UNIVERSIDADE ESTADUAL PAULISTA

"Julio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

Diana Gabriela de Sousa Soares

Novos parâmetros para o clareamento dental: avaliação da eficácia, citotoxicidade e efeitos moleculares

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Tese apresentada ao Programa de Pós-graduação em Reabilitação Oral – Área de Prótese, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista, para obtenção do título de Doutor em Reabilitação Oral.

Orientador: Prof. Dr. Carlos Alberto de Souza Costa

Araraquara

2014

DIANA GABRIELA DE SOUSA SOARES

NOVOS PARÂMETROS PARA O CLAREAMENTO DENTAL: AVALIAÇÃO DA EFICÁCIA, CITOTOXICIDADE E EFEITOS MOLECULARES

Comissão Julgadora

Tese para obtenção do grau de Doutor

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Araraquara, 28 de Março de 2014

DADOS CURRICULARES

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Dedicatória

Dedico este trabalho...

A Deus,

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"Nossa maior fraqueza está em desistir. A maneira mais segura de ter sucesso é sempre tentar mais uma vez"

Thomas Edison
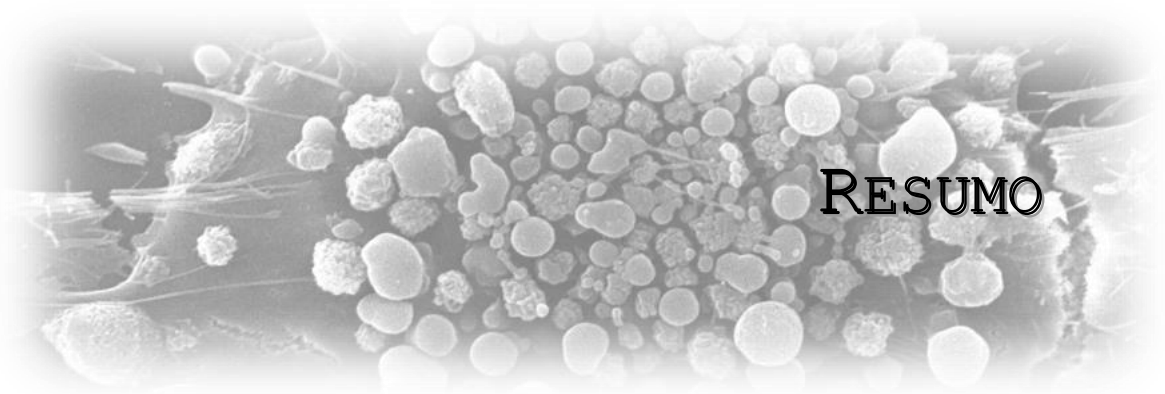
RESUMO

Soares DGS. Novos parâmetros para o clareamento dental: avaliação da eficácia, citotoxicidade e efeitos moleculares [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2014.

Resumo

Nesta pesquisa, foram analisadas a eficácia clareadora, citotoxicidade e características fenotípicas de células pulpares expostas a protocolos de clareamento de consultório experimentais. No primeiro estudo, discos de esmalte/dentina foram submetidos a 6 sessões clareadoras, caracterizadas por 1 ou 3 aplicações de peróxido de hidrogênio (H_2O_2) a 35% ou 17,5%, por 5 ou 15 min, ou de peróxido de carbamida (PC) a 37%, por 10 ou 20 min. A alteração de cor (ΔE) e quantificação da difusão de H_2O_2 (violeta leuco-cristal/peroxidase) foram avaliadas. Todos os protocolos testados promoveram alteração significativa de cor associados a redução na difusão de H_2O_2; no entanto, o gel com PC apresentou os piores resultados (Traço de Pilai; Bonferroni/$p<0,05$). Apenas os protocolos 35% H_2O_2/ 1x 15 e 3x 5 min, 17,5% H_2O_2/ 3x 15 min e 37% PC/ 3x 20 min apresentaram valores de ΔE similares ao protocolo tradicional (35%/ 3x 15 min) em até 6 sessões de clareamento (ANOVA; SNK e Tamhane/$p>0.05$). No segundo estudo, foi avaliada a citotoxicidade trans-amelodentinária causada pela aplicação de protocolos experimentais usando 35% H_2O_2/ 1x 15 e 1x 5 min e 17,5% H_2O_2/ 3x 15, 1x 15 e 1x 5 min. O protocolo 35% H_2O_2/ 3x 15 min foi empregado como controle positivo. Os discos de esmalte/dentina foram adaptados em dispositivos trans-well, os quais foram posicionados sobre células odontoblastóides (MDPC-23) e cultura primária de células pulpares humanas (HDPCs) previamente semeadas. A viabilidade (MTT) e morfologia celular (MEV) foram avaliadas imediatamente e 72 h pós-clareamento, bem como o estresse oxidativo e lesão à membrana celular (microscopia de fluorescência). Todos os protocolos experimentais avaliados reduziram significativamente o dano celular quando comparados ao controle positivo (Kruskal-Wallis; Mann-Whitney/$p<0,05$). Esta redução foi tempo/concentração dependente. As células expostas aos protocolos com o gel contendo 17,5% de H_2O_2 apresentaram capacidade de recuperação em torno de 50% três dias pós-clareamento. No terceiro estudo, células pulpares (MDPC-23 e HDPCs) em cultura foram submetidas aos protocolos experimentais, utilizando-se para isto um gel com 17,5% de H_2O_2, o qual foi aplicado por 3x 15; 1x 15; e 1x 5 min sobre o esmalte. Então, foi avaliada a expressão de marcadores de diferenciação odontoblástica (expressão gênica de DMP-1, DSPP e ALP, deposição de nódulos de mineralização e atividade de ALP), em períodos de 7, 14 e 21 dias pós-clareamento, bem como a expressão gênica de mediadores inflamatórios (IL-1β; TNF-α, IL-6 e COX-2) imediatamente após o procedimento clareador. Foi observado aumento significante na expressão gênica dos

mediadores inflamatórios nas células clareadas proporcional ao tempo de tratamento (Kruskal-Wallis; Mann-Whitney/p<0,05). Houve, ainda, redução tempo/dependente na expressão dos marcadores de diferenciação em relação ao controle negativo (sem tratamento); no entanto, as células foram capazes de se recuperar da agressão em períodos de até 21 dias pós-clareamento, com exceção para a deposição nódulos de mineralização pelas HDPCs no grupo clareado por 45 min (Kruskal-Wallis; Mann-Whitney/p<0,05). Pode-se concluir que a redução na concentração e/ou freqüência de aplicação de géis clareadores à base de H_2O_2 sobre o esmalte promove clareamento efetivo, associado a reduzida difusão deste radical livre pela estrutura dental, o que minimiza seu efeito tóxico sobre as células pulpares. No entanto, o gel com 17,5% de H_2O_2 aplicado por curtos períodos sobre o esmalte apresentou-se como a alternativa clareadora mais interessante, visto que as células apresentaram maior capacidade de recuperação, bem como mantiveram seu potencial para diferenciação odontoblástica e deposição de matriz mineralizada.

Palavras-chave: Polpa dentária, Toxicidade, Clareamento dental
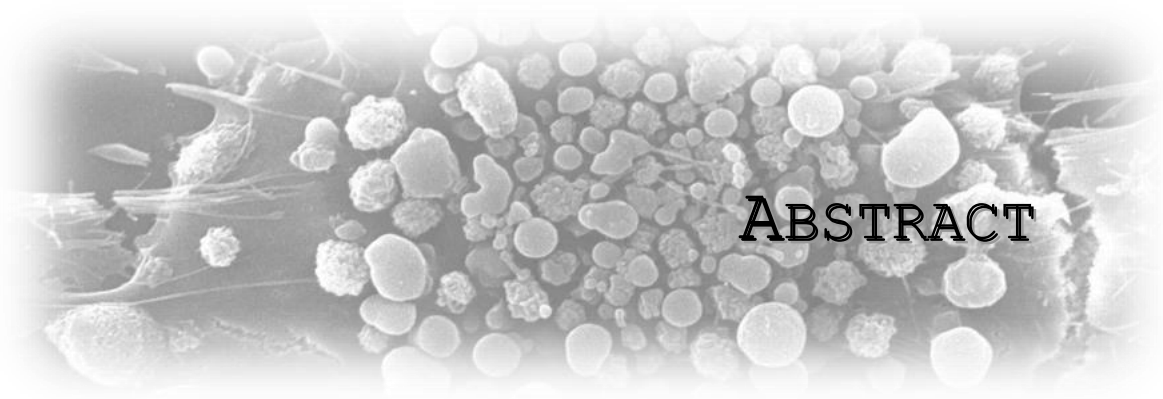
ABSTRACT

Soares DGS. New parameters for tooth bleaching: evaluation of bleaching effectiveness, cytotoxicity and molecular effects [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2014.

Abstract

In this study, the bleaching effectiveness, cytotoxicity and phenotypic characteristic of dental pulp cells exposed to experimental in-office bleaching protocols were analyzed. In the first study, enamel/dentin discs were subjected to 6 bleaching sessions, composed by 1 or 3 applications of a 35%- or 17.5%-H_2O_2 (hydrogen peroxide) gel, during 5 or 15 minutes, or by a 37%-CP (carbamide peroxide) gel, applied for 10 or 20 minutes. Color change (ΔE) and H_2O_2 diffusion (leucocrystal violet/ peroxidase) were analyzed. All the tested protocols promoted significant color alteration and reduction of H_2O_2 diffusion; however, the CP gel presented the worst results (Pillai's trace/ Bonferroni test; $p<0.05$). Only the protocols 35%-H_2O_2/ 1x 15, 35%-H_2O_2/ 3x 5 min, 17.5%-H_2O_2/ 3x 15 min and 37%-CP 3x 20 min presented similar ΔE values than traditional protocol (35%-H_2O_2/ 3x 15 min) up to 6 sessions (ANOVA; SNK and Tamhane tests/$p>0.05$). In the second study, the trans-enamel and trans-dentinal cytotoxicity on dental pulp cells of selected experimental bleaching protocols (35%-H_2O_2/ 1x 15 and 1x 5 min; 17.5%-H_2O_2/ 3x 15, 1x 15 and 1x 5 min) were tested. The protocol 35%-H_2O_2/ 3x 15 min was considered as the positive control group. The enamel/dentin discs were adapted to trans-wells devices, which were positioned over odontoblast-like cells (MDPC-23) and primary culture of human dental pulp cells (HDPCs) previously seeded on culture plates. The cell viability (MTT) and morphology (SEM) were evaluated immediately and 72 h post-bleaching, as well as oxidative stress and cell membrane damage (fluorescence microscopy). The experimental protocols promoted significant minimization of cell damage, for all evaluated parameters, when compared to positive control, in a time/concentration fashion (Kruskal-Wallis; Mann-Whitney/ $p<0.05$). Cells exposed to protocols with the 17.5%-H_2O_2 gel presented cell recovery capability in around 50% three days after bleaching. In the third study, the cells were exposed to the experimental protocols with the 17.5%-H_2O_2 gel (3x 15, 1x 15 and 1x 5 min), and the expression of odontoblastic differentiation markers (gene expression of DMP-1, DSPP and ALP, mineralized nodule deposition and ALP activity) was analyzed 7, 14 and 21 days after bleaching. Gene expression of inflammatory mediators (IL-1β; TNF-α, IL-6 e COX-2) was assessed immediately after bleaching. It was observed up-regulation of inflammatory mediators gene expression on bleached cells, which was proportional to treatment time. Reduction on differentiation markers expression related to negative control (no treatment) in a time/dependent fashion was also observed on bleached cells; however, the cells were able to recovery up to 21 days, excepting for mineralized nodule deposition by HDPCs on the 45-

minute group (Kruskal-Wallis; Mann-Whitney/$p<0.05$). It was concluded that the reduction on concentration and/or frequency of application of H_2O_2 based gels promotes effective bleaching, associated to reduction on H_2O_2 diffusion through dental structure, and consequently minimization of toxic effects over two cell lineages from pulp tissue. However, the 17.5%-H_2O_2 gel applied for short periods appeared to be an interesting alternative, as the cells presented capability to recovery its viability, as well as kept its potential for odontoblastic differentiation and deposition of mineralized matrix.

Key-words: Dental pulp, Toxicity, Tooth bleaching

SUMÁRIO

INTRODUÇÃO

1 Introdução

O clareamento de dentes vitais vem enfrentando críticas e desconfianças quanto a segurança biológica, especialmente nas últimas décadas[22,51], devido ao fato de cerca de 2/3 dos pacientes submetidos a esta terapia relatarem a ocorrência de sensibilidade dental[32]. A intensidade desta sensibilidade pode variar de sutil à intensa, sendo, em alguns casos, responsável pela interrupção do tratamento[1,3,13,27,32,37,38,52]. Desta forma, nos últimos anos, diversos pesquisadores buscaram entender a fisiopatologia relacionada a este efeito adverso.

Estudos laboratoriais iniciais demonstraram que o peróxido de hidrogênio (H_2O_2), o qual é o componente ativo principal dos agentes clareadores indicados para o tratamento de dentes vitais, é capaz de se difundir por toda a estrutura de esmalte e dentina, e atingir a câmara pulpar em dentes extraídos[2,6,7,21]. Apesar deste modelo de estudo não mimetizar as condições clínicas, tais como pressão intra-pulpar e presença de componentes orgânicos no interior dos túbulos dentinários[53], os quais interferem diretamente na difusão de componentes de materiais dentários na câmara pulpar[40], eles fornecem forte evidência de que o contato de produtos provenientes dos géis clareadores com o tecido pulpar pode apresentar papel crucial na sintomatologia clínica relatada pelos pacientes[48].

Esta correlação é decorrente do fato do H_2O_2 ser uma espécie reativa do oxigênio (ERO), o qual apresenta a capacidade de se dissociar em outras EROs com elevada atividade oxidativa, tais como íon peri-hidroxila (HO_2^-), ânion superóxido (O_2^-), oxigênio singleto (O^{-2}) e íon hidroxila (HO^-). Estas moléculas são altamente reativas e instáveis, devido a presença de um elétron desemparelhado na camada externa[9]. Evidências científicas demonstram que durante o procedimento clareador, as EROs atuam nas proteínas do esmalte, levando à desorganização da matriz orgânica deste tecido e perda do material cristalino envolto, o que promove a formação de porosidades[19,28,47]. Estes poros servem como via de acesso direto das EROs no tecido dentinário, que concentra a maioria dos componentes responsáveis pela coloração do elemento dental, os cromóforos[28]. Estas moléculas orgânicas de cadeias complexas, com presença de insaturações e anéis aromáticos, promovem intensa absorção da luz que incide sobre o elemento dental, resultando em uma coloração escurecida[51]. A oxidação dos cromóforos da dentina pelas EROs resulta na quebra das insaturações das suas cadeias, transformando-os em moléculas mais simples, o que reduz o índice de absorção de luz pelo elemento dental, clareando, conseqüentemente, a estrutura dentária[22].

No entanto, o tecido dentinário é altamente permeável quando comparado ao esmalte, devido, principalmente, à presença dos túbulos dentinários, os quais promovem uma comunicação direta com o tecido pulpar imediatamente subjacente[23]. Na periferia da polpa, em íntimo contato com a dentina, encontram-se os odontoblastos, células centrais envolvidas

na homeostasia e vitalidade do complexo dentino-pulpar. Estas células terminais altamente diferenciadas são responsáveis pela deposição da dentina[12], sendo que as mesmas apresentam seu prolongamento citoplasmático no interior dos túbulos dentinários, conferindo uma interconexão entre dentina superficial e polpa[23]. Os odontoblastos mantêm durante toda a vida do elemento dental uma produção constante de matriz de dentina não mineralizada, a pré-dentina, a qual impede o contato de proteínas da dentina mineralizada, que não são reconhecidas pelo sistema imunológico do tecido pulpar[48]. Tal contato resulta em ativação do sistema imune, com conseqüente desencadeamento do processo de reabsorção dentinária interna[16,48]. Os odontoblastos também estão diretamente envolvidos no sistema de defesa do complexo dentino-pulpar frente a uma injuria externa. Essas células apresentam receptores em seus prolongamentos, os quais iniciam a reação imuno-inflamatória pulpar desde o momento em que o patógeno/irritante encontra-se na dentina superficial[11,20]. Ainda, o processo de dentinogênese (reacional) é desencadeado frente a injurias externas, com o objetivo de afastar a polpa da fonte de agressão[48]. Assim, os odontoblastos são considerados como a primeira linha de defesa do complexo dentino-pulpar[20]. O tecido pulpar também apresenta uma reserva de células mesenquimais indiferenciadas (tronco), as quais, em caso de morte dos odontoblastos, de forma fisiológica ou em resposta a uma injuria intensa, são recrutadas, proliferam e se diferenciam em células odontoblastóides, mantendo a homeostasia do complexo dentino-pulpar[23,24,41].

Quando as concentrações de EROs em contato com células pulpares ultrapassam a capacidade do sistema antioxidante em inativa-las, o processo de estresse oxidativo instala-se[48,49]. Como conseqüência, as EROs promovem oxidação de macromoléculas celulares, como lipídeos, proteínas e ácidos nucleicos, sendo que a acumulação das mesmas gera sérios distúrbios no funcionamento celular[48]. Ainda, a interação das EROs com os ácidos graxos da membrana celular desencadeia o processo de peroxidação lipídica, podendo culminar com o rompimento da membrana citoplasmática e morte celular por necrose[9]. Este tipo de morte celular leva a um extenso dano tecidual, visto que as enzimas lisossomais são liberadas no tecido, gerando danos às células adjacentes[49]. O processo de estresse oxidativo também leva à ativação de proteases no tecido pulpar, tais como metaloproteinases e catepsinas, as quais promovem degeneração da matriz extracelular, intensificando, assim, a extensão da lesão tecidual promovida[39].

Acredita-se que durante o procedimento de clareamento dental, uma grande quantidade de EROs provenientes dos agentes clareadores entram em contato com as células pulpares no ambiente extra-celular, sendo capazes de causar dano direto às membranas celulares[48]. Ainda, como o H_2O_2 é permeável à membrana citoplasmática, o mesmo é capaz de penetrar no citoplasma celular e desencadear o processo de estresse oxidativo[9]. Em conseqüência do dano celular e tecidual promovido, uma reação inflamatória é desencadeada

no tecido pulpar[8,15,26]. Como a polpa apresenta a característica de ser envolta por tecido mineralizado em praticamente toda sua extensão, excetuando-se pelo forame apical, qualquer aumento na pressão intra-pulpar gera sensibilidade dental, visto que as fibras nervosas da periferia pulpar são estimuladas mecanicamente pelo aumento de pressão[35,48]. Associado a esta situação fisiológica, o desencadeamento da inflamação neurogênica promove proliferação das terminações nervosas pulpares, aumentando a capacidade de transmissão da sensibilidade dolorosa[35].

Os estudos clínicos demonstram que a prevalência e intensidade de sensibilidade dental pós-clareamento está diretamente relacionada com o protocolo clínico empregado[1,3,13,27,32,37,38,52]. Dependendo da técnica clareadora, a atuação das EROs sobre os cromóforos pode se limitar à dentina superficial[18] ou pode atingir a dentina que recobre a polpa[50], o que interfere diretamente na quantidade de EROs capazes de se difundir para a câmara pulpar[2,6,21]. Existem dados na literatura demonstrando prevalência de sensibilidade dental em 80 a 100% dos pacientes submetidos ao protocolo de clareamento de consultório tradicional, cuja intensidade varia de moderada a intolerável[3,13,37,52]. Este protocolo é baseado no emprego de géis com elevadas concentrações de H_2O_2 (35-40%) aplicados por períodos de 30 a 45 minutos na superfície dental, em cada sessão clínica[22,51]. Buscando entender os efeitos celulares relacionados a esta terapia, pesquisadores realizaram estudos in vitro empregando um substrato dental (esmalte e dentina) para o clareamento. De acordo com os resultados, quando os produtos capazes de se difundir pelos discos foram aplicados por 60 minutos sobre células odontoblastóides, redução em torno de 40 a 70% na viabilidade celular foi observada[14,17,43-45], sendo a mesma em torno de 96% quando células pulpares humanas foram expostas[14]. Na situação in vivo, foi constatado que este protocolo causa intensos danos ao tecido pulpar quando realizado em dentes com pequena espessura de esmalte e dentina[10,15]. De Souza Costa et al.[15] (2010) observaram áreas de necrose de coagulação na superfície pulpar de incisivos inferiores humanos submetidos ao protocolo de clareamento de consultório tradicional. Resultados similares foram encontrados por Cintra et al.[10] (2012), os quais observaram necrose em molares de rato submetidos a este protocolo. Já nos estudos de Kina et al.[26] (2010) e Caviedes-Buchelli et al.[8] (2009), a análise histológica da polpa de pré-molares submetidos a uma sessão de clareamento de consultório revelou apenas uma leve reação inflamatória. Estes dados são corroborados pelos achados de estudos clínicos recentes que observaram maior prevalência e intensidade de sensibilidade dental nos dentes anteriores quando a técnica de consultório é empregada[3,13].

A associação da técnica de clareamento de consultório previamente descrita com diferentes fontes de luz (diodo emissor de luz, laser e luz halógena) é outra alternativa bastante empregada para o clareamento de consultório, cujo objetivo é catalisar a degradação do H_2O_2 e aumentar a eficácia clareadora[25]. No entanto, estudos demonstram que o uso da luz

resulta em sensibilidade dental mais intensa e duradoura[13], maior penetração de H_2O_2 na câmara pulpar[7], bem como maior citotoxicidade trans-amelodentinaria sobre células odontoblastóides[17]. Ainda, Caviedes-Buchelli et al.[8] (2009) observaram aumento significativo de substância P, um neuropeptídeo associado com a dor, apenas nos pré-molares submetidos a técnica de consultório tradicional ativada com luz. Outros pesquisadores não observaram, em estudos in vitro e in vivo, aumento da eficácia clareadora quando a luz é utilizada[1,5,27]. Assim, a real necessidade da ativação do gel clareador com luz tem sido questionada, devido aos maiores riscos de danos ao complexo dentino-pulpar.

Diante de tais resultados, observa-se que o emprego da técnica de clareamento de consultório, da forma que vem sendo recomendada, apresenta elevados riscos de causar intensa lesão no tecido pulpar, o que envolve a morte dos odontoblastos primários e de células mesenquimais indiferenciadas, tal como observado nos cortes histológicos realizados por De Souza Costa et al.[15] (2010). Mesmo que a polpa apresente capacidade de se recuperar desta agressão, visto que a mesma apresenta intensa capacidade de regeneração tecidual, tais danos causam um envelhecimento precoce do tecido pulpar, limitando a capacidade de resposta do complexo dentino-pulpar frente a agressões futuras[48]. Ainda, em caso de necrose tecidual, o fino balanço existente entre a dentina superficial e a polpa é perdido, devido a morte dos odontoblastos primários, e a conseqüente perda dos seus prolongamentos citoplasmáticos no interior dos túbulos dentinários[48].

Atualmente, o clareamento caseiro empregando o peróxido de carbamida (PC) a 10% é considerado como a modalidade de tratamento mais segura para o clareamento dental[48]. Estudos demonstraram ausência de citotoxicidade significativa sobre células odontoblastóides e em cultura primária de polpa humana após 4 a 8 horas de aplicação deste gel clareador em discos de esmalte e dentina[15,42], bem como menor prevalência e intensidade de sensibilidade dental[1,13]. Ainda, estudo recente demonstrou que pacientes submetidos a esta técnica apresentaram reposta positiva para testes de sensibilidade dental, associado a ausência de alterações radiográficas pulpares e periapicais em períodos de até 18 anos pós-tratamento[4]. No entanto, a técnica de clareamento caseiro ainda apresenta certas limitações, sendo a principal delas relacionada ao fato da aplicação do gel clareador ser realizada pelo paciente em sua residência sem a supervisão direta do profissional. Existe, assim, o risco de uso inadvertido da moldeira, resultando em deglutição inapropriada do produto, e risco de uso indiscriminado[5,48]. Estudos recentes demonstram que o número de aplicações do PC 10% interfere diretamente com o potencial citotóxico deste técnica[31,44]. Em pacientes com áreas de exposição de dentina, como lesões de abrasão, abfração e recessão gengival, a realização desta técnica é contra-indicada devido ao risco de aplicação do produto diretamente sobre dentina, o que intensifica a difusão das EROs e os efeitos tóxicos[30]. Apesar das vantagens

relacionadas ao uso de géis com PC, sua utilização para a técnica de consultório ainda não está bem estabelecida.

Desta forma, o clareamento de consultório ainda apresenta-se como uma técnica necessária dentro da Odontologia, devido ao fato de todo o procedimento ser realizado sob supervisão profissional. No entanto, novos parâmetros devem ser pesquisados para que esta técnica além de efetiva, também seja biocompatível com o complexo dentino-pulpar. De acordo com os resultados de estudos prévios, fica claro que a redução na difusão de H_2O_2 pela estrutura dental, bem como a difusão lenta e gradual desta molécula, como proporcionado por géis à base de PC, podem minimizar os danos às células pulpares[42,44-46]. Ainda, alguns estudos demonstraram que o contato de células pulpares humanas e células odontoblastóides com baixas concentrações de H_2O_2[29,33,34] ou mesmo um processo inflamatório de baixa intensidade[11,36], podem induzir a diferenciação odontoblástica e deposição de matriz mineralizada.

No presente estudo, foram propostas modificações nos parâmetros concentração de H_2O_2, forma de apresentação (géis a base de H_2O_2 ou PC), e redução no tempo de contato e/ou freqüência de aplicação dos produtos clareadores em cada sessão clínica. Foram analisadas a eficácia clareadora em até seis sessões, sendo os dados comparados com a difusão de H_2O_2 pela estrutura dental. Aqueles protocolos considerados promissores foram analisados quanto a citotoxicidade trans-amelodentinária em duas linhagens de células pulpares (células odontoblastóides e cultura primária de células pulpares humanas – ANEXO 1) sendo estudados a viabilidade, morfologia, lesão a membrana e estresse oxidativo celular, bem como a capacidade de recuperação das células em um período de até 72 horas. Em seguida, para os protocolos com menor efeito tóxico, as características fenotípicas das células pulpares foram analisadas em períodos de até 21 dias pós-clareamento, sendo os dados comparados com a indução da expressão gênica de mediadores inflamatórios imediatamente após o clareamento.
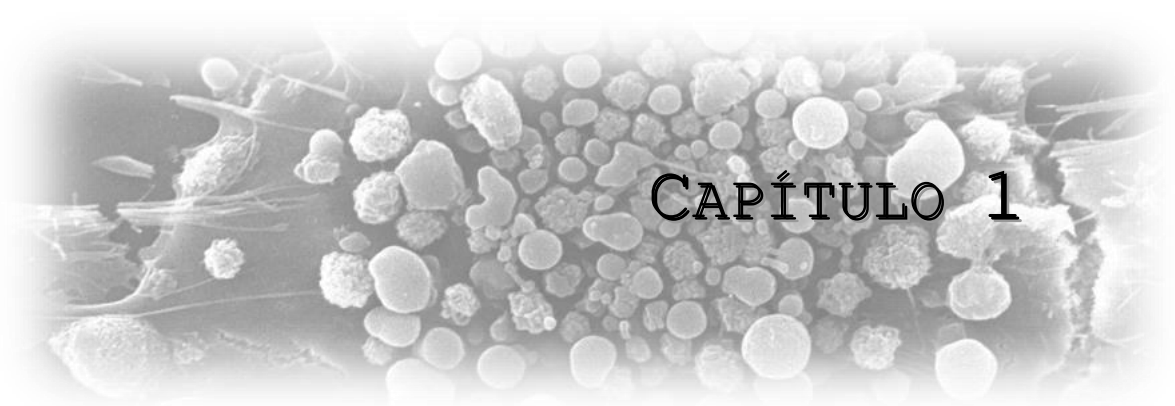
CAPÍTULO 1

2 Capítulo 1

Effective tooth-bleaching protocols capable of reducing H$_2$O$_2$ diffusion through enamel and dentin.

Short title: Effects of experimental bleaching protocols.

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Effective tooth-bleaching protocols capable of reducing H$_2$O$_2$ diffusion through enamel and dentin

ABSTRACT

Objectives. To evaluate the effects of experimental protocols on bleaching effectiveness and hydrogen peroxide (HP) diffusion through enamel and dentin. *Methods.* Enamel/dentin discs were subjected to six bleaching sessions, consisting of 1 or 3 applications of 17.5% or 35%-HP gel for 5/15 min, or 37% carbamide peroxide (CP) gel for 10/20 min. Discs undergoing the regular protocol (35%-HP; 3x15 min) constituted the positive control group. Color change (ΔE) was assessed (CIE *L*a*b** system) after each session. HP diffusion was quantified (sessions 1, 3, and 6) in enamel/dentin discs adapted to artificial pulp chambers. Data were analyzed by Pillai's Trace and Bonferroni test, or by one-way ANOVA and SNK/Tamhane's test (α=5%). *Results.* All tooth-bleaching protocols significantly increased the ΔE values. A reduction in HP diffusion and no significant difference in ΔE compared with the positive control were observed for the following bleaching protocols: 17.5%-HP 3x15 min, at the 4th session; and 35%-HP 1x15 and 3x5 min, at the 5th session. HP diffusion in the 37%-CP 3x20 min bleaching protocol was statistically similar to that in the positive control. The other experimental bleaching protocols significantly decreased HP diffusion through enamel/dentin discs, but the ΔE values were statistically lower than those observed in the positive control, in all sessions. *Conclusion.* Shortening the contact time of a 35%-HP gel or reducing its concentration produces gradual tooth color change and reduced HP diffusion through enamel and dentin.

Clinical Significance: A reduction in HP concentration, from 35% to 17.5%, in a bleaching gel or shortening its application time on enamel provides a significant tooth-bleaching improvement associated with decreased HP diffusion across hard dental tissues. Therefore, these protocols may be an interesting alternative to be tested in the clinical situation.

1. Introduction

An at-home tooth-bleaching procedure with 10% carbamide peroxide (CP) gel has been considered the safest method for bleaching teeth with minimal adverse effects (1-5). However, since this tooth-bleaching modality is patient-applied, there is a risk of gel application over exposed dentin in patients with gingival recession and abfraction/abrasion lesions. Also, the inadequate use of the tray may result in gel overflow, with extended soft-

tissue exposure and likely material ingestion (3,6). Therefore, it seems evident that this kind of esthetic therapy should be performed entirely under professional supervision.

For the in-office tooth-bleaching technique, in which the procedure is performed by clinicians, bleaching gels with high concentrations (35-38%) of hydrogen peroxide (HP) are applied in a 30- to 45-minute chair-side session (1). This therapy allows for a clinically perceptible dental color improvement even after one clinical appointment (6-10). However, tooth sensitivity is a side-effect commonly reported in the literature when this technique is applied to vital teeth (7-11). The low molecular mass of HP and its sub-products favors its diffusion through mineralized dental tissues to reach the pulp chamber (12-14). The contact of pulp cells with these reactive oxygen species (ROS) results in oxidative stress generation, due, at least in part, to an imbalance between the amount of ROS and endogenous/exogenous antioxidants (10,11). It has been shown that HP and its sub-products can reduce cell viability, as well as cause cell membrane damage and proteolytic enzyme activation, extracellular-matrix degradation, tissue inflammatory reaction, and even partial pulp necrosis (11, 15-22).

Previous studies demonstrated that the higher the concentration and the length of time of the bleaching agent application on enamel, the higher the HP penetration of the pulp chamber, and the more intense are the adverse effects to pulp cells (15). Thus, changes in in-office tooth-bleaching techniques may provide an interesting alternative for maintaining bleaching effectiveness, while preventing or at least minimizing the negative effects of this kind of esthetic therapy on pulp tissue. Therefore, the present study aimed to assess and correlate the bleaching effectiveness of different experimental in-office tooth-bleaching protocols and their capacity for HP diffusion through enamel and dentin.

2. Materials and methods

2.1. Sample preparation: Enamel/dentin discs (5.6 mm diameter, 3.5 mm thick) were obtained from the buccal surfaces of sound bovine central incisors (24- to 30-month-old bullocks) (9). An intrinsic stain model was carried out as described by Sulieman et al. (23). The dentin surfaces of experimental and control discs were etched with 37% phosphoric acid (3M ESPE, St. Paul, MN, USA) for 60 sec. The acidic product was then rinsed with distilled water spray. The discs remained in contact with a standardized solution of black tea (Leão Jr S.A., Fazenda Rio Grande, PR, Brazil), produced by filtration of 2 g of tea in 100 mL of boiling water for 5 min, for 6 days at 37ºC. The enamel was polished with a low-speed rubber cup (KG Sorensen, Barueri, SP, Brazil) and pumice stone/water solution. This procedure was performed to remove any undesirable external staining caused by the 6-day immersion of the sample in black tea. The enamel/dentin discs were then immersed in distilled water for 6 days to remove any non-adhering dentin pigments.

2.2. Bleaching procedure: Three bleaching gels were evaluated: a 37% CP gel (Whiteness super; FGM, Joinville, SC, Brazil); a 35% HP gel (Whiteness HP; FGM); and a 17.5% HP gel, which was obtained by dilution of the 35%-HP gel in distilled water immediately before the bleaching procedure (24). The gels were applied according to different protocols (Table 1). For each bleaching protocol, 6 sessions were performed within a 7-day interval. At the end of each session, the enamel surface was kept in contact with artificial saliva (3.9% monobasic potassium phosphate; 3.6% potassium chloride; 2% sodium chloride; 2% potassium chloride; 3.7% magnesium chloride; 0.2% phenochem; 10% natrosol gel; distilled water qsp), and the dentin surface was kept in a humid environment to prevent dehydration (19).

2.3. Color analysis: A color readout was performed by a UV-VIS spectrophotometry (Spectro Guide 45/0, BYK-Gardner GmbH, Geretsried, Germany), following the CIE L*a*b* system, which consists of two axes, a* and b*, which have right angles and represent the dimension of color. The third axis (L*) represents lightness, corresponding to the amount of light reflected from the object, and is expressed in numeric values ranging from 0 (black) to 100 (white). ΔL represents the difference in lightness. The closer to zero, the darker the color of the object. In turn, lighter samples present numeric values close to 100. Positive ΔL values indicate that the sample is lighter than the standard (baseline readout), with negative values indicating that the sample is darker than the standard. Discs with similar initial L* values were randomly distributed into the control and experimental groups (n=8) to obtain standardized samples. The discs were adapted in a white silicone matrix, with only the enamel surface left exposed (15). The spectrophotometer was positioned over the enamel surface, and color readouts were performed before bleaching (baseline), 24 h after each session, and 30 days after the last session. The mean values of each coordinate were calculated, and the color change (ΔE) between values obtained in the baseline and subsequent readouts was calculated according to the following equation: $\Delta E = ((\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2)1/2$, where: ΔE = color change; $\Delta L = L_{Final} - L_{Initial}$; $\Delta a = a_{Final} - a_{Initial}$ and $\Delta b = b_{Final} - b_{Initial}$. ΔE ≥ 3.3 were considered perceptible to the naked eye (25).

2.4. HP diffusion quantification: To quantify the amount of HP capable of diffusing through enamel and dentin substrates for each bleaching protocol, we mounted enamel/dentin discs in artificial pulp chambers (APC) (15). Each disc was placed between 2 silicon o-rings in the upper compartment of the APC, which was individually positioned in a well of a 24-well plate containing 1 mL of acetate buffer. The dentin surface was maintained in direct contact with the buffer, and the enamel was exposed to receive the bleaching agents. Immediately

after the bleaching procedures, an aliquot (100 μL) of the buffer was transferred to tubes containing 250 μL of leucocrystal violet (0.5 mg/mL, Sigma-Aldrich Corp., St. Louis, MO, USA) and 50 μL of horseradish peroxidase enzyme solution (1 mg/mL, Sigma-Aldrich Corp.). The final volume of the reaction was adjusted to 3 mL with distilled water. Then, three 100-μL aliquots of each tube were transferred to 96-well plates, and the optical density of the solutions was measured at a 600-nm wavelength in an ELISA plate reader (Tp Reader, Thermoplate, Nanshan District, Shenzhen, China). A standard curve of known HP concentrations was used for conversion of the optical density obtained in the samples into μg of HP, and the data were related to μg *per* mL of acetate buffer solution.

2.5. Statistical analysis: To analyze the effects of the bleaching protocols through the different time-points (sessions), we performed a Profile Analysis. When the flatness and parallelism hypotheses were rejected, Pillai's Trace supplemented by the Bonferroni test (pairwise comparison) was used. The mean values of the bleaching protocols in each session were analyzed by one-way ANOVA supplemented by SNK or Tamhane's test. All analyses were performed at the 5% significance level. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analysis.

3. Results

3.1. Bleaching effectiveness: The graphic representation of Δa, Δb, ΔL, and ΔE according to the different tooth-bleaching sessions is shown in Fig. 1. When each experimental group was analyzed during the sessions, a significant reduction in Δa values was observed for G2 (sessions 3-6), G4 (session 6), G3 (session 6), and G6 (sessions 5-6) ($p < 0.05$). No significant difference was observed in Δb through the sessions for all experimental groups ($p > 0.05$). All groups presented a significant increase in mean ΔL values, which mainly governed the overall color change (ΔE). Such a gain in lightness may be considered as 'test specimen bleaching', since positive ΔL values mean a trend toward white.

The ΔL and ΔE results are described in detail in Table 2. The positive control group (G2) presented the highest ΔE and ΔL values. When this group was analyzed through the sessions, significant increases in ΔE and ΔL were observed until the 4th session ($p < 0.05$). Otherwise, the experimental protocols promoted a gradual color change through all sessions (Table 2, rows). When the groups were compared in each session (Table 2, columns), no significant difference with G2 for ΔE and ΔL values was observed for G6, at the 4-6th sessions; for G3 and G4, at the 5-6th sessions; and for G10, at the 6th session ($p > 0.05$). The other experimental groups presented ΔE and ΔL values significantly lower than those of G2 in each session. Color change perceptible to the naked eye (ΔE ≥ 3.3) was observed after the

1st session for G2, G3, G4, G6, and G10; after the 2sd session for G5, G7, G8, G9, and G12; and after the 3rd session for G11 and G13. Analysis 30 days after the last session demonstrated decreased ΔE and ΔL values for all groups, which were significantly lower than those of the 6th session only for ΔL in the groups G3, G8, G10, and G13 ($p < 0.05$).

3.2. HP diffusion quantification: The results for HP diffusion quantification are presented in Table 3. No HP was detected in the negative control group, which was disregarded for statistical analysis. There were no significant differences in HP diffusion among the sessions in all groups ($p > 0.05$). However, significant differences were found among the bleaching protocols ($p < 0.05$). The highest values of HP diffusion through enamel and dentin were observed in G2 and G10, with no significant difference between them ($p > 0.05$). The experimental groups presented significantly lower HP diffusion than did groups G2 and G10. Considering the positive control group (G2) as having 100% of HP diffusion, groups G3, G4, G6, G11, and G12 presented around 60% less HP diffusion, with no significant differences among them ($p > 0.05$). Similar results were also found among groups G7, G8, and G13, which presented around 85% less diffusion than the positive control group. The smallest HP values were found for G9 (around 95% less than G2) and G13 (around 88% less than G2).

4. Discussion

Currently, post-operative sensitivity claimed by patients subjected to tooth-bleaching therapies is believed to be a consequence, at least in part, of HP diffusion through enamel and dentin into the pulp chamber. This phenomenon results in pulp inflammation, with release of inflammatory mediators and pulp sensory nerve stimuli (16,18). In some cases, the sensitivity is so intense that treatment is discontinued (7,11). Therefore, a reduction in enamel/dentin-HP diffusion and, consequently, its penetration into the pulp chamber is required to prevent, or at least decrease, the oxidative damage caused by HP and its toxic sub-products to pulp tissue. To prevent or minimize transenamel and transdentinal HP diffusion, in the present study, the authors decreased the bleaching gel-HP concentration from 35% to 17.5%, as well as reduced the application time of the product on enamel. The data obtained were compared with those from the condition in which a bleaching gel with a high concentration of CP was used. Color analysis was performed to demonstrate the bleaching potential for each tested protocol through 6 sessions.

The traditional in-office protocol (35%-HP gel; 3x15 min) was used in the present study as the positive control, since tooth-bleaching effectiveness (7,8,10) and transenamel and transdentinal toxicity (15,19,20) have been well-demonstrated in the literature. Despite the rapid saturation of dentin chromophores and a significant visual impact noted immediately

after the first bleaching session, which corroborated the results from previous studies (7,8,10), the highest HP diffusion occurred in this positive control group. Therefore, it may be suggested that, *in vivo*, the large number of non-reacted toxic molecules present in dentin is capable of reaching the pulp space (12,13) to cause tooth sensitivity (7-10) associated with intense inflammatory pulp reaction (18) or even partial necrosis in this specialized connective tissue (21).

When the contact time of the 35%-HP gel with enamel was reduced to 15 min (1x15 min; 3x5 min), a bleaching pattern similar to that of the traditional protocol (positive control) was obtained at the 5th session, with the advantage of reducing HP diffusion across enamel and dentin by 60%. Likewise, when the 5-minute protocol was performed, HP diffusion decreased by around 85%; however, even after 6 sessions, the color alteration did not reach the positive control parameters. In a recent study, Soares et al. (15) demonstrated that shortening the contact time of a 35%-HP gel with enamel to 15 min (1x15 min; 3x5 min) caused significantly less HP diffusion through enamel/dentin discs, with a consequent decrease in toxicity to cultured odontoblast-like cells compared with that of the traditional in-office bleaching procedure (3x15 min). The 35%-HP bleaching gel applied for only 5 min to enamel increased ALP activity, which is an enzyme that plays an important role in dentin matrix deposition. This finding indicates that a low concentration of HP in contact with pulp cells may stimulate differentiation and deposition of reactionary dentin matrix protein.

The reduction of HP concentration in the bleaching agent from 35% to 17.5%, which was applied to enamel for 45 min (3x15 min - 4 sessions), caused the same bleaching pattern of color change as observed in the positive control group. Also, a 60% reduction in HP diffusion through dental structure was observed. So, it may be assumed that by subjecting tooth structure gradually to less HP, the amount of non-reacted HP on tooth structure would be reduced, with a consequent decrease in HP and its sub-products capable of diffusing across enamel and dentin substrates. The effectiveness of gels with low concentrations of HP was previously evaluated by Sulieman et al. (24), who used teeth subjected to the same darkness process as used in the present study. Those authors reported that 1, 2, 4, 7, and 12 bleaching sessions (3x10 min) were required to obtain an optimal shade outcome (B1- VITA Classic Shade Guide) when gels with 35, 25, 15, 10, and 5% HP concentrations were used, respectively. Many current clinical studies have also demonstrated that bleaching gels with 15-20% of HP in their composition, when applied to enamel for 45-60 min, were capable of promoting a significant color change, similar to that caused by a 35%-HP bleaching gel. In these studies, the incidence of tooth sensitivity ranged from 24% to 78%, which was considered as mild in severity (9,26,27).

Gels with high concentrations of CP are encouraged for bleaching teeth in a chair-side session, combining the advantages of both in-office and at-home tooth-bleaching

techniques, chiefly, slow and gradual release of HP under a controlled environment (28). In the present investigation, a 37%-CP gel was evaluated following the manufacturer's instructions (3x20 min), and an esthetic outcome similar to that in the positive control group was observed only after 6 bleaching sessions. These data are similar to those provided previously by Patel et al. (28), who observed a slight color alteration after a single 30-minute session using a 35%-CP gel. It is known that 37%-CP gel has an HP concentration similar to that of the 17.5%-HP gel used in the present study. When both bleaching gels were applied following the traditional protocols (G6 and G10), no significant difference between them was observed until the 5th session. However, components released from 17.5%-HP resulted in higher ΔE values compared with those achieved from the 37%-CP gel. Previous studies showed that CP and HP gels with similar final concentrations applied to enamel for the same period yielded similar whitening effects (29,30). However, in these studies, a long-time treatment (up to 14 days) was carried out, which may explain the better results found for the CP gels compared with those of the present study. In this way, in the present investigation, the method of whitening gel delivery (CP x HP) influenced the tooth-bleaching effect. Other *in vitro* and *in vivo* studies also demonstrated significant color alteration when gels with high concentrations of CP were applied daily for up to 2 weeks (31,32). Therefore, it seems that, to be effective, the gels with high concentrations of CP should be applied to enamel for long periods of time, as required for at-home bleaching therapies. However, since the increased CP concentration causes higher toxic effects in the pulp cells (2,4,5), with no benefits in the final esthetic outcome (31), it appears that there is no advantage in performing at-home bleaching therapy with this kind of gel. Additionally, the use of this product for in-office tooth-bleaching therapy may be considered unsuitable, since a long-time application of the gel to enamel is necessary to produce only a slight color alteration.

To assess color stability over time, we evaluated the tooth bleaching achieved after the last session for each group 30 days later. The color rebound observed in all the experimental and control groups was neither significant nor clinically perceptible (ΔE ≤ 3.3). Moreover, L* coordinate variation appeared to be the most significant parameter in global color change, because the increase in lightness (whitening) may be interpreted as dental structure bleaching, as previously demonstrated (23,24,31). Therefore, it may be speculated that the quantity of oxidized molecules present in dentin structure after bleaching was reduced, since previous researchers showed that organic substances in artificial saliva may contribute to this process (33).

In the present investigation, the application of 35%-HP gel to enamel for only 15 min, or 17.5%-HP gel for 45 min (3x15 min), caused a gradual color alteration through the sessions. In both conditions, the optimal shade outcome was achieved with a few more bleaching sessions, which reduced by 60% the transenamel and transdentinal HP diffusion.

As the amount of HP that diffuses through enamel and dentin is directly related to the toxic effects to the pulp cells (15), one can expect that these bleaching protocols would be less toxic to the pulp cells. However, since this study used an *in vitro* model, the data obtained must be interpreted with caution. Vital teeth present a continuous outward dentin fluid movement through dentinal tubules. Conversely, extracted teeth devoid of dentinal fluid allow for a rapid inward movement of HP and its sub-products through enamel and dentin, which favors the tooth-bleaching outcome as well as intensifies HP diffusion (24). Also, in this study, the intrinsic stain model used only black tea, which constitutes a limitation to this *in vitro* study, because, in clinical situations, the intrinsic discolouration of teeth is caused by a combination of different chromogens that have been accumulated over a long period with different binding characteristics to the enamel and dentin, which, in turn, may result in diverse responses to the bleaching procedure (23). In addition, it is known that human teeth are more permeable to HP than are bovine teeth (12). Therefore, one can suggest that the same bleaching protocols evaluated in the present study may result in a greater HP diffusion in human teeth, increasing the risks of damage to pulp tissue. Thus, *in vivo* studies in vital human teeth are needed to assess tooth-bleaching effectiveness as well as pulp responses after the application of the bleaching protocols successfully evaluated in the present study.

5. Conclusions

Based on the data obtained in the present study, it can be concluded that the application of 35%-HP gel to enamel for only 15 min or 17.5%-HP gel for 45 min (3x15 min) produces gradual tooth color change and increases its lightness. Both protocols, which presented bleaching performance similar to that of the traditional in-office protocol (35%-HP gel - 3x15 min), significantly reduced HP diffusion through enamel and dentin. The 37%-CP gel presented the worst tooth-bleaching performance, requiring up to 6 sessions (60 min each) to achieve a tooth-bleaching effect similar to that of the in-office protocol, with no reduction in HP diffusion.

6. Acknowledgements

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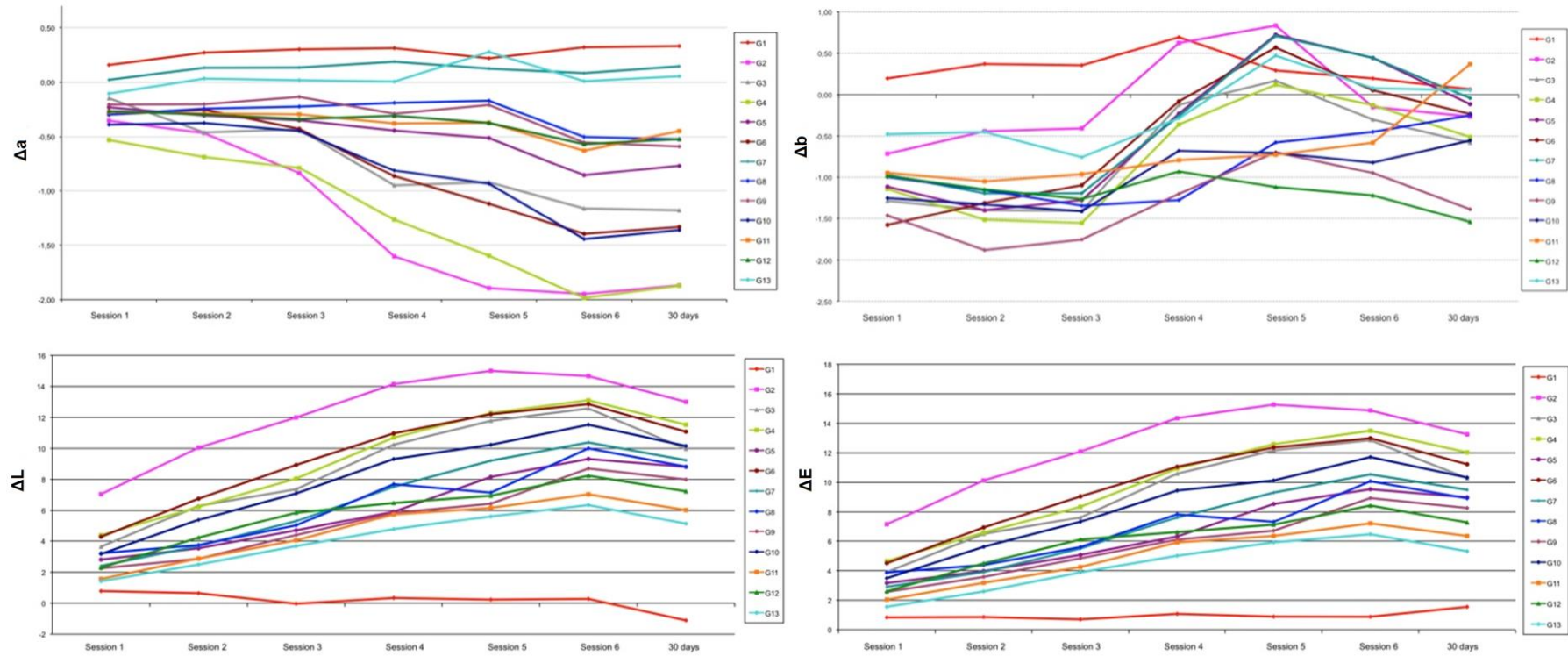
Figure 1. Graphic representation of variations in Δa, Δb, ΔL, and ΔE mean values through the different sessions and 30 days thereafter.

Table 1 - Experimental groups description according to the gel formulation and application protocol.

Groups	Bleaching Gel	Application Protocol
G1 (negative control)	None	None
G2 (positive control)	35%-HP gel	3x 15 min
G3	35%-HP gel	1x 15 min
G4	35%-HP gel	3x 5 min
G5	35%-HP gel	1x 5 min
G6	17.5%-HP gel	3x 15 min
G7	17.5%-HP gel	1x 15 min
G8	17.5%-HP gel	3x 5 min
G9	17.5%-HP gel	1x 5 min
G10	37%-CP gel	3x 20 min
G11	37%-CP gel	1x 20 min
G12	37%-CP gel	3x 10 min
G13	37%-CP gel	1x 10 min

Table 2 - Mean values and standard deviation for ΔE and ΔL of the different bleaching protocols at each session (S) and 30 days after the last session.

Protocol	S1	S2	S3	S4	S5	S6	30 d	S1	S2	S3	S4	S5	S6	30 d
				ΔE							ΔL			
(G1) Negative Control	0.84 (0.14)^{Aa}	0.86 (0.37)^{ABa}	0.70 (0.42)^{ABa}	1.07 (0.48)^{ABa}	0.89 (0.40)^{ABa}	0.88 (0.41)^{ABa}	1.55 (0.41)^{Ba}	0.78 (0.17)^{Aa}	0.64 (0.29)^{Aa}	-0.03 (0.63)^{Aa}	0.33 (0.43)^{Aa}	0.23 (0.48)^{Aa}	0.27 (0.76)^{Aa}	-1.11 (0.80)^{Aa}
(G2) 35%-HP gel 3x 15 min	7.14 (0.83)^{Ab}	10.13 (1.09)^{Bb}	12.09 (1.40)^{Cb}	14.36 (1.36)^{Db}	15.27 (1.27)^{DEb}	14.88 (1.30)^{DEFb}	13.26 (1.62)^{CDFb}	7.05 (0.81)^{Ab}	10.05 (1.07)^{Bb}	12.00 (1.40)^{Cb}	14.15 (1.32)^{Db}	15.00 (1.23)^{Db}	14.67 (1.30)^{Db}	13.01 (1.57)^{Db}
(G3) 35%-HP gel 1x 15 min	3.91 (0.98)^{Ac}	6.49 (0.96)^{Bcd}	7.62 (0.96)^{Cc}	10.59 (1.19)^{Dc}	12.17 (1.78)^{Ebc}	12.86 (1.06)^{Ebc}	10.28 (1.24)^{Dbc}	3.64 (1.03)^{Acd}	6.29 (0.88)^{Bceg}	7.37 (0.96)^{Bcf}	10.24 (0.94)^{CEc}	11.78 (1.54)^{CDbc}	12.58 (0.97)^{Dbd}	9.97 (1.14)^{Ecd}
(G4) 35%-HP gel 3x 5 min	4.65 (1.12)^{Ac}	6.57 (1.43)^{Bcd}	8.35 (1.87)^{Cc}	10.94 (2.10)^{Dbc}	12.59 (1.72)^{Ebc}	13.50 (1.03)^{DEbc}	12.03 (1.04)^{DEb}	4.38 (0.92)^{Ac}	6.23 (1.24)^{Bceg}	8.06 (1.73)^{Ccd}	10.70 (1.91)^{Dce}	12.29 (1.61)^{Ebc}	13.10 (1.00)^{DEb}	11.53 (1.07)^{DEc}
(G5) 35%-HP gel 1x 5 min	3.16 (0.46)^{Ad}	3.97 (0.44)^{Ag}	5.07 (0.25)^{Bdf}	6.32 (0.39)^{Cdf}	8.54 (1.37)^{CDd}	9.54 (1.36)^{Dd}	8.99 (1.20)^{Dcdg}	2.81 (0.45)^{Adef}	3.54 (0.22)^{Ad}	4.70 (0.29)^{Bde}	5.91 (0.69)^{Cdf}	8.16 (1.14)^{CEde}	9.32 (1.20)^{Ece}	8.81 (1.12)^{Edef}
(G6) 17.5%-HP gel 3x 15 min	4.51 (0.97)^{Ac}	6.93 (0.92)^{Bc}	9.05 (1.15)^{Cc}	11.06 (1.67)^{Dbc}	12.36 (1.69)^{Ebc}	13.00 (0.96)^{DEbc}	11.23 (1.25)^{DEbd}	4.28 (0.78)^{Ac}	6.76 (0.94)^{Beg}	8.93 (1.17)^{Cc}	10.97 (1.61)^{bc}	12.21 (1.61)^{Ebc}	12.86 (0.88)^{DEb}	11.08 (1.19)^{DEc}
(G7) 17.5%-HP gel 1x 15 min	2.90 (0.86)^{Adf}	3.91 (0.47)^{Ag}	5.50 (0.57)^{Bdf}	7.62 (1.20)^{Cedf}	9.31 (1.24)^{Dc}	10.54 (0.94)^{Dcd}	9.50 (0.76)^{CDcdg}	2.40 (0.30)^{Aef}	3.69 (0.42)^{Bd}	5.29 (0.53)^{Cde}	7.52 (1.22)^{Ddef}	9.21 (1.27)^{Ecdf}	10.38 (0.81)^{Ece}	9.24 (0.68)^{Ede}
(G8) 17.5%-HP gel 3x 5 min	3.27 (0.70)^{Acd}	4.38 (0.78)^{Aeg}	5.60 (0.87)^{Bdf}	7.84 (1.51)^{Ccdf}	8.31 (1.17)^{BCDde}	10.08 (1.39)^{Ede}	8.92 (1.20)^{Dcdf}	3.22 (0.36)^{Ade}	3.75 (0.64)^{Ad}	5.02 (0.74)^{Bde}	7.69 (1.50)^{Ccdf}	7.15 (1.13)^{CEde}	10.00 (1.38)^{Dcde}	8.82 (1.19)^{Edef}

(G9) 17.5% HP-gel 1x 5 min	2.56 (0.39) ^{Af}	3.58 (1.18) ^{Afg}	4.84 (1.47) ^{Bdf}	6.10 (0.92) ^{BDdf}	7.70 (0.61) ^{BDde}	8.94 (0.95) ^{Ce}	8.27 (0.80) ^{CDcef}	2.24 (0.67) ^{Afg}	2.86 (1.33) ^{Aadf}	4.39 (1.48) ^{Be}	5.84 (0.96) ^{Bdf}	6.43 (0.76) ^{BCef}	8.70 (0.84) ^{CDce}	7.99 (0.83) ^{BCef}
(G10) 37%-CP gel 3x 20 min	3.49 (0.81) ^{Ade}	5.61 (0.64) ^{Bcd}	7.32 (1.48) ^{Ccd}	9.45 (2.10) ^{CDcd}	10.13 (2.11) ^{Dc}	11.72 (1.84) ^{Ebde}	10.33 (1.71) ^{Dbde}	3.17 (0.64) ^{Ade}	5.38 (0.53) ^{Befg}	7.10 (1.31) ^{Ccde}	9.31 (2.09) ^{CDcd}	10.23 (2.05) ^{Dcde}	11.53 (1.77) ^{Ebc}	10.16 (1.71) ^{Dcd}
(G11) 37%-CP gel 1x 20 min	2.04 (0.62) ^{Afg}	3.18 (0.83) ^{Bfg}	4.24 (1.79) ^{ABCDf}	5.91 (1.51) ^{Cdf}	6.35 (1.71) ^{CDde}	7.20 (2.08) ^{Cde}	6.35 (1.19) ^{CDf}	1.56 (0.43) ^{Agh}	2.88 (0.79) ^{Bd}	4.05 (1.80) ^{Bef}	5.74 (1.56) ^{Cdf}	6.16 (1.76) ^{CDef}	7.04 (2.13) ^{De}	6.02 (1.36) ^{CDgh}
(G12) 37%-CP gel 3x 10 min	2.60 (0.58) ^{Af}	4.49 (1.02) ^{Beg}	6.11 (1.80) ^{BCcdf}	6.61 (2.63) ^{BCcdf}	7.12 (2.92) ^{BCde}	8.43 (2.20) ^{Ccde}	7.27 (1.47) ^{Cefg}	2.28 (0.62) ^{Afg}	4.22 (1.01) ^{Bcdg}	5.85 (1.82) ^{BCcde}	6.48 (2.67) ^{BCcdf}	6.95 (2.98) ^{CDcdf}	8.24 (2.27) ^{Dcde}	7.23 (1.64) ^{CDfg}
(G13) 37%-CP gel 1x 10 min	1.56 (0.66) ^{Ag}	2.59 (0.73) ^{Bf}	3.88 (1.59) ^{BCDfg}	5.01 (1.66) ^{BCDf}	5.92 (2.32) ^{BCDde}	6.47 (2.48) ^{Cde}	5.31 (2.38) ^{ACDafg}	1.41 (0.55) ^{Aah}	2.49 (0.69) ^{Ad}	3.68 (1.43) ^{BCDde}	4.77 (1.56) ^{BCDf}	5.61 (2.19) ^{BCDde}	6.35 (2.40) ^{Ce}	5.12 (2.34) ^{Dh}

Values represent mean (standard deviation), n=8.

Different uppercase letters in lines (Bonferroni test) and lowercase letters in columns (SNK or Tamhane's test) indicate statistically significant difference among groups ($p < 0.05$).

Table 3 - Data of HP diffusion quantification through enamel and dentin discs for the different bleaching protocols at the sessions (S).

Protocol	S1	S3	S6
(G1) Negative Control	n.d.	n.d.	n.d.
(G2) 35%-HP gel 3x 15 min	1.84 (0.20)[a]	1.82 (0.39)[a]	2.26 (0.65)[a]
(G3) 35%-HP gel 1x 15 min	0.79 (0.21)[b]	0.75 (0.18)[b]	0.95 (0.09)[b]
(G4) 35%-HP gel 3x 5 min	0.77 (0.25)[b]	0.68 (0.34)[bd]	0.84 (0.22)[b]
(G5) 35%-HP gel 1x 5 min	0.28 (0.05)[ce]	0.35 (0.12)[cde]	0.58 (0.08)[c]
(G6) 17.5%-HP gel 3x 15 min	0.73 (0.06)[b]	0.72 (0.27)[b]	0.98 (0.27)[b]
(G7) 17.5%-HP gel 1x 15 min	0.35 (0.11)[ce]	0.25 (0.09)[cd]	0.32 (0.08)[df]
(G8) 17.5%-HP gel 3x 5 min	0.36 (0.09)[ce]	0.30 (0.10)[cde]	0.36 (0.06)[df]
(G9) 17.5% HP-gel 1x 5 min	0.10 (0.05)[d]	0.15 (0.03)[df]	0.13 (0.01)[e]
(G10) 37%-CP gel 3x 20 min	1.53 (0.31)[a]	1.39 (0.28)[a]	1.36 (0.37)[a]
(G11) 37%-CP gel 1x 20 min	0.73 (0.04)[b]	0.70 (0.23)[b]	0.68 (0.22)[bd]
(G12) 37%-CP gel 3x 10 min	0.79 (0.18)[b]	0.85 (0,. 4)[b]	0.76 (0.12)[b]
(G13) 37%-CP gel 1x 10 min	0.21 (0.06)[de]	0.24 (0.06)[cf]	0.23 (0.07)[ef]

n.d. = not detected
Values represent mean (standard derivation), n=8.
Different lowercase letters in lines (SNK or Tamhane's test) indicate significant difference among groups ($p < 0.05$).
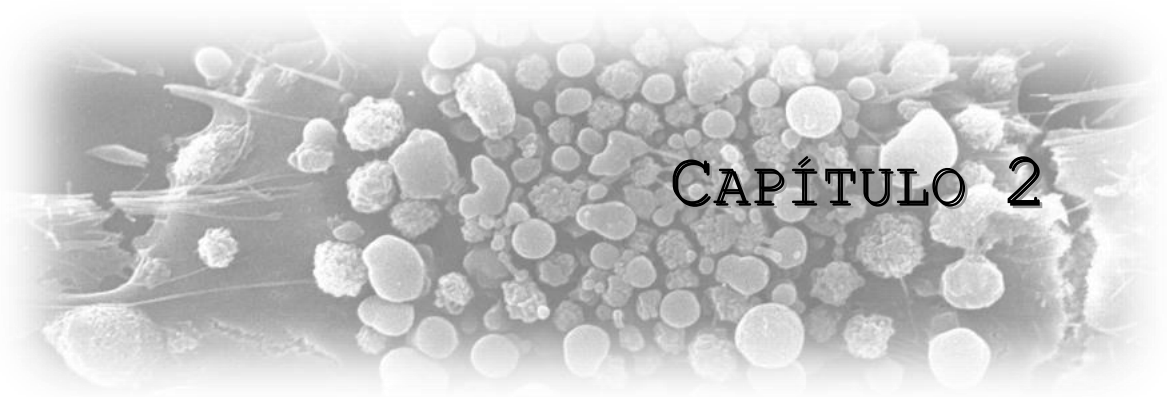
Capítulo 2

3 Capítulo 2

Concentrations of and application protocols for hydrogen peroxide bleaching gels: effects on pulp cell viability and whitening efficacy

 Short Title: Effects of experimental bleaching protocols

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Concentrations of and application protocols for hydrogen peroxide bleaching gels: effects on pulp cell viability and whitening efficacy

ABSTRACT

Objectives: to assess the whitening effectiveness and the trans-enamel/trans-dentinal toxicity of experimental tooth-bleaching protocols on pulp cells. *Methods:* Enamel/dentin discs individually adapted to trans-well devices were placed on cultured odontoblast-like cells (MDPC-23) or human dental pulp cells (HDPCs). The following groups were formed: G1 – no treatment (control); G2, G3, and G4 – 35% H_2O_2, 3x15, 1x15, and 1x5 min, respectively; and G5, G6, and G7 – 17.5% H_2O_2, 3x15, 1x15, and 1x5 min, respectively. Cell viability and morphology were evaluated immediately after bleaching (T1) and 72 h thereafter (T2). Oxidative stress and cell membrane damage were also assessed (T1). The amount of H_2O_2 in culture medium was quantified (Mann-Whitney; $\alpha=5\%$) and colour change (ΔE) of enamel was analysed after 3 sessions (Tukey's test; $\alpha=5\%$). *Results:* Cell viability reduction, H_2O_2 diffusion, cell morphology alteration, oxidative stress, and cell membrane damage occurred in a concentration-/time-dependent fashion. The cell viability reduction was significant in all groups for HDPCs and only for G2, G3, and G5 in MDPC-23 cells compared with G1. Significant cell viability and morphology recovery were observed in all groups at T2, except for G2 in HDPCs. The highest ΔE value was found in G2. However, all groups presented significant ΔE increases compared with G1.*Conclusion:* Shortening the contact time of a 35%- H_2O_2 gel for 5 min, or reducing its concentration to 17.5% and applying it for 45, 15, or 5 min produce gradual tooth colour change associated with reduced trans-enamel and trans-dentinal cytotoxicity to pulp cells.

Clinical Significance: The experimental protocols tested in the present study provided significant tooth-bleaching improvement associated with decreased toxicity to pulp cells, which may be an interesting alternative to be tested in clinical situations intended to reduce tooth sensitivity and pulp damage.

1. Introduction

Hydrogen peroxide (H_2O_2) has been widely used by clinicians to whiten darkened/coloured teeth.[1-4] However, the high incidence of post-operative sensitivity claimed by patients subjected to tooth-bleaching therapies[1-4] indicates that this kind of esthetic procedure

can damage pulp tissue.[5-7] Acute inflammation or even partial necrosis of the coronal pulp tissue has previously been shown in teeth subjected to highly concentrated in-office bleaching gels.[5,6] It has been reported that the inward diffusion of H_2O_2 into the pulp chamber is crucial for post-operative tooth sensitivity.[1,2] This phenomenon is directly related to the concentration of H_2O_2 in the bleaching gel as well as the period of application of this dental product to enamel.[8,9]

The low molecular weight of H_2O_2 and its subproducts favors the rapid diffusion of these reactive oxygen species (ROS) through mineralized dental tissues[10] to cause oxidative stress in pulp cells.[7] Intense cell death[11,12] or stimulus for odontoblastic marker over-expression and cell differentiation[13-15] may occur, depending on the amount of ROS that reaches the pulp cells. In this way, reducing the trans-enamel and trans-dentinal H_2O_2 diffusion during tooth-bleaching procedures may be an interesting alternative to minimize the oxidative damage caused in pulp cells by this esthetic procedure, whilst also stimulating pulpal healing. Therefore, the aim of this study was to assess the effects of H_2O_2 concentration in bleaching gels and their application periods on the trans-enamel and trans-dentinal diffusion of H_2O_2, and its influence on pulp cell viability, correlating these data with tooth-whitening effectiveness.

2. Materials and methods

2.1. Cell Culture: Two cell linage from pulp tissue were evaluated in this study. The odontoblast-like MDPC-23 cell is an immortalized cell linage from rat dental papilla, which exhibits an odontoblast phenotype. This cell linage has been extensively used to test the toxic effects of bleaching gels.[11,12,16] This is because in clinical situation the odontoblasts are the first cells that receive any component released from bleaching gels capable of diffusing through enamel and dentin to reach the pulp chamber. A primary culture of human dental pulp tissue was also used in this study since it represents a mixed culture from pulp cells containing a subpopulation of mesenchymal stem cells (MSC). The MSCs from pulp tissue, which have the ability to differentiate into odontoblast-like cells, play an important hole in the pulp-dentin complex healing and homeostasis.

The primary culture of human dental pulp cells (HDPCs) were obtained from freshly impacted third molars from donors who signed a corresponding form approved by the Ethics Committee of Araraquara School of Dentistry, SP, Brazil (Proc. nº 13/11). The pulp tissue was aseptically removed and minced into small fragments, which were subjected to enzymatic digestion by collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA). The cells released from digested tissue were then subcultured in complete DMEM. The identification of stem cells on HDPC culture was performed by immunofluorescence assay.

After fixation and permeabilisation, the cells were incubated with 1:50 Vimentin, Nestin, Nanog, and Oct3/4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies (12 h at 4°C), followed by incubation with 1:100 secondary antibody conjugated with fluorescein isothiocyanate (FITC). Nuclear fluorescence was performed with 4′,6′-diamino-2-phenylindole (DAPI). Positive staining for the primary antibodies tested (Fig. 1) indicated that the HDPC culture used in the present investigation contains a subpopulation of MSCs derived from the neural crest.[17,18] Cells from the 4th to 6th passages were used. The MDPC-23 cells were cultured and subcultured into cell culture flasks with complete DMEM at 37°C and 5% CO_2 until adequate numbers of cells were obtained.

2.2. Experimental Procedure: Enamel/dentin discs (5.6 mm diameter x 3.5 mm thickness)[16] obtained from 24- to 30-month-old bullocks were adapted to acrylic trans-wells (Corning Inc., Corning, NY, USA) by means of a fluid light-cured resin (TopDam, FGM, Joinville, SC, Brazil). Each disc/trans-well set was individually sterilized by ethylene oxide.[19] The HDPCS and MDPC-23 cells were seeded into 24-well plates (6 x 10^4 cells/cm²) and incubated at 37°C and 5% CO_2 for 24 h before the experiment (80% confluence). Thereafter, the culture medium was replaced by 300 μL DMEM without SFB, and the disc/trans-well set was placed on the cultured cells in such way that only the dentin surface was maintained in direct contact with DMEM. The DMEM without SFB was placed in contact with the cells prior the bleaching procedure in order to avoid interaction between SFB components and the HP that diffused through the disc/trans-well set. Two concentrations of HP were tested in this study, a 35% HP gel (Whiteness HP – FGM, Joinville, SC, Brazil); and a 17.5% HP gel, which was obtained by dilution of the 35% HP gel in distilled water immediately before the bleaching procedure. The bleaching procedure was performed on the central surface of enamel, according to the different protocols established, giving rise to the following groups: G1 - no treatment (negative control); G2 – three 15-minute applications of a 35%- H_2O_2 gel (traditional protocol - positive control); G3 - one 15-minute application of a 35%- H_2O_2 gel ; G4 - one 5-minute application of a 35%- H_2O_2 gel; G5 - three 15-minute applications of a 17.5%- H_2O_2 gel; G6 - one 15-minute application of a 17.5%- H_2O_2 gel; and G7 - one 5-minute application of a 17.5%- H_2O_2 gel. Immediately after the end of the bleaching procedure, the disc/trans-well set was removed, and cell analysis was performed at two experimental time-points: T1 – immediately after bleaching; and T2 – 72 h after bleaching (DMEM plus 10% SFB was changed daily).

2.3. Cell Viability Assay: Cell viability was assessed by the MTT assay as previously described.[16] The cells were incubated for 4 h with MTT solution (Sigma-Aldrich Corp.) at 37°C

and 5% CO_2. After that, the absorbance of formazan crystals in the viable cells was assessed in an ELISA microplate reader (570 nm) (Tp Reader, Thermoplate, Nanshan District, Shenzhen, China). Cell viability in the negative control group was considered as 100%, and the percentage values for experimental groups were calculated. This protocol was performed at T1 and T2 (n = 6).

2.4. Cell Morphology Analysis: Cell morphology (n = 2) was assessed according to a protocol previously described[11,12,16] at the T1 and T2 periods. At the end of each time-point, the culture medium was aspirated, and the cells attached to round coverslips were fixed with 2.5% buffered glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated in ethanol (30%, 50%, 70%, 95%, and 100%), and finally immersed in 1,1,1,3,3,3-hexamethyldisilazane (HMDS). The coverslips were mounted on metallic stubs, stored in a vacuum desiccator for 72 h at room temperature, and sputter-coated with a gold layer. Cell morphology was then examined by scanning electron microscopy (JEOL JSM 6610; JEOL Ltd., Akishima, Tokyo, Japan).

2.5. Oxidative Stress Measurements: Production of ROS (reactive oxygen species) in cultured cells was quantified by means of a cell-permeant fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Life Technologies, San Francisco, CA, USA) (n = 5). The cells were seeded over coverslips placed on the bottoms of the 24-well plates. Based on prior experimental procedure, the cells were incubated at 37ºC and 5% CO_2 with 5 μM H_2DCFDA for 10 min. Immediately after being bleached (T1), the cells were fixed in 2.5% buffered glutaraldehyde for 1 h, mounted over a glass sheet, and analysed by fluorescence microscopy. Photographs of 6 fields from each sample were taken, and the percentages of cells with positive fluorescence related to the total of cells counted in the brightfield were calculated. The average value was used for statistical analysis.

2.6. Cell Membrane Damage Measurements: This assay was performed with the Live/Dead Cell Viability/Cytotoxicity Kit (Invitrogen, San Francisco, CA, USA), which uses the fluorescence probe Ethyl homodimer-1 (Eth-1) that binds to DNA bands only in cells with cell membrane rupture. The second probe was the Calcein AM (CA), which is hydrolyzed by cytoplasmic esterases in viable cells. Immediately after cells were bleached (T1) (n = 5), the supernatant was centrifuged (4,000 rpm for 2 min) to remove the culture medium containing H_2O_2 (extract), re-suspended with DMEM, and returned to its original well. The plate was centrifuged (4,000 rpm for 2 min) to allow the cells to precipitate, and the cells were incubated for 15 min with 2 μM CA and 4 μM Eth-1, and Hoechst (1:5,000) for nuclear staining.

Fluorescence was assessed by the In Cell Analyzer 2000 (GE Healthcare Life Sciences, Freiburg, Germany) in 9 folds per well. The percentages of dead (positive Eth-1 staining) and live (positive CA staining) cells were calculated from the cells stained with Hoechst, with the software In Cell Investigation (GE Healthcare Life Sciences), and the average value of dead cells per well was used for statistical analysis.

2.7. Quantification of H_2O_2 Diffusion: A 100-μL aliquot of the extract from each group (n = 6) was transferred to tubes containing 900 μL of acetate buffer solution (2 mol/L, pH 4.5), to avoid H_2O_2 degradation. Then, a 100-μL quantity of buffer solution plus extract was transferred to experimental tubes to react with leucocrystal violet (0.5 mg/mL; Sigma-Aldrich Corp.) and horseradish peroxidase enzyme (1 mg/mL; Sigma-Aldrich Corp.). The final volume of reaction was adjusted to 3 mL with distilled water, and the optical density of the solutions was measured at 600-nm wavelength in an ELISA microplate reader. A standard curve of known H_2O_2 concentrations was used for conversion of the optical density obtained in the samples into μg/mL of H_2O_2, and the data were related to μg/mL of extract.

2.8. Bleaching Efficacy: For this analysis, prior to bleaching, the discs were subjected to a staining process with black tea to standardize the baseline colour as previously described.[16] Three bleaching sessions were performed for each protocol. Colour readout was performed before bleaching occurred and 24 h after the end of the bleaching treatment. The specimens (n = 6) were positioned in a white silicone matrix, leaving only the enamel surface exposed. A portable UV-VIS spectrophotometer (Spectro Guide 45/0; BYK-Gardner GmbH, Geretsried, Germany) was positioned over the sample, and three readings were made for calculation of the average. The values of L* a* b*, according to the Commission Internationale de l'Eclairage (CIE), were recorded, and the difference between L*, a*, and b * at the end of the experiment and at baseline was expressed as ΔL, Δa, and Δb. The overall colour change of each specimen, expressed as ΔE, was calculated according to the following equation: $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$.

2.9. Statistical Analysis: Data obtained by MTT, Live/Dead, ROS, and H_2O_2 diffusion assays were analyzed by Kruskal-Wallis and Mann-Whitney tests. The values of ΔE, ΔL, Δa, and Δb were analyzed by one-way ANOVA, complemented by Tukey's test for pairwise comparison. All statistical analyses were performed at the 5% significance level. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analysis. Two independent experiments were performed to demonstrate the reproducibility of data.[20]

3. Results

3.1. Cell Viability and Morphology: For both cell lineages, reductions in cell viability and alterations in cell morphology were proportional to the H_2O_2 concentration on the bleaching gel and periods of application of the products to enamel (Figs. 2, 3). For the HDPCs and MDPC-23 cells, the most intense alterations in morphology were observed in G2/G3 and only G2, respectively. With G1 considered as having 100% of HDPC viability, it was determined, in the T1 period for G2, G3, G4, G5, G6, and G7, that the cell viability was 3.1%, 6.9%, 17.1%, 13.9%, 23.4%, and 34.9%, respectively. All these values were significantly lower than in G1 ($p < 0.05$), but only G3 had no significant difference compared with G2 ($p > 0.05$). A significant increase in cell viability was observed in the T2 period compared with T1 for G3 (22.3%), G4 (37.7%), G5 (58.5%), G6 (55.4%), and G7 (92.7%) ($p < 0.05$). These values were significantly different from those in G1 and G2 at the T2 period ($p < 0.05$). Lower reductions in cell viability and alterations in cell morphology were observed for MDPC-23 cells immediately after being bleached (T1). Also, a significantly greater recovery in cell viability at 72 h thereafter (T2) occurred for all groups ($p < 0.05$). In the T1 and T2 periods, the cell viability determined for G2, G3, G4, G5, G6, and G7 was (for T1) 45.5%, 71.7%, 85.1%, 67%, 74.6%, and 83.9%, and (for T2) 58.7%, 96.2%, 132.6%, 97.1%, 164.8%, and 215.6%. In the T1 period, significant differences were observed for G2, G3, G5, and G6, compared with G1 (control, $p < 0.05$). In the T2 period, significant differences were observed only for G2, G3, and G5 compared with G1 ($p < 0.05$). Percentages of cell viability significantly higher than in G2 were observed for all groups in both the T1 and T2 periods ($p < 0.05$).

3.2. Oxidative Stress Measurement: The percentage of cells under oxidative stress and the representative images for each group are shown in Figs. 4 and 5. The negative control group (G1) had no cells under oxidative stress, and was discarded from statistical analysis. For the HDPCs and MDPC-23 cells, a greater percentage of stained cells was observed in G2/G3 and only G2, respectively. The percentages of cells under oxidative stress were similar for G3-G7.

3.3. Cell Membrane Damage: The percentages of cells stained with Eth-1 probe are demonstrated in Table 1. These data were similar to those obtained when the MTT assay was applied, demonstrating that the reduction in cell viability was a consequence of cell death associated with cell membrane disruption. Data on live/dead percentages and illustrative images from each group are shown in Figs. 6 and 7.

3.4. H₂O₂ Diffusion Quantification: G2 extract presented the highest H_2O_2 concentration, which was statistically significantly different compared with that in the other experimental groups ($p < 0.05$). G3 and G5 had intermediate H_2O_2 concentrations with no difference between them ($p > 0.05$). The lowest H_2O_2 concentration was found in G4, G6, and G7 extracts. H_2O_2 was not detected in the control group. Data on H_2O_2 concentrations in the extracts are shown in Table 2.

3.5. Bleaching Effectiveness: The results of ΔL, Δa, Δb, and ΔE for the specimens subjected to the bleaching procedures are shown in Table 3. For all groups, only a slight alteration in a* and b* variables was observed. The L* value presented the most intense alteration, having a major impact on overall tooth colour change (ΔE). All groups presented significant increases in ΔL and ΔE values when compared with the negative control ($p < 0.05$). However, none of them reached the same degree of colour change as G2, since all groups were significantly different from this group ($p < 0.05$). The G3 and G5 groups presented significantly higher ΔL and ΔE values than the G4, G6, and G7 groups ($p < 0.05$).

4. Discussion

Almost all patients (80-100%) undergoing in-office tooth-bleaching therapy have complained about tooth sensitivity, ranging in intensity from moderately severe to intolerable.[1-4] Despite the important role played by ROS in stress-mediated pulp inflammation,[14,21] bleaching agents with high concentrations of H_2O_2 are still widely used (35-38%; 30-60 min) to perform this kind of treatment.[22] However, tooth bleaching remains an interesting treatment modality for discoloured teeth,[16] because it is a relatively low-cost therapy in which no sound mineralized structure is removed and the esthetic outcome is quickly achieved.[22] Therefore, much effort has been spent in the development of tooth-bleaching protocols that are both clinically efficient and harmless to teeth.[16,23-25] In the present study, we assessed the decreased H_2O_2 concentration in the bleaching gel (from 35% to 17.5%) as well as reduced application time to enamel, to determine if different protocols could prevent or at least reduce the toxic effects of this kind of esthetic therapy to pulp cells. Since these changes in tooth-bleaching treatment may also reduce whitening effectiveness in a short time evaluation,[16] tooth colour analysis was performed to correlate toxicity and bleaching potential with time.

It is known that odontoblasts underlie dentin at the periphery of the pulp, and its protracted processes are anchored inside dentinal tubules, forming the pulp-dentin complex. Thus, these cells are the first to receive toxic components from dental materials capable of

diffusing through enamel and/or dentin. The main function of odontoblasts is to produce dentin throughout the lifespan of the tooth.[26] Odontoblasts are also involved in the initiation, development, and maintenance of the pulp inflammatory/immune response, representing the first line of defense for the host.[27] Therefore, MDPC-23 cells characterising an immortalised odontoblast-like cell line that presents an odontoblast phenotype[28] were used in the present investigation. Additionally, HDPCs representing a heterogeneous culture from pulp tissue, including a population of mesenchymal stem cells,[17,18] were also used in this study, because after lethal odontoblast damage, mesenchymal stem cells are recruited, proliferate, and differentiate into new odontoblast-like cells, allowing for dentin-pulp complex regeneration.[26]

All bleaching protocols evaluated in the present study resulted in trans-enamel and trans-dentinal H_2O_2 diffusion, which was directly related to the concentration of this ROS in the bleaching gel and the time of application to enamel. An intense reduction in HDPC viability immediately after bleaching was observed in those groups in which 35%- H_2O_2 gel was applied to enamel for 45 min (96.9%) or 15 min (93.1%). In those groups bleached with 35%- H_2O_2 gel for 5 min or with 17.5%- H_2O_2 gel, cell viability was reduced by 86.1% to 65.1%. In general, the viability reduction observed in the MDPC-23 cells was less intense than in the HDPCs. The traditional protocol (35%- H_2O_2; 3x15 min) caused about 54.5% cell viability reduction, which corroborates reports from previous studies.[11,16] In a recent study, Soares et al.[16] observed that the culture medium containing bleaching gel components (extract) of this same gel, applied for 15 or 5 min to enamel/dentin discs, promoted no significant cell viability reduction after 1 h of contact time with MDPC-23 cells. Also, the cell viability reductions for the 5- and 15-minute protocols in both gels were similar to those observed after 1-hour application of the extracts of a 10%- and 16%-CP gel (8-hour application) to MDPC-23 cells, respectively.[29,30] Thus, the experimental protocols evaluated in the present study successfully minimized the H_2O_2 diffusion and its toxic effects on pulp cells. However, the pulp cells from human tissue were still highly sensitive to all bleaching protocols tested in this investigation.

Cell viability results were confirmed in SEM images in which a reduced quantity of cells still adhered to the glass surface after the bleaching procedures, indicating intense toxicity. The cells that remained attached to the substrate exhibited morphological alterations. These findings occurred more frequently in G2 and G3 for HDPCs and in G2 for MDPC-23 cells. The use of a fluorescence assay with H₂DCFDA demonstrated that those cultured cells in contact with ROS were under oxidative stress. Squier et al.[31] reported that oxidative stress arises when concentrations of ROS exceed the cellular ability to remove highly oxidative molecules and repair cellular damage. This process leads to the accumulation of oxidized-damaged molecules and dysfunctional macromolecules, resulting in multiple compromises to cellular homeostasis.[32] In addition, the reaction of ROS with the polyunsaturated fatty acids from cell membranes

results in lipid peroxidation initiation, which can culminate in cell membrane damage and cell death.[31] In the present study, cell membrane damage was assessed by means of the Eth-1 probe, which binds to the DNA of cells with membrane disruption. Higher percentages of Eth-1-stained HDPCs were observed compared with MDPC-23 cells. However, cell death was observed in both cell lines, being higher in the positive control group (G2) and decreasing in G4 to G7. Thus, it may be assumed that the pulp cells in contact with H_2O_2 released from the bleaching gels suffered oxidative stress, resulting in alterations in cell morphology or even in cell death, such as observed in SEM images and by Live/Dead assay.

Previous studies demonstrated pulp cell oxidative stress induced by H_2O_2 in a time-/concentration-dependent fashion.[14,15] In vital teeth, oxidative stress causes inflammatory pulp response with intensity directly related to the thickness of enamel and dentin of bleached teeth.[6,26] Recently, Sato et al.[7] demonstrated that *in vivo* oxidative stress generated by a 35%-H_2O_2 gel in the pulp tissue of young human pre-molars increased the activity of metalloproteinases and cystein cathepsin B, which play an important role in protein matrix degradation. According to data from the present study, these negative side-effects may be minimized by shortening the contact time of the bleaching gel with enamel or reducing the concentration of H_2O_2 in this dental product. However, human pulp cells were more sensitive to oxidative stress than were the MDPC-23 cells, which may explain, at least in part, the intense damage caused by bleaching agents with high concentrations of H_2O_2 to pulps of human incisors.[6] According to Zhu et al.,[21] the cytotoxic effects of H_2O_2 depend on the types of cells affected. The authors observed that the same quantity of H_2O_2 (0.5 mmol/L) had no effect on cell viability in the primary culture of human gingival fibroblasts (HGF) and L929 mouse fibroblasts. However, an almost 100% reduction in cell viability was observed for HDPCs and MC3T3-E1 pre-osteoblast cells. Also, Min et al.[14] observed a 65% viability reduction in HDPCs after 1-hour contact time with 0.5 mM H_2O_2. In contrast, only 35% viability reduction occurred in MDPC-23 cells exposed to the same concentration of H_2O_2 for 24 h.[13] According to Ceçarini et al.,[32] the sensitivity of cells to undergo oxidative stress appears to be cell-type-specific, as the ability to repair the oxidative damage or sensitivity to the oxidized molecules. However, the basis for this cell-type specificity is still poorly understood.

In the present study, the capability of remaining cells to overcome oxidative stress and proliferate was assessed 72 h after the bleaching procedure. All groups presented cell viability recovery, except when the traditional protocol (G2) was tested in HDPCs. The cell viability recovery was more expressive for G4-G7, in which the percentage of cell viability at T2 was approximately twice that observed in T1, and cell morphology recovery was also observed. Analysis of these data confirms that pulp cells not lethally damaged by H_2O_2 have an inherent ability to regulate oxidative stress. Squier et al.[31] reported that cells present specific defense

mechanisms, such as production of superoxide dismutase, catalase, glutathione peroxidase, and vitamins C and E, which are capable of preventing oxidative stress and providing collective help to protect living organisms against oxidative cell damage. Min et al.[14] reported that H_2O_2 mediating cell viability reduction is associated with heme oxygenase-1 (HO-1) over-expression, which has been related to cytoprotection of pulp cells. The authors demonstrated that over-expressed HO-1 HDPCs significantly improved their viability against H_2O_2-induced cytotoxicity. Also, the activity and expression of odontoblast markers were increased in MDPC-23 and HDPCs exposed to low concentrations of H_2O_2.[13-15] Therefore, it may be speculated that low concentrations of H_2O_2 in contact with pulp tissue may play an important role in biostimulation and healing of the pulp-dentin complex.

To correlate cytotoxicity and whitening effectiveness, we assessed the colour alteration of enamel using a spectrophotometer according to the CIE L*a*b* system. In general, all bleaching protocols promoted significant alterations in tooth colour (ΔE) after three bleaching sessions compared with the negative control group (no bleaching). This colour alteration was regarded as clinically perceptible to the naked eye, as ΔE values obtained were above 3.3.[33] The L* parameter had the major influence on overall colour change, which is in line with previous investigations.[1-4,23-25,34,35] Such gains in luminosity may be considered as 'test specimen bleaching,' since positive ΔL values mean a trend toward white. Despite the fact that all groups presented significantly lower ΔE values than the traditional in-office protocol (G2), one can expect that additional tooth-bleaching sessions would produce an esthetic outcome as effective as that observed in G2. Sulieman et al.[36] reported that gels with 25, 15, 10, and 5% H_2O_2 were capable of achieving an optimal shade outcome (B1-VITA Classic Shade Guide) when the number of bleaching sessions was increased. Many current clinical studies have also demonstrated that bleaching gels with 15-20% of H_2O_2 applied to enamel for 45-60 min were capable of promoting a significant colour change, similar to that caused by a 35%- H_2O_2 bleaching gel. In these studies, the incidence of tooth sensitivity ranged from 24% to 78%, which was considered as mild in severity.[23-25] Thus, it is possible to perform an efficient and less aggressive in-office tooth-bleaching protocol by increasing the number of clinical sessions.[35] However, *in vivo* studies in vital human teeth are needed to assess tooth-bleaching effectiveness as well as pulp responses after the application of the bleaching protocols evaluated in the present study.

5. Conclusions

Based on the data obtained in the present study, it can be concluded that, in spite of the fact that traditional in-office tooth bleaching (35%-H_2O_2 gel applied for 45 min) results in a rapid and effective whitening outcome, it can produce strong oxidative stress on pulp cells, associated with an intense reduction in cell viability. Reducing contact time for 5 min, or decreasing the concentration to 17.5%, with 45, 15, or 5 min of application, produces gradual tooth colour change associated with a reduction in trans-enamel and trans-dentinal cytotoxicity to pulp cells.

6. Acknowledgements

7. References

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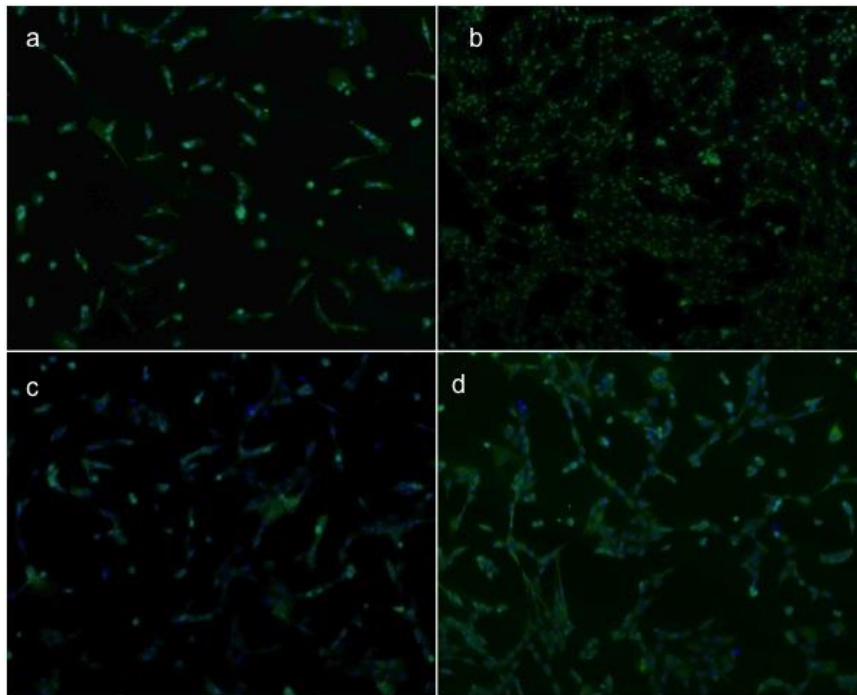
FIGURES

Fig. 1– Immunofluorescence stain for stem cell markers in HDPC culture (10x). (a, b) Nanog and Oct3/4 markers, respectively. Positive cells were found for both embryonic stem cell markers. (c, d) Nestin and Vimentin markers, respectively. The cells presented positive stain for Nestin and Vimentin, demonstrating neural crest and mesenchymal origins, respectively.
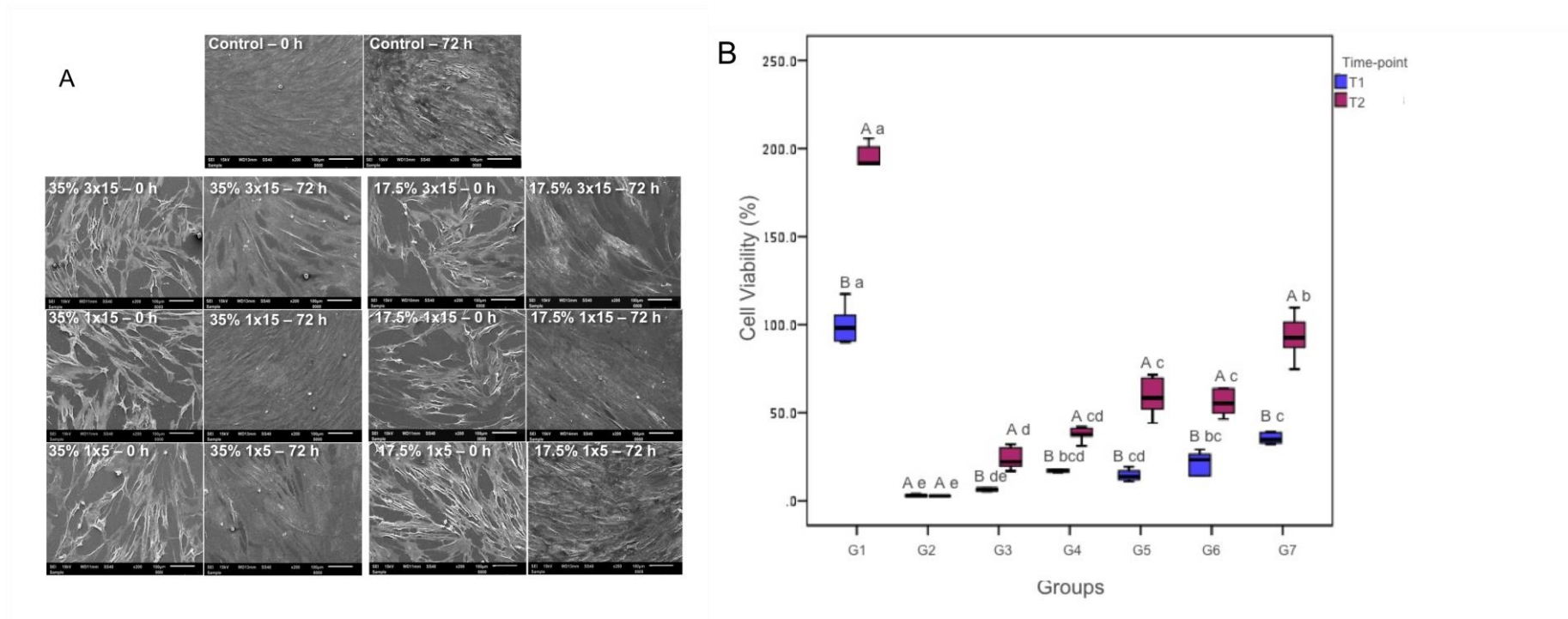
Fig. 2 – A - SEM, 500x. Composite of SEM micrographs at T1 (0 h) and T2 (72 h) for HDPCs. [control (G1)] Large numbers of cells were observed at T1 and T2. [35% 3x15 (G2) and 35% 1x15 (G3)] Remarkable shrinkage of the cytoplasm, intense alterations in cell morphology, and cell-free areas were observed at T1. At T2, the cells exhibited a large cytoplasm, covering almost the entire glass surface in G3. For G2, cells with large cytoplasm were observed, but a lower number of cells was observed. [35% 1x5 (G4) and 17.5% 3x15 (G5), 1x15 (G6) and 1x5 (G7)] Reduced numbers of cells presenting shrunken cytoplasm were observed in all groups at T1. At T2, cells with morphology similar to that of G1 at T1 were observed in G4, G5, and G6. The most evident cell morphology recovery was observed for G7, which presented morphology similar to that observed for G1, at T2. B - Box-whisker plot of MTT Assay: Vertical axis represents percentage of cell viability, and horizontal axis represents the experimental groups. Blue boxes represent the cell viability results at T1, whilst green boxes represent its values at T2. Upper and lower limits of boxes represent, respectively, percentiles 25 and 75, and the horizontal line represents the median. Upper and lower lines indicate the maximum and minimum values, respectively. Lower-case letters allow for comparisons within the groups at each time-point; upper-case letters allow for comparisons within the time-points for each group. Different letters indicate a statistically significant difference among groups (Mann-Whitney, $p > 0.05$).
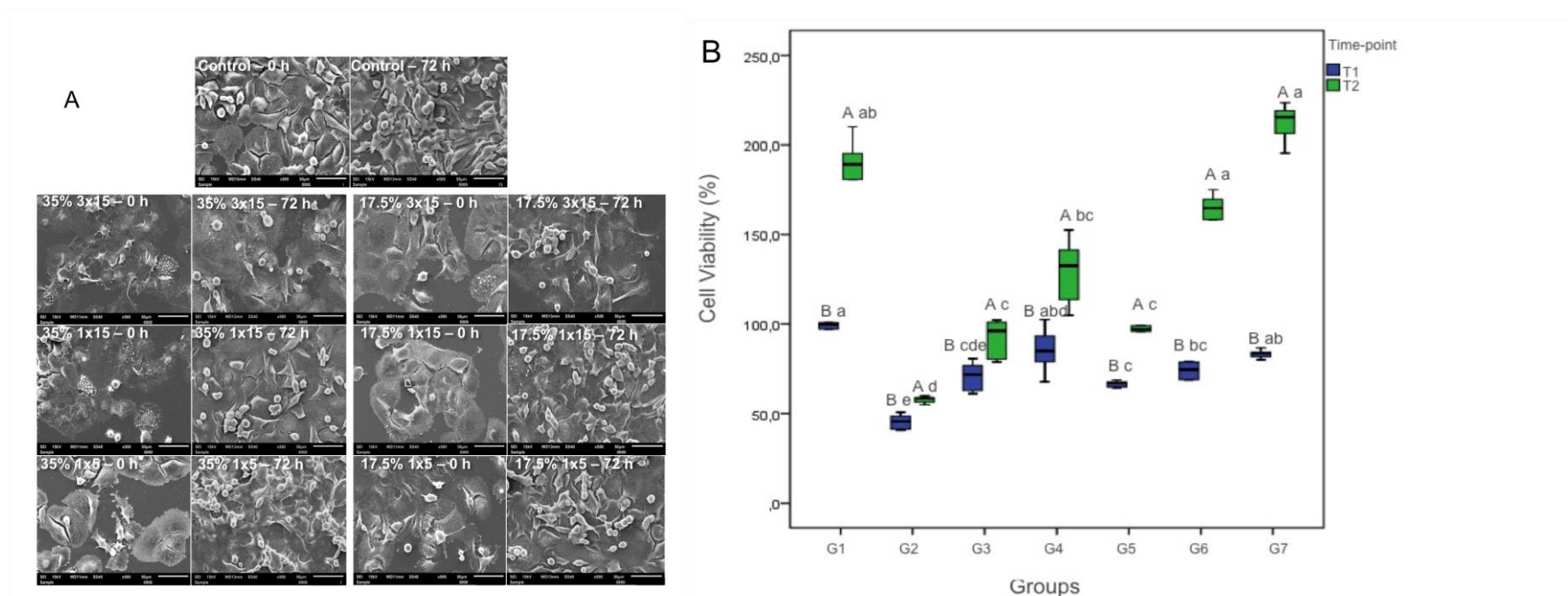
Fig. 3 – A - SEM, 500x. Composite of SEM micrographs at T1 (0 h) and T2 (72 h) for MDPC-23 cells. [control (G1)] A large number of cells near confluence can be observed on the coverglass at T1. An increased number of cells was observed at T2, with cells presenting significant mitosis. [35% 3x15 (G2) and 35% 1x15 (G3)] The cells at T1 exhibited remarkable shrinkage of the cytoplasm and intense alterations in cell morphology. Also, cell-free areas demonstrated that some cells were lethally damaged and detached from the coverglass. At T2, cells with a large cytoplasm were observed, covering almost the entire glass surface. Several areas of mitosis were also observed. These findings were more evident in G3. [35% 1x5 (G4) and 17.5% 3x15 (G5), 1x15 (G6) and 1x5 (G7)] A reduction in the number of cells that continued to adhere to the glass surface was observed; however, in these groups, almost all the remaining cells presented a wide cytoplasm. At 72 h thereafter (T2), large numbers of cells and mitosis areas were observed, which were more evident in G4, G6, and G7. B-Box-whisker plot of MTT Assay: The vertical axis represents the percentage of cell viability, and the horizontal axis represents the experimental groups. Blue boxes represent the cell viability results at T1, whilst green boxes represent its values at T2. Upper and lower limits of boxes represent, respectively, percentiles 25 and 75, and the horizontal line represents the median. Upper and lower lines indicate the maximum and minimum values, respectively. Lower-case letters allow for comparisons within the groups at each time-point; upper-case letters allow for comparisons within the time-points for each group. Different letters indicate statistically significant difference among groups (Mann-Whitney, $p > 0.05$).
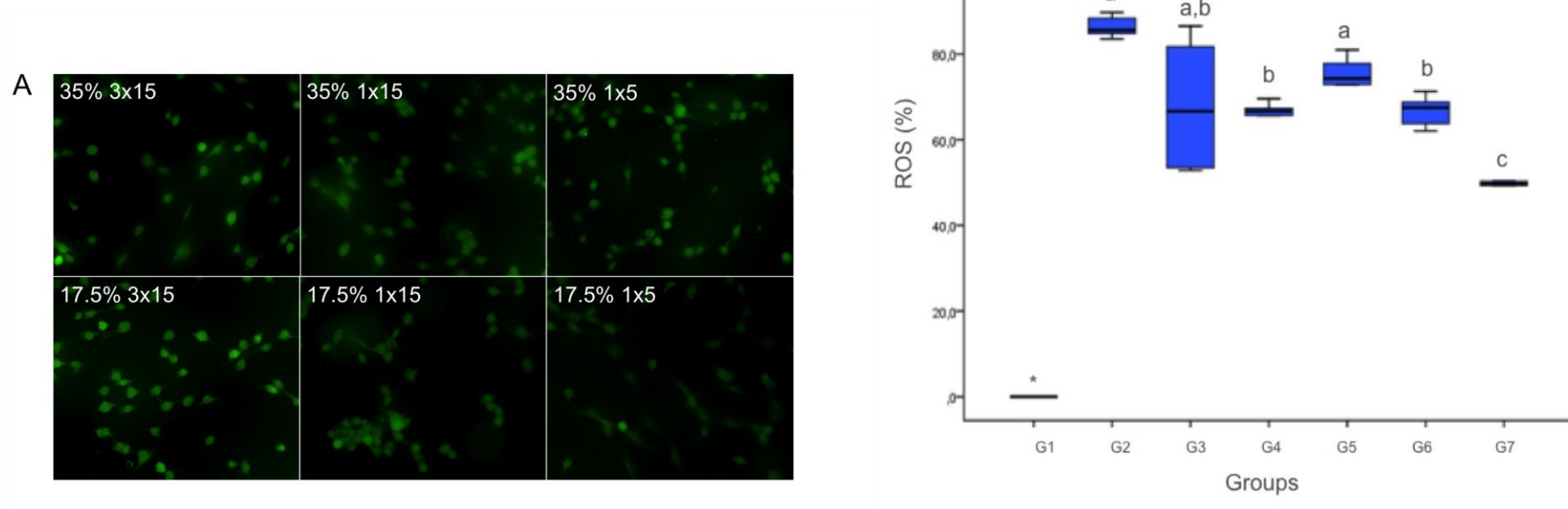
Fig. 4 – A - Images obtained by fluorescence microscopy of HDPCs stained with H_2DCFDA (20x). Cells presented positive stain for H_2DCFDA, indicating that they are under oxidative stress. B - Box-whisker plot of ROS percentage: The vertical axis represents the percentage of positively H_2DCFDA stained cells, and the horizontal axis represents the experimental groups. Upper and lower limits of boxes represent, respectively, percentiles 25 and 75, and the horizontal line represents the median. Upper and lower lines indicate the maximum and minimum values, respectively. Different letters indicate statistically significant difference among groups (Mann-Whitney, $p > 0.05$). * Not detected.

Fig. 5 – A - Images obtained by fluorescence microscopy of MDPC-23 cells stained with H_2DCFDA (20x). Cells of the bleached groups presented positive stain for H_2DCFDA, indicating that they were under oxidative stress. B - Box-whisker plot of ROS percentage: The vertical axis represents the percentage of positively H_2DCFDA stained cells, and the horizontal axis represents the experimental groups. Upper and lower limits of boxes represent, respectively, percentiles 25 and 75, and the horizontal line represents the median. Upper and lower lines indicate the maximum and minimum values, respectively. Different letters indicate statistically significant difference among groups (Mann-Whitney, $p > 0.05$). * Not detected.
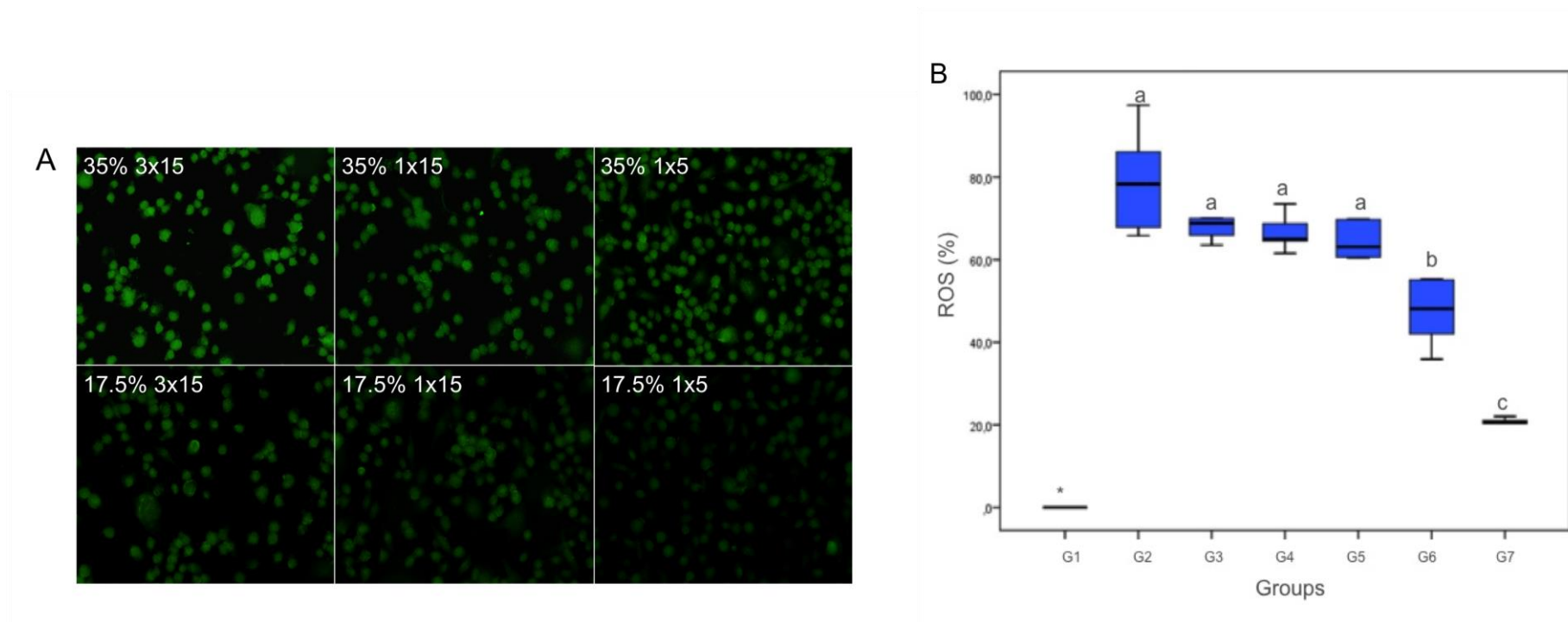
Fig. 6 – A - Fluorescence images obtained by In Cell Analyzer of HDPC cells stained with the Live/Dead viability/cytotoxicity kit (20x). Green fluorescence (positive stain for CA) indicates live cells, and red fluorescence (positive stain for Eth-1) indicates dead cells with cell membrane rupture. It is possible to observe a large number of cells stained positively with Eth-1 in the bleached groups, which represents cell membrane damage. B - Illustrative bar graph of Live and Dead cells percentage: The vertical axis represents the percentage of Live/Dead cells, and the horizontal axis represents the experimental groups.
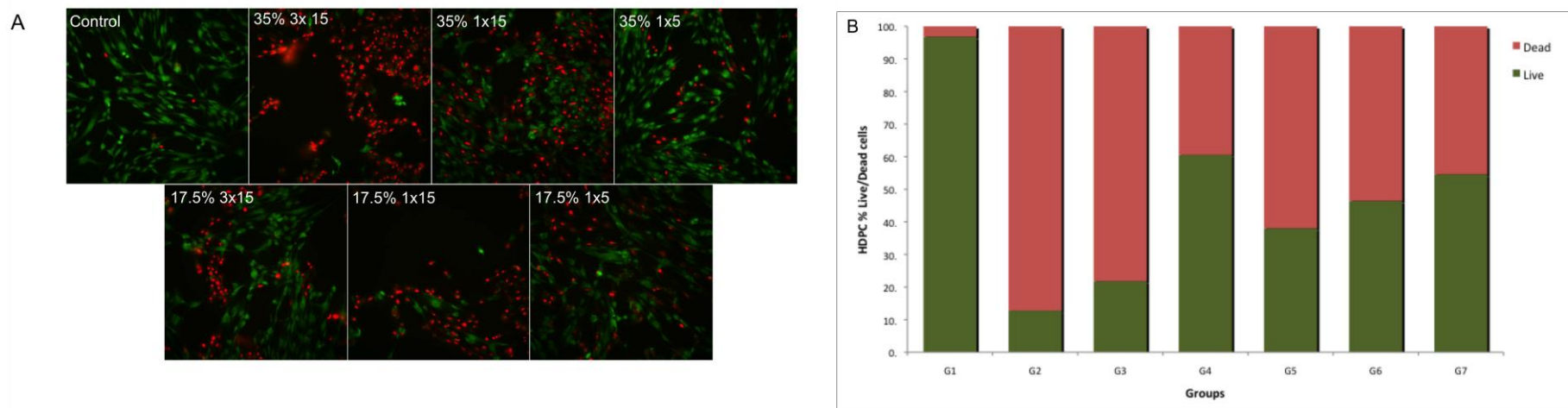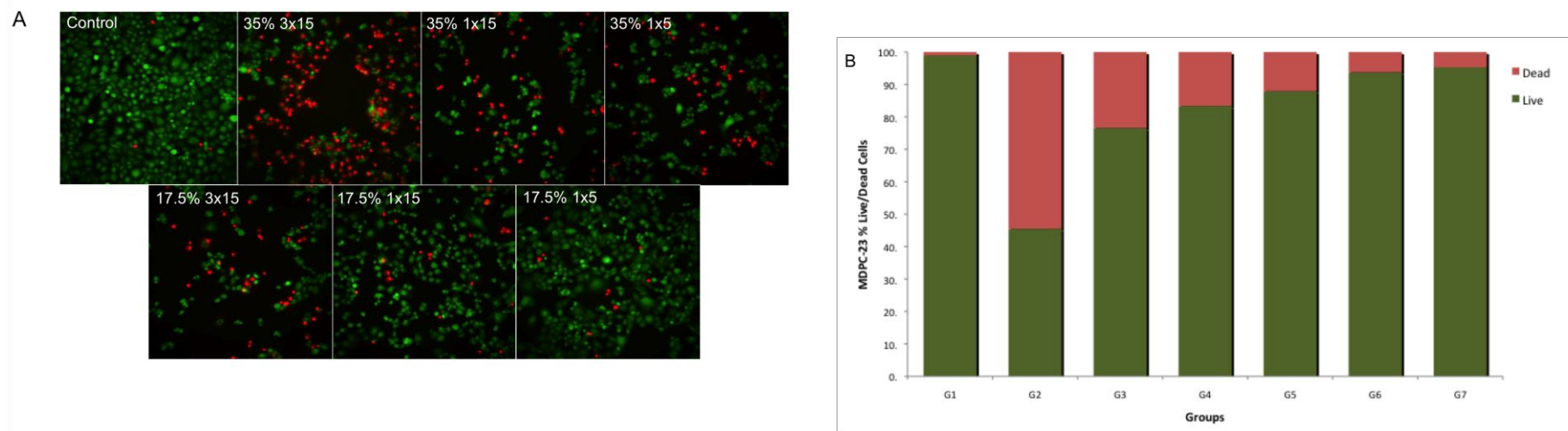
Fig. 7 – A - Fluorescence images obtained by In Cell Analyzer of MDPC-23 cells stained with the Live/Dead viability/cytotoxicity kit (20x). Green fluorescence (positive stain for CA) indicates live cells, and red fluorescence (positive stain for Eth-1) indicates dead cells with cell membrane rupture. It is possible to observe, in G2 (35% 3x15), a large number of cells stained positively with Eth-1, indicating that, in these cells, the cell membrane was damaged. A smaller number of cells staining positive for Eth-1 was observed in the other bleached groups. B - Illustrative bar graph of Live and Dead cells percentage: The vertical axis represents the percentage of Live/Dead, and the horizontal axis represents the experimental groups.

Table 1. Percentage of dead cells (Eth-1 positive staining) for HDPC and MDPC-23.

Groups	% Dead Cells	
	MDPC-23	HDPC
G1	1.1 (1.0-1.2) [d]	3.1 (2.4-4.0) [d]
G2	49.8 (35.6-53.3) [a]	88.0 (87.8-88.0) [a]
G3	22.4 (20.1-23.3) [ab]	78.0 (77.5-79.3) [a]
G4	15.9 (12.3-16.9) [bc]	40.8 (31.5-41.3) [c]
G5	11.9 (7.9-12.0) [bcd]	64.9 (63.1-67.8) [ab]
G6	6.1 (5.2-7.1) [cd]	57.6 (55.4-66.7) [b]
G7	4.6 (2.8-5.5) [d]	47.5 (35.8-49.4) [bc]

Values are median (percentile 25-percentile 75), n=5. Groups identified by the same letter do not differ statistically (Mann-Whitney, $p>0.05$).

Table 2 – H_2O_2 content (µg/mL) detected in the extracts after the bleaching protocols.

Groups	Hydrogen peroxide (µg/mL)
G1	n.d.
G2	1.69 (1.58-2.04) [a]
G3	0.71 (0.66-0.80) [b]
G4	0.35 (0.22-0.47) [bc]
G5	0.74 (0.60-0.83) [b]
G6	0.21 (0.18-0.25) [c]
G7	0.13 (0.09-0.15) [c]

Numbers are medians (percentile 25 – percentile 75), n = 6. Groups identified by the same letter do not differ statistically (Mann-Whitney, $p > 0.05$). n.d. = not detected.

Table 3 – Colour change (ΔL, Δa, Δb, and ΔE) of enamel after the bleaching protocols.

Groups	Enamel colour change (CIE *L*a*b** colour system)$^{\Omega}$			
	Δa	**Δb**	**ΔL**	**ΔE**
G1	0.25 ± 0.18 [c]	0.29 ± 0.24 [b]	0.13 ± 0.55 [d]	0.62 ± 0.36 [d]
G2	-0.72 ± 0.42 [a]	-0.54 ± 1.36 [ab]	11.77 ± 1.57 [a]	11.87 ± 1.59 [a]
G3	-0.17 ± 0.40 [abc]	-1.88 ± 1.21 [a]	7.25 ± 1.08 [b]	7.58 ± 1.11 [b]
G4	-0.39 ± 0.44 [ab]	-1.82 ± 1.15 [a]	4.68 ± 0.34 [c]	5.16 ± 0.21 [c]
G5	-0.37 ± 0.23 [abc]	-0.95 ± 0.84 [ab]	8.56 ± 0.99 [b]	8.66 ± 0.95 [b]
G6	0.14 ± 0.43 [bc]	-1.05 ± 0.79 [ab]	5.20 ± 0.48 [c]	5.36 ± 0.58 [c]
G7	-0.17 ± 0.25 [abc]	-2.06 ± 0.97 [a]	3.86 ± 0.98 [c]	4.40 ± 1.19 [c]

$^{\Omega}$ The maximum for L* is 100, which represents a perfect reflecting diffuser. The minimum for L* is zero, which represents black. The a* and b* have no specific numerical limits. Positive a* is red; negative a* is green. Positive b* is yellow; negative b* is blue. ΔL, Δa, and Δb indicate how much the baseline and post-treatment numbers differ from one another in L*, a*, and b*. ΔE is the total colour difference. Values are mean ± standard deviation. N = 6. [a] Within each column, groups identified by the same letter do not differ statistically significantly (Tukey's test, $p > 0.05$).
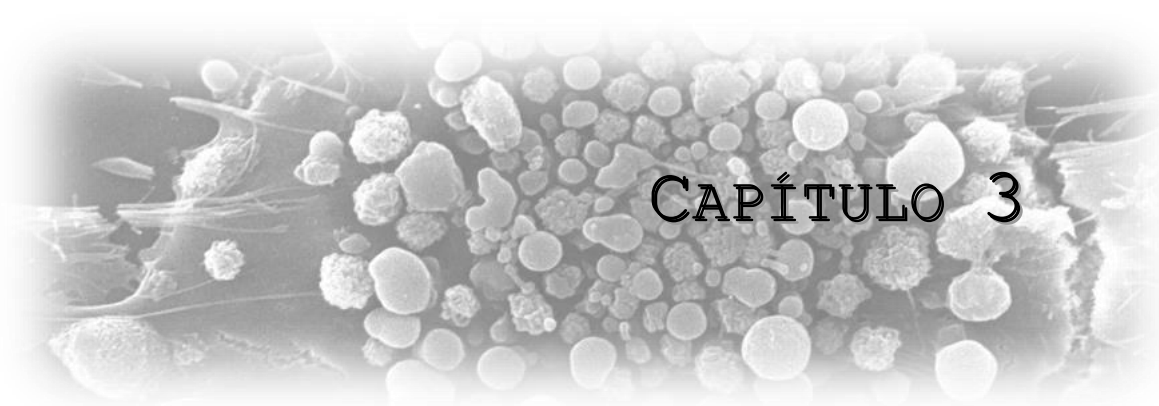
CAPÍTULO 3

4 Capítulo 3

Effects of professional tooth bleaching strategies on odontoblastic phenotype and gene expression of inflammatory mediators

Short Title: Effects of bleaching on odontoblastic differentiation

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Effects of professional tooth-bleaching strategies on odontoblastic phenotype and gene expression of inflammatory mediators

ABSTRACT

Objectives: This study aimed to assess the phenotype characteristics and inflammatory mediator expression by pulp cells subjected to less aggressive in-office bleaching therapies.

Methods: A 17.5% hydrogen peroxide (H_2O_2) gel was applied for 45, 15 or 5 minutes to enamel/dentin discs adapted to transwells positioned over cultured odontoblast-like cells (MDPC-23) or human dental pulp cells (HDPCs). In the negative control group, no treatment was performed. Alkaline phosphatase activity (ALP, thymolphthalein assay), mineralised nodule deposition (MND, Alizarin Red) and mRNA gene expression (real-time PCR) of odontoblast markers (DMP-1, DSPP and ALP) were assessed at 7, 14 or 21 days post-bleaching. Immediately after the bleaching procedures, mRNA gene expression of inflammatory mediators (TNFα, IL-1β, IL-6 and COX-2) was also evaluated (real-time PCR) (Mann-Whitney; $\alpha = 5\%$). *Results:* Gene expression of inflammatory mediators was upregulated in all bleached groups, being directly related to the treatment time. In all bleached groups, the cells presented ALP, DMP-1 and DSPP gene expression throughout the periods of analysis. However, the alteration in ALP activity and reduced MND that occurred in all bleached groups were also directly related to the bleaching time. HDPCs exposed to a 45-minute bleaching treatment presented no deposition of mineralised nodules. The 5- and 15-minute groups showed significant cell recovery capability with time. *Conclusions:* Long-term bleaching procedures with a 17.5%-H_2O_2 gel upregulate inflammatory mediator expression and cause alterations of odontoblast marker expression and mineralised matrix deposition by pulp cells. However, cells subjected to short-term bleaching are capable of recovering with time.

Clinical Significance: Pulp cells maintained their odontoblastic phenotype after the application of a 17.5%-H_2O_2 bleaching gel to enamel. However, as treatment time increases, odontoblast-like cell upregulation and odontoblastic differentiation are delayed. Therefore, short bleaching therapies with low concentrations of H_2O_2 gels may be an interesting alternative to prevent damage to pulp cells and improve the healing capacity of pulp tissue.

1. Introduction

Professional in-office bleaching therapy is performed with highly concentrated hydrogen peroxide (H_2O_2)-based gels (35-38%), applied to dental structure for 45-60 minutes at each chair-side session. However, around 80-100% of patients subjected to this kind of esthetic therapy report post-operative tooth sensitivity.[1-5] Clinical studies recently demonstrated that this negative side-effect takes place mainly in anterior teeth and is more intense and prevalent in thinner teeth, such as incisors.[3,5]

Several in vitro studies in which the enamel/dentin barrier was used to simulate the thickness of incisors (3.5 mm) demonstrated that professional in-office protocols cause intense oxidative stress on pulp cells, characterised by disruption of cell membranes and cell death by necrosis.[6,7] The histological features of human incisors subjected to this kind of esthetic therapy showed a wide zone of coagulation necrosis in coronary pulp, as well as the occurrence of moderate inflammatory response and the deposition of reactionary dentin in the root pulp.[8] However, bleached premolars presented a lack of inflammatory response, no tissue disorganisation and the absence of post-operative tooth sensitivity.[8,9] Therefore, it seems that the laboratory and in vivo intensity of pulp cell damage depends on the amount of H_2O_2 and other toxic by-products released from the bleaching gel and capable of diffusing across enamel and dentin to reach the pulp chamber.[8-12] It has been shown that this transenamel and transdentinal diffusion of bleaching gel components is influenced by three main factors: 1) H_2O_2 concentration in the bleaching gel[13]; 2) contact time of product with dental enamel[6,13]; and 3) enamel/dentin thickness of bleached teeth.[8]

Based upon the intense damage to the pulp-dentin complex caused by professional in-office tooth-bleaching therapies, new strategies have been suggested to prevent or at least reduce this negative side-effect.[6,7,13] It was recently demonstrated that a 17.5%-H_2O_2 gel promoted significant colour enhancement, associated with a reduction of around 60-90% in H_2O_2 diffusion through enamel and dentin, depending on the application regimen.[13] In addition, around 50% of the damaged cells were able to overcome the aggression and improve viability, three days after bleaching.[7] Many researchers have also demonstrated that low concentrations of H_2O_2 upregulated odontoblast-like cells' secretory activity and odontoblastic differentiation of mesenchymal stem cells (MSCs) obtained from human pulp tissue.[14-16] Therefore, in the present study, the effects of different bleaching protocols (with a 17.5%-H_2O_2 gel) on odontoblastic marker regulation and mineralised matrix deposition by cultured pulp cells were assessed up to 21 days post-bleaching. These data were compared with the intensity of inflammatory mediator gene expression by pulp cells immediately after the bleaching protocols were performed.

2. Materials and methods

2.1. Cell Culture: Two cell lineages from pulp tissue were evaluated in this study: the primary culture of human dental pulp cells (HDPCs), which represents a mixed culture containing a subpopulation of MSC,[7] and odontoblast-like MDPC-23 cells, an immortalised cell culture with an odontoblastic phenotype.[17] The HDPCs were obtained by enzymatic digestion of pulp tissue from freshly impacted third molars (Proc. nº 13/11; Ethics Committee of Araraquara School of Dentistry, SP, Brazil) after incubation for 24 h in complete Dulbecco's Modified Eagle Medium (DMEM; supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) and 200 units/mL of type II collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA). After this period, the released cells were subcultured in complete DMEM with 10% SFB. The identification of MSCs on this HDPC culture was previously demonstrated by Soares et al.,[7] by immunofluorescence assay. Positive stain was observed for Nanog, Oct3/4, Vimentin and Nestin, demonstrating that HDPC culture presents a subpopulation of MSCs from the neural crest.[7] Cells from the 4th to 6th passages were used. The MDPC-23 cells were cultured and subcultured into cell culture flasks with complete DMEM at 37ºC and 5% CO_2 until adequate numbers of cells were obtained.

2.2. Experimental Procedure: Standardised enamel/dentin discs (from 24- to 30-month-old bullocks), 5.6 mm in diameter and 3.5 mm in thickness, were adapted to acrylic transwells (Corning Inc., Corning, NY, USA) by means of a fluid light-cured resin (TopDam, FGM, Joinville, SC, Brazil), and sterilised by ethylene oxide.[7] The cells (HDPC and MDPC-23) were seeded into 24-well plates at 80% confluence (6 x 10^4 cells/cm²; 24 h). Thereafter, the culture medium was replaced by 300 μL of complete DMEM with no SFB, and the disc/transwell set was placed over previously cultured cells, in such a way that only the dentin was in direct contact with the culture medium, and the enamel surface remained exposed to receive the bleaching procedure. A bleaching gel with 17.5%- H_2O_2 was freshly prepared by dilution of the liquid phase of a commercial 35%-H_2O_2 gel (Whiteness HP; FGM, Joinville, SC, Brazil).[7,13] The gel was applied to dental enamel for different times, giving rise to the following groups: *45-minute group* – three applications of 15 min; *15-minute group* – one application of 15 min; *5-minute group* – one application of 5 min; and *control group* – no treatment. For gene expression of inflammatory mediators (TNFα, IL-1β, IL-6 and COX-2; real-time PCR), immediately after bleaching, the culture medium was replaced by fresh

DMEM, and total RNA was extracted after 6 h. For analysis of the odontoblastic phenotype, immediately after bleaching, the cells were cultured with complete DMEM supplemented with 10 nmol/L β-glycerophosphate and 50 μg/mL sodium ascorbate (Sigma Chemical Co., St. Louis, MO, USA). The medium was changed every day up to 21 days. The ALP activity (thymolphthalein assay), mineralised nodule formation (Alizarin Red) and mRNA gene expression of DMP-1, DSPP and ALP (real-time PCR) were assessed 7, 14 and 21 days after bleaching.

2.3. Real-time PCR: Total RNA was extracted with an RNAqueous®-micro kit (Ambion®, Austin, TX, USA) by following the manufacturer's protocols. One microgram of total RNA, following DNase I treatment, was reverse-transcribed into single-stranded cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the recommended protocol [25ºC (10 min), 37ºC (120 min), 85ºC (5s), 4ºC]. For relative quantification of inflammatory mediators and odontoblastic marker mRNA levels, Syber Green primers (Sigma) and TaqMan Assays (Applied Biosystems) were used (Table 1). Amplification assays were performed with Applied Biosystems Master Mix, and fluorescence was determined with StepOne Plus equipment (Applied Biosystems). The CT values for each sample were normalised by an endogenous control gene (β-actin). Thereafter, the mean CT value of the control group at 7 days was used to normalise the CT value of both control and experimental groups at all periods.

2.4. ALP activity: After each time-point, cell lysis was performed with 0.1% sodium lauryl sulfate (Sigma). ALP activity was assessed with a kit (End point assay; Labtest, Lagoa Santa, MG, Brazil) as previously described by Soares et al.[6] This assay is based on the reactivity of ALP with the thymolphthalein monophosphate substrate. Released thymolphthalein acquires a purple colour in the presence of sodium carbonate and sodium hydroxide. Absorbance was then read at a 590-nm wavelength with an ELISA microplate reader (Tp Reader; Thermoplate, Nanshan District, Shenzhen, China), and converted into U/L by means of a standard curve with known amounts of ALP. Total protein (TP) dose was performed for normalisation of ALP according to the Read and Northcote[18] protocol, as previously described.[6] The absorbance of the test and blank tubes was measured at a 655-nm wavelength with the ELISA microplate reader. The absorbance value obtained was converted into mg/L by a standard protein curve. The final value of ALP was normalised by total protein obtained from each well, with the value of ALP activity as U/mg of protein.

2.5. Alizarin Red Staining: For assessment of the quantity of mineralised nodules deposited, the cell cultures at each time-point were washed twice with PBS, fixed with cold 70% ethanol for 1 h, washed 3 times with deionised water and then stained with Alizarin Red dye (40 mM, pH 4.2; Sigma) for 20 min, under gentle shaking (VDR Shaker, Biomixer, Ribeirão Preto, SP, Brazil). After aspiration of unincorporated dye, the cells were washed twice with deionised water for the removal of excess stain, and representative photographs from each group were taken by light microscopy (Olympus BX51, Olympus, Miami, FL, USA). The cells were then incubated with 10% cetylpyridinium chloride (Sigma) for 15 min under agitation to solubilise the nodules. The absorbance of the resulting solution was measured at 570 nm by means of an ELISA microplate reader. The percentage of calcium deposition for each experimental group was calculated based on the mean value of the control group at 7 days as 100% of staining.

2.6. Statistical Analysis: To verify the reproducibility of data, two independent experiments were performed for all protocols in this study. Thereafter, data were compiled and subjected to Levene's test to verify homoscedasticity. mRNA gene expression, ALP activity and percentages of Alizarin Red staining for HDPC and MDPC-23 cells were subjected to the Kruskal-Wallis and Mann-Whitney tests at a significance level of 5%.

3. Results

3.1. Inflammatory mediator gene overexpression: The results for inflammatory mediator mRNA overexpression are presented in Fig. 1. In both cell lineages, significant increases in IL-6, TNF-α, COX-2 and IL-1β mRNA were observed for the 45- and 15-minute groups compared with the negative control. A discrete increase in the expression of these genes was observed for the 5-minute group; however, a significant difference from the negative control was observed only for IL-6 in the HDPCs.

3.2. Odontoblastic marker gene expression: The mRNA gene expression of odontoblastic markers is presented in Fig. 2. For the HDPCs, gene expression of ALP was significantly reduced in the 45-minute group relative to the negative control at 7 and 14 days, having no significant difference at 21 days. The other bleached groups presented significant differences

compared with the control group only at 14 days, when the control group reached peak gene expression, which was not observed in the bleached groups. Reduced DMP-1 gene expression was observed in the bleached groups compared with the control group, and a gradual increase was observed through time. For DSPP, the expression was significantly reduced only for the 45-minute group at 21 days. The 5-minute group had a significant upregulation of DSPP compared with the control group at 14 and 21 days. Concerning the MDPC-23, ALP gene expression was not reduced in the bleached groups; however, significant increases in this gene expression were observed for the 15- and 5-minute groups compared with the control group at 14 days and for the 5-minute group at the 21-day period. Significantly increased DMP-1 and DSPP mRNA gene expression was observed for the control and bleached groups with time. The 45-minute group presented the lowest gene expressions at all periods of analysis. The 15-minute group showed no significant difference compared with negative control at 21 days for DMP-1. No significant difference from negative control for DMP-1 and DSPP was found for the 5-minute group.

3.3. ALP activity and mineralised nodule deposition: The ALP activity of HDPCs was not significantly affected in bleached groups. All groups presented low ALP activity, which reached a peak at 14 days. Mineralised nodule deposition occurred in the control group only after 14 days, as observed in Fig. 3. In bleached groups, intense reduction in the percentage of Alizarin Red dye relative to negative control was observed at the 7- and 14-day periods, being more intense for the 45-minute group. At the 21-day period, only the 45-minute group presented a significantly lower percentage than the negative control (Table 2). A remarkable increase in mineralised matrix deposition was observed in negative control and 5-minute groups at 21 days. The 45-minute group presented no mineralised nodule deposition at any period of analysis. In general, the MDPC-23 cells presented more intense ALP activity and mineralised matrix deposition than HDPCs. This was expected, since the MDPC-23 cells present the odontoblastic phenotype. The negative control group presented peak ALP activity at 7 days, which was significantly decreased at 14 and 21 days. The bleached groups showed a significant reduction in ALP activity at 7 days compared with negative control; however, a significant increase was observed at 21 days, which was more expressive for the 5-minute group. Mineralised nodule deposition was significantly reduced at 7 days only for the 45- and 15-minute groups compared with negative control; however, these groups reached the same pattern as negative control at 21 and 14 days, respectively. The 5-minute group presented no significant difference when compared with negative control at all periods of analysis (Table 3 and Fig. 4).

4. Discussion

Dental pulp is a highly specialised connective tissue with inherent regenerative potential. It is known that odontoblasts play a role in pulp regeneration, having the main functions of deposition and mineralisation of dentin matrix throughout tooth life, physiologically or in response to injury.[19,20] These highly differentiated terminal cells are considered the first host defense line, as they act as a barrier at the periphery of pulp, activating the immune system, angiogenesis and neurogenic inflammation against noxious signals applied to dentin.[21,22] MSCs represent another important population of pulp cells related to pulp-dentin complex regeneration. These cells have the potential to differentiate into odontoblast-like cells to replace apoptotic odontoblasts, recovering homeostasis in the pulp-dentin complex after intense injury.[19,20] Therefore, the odontoblast-like MDPC-23 cells and the primary culture of human dental pulp cells (HDPCs) have been widely used to evaluate the toxic effects of different bleaching agents.[6,7] These specific pulp cells were lethally affected when traditional in-office tooth-bleaching protocols (35% H_2O_2 applied to enamel for 45 min) were performed in sound teeth.[6-8,10,12] In this specific situation, the high concentration of H_2O_2 present in the bleaching agents widely used for professional in-office procedures can cause intense cytopathic effects in cultured pulp cells[6,7] and irreversible damage to pulp tissue.[8,10,12] A recent in vitro study demonstrated the occurrence of moderate to slight toxicity to MDPC-23 and HDPCs when a 17.5%-H_2O_2 bleaching gel was applied to enamel for 45, 15 or 5 min.[7] The authors reported that the cells damaged by the components released from the bleaching agent had the capability to overcome oxidative stress and proliferate significantly 72 h after the bleaching procedure.

It is known that host pulpal cells, such as odontoblasts, fibroblasts and MSCs exposed to aggression, play key regulatory roles within the innate immune system because of their recognised expression of inflammatory mediators in response to cellular stress.[23] The expression of these molecules promotes regulatory functions for lymphocytes, macrophages and neutrophils, activating the inflammatory cascade. Depending on the intensity of immune cell recruitment, intense tissue damage may take place, as these cells release proteolytic enzymes as metalloproteinases, which break down the extracellular matrix components and cellular contacts.[23,24] However, it has been shown that inflammation is essential for pulp tissue healing after aggression, which depends on the period and intensity of tissue damage after injury.[25] The main role of the pulp healing process involves upregulation of odontoblast secretory activity. Depending on the intensity of injury, the recruitment of MSCs, their differentiation into odontoblast-like cells and subsequent upregulation of secretory activity

may also occur.[26] The activation of dentinogenesis results in the deposition of an organic matrix by odontoblasts or odontoblast-like cells, followed by its biomineralisation. Several non-collagenous proteins and enzymes secreted by these cells are directly related to the biomineralisation process. Alkaline phosphatase (ALP) is involved with the initial phase of dentin matrix biomineralisation, as it promotes dephosphorylation of extracellular matrix proteins, providing inorganic phosphate.[19] Other proteins, such as DMP-1, DSP (dentin sialoprotein) and DPP (dentin phosphoprotein), are implicated in the nucleation and growth of the mineral phase, being expressed in mature odontoblasts, and upregulated during the active mineralisation phase.[19,27,28] DSP and DPP are encoded by the DSPP gene, which is cleaved immediately after secretion.[19] These proteins are rapidly discharged in the odontoblastic process on the mineralisation front after the deposition of dentin matrix,[29] having direct participation in calcium binding to previously synthesised collagenous matrix, initiating the formation of hydroxyapatite crystals within collagen fibers, and controlling the rate of crystal growth.[19] Since ALP, DMP-1 and DSPP are considered key regulators of dentin mineralisation and markers of odontoblast differentiation,[19,22,27,28] the authors decided to evaluate the expression of these proteins in pulp cells subjected to alternative in-office bleaching protocols.

In the present in vitro study, the effects of experimental in-office bleaching protocols on immediate inflammatory mediator gene expression and late upregulation of odontoblastic phenotypes in odontoblast-like cells (MDPC-23) and primary culture of human pulp tissue (HDPC) were evaluated. Analysis of the data obtained from all bleached groups showed that the cultured pulp cells had increased gene expression of IL-6, TNF-α, IL-1β and COX-2, which are key inflammatory mediators of pulp tissue.[23] This over-expression of specific inflammatory mediators was proportional to the contact time of the product with dental structure. All bleached groups showed ALP, DMP-1 and DSPP mRNA gene expression at all periods of analysis, which means that pulp cells maintained their odontoblastic phenotype after contact with the components released from the bleaching agent capable of diffusing across enamel and dentin. However, a long contact period of bleaching agent application to enamel, e.g. 45 min, resulted in the more intense alteration of these gene expressions in comparison with those in the negative control group. Reducing the contact time to 15 or 5 min allowed cells to reach a pattern similar to that of the negative control group at 14 or 21 days. Therefore, it seems that not only the concentration of H_2O_2 in the bleaching agent but also its period of application to enamel is directly related to the toxicity of this kind of esthetic procedure to pulp cells.

After the bleaching procedures were performed, both cell lines showed ALP activity and deposition of mineralised nodules, except for the HDPCs subjected to the 45-minute

protocol. Peak ALP activity in the control group was observed at 7 and 14 days for MDPC-23 and HDPCs, respectively. Deposition of mineralised nodules was found in all periods for MDPC-23, being more intense after 14 days in culture. Some nodules were found in the HDPC control culture at 14 days; however, intense deposition was found only at 21 days. These data are in line with those from other studies that found downregulation of ALP activity during the odontoblast maturation process, followed by intense secretory activity with time.[30] Long bleaching treatments (45- and 15-minute groups) caused the most intense reduction in mineralised nodule production by MDPC-23 cells at 7 days. However, these activities were significantly recovered with time, reaching a pattern similar to that in the negative control group at the 21-day period. The mineralised nodule deposition by HDPCs was intensely affected in the 45-minute group. Microscopic analysis showed the absence of nodule formation even after 21 days. The 5- and 15-minute bleaching treatments reduced mineralised nodule deposition at 7 days; conversely, a gradual increase was observed up to 21 days.

The intense alteration in mineralisation nodule deposition observed for the 45-minute group was probably a consequence of a more intense cell death by necrosis promoted by this protocol, as previously observed.[7] The regeneration process of pulp tissue involves the following steps: (1) migration of MSCs to the affected area; (2) proliferation; (3) differentiation into odontoblast-like cells; (3) deposition of organic dentin matrix; and (4) biomineralisation of dentin matrix.[19,20,26] In this study, a similar process was observed. The deposition of mineralised nodules occurred only after the cell confluence into the wells, demonstrating that numerous cells are necessary for mineralisation to occur. Therefore, as a reduced number of cells remained in the wells immediately after bleaching in the 45-minute group, longer periods were necessary for the cells to reach confluence and deposit mineralised matrix (Figs. 3 and 4). Also, the recovery time may be longer, as this protocol also promoted intense inflammatory mediator gene expression in the remaining cells. Along et al.[32] observed that exposure of MSCs from normal tissue to high doses of TNF-α and IL1-β for 48 h reduced their odontoblastic differentiation capacity and mineralisation rate in short evaluation periods. Soares et al.[7] also observed that the HDPCs were much more sensitive to the bleaching protocols than were MDPC-23 cells, which may explain the impaired deposition of mineralised nodules by these cells. However, instead of finding no mineralised nodules in HDPCs up to 21 days in culture, it was possible to observe (Fig. 3) that these cells had the capability to proliferate and deposit organic matrix over time. As the other odontoblastic markers were expressed by HDPCs exposed to the 45-minute protocol, one can conclude that these cells would need longer periods to deposit mineralised matrix.

For both pulp cell cultures, the highest values of ALP, DMP-1 and DSPP mRNA gene expression were found for the 5-minute group at the 21-day period in comparison with the other bleached groups. This short-term bleaching group also had the best mineralisation nodule deposition recovery rate associated with the less intense upregulation of inflammatory mediator genes. According to Cooper et al.,[23] there is a fine balance between inflammatory mediator dose and pulp tissue regeneration. Paula Silva et al.[25] applied low-dose TNF-α to HDPCs and observed notable over-expression of DPP, DSP and DMP-1 after a short treatment time (6 h). Min et al.[32] observed increased ALP activity and dentin matrix non-collagenous protein over-expression after 72-hour cultivation of HDPCs with pro-inflammatory cytokines (IL-1α and TNFα). However, the contact of the cells with these cytokines for 14 days significantly decreased ALP activity and odontoblastic marker expression. Additionally, Spoto et al.[33] showed increased ALP activity in reversible pulpitis in comparison with healthy or irreversible pulpitis samples. These data corroborate those found by Alongi et al.,[31] who verified that MSCs from teeth diagnosed with irreversible pulpitis presented lower expression of odontoblastic markers (DSPP, ALP and osteocalcin) than normal pulp tissue MSC-derived cells. One can conclude that the contact of pulp cells with low doses of inflammatory mediators in a short period may play a regulatory role in the pulp regeneration process. Also, it may be suggested that, rather than irreversible pulp cell damage, the increased gene expression of IL-6, TNF-α, IL-1β and COX-2, observed in the present study, may be responsible, at least in part, for the long-term cell recovery. Based upon the data obtained in the present investigation, it may be speculated that pulp cells subjected to the bleaching procedures tested retained their regenerative capability. The odontoblast-like MDPC-23 cells maintained their phenotype and were able to deposit and mineralise dentin matrix in vitro. In addition, the MSC population of HDPCs had the ability to differentiate into cells with an odontoblastic phenotype as well as deposit mineralisation nodules. However, the intensity of odontoblastic marker expression recovery with time depended on the period of application of the bleaching agent to enamel, which was also directly related to the level of inflammatory mediator mRNA expression immediately after bleaching. The lower the contact time of gel with the enamel/dentin substrate, the lower the inflammatory mediator over-expression and the higher the odontoblastic phenotype regulation.

The toxic effects of bleaching protocols on pulp cells are directly related to the amount of H_2O_2 and its by-products able to diffuse through enamel and dentin to reach the pulp chamber.[7,13] According to the results found by Soares et al.,[7] who used an experimental design similar to that used in the present investigation, reducing the bleaching agent (17.5%-H_2O_2) application time from 45 min to 5 or 15 min decreased the transenamel and transdentinal diffusion of H_2O_2 by 83% or 72%, respectively. In this way, one may

hypothesize that the low oxidative stress caused by H_2O_2 and its by-products allowed the cultured pulp cells to achieve a high potential for recovery, maintaining their odontoblastic phenotype, such as observed in the present study for the 15- and 5-minute groups. Other researchers showed that low doses of H_2O_2 applied to MDPC-23 or HDPCs resulted in oxidative stress associated with over-expression of odontoblastic markers and increased mineralized nodule deposition in vitro. Matsui et al.[16] observed that a short treatment (5-10 min) of HDPCs with low amounts of H_2O_2 (0.9 mmol/L) promoted odontoblastic differentiation and increased calcified nodule deposition in vitro. Similarly, a low H_2O_2 dose (0.2 mmol/L) applied for 24 h to cultured HDPCs strongly induced DSPP mRNA expression.[15] Lee et al.[14] observed that 0.1-0.3 mmol/L of H_2O_2 applied on alternate days for 4 days promoted oxidative stress associated with significantly increased ALP activity and mineralized nodule deposition by MDPC-23 cells. Soares et al.[6] also demonstrated an increase in MDPC-23 ALP activity when a 35%-H_2O_2 gel was applied for 5 min to enamel/dentin discs, and the extracts (culture medium containing the bleaching gel by-products that diffused through the hard dental structures) were applied to the cultured cells for 1 h. Nevertheless, high H_2O_2 doses released from bleaching gels in contact with pulp cells led to intense cell death and impairment of proliferative capability.[7]

The data obtained in the present in vitro study should be considered with caution, because the toxicity observed in laboratory tests (in vitro studies) may be minimized in clinical conditions, since, in vital teeth, there is outward dentinal fluid movement as well as the presence of collagen and odontoblast cytoplasmic processes inside dentinal tubules. All these factors interfere with the inward displacement of components released from dental materials, such as bleaching agents.[19] In addition, the presence of extracellular matrix, anti-oxidant and lymphatic systems in pulp tissue protects pulp cells against external aggression.[34-36] Therefore, short treatments with low concentrations of H_2O_2 gels may be an interesting alternative to in-office tooth bleaching to minimize cell damage and improve pulp tissue healing capability. These less-invasive protocols may be safely recommended for the bleaching of small teeth (incisors), which would need less bleaching time than large teeth (canines and premolars) to achieve the desired esthetic outcome; however, more studies are necessary to verify this hypothesis in vivo.

5. Conclusions

According to the results found in the present investigation, a bleaching gel with 17.5% H_2O_2 applied to enamel for 45, 15 or 5 min promotes immediate over-expression of inflammatory

mediators by pulp cells, which is directly related to treatment time. Despite the fact that pulp cells maintain their odontoblastic phenotype up to 21 days after bleaching, the upregulation of odontoblastic markers is also related to the contact time of the product with enamel. Therefore, short-time tooth-bleaching procedures using low concentrations of gels appear to be an interesting alternative to prevent or minimize pulp cell damage.

6. **Acknowledgements**

7. References

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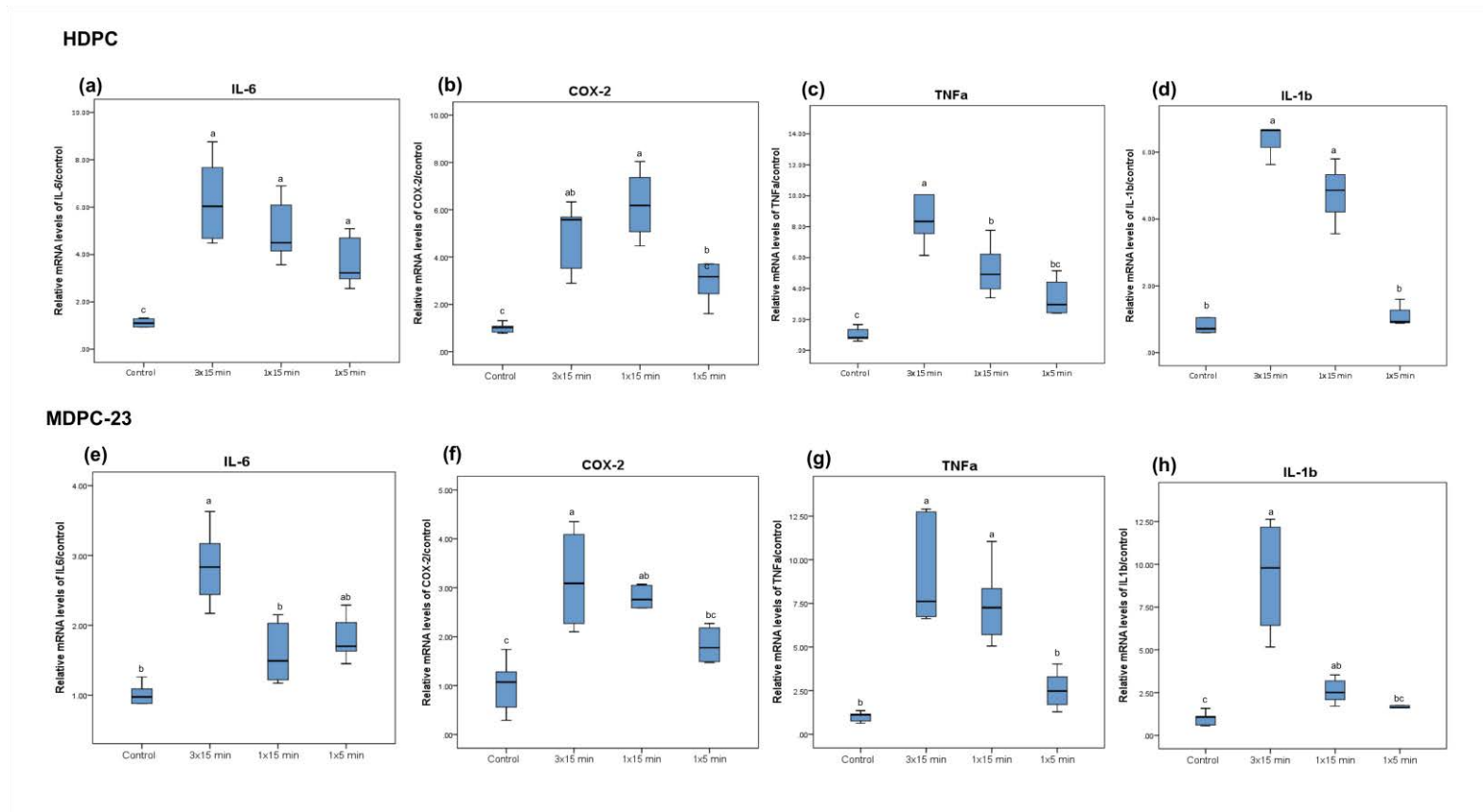
FIGURES

Fig. 1 – Box-whisker plot of inflammatory mediators for HDPC (a-d) and MDPC-23 (e-f). Vertical axis represents relative gene expression normalised by the negative control group, and horizontal axis represents the experimental groups. Upper and lower limits of boxes represent, respectively, percentiles 25 and 75, and the horizontal line represents the median. Upper and lower lines indicate the maximum and minimum values, respectively. Different letters indicate a statistically significant difference among groups (Mann-Whitney, $p > 0.05$).
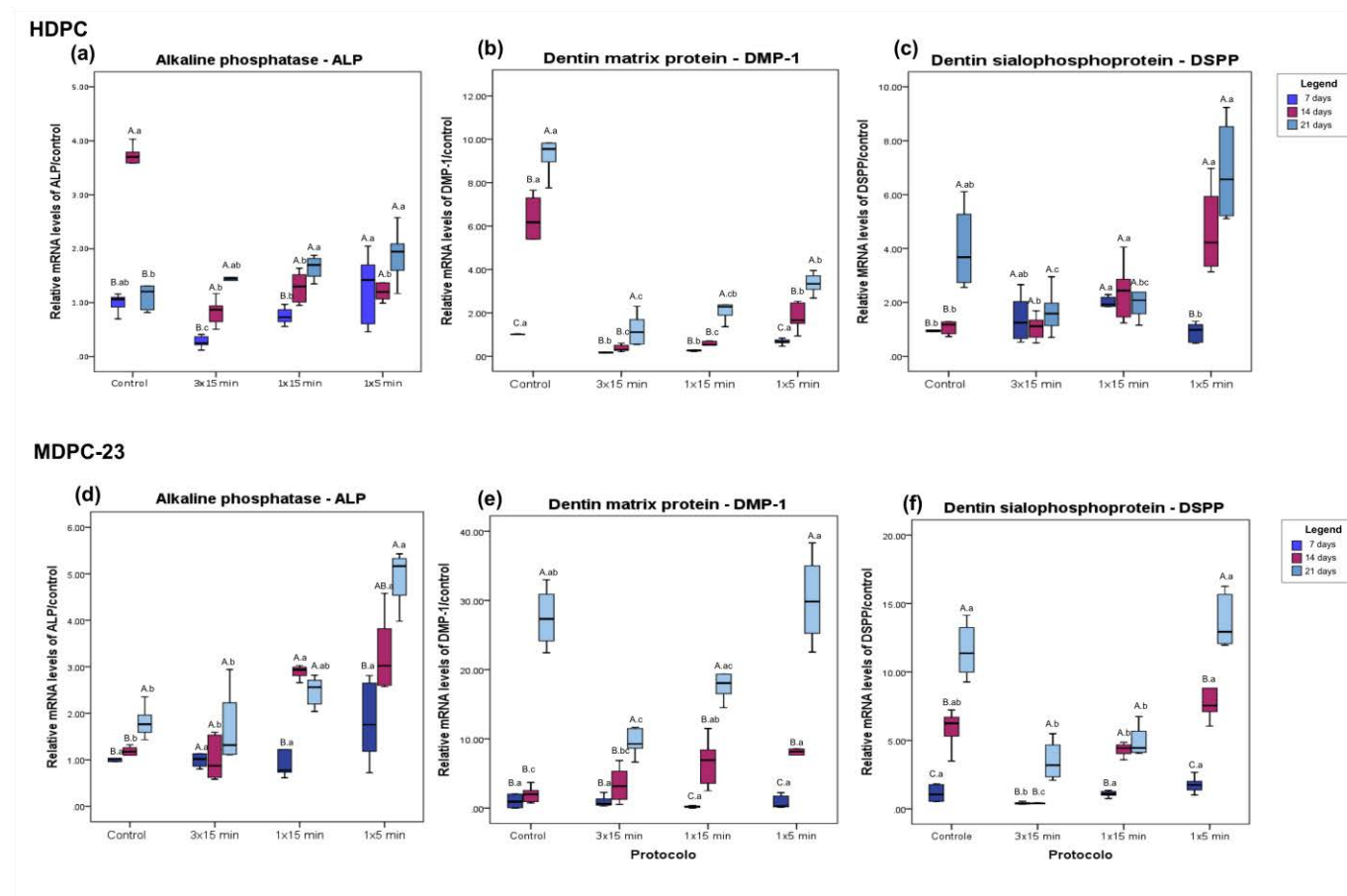
Fig. 2 – Box-whisker plot of odontoblastic mediators for HDPC (a-d) and MDPC-23 (e-f). Vertical axis represents the percentage of cell viability, and horizontal axis represents the experimental groups. Upper and lower limits of boxes represent, respectively, percentiles 25 and 75, and the horizontal line represents the median. Upper and lower lines indicate the maximum and minimum values, respectively. Lower-case letters allow for comparisons within the groups at each time-point; upper-case letters allow for comparisons within the time-points for each group. Different letters indicate a statistically significant difference among groups (Mann-Whitney, $p > 0.05$).
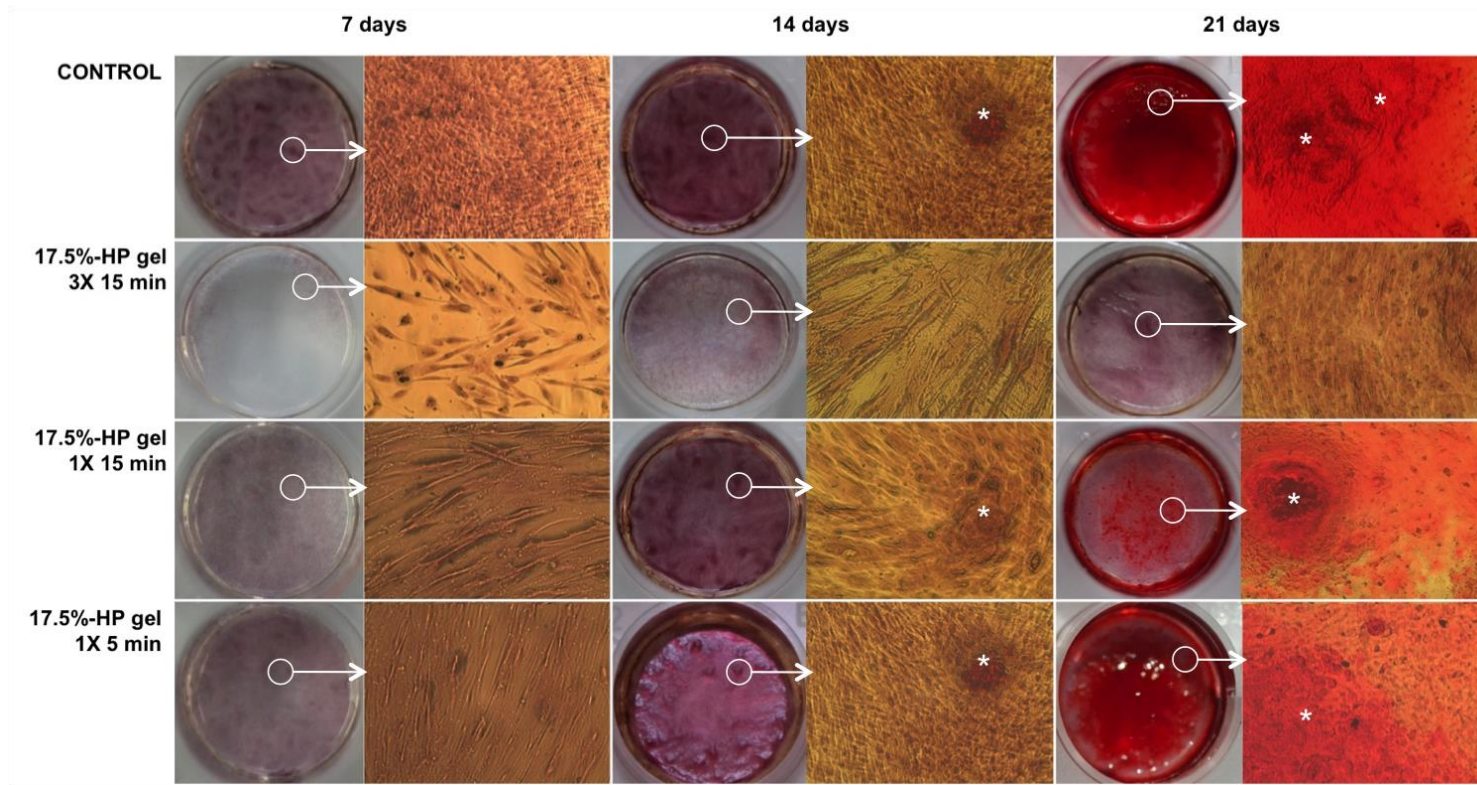
Fig. 3 – Panel of mineralised nodule deposition through periods of analysis for HDPCs. For each group and period, the left image represents a digital photograph of the well, and at right is a light-microscopic (20x) image from the delimited area of the well. The mineralised nodule is demarcated with asterisks (*). Absence of nodule deposition was observed in all groups at 7 days. Some nodules were observed on the control group and the 15- and 5-minute groups at 14 days. At the 21-day period, the control and 5-minute groups presented an intense deposition of mineralised nodules, and increased numbers of nodules were observed on the 15-minute group. Absence of nodule deposition was observed for the 45-minute group at all periods of analysis.
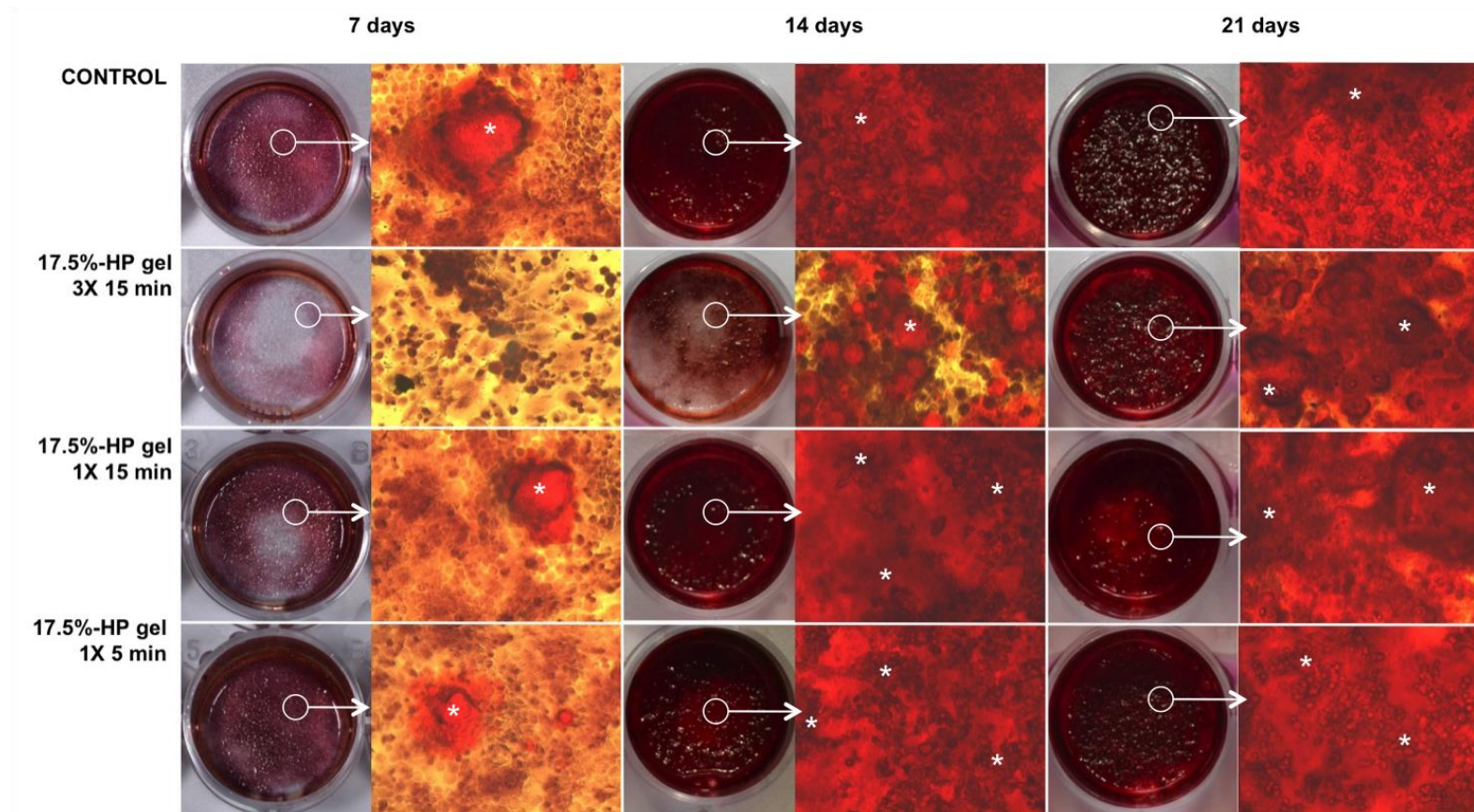
Fig. 4 – Panel of mineralised nodule deposition through periods of analysis for MDPC-23. For each group and period, the left image represents a digital photograph of the well, and at right is a light microscopic (20x) image from the delimited area of the well. The mineralised nodule is demarcated with asterisks (*). A peak of mineralised nodule deposition was observed at 14 days for the control group and for the 15- and 5-minute groups. The 45-minute group showed no mineralisation nodule deposition at 7 days, and a reduced number at 14 days; however, this group reached a pattern similar to that of the negative control at 21 days. The other bleached groups showed mineralised nodules from the 7-day period**.**

Table 1 – Assay ID and primer sequences for each selected gene.

System	Species	Gene	Identification
TaqMan Assay ID	Human	DMP-1	Hs01009391_g1
	Human	DSPP	Hs00171962_m1
	Human	ALP	Hs01029144_m1
	Human	IL-1β	Hs01555410_m1
	Human	IL-6	Hs00985639_m1
	Human	TNF-α	Hs00174128_m1
	Human	COX-2	Hs00153133_m1
	Human	β-actin	4333762T
	Rat	DMP-1	Rn01450122_m1
	Rat	DSPP	Rn02132391_s1
Syber Green Sequences	Rat	ALP	_Forward:5'-GCTGATCATTCCCACGTTTT-3'_ _Reverse:5'-CTGGGCCTGGTAGTTGTTGT-3'_
	Rat	IL-1β	_Forward:5'-AAAGCCTCGTCGTGTCGG -3'_ _Reverse:5'-CCTTTGAGGCCCAAGGGC-3'_
	Rat	IL-6	_Forward:5'-GAGGATACCACTCCCAACAGACC-3'_ _Reverse:5'-AAGTGCATCATCGTTGTTCATACA-3'_
	Rat	TNF-α	_Forward:5'-CCCTCCTGGCCAACGGCA-3'_ _Reverse:5'-TCGGGGCAGCCTTGTCCC-3'_
	Rat	COX-2	_Forward:5'-ACCCTGCCTACGAAGGAACT-3'_ _Reverse:5'-ACCACGGTTTTGACATGGGT-3'_
	Rat	β-actin	_Forward:5'-GGACCTGACGGACTACCTCATG-3'_ _Reverse:5'-TCTTTGATGTCACGCACGATTT-3'_

Legend: DMP-1, dentin matrix phosphoprotein 1; DSPP, dentin phosphoprotein; ALP, alkaline phosphatase; IL-1β, interleukin 1 beta; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; β-actin, beta actin; and COX-2, cyclooxygenase 2.

**Table 2 –** ALP activity (U/mg) and Alizarin Red staining (%) for HDPCs.

	Groups	Periods of Analysis		
		7 days	14 days	21 days
ALP (U/mg)	Control	0.08 (0.07-0.09) [b A]	0.17 (0.16-0.19)[a A]	0.13 (0.11-0.14) [a A]
	45-minute	0.03 (0.03-0.04) [b A]	0.13 (0.12-0.14) [a A]	0.16 (0.15-0.18) [a A]
	15-minute	0.10 (0.10-0.12) [b A]	0.13 (0.11-0.17) [a A]	0.18 (0.12-0.24) [a A]
	5-minute	0.07 (0.06-0.07)[b A]	0.15 (0.14-0.18) [a A]	0.16 (0.16-0.16) [a A]
Alizarin stain (%)	Control	100.4 (100.0- 100.7) [c A]	392.5 (382.7-414.6)[b A]	557.1 (557.0-557.9) [a A]
	45-minute	9.7 (9.1-11.2) [b C]	54.5 (51.0-57.1) [a C]	71.0 (70.4-71.1) [a B]
	15-minute	40.7 (37.9-44.5) [c B]	77.3 (70.8-87.2) [b C]	427.7 (427.7-512.2) [a A]
	5-minute	57.6 (54.9-62.7) [b B]	120.5 (104.3-133.2) [b B]	570.9 (554.5-574.9) [a A]

Numbers are medians (percentile 25-percentile 75), n = 6; lower-case letters allow for comparisons in rows and upper-case letters comparisons in columns. Comparisons are possible for each cellular product. Groups identified by the same letter do not differ statistically significantly (Mann-Whitney, p > 0.05).

Table 3 – ALP activity (U/mg) and Alizarin Red staining (%) for MDPC-23.

	Groups	Periods of Analysis		
		7 days	14 days	21 days
ALP (U/mg)	Control	3.28 (3.17-3.64) [a A]	1.15 (1.03-1.44) [b C]	1.46 (1.45-1.88) [b C]
	45-minute	1.62 (1.62-2.09) [b C]	1.68 (1.62-2.15) [b BC]	3.03 (3.02-3.97) [a B]
	15-minute	2.22 (2.21-2.34) [b CB]	2.34 (2.33-2.43) [b A]	3.46 (3.38-3.69) [a B]
	5-minute	2.57 (2.57-2.81) [b AB]	2.28 (2.20-2.55) [b AB]	5.57 (5.39-5.97) [a A]
Alizarin stain (%)	Control	100.0 (100.0-100.1) [c A]	735.1 (706.5-735.8) [b A]	1246.3 (1212.6-1246.8) [a A]
	45-minute	51.6 (50.9-51.8) [c C]	142.1 (142.0-148.9) [b B]	1163.1 (1161.3-1182.8) [a B]
	15-minute	77.2 (76.3-77.4) [c BC]	755.3 (755.1-757.3) [b A]	1160.8 (1145.4-1161.4) [a B]
	5-minute	95.5 (95.4-95.9) [c AB]	710.4 (710.3-726.3) [b A]	1198.4 (1195.9-1198.8) [a AB]

Numbers are medians (percentile 25-percentile 75), n = 6; lower-case letters allow for comparisons in rows and upper-case letters comparisons in columns. Comparisons are possible for each cellular product. Groups identified by the same letter do not differ statistically significantly (Mann-Whitney, $p > 0.05$).
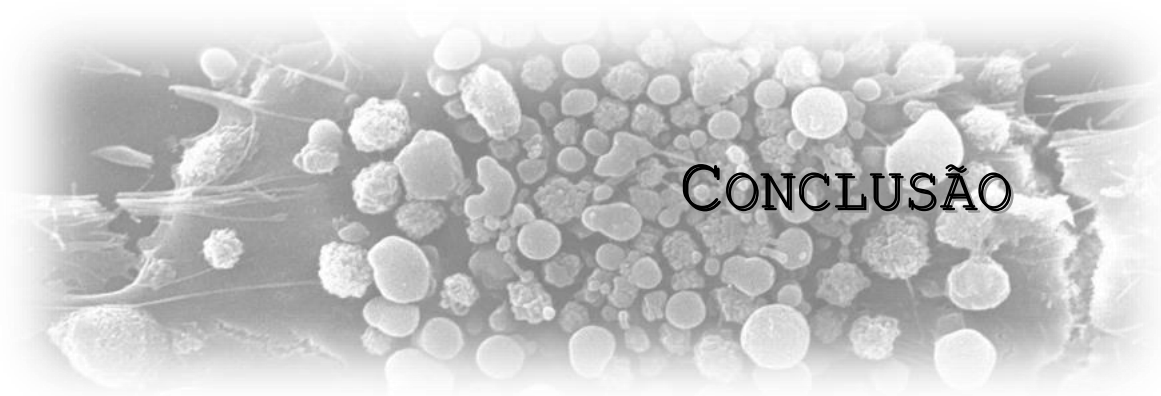
CONCLUSÃO

5 Conclusão

- O protocolo de clareamento de consultório tradicional (35% H_2O_2; 3x 15 minutos) promove intensa alteração de cor da estrutura dental após uma única sessão, causando rápida saturação dos cromóforos presentes nos tecidos. Porém, a difusão de H_2O_2 associada a este protocolo gera intensa redução na viabilidade das células pulpares, caracterizada por indução de estresse oxidativo com conseqüente lesão à membrana citoplasmática e morte celular por necrose;

- No clareamento de consultório, empregando géis com elevadas concentrações de PC, apesar de reduzir a difusão de H_2O_2 pela estrutura dental, este procedimento não se apresentou como uma boa alternativa clínica. Isto se deve, particularmente, a necessidade de se empregar longos períodos de tratamento em uma mesma sessão para se obter alteração de cor significativa da estrutura dental;

- A redução no tempo de contato com o esmalte e/ou concentração de H_2O_2 no gel clareador, são alternativas interessantes para o clareamento dental de consultório devido ao fato destes protocolos promoverem alteração de cor gradual associada à redução na difusão de H_2O_2 pela estrutura dental. Como conseqüência, uma importante minimização no efeito tóxico é observado. No entanto, quanto menor a concentração de H_2O_2 no gel clareador e seu tempo de contato com o esmalte, maior o número de sessões clínicas necessárias para que a alteração de cor seja significativa;

- As células pulpares expostas aos protocolos experimentais utilizando gel contendo 17,5% de H_2O_2 mantiveram suas características fenotípicas. No entanto, a regulação dos marcadores de diferenciação foi proporcional ao tempo de contato do produto com a superfície do esmalte, parâmetro este que também esteve associado à indução na expressão gênica de mediadores inflamatórios. Desta forma, o uso de géis clareadores com baixas concentrações de H_2O_2, aplicados por um curto período de tempo sobre o esmalte, caracteriza uma interessante alternativa para ser testada em estudos clínicos para realização do clareamento de consultório.

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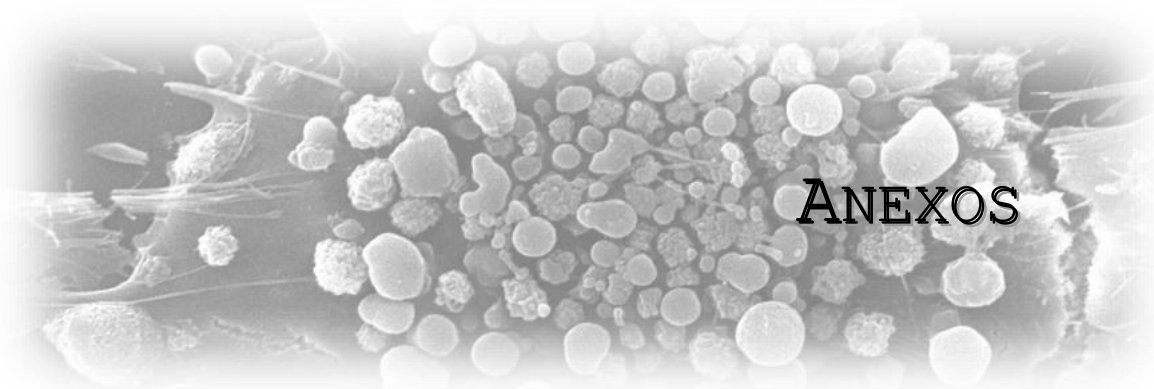
ANEXOS

ANEXO 1 - CERTIFICADO DO COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE ESTADUAL PAULISTA " JÚLIO DE MESQUITA FILHO"

FACULDADE DE ODONTOLOGIA DE ARARAQUARA

Comitê de Ética em Pesquisa

Certificado

Certificamos que o projeto de pesquisa intitulado *"NOVOS PARÂMETROS PARA O CLAREAMENTO DENTAL: AVALIAÇÃO DA EFICÁCIA, CITOTOXICIDADE E EFEITOS MOLECULARES"* sob o protocolo nº 13/11, de responsabilidade do Pesquisador (a) *CARLOS ALBERTO DE SOUZA COSTA* está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa–FOAr, com validade de 03 (três) ano, quando será avaliado o relatório final da pesquisa.

Certify that the research project titled *"NEW PARAMETERS FOR TOOTH BLEACHING: AN EVALUATION OF EFFICACY, AND CYTOTOXICITY MOLECULAR EFFECTS"*, protocol number 13/11, under *Dr CARLOS ALBERTO DE SOUZA COSTA* responsability, is under the terms of Conselho Nacional de Saúde/MS resolution # 196/96, published on May 10, 1996. This research has been approved by Research Ethic Committee, FOAr-UNESP. Approval is granted for 03 (three) years when the final review of this study will occur.

Araraquara, 13 de junho de 2011.

MMSNagle

Prof Dr Maurício Meirelles Nagle
Coordenador

ANEXO 2 – ARTIGO PUBLICADO REFERENTE AO CAPÍTULO 1

Soares DG, Basso FG, Pontes EC, Garcia LD, Hebling J, de Souza Costa CA. Effective tooth-bleaching protocols capable of reducing H2O2 diffusion through enamel and dentine. J Dent. 2014; 42: 351-8. doi: 10.1016/j.jdent.2013.09.001. [Epub ahead of print].

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Effective tooth-bleaching protocols capable of reducing H_2O_2 diffusion through enamel and dentine

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ARTICLE INFO

ABSTRACT

Objectives: To evaluate the effects of experimental protocols on bleaching effectiveness and hydrogen peroxide (HP) diffusion through enamel and dentine.
Methods: Enamel/dentine discs were subjected to six bleaching sessions, consisting of 1 or 3 applications of 17.5% or 35%-HP gel for 5/15 min, or 37% carbamide peroxide (CP) gel for 10/20 min. Discs undergoing the regular protocol (35%-HP; 3 × 15 min) constituted the positive control group. Colour change (ΔE) was assessed (CIE *L**a*b* system) after each session. HP diffusion was quantified (sessions 1, 3, and 6) in enamel/dentine discs adapted to artificial pulp chambers. Data were analysed by Pillai's Trace and Bonferroni test, or by one-way ANOVA and SNK/Tamhane's test (α = 5%).
Results: All tooth-bleaching protocols significantly increased the ΔE values. A reduction in HP diffusion and no significant difference in ΔE compared with the positive control were observed for the following bleaching protocols: 17.5%-HP 3 × 15 min, at the 4th session; and 35%-HP 1 × 15 and 3 × 5 min, at the 5th session. HP diffusion in the 37%-CP 3 × 20 min bleaching protocol was statistically similar to that in the positive control. The other experimental bleaching protocols significantly decreased HP diffusion through enamel/dentine discs, but the ΔE values were statistically lower than those observed in the positive control, in all sessions.
Conclusion: Shortening the contact time of a 35%-HP gel or reducing its concentration produces gradual tooth colour change and reduced HP diffusion through enamel and dentine.
Clinical significance: A reduction in HP concentration, from 35% to 17.5%, in a bleaching gel or shortening its application time on enamel provides a significant tooth-bleaching improvement associated with decreased HP diffusion across hard dental tissues. Therefore, these protocols may be an interesting alternative to be tested in the clinical situation.

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ANEXO 3 - ARTIGO PUBLICADO REFERENTE AO CAPÍTULO 2

Soares DG, Basso FG, Hebling J, de Souza Costa CA. Concentrations of and application protocols for hydrogen peroxide bleaching gels: Effects on pulp cell viability and whitening efficacy. J Dent. 2014;42:185-98. doi: 10.1016/j.jdent.2013.10.021

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Concentrations of and application protocols for hydrogen peroxide bleaching gels: Effects on pulp cell viability and whitening efficacy

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ARTICLE INFO

ABSTRACT

Objectives: To assess the whitening effectiveness and the trans-enamel/trans-dentinal toxicity of experimental tooth-bleaching protocols on pulp cells.

Methods: Enamel/dentine discs individually adapted to trans-well devices were placed on cultured odontoblast-like cells (MDPC-23) or human dental pulp cells (HDPCs). The following groups were formed: G1 – no treatment (control); G2 to G4 – 35% H_2O_2, 3×15, 1×15, and 1×5 min, respectively; and G5 to G7 – 17.5% H_2O_2, 3×15, 1×15, and 1×5 min, respectively. Cell viability and morphology were evaluated immediately after bleaching (T1) and 72 h thereafter (T2). Oxidative stress and cell membrane damage were also assessed (T1). The amount of H_2O_2 in culture medium was quantified (Mann–Whitney; $\alpha = 5\%$) and colour change (ΔE) of enamel was analysed after 3 sessions (Tukey's test; $\alpha = 5\%$).

Results: Cell viability reduction, H_2O_2 diffusion, cell morphology alteration, oxidative stress, and cell membrane damage occurred in a concentration-/time-dependent fashion. The cell viability reduction was significant in all groups for HDPCs and only for G2, G3, and G5 in MDPC-23 cells compared with G1. Significant cell viability and morphology recovery were observed in all groups at T2, except for G2 in HDPCs. The highest ΔE value was found in G2. However, all groups presented significant ΔE increases compared with G1.

Conclusion: Shortening the contact time of a 35%-H_2O_2 gel for 5 min, or reducing its concentration to 17.5% and applying it for 45, 15, or 5 min produce gradual tooth colour change associated with reduced trans-enamel and trans-dentinal cytotoxicity to pulp cells.

Clinical significance: The experimental protocols tested in the present study provided significant tooth-bleaching improvement associated with decreased toxicity to pulp cells, which may be an interesting alternative to be tested in clinical situations intended to reduce tooth sensitivity and pulp damage.

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ANEXO 4 - ARTIGO EM REVISÃO REFERENTE AO CAPÍTULO 3

Soares DG, Basso FG, Hebling J, de Souza Costa CA. Effects of professional tooth bleaching strategies on odontoblastic phenotype and gene expression of inflammatory mediators. J Dent. 2014. (in review). JJOD-D-14-00106.

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Abstract: Objectives: This study aimed to assess the phenotype characteristics and inflammatory mediator expression by pulp cells subjected to less aggressive in-office bleaching therapies.
Methods: A 17.5% hydrogen peroxide (H_2O_2) gel was applied for 45, 15 or 5 minutes to enamel/dentin discs adapted to trans-wells positioned over cultured odontoblast-like cells (MDPC-23) or human dental pulp cells (HDPCs). In the negative control group, no treatment was performed. Alkaline phosphatase activity (ALP, thymolphthalein assay), mineralised nodule deposition (MND, Alizarin Red) and mRNA gene expression (real-time PCR) of odontoblast markers (DMP-1, DSPP and ALP) were assessed at 7, 14 or 21 days post-bleaching. Immediately after the bleaching procedures, mRNA gene expression of inflammatory mediators (TNFα, IL-1β, IL-6 and COX-2) was also evaluated (real-time PCR) (Mann-Whitney; α = 5%).
Results: Gene expression of inflammatory mediators was up-regulated in all bleached groups, being directly related to the treatment time. In all bleached groups, the cells presented ALP, DMP-1 and DSPP gene expression throughout the periods of analysis. However, the alteration in ALP activity and reduced MND that occurred in all bleached groups were also directly related to the bleaching time. HDPCs exposed to a 45-minute bleaching treatment presented no deposition of mineralised nodules. The 5- and 15-minute groups showed significant cell recovery capability with time.
Conclusions: Long-term bleaching procedures with a 17.5%-H_2O_2 gel upregulate inflammatory mediator expression and cause alterations of odontoblast marker expression and mineralised matrix deposition by pulp cells. However, cells subjected to short-term bleaching are capable of recovering with time.

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