UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

Luciana Louzada Ferreira

TESE DE DOUTORADO

INFLUÊNCIA DA CLAREAÇÃO DENTÁRIA SOBRE O TECIDO PULPAR DE RATOS DIABÉTICOS: ASPECTOS HISTOLÓGICOS E IMUNOLÓGICOS

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Tese apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP como parte dos requisitos para obtenção do título de Doutora em Ciência Odontológica, área de concentração em Endodontia.

Orientador: Prof. Dr. Luciano Tavares Angelo Cintra

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Dedicatória

Aos meus pais, Vera e Aníbal

À minha avó Maria Luiza Louzada

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Resumo e Abstract

Influência da clareação dentária sobre o tecido pulpar de ratos diabéticos: aspectos histológicos e imunológicos

RESUMO

Objetivo: Avaliar a influência da clareação dentária sobre o tecido pulpar de ratos diabéticos por meio da observação dos aspectos histológicos e imunológicos.

Material e Método: Vinte e oito ratos Wistar foram divididos em quatro grupos: (N) normoglicêmicos; (NCla) normoglicêmicos-clareados; (D) diabéticos; (DCla) diabéticos-clareados. Quatorze animais receberam uma dose de 150mg/kg de aloxano para indução da diabetes, via IM. Após 3 dias, todos os animais foram anestesiados e realizado o procedimento clareador com gel de peróxido de hidrogênio (H_2O_2) a 35% (Whiteness HP Maxx) aplicado uma vez nos molares superiores direitos por 30 minutos. Os molares superiores esquerdos foram usados como controle. Após 2 e 30 dias os animais foram eutanaziados e as maxilas processadas para análise histológica e histoquímica. Foram realizadas análises qualitativas para a reação inflamatória e imunomarcação, e quantitativas para a redução da área da camara pulpar e fibras colágenas. Os resultados foram submetidos à análise estatística com nível de significância de 5% ($p<0.05$).

Resultados: Aos 2 dias observou-se infiltrado inflamatório leve no grupo NCla e severo no grupo DCla ($p<0.05$). Quanto ao grau de maturação das fibras colágenas, aos 2 dias observou-se maior quantidade de fibras imaturas no grupo N, com diferença estatística para os demais grupos ($p<0.05$), e, menor quantidade de fibras maduras no grupo N com diferença significante para os demais grupos ($p<0.05$). Aos 30 dias não foi observado concentração de células inflamatórias e não houve diferença estatística entre os grupos para ambas as fibras colágenas ($p>0.05$). Entretanto, houve diferença significante quando comparados os grupos NCla e DCla em relação à redução da área da câmara pulpar devido a deposição de dentina

reacionária ($p<0.05$). Quanto a análise imunoistoquímica, observou-se aos 2 dias aumento da imunomarcação para IL-6 nos grupos submetidos à clareação dentária (NCla e DCla) ($p<0.05$) e, aos 30 dias houve diferença estatística apenas entre os N e DCla ($p<0.05$). Para TNF-α, observou-se aumento da imunomarcação nos grupos submetidos à clareação dentária aos 2 dias, com diferença estatística quando comparados aos grupos não clareados (N e D) ($p<0.05$). Já aos 30 dias, houve diferença estatística apenas entre os N e DCla ($p<0.05$). Para IL-17, aos 2 dias houve superior imunomarcação apenas no grupo NCla, com diferença estatística quando comparado aos grupos N e D ($p< 0.05$). Trinta dias após, não observou-se diferença significante entre os grupos para IL-17.

Conclusão: A diabetes potencializa a reação inflamatória do tecido pulpar decorrente do procedimento clareador e contribui para o aumento da deposição de dentina reacionária. A diabetes também promove o aumento de fibras colágenas maduras, independente do procedimento clareador. A clareação dentária é capaz de promover um aumento de IL-6 e TNF-α no tecido pulpar de ratos, independentemente da presença da diabetes, no entanto a diabetes contribuiu para a manutenção da expressão de IL6 e TNF-α por um maior período. Ainda, a clareação dentária influenciou no aumento de IL-17 no período inicial em ratos normoglicêmico.

Palavras-Chave: Clareamento dental, diabetes mellitus, inflamação, colágeno, citocinas.

Influence of dental bleaching on the pulp tissue of diabetic rats: histological and immunohistochemical aspects.

ABSTRACT

Objective: To evaluate the influence of dental bleaching on the pulp tissue of diabetic rats over the histological aspects and proinflammatory cytokines regulation.

Methods: Twenty-eight Wistar rats were divided into four groups: (N) normoglycemic; (NBle) normoglycemic-bleached, (D) diabetic, (DBle) diabetic-bleached. Fourteen animals reveiced a 150mg/kg dose of alloxan for diabetes induction, via IM. After 3 days, all animals were anesthetized and the dental bleaching performed using 35% hydrogen peroxide (H_2O_2) gel (Whiteness HP Maxx) in the right maxillary molars for 30 minutes. Left upper molars were used as control. Two and 30 days after the animals were euthanized, and the hemi-maxillae processed for histological and histochemistry analysis. Qualitative analyses were performed for inflammatory reaction and immunolabelling, and quantitative for pulp chamber area reduction and collagen fibers. The results were submitted to statistical analysis ($p<0.05$).

Results: At two days a mild inflammation was observed in NBle group and severe for DCla ($p<0.05$). For the maturation of collagen fibers, at two days was observed an increase amount of immature fibers in the N group, with statistical difference to the other groups ($p<0.05$), and a reduced amount of mature fibers in the N group with significant diference to the other groups ($p<0.05$). At 30 days no inflammatory cells were observed and there was no statistical difference between the groups for both fibers type ($p>0.05$). However, there was a significant difference when NBle and DBle groups were compared with regards to reduction of the pulp chamber area due the presence of reactionary dentin deposition ($p<0.05$). With regards to immunoistochemistry, was observed for IL-6 at 2 days an increased immunolabelling

in the bleached groups (NBle and DBle) ($p<0.05$) and, at 30 days, there was a significant difference only between N and DBle groups ($p<0.05$). For TNF-α, was observed an increase in the immunolabelling of bleached groups at 2 days, with significant difference when compared to non-bleached groups (N and D) ($p<0.05$). At 30 days, the significant difference was found only between N and ($p<0.05$). For IL-17, at 2 days, there was a superior immunolabelling only in NBle, with significant difference when compared to N and D ($p<0.05$). Thirty days after, no statistical difference was obsered between the groups.

Conclusion: The diabetes potencialize the inflammatory reaction on pulp tissue due to the dental bleaching and contributes to the increase of reactionary dentin deposition. The diabetes also promotes an increse of mature collagen fibers regardless the bleaching procedure. The dental bleaching is capable of promoting an increase of IL-6 and TNF-α in the pulp tissue regardless the presence of diabetes, however the dibetes contributed to the maintenance of IL-6 and TNF-α for a longer period. Still, the dental bleaching influenced the increse of IL-17 in the early periods in nomoglycemic rats.

Key-words: Dental bleaching, diabetes mellitus, inflammation, collagen, cytokines.

S U M Á R I O

Páginas

Introdução

A clareação dentária é considerada um dos principais recursos que o profissional dispõe para oferecer aos pacientes um sorriso com dentes “brancos”, satisfazendo os padrões de beleza valorizados pela sociedade (Agostinho et al. 2003). Apesar da clareação caseira ser precursora à de consultório, ela continua sendo a forma mais aceita e documentada na literatura, além de ser uma técnica considerada segura e eficaz (Fugaro et al. 2004, Auschill et al. 2005), apontada como o padrão ouro quando comparada a outras terapias clareadoras (Buchalla & Attin 2007).

O peróxido de carbamida (PC) em contato com os tecidos dentais se dissocia em uréia e peróxido de hidrogênio (H_2O_2). O processo clareador ocorre, pelo menos em parte, devido ao baixo peso molecular do H_2O_2, que se difunde facilmente por esmalte e dentina (Gokay et al. 2000; Benetti et al. 2004), liberando espécies reativas de oxigênio (ROS), que por serem instáveis, buscam a reação com outras substâncias livres ou fracamente ligadas, reestabelecendo sua estabilidade molecular. Esse fenômeno oxidante explica parcialmente o mecanismo complexo da clareação dentária (Kawamoto & Tsujimoto 2004).

Além do tempo e da concentração que o clareador permanece sobre o esmalte (Tredwin et al. 2006), outros fatores podem influenciar na penetração dos compostos químicos presentes em formulações distintas como: catalização com calor/luz (De Moor et al, 2015), a espessura do esmalte e da dentina variável em cada grupo de dentes (Camargo et al. 2007; de Souza Costa et al. 2010), bem como o número de sessões clareadoras (Cintra et al, 2013) podem influenciar na penetração de radicais livres derivados do oxigênio na câmara pulpar (Benetti et al, 2004; Coldebella et al. 2009). Esta ocorrência pode estar associada à inflamação da polpa e à sensibilidade dentária (Lee et al. 2006).

O tecido pulpar é composto por fibroblastos, odontoblastos e células mesenquimais indiferenciadas em contato direto com uma complexa cadeia de

macromolécula, secretada para o meio extrcelular, dando origem à matriz extracelular (MEC) (Linde, 1985). As principais macromoléculas presente no tecido pulpar são as proteínas colágenas (aproximadamente 34% de toda a MEC) (Abrahão et al, 2006), proteínas não-colagenosas e proteoglicanas (Goldberg & Lasfargues, 1995; Moure et al, 2011). Esse colágeno presente na matriz extracelular (MEC) do tecido pulpar mostra variações em número, espessura e distribuição, como observado em polpas humanas maduras (Pereira et al, 2014).

As fibras colágenas podem ser vistas sob várias colorações teciduais, como Van Gieson, tricromo de Masson (Puchtler et al, 1973; Rich & Whittaker, 2005) e picrosiruis red sob luz polarizada (Junqueira et al, 1982; Pereira et al, 2014). No entanto, em fibras muito finas, a visualização se torna mais difícil, sendo necessária a coloração por picrosirius red, pois a visualização sob luz polarizada vai permitir a observação dessas fibras em diferentes tons, permitindo a diferençiação entre elas. Fibras imaturas e mais finas são vistas sob polarização na cor verde-amarelada, enquanto que as mais grossas e maduras mostram um comprimento de onda maior e são vistas sob polarização nas cor amarelo-avermelhada (Junqueira et al, 1982). A distribuição e estrutura das fibras colágenas muda com a idade, resultando em feixes fibrosos (Nanci 2009), promovendo características de envelhecimento à esse tecido.

Os odontoblastos são células especializadas, pré-mitóticas e organizadas em paliçada ao longo do tecido pulpar e dentinário, sendo primeiro tipo celular a sofrer as agressões do meio externo Dentre suas funções, está a formação da dentina (Ruch et al, 1995). Essas células secretam pelo seu polo apical uma matrix de colágeno tipo-1, proteínas não-colagenosas e proteoglicanas (Bleicher, 2014). Frente a estímulos nocivos, os odontoblastos são capazes de detectar e responder à injurias, regulando sua atividade com o objetivo de promover a deposição de uma

espessa camada de matriz de dentina terciária para proteção do tecido pulpar contra as agressões externas (Smith et al, 1994).

O processo de secreção da dentina terciária pode ser classificada dependendo de sua origem, como reacionária ou reparadora, dependendo da severidade da resposta inicial e das condições sob a qual a matriz de dentina será formada. A dentina reacionária é secretada por odontoblastos originais em resposta a um atrito ou estímulo, enquanto que a reparadora, por células odontoblasticas recém-diferenciadas (Smith et al, 1995).

A formação de dentina reacionária é estimulada por pequenas quantidades de citocinas pró-inflamatória ou por moléculas biologicamente ativadas, como os fatores de crescimento (TGB-β e BMPs) e pos componentes da matriz extracelular, para a indução da diferençiação dos odontoblastos. Contudo, a formação da dentina reacionária é inibida na presença de reação inflamatória intensa (Cooper et al, 2010), fazendo com que o metabolismo dos odontoblastos se oriente apenas para a produção de citocinas pró-inflamatórias, como o TNF-α, e efetores da imunidade inata, aumentando assim a capacidade de defesa do tecido pulpar (Bleicher, 2014).

Em estudo prévio, a secreção de dentina reacionária se mostrou quase completamente formada 28 dias após a injuria ao tecido dentário, sugerindo que a após o fim da secreção de dentina, a razão do tamanho núcleo/citoplasma dos odontoblastos reduziu bruscamente com o tempo, retornando à sua morfologia (Murray et al, 2003). Outros estudos demonstram a formação de dentina reacionária em dentes humanos após a clareação dentária (Costa et al, 2010; Kina et al, 2010).

Assim como outros tecidos do corpo, polpa dentária está em constante mudança. E uma das mudanças mais evidentes é a redução da área da câmara pulpar e canais radiculares devido a deposição de dentina. Em estudo prévio foi observado que a dentina reacionária apresentou tubulos dentinários em menor número, tamanho e mais constritos (Charadram et al, 2013).

O sistema imune é formado por células que tem como função principal o reconhecimento de antígenos, neutralizando e destruindo-os (Abbas 2007). Os odontoblastos são capazes de desencadear uma resposta imune inata no tecido pupar (Bleicher 2014). Quimiocinas e citocinas pró-inflamatórias não são as únicas moléculas envolvidas na resposta inata. As beta-defensinas são pequenos peptídeos antomicrobianos que matam os microrganismos atravéz de falhas na integridade de suas membranas (Dommish et al, 2005).

Considerando a literatura existente, não se sabe o suficiente quanto às alterações pulpares decorrentes de procedimentos de clareação dentária quando associada à presença de doenças sistêmicas. Algumas alterações teciduais, especialmente as vinculadas a processos inflamatórios, podem ser avaliadas por meio da quantificação de citocinas pró-inflamatórias (Schenkein et al. 2010, Nibali et al. 2012, Aggarwal et al. 2012). Além dos fatores locais, estudos recentes têm revelado alterações na produção de algumas destas citocinas devido à presença da diabetes mellitus (DM) (Triñanes et al. 2012, Amir et al. 2011, Sun et al. 2011).

A DM tem aparecido na última década como uma doença comum, que afeta o metabolismo de carboidratos, lipídios e proteínas, resultando em hiperglicemia (Sun et al. 2011), e quando não controlada, pode se manifestar através de inflamações crônicas ou micro-inflamações sistêmicas (Mirza et al. 2012).

O estresse oxidativo produzido pela inflamação é capaz de prejudicar vários orgãos e tecidos (Kajitani et al. 2010). Por esta razão, pacientes diabéticos são mais suscetíveis a apresentarem complicações orais (Bender e Bender 2003, Fouad 2003). Além disso, a integridade da polpa dentária é diretamente afetada (Leite et al. 2008, 2010, Inagaki et al. 2010, Claudino et al, 2015), causando diversas alterações na estrutura do tecido pulpar, como presença de nódulos de calcificação e redução do colágeno (Bender e Bender 2003, Leite et al. 2008), além da circulação colateral

prejudicada, resultando em aumento no risco de necrose (Bender e Bender 2003, Catanzaro et al. 2006).

A hiperglicemia pode impedir a ação macrofágica, resultando em um estado inflamatório que prejudica a proliferação celular e o processo cicatricial do hospedeiro, essencial para o tecido pulpar (Garber et al. 2009). A migração celular para o local da inflamação faz com que o recrutamento de células do sistema imune ocorra de maneira diferente quando comparado à tecidos saudáveis (Stashenko et al. 1998).

Altos níveis de citocinas pró-inflamatórias são considerados fatores predisponentes para um futuro desenvolvimento da diabetes, inclusive em indivíduos com a glicose alterada em jejum (Mirza et al. 2012). Elevados níveis séricos de glicose na resposta inflamatória podem estar associados à redução de algumas citocinas e marcadores de mineralização, ou ao aumento na expressão de marcadores pro-inflamatórios como interleucina (IL)-6, IL-8 e TNF-α (Wang et al, 2012; Alexandraki et al, 2006). No entanto, sabemos que elevados níveis circulantes de IL-6 e TNF-α podem aumentar a resistência à insulina, impedindo o controle glicêmico (Correa et al. 2010).

A IL-6 é uma citocina pleotrópica, presente em processos fisiológicos e patológicos. Possui várias formas moleculares, com diferentes funções quando secretadas por diferentes células em situações distintas (Prso et al, 2007; Hirano 2014). Alterações em sua produção implicam na patogênese de várias doenças auto-imunes e inflamatórias crônicas (Tanaka et al. 2012). É rapidamente expressa no tecido pulpar em resposta ao trauma, à infecção (Nibali et al. 2012) e ao desenvolvimento e progressão de processos inflamatórios, como pulpite (Farges et al. 2011).

Tradicionalmente, a expressão de IL-6 é induzida por IL-1 e TNF-α nos períodos iniciais da cascata inflamatória (Balto et al. 2001). Juntas, IL-6, IL-1 e TNF-α, possuem um amplo espectro de ação e participam da fase inflamatória consistindo

em febre, aumento na sedimentação de eritrócitos e alterações nas proteínas séricas sintetizadas no fígado (Radics et al. 2003), pertencendo ao principal grupo de citocinas pró-inflamatórias (Yamano et al. 1999).

Na presença de IL-6, o TGF-β1 contribui para o direcionamento de células T helper (Th)17, que irão mediar o recrutamento de neutrófilos no processo inflamatório (Torchinsky & Blander 2010). As principais fontes de produção de IL-6 são monócitos, macrófagos, linfócitos Th2 e células polimorfonucleares. Células epiteliais, endoteliais e fibroblastos também tem mostrado a expressão de IL-6 (Baqui et al. 1998, Takeichi et al. 2000).

A IL-17 é uma citocina pró-inflamatória, membro de uma familia de 6 citocinas (IL-17A-F) (Kolls & Linden 2004) secretada principalmente por células T, denominadas Th 17 (Gaffen, 2011); à exceção da IL-17E, que é secretada por células Th12. No entanto, a família da IL-17 é a mais nova e menos compreendida das subclasses de citocinas. É ativa em respostas inflamatórias, auto-imunes e antimicrobianas (Schenkein et al. 2010).

Há evidências de que a IL-17 atua ativamente na patogênese da diabetes (Honkanen et al. 2010, Silva et al. 2012). Possui características únicas, estruturais e funcionais (Gaffen, 2011), e elevados níveis séricos de IL-17 estão associados à inflamação crônica (Van Belle et al. 2011), contribuindo para a patogênese de várias doenças como câncer (Chen et al. 2012), lúpus eritematoso sistêmico (Dolff et al. 2011), psoríase (Ariza & Williams 2011), artrite reumatóide (Yu & Ibrahim 2011). Nas doenças da cavidade oral humana, as pesquisas se iniciaram avaliando a presença da IL-17 em periodontites e lesões periapicais (Cólic et al, 2007, Xiong et al. 2009, Marçal et al, 2010).

Hoje sabemos que a IL-17 é capaz de ativar fibroblastos, células endodoteliais e epiteliais e osteoclastos a produzir citocinas pro-inflamatórias, como IL-6 e IL-8, e matriz metaloproteinase (Yu e Gaffen 2008). Considerando que os fibroblastos são

as células predominantes no tecido pulpar, é provavel que a IL-17 os estimule a secretar outras citocinas pró-inflamatórias (Xiong et al. 2015), atuando na patogênese da pulpite. Estudos recentes mostraram que o TGF-β sozinho ou acompanhado da IL-6 são necessários o início da diferenciação da IL-17, sendo IL-1β e TNF-α necessários para amplificar a resposta das células Th17 (Bettelli et al. 2006, Mangan et al. 2006, Veldhoen et al. 2006)

Também se relaciona com o processo inflamatório o fator de necrose tumoral-alfa (TNF-α). O TNF-α é uma citocina pró-inflamatória que atua como um importante mediador de lesões teciduais (Tansey & Szymkowski 2009). É secretado por macrófagos e uma variedade de outras células (Giemeno & Klaman 2005), sendo capaz de aumentar a toxicidade de leucócitos, estimular a síntese de proteínas inflamatórias e induzir a expressão de outras citocinas, como o IL-6 (Haynes & Fauci 2001). Disturbios no metabolismo do TNF-α tem sido associado a desordens metabólicas, como desenvolvimento e progressão da DM e obesidade (Swaroop et al. 2012), processos fisiológicos, como a proliferação e a diferenciação das células β, mas também a uma variedade de outras doenças, incluindo câncer, problemas cardiovasculares, neurológicos e distúrbios pulmonares, (Làinez 2004, Aggarwal et al. 2012).

Com relação às doenças ou inflamações bucais, sabe-se que o TNF-α interfere na patogênese da doença periodontal (Fagundes et al. 2011, Yamamoto et al. 2011). Na periodontite há uma resposta inflamatória sistêmica à endotoxina bacteriana (Shaddox et al. 2011) e evidências que essa inflamação aumenta os níveis séricos de TNF-α, interferindo negativamente no controle glicêmico de pacientes diabéticos (Liu et al. 2011, Sun et al. 2011, Marigo et al. 2011).

Com relação à polpa dentária, há evidencias quanto à participação do TNF-α em pulpites (Li et al. 2015), uma correlação com seu estado de irreversibilidade (Kokkas et al. 2007) e com a gravidade dos sintomas clínicos (Kawashima et al.

2005). Contudo, os níveis de expressão dessa citocina se mostraram reduzidos em amostras de polpas saudáveis (Pezelj-Ribaric et al. 2002).

Até o presente momento não há relatos na literatura científica relacionando os efeitos na clareação dentária em tecido pulpar diabético e sua relação com o a expressão de citocinas pró-inflamatórias. Diante do exposto, foi objetivo do estudo analisar a influência da diabetes e da clareação dentária no tecido pulpar de ratos Wistar, investigando por meio da análise histopatológica a resposta tecidual inflamatória, o padrão de estruturação da MEC e o nível de maturação das fibras colágenas, além da análise imunohistoquímica para as citocinas pró-inflamatórias IL-6, IL-17 e TNF-α.

ARTIGO 1

International Endodontic Journal

Diabetes influence pulpal tissue response after dental bleaching

Running Title: Dental bleaching in diabetic pulp

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Diabetes influence pulpal tissue response after dental bleaching

Abstract

Aim: The aim of this study was to evaluate the influence of diabetes mellitus (DM) on the pulpal tissue response after dental bleaching.

Methods: Twenty-eight rats were divided into four groups of seven hemi-maxillae each: (N) normoglycemic; (NBle) normoglycemic-bleached, (D) diabetic, (DBle) diabetic-bleached. DM was induced with alloxan in fourteen animals. After confirmation of DM, all animals were anesthetized and the dental bleaching performed using 35% hydrogen peroxide (H_2O_2) in the right maxillary molars for 30 minutes. Left molars were used as control (N and D). After 2 and 30 days, the animals were euthanized, the hemi-maxillae removed and processed in convencional manner for histopathological analysis and stained with hematoxylin-eosin (HE), Masson's Trichrome (MT) and picrosirius red staining technique (PSR).

Results: At 2 days, the NBle group showed a mild inflammatory infiltrate in pulpal tissue, and the DBle group showed severe or necrosis ($p<0.05$). At 30 days, no inflammatory infiltrate was present. However, regarding pulp chamber area reduction by reactionary dentin deposition, significant difference was found between NBle and DBle ($p<0.05$). With regarding collagen fibers, at 2 days it was showed a reduced amount of immature fibers and larger amount of mature collagen fibers in the NBle, D and DBle groups with statistical difference when compared to N ($p<0.05$). However, at 30 days no statistical difference was observed ($p>0.05$).

Conclusion: The DM influences the inflammatory tissue response in rats teeth after dental bleaching and increase reactionary dentin deposition. The DM promoted an increase of mature collagen fibers, despite the dental belaching procedure.

Key-words: Dental bleaching, inflammatory response, reactionary dentin, collagen fibers.

Introduction

Diabetes mellitus (DM) is a complex multisystemic disorder, with an elevated prevalence, defined as a metabolic disease, characterized by hyperglycemia as a result of defects in insulin secretion and/or action (Manfredi *et al*. 2004, Nakajima *et al*. 2013). The hyperglycemia is associated to a wide spectrum of oral complications (Manfredi *et al*. 2004) and pulp healing (Garber *et al*. 2009).

Diabetic patients have a reduced resistance to bacterial infecction and tissue repair ability, contribuiting for an increased susceptibility of inflammatory reactions and infections (Iacopino 2001). DM is capable of modify the parameters of antioxidant system in the pulp tissue, (Leite *et al*. 2008), induce the formation of pulp stones and thickened predentin (Inagaki *et al*. 2010) and an increase in the detection of anaerobic bacteria (Iwama *et al*. 2006). In long periods, DM causes reduction in the collagen concentration and an increase of alkaline phosphatase activity, which might be related to irreversible damage to the dental pulp (Catanzaro *et al*. 2006).

Despite of few studies about the effects of DM in the pulp tissue (Leite *et al*. 2008, Inagaki *et al*. 2010, Claudino *et al.* 2015) some results as inflammation, dentin resorption, fibrosis, circulatory disturbances and pulp aging remains unclear (Catanzaro *et al*. 2006). It is known that an uncontrolled diabetic organism has more inflammatory reactions due to capilary leaks and cell dehydration (Bender & Bender 2003), and that diabetic pulp tisses have shown methabolic alterations such as altered levels of nitrite and kallikrein (Catanzaro *et al.* 2006).

Evidences show a reduction in the antioxidant defenses of diabetic patients (West 2000). The hyperglycemia produces superoxides, contributing to vascular complications (Rolo & Palmeira 2006). The self-defense against the superoxides is the enzimatic system, consisting in catalase, dismutase and peroxidase (Gaté *et al*. 1999), produced as a result of hydrogen peroxide (H_2O_2) degradation in the mitochondia.

H_2O_2 has also been employed as bleaching agent in the esthetic dentistry. The bleaching efficacy is due to the ability of H_2O_2 to penetrate in the tooth structures, oxidizing the tooth organic molecules (Kina *et al*. 2010, Toledano *et al*. 2011).

Studies have demonstrated the presence of pulp inflammatory reactions (Seale & Wilson 1985, Fugaro *et al*. 2004, de Souza Costa *et al*. 2010, Kina *et al*. 2010), and pulp necrosis (Cintra *et al*. 2013) caused by H_2O_2 gels during dental bleaching procedures. A previous research reported that the H_2O_2 and its degradation products could increase matrix metalloproteinase (MMP)-mediated collagen degradation in dentin (Toledano *et al*. 2011), nevertheless their effect in the pulp collagen are still unclear. As that, the effects of dental bleaching in the pulp tissue of a diabetic subject are not yet stablished. The hypothesis is that the diabetic condition intensify the inflammatory reaction and the maturation of collagen fibers on pulp tissue after dental bleaching when compared to normoglicemic rats. Thus, the purpose of this study was to evaluate the influence of DM on the pulp tissue response, the structuring pattern of pulp ECM and the maturation level of collagen fibers after dental bleaching.

Material and Methods

A total of twenty-eight male Wistar albino rats (200-250 g) with two months-old were used in this study. The animals were housed in a temperature-controlled enviroment (22°C ± 1°C, 70% humidity, and 12h light–dark cycle) and received water and food *ad libitum*. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD). The experimental protocol (CEUA-00886) was approved by the local Ethics Committee.

Fourteen animals were randomly selected and received via intramuscular (IM), a single dose of alloxan (150 mg/kg), dilluted in citrate buffer. The other animals

received an injection of citrate buffer, via IM, to simulate a fictional induction of DM. After 72h, blood samples were collected from a small puncture in the tail from each animal to determine their blood glucose levels. Animals with glycemic levels over 200 mg/dl were considered hyperglycemic (Cintra *et al*. 2014).

All animals were anesthetized by intramuscular injection of ketamine (87 mg/kg, Francotar; Virbac do Brasil Ind e Com Ltda; Roseira, SP, Brazil) and xylazine (13 mg/kg, Rompum; Bayer SA, São Paulo, SP, Brazil). The animals were assigned into 4 groups of 7 hemi-maxillae each: N, normoglycemic; NBle, normoglycemic-bleached; D, diabetic, DBle, diabetic-bleached. About 0.01 ml of 35% H_2O_2 gel (Whiteness HP Maxx, FGM Produtos Odontológicos, Joinville, SC, Brazil) was applied on the upper right molars, once, for 30 minutes and then washed. The left molars were used as control.

Two and 30 days after dental bleaching procedure, the animals were euthanized with an overdose of anesthetic solution. The right and left maxillae were separated, dissected, and fixed in a solution of 4% buffered formaldehyde for 24 hours and decalcified in 10% EDTA for 75 days. The tissues processed in a conventional manner and embedded in paraffin. Five-microns sections were cut in the mesiosagittal plane and stained with Hematoxylin-Eosin (H.E.), Masson's trichrome (MT), and Picrosirius Red (PSR).

For the microscopic analysis, five serial histological sections of each specimen were examined by light microscopy (400x magnification; DM4000 B, Leica Microsystems, Wetzlar, Germany). The histological sections stained by hematoxylin-eosin (HE) were used for histopathological and histometric analysis. The intensity of inflammation was scored according to the inflammatory infiltrate, as follows: 0, no or few cells (normal); 1, <25 cells (mild); 2, 25–125 cells (moderate); and 3, >125 cells (severe or necrosis) (Cintra *et al*. 2013). For the histometric analysis of pulp chamber area was measured with the aid of an image processing software (100x

magnification; Leica QWin V3, Leica Microsystems), obtaining the total area in the control and experimental groups. Based on obtained data, the percentage area reduction of the pulp chamber was calculated in the treated groups (NBle and DBle).

The maturation levels of the collagen fibers were analyzed in the sections stained by PSR, seen under polarized light microscopy. The program QWin was used (x400 magnification; Leica QWin V3, Leica Microsystems), allowing the selection of correspondent colors to each type of collagen fibers in the pulp tissue. After the color selection, the program automatically calculated the marked area of each collagen type inside the pulp chamber. The greenish-yellow fibers are considered immature and thin, and the yellowish-red, mature fibers and thick (Junqueira *et al*. 1982).

Statistical analysis

Data were collected and analyzed by a single calibrated blinded-operator. One-way analysis of variance (Anova) and Kruskal-Wallis test were used for statistical comparisons of pulp damage and inflammatory response. For the reduction of pulp chamber area and collagen fibers quantification, the Anova and Tukey test. All statistical analysis were applied at a significance level of 5% ($p<0.05$).

Results

Effects of dental bleaching were compared by the same parameters between the groups. The scores atributed to each group are present in table 1 and the sample photomicrographs analysis in the different experimental groups are shown in figure 1.

At 2 days, a high inflammatory response was observed in the bleached groups, with disorganization of the odontoblastic and sub-odontoblastic layer and ECM, congested blood vessels and destruction of the loose connective tissue of the pulp central area (Fig 1 NBle, DBle). The NBle group showed a predominant mild

inflammation, while the DBle group showed a higher inflammation ($p < 0.05$), with 4 specimens presenting necrosis in the pulp horns. These alterations were not seen in any specimen of N and D groups, that presented histological characteristics of normal pulp tissue, with predentin layer, a well-defined acellular and cell-rich layer underneath an intact odontoblastic layer, blood vessels and ECM structured at both experimental periods.

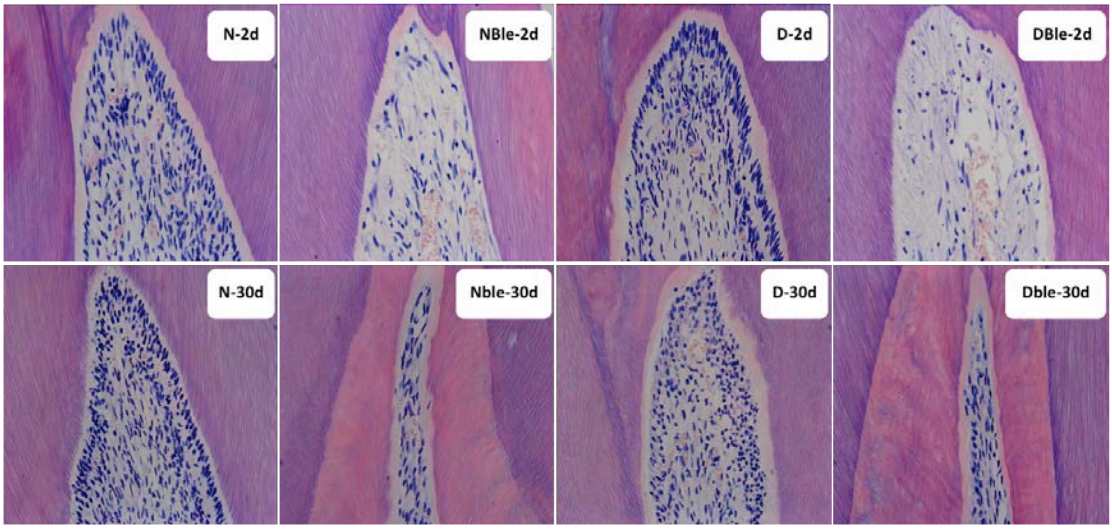

Figure 1 Histological sections of the pulp horns in experimental groups at 2 and 30 days. At 2 days, normoglycemic (N-2d) and diabetic (D-2d) groups present a well-defined ECM structure, the presence of a thin predentin, odontoblastic layer and blood vessels; normoglycemic-bleached (NBle-2d) and diabetic-bleached (DBle-2d) groups, presented inflammatory infiltrate, absence of predentin and odontoblastic layer and areas of necrosis underneath the pulp horns. At 30 days, the control groups (N-30d and D-30d) did not present any alterations, however, the presence of reactionary dentin was observed in the bleached specimens (NBle-30d and DBle-30d), with no inflammatory reaction (HE staining, 400x magnification).

Table 1 Scores observed for pulp tissue inflammatory reaction according to the groups

Period	Score	Groups			
		N	NBle	D	DBle
2 days	0	7/7	0/7	7/7	0/7
	1	0/7	5/7	0/7	1/7
	2	0/7	2/7	0/7	1/7
	3	0/7	0/7	0/7	5/7
	Median	0^{a}	1^{b}	0^{a}	3^{c}
30 days	0	7/7	7/7	7/7	7/7
	1	0/7	0/7	0/7	0/7
	2	0/7	0/7	0/7	0/7
	3	0/7	0/7	0/7	0/7
	Median	0^{a}	0^{a}	0^{a}	0^{a}

*Different letters indicate significant statistical differences ($p < 0.05$)

N: normoglycemic, NBle: normoglicemic-bleached, D: diabetic, DBle: diabetic-bleached

Thirty days after the dental bleaching, the NBle and DBle groups presented the formation of reactionary dentin in the entire pulp chamber. The group DBle showed the lower mean value for the pulp chamber area and the higher percentage reduction, with significant difference when compared to NBle group ($p<0.05$). The N and D groups did not present the formation of reactionary dentin. The mean area values and percentage reduction, of all groups, are shown in table 2.

Table 2 Pulp chamber mean area ($x10^4$ mm^2) and reduction (%) 30 days after dental bleaching session

Groups	Mean ± SD*		Reduction	n
	2 dias	30 dias		
N	72,10 ± 21,53^{Aa}	73,74 ±18,14 ^{Aa}	-	7
NBle	71,79 ± 18,69 ^{Aa}	35,26 ±5,81^{Bb}	52,18%	7
D	70,79 ± 18,16 ^{Aa}	74,91 ±14,88^{Aa}	-	7
DBle	78,00 ± 33,30 ^{Aa}	15,41 ±03,05^{Cb}	79,43%	7

*Different uppercase at the same collum and lowercase letters at the same line indicate significant statistical differences ($p< 0.05$);
N: normoglycemic, NBle: normoglicemic-bleached, D: diabetic, DBle: diabetic-bleached.

When observed the predominance of collagen fibers (Fig 2), the statistical analysis of immature fibers showed that the N group presented a higher percentual number of birrefringent structures in the pulp tissue at 2 days, with significant difference when compared to the other groups ($p< 0.05$). However, the percentual number of mature fibers was higher for the diabetic group, followed by DBle and NBle groups, with significant difference when compared to N group ($p< 0.05$), that presented the lowest percentual value. At 30 days, no significant difference was observed between the groups, for both fiber types ($p> 0.05$) (Table 3).

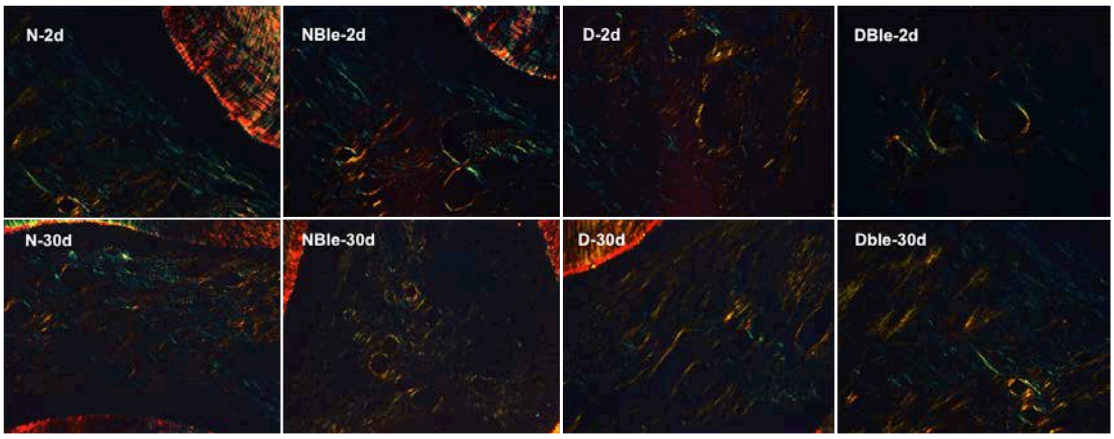

Figure 2 An overview of the histological sections of experimental groups, stained with picrosirius red (PSR) and analyzed under polarized light microscopy. Collagen fibers are shown as birrefringent structures in the pulp tissue. Greenish-yellow fibers indicate immature collagen, yellowish-red fibers indicate mature collagen. Groups: normoglycemic (N), normoglycemic-bleached (NBle), Diabetic (D), diabetic-bleached (DBle). Experimental periods: 2 and 30 days. (PSR staining, 400x magnification).

The MT stainnig allows the expression of collagen to be visualized in the dental pulp of rats as blue-stained tissue in all groups in both experimental periods (Fig 3), however it not allow the differentiation between immature and mature collagen fibers. This technique also enables other tissue structures to be visualized as nucleos in black and citoplasm and queratin in red. The collagen fibers were not seen in the same parallel disposition. NBle and DBle groups at 2 days presented an tissue disorganization and necrosis areas in the pulp horns, with a loose arrangement of collagen fibers in the adjaent tissue. The arrangement of fibers in the N and D groups were still loose, but with well-defined structures of a health and organized tissue.

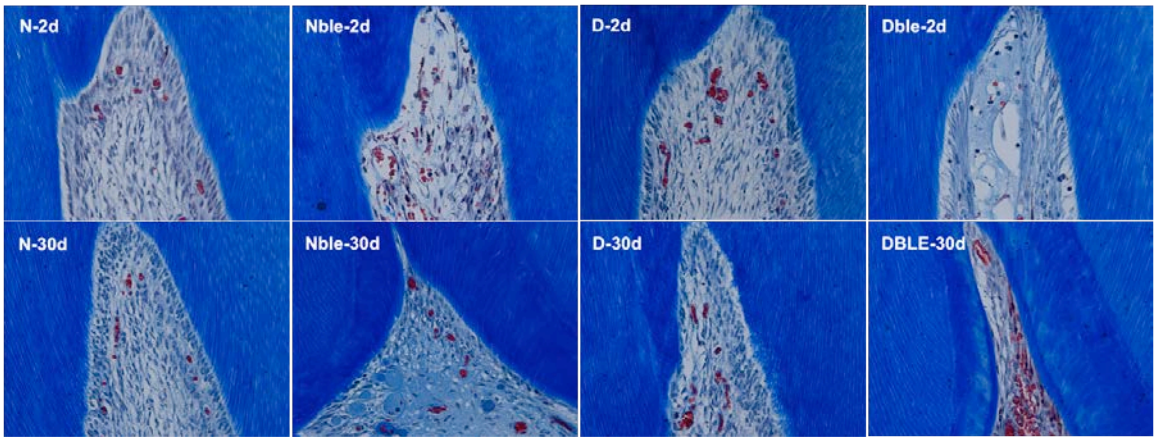

Figure 3 An overview of the histological sections of experimental groups normoglycemic (N), normoglycemic-bleached (NBle), diabetic (D), diabetic-bleached (DBle). Experimental periods: 2 days and 30 days (MT staining, 400x magnification).

Table 3 Percentual values of birrefringent fibers collagen in the pulp tissue of the groups, in both experimental periods.

Groups	Fibers (%)				n
	2 days		30 days		
	Immature	Mature	Immature	Mature	
N	83.57 ± 11.98 [a]	16.43 ± 11.98 [a]	45.00 ± 26.01 [a]	55.00 ± 26.01 [a]	7
NBle	54.71 ± 14.85 [b]	45.29 ± 14.85 [b]	31.71± 12.24 [a]	68.29 ± 12.24 [a]	7
D	41.57 ± 08.40 [b]	58.43 ± 08.40 [b]	53.85 ± 20.42 [a]	46.15 ± 20.42 [a]	7
DBle	42.85 ± 05.70 [b]	57.15 ± 05.78 [b]	45.42 ± 09.65 [a]	54.28 ± 09.65 [a]	7

*Different letters indicate significant statistical differences ($p < 0.05$)

Discussion:

The results showed that DM influences the severity of inflammatory response, with necrosis in the pulp horns of some specimens and a greater formation of reactionary dentin in the pulp tissue of rats submitted to bleaching procedures. The hypothesis was accepted, since the DM induced an intense inflammatory reaction in the bleached group and more mature collagen fibers in the pulp tissue, indicating the pulp fibrosis.

Cell damages caused by H_2O_2 gels have been widely studied, since this product, used in the dental bleaching therapy at high concentrations, has become one of the most common product in clinical procedures in the esthetic dentistry (Coldebella *et al*. 2009). Studies reported that H_2O_2 can penetrate through enamel and dentin, reaching the pulp tissues (Benetti *et al*. 2004, Marson *et al*. 2015).

The intense and fast H_2O_2 penetration in dental tissues may be further the repair capacity of pulp tissue, resulting in immediate response of pulp to this agressive agent, not knowing exactly the amount of H_2O_2 that pulp tissue can tolerate without suffer damages (Seale *et al*. 1985).

As noted in this study, histological sections stained with HE and MT showed the major inflammatory reactions, tissue destruction and loss of collagen in the pulp horns of rats teeth submited to dental bleaching, especially in the DBle group followed by NBle.

Diabetic subjects have an impared oral wound healing, especially with an uncontrolled DM, which might be explained by the disruption of the vasculature and factors as apoptosis (Nagy *et al*. 2001), leading to chronical irritation of the dental pulp (Garber *et al*. 2009) and greater inflammatory reactions (Bender & Bender 2003). The pulp defense system is activated after an injury, releasing antioxidant agents, such as catalases and peroxidases, promoting H_2O_2 degradation, avoiding excessive tissue damage (Esposito *et al*. 2003). The dental pulp is densely innervated, being capable of mediate signals of external tooth damage to the

odontoblastic cells (Norlin *et al*. 1999). The present study observed that health and diabetic dental pulps are capable of promoting a continuous deposition of reactionary dentin over the time, after the use of bleaching agents. However, diabetic group provide more deposition of reactionary dentin. Diabetic pulps are more susceptible to pathologic calcifications as a response to local and systemic stimuli (Inagaki *et al*. 2010). A study with diabetic rats showed that they do not respond to pulp capping procedures as health rats, with no formation of dentin bridges (Garber *et al*. 2009), leading the tissue more susceptible to infections.

The dental pulp is formed by connective tissue, cells and a complex chain of macromolecules, forming the ECM (Linde 1985). One of the main macromolecules of dental pulp ECM are the collagenous proteins. The collagen consisting nearly 34% of the ECM proteins, being the mature and immature collagens the most predominants (Abrahão *et al*. 2006).

The concomitant analysis of immature and mature collagen bundles in the pulp tissue of health and diabetic rats submitted to dental bleaching, has not yet been done. Studies that evaluated the distribution of collagen in the pulp tissue have found predominantly collagen type I and III or mature and immature respectively (Garcia *et al*. 2003, Pereira *et al.* 2014). Among the available techniques for collagen fibers detection, there are two widely used, the PSR and MT. However, MT technique is not capable of distinguish the collagen fiber types, specially when this protein is in small amounts or too thin, showing the collagen as a total, differing from the PSR staining, which is an specific method for collagen fibers detection. The difference of fiber colors are due to the pack of collagen fibers. Greenish-yellow colors sugest that the collagen is poorly packed, while yellowish-red color are originated from tightly packed fibers (Junqueira *et al*. 1982).

Studies demonstrated that home or in-office bleaching agents are capable to induce collagen degradation in the hard tissues of teeth (Toledano *et al*. 2011), promoting structural and biochemical changes (Sato *et al*. 2013). In most specimens

of the present study, the collagens fibers were seen predominantly in the middle and cervical thirds of the pulp chamber. This might be due the ECM destruction after the dental bleaching, specially in the period of 2 days. The groups submitted to dental bleaching or only with DM presented a significant increase in the quantity of mature fibers when compared to a normal and health pulp at 2 days.

Conclusion

The DM influences the inflammatory tissue response in rats teeth after dental bleaching and increase reactionary dentin deposition. The DM promoted an increase of mature collagen fibers, despite the dental belaching procedure.

Acknowledgments

The authors deny any conflict of interent.

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ARTIGO 2

Journal of Endodontics

Dental bleaching influences the expression of proinflammatory cytokines on the pulp tissue of diabetic rats

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Dental bleaching influences the expression of proinflammatory cytokines on the pulp tissue of diabetic rats

Introduction: Evaluate the influence of dental bleaching on the expression of IL-6, TNF-α and IL-17 in the pulp tissue of normoglicemic and diabetic rats. **Methods:** Twenty-eight rats were divided into groups: (N) normoglycemic; (NBle) normoglycemic-bleached, (D) diabetic, (DBle) diabetic-bleached. Diabetes mellitus (DM) was induced with alloxan in fourteen animals. After confirmation of DM, all animals were sedated and the dental bleaching performed using 35% hydrogen peroxide in the right maxillary molars for 30 minutes. Left molars were used as control. After 2 and 30 days, the animals were euthanized, and the hemi-maxillae processed for histological and immunohistochemical analysis. **Results:** At two days, mild inflammation was observed in NBle and severe in DBle ($p<0.05$), reducing to absent after 30 days in both groups ($p>0.05$). On immunoistochemistry, was observed for IL-6 at 2 days an increased immunolabelling in the bleached groups ($p<0.05$) with significant difference between N and DBle after 30 days ($p<0.05$). For TNF-α, was observed a high immunolabelling of bleached groups at 2 days, with significant difference compared to non-bleached groups ($p<0.05$). At 30 days, the difference was found between N and DBle ($p<0.05$). For IL-17, at 2 days, there was a superior immunolabelling in NBle, with significant difference compared to N and D ($p<0.05$). Thirty days after, no statistical difference was observed ($p>0.05$).

Conclusion: DM potencializes the inflammatory pulp reaction as a result of dental bleaching. The dental bleaching is capable of promoting an increase of IL-6 and TNF-α in the pulp tissue regardless the DM, however the DM contributed to the maintenance of IL-6 and TNF-α for longer period. Still, the dental bleaching influenced the increse of IL-17 in the early periods in nomoglycemic rats.

Key-words: Inflammatory response, diabetes mellitus, dental bleaching, proinflammatory cytokines.

Introduction

The dental bleaching is based on the diffusion capacity of H_2O_2 due its low molecular weight (1). As higher the concentration and application time of H_2O_2, higher will be the release of reactive oxygen species (ROS), promoting damages to the hard tissues of teeth and dental pulp (1). The products of H_2O_2 degradation results in celullar and pulp damages, specially in the odontblastic layer (2) promoting a reduction in the cell proliferation and necrosis (3). Studies have shown the deleterious effects of dental bleaching in the pulp tissue (4,5). However, the effects of dental bleaching on uncontrolled diabetic patients are still unknown.

The DM is a metabolic disorder capable of causing per se structural alterations in the pulp tissue (6). It has become a common disease, affecting the proteins, carbohydrates and lipids methabolism resulting in hyperglycemia due to defects in insulin production or function (7). This poor glycemic control might result in chronical inflammation, inhibition of of defense cells, impaired wound healing and damaged organs and tissues (8).

Research studies demonstrated that DM in animal model impair the dentine bridge formation (8), modify the paramenters of antioxidant system of dental pulp tissues (6), promote more calcifications in the dental pulp (9) and increase and exacerbate the expression of proinflammatory mediators (10).

Among the proinflammatory mediators, cytokines have low molecular weight (11), which main function is the recognition, neutralization and destruction of the antigens (12). Elevated blood levels of glucose might be associated to decrease or increase of proinflammatory cytokines, as interleukin (IL)-6, tumor necrosis factor (TNF)-α (10,13) and IL-17 (14).

The IL-6 is a pleotropic and proinflammatory cytokine. TNF-α (15) and IL-17 (16) act sinergistically in the production of IL-6 , being associated with high levels of blood glucose (17). The IL-6 (18) and TNF-α (19) are often associated with the

severity of clinical symptoms, whereas IL-17 acts in the DM pathogenesis (14), inflammatory response and cells recruitment (20).

Alterations resulted of DM have been widely studied in dentistry, however to our knowledge, no previous study compared the effects of dental bleaching in the pulp tissue of diabetic rats. The objective of this study was to analyze the influence of dental bleaching on the inflammatory cell migration and production of IL-6, TNF-α and IL-17 in the pulp tissue of normoglycemic and diabetic rats.

Material and Methods

A total of twenty-eight male Wistar albino rats (200-250g) of two months-old were used in this study. The animals were housed in a temperature-controlled enviroment (22°C ± 1°C, 70% humidity, and 12 h light–dark cycle) and received water and food *ad libitum*. All the animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD). The experimental protocol (CEUA-00886) was approved by the local Ethics Committee.

Diabetes mellitus induction

Fourteen animals were randomly selected and received a single dose of (150mg/kg) of alloxan (Sigma-Aldrich Co, St Louis, Mo, USA), via intramuscular, dilluted in citrate buffer. After 72 h, blood samples were collected (Accu-Check Performa, Roche Diagnostics Corporation, Indianapois, IN, USA) from each animal to determine their glycemic levels. Animals with blood glucose levels over 200mg/dl were considered the threshold for hyperglycemia (21).

Dental bleaching

All animals were anesthetized by intramuscular injection of ketamine (87 mg/kg, Francotar; Virbac do Brasil Ind e Com Ltda; Roseira, SP, Brazil) and xylazine

(13 mg/kg, Rompum; Bayer SA, São Paulo, SP, Brazil).

The rats were assigned into 4 groups of 7 hemi-maxillae as follows: (N) normoglycemic; (NBle) normoglycemic-bleached, (D) diabetic, (DBle) diabetic-bleached. It was applied about 0.01 ml of 35% H_2O_2 gel (Whiteness HP Maxx, FGM Produtos Odontológicos, Joinville, SC, Brazil) on the upper right molars, once, for 30 min and then washed. The left upper molars were used as control.

Laboratory procedures

Two and 30 days after dental bleaching, the animals were euthanized with an overdose of anesthetic solution. The right and left maxillae were separated, dissected, and fixed in 4% buffered formaldehyde for 24 h and decalcified in 10% EDTA for 75 days. The tissues were prepared in a conventional manner and embedded in paraffin. Five-microns sections were cut and stained with Hematoxylin-Eosin (H.E.) or submitted to immunohistochemistry for IL-6, TNF-α and IL-17.

For immunohistochemical reactions, the histological sections were deparaffinized in xylene and hydrated in a decreasing ethanol series. Antigen retrieval was achieved by immersing the histological slides in buffer citrate solution (Antigen Retrieval Buffer, Spring Bioscience, Pleasanton, CA, USA) in a pressurized chamber (Decloaking Chamber®; Biocare Medical) at 95°C for 10 min. The slides were rinsed with phosphate-buffered saline (PBS) at the end of each stage of the immunohistochemical reaction. The histological sections were immersed in 3% H_2O_2 solution for 1 h 20 min and in 1% bovine serum albumin for 12 h to block the endogenous peroxidase activity and the nonspecific sites, respectively. The histological slides were divided and incubated with one of the following primary antibodies: rabbit anti-IL-6 (1:100, rabbit anti-IL-6, SC 1265; Santa Cruz Biotechnology, Santa Cruz, CA, USA); goat anti-TNF-α (1:100, goat anti-TNF-α, SC 1350; Santa Cruz Biotechnology); or rabbit anti-IL-17 (1:100, rabbit anti-IL-17, SC

7927; Santa Cruz Biotechnology). The primary antibodies were diluted (Antibody Diluent with Background Reducing Components, Dako Laboratories, Carpinteria, CA, USA), and placed in a moist chamber for 24 h. The histological sections were incubated with a biotinylated secondary antibody for 1 h 30 min and were subsequently treated with streptavidin–horseradish peroxidase conjugate for 1 h 30 min (Universal Dako Labeled Streptavidin-Biotin kit®; Dako Laboratories, Carpinteria, CA, USA). The slides were rinsed with PBS and the reaction was developed using the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB Chromogen kit®; Dako Laboratories, Carpinteria, CA, USA) and was counterstained with Harris's Hematoxylin. The negative controls consisted of specimens submitted to the procedures previously mentioned but without the primary antibodies.

Positive immunostaining was defined as brownish color in the cell cytoplasm and extracellular matrix (ECM). The analysis was performed under 400x magnification in light microscopy (DM 4000 B, Leica, Wetzlar, Germany). The criteria for inflammatory reaction was adapted from Cintra et al. (5) as follows: 0, inflammatory cells absent or negligible in number; 1, mild inflammatory infiltrate, 2, moderate inflammatory infiltrate, 3, severe inflammatory infiltrate or necrosis; and the immunohistochemistry was adapted from Garcia et al (22): 0, absent immunolabeling; 1, low immunolabeling; 2, moderate immunolabeling; 3, high immunolabeling and 4, extremely high immunolabeling. The inflammatory reaction and immunohistochemistry were scored in accordance with the average number of inflammatory cells and immunolabelled cells presented in the coronal pulp of the teeth.

Statistical analysis

Data were collected and analyzed by a single calibrated blinded-operator. One-way analysis of variance and Kruskal-Wallis test were used for statistical

comparisons of pulp damage, inflammatory response and immunohistochemistry at the significance level of 5% ($p<0.05$).

Results

Inflammatory response:

The groups N and D exhibited well-defined odontoblastic and sub-odontoblastic layer (-poor and cell-rich layers), and in the central area, a loose connctive tissue, with blood vessels and well-structured ECM ($p>0.05$) (Fig. 1, 2-HE) during both experimental periods.

Two days after the dental bleaching, the pulp tissue of NBle and DBle groups presented discontinuity in the odontoblastic layer, disorganized ECM in the central pulp, with an inflammatory infiltrate predominantly composed by neutrophils and eosinophils. Necrosis was observed in the pulp horns of some specimens of DBle group (Fig.1). The inflammatory response was predominantly mild for NBle, however four specimens of DBle group showed score 3, as a results of severe inflammation/necrosis in the pulp horns ($p<0.05$).

At 30 days, the repair of the pulp tissue was observed, with absence of inflammatory reaction or areas of pulp necrosis in the NBle and DBle groups respectively ($p>0.05$) (Table 1). All experimental groups showed a well-defined odontoblastic and sub-odontoblastic layer, a well-structured connective tissue and the presence of thicker predentin in all specimens of NBle, D and DBle groups. The formation of reactionary dentin was observed in the coronal pulp of all bleached teeth (Fig. 2).

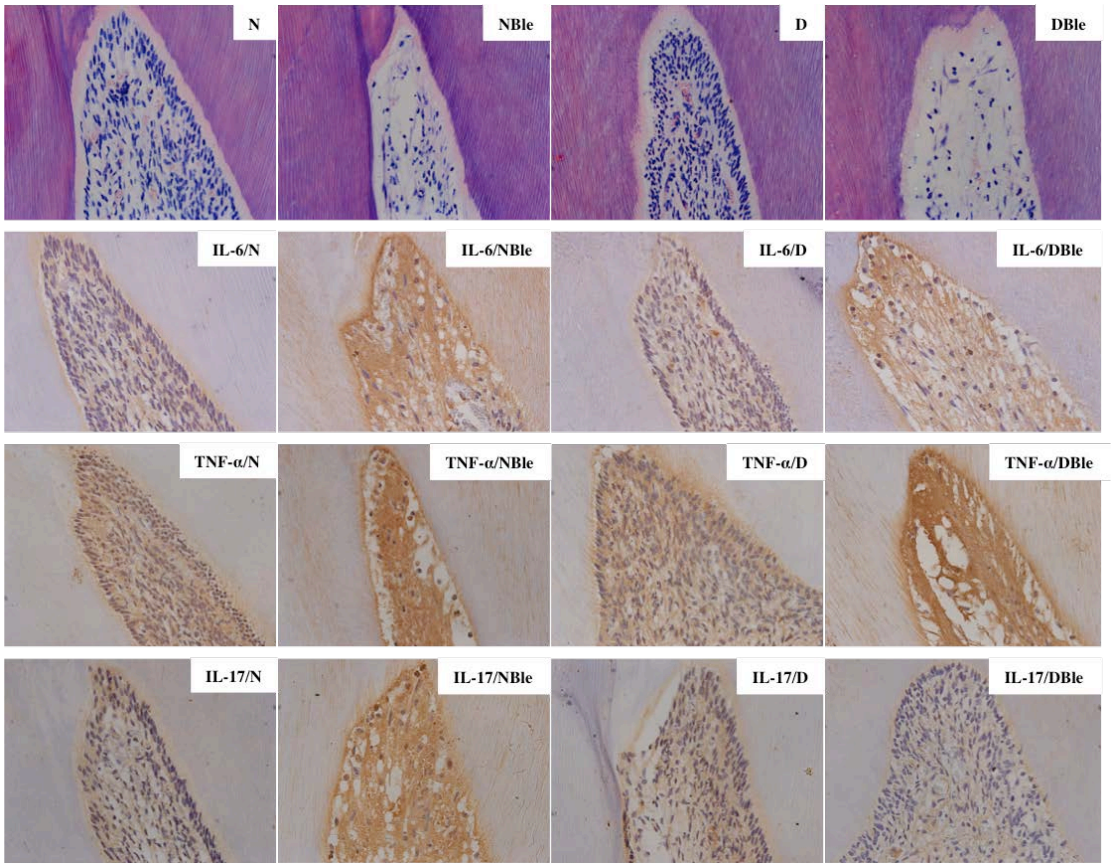

Fig. 1: Representative images of specimens pulp horns at experimental period of 2 days: HE, IL-6, TNF-a, IL-17 respectively (400x magnification). Groups: Normoglicemic (N), Normoglicemic-bleached (NBle), Diabetic (D), Diabetic-bleached (DBle).

Immunohistochemistry:

The immunoistochemistry technique employed for IL-6, TNF-α e IL-17 detection, presented high specificity, since the negative controlremained unstained (data not shown). The cytokines immunolabeling was present predominantly in inflammatory cells and ECM. All score values atributed for proinflammatory cytokines are presented in table 1.

For IL-6, specimens of N and D groups showed low standard immunolabeling in both experimental periods. At two days, the groups NBle and DBle showed

immunolabeled cells, with moderate predominance for NBle and varing from low to high for DBle, with significant difference when compared NBle and DBle to N and D groups ($p<0.05$) (Fig 1). Thirty days after, there was a moderate immunolabeling for NBle and DBle (Fig 2), with significant difference for DBle and N groups.

Specimens of N and D groups presented immunolabeling varing from low to moderate in both experimental times for TNF-α. Intense immunolabeling was present for NBle and high to extremelly high for DCla, with significant difference when compared the bleached groups to N and D groups ($p<0.05$) (Fig 1). At 30 days, the TNF-α presented an immunolabeling ranging from low to moderate for NBle and low to high for DBle (Fig 2), with significant difference between DBle and N groups ($p<0.05$).

The IL-17 presented a predominant low immunolabeling for N, D and DBle ($p>0.05$), nevertheless the NBle group presented five specimens with moderate immunolabeling, with significant difference when compared to the other groups ($p<0.05$) (Fig 1). Thirty days after, IL-17 immunoreaction was mainly low for all groups ($p>0.05$) (Fig 2).

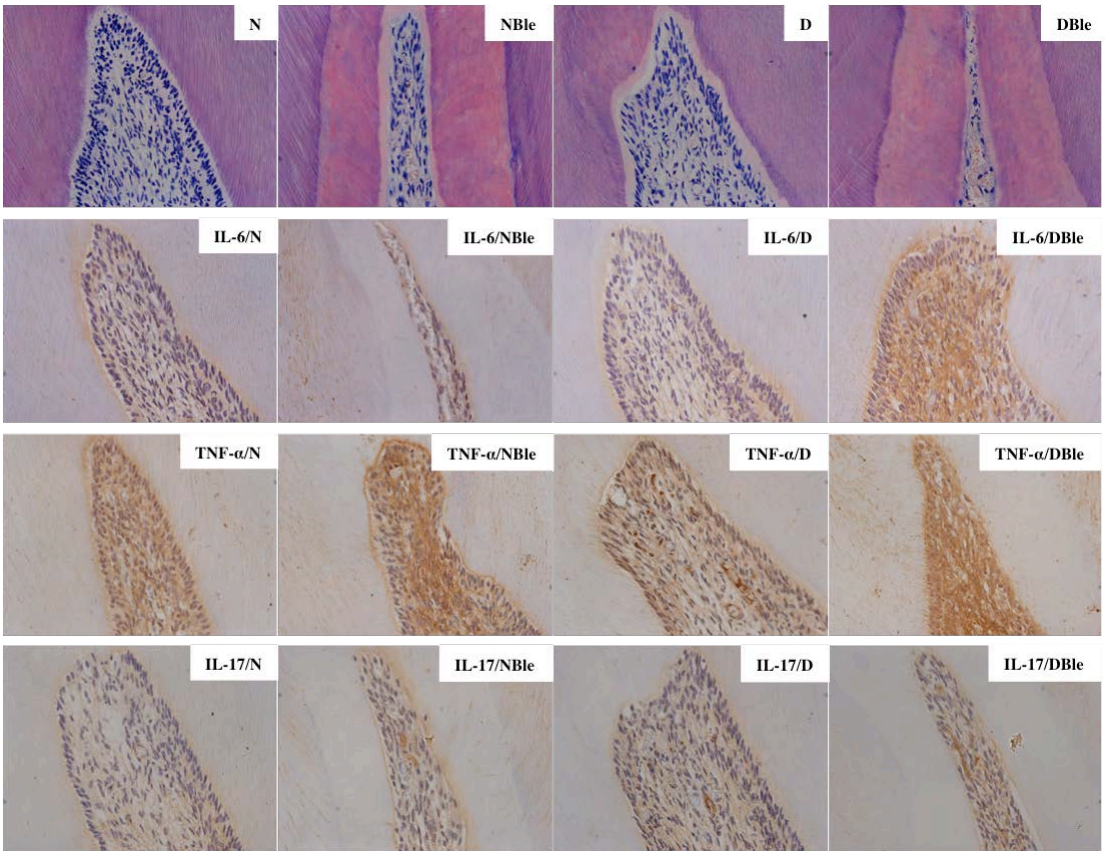

Fig. 2: Representative images of specimens pulp horns at experimental period of 30 days: HE, IL-6, TNF-a, IL-17 respectively (400x magnification). Groups: Normoglicemic (N), Normoglicemic-bleached (NBle), Diabetic (D), Diabetic-bleached (DBle).

Table 1: Scores observed for inflammatory response and immunohistochemistry according to the groups

Staining	Score	Groups				Statistical analysis
		N	NBle	D	DBle	
2D						
H.E	0	7/7	0/7	7/7	0/7	
	1	0/7	5/7	0/7	1/7	Kruskal-wallis
	2	0/7	2/7	0/7	2/7	
	3	0/7	0/7	0/7	4/7	*P= 0.000014*
	Median	0^{a}	1^{b}	0^{a}	3^{c}	
IL-6	0	0/7	0/7	0/7	0/7	
	1	7/7	0/7	7/7	1/7	
	2	0/7	6/7	0/7	3/7	Kruskal-wallis
	3	0/7	1/7	0/7	3/7	*P= 0.000080*
	4	0/7	0/7	0/7	0/7	
	Median	1^{a}	2^{b}	1^{a}	2^{b}	
TNF-a	0	0/7	0/7	0/7	0/7	
	1	5/7	0/7	5/7	0/7	
	2	2/7	1/7	2/7	0/7	Kruskal-wallis
	3	0/7	6/7	0/7	4/7	*P= 0.000053*
	4	0/7	0/7	0/7	3/7	
	Median	1^{a}	3^{b}	1^{a}	3^{b}	
IL-17	0	0/7	0/7	0/7	0/7	
	1	6/7	0/7	6/7	4/7	
	2	1/7	5/7	1/7	3/7	Kruskal-wallis
	3	0/7	2/7	0/7	0/7	*P= 0.001932*
	4	0/7	0/7	0/7	0/7	
	Median	1^{a}	2^{b}	1^{a}	1^{ab}	
30D						
H.E	0	7/7	7/7	7/7	7/7	
	1	0/7	0/7	0/7	0/7	Kruskal-wallis
	2	0/7	0/7	0/7	0/7	*P=1*
	3	0/7	0/7	0/7	0/7	
	Median	0^{a}	0^{a}	0^{a}	0^{a}	
IL-6	0	0/7	0/7	0/7	0/7	
	1	7/7	4/7	6/7	1/7	
	2	0/7	3/7	1/7	6/7	Kruskal-wallis
	3	0/7	0/7	0/7	0/7	*P* = 0.005586
	4	0/7	0/7	0/7	0/7	
	Median	1^{a}	1^{ab}	1^{ab}	2^{b}	
TNF-a	0	0/7	0/7	0/7	0/7	
	1	6/7	3/7	5/7	1/7	
	2	1/7	4/7	2/7	3/7	Kruskal-wallis
	3	0/7	0/7	0/7	3/7	*P* = 0.015696
	4	0/7	0/7	0/7	0/7	
	Median	1^{a}	2^{ab}	1^{ab}	2^{b}	
IL-17	0	0/7	0/7	0/7	0/7	
	1	6/7	4/7	6/7	4/7	
	2	1/7	3/7	1/7	3/7	
	3	0/7	0/7	0/7	0/7	Kruskal-wallis
	4	0/7	0/7	0/7	0/7	*P=0.440227*
	Median	1^{a}	1^{a}	1^{a}	1^{a}	

*Same letters on the line indicate no statistical difference among the groups (p> 0.05).

Discussion

Pulp inflammation is characterized by an intense inflammatory infiltrate, and cytokines, capable of regulate the immune response with a protective or detructive role in the progression of a disease (23). It was observed that DM associated with dental bleaching promoted an intense inflammatory response and necrotic areas during the initial phase, with a huge difference in the pulp damages when compared to the NBle group, that showed a less intense inflammatory response with more tissue and cellular disorganization just below the pulp horns. Since there was no total disorganization or necrosis of the entire coronal pulp tissue of NBle and DBle group respectivelly, at 30 days was observed a tissue repair of the pulp, followed by the formation of reactionary dentin, as a response to an injury (24) caused by the bleaching agents.

Inflammatory tissue alterations can also be evaluated by the expression of proinflammatory cytokines (25), and in the presence of DM these cytokines might be altered (7). The glycemic levels and the expression of cytokines involved in the inflammatory response are a two-way relationship. One cytokine can act on several target cells and many cytokines can interact on only one target cell, showing synergistic or antagonistic affects, depending on the microenvironment (26).

The high glucose serum levels may be associated to the upregulation of IL-6, TNF-α (13) and IL-17 (15,21), while the increase expression of these cytokines may promote an insulin resistance impairing the glycemic control (27).

IL-6 is syntetized during the inflammatory reaction as a response to trauma or infecctions (28). A study quantified the expression of IL-6 in apical periodontitis, inflamed pulp and health pulp, and detected significant levels of this interleukin in the apical periodontitis and inflamed pulp when compared with healthy pulps (18). High

levels of IL-6 expression are related to the worsening of inflammation, clinical symptoms (29), and pulpitis (30); high levels of TNF-α in teeth are present in teeth with irreversible pulpitis (31). The present study observed high levels of IL-6 and TNF-α in NBle and DBle groups at 2 days; moreover, 30 days later, the expression of IL-6 was still moderate for the same groups, while the TNF-α presented a slight reduction, which is in accordance with other studies that shows the inhibition of TNF-α and IL-1 expression after IL-6 secretion (32). Elevated levels of proinflammatory cytokines, such as IL-6 and TNF-α are highly expressed in individuals with DM (27), and IL-6 may be considered a strong predictor of DM in at-risk individuals (17).

Once produced, IL-6 and TNF-a may exert a beneficial or deleterious effect, depending on the amount and time for which its production remains active (33). TNF-a and others cytokines were detected in the interstitial fluid of an inflammatory response triggered by LPS (34). A recent study evaluated the expression of inflammatory cytokines in an inflamed pulp tissue, and observed that the expression of proinflammatory cytokines, including TNF-α, were elevated, reaching the highest peak one day after stablished the pulpitis (19).

Interleukin-17 is a cytokine produced by actived Th17 cells, being responsible for the recruitment of inflammatory cells, induction of other proinflammatory cytokines (20) and increase of neuthophils number (21). IL-1β and TNF-α cytokines are responsable for increase the IL-17 response, induced by TGF-β and IL-6, however, they cannot substitute none of these cytokines (35).

Human dental pulp fibroblasts showed an increase of IL-17 in inflamed pulp tissue, and the stimuli of those cells with IL-17 caused an increase in the IL-6 expression (16). These results are in accordance with the present results, observing a positive correlation between the immunolabeling of IL-17 on the pulp tissue at 2 days in the NBle group, and 30 days after the levels of IL-17 were reduced in all

groups, since there was no more inflammation, with a predominantly low immunolabeling for all groups ($p>0.05$). However, the strong action of H_2O_2 in the pulp tissue might have affected the expression of IL-17 in the DBle in the inicial inflammatory period, explaing the low immunolabeling for this group. Low levels of Th1/Th2 response in the presence of DM can indicate a prevalence of Th17 cells, and, high levels of Th1/Th2 can interfere the development of Th17 cells (14).

The transition of acute to chronic may be associated with the late increase of other cytokines involved in the inflammatory response (21). Nevertheless, more researches are necessary for a better comprehention of cytokines involved in the inflammatory process after dental bleaching in the presence of DM.

Conclusion

The diabetes potencialize the inflammatory reaction on pulp tissue due to the dental bleaching. The dental bleaching is capable of promoting an increase of IL-6 and TNF-α in the pulp tissue regardless the presence of diabetes, however the dibetes contributed to the maintenance of IL-6 and TNF-α for a longer period. Still, the dental bleaching influenced the increse of IL-17 in the early periods in nomoglycemic rats.

Acknowledgments

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ANEXOS

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

Comitê de Ética no Uso de Animais (CEUA)
Committee for Ethical Use of Animals (CEUA)

CERTIFICADO

Certificamos que o Projeto **"Influência da clareação dentária na quantificação das citocinas pró-inflamatórias IL-6, IL-17 e TNF-α em tecido pulpar de ratos diabéticos"** sob responsabilidade do Pesquisador **LUCIANO TAVARES ANGELO CINTRA** e colaboração de Luciana Louzada Ferreira, Renata Oliveira Samuel, Aguinaldo Candido da Silva Facundo, Annelise Katrine Carrara Prieto e Mariane Maffei Azuma está de acordo com os Princípios Éticos da Experimentação Animal (COBEA) e foi aprovado pelo CEUA, de acordo com o processo **00886-2012**.

CERTIFICATE

We certify that the research **"Dental bleaching influence in quantification of proinflammatory cytokines IL-6, IL-17 and TNF-α in the pulp tissue of diabetics rats"**, process number **00886-2012**, under responsibility of **LUCIANO TAVARES ANGELO CINTRA** and with collaboration of Luciana Louzada Ferreira, Renata Oliveira Samuel, Aguinaldo Candido da Silva Facundo, Annelise Katrine Carrara Prieto and Mariane Maffei Azuma agree with Ethical Principles in Animal Research (COBEA) and was approved by CEUA.

Prof. Dr. Edilson Ervolino
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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 www.consort-statement.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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Journal's compliant self-archiving policy please visit: http://www.wiley.com/go/funderstatement.

3. OnlineOpen

OnlineOpen 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 OnlineOpen, 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

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Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper OnlineOpen if you do not wish to. All OnlineOpen 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 e-mail server. Also, the e-mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their whitelist.

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.asp

In 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 book

Wesselink 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 bold ruling 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), 1.5 column 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 typeface 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 instead. 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-dimensional skyscraper 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 typesize 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 a thin space (1 000). Unusual units or abbreviations should be spelled out in full or defined 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 explanations in 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.

Permissions: If all or part of previously published illustrations are to be used, permission must be obtained from the copyright holder concerned. This is the responsibilty of the authors before submission.

Preparation of Electronic Figures for Publication: Although low quality images are adequate for review purposes, print publication requires high quality images to prevent the final product being blurred or fuzzy. Submit EPS (lineart) or TIFF (halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Do not use pixel-oriented programmes. Scans (TIFF only) should have a resolution of 300 dpi (halftone) or 600 to 1200 dpi (line drawings) in relation to the reproduction size (see below). EPS files should be saved with fonts embedded (and with a TIFF preview if possible). For scanned images, the scanning resolution (at final image size) should be as follows to ensure good reproduction: lineart: >600 dpi; half-tones (including gel photographs): >300 dpi; figures containing both halftone and line images: >600 dpi.

Further information can be obtained at Wiley Blackwell's guidelines for figures: http:/authorservices.wiley.com/bauthor/illustration.asp.

Check your electronic artwork before submitting it: http://authorservices.wiley.com/bauthor/eachecklist.asp.

5.5. Supporting Information

Publication in electronic formats has created opportunities for adding details or whole sections in the electronic version only. Authors need to work closely with the editors in developing or using such new publication formats.

Supporting information, such as data sets or additional figures or tables, that will not be published in the print edition of the journal, but which will be viewable via the online edition, can be submitted. It should be clearly stated at the time of submission that the supporting information is intended to be made available through the online edition. If the size or format of the supporting information is such that it cannot be

accommodated on the journal's website, the author agrees to make the supporting information available free of charge on a permanent Web site, to which links will be set up from the journal's website. The author must advise Wiley Blackwell if the URL of the website where the supporting information is located changes. The content of the supporting information must not be altered after the paper has been accepted for publication.

The availability of supporting information should be indicated in the main manuscript by a paragraph, to appear after the References, headed 'Supporting Information' and providing titles of figures, tables, etc. In order to protect reviewer anonymity, material posted on the authors Web site cannot be reviewed. The supporting information is an integral part of the article and will be reviewed accordingly.

Preparation of Supporting Information: Although provision of content through the web in any format is straightforward, supporting information is best provided either in web-ready form or in a form that can be conveniently converted into one of the standard web publishing formats:

• Simple word-processing files (.doc or .rtf) for text.

• PDF for more complex, layout-dependent text or page-based material. Acrobat files can be distilled from Postscript by the Publisher, if necessary.

• GIF or JPEG for still graphics. Graphics supplied as EPS or TIFF are also acceptable.

• MPEG or AVI for moving graphics.

Subsequent requests for changes are generally unacceptable, as for printed papers. A charge may be levied for this service.

Video Imaging: For the on-line version of the Journal the submission of illustrative video is encouraged. Authors proposing the use such media should consult with the Editor during manuscript preparation.

Guidelines for Publishing Papers in the Journal of Endodontics

Writing an effective article is a challenging assignment. The following guidelines are provided to assist authors in submitting manuscripts.

The *JOE* publishes original and review articles related to the scientific and applied aspects of endodontics. Moreover, the *JOE* has a diverse readership that includes full-time clinicians, full-time academicians, residents, students and scientists. Effective communication with this diverse readership requires careful attention to writing style.

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 highly discouraged. Instead, the information of the original research should be expressed by new manuscript author's own words, and a proper citation given at the end of the sentence. Plagiarism will not be tolerated and manuscripts will be rejected, or papers withdrawn after publication based on unethical actions by the authors. In addition, authors may be sanctioned for future publication.

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 rejected from publication. All Figures, Charts and Tables should be described in their order of numbering with a brief description of the major findings. Author may consider the use of supplemental figures, tables or video clips that will be published online. Supplemental material is often used to provide additional information or control experiments that support the results section (*e.g.*, microarray data).

6. **Figures:** There are two general types of figures. The first type of figures includes photographs, radiographs or micrographs. Include only essential figures, and even if essential, the use of composite figures containing several panels of photographs is encouraged. For example, most photo-, radio- or micrographs take up one column-width, or about 185 mm wide X 185 mm tall. If instead, you construct a two columns-width figure (*i.e.*, about 175 mm wide X 125 mm high when published in the *JOE*), you would be able to place about 12 panels of photomicrographs (or radiographs, etc.) as an array of four columns across and three rows down (with each panel about 40 X 40 mm). This will require some editing to emphasize the most important feature of each photomicrograph, but it greatly increases the total number of illustrations that you can present in your paper. Remember that each panel must be clearly identified with a letter (*e.g.*, "A," "B," etc.), in order for the reader to understand each individual panel. Several nice examples of composite figures are seen in recent articles by Jeger et al (J Endod 2012;38:884–888); Olivieri et al., (J Endod 2012;38:1007 1011); Tsai et al (J Endod 2012;38:965–970). Please note that color figures may be published at no cost to the authors and authors are encouraged to use color to enhance the value of the illustration. Please note that a multipanel, composite figure only counts as one figure when considering the total number of figures in a manuscript (see section 3, below, for maximum number of allowable figures).

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, consultantships, 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

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