UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

Francine Benetti

DISSERTAÇÃO

ANÁLISE HISTOLÓGICA E IMUNOISTOQUÍMICA DO TECIDO PULPAR DE RATOS WISTAR SUBMETIDOS A PROCEDIMENTO CLAREADOR COM DUAS CONCENTRAÇÕES DE PERÓXIDO DE HIDROGÊNIO

ARAÇATUBA-SP

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Orientador: Prof. Dr. Luciano Tavares Angelo Cintra

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Análise histológica e imunoistoquímica do tecido pulpar de ratos Wistar submetidos a procedimento clareador com duas concentrações de peróxido de hidrogênio.

Resumo

Estudos demonstram danos causados à polpa dentária, e sua capacidade de reparo tecidual, após procedimento de clareação dentária. Este estudo investigou o processo de necrose, o nível de proliferação celular (imunomarcação com PCNA) e apoptose (Caspase-3-clivada), e a resposta imune inflamatória (IL-17, IL-6 e CD5) no tecido pulpar após este procedimento. Foram utilizados dois protocolos de clareação dentária com peróxido de hidrogênio (H_2O_2) nos molares superiores de 40 ratos Wistar, formando os grupos Controle (gel placebo), BLUE (H_2O_2 a 20% 1x50min) e MAXX (H_2O_2 a 35%, 3x15min). Após 2 e 30 dias, os ratos foram eutanasiados e as maxilas processadas para avaliação histológica (H.E.) e imunoistoquímica. Foram realizados os testes estatísticos referentes a cada caso, considerando $p<0,05$. Aos dois dias, foi observada necrose no terço oclusal do grupo MAXX, e infiltrado inflamatório moderado no BLUE ($p<0,05$). Aos 30 dias, não houve infiltrado inflamatório nos grupos, e grande área da câmara pulpar foi ocupada por dentina terciária. A marcação para PCNA aos 2 dias, demonstrou maior nível de proliferação celular no terço médio do grupo BLUE ($p<0,05$) e no terço cervical do MAXX ($p<0,05$), com redução significativa aos 30 dias no terço cervical ($p<0,05$). Caspase-3-clivada foi presente em todos os grupos, apresentando maior nível de apoptose nos grupos clareados quando comparados com o controle, nos dois períodos de análise ($p<0,05$); quando comparados os grupos aos 2 e 30 dias, foi observada redução significativa do nível de apoptose apenas no grupo BLUE ($p<0,05$). Células CD5 positivas foram presentes em todos os grupos, em ambos períodos de análise; aos dois dias, o grupo BLUE apresentou maior imunomarcação para CD5 no terço oclusal quando comparado aos demais grupos ($p<0,05$); nos demais terços, ambos grupos clareados apresentaram maior imunomarcação quando comparados com o grupo controle ($p<0,05$); não foi observada diferença significativa na comparação de cada grupo aos 2 e 30 dias ($p>0,05$). O grupo MAXX apresentou maior imunomarcação para IL-17 aos 2 dias, quando

comparado aos demais grupos ($p<0,05$); em 30 dias não foi observada diferença significativa entre os grupos ($p>0,05$). Houve maior imunomarcação para IL-6 nos grupos clareados aos 2 dias, quando comparados com o controle ($p<0,05$); aos 30 dias, não foi observada diferença significativa entre os grupos ($p>0,05$). Conclui-se que o H_2O_2 35% gera necrose em aéreas mais superficiais do tecido pulpar e infiltrado inflamatório moderado em áreas subjacentes, e que o H_2O_2 20% ocasiona infiltrado inflamatório moderado, proliferação celular e apoptose. Ainda, que o processo inflamatório é acompanhado do recrutamento e ativação de células CD5 positivas, e que IL-17 e IL-6 participam deste processo. Assim, deve-se optar por géis clareadores com concentrações mais baixas de H_2O_2 para o uso clínico.

Palavras-Chave: Clareamento dental, pulpite, peróxido de hidrogênio.

Histological and immunohistochemical analysis of the pulp tissue of Wistar rats submitted to dental bleaching with two hydrogen peroxide concentrations.

Abstract

Studies have shown damage to tooth pulp, and your capacity of tissue repair, after dental bleaching. This study investigated the necrosis process, the level of cell proliferation (immunolabeling with PCNA) and apoptosis (Caspase-3, cleaved), and inflammatory response (IL-17, IL-6 and CD5) generated in the pulp tissue after this procedure. Wistar rats were divided into Control (placebo gel), BLUE (20% H_2O_2,1x50min), and MAXX (35% H_2O_2, 3x15min) groups. At 2 and 30 days, the rats were killed (n=10).The jaws were removed and processed for histology analysis (H&E) and immunohistochemistry. Statistical tests were performed for each case, considering $p<0.05$. At two days, necrosis was observed on the occlusal third MAXX group and moderate inflammatory infiltrate in BLUE ($p<0.05$). At 30 days, there was no inflammatory infiltrate in groups, and large area of the pulp chamber was occupied by tertiary dentin. The immunolabeling for PCNA, at two days, was more expressive in the middle third of the BLUE group ($p<0.05$) and the cervical third of the MAXX ($p<0.05$), with significant reduction to 30 days in the cervical third ($p<0.05$). Caspase-3-cleaved was present in all groups, showing a higher level of apoptosis in the bleached groups compared with the control in both analysis periods ($p<0.05$); comparing the groups at 2 and 30 days, significant reduction in the level of apoptosis was observed only in BLUE group ($p<0.05$). CD5 positive cells were found in all groups in both analysis periods; at two days, the BLUE group had higher immunolabeling for CD5 in the occlusal third when compared to the other groups ($p<0.05$); in other thirds, both groups bleached showed most immunolabeling compared with the control group ($p<0.05$); there was no significant difference in the comparison of each group at 2 and 30 days ($p>0.05$). The MAXX group presented higher immunolabeling for IL-17 at two days when compared to the other groups ($p<0.05$); at 30 days there was no significant difference between groups ($p>0.05$). There was a larger immunolabeling for IL-6 in the bleached groups at 2 days when compared with control ($p<0.05$); at 30 days, there was no significant difference between groups

($p>0.05$). It concludes that the 35% H_2O_2 generates necrosis in the superficial air of the pulp tissue and moderate inflammatory infiltrate in surrounding areas, and that 20% H_2O_2 causes moderate inflammatory infiltrate, cell proliferation and apoptosis. Furthermore, the inflammatory process is accompanied by recruitment and activation of CD5 positive cells, and that IL-17 and IL-6 participate in this process. Thus, we must choose bleaching agents at lower concentrations of H_2O_2 for clinical use.

Keywords: Dental bleaching, pulpits, hydrogen peroxide.

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I. *Introdução*

I. INTRODUÇÃO

O peróxido de hidrogênio (H_2O_2) é amplamente utilizado em diferentes concentrações como agente clareador. Embora seja eficaz como clareador, seu efeito oxidativo tem sido estudado como possível responsável por alterações morfológicas (Hegedus *et al.* 1999) e histoquímicas (Rotstein *et al.* 1996, Fattibene *et al.* 2005, Bistey *et al.* 2007) na estrutura dos tecidos dentários.

Seu baixo peso molecular faz com que tenha difusão rápida pelos prismas do esmalte e espaços interprismáticos (Park *et al.* 2004), tendo a capacidade de atingir a polpa, através dos túbulos dentinários, causando diminuição da celularidade, do metabolismo (Seale *et al.* 1981, Min *et al.* 2008, Cintra *et al.* 2013, Soares *et al.* 2014), e da capacidade reparadora do tecido pulpar (Goldberg & Smith 2004), podendo levá-lo à necrose (Costa *et al.* 2010, Cintra *et al.* 2013). Estas podem ser possíveis causas da sensibilidade dentária gerada após tratamento clareador (Lu *et al.* 2001, Kugel *et al.* 2006).

O H_2O_2 sofre dissociação nas condições de uso clínico, dando origem às espécies reativas de oxigênio (EROs), responsáveis pelo processo de clareação (Korytowski & Sarna 1990). O mecanismo clareador é explicado como sendo uma reação de oxidação, onde a substância a ser clareada doa elétrons ao agente clareador (Goldstein *et al.* 1994). Quanto maior a concentração e o tempo de exposição do tecido dentário ao produto clareador, maior é a liberação das EROs, e seus efeitos sobre os tecidos (Benetti *et al.* 2004, Tredwin *et al.* 2006, Buchalla & Attin 2007, Costa *et al.* 2010, Cintra *et al.* 2015, Cintra *et al.* 2015a).

Cada tipo de lesão ou irritação sobre a polpa terá diferentes efeitos, que são caracterizados por inflamação aguda, crônica ou necrose. A inflamação se

desenvolve como um mecanismo de defesa biológica em resposta a qualquer tipo de injúria (Bagis *et al.* 2007, Yu *et al.* 2007).

Em um primeiro estudo (Processo Fapesp nº 2011/13709-2) investigamos os efeitos do número de sessões clareadoras sobre o tecido pulpar, e observamos a presença de necrose na região de cornos pulpares no grupo de apenas uma sessão (Cintra *et al.* 2013). Esses resultados foram semelhantes ao encontrado no trabalho de Costa *et al.* 2010, realizado em incisivos inferiores humanos. Assim, inferimos que resultados encontrados em molares de ratos podem ser utilizados para prever resultados em incisivos inferiores humanos, os mais afetados pelo gel clareador devido à menor espessura de esmalte e dentina (Costa *et al.* 2010).

A facilidade de padronização e controle, e a possibilidade de realizar outros testes, nos fez caracterizar o modelo experimental animal (Processo Fapesp nº 2011/13709-2/renovação da bolsa de IC). Assim, tornou-se possível estudar diferentes variáveis, para que possamos em um segundo momento, com resultados já padronizados e avaliados em animais, propor a validação desses resultados em humanos, com grupos mais reduzidos, seguindo os princípios éticos. Neste estudo, avaliamos a influência do tempo de aplicação e da concentração do agente clareador sobre a polpa dentária dos ratos, utilizando H_2O_2 20% e H_2O_2 35%, por 5, 10, 15, 30 e 45 minutos. Após 2 dias, encontramos um aumento gradual de infiltrado inflamatório, variando de leve a ausente nos grupos de 5 e 10 min, de ambas as concentrações, à necrose no grupo de 45 min com H_2O_2 35%. Aos 30 dias, observamos que os danos causados ao tecido pulpar foram reparados, porém houve redução da área da câmara pulpar ocupada por dentina terciária (Cintra *et al.* 2015).

Avaliamos ainda a penetração destas concentrações de H_2O_2 e a alteração de cor em blocos de dentes bovinos e câmaras pulpares artificiais, e o mesmo protocolo de clareação em molares de ratos, para a análise histológica. Os grupos que receberam H_2O_2 35% apresentaram maior penetração do agente clareador, e maiores danos causados a polpa, quando comparados com os grupos que receberam H_2O_2 20%. No entanto, não houve diferença entre os grupos quanto à alteração de cor, indicando que o H_2O_2 20% pode ser uma alternativa menos prejudicial à polpa, sem prejudicar o resultado estético final (Cintra *et al.* 2015a).

Assim, após estes estudos sobre a citotoxicidade dos agentes clareadores em função do número de sessões, da concentração e do tempo de ação do agente clareador, algumas novas dúvidas surgiram sobre os mecanismos celulares e moleculares envolvidos com a resposta inflamatória da polpa após procedimento clareador. Para esclarecer estas dúvidas, lançamos mão da técnica de imunoistoquímica, utilizando marcadores que representam os fenômenos que envolvem uma resposta tecidual.

A apoptose é a morte celular programada mediada via mitocondrial, processo ativado por uma variedade de estímulos estressantes (Cappello *et al.* 2002, Mak *et al.* 2008, Bellmann *et al.* 2010, Tuan 2011). Esta morte celular é elemento fundamental no mecanismo de homeostase do tecido. Durante a apoptose, as células mantêm a integridade da membrana plasmática, o que ajuda a prevenir a inflamação (Munoz-Pinedo 2012).

As caspases são proteases de cisteínas responsáveis por iniciar e promover a sinalização apoptótica. A caspase-9, quando induzida por um estresse, ativa outras caspases, como pró-caspase-3 em Caspase-3-clivada,

que cliva moléculas de reparo do DNA, levando ao processo de apoptose (Li *et al.* 1997). Vários fatores estressantes podem acelerar a clivagem da pró-caspase-3 e ativar a apoptose (Samali *et al.* 1999, Xanthoudakis *et al.* 1999, Chandra *et al.* 2007). O H_2O_2 funciona como um desses fatores (Wu *et al.* 2013).

Por outro lado, o H_2O_2 também pode induzir a necrose, impedindo a ativação da caspase, ou inibindo sua atividade (Davies 1999, Saito *et al.* 2006). Dessa maneira, é importante que se realizem estudos de aplicação *in vivo* com o intuito de determinar se durante um processo de clareação dentária ocorre a apoptose das células pulpares, ou processo de necrose, para que se compreenda melhor os danos causados à polpa após clareação dentária.

Uma vez que a atividade inflamatória depende do equilíbrio entre a proliferação e a morte celular, pode-se verificar a taxa de crescimento celular em meio a um processo inflamatório. O Antígeno Nuclear de Proliferação Celular (PCNA) pode ser utilizado como um indicador desse processo (Suzuki *et al.* 2005, Cappello *et al.* 2006), por funcionar como coordenador central na via que conduz à síntese de DNA e proliferação celular (Takasaki *et al.* 1981, Celis *et al.* 1984). Possui forma de anel que desliza sobre o DNA para o recrutamento de outras proteínas de replicação e reparação deste (Kubota *et al.* 2013). Assim, são úteis para fornecer informações sobre diferenciação, proliferação e prognóstico (Coltrera *et al.* 1992, McCormick & Hall 1992, Huang *et al.* 1994). Sua imunomarcação em polpas dentárias poderá nos dar informações sobre o nível de proliferação celular após procedimento clareador, durante o processo de reparo.

A polpa dentária é composta por vários tipos de células incluindo fibroblastos, odontoblastos, células mesenquimais indiferenciadas que estão em meio com a matriz extracelular (Abrahão *et al.* 2006), vasos sanguíneos e nervos, macrófagos, linfócitos e outros componentes celulares menores (Ten Cate 1988). Segundo Okiji *et al.* 1992, a polpa dentária normal contém uma variedade de células imunocompetentes.

As citocinas, secretadas por leucócitos e outras células, podem atuar como moduladores da resposta inflamatória (Ashida *et al.* 2011). Essas citocinas podem ser divididas em anti-inflamatórias, e pró-inflamatórias, onde estão incluídas IL-17 e IL-6 (Dong 2008, ElSalhy *et al.* 2013).

A IL-17 é produzida por células T-helper-17 (Th17) (Xiong *et al.* 2015), e age induzindo outras citocinas pró-inflamatórias, quimiocinas, e neutrófilos (Chang & Dong 2009, Tong *et al.* 2014). Estudos mostram que a IL-17 pode estimular a produção de células T (Chauhan *et al.* 2011, Chen *et al.* 2011). Sua presença foi associada à exacerbação da resposta inflamatória (Xiong *et al.* 2009) e aumento da expressão de IL-6 e IL-8 em fibroblastos da polpa dentária humana (HDPFs) (Xiong *et al.* 2015).

Já a IL-6 atua promovendo o recrutamento de neutrófilos e intensificando o edema inflamatório pelo aumento da permeabilidade vascular, sendo que este efeito já foi demonstrado na câmara pulpar (ElSalhy *et al.* 2013, Xiong *et al.* 2015). Ainda, níveis elevados de IL-6 estão relacionados com a piora do processo inflamatório e dos sintomas clínicos (Prso *et al.* 2007).

Estudos anteriores que quantificaram a imunomarcação de IL-6 em polpa saudável e inflamada de dentes humanos, diagnosticaram níveis significantes desta interleucina na polpa dentária inflamada quando comparada

ao tecido saudável (Barkhodar *et al.* 1999). Segundo Balto *et al.* 2001, a produção de IL-6 pode exercer um efeito benéfico ou deletério, estando dependente da quantidade produzida e do tempo de sua ação.

Por sua vez, o linfócito, é uma célula de defesa responsável pela resposta específica (Luisi *et al.* 2004). A homeostase de linfócitos depende do balanço entre proliferação e morte celular. A ruptura deste equilíbrio pode fazer com que ocorra a progressão da enfermidade (Gillet *et al.* 2007).

O CD5 é um receptor presente em todos os timócitos ou linfócitos T maduros, e em células B, sendo um importante regulador da ativação destas células (Lozano *et al.* 2000, Brown & Lacey 2010, de Wit *et al.* 2011). Durante uma interação entre células T e apresentadoras de antígeno, o CD5 forma parte da sinapse imunológica (Brossard *et al.* 2003, Gimferrer *et al.* 2003, de Wit *et al.* 2011). Acredita-se que a verificação da proliferação do marcador CD5 em células de polpas de dentes que sofreram clareação dentária poderá nos ajudar no entendimento da resposta imunológica, indicando a presença de linfócitos.

Assim, com o objetivo de contribuir com os dados já obtidos nos estudos anteriores e melhorar o entendimento dos efeitos dos agentes clareadores sobre a polpa dentária, este estudo avaliou a influência da concentração do agente clareador sobre: a resposta tecidual inflamatória; a presença de necrose;e os níveis de proliferação celular e apoptose na polpa, através quantificação de PCNA e Caspase-3-ativada, respectivamente. Ainda, o recrutamento de linfócitos e o comportamento assumido por IL-17 e IL-6 durante o processo de reação pulpar após clareação dentária.

II. *Artigo 1*

Journal of Endodontics

Hydrogen peroxide induces cell proliferation and apoptosis in pulp after dental bleaching in vivo.

Abstract

Inflammatory events in different degrees occur in the pulp tissue after dental bleaching with hydrogen peroxide (H_2O_2). The tissue reaction depends on the balance between cell proliferation and death. This study evaluated *in vivo* the inflammatory response, levels of cell proliferation and apoptosis, and the presence of tissue necrosis after dental bleaching with two concentration of H_2O_2. Wistar rats were divided into Control (placebo gel), BLUE (20% H_2O_2, 1x50 min), and MAXX (35% H_2O_2, 3x15 min) groups. At 2 and 30 days, the rats were killed (n=10). The jaws were processed for histology analysis and immunohistochemistry of PCNA and Caspase-3-cleaved, and data were submitted to Mann-Whitney or ANOVA test (P<0.05). At 2 days, the MAXX group showed necrosis and the BLUE group moderate inflammation on the oclusal third of the crown (P<0.05). At 30 days, there was formation of tertiary dentin and absence of inflammatory infiltrate. The level of cell proliferation was higher in the middle third of the BLUE group (P<0.05), and cervical of MAXX at 2 days (P<0.05), with a decrease at 30 days. The apoptosis was present at 2 days, particularly in the cervical third of the crown in the bleached groups (P<0.05), with decrease at 30 days only in BLUE group (P<0.05). It can be concluded that higher concentration of H_2O_2 cause necrosis in pulp at 2 days and prolonged effect of the apoptotic process; and that lower concentrations of H_2O_2, provide moderate inflammatory infiltrate, cell proliferation and apoptosis, with reduction of these processes at 30 days.

Key-words: hydrogen peroxide, pulp tissue, tooth bleaching.

The H_2O_2 is widely used in different concentrations as bleaching agent. It acts through the releasing reactive oxygen species (ROS) that provides the change in surface roughness and hardness of the enamel [1], and in chemistry composition of enamel and dentin [2]. Moreover, it has the ability to reach the pulp through the dentinal tubules causing decreased of cellularity and cellular metabolism [3-6], DNA damage in cells [7], leading to apoptosis [8] or necrosis [5,9]. These can be possible causes of tooth sensitivity after bleaching treatment [10].

In previous study, the H_2O_2 caused necrosis in oclusal third of coronary pulp after one bleaching session [5]. The same was found in study with human tooth [9]. However, some *in vivo* [3] and *in vitro* studies [11] showed that after a certain period, the pulp tissue recovering your organization, or pulp cells, the viability values. Recent attention is being given to fact of small amount of ROS raise proliferation and cell differentiation [12]. It is believed that at low concentration, the H_2O_2 stimulates cells to proliferate and to perform functions [13], with role more complex in cell physiology, than just cause oxidative damages [12,14-16].

The Proliferation of Cell Nuclear Antigen (PCNA) is a sensitive marker detectable during the DNA synthesis or cell division [17]. This measurement information about cell proliferation [18], being give us information about repair process of pulp tissue after bleaching procedure.

The apoptosis is cell death mediated by mitochondrial [19], and is key element in tissue homeostatic mechanisms. Various stress factors can speed up cleavage of pro-caspase-3 in Caspase-3-cleaved (C3C) to apoptosis activation [20], as H_2O_2. Once that inflammatory activity depends of equilibrium

between proliferation and cell death, can check occurrence these two mechanisms to determine the prognosis of installed inflammation in pulp tissue after bleaching procedure with different H_2O_2 concentrations.

In vitro studies have shown that 50mM of H_2O_2 cause apoptosis in cell culture, and 500mM, necrosis [21], and low H_2O_2 dose facilitate of differentiation of odontoblastics [4]. However, there are no *in vivo* studies about the effects of bleaching procedure in pulp tissue regards the levels of cell proliferation and apoptosis. The study of the pulp tissue response would give important collaboration to understanding of mechanisms that involve pulp after this esthetics procedure. Thus, this study evaluated *in vivo* the inflammatory response, levels of cell proliferation and apoptosis, and the presence of tissue necrosis after dental bleaching with two concentration of H_2O_2. Our hypothesis is that different H_2O_2 concentrations will not promote different responses in the pulp tissue.

Materials and Methods

Were used 40 male Wistar albino rats (250g), housed in a temperature-controlled environment (22ºC±1ºC) on a standard light/dark schedule with access to food and water *ad libitum*. The experimental protocol was approved by the institutional ethics committee of UNESP-Univ. Estadual Paulista (São Paulo, Brazil) and conducted in accordance with relevant guidelines.

Tooth Bleaching

The rats were anesthetized by intramuscular injections of ketamine (80 mg/kg, Ketamina Agener 10%, União Química Farmacêutica Nacional S/A –

Embu-Guaçu, São Paulo, Brasil) and xylazine (10 mg/kg, Xilazin, Syntec do Brazil LTDA – Cotia, São Paulo, Brasil). The upper hemi-maxillae were randomly divided into (n=10): Control group (placebo gel); BLUE group, bleached with 20% H_2O_2 (Whiteness HP Blue®); and MAXX group, with 35% H_2O_2 (Whiteness HP Maxx®). The application of the bleaching gel (0.01 mL) was performed in the molars, as the manufacturer recommends (FGM Dental Products, Joinville, SC, Brazil): 1 application for 50 min to BLUE group; 3 followed applications of 15 min each to MAXX group.

Histology and immunohistochemical analysis

At 2 and 30 days (n=10), the animals were killed with an overdose of the Tiopental anesthetic (Thipentax, Cristália – Produtos Químicos Farmacêuticos Ltda – Itapira, Brasil). The hemi-maxillae were separated and fixed in a solution of 4% buffered formaldehyde for 24 hours. The tissues were then dehydrated, clarified and embedded in paraffin. Five-micron sections were cut in the vestibular-lingual plane and stained with hematoxylin-eosin, or submitted to immunohistochemistry by using an indirect immunoperoxidase technique [22] for PCNA (primary antibody mouse anti-PCNA, VP-P980, Vector Laboratories Inc., Burlingame, CA, EUA) and C3C (primary antibody rabbit anti-C3C, Asp175, CellSignaling Technology, Bervely, MA). The specimens were submitted to the previously described procedures suppressing the use of primary antibodies to negative control.

The analyses were performed by a single calibrated operator in a blinded manner, under light microscopy (400X, DM 4000 B; Leica, Wetzlar, Germany). The pulp chamber was divided into thirds (occlusal, middle and cervical) as was

the root pulp (cervical, middle and apical) [5]. The inflammation was scored considering the intensity and cell distribution in accordance with the approximate average number of inflammatory cells present in each third of the same specimen (Table 1).

Table 1 - Scores attributed to the intensity of inflammatory cell infiltration

Score	Pulpar tissue condition
1	Inflammatory cells absent or negligible in number
2	Mild inflammatory infiltrate (<25 cells per field)
3	Moderate inflammatory infiltrate (between 25 and 125 cells per field)
4	Severe inflammatory infiltrate (>125 cells per field)
5	Necrosis

The cell with brownish present in the nucleus, in the case of PCNA, and in cytoplasm, in the case of C3C, were counted and expressed in number of cells per mm^2. The area corresponding to the total volume, and each third of the pulp chamber and root canal, was performed by the software of Leica Qwin Plus (Leica®, Germany).

Statistical Analysis

Histological data were subjected to Mann-Whitney statistical test, and number of immunostained cells to Two Way ANOVA statistical test (P<0.05).

Results

Histological analysis at 2 days

Control Group

The specimens showed pulp tissue in homeostasis: continuous odontoblastics layers around the entire pulp, in continuity with pre-dentine and dentine layers; other layers organized; negligible number inflammatory cells; blood vessels and fiber density indicates repair potential (Figure 1. A,d). This group served as parameter for analysis of experimental groups.

BLUE group – 20% H_2O_2

Most specimens showed moderate inflammation in the occlusal third of coronal pulp; three specimens, severe inflammation, and one, necrosis, with mild or moderate inflammation in the middle third of the coronal pulp; most of the specimens showed mild inflammation in the cervical third of the crown; two specimens showed mild inflammation in the cervical third of the root (Figure 1. B,e).

MAXX group – 35% H_2O_2

In most specimens there was necrosis in the region of pulp horns; one showed necrosis in the middle third; two, severe inflammation; and five, moderate; most specimens showed mild inflammation in the cervical third of the coronal pulp; there was mild inflammatory infiltrate in the cervical third of the root pulp in three specimens (Figure 1. C;f).

Histological analysis at 30 days

The specimens of all groups showed no inflammatory infiltrate; the odontoblasts layer was organized around the entire pulp and the remaining layers with aspect of normality. However, the groups that received the bleaching agent had tertiary dentin occupying area of the pulp tissue, especially in the coronal pulp region (Figure 1. H,k; I,L), which was not observed in the control group (Figure 1. G,j).

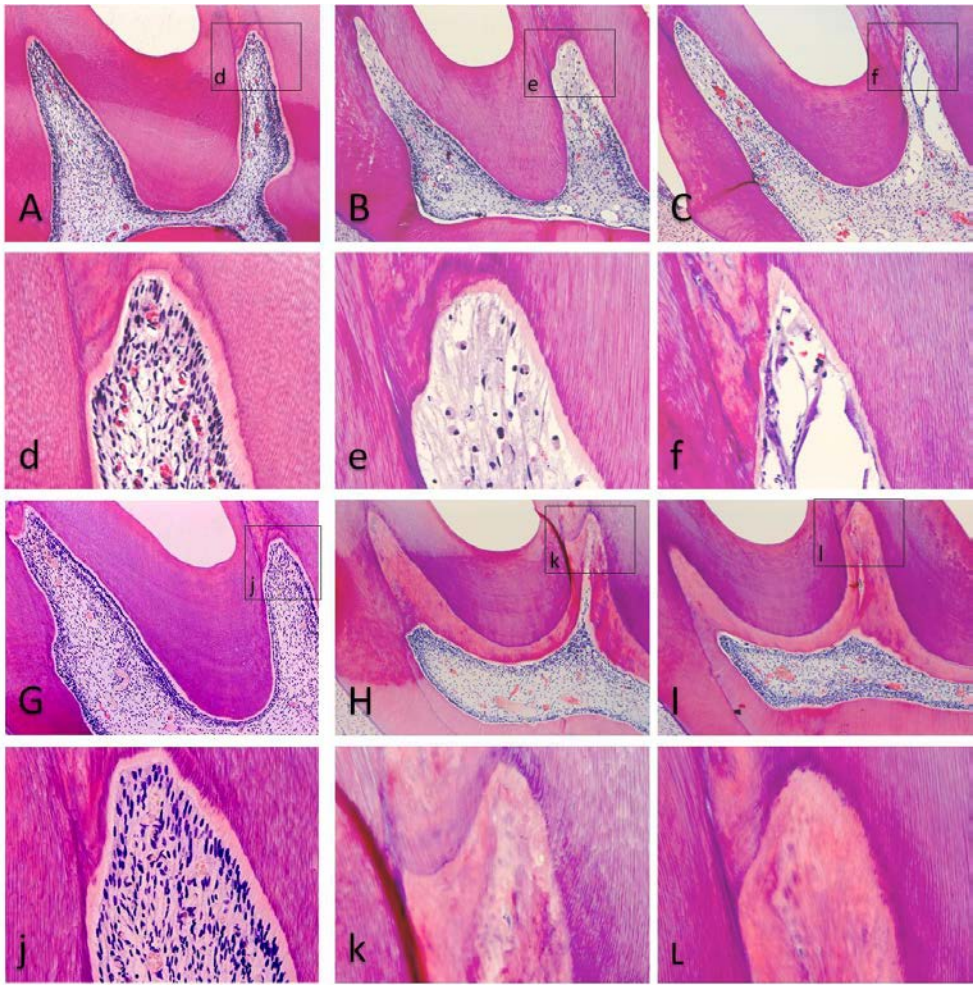

Figure 1. Photomicrographs of the specimens in the Control group (A,d; G,j); BLUE group (B,e;H,k); and the MAXX group (C,f; I,L). The images (A-f) represent the groups at 2 days after bleached procedure, and (G-L) at 30 days after bleached procedure. Staining with hematoxylin-eosin. [A-C,G-I: 100X; d-f,j-L: 400X].

Immunohistochemical analysis

The positive immunolabeling for PCNA and C3C was found in diffusely distributed cells over the pulp. The level of proliferation was higher in the middle third of the coronal pulp in BLUE group (Figure 2,F), and in the cervical third in MAXX group (Figure 2,K). There was a lower level of proliferation in root thirds (unexposed data) and at 30 days (Table 3). Higher level of apoptosis was found in bleached groups at 2 days, particularly in cervical third (Figure 2,N,O),and at 30 days, remained almost at the same level in MAXX group, and decreased considerably in BLUE group (Figure 2,P,Q), as well as in root thirds (unexposed data). At 30 days, the number of immunolabeling cells was performed only in the cervical third of the pulp chamber, because the occlusal and middle thirds of most specimens were occupied by tertiary dentin.

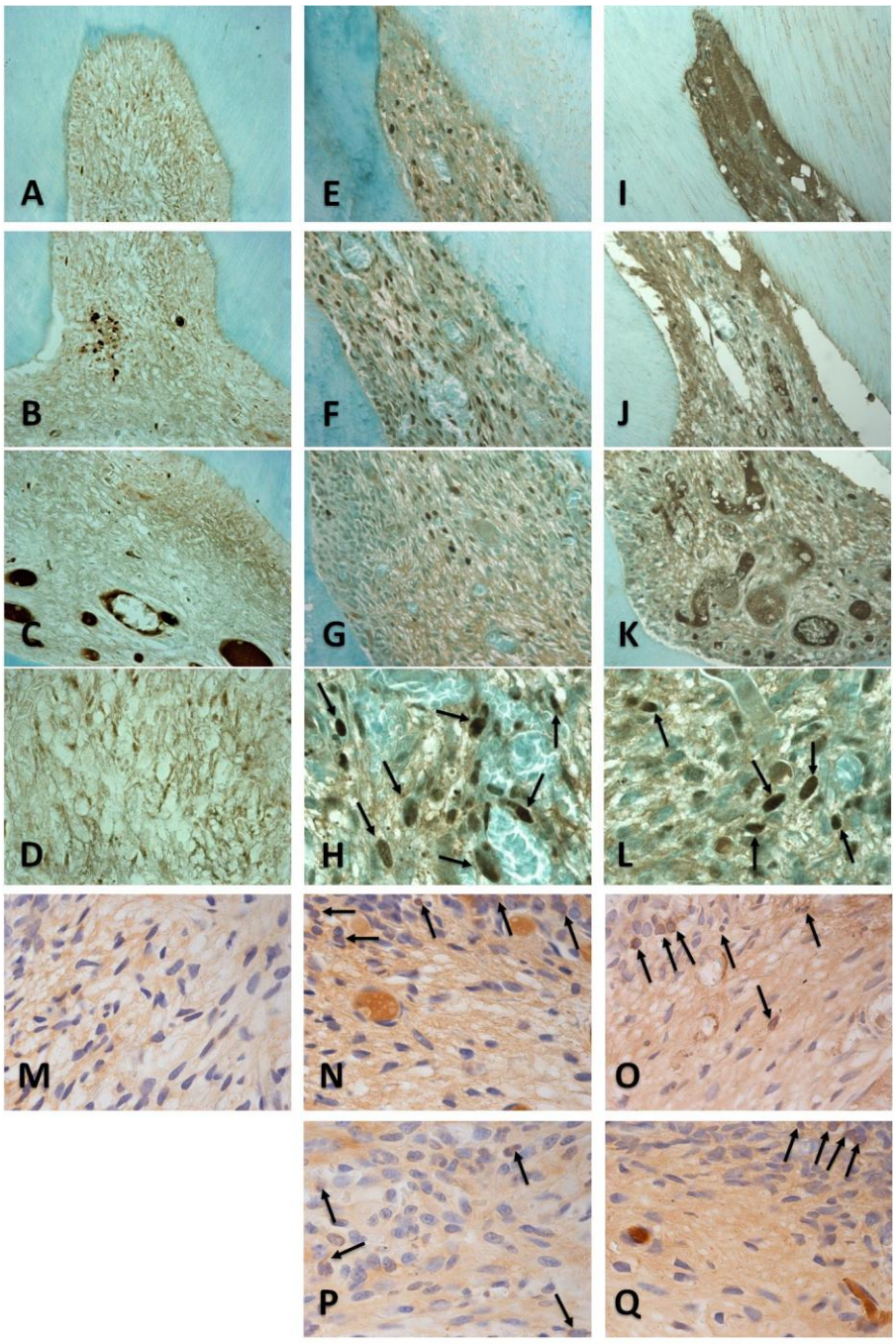

Figure 2. Photomicrographs of the specimens of the Control group (A-D,M); BLUE group (E-H,N,P); and the MAXX group (I-L,O,Q). The images (B,F,J) represent the oclusal third; (C,G,K), middle third; (D,H,L), cervical third. Images (A-L) represent labeling to PCNA; and (M-Q), to C3C. Images (A-O) represent groups at 2 days after bleached procedure, and (P,Q), at 30 days. The arrows point to the immunolabeling cells. [A-C;E-G;I-K: 400X; D,H,L;M-Q: 1000X].

Comparison among the Groups

At 2 day, the BLUE and MAXX groups showed significant difference in occlusal and middle thirds of the coronal pulp with more intense response in MAXX group (*P*<0.05, Table 2). In the cervical third there was no significant difference(*P*>0.05, Table 2). There were no inflammation in the middle and apical thirds of the root (unexposed data).

Table 2 - Scores Observed for Inflammatory Cell Response According to the Groups

Third		Score 2 days	Groups			P
			Control	BLUE	MAXX	
Coronal	**Occlusal**	1	10/10	0/10	0/10	
		2	0/10	1/10	0/10	Mann-Whitney
		3	0/10	5/10	1/10	BLUE X MAXX
		4	0/10	3/10	3/10	P=0,010
		5	0/10	1/10	6/10	
		Median*	1	3[a]	5[b]	
	Middle	1	10/10	2/10	0/10	
		2	0/10	5/10	2/10	Mann-Whitney
		3	0/10	3/10	5/10	BLUE X MAXX
		4	0/10	0/10	2/10	P=0,013
		5	0/10	0/10	1/10	
		Median*	1	2[a]	3[b]	
	Cervical	1	10/10	2/10	2/10	
		2	0/10	8/10	5/10	Mann-Whitney
		3	0/10	0/10	3/10	BLUE X MAXX
		4	0/10	0/10	0/10	P=0,304
		5	0/10	0/10	0/10	
		Median*	1	2[a]	2[a]	
Root	**Cervical**	1	10/10	8/10	7/10	
		2	0/10	2/10	3/10	Mann-Whitney
		3	0/10	0/10	0/10	BLUE X MAXX
		4	0/10	0/10	0/10	P=0,651
		5	0/10	0/10	0/10	
		Median*	1	1[a]	1[a]	

*Same letters on the line indicate no statistical difference among the groups (P>.05).

The BLUE group showed higher immunolabeling for PCNA in the occlusal third at two days (*P*>0.05); in the middle and cervical third where

significant difference among all groups, being higher in BLUE group in middle third (P<0.05), and higher in MAXX in cervical third (P<0.05). In coronary and middle root thirds, there was a significant difference when comparing the groups bleached with control, at two days. There was significant decrease of immunolabeling cells in cervical third at 30 days of bleached groups.

The immunolabeling for C3C showed significant difference between bleached and control groups, with higher immunolabeling in bleached groups. Except in the occlusal third to two days for MAXX and control group, due to decrease of cellularity in MAXX group. At 30 days, the immunolabeling of cervical third of the BLUE group showed a significant decrease to found at 2 days (P<0.05); this was not observed in MAXX group (P>0.05).

Table 3 – Mean of count immunolabeling cells to PCNA and C3C in groups, expressed in number of cells per mm^2.

Time	Third	PCNA			Caspase-3-cleaved		
		Control	BLUE	MAXX	Control	BLUE	MAXX
2 days	**Occlusal**	$1,5^{a}$	$121,5^{b}$	$26,2^{a}$	$3,8^{a}$	$20,8^{b}$	$6,9^{a}$
	Middle	$2,1^{a}$	$172,5^{b}$	$65,0^{c}$	$6,3^{a}$	$20,4^{b}$	$27,5^{b}$
	Cervical	0^{aA}	$113,0^{bA}$	$167,0^{cA}$	$9,0^{aA}$	$40,5^{bA}$	$37,5^{bA}$
30 days	**Cervical**	3^{aA}	$20,7^{aB}$	$7,1^{aB}$	$6,5^{aA}$	$24,4^{bB}$	$45,9^{cA}$

*Different small letters represent statistical difference in lines; different uppercase letters represent statistical difference between 2 and 30 days of the cervical third (P>.05).

Discussion

This study evaluated the *in vivo* effects of two concentrations of bleaching agents in the inflammatory response, levels of cell proliferation and apoptosis, and the presence of tissue necrosis in pulp tissue after the bleaching procedure. It was observed that the concentration of the bleaching agent influences the pulpal tissue response, rejecting the null hypothesis.

The *in vivo* model has important characteristics to study the reactions of the pulp tissue against aggressors, due to cytoplasm extensions of odontoblast and dentin fluid, acting as a physical barrier to the penetration of substances [23]. In addition, there are H_2O_2 degrading enzymes, minimizing damage to pulp tissue [24].

Studies in human tooth [9], in animals tooth [3,5,25], and cell culture [26,27], shows that pulp tissue of bleached tooth exhibits intense change, as severe inflammation or necrosis, in first days after the bleaching procedure, as we observed in this study. Other studies show that the concentration of the bleaching gel can influence in the capacity to penetrate of H_2O_2 in the pulp chamber [28]. In the present study, we found that with the use of 35% H_2O_2, 60% of the specimens showed necrosis in the occlusal third, while only 10% of the specimens that received bleaching gel with 20% H_2O_2 showed necrosis, and 50%, moderated inflammatory infiltrate.

These changes were shown to be reversible after a certain period, where the pulp tissue is restructured, and shows the formation of tertiary dentin. Other studies also show the reversal of such damages [3].

We observe immunolabeling cells for PCNA in the pulp tissue, indicating proliferation of these. Most immunolabeling was observed in regions where probably there was lower penetration of ROS. These data corroborate studies showing that low oxidizing agents concentrations stimulate the growth and cell division [4,13], which explains the proliferation observed in the pulp tissue after the bleaching procedure.

The PCNA labeling was present after topical application of carbamide peroxide or hydrogen in the oral mucosa of rats [29,30], where there was

hyperplasia and hyperkeratosis [29]. The proliferative effect of low doses of H_2O_2 was reported in odontoblasts, such as induction of dentin formation [4]. Also increased the expression of osteogenic markers in human dental pulp cells [15]. ROS generated by the low power laser promoted wound healing [31], and the differentiation of pre-odontoblastics cells of rats, increases the amount of tertiary dentin after 12 weeks of treatment and increase of H_2O_2 in cells [32].

Already at higher concentrations, the cells simply disintegrate or become necrotic due to disruption of the cell membrane [13]. The same we see when we analyze histologically the occlusal third of the coronal pulp, especially in specimens of MAXX group. At 30 days, cells were immunolabeling for PCNA not observed, indicating absence of cell proliferation process.

We reveal at two days increase of immunolabeling cells to C3C in bleached specimens, except in the occlusal third of MAXX group, due to the presence of necrosis in this region. Studies show that moderate concentrations of H_2O_2 lead the cells to apoptosis, and high concentrations, lead directly to the necrotic process [13,21]. In this study, the apoptotic cells were found in greater amounts in the most distant regions of the bleaching gel, with likely lower penetration of H_2O_2; in the most superficial areas of pulp tissue, there was the direct process of necrosis. This can be confirmed by comparing the effects of two concentrations of bleaching agents on the pulp tissue, where the occlusal third showed necrosis MAXX group, possibly the highest concentration of H_2O_2, and higher amounts of immunolabeling cells to C3C in the group BLUE. Both mechanisms of cell death occur independently of each other, and dependent on contact time with H_2O_2, apoptosis can occur in the presence of necrotic cells [21], as well as occurred in this study.

Apoptosis is necessary to odontoblasts maintain an appropriate dentin deposition rate [33] and leads to formation of mature dental pulp when exposed to extraneous stimuli [34]. This may indicate the presence of immunolabeling for C3C is related to the aging of the pulp tissue.

We note that the MAXX group showed no significant difference when comparing the cervical thirds of the coronal pulp at 2 and 30 days, indicating that the highest concentration of H_2O_2 was able to keep apoptotic effect on pulp cells, even after long time. It may be that the pulp tissue is further completed by tertiary dentine due to extended effect of bleaching gel.

This study contributes to the understanding of H_2O_2 the effects of bleaching gel on the pulp tissue, demonstrating *in vivo* the presence of cell proliferation and apoptosis when the use of 20% H_2O_2, and areas of necrosis in the use of 35% H_2O_2. Moreover, even after a certain period of time, there cells in apoptotic state in the group with the greatest concentration of H_2O_2, even with the organization of the pulp tissue. This gradual increase in the level of apoptosis in 35% H_2O_2 group indicates prolonged impairment of tissue viability.

These facts show that bleaching gels with higher concentration of H_2O_2 should be used with caution since, apart from necrosis, maintains prolonged level of apoptosis. Perhaps, 20% H_2O_2 is the limit concentration for clinical use. Other in vivo studies are needed to elucidate the understanding of the mechanisms pulp after dental bleaching procedure.

Conclusion

It can be concluded that most superficial area of the dental pulp of group that receives 35% H_2O_2 shows necrosis at 2 days and prolonged effect of the

apoptotic process; and that 20% H_2O_2 shows moderate inflammatory infiltrate, cell proliferation and apoptosis at 2 days, with reduction of these processes at 30 days. According to the histological findings, it is preferable for clinical use bleaching gels with low concentrations of H_2O_2.

Acknowledgments

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III. *Artigo 2*

International Endodontic Journal

Concentration-dependent effect of bleaching agents on the expression of interleukin-17, interleukin-6, and CD5 positive cells in the pulp of Wistar rats.

Abstract

Aim: To evaluate *in vivo* leukocyte recruitment and activation, and behavior of the IL-17 and IL-6 pro-inflammatory cytokines in the pulp tissue after tooth bleaching with two concentrations of hydrogen peroxide (H_2O_2).

Methodology: Molars Wistar rats were treated with: 20% H_2O_2 (BLUE group); 35% H_2O_2 (MAXX group); or placebo gel (Control group). It was made histological and immunohistochemical analysis of IL-17, IL-6 and CD5 expression, at 2 and 30 days. The data were subjected to Mann-Whitney, Two Way ANOVA, Kruskal-Wallis and Dunn statistical test ($P<0.05$).

Results: The BLUE group showed moderate inflammatory infiltrate in the regions of pulp horns, and the MAXX groups, necrosis ($P<0.05$). At 30 days, the pulp recovered your organization and there was great formation of tertiary dentin. Low immunolabeling standard to IL-17 was present in BLUE and Control groups, and moderate standard in MAXX at 2 days ($P<0.05$), reducing at 30 days ($P>0.05$). The bleached groups had moderate immunolabeling standard to IL-6 at 2 days ($P<0.05$); there was no significant difference between the groups at 30 days ($P>0.05$). CD5 positive cells was present at 2 and 30 days in greater quantity in bleached groups ($P<0.05$), with no significant difference to each group between the experimental periods ($P>0.05$).

Conclusion: IL-17 and IL-6 participates in the inflammatory process generated in the pulp of Wistar rats after dental bleaching, and the immunolabeling is greater with increasing H_2O_2 concentration. This process is accompanied by the prolonged recruitment and activation of CD positive cells.

Key-words: hydrogen peroxide, pulp tissue, tooth bleaching, IL-17, IL-6, CD5.

The H_2O_2 bleaching gel dissociates the clinical conditions, giving rise to reactive oxygen species (ROS), responsible for the bleaching process (Korytowski & Sarna 1990). The ROS are unstable and react with other substances to achieve molecular stability (Kawamoto & Tsujimoto 2004).

Studies have reported diffusion of H_2O_2in enamel and dentin (Benetti *et al.* 2004, Trindade *et al.* 2009) reaching the dentin-pulp complex and react with the pulp cells (Costa *et al.* 2010, Cintra *et al.*2013). This would explain the fact that most of the patients who submit to this treatment have reported tooth sensitivity (Haywood *et al.* 1994, Charakorn *et al.* 2009).

In our previous study, we observed that the more bleaching sessions are carried out, the greater the damage found in the pulp tissue (Cintra *et al.* 2013). It is also known that the time that the bleaching agent remains on the surface of enamel, the H_2O_2 concentration (Benetti *et al.* 2004, Buchalla & Attin 2007, Costa *et al.* 2010), the use of light (Baik *et al.* 2001), and the thickness of enamel and dentin (Camargo *et al.* 2007, Costa *et al.* 2010), can influence the penetration of ROS in the pulp chamber.

The pulp condition is very dependent on the state of dentin and of enamel, and any injury to these structures can affect it, and will respond by defensive reactions (Smith *et al.*1995). The cytokines, secreted by leukocytes and other cells, are generally excellent markers of inflammation in acting as modulators of inflammatory responses (Ashida *et al.* 2011). They can be divided into inflammatory and anti-inflammatory, where the first group includes IL-17 and IL-6 (Dong 2008, ElSalhy *et al.* 2013).

The IL-17 produced by T-helper-17 cells (Th17) (Xiong *et al.* 2015), acting on the inflammatory response to infection by inducing the expression of

other proinflammatory cytokines such as IL-6, chemokines (Chang & Dong 2009) and the neutrophil recruitment (Tong *et al.* 2014). Studies have shown that IL-17 can stimulate the production of T cells (Chauhan *et al.* 2011, Chen *et al.* 2011). The presence of IL-17 is associated with an exacerbation of inflammatory response (Xiong *et al.* 2009) and an increase of IL-6 and IL-8 expression in fibroblasts of human dental pulp (HDPFs) (Xiong *et al.* 2015). Considering that IL-17 has potent effects on numerous cells, it is likely to stimulate pulp cells to secrete other cytokines after dental bleaching procedure, and may mediate the inflammatory process.

The IL-6 generation front to traumas and infections (Schindler *et al.* 1990) can promote the attraction of neutrophils and increase vascular permeability in the pulp chamber (ElSalhy *et al.* 2013, Xiong *et al.* 2015). Consequently generates increased inflammatory edema (ElSalhy *et al.* 2013). These actions can contribute to inflammation of the pulp tissue, and is involved in acute inflammation (Azuma *et al.* 2014). Thus generates initial tissue destruction for chemotaxis of immune cells that assist in the repair process, would prevent the establishment of chronic condition (ElSalhy *et al.* 2013).

The CD5 is a receptor present in mature T lymphocytes, and B cells. It is an important regulator of activation of these cells (de Wit *et al.* 2011). The lymphocyte is a defense cell responsible for specific response, and the balance between proliferation and cell death are responsible for the homeostasis these cells. Disruption of this balance may cause the occurrence of disease progression (Gillet *et al.* 2007).

Already inflammatory varying degrees events were demonstrated in pulp tissue teeth submitted to bleaching procedure (Seale *et al.* 1981, Frigo *et al.*

2009, Costa *et al.* 2010, Cintra *et al.* 2013, Ferreira *et al.* 2013). However, no one knows for sure the consequences of this inflammatory process in the pulp tissue. Thus, this study investigated *in vivo* the leukocyte recruitment and activation, and behavior of the IL-17 and IL-6 pro-inflammatory cytokines in the pulp tissue after dental bleaching with two concentrations of H_2O_2. These immunolabeling can give us information if the inflammation generated in the pulp tissue of dental bleached tend to process of chronic inflammation or repair. We hypothesized that IL-17 and IL-6 has a higher standard immunolabeling, as well as the higher amount of CD positive cells, in the presence of higher H_2O_2 concentration. The presence of these markers in the inflammatory process generated after dental bleaching, could indicate them as potential targets for further therapeutic agents.

Materials and Methods

Were used 40 male Wistar albino rats (250g), housed in a temperature-controlled environment (22ºC±1ºC) on a standard light/dark schedule with access to food and water *ad libitum*. The experimental protocol was approved by the institutional ethics committee of UNESP-Univ. Estadual Paulista (São Paulo, Brazil) and conducted in accordance with relevant guidelines.

Tooth Bleaching

The rats were anesthetized by intramuscular injections of ketamine (80 mg/kg, Ketamina Agener 10%, União Química Farmacêutica Nacional S/A – Embu-Guaçu, São Paulo, Brasil) and xylazine (10 mg/kg, Xilazin, Syntec do Brazil LTDA – Cotia, São Paulo, Brasil). Randomly, the upper molars (Cintra *et*

al. 2013) were divided in groups (n=10): BLUE group, which received bleaching gel with 20% H_2O_2 (Whiteness HP Blue; FGM Dental Products, Joinville, SC, Brazil); MAXX group, which received bleaching gel with 35% H_2O_2 (Whiteness HP Maxx; FGM Dental Products, Joinville, SC, Brazil); and Control group, which received placebo gel (thickener of the bleaching gel). The bleaching gels (0.01mL) were applied as the manufacturer recommends: 1 application of 50 min to BLUE group, and 3 following applications of 15 min each to MAXX group. The placebo gel was maintained by 50 min.

Histology and immunohistochemical analysis

At 2 and 30 days (n=10), the animals were killed with an overdose of the Tiopental anesthetic (Thipentax, Cristália – Produtos Químicos Farmacêuticos Ltda – Itapira, Brasil), and the hemi-maxillae were separated and fixed in a solution of 4% buffered formaldehyde for 24 hours. The tissues were then dehydrated, clarified and embedded in paraffin. Five-micron sections were cut in the vestibular-lingual plane and stained with hematoxylin-eosin, or submitted to immunohistochemistry by using an indirect immunoperoxidase technique (Garcia *et al.* 2013) for IL-17 (primary antibody rabbit,SC-7927, Santa Cruz Biotechnology, CA, USA), IL-6 (primary antibody rabbit, SC-1265, Santa Cruz Biotechnology, CA, USA) and CD5 (primary antibody goat, SC-6986, Santa Cruz Biotechnology, CA, USA).The specimens were submitted to the previously described procedures suppressing the use of primary antibodies to negative control.

The analyses were performed by a single calibrated operator in a blinded manner, under light microscopy (400X, DM 4000 B; Leica, Wetzlar, Germany).

The pulp chamber was divided into thirds (occlusal, middle and cervical) (Cintra *et al.* 2013). The inflammation was scored considering the intensity and cell distribution in accordance with the approximate average number of inflammatory cells present in each third of the same specimen. The scores were: 1, Inflammatory cells absent or negligible in number; 2, Mild inflammatory infiltrate (<25 cells per field); 3, Moderate inflammatory infiltrate (between 25 and 125 cells per field); 4, Severe inflammatory infiltrate (>125 cells per field); and 5, tissue necrosis (adapted from Cintra *et al.* 2013).

The immunochemical to IL-17 and IL-6 received the scores: 0, immunolabeling missing; 1, low standard of immunolabeling; 2, moderate standard of immunolabeling; 3, severe standard of immunolabeling; 4, very severe standard of immunolabeling. To analysis of CD5, the cell with brownish present in the cytoplasm were counted and expressed in number of cells per mm^2.

The area corresponding of the total volume, and each third of the pulp chamber, at 2 and 30 days, was performed by the software of Leica Qwin Plus (Leica®, Germany).

Histological data were subjected to Mann-Whitney statistical test, scores immunolabeling to Kruskal-Wallis and Dunn, and number of immunolabeling CD5 cells to Two Way ANOVA statistical test ($P<0.05$). The values of the area of the pulp chamber at 2 and 30 days were subjected to One Way ANOVA statistical test ($P<0.05$).

Results

Presence of Inflammatory Cells - at 2 days

The Control group exhibited intact pulp tissue, with odontoblastics layer intact, cellular organization and absence of inflammatory cells (Figure 1; A,d). The BLUE group showed cellular disorganization especially in pulp horn region with decreased cellularity; most specimens showed moderate inflammatory infiltrate in this region, whereas in the middle and cervical third mild inflammatory infiltrate was observed (Figure 1; B,e). The MAXX group there was necrosis in the occlusal third in most specimens, moderate in the middle third, and mild in the cervical third (Figure 2; C,f). The bleached groups showed significant difference in occlusal and middle thirds of the coronal pulp (Table 1).

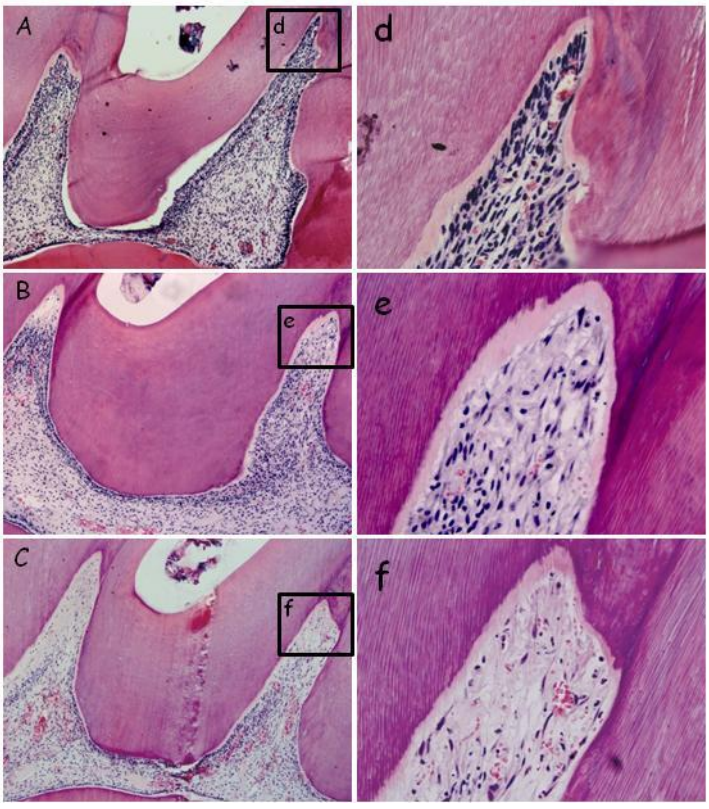

Figure 1. H&E staining. Photomicrographs of the Control group (A, d) show organized pulp tissue and odontoblastic intact layer; BLUE group (B, e) presents cellular disorganization and severe inflammatory infiltrate in the occlusal third; and MAXX group (C, f) shows areas of necrosis and a decrease in cellularity occlusal third. [A-C: 100X; a-c: 400X].

Presence of Inflammatory Cells and tertiary dentin - at 30 days

All specimens showed cellular organization and absence of inflammatory infiltrate (Figure 2). There was large formation of tertiary dentin, which occupied a large part of pulp, especially in pulp chamber area. The analysis of the tertiary dentin was evaluated by measuring the area corresponding to the pulp chamber of each group using Qwin Leica Plus (Leica® type, Germany) and exposed in Table 1.

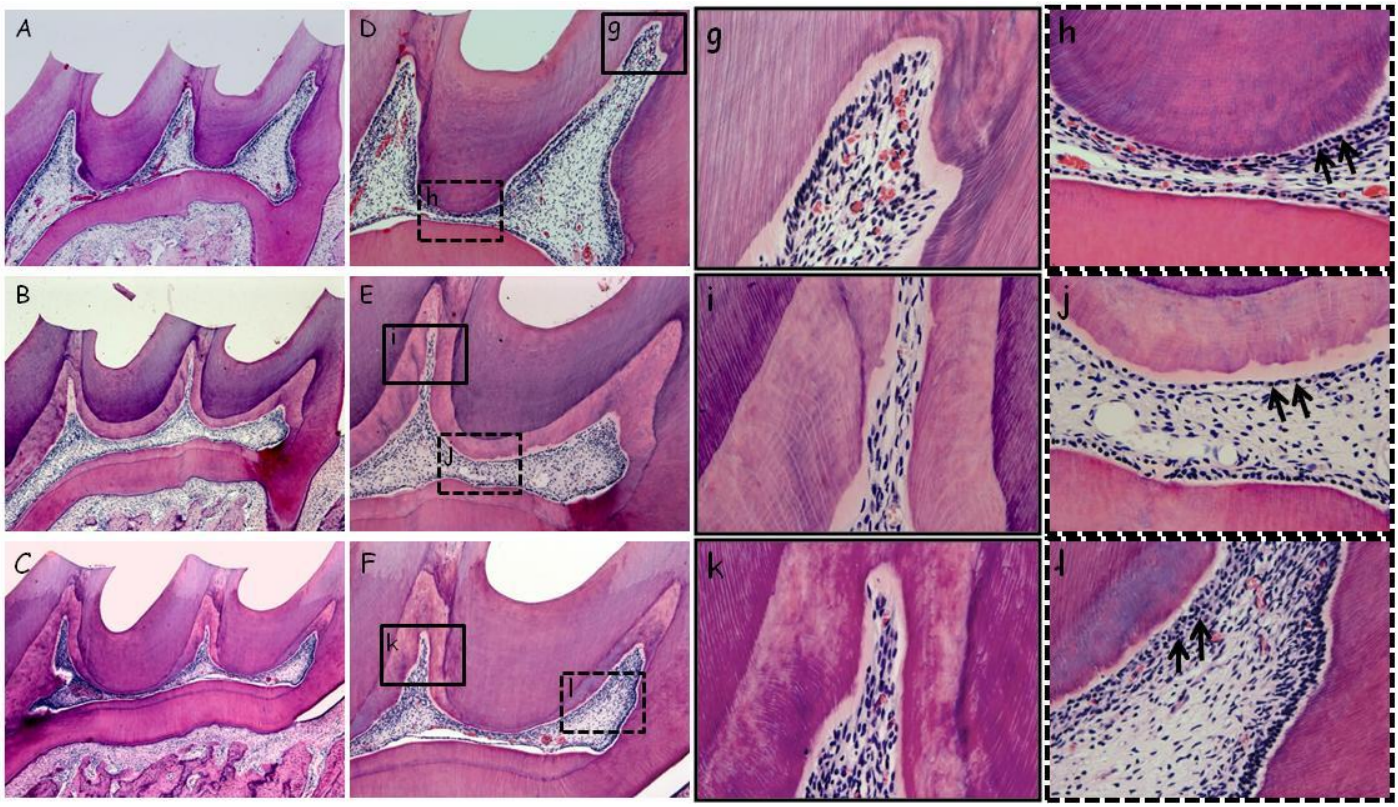

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Figure 2. H&E staining. Photomicrographs of Control (A, D, g, h); BLUE (B, E, i, j); and MAXX (C , F, k, l) groups at 30 days. In panoramic photomicrographs (A-C) can observed of the pulp chamber as a whole, showing the formation of tertiary dentin in bleached groups; at an intermediate magnification (D-F), it can be seen the organization of the pulp tissue in all groups; the largest magnification(g-l) shows the dentin layer occupying much of the oclusal third, and the formation of the odontoblast layer (black arrows) in areas that had cellular disorganization in the bleached groups. [A-C: 50X; D-F: 100X; g-l: 400X].

Table 1. Scores to inflammatory infiltrate at 2 days, and area of the pulp chamber at 30 days.

Third		Score 2 days	Groups (n=10)			P
			Control	BLUE	MAXX	
Coronal	**Occlusal**	1	10	0	0	
		2	0	2	0	Mann-Whitney
		3	0	6	2	BLUE X MAXX P=0.007
		4	0	1	2	
		5	0	1	6	
		Median*	**1**	**3[a]**	**5[b]**	
	Middle	1	10	0	0	
		2	0	6	2	Mann-Whitney
		3	0	4	6	BLUE X MAXX P=0.049
		4	0	0	1	
		5	0	0	1	
		Median*	**1**	**2[a]**	**3[b]**	
	Cervical	1	10	4	3	
		2	0	6	5	Mann-Whitney
		3	0	0	2	BLUE X MAXX P=0.372
		4	0	0	0	
		5	0	0	0	
		Median*	**1**	**2[a]**	**2[a]**	
Área of pulp chamber at 30 days (µm²)		**Mean** (10^5)*	19.86[a]	9.25[b]	7.00[c]	One Way Anova P<0.001
		SD (10^5)	2.35	0.59	0.25	

*Different letters in the same line indicate statistically significant differences between groups.

Presence of Inflammatory Mediators

Immunohistochemical analysis for IL-17 e IL-6

At 2 days, IL-17 analysis (Figure 3) showed that the control group presented low standard of immunolabeling; BLUE group exhibited low and moderate standard of immunolabeling; specimens of MAXX groups had a moderate standard immunolabelingin their majority. At 30 days, all groups showed lower standard of immunolabeling for IL-17. There was statistical difference only between MAXX group and the others groups at two days.

In the analysis of IL-6 to two days (Figure 3), the control group specimens showed low standard of immunolabeling; BLUE group showed in most specimens, a moderate immunolabeling standard; while the MAXX group, presented moderate and high standard of immunolabeling. At 30 days, the control group specimens showed low standard of immunolabeling; part of the BLUE group, moderate immunolabeling standard; and MAXX group, presented moderate standard of immunolabeling in most specimens. There were significant differences only for two days, between the bleached groups and control group (Table 2).

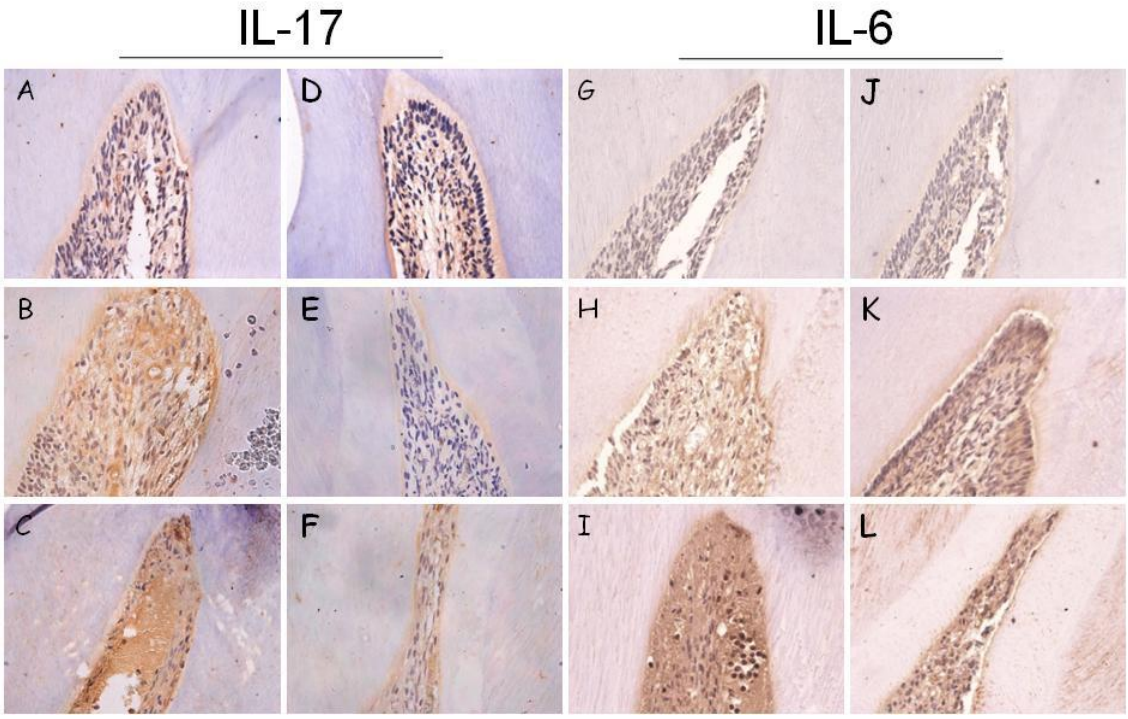

Figure 3. Immunohistochemical labeling to IL-17 and IL-6. Photomicrographs of the Control group (A,D;G,J); BLUE group (B,E;H,K); and MAXX group (C,F;I,L), at 2 days (A-C;G-I) and 30 days (D-F;J-L). [400X].

Table 2. Scores to immunohistochemical labeling of IL-17 and IL-6, at 2 and 30 days.

Immunochemistry		Groups						P
		Control		BLUE		MAXX		
	Score/Days	2d	30d	2d	30d	2d	30d	P
IL-17	0	0	0	0	0	0	0	Kruskal-Wallis 2d: P<0.001
	1	9	10	5	10	0	10	
	2	1	0	5	0	6	0	
	3	0	0	0	0	4	0	
	4	0	0	0	0	0	0	
	Median*	1^{a}	1^{A}	1^{a}	1^{A}	2^{b}	1^{A}	
IL-6	0	0	0	0	0	0	0	Kruskal-Wallis 2d: P<0.001 30d: P=0.014
	1	10	10	1	5	0	4	
	2	0	0	9	5	5	6	
	3	0	0	0	0	5	0	
	4	0	0	0	0	0	0	
	Median*	1^{a}	1^{A}	2^{b}	1^{A}	2^{b}	2^{A}	

*Different superscript lowercase letters in the same line indicate statistically significant differences between groups at 2 days, and superscript uppercase letters, at 30 days.

Immunohistochemical analysis for CD5

The analysis for CD5 was performed by counting cells that had brown citosplasma, and labeling was observedin diffusely distributed cells over the pulp. At 30 days, the number of immunolabeling cells was performed only in the cervical third of the crown because the oclusal and middle thirds were occupied by tertiary dentin. The images and data to CD5 are shown in Figure 4 and 5, respectively.

After 2 days, there was a significant difference between the BLUE group and other groups in occlusal third. From the middle third and cervical third, both bleached groups showed significant difference with control group. The immunolabeling was present at 30 days, with no significant difference compared at two days.

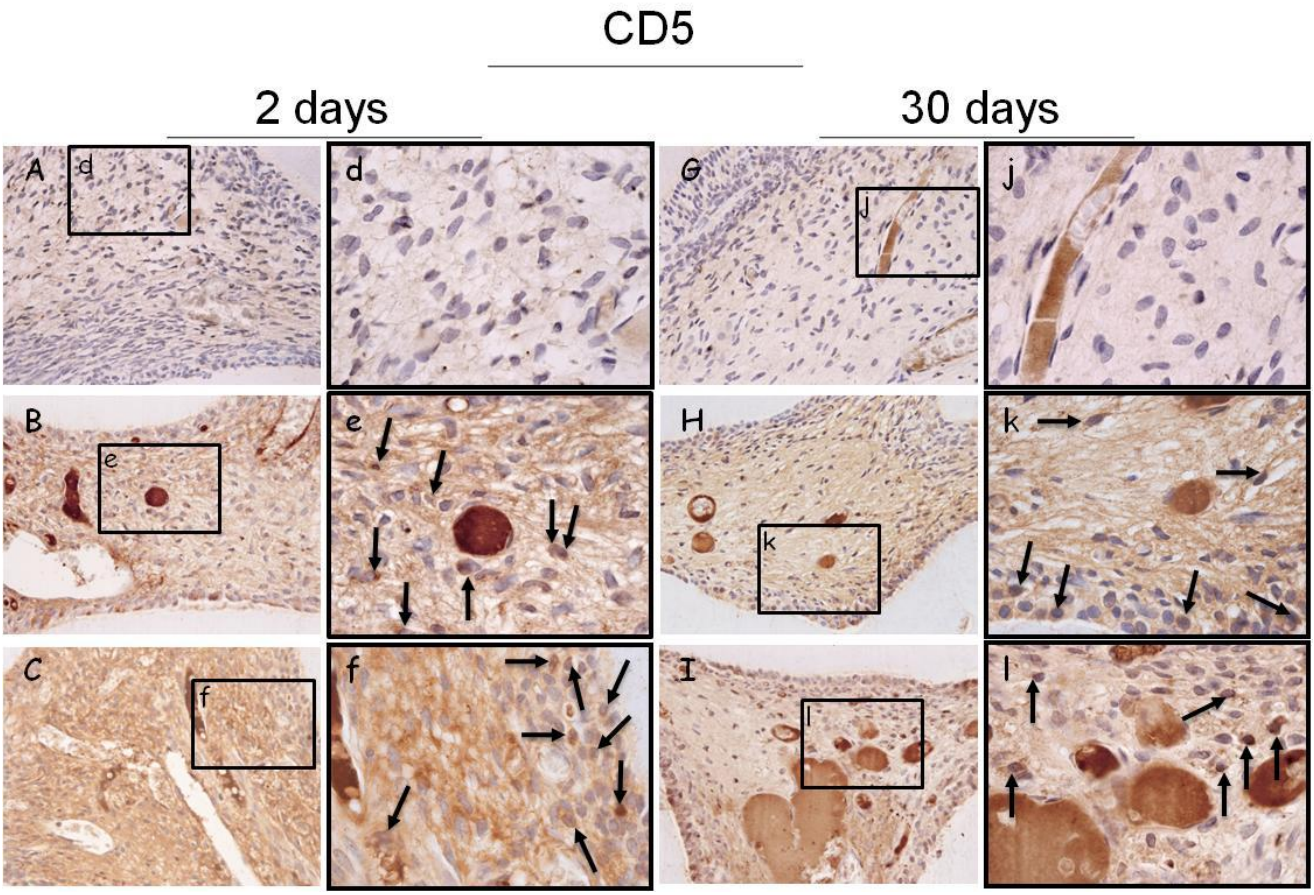

Figure 4. Immunohistochemical labeling to CD5. Photomicrographs of the cervical third of Control (A,d;G,j), BLUE (B,e;H,k), and MAXX (C,f;I,l) groups, at 2 (A-f) and 30 (G-l) days. Photomomicrographs in largest magnification are represented for lowercase (Controle: d,j; BLUE: e,k; and MAXX: f,l). The black arrows appoint labeling cells. [400X; 1000X].

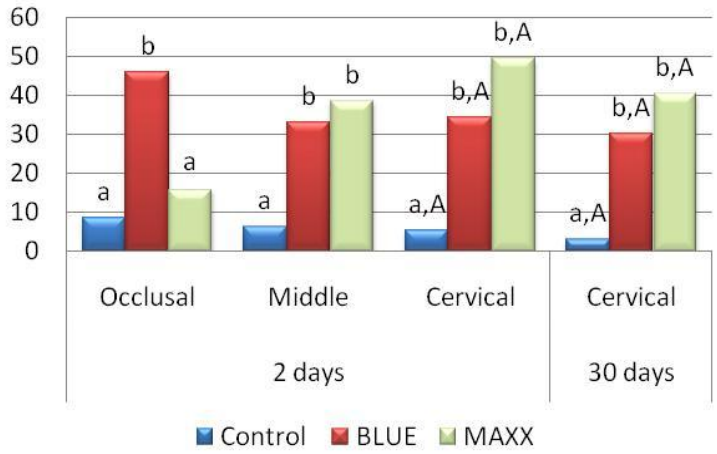

Figure 5. The graphic showed the cells count to CD5, expressed in number of cells per mm^2, and analysis statistical. Differents upperscript lowercase letters in the indicate statistically significant differences between groups in each analysis time, and superscript uppercase letters, between 2 and 30 days in cervical third.

Discussion

This study investigated the leukocyte recruitment and activation, and behavior of the IL-17 and IL-6 pro-inflammatory cytokines in the pulp tissue after tooth bleaching with two concentrations of H_2O_2, and accepts the hypothesis presented.

Previously, we conducted a study with Wistar rat molars, where we observe that only one bleaching session with 35% H_2O_2 was capable of causing necrosis in the occlusal third of the coronal pulp (Cintra *et al.* 2013). A similar study in human mandibular incisors (Costa *et al.* 2010), showed similar pulp reactions, and suggested that this experimental model may be useful to predict outcomes of bleaching procedures performed in human mandibular incisors (Cintra *et al.* 2013).

We observed that the concentration of the bleaching gel can influence the damage to pulpal tissue, probably due to the greater penetration of H_2O_2 in the tissue, as observed in previous studies (Benetti *et al.* 2004). We have seen that approximately 60% of the specimens of group that received bleaching gel with H_2O_2 20% exhibited moderate inflammatory infiltrate in the occlusal third of the coronal pulp, while 60% of the specimens of the groups that received bleaching gel with H_2O_2 35%, showed necrosis in this area.

Considering the search for the bleaching procedure at the clinic, and the effects it can generate the pulp tissue, this study expanded the knowledge of the H_2O_2 mechanism of action on the pulp cells, according to the concentration of H_2O_2. It was observed that after severe damage caused to the pulp tissue in two days by the bleaching procedure, the pulp tissue was shown to be able to recover their organization, forming the odontoblast layer around the entire pulp.

However, a large amount of tertiary dentin was produced, which reduced the volume of the pulp chamber.

After an injury in the tooth tissue, pulp progenitor cells are recruited to the repair process, and differentiate into odontoblasts second generation to produce the reparative dentin (Kitamura *et al.* 1999). Odontoblasts that persist in the tissue also participate in the repair process secreting reactionary dentin (Smith *et al.* 1995). Thus, the dentin gradually increases in thickness over the space formerly occupied by pulp tissue (Goldberg & Smith 2004), as occurred in the present study.

Several studies show that H_2O_2 is capable of inducing mineralization of pulp (Lee *et al.* 2006, Matsui *et al.* 2009, Soares *et al.* 2014). Lower H_2O_2 concentrations strongly induced the expression of proteins associated with the mineralization process, whereas these proteins were adjusted according to the concentration of H_2O_2. This study showed that the MAXX group was associated with a greater decrease in area of the pulp chamber. This indicates that the highest concentration of H_2O_2 is capable of stimulating more odontoblasts to produce tertiary dentin, as a form of protection to ROS. This fact leads to aging of the pulp tissue and impairs their ability to defend face to new aggressors.

Most immunolabeling found for IL-17 in MAXX group may indicate that this cytokine acts on the pulp inflammatory process during oxidative stress by H_2O_2. As the labeling has been present in various types of pulp cells, it is likely that it has stimulated cells of the pulp tissue to secrete other cytokines. The treatment of human dental pulp cells with IL-17 showed that it is able to induce IL-6 in a dose dependent manner. Thus, the authors suggest that IL-17 can exarceber inflammatory response in pulpits (Xiong *et al.* 2015). Studies also

show that IL-17 promoted bone remodeling, as well as increased osteogenic differentiation markers on human mesenchymal stem cells (Huang *et al.* 2009).

IL-6 was present most strongly in the tissue, both in BLUE group, as the MAXX. Significant levels were identified in this interleukin inflamed dental pulp (Barkhodar *et al.* 1999). This IL-6 production can have a beneficial or deleterious effect, depending on the quantity produced and the time of their action (Balto*et al.* 2001). Elevated levels of IL-6 are associated with increased inflammation and clinical symptoms (Prso *et al.* 2007). The immunolabeling to IL-6 were most intense at 2 days than at 30 days, suggesting that inflammation and symptoms decrease after some time.

CD5 positive cells were present both in the specimens analyzed two days after the bleaching procedure such as those analyzed 30 days, with no statistical difference between these. The CD5 is a receptor present on T and B cells, related to the progression of diseases that involve the pulp tissue (Cooper *et al.* 2010). However, this immunolabeling was also found in other cells. These data are consistent with studies that show that H_2O_2 bleaching gel, when used in low concentrations, is capable of promoting expression of inflammatory mediators by odontoblasts, which are the first defense cells of the pulp tissue due to peripheral location and intimate contact with dentin (Cooper *et al.* 2010). Thus, the odontoblasts are seen as immunocompetent cells, capable of activating an immune response (Cooper *et al.* 2010).

With the increase of the pathogenic agent, occur the expression of inflammatory markers by other cells. This fact can be observed during infection, where the progression of the disease involves the release of cytokines and chemokines by fibroblasts, stem cells, endothelial cells, and finally, cells of the

immune system (Cooper *et al.* 2014). In the present study, the effects of H_2O_2 were intense, so that there was the release of inflammatory mediators by other cells of the pulp tissue, and these caused the attraction of immune cells.

Positive immunolabeling for CD5 to 30 days, indicates that the pulp tissue remains in an inflammatory state, even though organized. This data can be confirmed in future studies, with markers showing the permanence of oxidative stress in the pulp after bleaching procedure.

Conclusion

We conclude that bleaching gel with 35% H_2O_2 cause of necrosis in pulp of Wistar rats, and bleaching gel with 20% H_2O_2, moderate inflammation. Still, that IL-17 and IL-6 participates this inflammatory process, and the immunolabeling is greater with increasing H_2O_2 concentration. This process is accompanied by the prolonged recruitment and activation of CD positive cells. Therefore, it should be considered bleaching gels with lower concentrations of hydrogen peroxide for clinical use.

Acknowledgments

The authors deny any conflict of interent.

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IV. *Anexos*

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UNIVERSIDADE ESTADUAL PAULISTA
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CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Presença de Caspase, PCNA e CD5 em polpas de ratos submetidos ao tratamento clareador com peróxido de hidrogênio em diferentes concentrações. Um estudo histológico e imunohistoquímico"**, Processo FOA nº 2014-00591, sob responsabilidade de Luciano Tavares Angelo Cintra apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 26 de junho de 2014.

VALIDADE DESTE CERTIFICADO: 02 de Janeiro de 2016.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 02 de Fevereiro de 2016.

CERTIFICATE

We certify that the study entitled **"Presence of Caspase, PCNA and CD5 in the pulp of rats subjected bleaching with different concentrations hydrogen peroxide. Study histological and immunohistochemical"**, Protocol FOA nº 2014-00591, under the supervision of Luciano Tavares Angelo Cintra presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on June 26, 2014.

VALIDITY OF THIS CERTIFICATE: January 02, 2016.
DATE OF SUBMISSION OF THE FINAL REPORT: February 02, 2016.

Profa. Dra. Maria Gisela Laranjeira
Vice-Coordenadora da CEUA
CEUA Vice-Coordinator

CEUA - Comissão de Ética no Uso de Animais
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1. **General Points on Composition**

    1. Authors are strongly encouraged to analyze their final draft with both software (*e.g.*, spelling and grammar programs) and colleagues who have expertise in English grammar. References listed at the end of this section provide a more extensive review of rules of English grammar and guidelines for writing a scientific article. Always remember that clarity is the most important feature of scientific writing. Scientific articles must be clear and precise in their content and concise in their delivery since their purpose is to inform the reader. The Editor reserves the right to edit all manuscripts or to reject those manuscripts that lack clarity or precision, or have unacceptable grammar or syntax. The following list represents common errors in manuscripts submitted to the *JOE*:

    2. The paragraph is the ideal unit of organization. Paragraphs typically start with an introductory sentence that is followed by sentences that describe additional detail or examples. The last sentence of the paragraph provides conclusions and forms a transition to the next paragraph. Common problems include one-sentence paragraphs, sentences that do not develop the theme of the paragraph (see also section “c” below), or sentences with little to no transition within a paragraph.

    3. Keep to the point. The subject of the sentence should support the subject of the paragraph. For example, the introduction of authors’ names in a sentence changes the subject and lengthens the text. In a paragraph on sodium hypochlorite, the sentence, “In 1983, Langeland *et al.*, reported that sodium hypochlorite acts as a lubricating factor during instrumentation and helps to flush debris from the root canals” can be edited to: “Sodium hypochlorite acts as a lubricant during instrumentation and as a vehicle for flushing the generated debris (Langeland *et al.*, 1983)." In this example, the paragraph’s subject is sodium hypochlorite and sentences should focus on this subject.

    4. Sentences are stronger when written in the active voice, *i.e.*, the subject performs the action. Passive sentences are identified by the use of passive verbs such as “was,” “were,” “could,” etc. For example: “Dexamethasone was

found in this study to be a factor that was associated with reduced inflammation," can be edited to: "Our results demonstrated that dexamethasone reduced inflammation." Sentences written in a direct and active voice are generally more powerful and shorter than sentences written in the passive voice.

5. Reduce verbiage. Short sentences are easier to understand. The inclusion of unnecessary words is often associated with the use of a passive voice, a lack of focus or run-on sentences. This is not to imply that all sentences need be short or even the same length. Indeed, variation in sentence structure and length often helps to maintain reader interest. However, make all words count. A more formal way of stating this point is that the use of subordinate clauses adds variety and information when constructing a paragraph. (This section was written deliberately with sentences of varying length to illustrate this point.)

6. Use parallel construction to express related ideas. For example, the sentence, "Formerly, endodontics was taught by hand instrumentation, while now rotary instrumentation is the common method," can be edited to "Formerly, endodontics was taught using hand instrumentation; now it is commonly taught using rotary instrumentation." The use of parallel construction in sentences simply means that similar ideas are expressed in similar ways, and this helps the reader recognize that the ideas are related.

7. Keep modifying phrases close to the word that they modify. This is a common problem in complex sentences that may confuse the reader. For example, the statement, "Accordingly, when conclusions are drawn from the results of this study, caution must be used," can be edited to "Caution must be used when conclusions are drawn from the results of this study."

8. To summarize these points, effective sentences are clear and precise, and often are short, simple and focused on one key point that supports the paragraph's theme.

9. Authors should be aware that the *JOE* uses iThenticate, plagiarism detection software, to assure originality and integrity of material published in the *Journal*. The use of copied sentences, even when present within quotation marks, is

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2. **Organization of Original Research Manuscripts**

Please Note: *All abstracts should be organized into sections that start with a one-word title (in bold),* i.e.*, Introduction, Methods, Results, Conclusions, etc., and should not exceed more than 250 words in length.*

1. **Title Page:** The title should describe the major emphasis of the paper. It should be as short as possible without loss of clarity. Remember that the title is your advertising billboard—it represents your major opportunity to solicit readers to spend the time to read your paper. It is best not to use abbreviations in the title since this may lead to imprecise coding by electronic citation programs such as PubMed (*e.g.*, use "sodium hypochlorite" rather than NaOCl). The author list must conform to published standards on authorship (see authorship criteria in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals at ***www.icmje.org***). The manuscript title, name and address (including email) of one author designated as the corresponding author. This author will be responsible for editing proofs and ordering reprints when applicable. The contribution of each author should also be highlighted in the cover letter.
2. **Abstract:** The abstract should concisely describe the purpose of the study, the hypothesis, methods, major findings and conclusions. The abstract should describe the new contributions made by this study. The word limitations (250 words) and the wide distribution of the abstract (*e.g.*, PubMed) make this section challenging to write clearly. This section often is written last by many authors since they can draw on the rest of the manuscript. Write the abstract in past tense since the study has been completed. Three to ten keywords should be listed below the abstract.
3. **Introduction:** The introduction should briefly review the pertinent literature in order to identify the gap in knowledge that the study is intended to address and the limitations of previous studies in the area. The purpose of the study, the tested hypothesis and its scope should be clearly described. Authors should realize that this section of the paper is their primary opportunity to establish communication with the diverse readership of the *JOE*. Readers who are not expert in the topic of the manuscript are likely to skip the paper if the

introduction fails to succinctly summarize the gap in knowledge that the study addresses. It is important to note that many successful manuscripts require no more than a few paragraphs to accomplish these goals. Therefore, authors should refrain from performing extensive review or the literature, and discussing the results of the study in this section.

4. **Materials and Methods:** The objective of the materials and methods section is to permit other investigators to repeat your experiments. The four components to this section are the detailed description of the materials used and their components, the experimental design, the procedures employed, and the statistical tests used to analyze the results. The vast majority of manuscripts should cite prior studies using similar methods and succinctly describe the essential aspects used in the present study. Thus, the reader should still be able to understand the method used in the experimental approach and concentration of the main reagents (*e.g.*, antibodies, drugs, etc.) even when citing a previously published method. The inclusion of a "methods figure" will be rejected unless the procedure is novel and requires an illustration for comprehension. If the method is novel, then the authors should carefully describe the method and include validation experiments. If the study utilized a **commercial product**, the manuscript must state that they either followed manufacturer's protocol *or* specify any changes made to the protocol. If the study used an ***in vitro*** **model** to simulate a clinical outcome, the authors must describe experiments made to validate the model, or previous literature that proved the clinical relevance of the model. Studies on **humans** must conform to the Helsinki Declaration of 1975 and state that the institutional IRB/equivalent committee(s) approved the protocol and that informed consent was obtained after the risks and benefits of participation were described to the subjects or patients recruited. Studies involving **animals** must state that the institutional animal care and use committee approved the protocol. The statistical analysis section should describe which tests were used to analyze which dependent measures; p-values should be specified. Additional details may include randomization scheme, stratification (if any), power analysis as a basis for sample size computation, drop-outs from clinical trials, the effects of important confounding variables, and bivariate versus multivariate analysis.
5. **Results:** Only experimental results are appropriate in this section (*i.e.*, neither methods, discussion, nor conclusions should be in this section). Include only those data that are critical for the study, as defined by the aim(s). Do not include all available data without justification; any repetitive findings will be

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   The second type of figures are graphs (*i.e.*, line drawings including bar graphs) that plot a dependent measure (on the Y axis) as a function of an independent measure (usually plotted on the X axis). Examples include a graph depicting pain scores over time, etc. Graphs should be used when the overall trend of the results are more important than the exact numerical values of the results. For example, a graph is a convenient way of reporting that an ibuprofen-treated group reported less pain than a placebo group over the first 24 hours, but was the same as the placebo group for the next 96 hours. In this case, the trend of

the results is the primary finding; the actual pain scores are not as critical as the relative differences between the NSAID and placebo groups.

7. **Tables:** Tables are appropriate when it is critical to present exact numerical values. However, not all results need be placed in either a table or figure. For example, the following table may not be necessary:

% NaOCl	N/Group	% Inhibition of Growth
0.001	5	0
0.003	5	0
0.01	5	0
0.03	5	0
0.1	5	100
0.3	5	100
1	5	100
3	5	100

8. Instead, the results could simply state that there was no inhibition of growth from 0.001-0.03% NaOCl, and a 100% inhibition of growth from 0.03-3% NaOCl (N=5/group). Similarly, if the results are not significant, then it is probably not necessary to include the results in either a table or as a figure. These and many other suggestions on figure and table construction are described in additional detail in Day (1998).
9. **Discussion:** This section should be used to interpret and explain the results. Both the strengths and weaknesses of the observations should be discussed. How do these findings compare to the published literature? What are the clinical implications? Although this last section might be tentative given the nature of a particular study, the authors should realize that even preliminary clinical implications might have value for the clinical readership. Ideally, a review of the potential clinical significance is the last section of the discussion. What are the major conclusions of the study? How does the data support these conclusions
10. **Acknowledgments:** All authors must affirm that they have no financial affiliation (*e.g.*, employment, direct payment, stock holdings, retainers, consultant ships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements

existed in the past three years. Any other potential conflict of interest should be disclosed. Any author for whom this statement is not true must append a paragraph to the manuscript that fully discloses any financial or other interest that poses a conflict. Likewise the sources and correct attributions of all other grants, contracts or donations that funded the study must be disclosed

11. **References:** The reference style follows Index Medicus and can be easily learned from reading past issues of the *JOE*. The *JOE* uses the Vancouver reference style, which can be found in most citation management software products. Citations are placed in parentheses at the end of a sentence or at the end of a clause that requires a literature citation. Do not use superscript for references. Original reports are limited to 35 references. There are no limits in the number of references for review articles.

3. **Manuscripts Category Classifications and Requirements**

Manuscripts submitted to the *JOE* must fall into one of the following categories. The abstracts for all these categories would have a maximum word count of 250 words:

1. CONSORT Randomized Clinical Trial-Manuscripts in this category must strictly adhere to the Consolidated Standards of Reporting Trials-CONSORT-minimum guidelines for the publication of randomized clinical trials. These guidelines can be found at ***www.consort-statement.org/***. These manuscripts have a limit of 3,500 words, [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.
2. Review Article-Manuscripts in this category are either narrative articles, or systematic reviews/meta-analyses. Case report/Clinical Technique articles even when followed by extensive review of the literature will should be categorized as “Case Report/Clinical Technique”. These manuscripts have a limit of 3,500 words, [including abstract, introduction, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.
3. Clinical Research (*e.g.*, prospective or retrospective studies on patients or patient records, or research on biopsies, excluding the use of human teeth for technique studies). These manuscripts have a limit of 3,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.

4. Basic Research Biology (animal or culture studies on biological research on physiology, development, stem cell differentiation, inflammation or pathology). Manuscripts that have a primary focus on biology should be submitted in this category while manuscripts that have a primary focus on materials should be submitted in the Basic Research Technology category. For example, a study on cytotoxicity of a material should be submitted in the Basic Research Technology category, even if it was performed in animals with histological analyses. These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or 4 tables*.
5. Basic Research Technology (Manuscripts submitted in this category focus primarily on research related to techniques and materials used, or with potential clinical use, in endodontics). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 3 figures and tables *.
6. Case Report/Clinical Technique (*e.g.*, report of an unusual clinical case or the use of cutting-edge technology in a clinical case). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or tables*.

* Figures, if submitted as multipanel figures must not exceed 1 page length. Manuscripts submitted with more than the allowed number of figures or tables will require approval of the *JOE* Editor or associate editors. If you are not sure whether your manuscript falls within one of the categories above, or would like to request preapproval for submission of additional figures please contact the Editor by email at ***jendodontics@uthscsa.edu***.

Importantly, adhering to the general writing methods described in these guidelines (and in the resources listed below) will help to reduce the size of the manuscript while maintaining its focus and significance. Authors are encouraged to focus on only the essential aspects of the study and to avoid inclusion of extraneous text and figures. The Editor may reject manuscripts that exceed these limitations.

Available Resources:

Strunk W, White EB. The Elements of Style. Allyn & Bacon, 4th ed, 2000, ISBN 020530902X.

Day R. How to Write and Publish a Scientific Paper. Oryx Press, 5th ed. 1998. ISBN 1-57356-164-9.
Woods G. English Grammar for Dummies. Hungry Minds: NY, 2001 (an entertaining review of grammar).
Alley M. The Craft of Scientific Writing. Springer, 3rd edition 1996 SBN 0-387-94766-3.
Alley M. The Craft of Editing. Springer, 2000 SBN 0-387-98964-1.

Guidelines for Publishing Papers in the International Endodontic Journal

1. GENERAL

International Endodontic Journal publishes original scientific articles, reviews, clinical articles and case reports in the field of Endodontology; the branch of dental sciences dealing with health, injuries to and diseases of the pulp and periradicular region, and their relationship with systemic well-being and health. Original scientific articles are published in the areas of biomedical science, applied materials science, bioengineering, epidemiology and social science relevant to endodontic disease and its management, and to the restoration of root-treated teeth. In addition, review articles, reports of clinical cases, book reviews, summaries and abstracts of scientific meetings and news items are accepted.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in International Endodontic Journal. Authors are encouraged to visit Wiley Blackwell Author Services for further information on the preparation and submission of articles and figures.

2. ETHICAL GUIDELINES

International Endodontic Journal adheres to the below ethical guidelines for publication and research.

2.1. Authorship and Acknowledgements

Authors submitting a paper do so on the understanding that the manuscript has been read and approved by all authors and that all authors agree to the submission of the manuscript to the Journal. International Endodontic Journal adheres to the definition of authorship set up by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE, authorship criteria should be based on 1) substantial

contributions to conception and design of, or acquisiation of data or analysis and interpretation of data, 2) drafting the article or revising it critically for important intellectual content and 3) final approval of the version to be published. Authors should meet conditions 1, 2 and 3.

Acknowledgements: Under acknowledgements please specify contributors to the article other than the authors accredited. Please also include specifications of the source of funding for the study and any potential conflict of interests if appropriate.

2.2. Ethical Approvals

Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included.

Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used. When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations. All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study. The authors MUST upload a copy of the ethical approval letter when submitting their manuscript. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

2.3 Clinical Trials

Clinical trials should be reported using the guidelines available at ww.consortstatement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material. The International Endodontic Journal encourages authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials/, http://isrctn.org/. The

clinical trial registration number and name of the trial register will then be published with the paper.

2.4 Systematic Reviews

Systematic reviews should be reported using the PRISMA guidelines available at http://prisma-statement.org/. A PRISMA checklist and flow diagram (as a Figure) should also be included in the submission material.

2.5 DNA Sequences and Crystallographic Structure Determinations

Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors directly.

2.6 Conflict of Interest and Source of Funding

International Endodontic Journal requires that all sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential conflicts of interest noted. Grant or contribution numbers may be acknowledged, and principal grant holders should be listed. Please include the information under Acknowledgements.

2.7 Appeal of Decision

The decision on a paper is final and cannot be appealed.

2.8 Permissions

If all or parts of previously published illustrations are used, permission must be obtained from the copyright holder concerned. It is the author's responsibility to obtain these in writing and provide copies to the Publishers.

2.8 Copyright Assignment

If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services; where via the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper. Your article cannot be published until this has been done.

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3. Online Open

Online Open is available to authors of primary research articles who wish to make their article available to non-subscribers on publication, or whose funding agency requires grantees to archive the final version of their article. With Online Open, the author, the author's funding agency, or the author's institution pays a fee to ensure that the article is made available to non-subscribers upon publication via Wiley Online Library, as well as deposited in the funding agency's preferred archive. For the full list of terms and conditions, see http://wileyonlinelibrary.com/onlineopen#OnlineOpen_Terms Any authors wishing to send their paper Online Open will be required to complete the payment form available from our website at:
https://authorservices.wiley.com/bauthor/onlineopen_order.asp

Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper Online Open if you do not wish to. All Online Open articles are treated in the same way as any other article. They go through the journal's standard peer-review process and will be accepted or rejected based on their own merit.

3.1 MANUSCRIPT SUBMISSION PROCEDURE

Manuscripts should be submitted electronically via the online submission site http://mc.manuscriptcentral.com/iej. The use of an online submission and peer review site enables immediate distribution of manuscripts and consequentially speeds up the review process. It also allows authors to track the status of their own manuscripts.

Complete instructions for submitting a paper is available online and below. Further assistance can be obtained from iejeditor@cardiff.ac.uk.

3.2. Getting Started

• Launch your web browser (supported browsers include Internet Explorer 5.5 or higher, Safari 1.2.4, or Firefox 1.0.4 or higher) and go to the journal's online Submission Site: http://mc.manuscriptcentral.com/iej

• Log-in, or if you are a new user, click on 'register here'.

• If you are registering as a new user.

- After clicking on 'register here', enter your name and e-mail information and click 'Next'. Your e-mail information is very important.
- Enter your institution and address information as appropriate, and then click 'Next.'
- Enter a user ID and password of your choice (we recommend using your e-mail address as your user ID), and then select your areas of expertise. Click 'Finish'.

• If you are registered, but have forgotten your log in details, please enter your e-mail address under 'Password Help'. The system will send you an automatic user ID and a new temporary password.

• Log-in and select 'Author Centre '

3.3. Submitting Your Manuscript

• After you have logged into your 'Author Centre', submit your manuscript by clicking on the submission link under 'Author Resources'.

• Enter data and answer questions as appropriate. You may copy and paste directly from your manuscript and you may upload your pre-prepared covering letter.

• Click the 'Next' button on each screen to save your work and advance to the next screen.

• You are required to upload your files.

- Click on the 'Browse' button and locate the file on your computer.
- Select the designation of each file in the drop down next to the Browse button.
- When you have selected all files you wish to upload, click the 'Upload Files' button.

• Review your submission (in HTML and PDF format) before completing your submission by sending it to the Journal. Click the 'Submit' button when you are finished reviewing.

3.4. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are

acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the abstract, main text, references, tables, and figure legends, but no embedded figures or Title page. The Title page should be uploaded as a separate file. In the main text, please reference figures as for instance 'Figure 1', 'Figure 2' etc to match the tag name you choose for the individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below.

3.5. Blinded Review

Manuscript that do not conform to the general aims and scope of the journal will be returned immediately without review. All other manuscripts will be reviewed by experts in the field (generally two referees). International Endodontic Journal aims to forward referees´ comments and to inform the corresponding author of the result of the review process. Manuscripts will be considered for fast-track publication under special circumstances after consultation with the Editor. International Endodontic Journal uses double blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper and the name(s) of the author(s) will not be disclosed to the reviewers.

To allow double blinded review, please submit (upload) your main manuscript and title page as separate files. Please upload:

• Your manuscript without title page under the file designation 'main document'

• Figure files under the file designation 'figures'

• The title page and Acknowledgements where applicable, should be uploaded under the file designation 'title page'

All documents uploaded under the file designation 'title page' will not be viewable in the html and pdf format you are asked to review in the end of the submission process. The files viewable in the html and pdf format are the files available to the reviewer in the review process.

3.6. Suspension of Submission Mid-way in the Submission Process

You may suspend a submission at any phase before clicking the 'Submit' button and save it to submit later. The manuscript can then be located under 'Unsubmitted Manuscripts' and you can click on 'Continue Submission' to continue your submission when you choose to.

3.7. E-mail Confirmation of Submission

After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may be caused by some sort of spam filtering on your email server. Also, the e-mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their white list.

3.8. Manuscript Status

You can access ScholarOne Manuscripts any time to check your 'Author Centre' for the status of your manuscript. The Journal will inform you by e-mail once a decision has been made.

3.9. Submission of Revised Manuscripts

To submit a revised manuscript, locate your manuscript under 'Manuscripts with Decisions' and click on 'Submit a Revision'. Please remember to delete any old files uploaded when you upload your revised manuscript.

4. MANUSCRIPT TYPES ACCEPTED

Original Scientific Articles: must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically evaluated and, if necessary, repeated. Original Scientific Articles must conform to the highest international standards in the field.

Review Articles: are accepted for their broad general interest; all are refereed by experts in the field who are asked to comment on issues such as timeliness, general interest and balanced treatment of controversies, as well as on scientific accuracy.

Reviews should generally include a clearly defined search strategy and take a broad view of the field rather than merely summarizing the authors´ own previous work.

Extensive or unbalanced citation of the authors´ own publications is discouraged. Mini Review Articles: are accepted to address current evidence on well-defined clinical, research or methodological topics. All are refereed by experts in the field who are asked to comment on timeliness, general interest, balanced treatment of controversies, and scientific rigor. A clear research question, search strategy and balanced synthesis of the evidence is expected. Manuscripts are limited in terms of word-length and number of figures.

Clinical Articles: are suited to describe significant improvements in clinical practice such as the report of a novel technique, a breakthrough in technology or practical

approaches to recognised clinical challenges. They should conform to the highest scientific and clinical practice standards.

Case Reports: illustrating unusual and clinically relevant observations are acceptable but they must be of sufficiently high quality to be considered worthy of publication in the Journal. On rare occasions, completed cases displaying non-obvious solutions to significant clinical challenges will be considered. Illustrative material must be of the highest quality and healing outcomes, if appropriate, should be demonstrated.

Supporting Information: International Endodontic Journal encourages submission of adjuncts to printed papers via the supporting information website (see submission of supporting information below). It is encouraged that authors wishing to describe novel procedures or illustrate cases more fully with figures and/or video may wish to utilise this facility. Letters to the Editor: are also acceptable. Meeting Reports: are also acceptable.

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

Language: The language of publication is English. It is preferred that manuscript is professionally edited. A list of independent suppliers of editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Presentation: Authors should pay special attention to the presentation of their research findings or clinical reports so that they may be communicated clearly.

Technical jargon should be avoided as much as possible and clearly explained where its use is unavoidable. Abbreviations should also be kept to a minimum, particularly those that are not standard. The background and hypotheses underlying the study, as well as its main conclusions, should be clearly explained. Titles and abstracts especially should be written in language that will be readily intelligible to any scientist.

Abbreviations: International Endodontic Journal adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

5.2. Structure

All manuscripts submitted to International Endodontic Journal should include Title Page, Abstract, Main Text, References and Acknowledgements, Tables, Figures and Figure Legends as appropriate Title Page: The title page should bear: (i) Title, which

should be concise as well as descriptive; (ii) Initial(s) and last (family) name of each author; (iii) Name and address of department, hospital or institution to which work should be attributed; (iv) Running title (no more than 30 letters and spaces); (v) No more than six keywords (in alphabetical order); (vi) Name, full postal address, telephone, fax number and e-mail address of author responsible for correspondence.

Abstract for Original Scientific Articles should be no more than 250 words giving details of what was done using the following structure:

• Aim: Give a clear statement of the main aim of the study and the main hypothesis tested, if any.

• Methodology: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and statistical tests.

• Results: Give the main results of the study, including the outcome of any statistical analysis.

• Conclusions: State the primary conclusions of the study and their implications.

Suggest areas for further research, if appropriate.

Abstract for Review Articles should be non-structured of no more than 250 words giving details of what was done including the literature search strategy.

Abstract for Mini Review Articles should be non-structured of no more than 250 words, including a clear research question, details of the literature search strategy and clear conclusions.

Abstract for Case Reports should be no more than 250 words using the following structure:

• Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.

• Summary: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and analysis if any.

• Key learning points: Provide up to 5 short, bullet-pointed statements to highlight the key messages of the report. All points must be fully justified by material presented in the report.

Abstract for Clinical Articles should be no more than 250 words using the following structure:

• Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.

• Methodology: Describe the methods adopted.

• Results: Give the main results of the study.

• Conclusions: State the primary conclusions of the study.

Main Text of Original Scientific Article should include Introduction, Materials and Methods, Results, Discussion and Conclusion Introduction: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested.

Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced.

(i) Clinical Trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material.

(ii) Experimental Subjects: experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study, if applicable. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

(iii) Suppliers: Suppliers of materials should be named and their location (Company, town/city, state, country) included.

Results: should present the observations with minimal reference to earlier literature or to possible interpretations. Data should not be duplicated in Tables and Figures.

Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The Discussion

section should progress with a review of the methodology before discussing the results in light of previous work in the field. The Discussion should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.
Conclusion: should contain a summary of the findings.
Main Text of Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should be focused to place the subject matter in context and to justify the need for the review. The Review section should be divided into logical sub-sections in order to improve readability and enhance understanding. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The use of tabulated and illustrative material is encouraged. The Conclusion section should reach clear conclusions and/or recommendations on the basis of the evidence presented. Main Text of Mini Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should briefly introduce the subject matter and justify the need and timeliness of the literature review. The Review section should be divided into logical sub-sections to enhance readability and understanding and may be supported by up to 5 tables and figures. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The Conclusions section should present clear statements/recommendations and suggestions for further work. The manuscript, including references and figure legends should not normally exceed 4000 words. Main Text of Clinical Reports and Clinical Articles should be divided into Introduction, Report, Discussion and Conclusion,. They should be well illustrated with clinical images, radiographs, diagrams and, where appropriate, supporting tables and graphs. However, all illustrations must be of the highest quality Acknowledgements: International Endodontic Journal requires that all sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential conflicts of interest noted. Grant or contribution numbers may be acknowledged, and principal grant holders should be listed. Acknowledgments should be brief and should not include thanks to anonymous referees and editors. See also above under Ethical Guidelines.

5.3. References

It is the policy of the Journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting. The EndNote reference style can be obtained upon request to the editorial office (iejeditor@cardiff.ac.uk). Reference Manager reference styles can be searched for here: www.refman.com/support/rmstyles.aspIn the text: single or double authors should be acknowledged together with the year of publication, e.g. (Pitt Ford & Roberts 1990). If more than two authors the first author followed by *et al.* is sufficient, e.g. (Tobias *et al.* 1991). If more than 1 paper is cited the references should be in year order and separated by "," e.g. (Pitt Ford & Roberts 1990, Tobias *et al.* 1991).

Reference list: All references should be brought together at the end of the paper in alphabetical order and should be in the following form.

(i) Names and initials of up to six authors. When there are seven or more, list the first three and add *et al.*

(ii)Year of publication in parentheses

(iii) Full title of paper followed by a full stop (.)

(iv) Title of journal in full (in italics)

(v) Volume number (bold) followed by a comma (,)

(vi) First and last pages

Examples of correct forms of reference follow:

Standard journal article

Bergenholtz G, Nagaoka S, Jontell M (1991) Class II antigen-expressing cells in experimentally induced pulpitis. *International Endodontic Journal* **24**, 8-14.

Corporate author

British Endodontic Society (1983) Guidelines for root canal treatment. *International Endodontic Journal* **16**, 192-5.

Journal supplement

Frumin AM, Nussbaum J, Esposito M (1979) Functional asplenia: demonstration of splenic activity by bone marrow scan (Abstract). *Blood* **54** (Suppl. 1), 26a.

Books and other monographs

Personal author(s)

Gutmann J, Harrison JW (1991) Surgical Endodontics, 1st edn Boston, MA, USA: Blackwell Scientific Publications.

Chapter in a bookWesselink P (1990) Conventional root-canal therapy III: root filling. In: Harty FJ, ed. Endodontics in Clinical Practice, 3rd edn; pp. 186-223. London, UK: Butterworth.

Published proceedings paper

DuPont B (1974) Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. Proceedings of the Third Annual Meeting of the International Society for Experimental Rematology; pp. 44-46. Houston, TX, USA: International Society for Experimental Hematology.

Agency publication Ranofsky AL (1978) Surgical Operations in Short-Stay Hospitals: United States- 1975. DHEW publication no. (PHS) 78-1785 (Vital and Health Statistics; Series 13; no. 34.) Hyattsville, MD, USA: National Centre for Health Statistics.8

Dissertation or thesis

Saunders EM (1988) In vitro and in vivo investigations into root-canal obturation using thermally softened gutta-percha techniques (PhD Thesis). Dundee, UK: University of Dundee.

URLs

Full reference details must be given along with the URL, i.e. authorship, year, title of document/report and URL. If this information is not available, the reference should be removed and only the web address cited in the text.

Smith A (1999) Select committee report into social care in the community [WWW document]. URL http://www.dhss.gov.uk/reports/report015285.html [accessed on 7 November 2003]

5.4. Tables, Figures and Figure Legends

Tables: Tables should be double-spaced with no vertical rulings, with a single boldruling beneath the column titles. Units of measurements must be included in the column title.

Figures: All figures should be planned to fit within either 1 column width (8.0 cm), 1.5column widths (13.0 cm) or 2 column widths (17.0 cm), and must be suitable for photocopy reproduction from the printed version of the manuscript. Lettering on figures should be in a clear, sans serif typeface (e.g. Helvetica); if possible, the same type face should be used for all figures in a paper. After reduction for publication, upper-case text and numbers should be at least 1.5-2.0 mm high (10 point Helvetica). After reduction, symbols should be at least 2.0-3.0 mm high (10 point). All half-tone photographs should be submitted at final reproduction size. In general, multi-part figures should be arranged as they would appear in the final version. Reduction to the scale that will be used on the page is not necessary, but any special requirements (such as the separation distance of stereo pairs) should be clearly specified.

Unnecessary figures and parts (panels) of figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text in stead. Figures should not contain more than one panel unless the parts are logically

connected; each panel of a multipart figure should be sized so that the whole figure can be reduced by the same amount and reproduced on the printed page at the smallest size at which essential details are visible.

Figures should be on a white background, and should avoid excessive boxing, unnecessary colour, shading and/or decorative effects (e.g. 3-dimensionalskyscraper histograms) and highly pixelated computer drawings. The vertical axis of histograms should not be truncated to exaggerate small differences. The line spacing should be wide enough to remain clear on reduction to the minimum acceptable printed size.

Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same type size as used elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field. Thousands should be separated by athin space (1 000). Unusual units or abbreviations should be spelled out in full ordefined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general, visual cues (on the figures themselves) are preferred to verbal explanation sin the legend (e.g. broken line, open red triangles etc.)Figure legends: Figure legends should begin with a brief title for the whole figure and continue with a short description of each panel and the symbols used; they should not contain any details of methods.

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