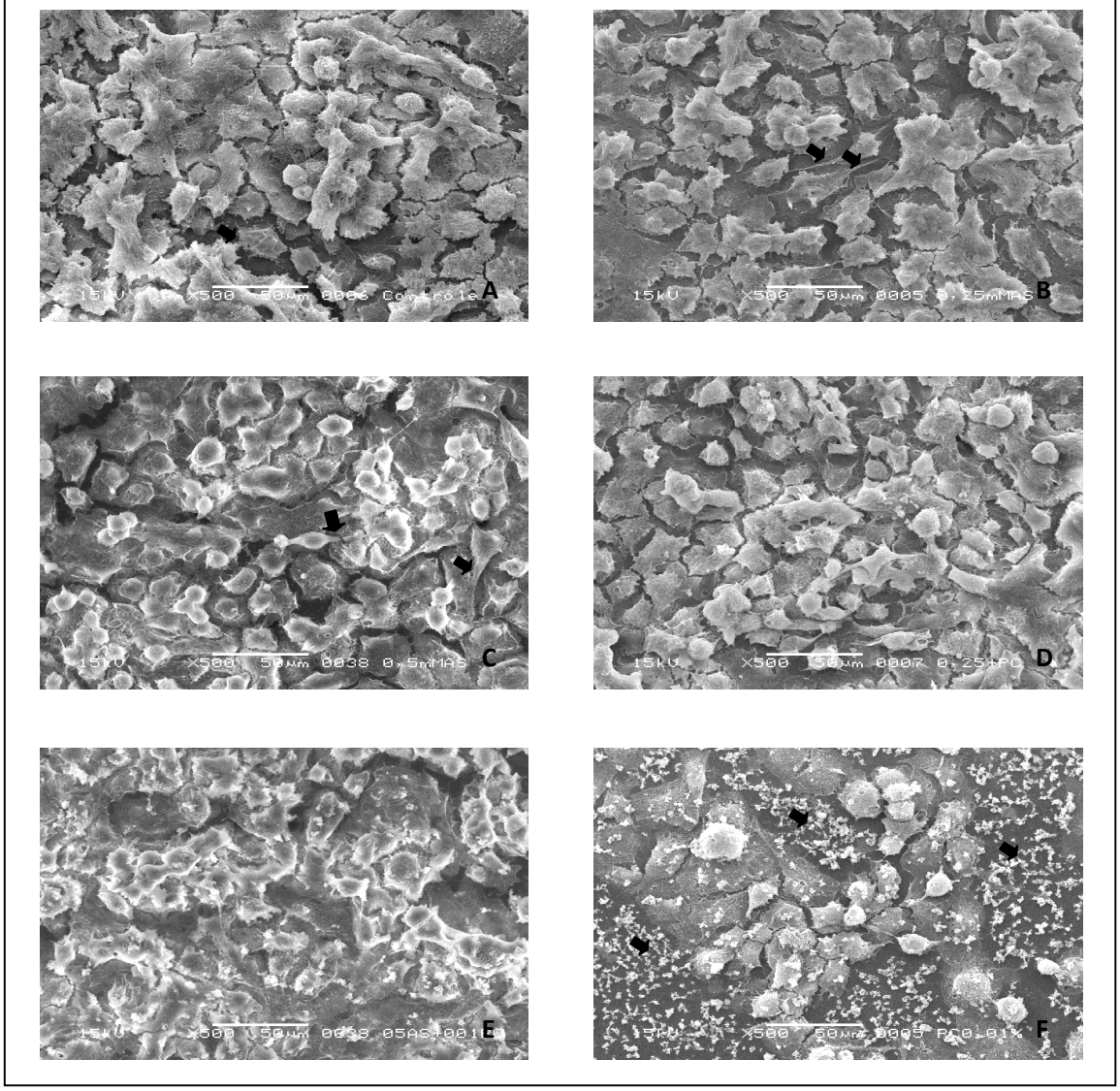

Figure 2 – The MDPC-23 cells, which are organized in epithelioid nodules, present large plasma membrane with several small cytoplasmic processes (arrows). Experimental groups G1(a), G2(b), G3(c), and G4(d). SEM, original magnification ×500. Odontoblasts with morphological changes characterized by small size and a few cytoplasmic processes are observed in group G5(e). SEM, original magnification ×500. In group G6(f), a number of MDPC-23 cells detached from the glass substrate, in which residual fragments of death cells are observed (arrows). The cells that remained attached to the substrate are organized in epithelioid nodules. SEM, original magnification ×500.

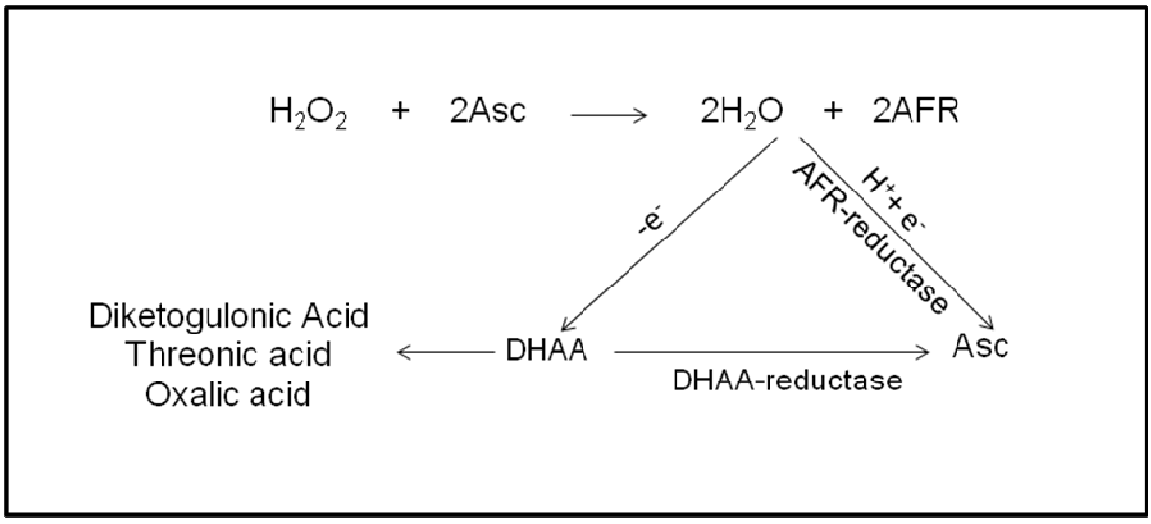

Figure 3 - The hydrogen peroxide (H2O2) is neutralized by transfer of a single-electron of ascorbates (Asc) producing water and ascorbyl free radicals (AFR). The pairs of AFR form one molecule of dehydroascorbic acid (DHAA) and one Asc. The lactone ring of DHAA is breaking down forming the inert products, diketogulonic acid, threonic acid and oxalic acid; alternatively, DHAA can be reduced to the useful Asc.

CAPÍTULO 3

Transdentinal Protection of Odontoblastic-Like Cells MDPC-23 Exposed to Bleaching Agents

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Running Title: Cytotoxicity of carbamide peroxide gel on odontoblast-like cells.

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Abstract

The aim of this study was to evaluate the transdentinal cytotoxicity of 10% and 16% carbamide peroxide gel (CP) on pulp cell in culture, as well as the antioxidant potential of 10% sodium ascorbate (SA) on the protection of these cells. Also, it was possible to determine the amount of hydrogen peroxide (H_2O_2) that diffuses through the human dentin. Dentin disks with 0.5mm thickness, which were obtained from intact human molars, had their permeability evaluated and were adjusted in artificial pulp chambers (APCs). Odontoblast-like cells MDPC-23 were seeded on the pulp surface of the disks and the following groups were established: G1-No Treatment (control); G2-SA 10%/6hs; G3-SA 10%/6hs+CP10%/6hs; G4-SA10%/6hs+CP10%/6hs; G5-CP16%/6hs; and G6-SA10%/6hs+CP16%/6hs. The cell viability was measured by metiltetrazolium (MTT) test, and diffusion of H_2O_2 analyzed by leukocrystal violet/enzyme horseradish peroxidase (LCV/HRP) assay. In groups where CP 16% was used, there was the lowest cell viability and it was determined the largest amount of H_2O_2 in the culture medium in the APCs pulp space. On the other hand, the SA 10% application on the dentin disks before the use of the bleaching agents reduced the cytopathic effects caused by reactive oxygen species (ROS) on the MDPC-23 cells. It was concluded that the ROS released by the carbamide peroxide gels evaluated, can diffuse by the dentin to cause significant reduction in the MDPC-23 cells. Therefore, the intensity of the toxic effects caused by the bleaching gels can be partially prevented by the presence of SA 10%.

Introduction

Dental bleaching is one of the most requested cosmetic procedures in aesthetic dentistry. Nevertheless, this treatment may cause some side effects, such as: 1) decrease in the bond strength of composite resin to the bleached substrate (1, 2); 2) morphological changes in dental structure (3, 4); 3) dentin hypersensitivity (5, 6); and 4) modifications in the pulp enzymes activities (7).

During the tooth bleaching, the carbamide peroxide gel breaks down to hydrogen peroxide, urea, ammonia and carbon dioxide. The degradation of H_2O_2 by the reactions of Fenton and Harber-Weiss, which occur in the tissues and cells containing metal ions and copper, can produce the hydroxyl radical. This free radical oxygen-derived, in addition of being able to bleach the tooth structures, has important biological significance due to its high reactivity and toxicity (8, 9).

Due to the carbamide peroxide remains in contact to the teeth during the treatment period, H_2O_2 and some reactive oxygen species are released, which can diffuse in the hard tissues and reach more vulnerable structures like the pulp tissue (10-12). In this specialized conjunctive tissue, the ROS can cause an inflammation (5) consequently increasing the intra-pulp pressure, and these effects could result in dentin hypersensitivity.

Dental materials or its components that are able to diffuse through the dentinal tubules can cause irreversible damage to the pulp or even induce the process of death and

tissue necrosis (13). In these cases, the first cells to be damaged are the odontoblast cells. Considering this fact, for the development of the *in-vitro* studies on the influence of dental materials to pulp tissue, cell types with odontoblast phenotype are the most appropriate for use (14), it could be possible to simulate, in laboratory, certain clinical conditions.

The sodium ascorbate 10% has been used to restore the bond strength of composite resin to the bleached surface (2, 15). This chemical agent, which is a powerful hydro soluble antioxidant found in biological fluids, has a remarkable ability to promote reduction of reactive species derived from oxygen and nitrogen, thus, is able to prevent oxidative damage to important biological macromolecules, such as DNA, proteins and lipids (16, 17). Therefore, the use of antioxidant substances, like sodium ascorbate, during the clinical procedure of tooth bleaching, may be important not only to restore the bond strength after the treatment, but also for the possible protection of pulp cells against aggression imposed by toxic components in bleaching materials with the ability of transdentinal diffusion. Therefore, the objectives of this study were: a) evaluate the possible transdentinal cytotoxic effects of bleaching agents based on carbamide peroxide (CP) 10% and 16%; b) examine whether the antioxidant property of sodium ascorbate 10% (SA) is able to protect the odontoblastic-like cells against possible cytotoxic effects of bleaching agents, and c) evaluate the amount of hydrogen peroxide that is able to diffuse through the human dentin.

Materials and Methods

After this research's approval by the Ethics Committee of the Dentistry University of Piracicaba/UNICAMP, Brazil (protocol 01/2007), seventy dentin disks with approximately 0.6 mm thickness were obtained from intact human third molars. For this, the teeth were fixed in the acrylic plates, being that transverse cuts of the crown were performed, one above the projection of the pulp horns and another below the enamel-dentin junction, using a diamond disc (11-4254, 4"x 0012"/15LC series, Diamond Blade, Burhker Ltd., Lake Bluf, IL, USA) on a metallographic precision saw (ISOMET 1000, Buehlar Ltd, USA). The discs were further manually ground with 400 and 600-grit abrasive papers (T469-SF-Noton, Saint-Gobam Abrasivos Ltda. Jundiai, Brazil) to a 0.5mm final thickness. The smear layer produced on both sides of the discs was removed by the active application of 0.5 M EDTA (pH 7.4) for 30s, followed by abundant rinse with distilled water (18). Thus, dentin disks with a thickness standardized (0.5mm) were mounted on a filtering camera connected to a water column of 180cm (19) and the hydraulic conductance calculated and determined for each disc. Finally, the dentin disks were divided between the groups, in a way that the permeability average values were not statistically different between them (Kruskal-Wallis, $p > 0.05$).

Transdentinal Cytotoxic Effect

For the cell viability analysis, it was used a device simulating an artifical pulp chamber (APC), with a dentin disk attached, to evaluate transdentinal dentin diffusion and

cytotocity analysis of the bleaching agent. For this, the lateral of the discs were worn with diamond bur #1095 (KG Sorensen, Barueri, Brazil) resulting in a diameter of 8mm. They were positioned with the pulp surface facing to the APC internal region (pulp space) between two rings of sterilized silicone (Orion-Sao Paulo, SP, Brazil), which, in addition to accommodate the dentin disks in place, avoid the materials diffusion through the sides of discs.

The APCs were individually placed in wells of sterile acrylic plates of 24-wells (Coaster Corp. Crambridge, MA, USA) with the pulp surface of dentin disks facing up. Immortalized cells of the MDPC-23 cell line (20, 21) were seeded (50,000 cells/cm2) on the dentin surface. The APCs with discs and cells were maintained for 48 hours in a humidified incubator at 37˚C with 5% CO2 and 95% air. The culture medium used in this experiment was Dulbecco"s Modified Eagles's Medium (DMEM, SIGMA Chemical Co., St. Louis, MO, USA) complemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil), supplemented with 100 IU/ mL of penicillin, 100 mg/mL streptomycin and 2 mmol/L glutamine (GIBCO, Grand Island, NY, USA). After this period, the APCs with dentin discs and cells were inverted and individually placed into a new 24-acrylic-well plate, with the cells immersed in the culture medium. In each well, 1mL of complete DMEM was immediately applied, so that cells adhered to the pulp surface of the discs were kept in contact with the culture medium. Moreover, the occlusal surface of the dentin disks was kept up, allowing the placement of products on this tubular substrate. The groups were formed according to the different treatment (n=10):

Group 1 – No Treatment (Control)

Group 2 – Sodium ascorbate (Sigma Chemical Co. St. Louis, MO, USA) 10% (SA)

Group 3 – Carbamide Peroxide (CP) 10% implemented during 6 hours;

Group 4 – SA 10%/6hours + washing the discs + CP 10%/6hours;

Group 5 – CP 16% implemented during 6 hours;

Group 6 – SA 10%/6hours + washing the discs + CP 16%/6 hours.

Of the total of 10 disks per group, eight were used to evaluate the cell viability, while two were used for the evaluation of cell morphology by scanning electron microscopy (SEM).

For groups 4 and 6, after 6 hours implementing sodium ascorbate 10% (3µL), the dentinal substrate was vigorous rinsed, to completely remove the antioxidant substance of the dentin, enabling the treatment of occlusal surface of discs with 18mg of bleaching gels containing 10% or 16% of carbamide peroxide (Whiteness, FGM, Joinvile, SC, Brazil). After a period of 6 hours implementing the bleaching gels, they were removed from the occlusal surface of the discs through vigorous rinsing with distilled water. Then, the dentin discs were removed from the APCs and placed individually in wells, in a way that their pulp surface, where the cells were cultivated, were kept facing up. These cells were submitted to cell metabolic activity analysis by metiltetrazolium assay (MTT Assay) (22). In a vertical laminar flow hood, the culture media in contact with cells was aspirated and 900 µL of complete DMEM plus 100µL of the MTT stock solution (Sigma Cemical Co., USA) at concentration of 5mg/ml of phosphate-buffered saline solution (PBS). The cells in contact

with the DMEM/MTT solution were incubated at 37^{o}C for additional 4 h. After this period, the DMEM/MTT solution was aspirated and replaced by 600 µL of acidified isopropanol solution in HCL 0.04N, to dissolve the blue crystals of formazan present in the cells resulting from the cleavage methyltetrazolium salt by the succinate dehydrogenase (SDH) enzyme produced in mitochondria of viable cells. After this procedure, two aliquots of 100 µL of each well were transferred to compartments of 96-well-dish (Costar Corp., Cambridge, MA, USA). The cell viability was determined as being proportional to the absorbance measured at 570nm in ELISA reader (Multiskan, Ascent 354, EC Labsystems, Les Ulis, France).

Measurement of H2O2 Transdentinal Diffusion

To the groups 3 and 6 only, five additional disks were used to measure the concentration of hydrogen peroxide that diffused through the dentin to reach the pulp space of the APCs. The measurement of hydrogen peroxide within the APC was performed using the leukocrystal violet/enzyme horseradish peroxidase (LCV/HRP) assay (23). In each well of a microtiter plate, 900 uL of acetate buffer 2M and pH 4.5 was added and the APCs were immersed with dentin, with the pulp surface facing down and in contact with the buffer solution.

The bleaching gels were applied on the occlusal of the disks and incubated for 6 hours at 37˚C. After incubation period, 100µL of the acetate buffer was removed from the wells and transferred to glass tubes. Afterwards, 100 µL of LCV 0.5mg/mL solution was added in

each tube (Sigma Chemical Co., St. Louis, MO) and 50µL of HRP solution with a concentration of 1mg/ml (Sigma Chemical Co., St. Louis, MO).

After 5min of reaction, the optical density of the resultant blue color in the tubes was measured by a UV spectrophotometer (UV-Vis Spectrophotometer UV-1203; Shimadzu, Kyoto, Japan) at the wavelength of 596 nm. The optical density values obtained from the samples were converted into micrograms equivalent to the H_2O_2 concentration in each solution using a calibration curve obtained with hydrogen peroxide in the range of 0.5 to 4.5mg.

Cell Morphology Analysis in Scanning Electronic Microscope (SEM)

For each group, two representative samples were prepared to be analyzed in SEM. For this, the dentin disks with cells present on the pulp surface were carefully removed from the APCs and fixed in 1 mL of buffered 2.5% glutaraldehyde for 24 hours. Next, the cells were submitted to three 5-minute rinses with 1 mL PBS, post-fixed in 1% osmium tetroxide for 60 min. Afterwards, the discs with cells were dehydrated in increasing concentrations of ethanol solutions (30%, 50%, 70%, 90%, 100%). Finally, the cells on discs were subjected to drying by low surface tension hexamethyl disilazane 98% (HMDS) solvent (Acros Organics, New Jersey, USA) and kept in desiccators for 12 hours. The dentin discs, with the MDPC-23 cells that remained attached to its pulp surface were fixed on metal stubs and gold sputtered. These procedures allowed the cell morphology analysis in MEV. (JEOL-JMS-T33A Scanning Microscope, JEOL-USA Inc., Peabody, MA, USA).

Statistical Analysis

The numeric values obtained by the MTT technique, referring to the production of SDH, were subjected to statistical analysis of Kruskal-Wallis complemented by Mann-Whitney test to compare the groups in pairs. For the values obtained for the transdentinal diffusion of carbamide peroxide a student-t test was used. All statistical tests were considered to the pre-established level of significance of 5%. Statistical analysis was carried out in the statistical software SPSS (SPSS Inc., Chicago, Illinois, USA) with a confidence interval of 95%.

Results

Dentin Permeability

The permeability of the disks was measured through a filtering chamber, and the data obtained, which were tabulated and subjected to statistical analysis (Kruskal-Wallis $p > 0.05$), are presented in Table 1.

Cell Viability

Median (P25/P75) for production of SDH, detected by MTT assay, is showed in Table 1 according to the experimental groups. The CP 16% application for 6 hours resulted in a lower values of SDH production ($p<0.05$), while the SA 10%/6h use resulted in increased production of this enzyme, although not statistically higher than control group ($p>0.05$). The groups 2, 3, 4 and 6 did not differ among themselves, as well as when

compared to control ($p>0.05$). Considering the control group as 100% of cell viability, it was possible to calculate the percentage of MDPC-23 cells viability for the different experimental groups. These inferences can be graphically displayed in Figure 1.

H_2O_2 Transdentinal Diffusion

The results obtained on H_2O_2 transdentinal diffusion are shown in Table 2. According to results, group 5 presented a statistically higher diffusion of hydrogen peroxide than group 3 (T-student Test, $p<0.01$).

Cell Morphology (SEM)

In groups 1 and 2, it was observed a large number of MPDC-23 cells remained attached to the dentinal substrate, which was near confluence. In both groups, the MDPC-23 cells presented large cytoplasm and numerous cytoplasmatic prolongations originating from the cell membrane, which seemed to adhere to dentin (Figure 2A/B). For all the other groups, a decrease in the number of cells that remained attached to the dentin substrate was observed. However, it was still possible to observe that the cells presented morphologic changes, characterized by the decrease in its size and reduction of the amount and extension of their cytoplasmatic prolongations. (Figures 2C/D/E/F). These important morphological changes observed in the MDPC-23 cells that remained attached to the dentin, made them take a round-shaped morphology. Nevertheless, the morphological changes of cells (reduction of size and decrease in number) were more evident in group 5, where large areas of the dentin with dentinal tubules exposed

remained open, which marked the occurrence of significant cell death followed by its detaching from substrate (Figure 2F).

Discussion

The dentin permeability was first measured so that all groups were statistically similar to each other. This initial trial ensured that at the time of evaluation of the H_2O_2 diffusion, as well as the cell viability, the dentin permeability would not have an influence on the results.

Due to the transdentinal diffusion of H_2O_2, which occurred for all the groups where the bleaching gels were applied, the cells cultured on the pulp surface of the dentin disks had direct toxic effect. An intense decrease in cell viability was observed in the group 5 (PC 16%), which was statistically different from group 1 (control) and all the other experimental groups. These negative results observed for group 5 were at least partially, due to the fact that a larger amount of H_2O_2 diffused through the dentinal tubules to reach the pulp area of the APCs, where the cells MDPC-23 were cultivated. This increased concentration of the H_2O_2 was clearly observed through the use of the technique of violet leucocristals and horseradish peroxidase enzyme (LCV/HRP). On the other hand, to group 3 (CP 10%) approximately 1.7 g/mL of H2O2 was detected, diffusion statistically lower than group 5. This fact determined that when higher concentration of carbamide peroxide gel is applied to dentin substrate, a larger amount of H_2O_2 diffuse through the dentin to cause cellular damage. Thus, despite the dentin acting as an organic barrier to protect the

pulp tissue against external toxic agents (24), this tubular tissue, with width standardized 0.5mm, does not prevent that components released from the bleaching, such as H_2O_2, to diffuse causing decrease in the metabolic activity.

The H_2O_2 and its sub-products, such as the hydroxyl radical (OH-), are considered reactive oxygen species, able to cause damage to several cellular components, such as cell membrane and DNA, by direct action or reacting with other cellular components generating new ROS (25). This damage begins in the membrane, triggering an autocatalytic reaction, denominated as lipid peroxidation (26). Damages to the cell membrane results in decrease of fluidity of this structure, increase of its permeability, as well as the inactivation of receptor, enzymes and ion channels (26). These changes in cell membrane allow a marked and sustained increase in cytosolic free Ca2+ concentration, which can cause calcium-dependent catabolic processes, such as activation of phospholipases, endonucleases and proteases (27). These changes may cause, since extensive damage to the cell membrane and even cell death (26).

To the group 5, most cells that were attached to the pulp surface of the dentin disks were lethaly damaged after application of bleaching gel with 16% of CP. This was confirmed by the SEM analysis. For this group, after treatment, several dead cells were detached from the dentinal substrate. Thus, only few MDPC-23 cells with round-shaped morphology and reduced size remained on broad areas of tubular dentin (Figure 2E). These morphological changes of cells were less significant for group 3 (10% CP), which

clearly shows that the damages of the MDPC-23 cells are directly related to the H_2O_2 concentration released by the CP gel applied on the dentin.

The oxidative stress, which occurs due to the severe unbalance between the production of ROS and the presence of endogenous antioxidants (28), was not very expressive in group 3. This indicates that perhaps the endogenous enzymatic antioxidants such as catalase, superoxide dismutase and glutathiose peroxidase, could have been sufficient to prevent a serious cellular damage. Moreover, it can be speculated that the concentration of approximately 1.7 g/mL of H_2O_2 in the APCs pulp space, where the cells were cultivated, has not been sufficient to cause cell death, as observed in group 5. However, for group 3, despite the small H_2O_2 diffusion through the dentin, the cell analysis in SEM showed significant morphological changes, without a remarkable decrease in cells number that remained attached to the dentin substrate. In this study, with the objective of simulating the daily period of application of carbamide peroxide in the nightguard vital bleaching, cell viability was assessed after 6 hours of the bleaching gels being applied on the tooth structure. Nevertheless, the nightguard vital bleaching technique has an average period of application of two weeks. With this increased time of applications, the discrete cytotoxicity of CP gel 10% on the MDPC-23 cells presented in this study, could be aggravated due to increase of transdentinal diffusion of oxidizing agents, which can cause major damage or cell death.

In group 2 (SA 10%), it was observed a percentage of a slightly higher cell viability, however, not statistically different from the control group. For this experimental group,

the morphological characteristics of the cells were similar with the normality, showing that concentration of 10% SA applied on the occlusal surface of the dentin disks did not cause toxic effects to the MDPC-23 cells. In groups 4 and 6, whose solution of 10% SA was applied to the dentin prior to the use of CP gel at 10% or 16%, respectively, it was verified that there was less damage to the MDPC-23 cells, which suggests the protective effect of SA against the deleterious effects of H_2O_2.

The dentinal diffusion of sodium ascorbate was not directly evaluated in this study. Nevertheless, it should be considered the capacity of resin monomers like TEGDMA (molecular weight = 286.3g/mol) and HEMA (molecular weight = 130g/mol) of diffuse through the dentin and reaching the pulp tissue on different situations (29-32). The sodium ascorbate, antioxidant agent used in this study, has a molecular weight of 198.11g/mol, intermediate when compared to resin components TEGDMA and HEMA. Therefore, could be speculate that the sodium ascorbate may have diffused through the dentin disk, reaching the pulp space of the APC. This fact might explain the lower cell damage observed in groups where the dentin tissue was previously treated with SA 10%. This is due to that SA is able to interact with the ROS, making them stable (Figure 3), decreasing its reactivity (33), and consequently preventing and/or decreasing cell damage caused by free radicals. The protection ability of sodium ascorbate was also observed in a previous study, where this antioxidant agent was applied with eluate of composite resins on fibroblast in culture, and the toxicity of these resin products were clearly reduced (16).

The dentin thickness of 0.5mm used in this study, can be mainly found in regions that have abrasion or erosion injuries, and this thickness has already been used in previous studies. (24). The results of the H_2O_2 diffusion for groups 5 (PC16%) were much larger than presented by group 3 (PC10%). The release of hydrogen peroxide is 4.7% for 16% CP and 3.5% for 10% CP, approximately (34), which explains the wide diffusion and greater cytotoxicity of the bleaching agent.

In this study, it was observed that the two concentrations of carbamide peroxide tested diffuse themselves through the human dentin, and 16% carbamide peroxide shows transdentinal toxicity to the odontoblastic cells. The 10% sodium ascorbate, when used before applying the bleaching gel on dentin substrate, can prevent the harmful effects of bleaching agents to these pulp cells. Nevertheless, results of in vitro studies cannot be directly extrapolated to clinical situations. More studies are necessary to know the effects of bleaching agents on pulp tissue, and analyze alternative ways to reduce the side effects of those agents, to dental bleaching, which has proven efficiency in the literature (35), has increased its security to pulp tissue.

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Table 1 – Median (range) of hydraulic condutance and succinic dehydrogenase enzyme (SDH) activity according to the experimental groups (n=8)

Groups	**Hydraulic Conductance*** **(**gL/cm^2/min/cm H_2O**)**	**SDH activity**	**Statistical Comparison*** **
1	0,01416	0,1989 (0,0975-0,2398)**	AB
2	0,01446	0,2002 (0,1807-0,2540)	A
3	0,01420	0,1733 (0,0629-0,2064)	B
4	0,01404	0,1897 (0,0714-0,2298)	B
5	0,01416	0,0697 (0,0595-0,1485)	C
6	0,01422	0,1605 (0,0796-0,1914)	B

* no statistical difference between the groups (Kruskal-Wallis, p>0,05)

** values represent the median (P25-P75)

** * Different letters indicate statistically significant difference (Mann-Whitney, p<0,05)

Table 2 – Mean of the transdentinal diffusion of hydrogen peroxide on the groups 3 and 5

Concentração	**µg/mL de H_2O_2**	
PC 10%	1,699	A
PC 16%	4,2314	B

* Different letters indicate statistically significant difference (T-Student Test, $p<0,01$)

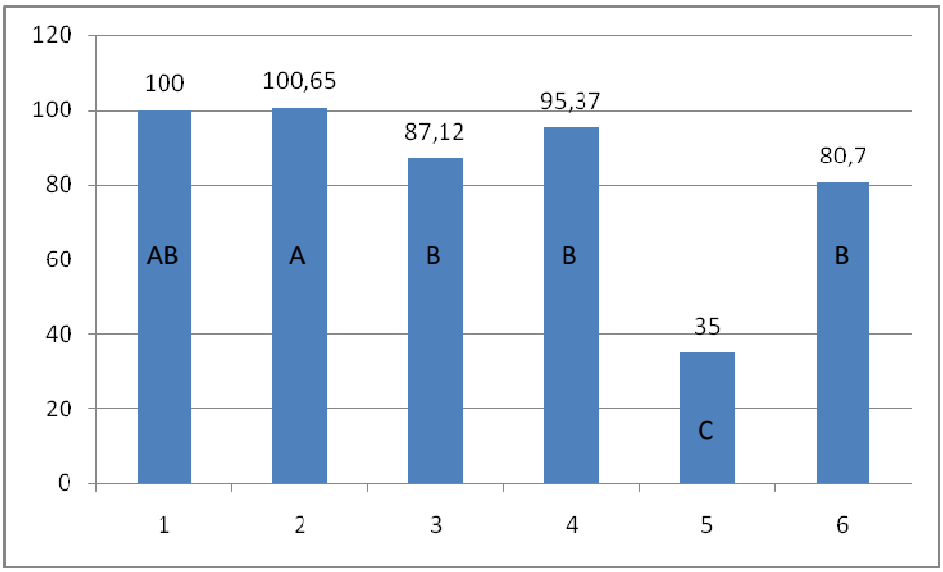

Figure 1 – Graphical representation in percentage of the cell viability in function of different experimental groups

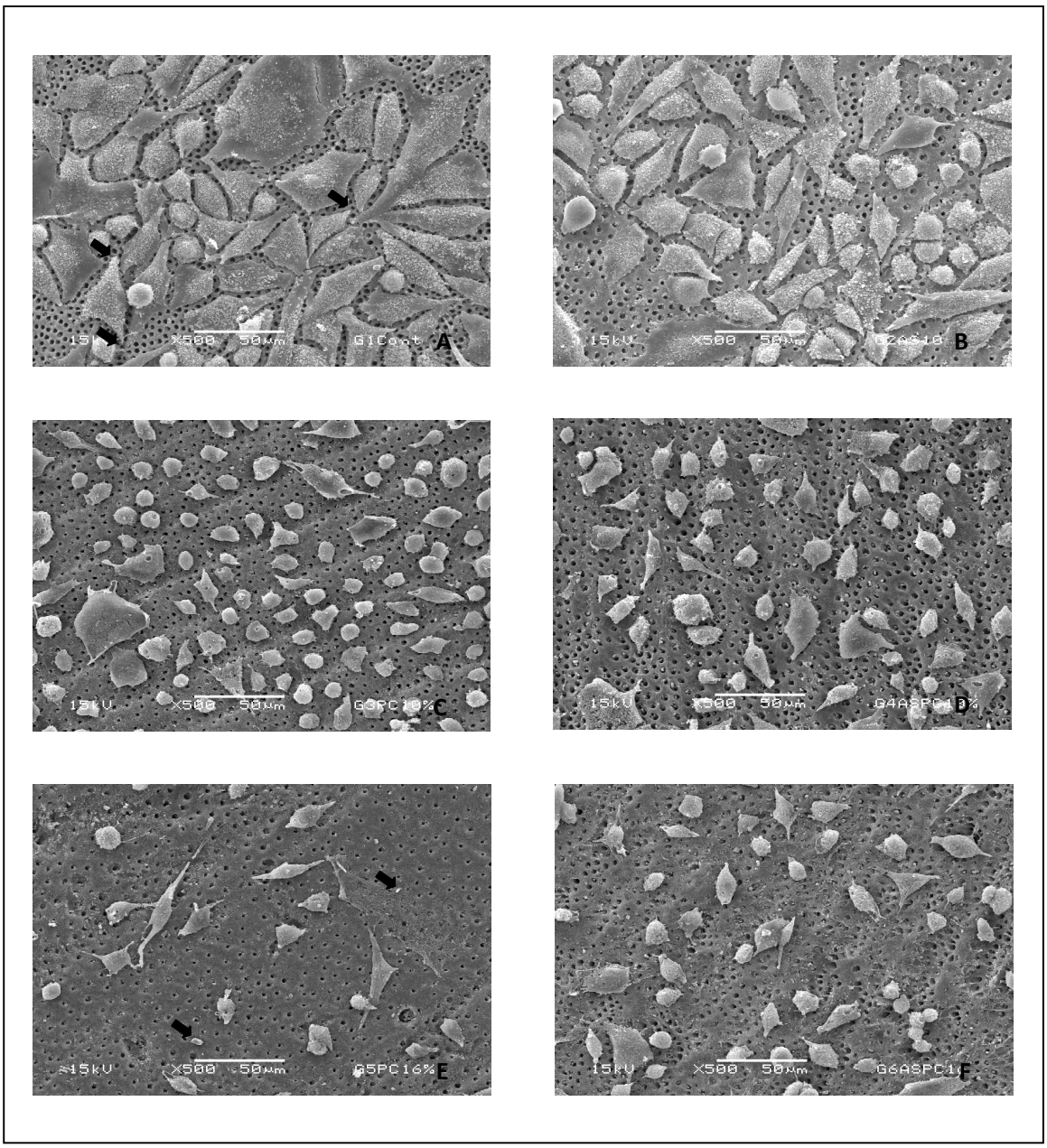

Figure 2 – Representative fotomicrografias in SEM (increased from original ×500) of experimental and control groups used in this study. A – Group 1 (control): Large number of odontoblastic cells near confluence are observed on the pulp surface of the dentin disk. Note that the cells exhibited a large cytoplasm with numerous short cytoplasmatic prolongations originating from the cell membrane (arrows) B – Groups 2 (AS):

morphological characteristics of MDPC-23 cells similar to those observed for the control group. C-Group 3 and D-Group 4: A large number of cells presented a round-shaped morphology scattered throughout the dentin disk, which lost cytoplasmatic prolongations originating from the cell membrane. Note that despite of some cells still show large cytoplasm, most of them have reduced size when compared to the control group. E-Group 5: Outstanding reduction in the number of cells attached to the substrate, and a large amount of cell membrane debris (arrows) due to oxidative stress. F-Group 6: Observe MPDC-23 cell with important morphological changes, as observed in Group 6. However, a greater number of cells remained attached to the dentin.

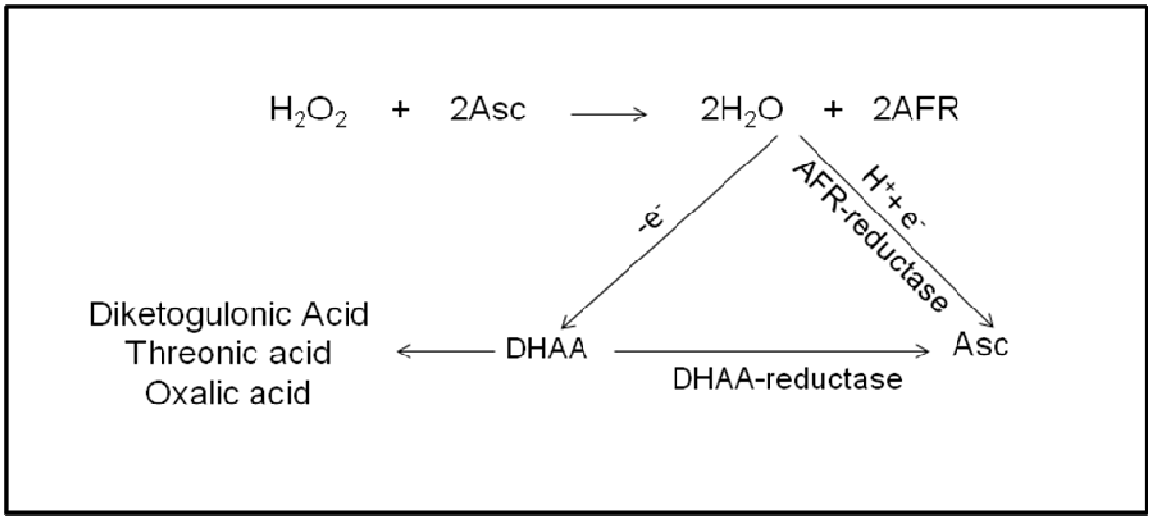

Figure 3 - The hydrogen peroxide (H2O2) is neutralized by transfer of a single-electron of ascorbates (Asc) producing water and ascorbyl free radicals (AFR). The pairs of AFR form one molecule of dehydroascorbic acid (DHAA) and one Asc. The lactone ring of DHAA is breaking down forming the inert products, diketogulonic acid, threonic acid and oxalic acid; alternatively, DHAA can be reduced to the useful Asc.

CONSIDERAÇÕES GERAIS

O procedimento clareador tem sido amplamente empregado na odontologia estética, porém sua ação sobre as células odontoblásticas ainda não havia sido estudada. O peróxido de carbamida, promove a liberação de peróxido de hidrogênio e uréia. Este peróxido de hidrogênio, quando em contato com as células odontoblásticas, promove um estresse oxidativo, que pode provocar danos celulares reversíveis e até mesmo irreversíveis (Halliwell, 2007).

No presente estudo, estes danos foram evidentes, em algumas concentrações cujas células foram expostas. As baixas concentrações do gel clareador utilizadas no Capítulo 1, foram selecionadas para análise da citotoxicidade, com o objetivo de simular a reduzida quantidade dos produtos do agente que atinge o tecido pulpar após sua aplicação sobre os tecidos duros do dente, confirmada por diversos estudos (Gokay *et al.*, 2000a; Gokay *et al.*, 2000b; Camargo *et al.*, 2007).

As concentrações de 0,01% e 0,1% (2,21µg/mL e 29.74µg/mL de H_2O_2, respectivamente) demonstraram a maior toxicidade para as células odontoblastóides MDPC-23. Essa ação deletéria destes agentes sobre as células se dá pela peroxidação lipídica, que consiste na quebra de ligações duplas de ácidos graxos insaturados dos lipídios da membrana plasmática, pela ação dos radicais OH^- liberados pelos agentes clareadores, causando lesão oxidativa imediata no local (Halliwell, 2006). As interações lipídio-radical geram peróxidos, que são instáveis e reativos, desencadeando uma reação autocatalítica, a qual pode causar lesões extensas na membrana das células ou mesmo determinar a morte celular (Halliwell, 2006).

Estas lesões podem causar uma diminuição da fluidez da membrana plasmática, aumento de sua permeabilidade, bem como inativação de receptores, enzimas e canais iônicos (Halliwell, 2006). São capazes de causar um aumento da concentração de cálcio intracelular, o qual é responsável pela ativação de processos

catabólicos cálcio dependentes, como ativação de fosfolipases, endonucleases e proteases (Bellomo *et al.*, 1987).

Buscando reduzir os efeitos citotóxicos causados pelos materiais odontológicos, alguns estudos realizaram a aplicação de agentes antioxidantes, tais como a N-acetilcisteína, o ascorbato de sódio e o Trolox, um análogo sintético da vitamina E, em meio contendo eluatos de agentes resinosos, para a avaliação da ação antioxidantes frente a esses materiais na proteção de fibroblastos gengivais humanos (Stanislawski *et al.*, 2000; Soheili Majd *et al.*, 2003). Os resultados destes estudos demonstraram a proteção das células fibroblásticas frente a alguns materiais, quando os agentes antioxidantes foram aplicados.

Objetivando a proteção das células odontoblásticas frente aos agentes clareadores, no Capítulo 2 foram utilizadas duas concentrações de ascorbato de sódio, e aplicadas juntamente com uma solução de 0,01% de peróxido de carbamida. A solução de 0,01% de peróxido de carbamida foi utilizada por apresentar efeitos tóxicos às células odontoblásticas, assim como uma liberação de peróxido de hidrogênio semelhante àquela encontrada no espaço pulpar, após a realização do clareamento utilizando diferentes concentrações dos agentes clareadores (Gokay *et al.*, 2000a; Gokay *et al.*, 2000b; Camargo *et al.*, 2007). As concentrações do ascorbato de sódio utilizadas foram baseadas em um estudo anterior (Soheili Majd *et al.*, 2003).

Quando o ascorbato foi aplicado isoladamente, não foi observada nenhuma diferença estatística nos valores da produção de SDH, quando comparados ao grupo controle. Já o peróxido de carbamida 0,01%, demonstrou uma redução na viabilidade celular de 38% e 33%, nos capítulos 1 e 2, respectivamente, por motivos previamente esclarecidos, na descrição e discussão de ambos os Capítulos. Quando avaliamos a efetividade das concentrações de 0,25mM e 0,5mM de ascorbato de sódio, na proteção das células odontoblásticas frente à solução de 0,01% de peróxido de carbamida,

observamos leve diminuição na viabilidade celular, não diferindo do grupo controle, e estatisticamente superior quando comparado ao grupo 6 (PC 0,01%).

No Capítulo 2, quando o agente clareador à base de peróxido de carbamida 16% foi aplicado sobre o disco de dentina na CPA. Após a aplicação e a manutenção daquele agente por seis horas, houve uma difusão de 4,23µg/mL de peróxido de hidrogênio pelo disco, atingindo o interior da CPA, e entrando em contato com as células odontoblásticas.

A dentina apresenta-se como uma barreira de proteção para o tecido pulpar, contra os agentes agressores externos (Hanks *et al.*, 1993), sendo que esta proteção foi mais efetiva no grupo onde o peróxido de carbamida 10 % foi aplicado, quando comparado ao peróxido de carbamida 16%, possivelmente devido à menor concentração do gel clareador utilizado no primeiro grupo, menor liberação e consequente menor difusão de peróxido de hidrogênio para o interior da CPA (1,6 µg/L no grupo 3).

A proteção promovida pelo ascorbato de sódio é devida à inativação das EROs difundidas pelo disco de dentina, advindas do gel clareador. O ascorbato age reagindo com as moléculas dos EROs, tornando-as estáveis, diminuindo sua reatividade (Chaudiere *et al.*, 1999), e conseqüentemente evitando e/ou diminuindo a lesão às células. Assim, pode-se sugerir que o ascobato de sódio nas concentrações de 0,25mM (G4) e 0,5mM (G5) aplicados sobre as células juntamente com a concentração de 0.01% de peróxido de carbamida, ou o ascorbato de sódio 10%, quando aplicado sobre os discos de dentina previamente à aplicação dos agentes clareadores, realmente pode prevenir danos celulares.

Pode-se observar nas MEVs realizadas nos três estudos apresentados, grandes modificações na morfologia das células expostas às diferentes concentrações de peróxido de carbamida, com perda dos prolongamentos odontoblásticos, morfologia arredondada, e presença de restos celulares devido à lise provocada pelos EROs liberadas pelos agentes

clareadores. Leves alterações morfológicas foram notadas quando o ascorbato de sódio foi aplicado juntamente ou previamente ao peróxido de carbamida, além de uma maior quantidade de células observada quando comparada aos grupos onde o gel clareador foi aplicado isoladamente. Características como a apresentação das células em nódulos, pequenos prolongamentos odontoblásticos e amplo citoplasma, ou seja, características condizentes com a normalidade, foram encontradas nos grupos controle, e onde o ascorbato, tanto nas concentrações de 0,25mM e 0,5mM, como na concentração de 10%, foram aplicados isoladamente, demonstrando que estas soluções antioxidantes não apresentam efeitos deletérios às células odontoblásticas MDPC-23.

Observamos através deste estudo, que as EROs liberadas pelos agentes clareadores, podem lesar de modo irreversível as células odontoblásticas, devido ao estresse oxidativo gerado; e que a aplicação de um agente oxidante, como o ascorbato de sódio, quando em contato com essas células, pode levar a uma diminuição desta toxicidade. Mais estudos devem ser realizados para o conhecimento sobre os danos ao tecido pulpar causado pelos agentes clareadores, assim como meios para que estes danos sejam diminuídos ou até mesmo evitados.

CONCLUSÃO

De acordo com as condições experimentais e com base nos resultados obtidos foi possível concluir que:

- baixas concentrações do gel de peróxido de carbamida (0,01% e 0,1%) apresentam significantes efeitos citotóxicos para células odontoblastóides MDPC-23;

- a concentração de 0,1% de PC foi altamente citopática, reduzindo em quase 80% o metabolismo celular;

- O gel de peróxido de carbamida 16% apresenta toxicidade transdentinária às células odontoblásticas MDPC-23 quando aplicados sobre discos de dentina com 0,5mm de espessura;

- O ascorbato de sódio nas concentrações de 0,25mM e 0,5mM pode proteger as células odontoblásticas dos efeitos nocivos dos agentes clareadores;

- O ascorbato de sódio na concentração de 10%, quando aplicado sobre os discos de dentina, foi capaz de proteger as células da toxicidade trandentinária do peróxido de carbamida 16%;

- O gel de peróxido de carbamida 16% apresenta maior difusão pela dentina quando comparado ao mesmo gel na concentração de 10%.

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ANEXO 1

Carta de aceitação do artigo referente ao Capítulo 1

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From: appliedbiomaterials@orthogencorp.com

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Subject: Accept - Journal of Biomedical Materials Research: Part B - Applied Biomaterials - Manuscript JBMR-B-08-0505.R1

Body: Date:30-Dec-2008
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Dear Mr. Lima:

I am pleased to inform you that your manuscript entitled "Cytotoxic Effects of Different Concentrations of a Carbamide Peroxide Bleaching Gel on Odontoblast-Like Cells MDPC-23" has been accepted for publication in the Journal of Biomedical Materials Research: Part B - Applied Biomaterials.

We no longer ask that you send us a hard copy of your manuscript. We will automatically forward your files to the publisher from our website. Page proofs will be emailed to you shortly.

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ANEXO 2

Certificado de Aprovação do Comitê de Ética em Pesquisa

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Avaliação da atividade protetora do ascorbato de sódio estabilizado (antioxidante) sobre o efeito citotóxico transdentinário de um agente clareador contendo 10% de peróxido de carbamida**", protocolo nº **001/2007**, dos pesquisadores **ADRIANO FONSECA DE LIMA, CARLOS ALBERTO DE SOUZA COSTA e GISELLE MARIA MARCHI BARON**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 14/02/2007.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Evaluation of the protective activity of sodium ascorbates stabilized (antioxidizing) on trandentinal cytotoxic effect of 10% carbamide peroxide bleaching agent**", register number **001/2007**, of **ADRIANO FONSECA DE LIMA, CARLOS ALBERTO DE SOUZA COSTA and GISELLE MARIA MARCHI BARON**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 14/02/2007.

Profa. Cecilia Gatti Guirado
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 3

Materiais e Métodos referentes ao Capítulo 3

Após aprovação da presente pesquisa pelo Comitê de Ética da Faculdade de Odontologia de Piracicaba/UNICAMP, Brasil (protocolo 01/2007), setenta discos de dentina com aproximadamente 0,6 mm de espessura foram obtidos de terceiros molares humanos íntegros. Para isso, os dentes foram fixados em placas de acrílico, sendo que cortes transversais da coroa foram realizados, um acima da projeção dos cornos pulpares e outro abaixo da junção amelo-dentinária, utilizando-se um disco de diamante (11-4254, 4"x 0,012"/ série 15LC, Diamond Blade, Buehler Ltd., Lake Bluf, IL, USA) montado numa cortadeira metalográfica (ISOMET 1000, Buehler Ltd., USA). A espessura dos discos de dentina foi reduzida através de desgaste manual em lixas d'água granulação 400 e 600 (T469-SF- Noton, Saint-Gobam Abrasivos Ltda., Jundiaí, SP, Brasil). Assim, discos de dentina com espessura final padronizada em 0,5 mm foram montados em câmara de filtragem (Fig. 1A) conectada a uma coluna d'água de 180 cm (Outhwaite *et al.*, 1974), sendo a condutância hidráulica (Lp; $\mu l/cm^2/min/cm\ H_2O$) calculada e determinada para cada disco. Finalmente, os discos de dentina foram distribuídos entre os grupos, de tal maneira que os valores médios de permeabilidade não fossem estatisticamente diferente entre eles (Kruskal-Wallis, $p>0,05$).

Efeito Citotóxico Transdentinário

Para a análise da viabilidade celular, foi utilizado um dispositivo simulando uma câmara pulpar artificial (CPA) (Fig. 1B), ao qual foi acoplado um disco de dentina para o teste de difusão transdentinária e citotoxicidade do agente clareador. Para isso, as laterais dos discos foram desgastadas com ponta diamantada #1095 (KG Sorensen, Barueri, SP, Brasil) obtendo-se um diâmetro de 8mm. Estes foram posicionados com a superfície pulpar voltada para a região interna da CPA (espaço pulpar), entre dois anéis de silicone esterilizados (Fig. 1C e 1D) (Orion – São Paulo, SP, Brasil), os quais além de

acomodarem o disco de dentina em posição, evitam a difusão dos materiais pelas laterais do disco. As CPAs foram individualmente posicionadas em *wells* de placas de acrílico esterilizados de 24-*wells* (Coastar Corp. Cambridge, MA, USA), com a superfície pulpar dos discos de dentina voltada para cima (Fig. 2A). Então, células imortalizadas de linhagem odontoblástica MDPC-23 (Hanks *et al.*, 1998; Sun *et al.*, 1998) foram semeadas (50.000 cells/cm^2) sobre a superfície dentinária. As CPAs com os discos e células foram mantidas por 48 horas em incubadora com atmosfera úmida à 37°C, com 5% de CO_2 e 95% de ar. O meio de cultura usado neste experimento foi o Dulbecco´s Modified Eagle´s Medium (DMEM, SIGMA Chemical Co., St. Louis, MO, USA) complementado com 10% de soro fetal bovino (FBS, Cultilab, Campinas, SP, Brasil), suplementado com 100 IU/mL de penicilina, 100 μg/mL de estreptomicina e 2 mmol/L de glutamina (GIBCO, Grand Island, NY, USA).

A seguir, as CPAs com os discos de dentina e células foram invertidos e posicionados, de maneira individual, em nova placa de acrílico de 24-*wells,* com as células imersas em meio de cultura. Em cada *well* foi imediatamente aplicado 1mL de DMEM completo, de tal maneira que as células aderidas à superfície pulpar dos discos fossem mantidas em contato com o meio de cultura. Por outro lado, a superfície oclusal dos discos de dentina foi mantida para cima, permitindo a aplicação dos produtos sobre este substrato tubular (Fig 2B). A realização ou não do tratamento da superície oclusal dos discos de dentina deram origem aos seguintes grupos (n = 10):

Grupo 1 - Sem tratamento (Controle);

Grupo 2 - Antioxidante 10% (AS);

Grupo 3- Peróxido de Carbamida (PC) 10% aplicado por 6 horas;

Grupo 4- Antioxidante 10% (AS)/6horas + lavagem dos discos + PC 10%/6horas;

Grupo 5- PC 16% aplicado por 6 horas;

Grupo 6- Antioxidante 10% (AS)/6horas + lavagem dos discos + PC 16%/6horas.

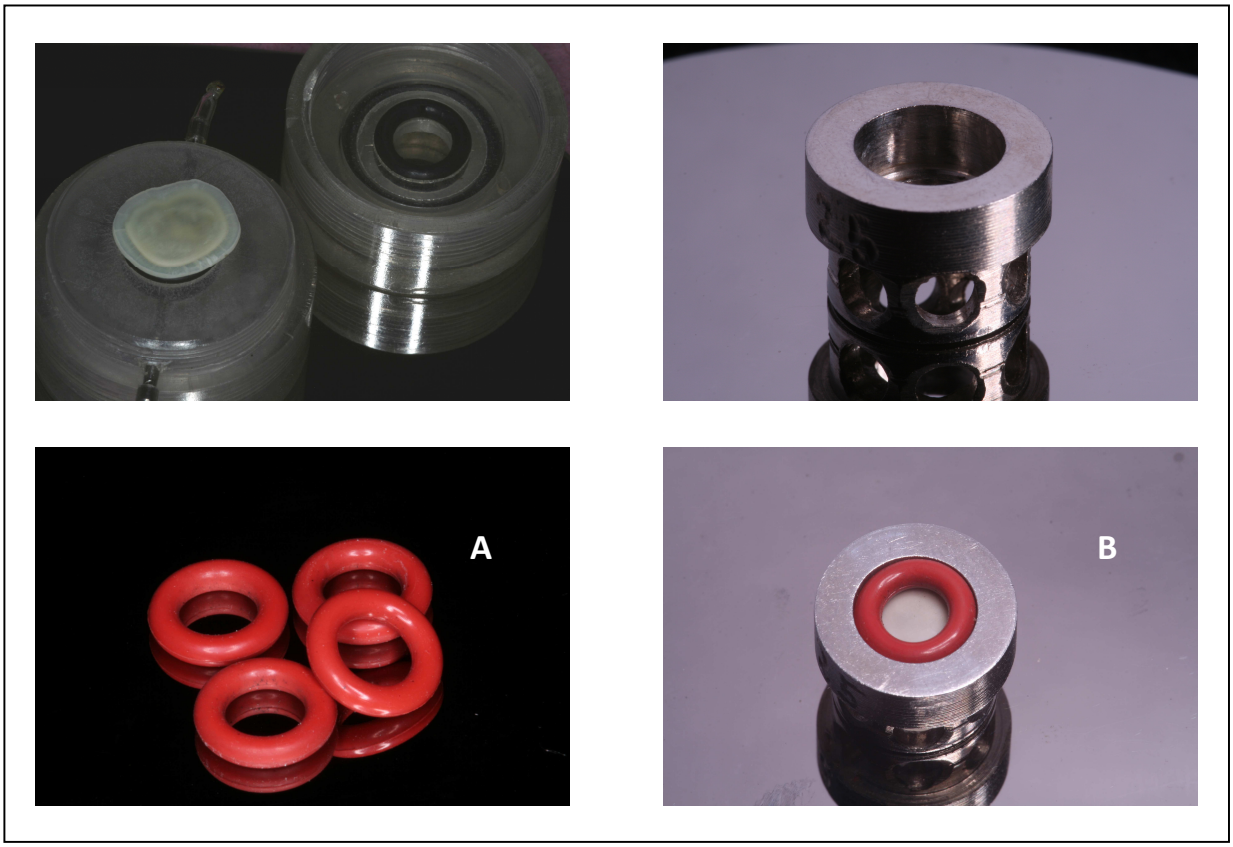

Figura 1: Câmara de filtragem (A); CPA (B); anéis de silicone (C) e CPA após acomodação do disco de dentina (D).

Do total de 10 discos por grupo, oito foram utilizados para avaliação da viabilidade celular, sendo que dois foram destinados à avaliação da morfologia celular em microscopia eletrônica de varredura (MEV). Apenas para os grupos 3 e 6, foram utilizados mais cinco discos para a mensuração da concentração de peróxido de hidrogênio que se difundiu através da dentina para alcançar o espaço pulpar das CPAs.

Para os grupos 4 e 6, após 6 horas da aplicação do ascorbato de sódio 10% (3µL) (Fig. 2C), este agente antioxidante foi lavado, permitindo o tratamento da superfície oclusal dos discos com 18mg dos géis clareadores (Fig. 2D) contendo 10% ou 16% de peróxido de carbamida (WHITENESS, FGM, JOINVILE, SC, BRASIL). Após o período de 6 horas de aplicação dos géis clareadores, estes foram removidos da superfície oclusal dos discos através de vigorosa lavagem com água destilada esterilizada. Os discos de dentina foram

removidos das CPAs e posicionados individualmente em *wells*, de tal maneira que sua superfície pulpar, onde as células foram cultivadas, fosse mantida voltada para cima. Então, estas células foram submetidas à análise de viabilidade celular através da aplicação da técnica do metiltetrazolium (MTT Assay) (Mosmann, 1983).

Em capela de fluxo laminar vertical, o meio de cultura foi substituído por 900µL de DMEM, ao qual foi adicionado 100µL de solução de MTT (Sigma Chemical Co., USA) em concentração de 5mg/mL de PBS. As células foram incubadas por 4 horas a 37^{o}C. Em seguida, o meio de cultura foi removido e cada compartimento recebeu 600 µL de solução de isopropanol acidificado em HCL a 0,04N. Após este procedimento, duas alíquotas de 100µL de cada compartimento foram transferidas para compartimentos de uma placa de 96-*wells* (Costar Corp., Cambridge, MA, USA). A viabilidade celular foi avaliada de maneira proporcional à absorbância determinada a 570 nm em leitor de ELISA (Multiskan, Ascent 354, Labsystems CE, Lês Ulis, France).

Dosagem da Difusão Transdentinária do H_2O_2

A quantificação do peróxido de hidrogênio no interior da CPA foi realizada através da técnica de leucocristais violeta e enzima horseradish peroxidase (LCV/HRP) (Mottola *et al.*, 1970). Em cada well de uma placa de microtitulação, foram adicionados 900 µL de tampão acetato 2M e pH 4,5 e imersas as CPAs com os discos de dentina, com a superfície pulpar voltada para baixo e em contato com a solução tampão.

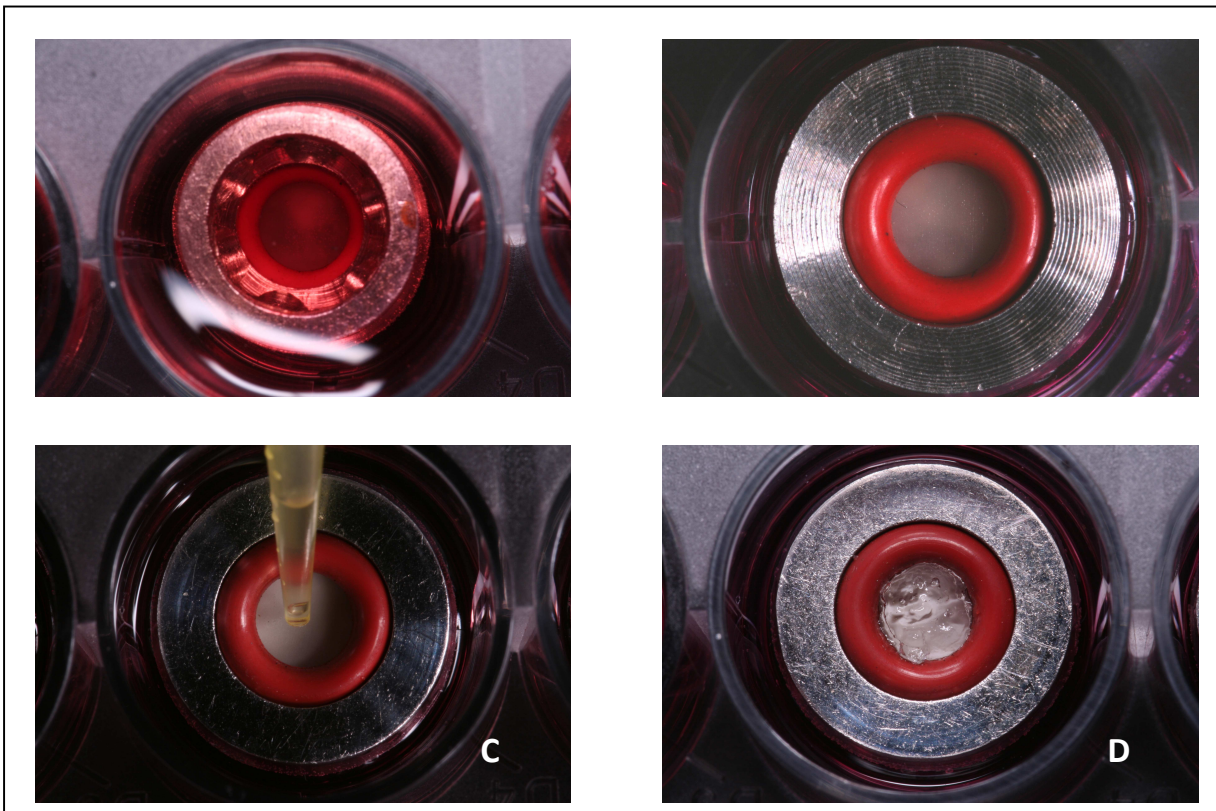

Figura 2: CPA com a superfície pulpar voltada para cima, permitindo a cultivo das células na superfície pulpar dos discos (A); CPA com a superfície oclusal voltada para cima (B); aplicação do ascorbato de sódio 10% (C) e gel clareador sobre o disco de dentina (D).

Os géis clareadores foram aplicados sobre a oclusal dos discos e incubados por 6 horas a 37 ^{o}C. Após o período de incubação, 100 µL do tampão acetato foram removidos dos *wells* e transferidos para tubos de vidro. A seguir, foram adicionados em cada tubo 100 µL de solução de LCV 0.5mg/mL (Sigma Chemical Co., St. Louis, MO) e 50µL de solução de HPR 1mg/mL (Sigma Chemical Co., St. Louis, MO)

Decorridos 5 minutos de reação, as densidades ópticas das soluções de coloração azul foram registradas, em 596nm, no espectrofotômetro UV (UV-Vis Spectophotometer UV-1203 – Shimadzu, Kyoto, Japan) e convertidas em microgramas de

H_2O_2 utilizando uma curva de calibração obtida com peróxido de hidrogênio no intervalo de 0,5 a 4,5 μg.

Análise da Morfologia Celular em Microscópio Eletrônico de Varredura (MEV)

Para cada grupo, foram preparadas duas amostras representativas para serem analisadas em MEV. Para isto, os discos de dentina com as células presentes sobre sua superfície pulpar foram cuidadosamente removidos das CPAs e imersos em solução fixadora de glutaraldeído 2,5% por 24 horas. Então, as células foram pós-fixadas por 60 minutos em tetróxido de ósmio 1%. Em seguida, os discos com as células foram submetidos à desidratação através de trocas periódicas de etanol em concentrações ascendentes (30%, 50%, 70%, 90% e 100%). Finalmente, as células sobre os discos foram submetidas à secagem por meio do solvente de baixa tensão superficial 1,1,1,3,3,3-hexamethyldisilazane 98% (HMDS - ACROS Organics, New Jersey, USA) e mantidas em dessecador durante 12 horas. Os discos de dentina, com as células MDPC-23 que permaneceram aderidas na sua superfície pulpar, foram fixados em *stubs* metálicos e cobertos com ouro, sendo que estes procedimentos permitiram a análise da morfologia celular em MEV (JEOL-JMS-T33A Scanning Microscope, JEOL – USA Inc., Peabody, MA, USA).

Análise Estatística

Os dados numéricos obtidos pela técnica de MTT, referentes à produção de SDH, foram submetidos à análise estatística de Kruskal-Wallis complementada por testes de Mann-Whitney para comparação dos grupos aos pares. Já para os valores obtidos pela difusão trandentinária do peróxido de carbamida, foi aplicado o Teste T-Student. Todos os testes estatísticos foram considerados ao nível pré-estabelecido de significância de 5%.