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Rafael Pillar

EFEITO DA SOLUÇÃO DE HIPOCLORITO DE SÓDIO EM PH ALTO E SUA INTERAÇÃO COM CLOREXIDINA NAS PROPRIEDADES VISCOELÁSTICAS DE UM BIOFILME EXPERIMENTAL

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Odontológicas, Área de Concentração em Odontologia, ênfase em Endodontia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Ciências Odontológicas**.

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Renata Dornelles Morgental, Dra. (UFSM)

Santa Maria, RS
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“Grandes realizações não são feitas por impulso,
mas por uma soma de pequenas realizações.”

(Vincent van Gogh)

RESUMO

EFEITO DA SOLUÇÃO DE HIPOCLORITO DE SÓDIO EM PH ALTO E SUA INTERAÇÃO COM CLOREXIDINA NAS PROPRIEDADES VISCOELÁSTICAS DE UM BIOFILME EXPERIMENTAL

AUTOR: Rafael Pillar
ORIENTADOR: Carlos Alexandre Souza Bier

A presente tese é constituída por dois artigos que avaliaram a influência das soluções irrigadoras nas propriedades viscoelásticas do biofilme. O primeiro artigo investigou a influência do NaOCl 3% com pH alcalino, estabilizado com adição de álcali ou solução tampão, nas características viscoelásticas de um modelo de biofilme (CDFF). Cepas bacterianas de *S. oralis* J22 e *A. naeslundii* T14v – J1 foram cultivadas em placas de ágar e introduzidos no sistema CDFF onde o biofilme foi cultivado durante 96h a 37 °C sob o fornecimento contínuo de caldo BHI modificado. O sistema estava equipado com 15 porta-amostras que continham 05 discos de hidroxiapatita (HA), com um recesso de 250 μm. Os discos de HA foram previamente cobertos, durante 14h a 4°C, com uma solução tampão adesiva preparada a partir de saliva humana liofilizada (1,5 g/l). As amostras foram divididas em 05 grupos (n=05): Grupo Controle – sem tratamento; Grupo NaOH – proporção de 1:1 de 2 mol/l de NaOH com água Milli-Q; Grupo NaOCl 3% padrão; Grupo NaOCl 3% estável – (1:1) de NaOCl 6% com 2 mol/l de NaOH e Grupo NaOCl 3% tamponado – (1:1) de NaOCl 6% com tampão de fosfato dissódico (Na_2HPO_4). Foram aplicados 20 μl de cada solução sobre o biofilme em 02 tempos: 60s e 300s. Os espécimes foram submetidas ao teste de compressão de baixa carga (LLCT) com deformações induzidas de 20% e 50% aplicadas em 01s. O relaxamento (%) e os elementos E_1 (elemento rápido: intervalo de tempo t_1 = 0,01s a 0,5s), E_2 (intermediário: t_2 = 0,5s a 3s) e E_3 (lento: t_3 = 3s a 100s) foram monitorados durante 100s. As amostras também foram submetidos a tomografia de coerência ótica (OCT), onde a espessura do biofilme foi mensurada antes e após. Os dados foram submetidos a análise de variância. Nenhuma diferença estatística foi observada nos valores do relaxamento de tensão, não houve diferença entre os elementos e entre a espessura do biofilme antes e depois do tratamento, independentemente do tempo ($p > 0.05$). Concluiu-se que o NaOCl, com uma maior capacidade alcalina, não foi capaz de alterar as propriedades viscoelásticas do biofilme. O segundo estudo avaliou a interação entre o NaOCl 3% com a clorexidina (CHX) 2% sobre as propriedades viscoelásticas descritas acima. O mesmo biofilme experimental foi usado, diferindo nos grupos como segue (n=05): Grupo NaOCl 3% (controle); Grupo CHX 2% (controle 2); Grupo CHX 2% + NaOCl 3% e Grupo NaOCl 3% + CHX 2%. Nos grupos combinados, um neutralizante foi usado entre as trocas. 20 μl foram aplicados, porém apenas por 60s. As amostras foram submetidas as análises por LLCT e OCT, sendo as mesmas variáveis avaliadas. Nenhuma diferença estatística foi encontrada para os grupos em relação ao relaxamento, elementos e espessura do biofilme ($p > 0.05$). Para este estudo, concluiu-se que o uso combinado do NaOCl com a CHX não foi capaz de alterar a viscoelasticidade do biofilme. Diante dos resultados apresentados nos dois trabalhos, compreendemos que o uso do NaOCl com pH alcalino estável ou sua associação com CHX, não foi capaz de modificar as características viscoelásticas de um biofilme dupla-espécie.

Palavras-chave: Biofilmes. Clorexidina. Endodontia. Hipoclorito de Sódio. Matriz Extracelular. Preparo de Canal Radicular.

ABSTRACT

EFFECT OF SODIUM HYPOCHLORITE AT HIGH PH AND ITS INTERACTION WITH CHLORHEXIDINE ON VISCOELASTIC PROPERTIES OF AN EXPERIMENTAL BIOFILM

AUTHOR: Rafael Pillar
ADVISOR: Carlos Alexandre Souza Bier

The present thesis consists of two articles that evaluated the influence of irrigation solutions on the viscoelastic properties of a double-species biofilm. The first study investigated the NaOCl 3% at alkaline pH stabilized by alkali or buffer on viscoelastic characteristics of an experimental biofilm (CDFF). Bacterial strains of *S. oralis* J22 and *A. naeslundii* T14v-J1 were grown on agar plates and introduced into the CDFF system where the biofilm was cultured for 96h at 37°C under continuous supply of modified BHI broth. The system was equipped with 15 samples-holder containing 05 hydroxyapatite (HA) discs, with a recess of 250 μm. HA discs were pre-coated for 14h at 4°C with an adhesion buffer prepared from lyophilized human saliva (1.5 g/l). The samples were divided into 05 groups (n = 05): Control Group - no treatment; NaOH group - 1: 1 ratio of 2 mol/l NaOH with Milli-Q water; 3% NaOCl standard group; 3% NaOCl stabilized group – (1:1) of 6% NaOCl with 2 mol/l NaOH and 3% NaOCl buffered group – (1:1) of 6% NaOCl with disodium phosphate buffer (Na_2HPO_4). A total of 20 μl were applied for 02 times: 60s and 300s. The samples were submitted to the low load compression testing (LLCT) with 20% and 50% deformations applied in 01s. The stress relaxation (%) and the elements: E1 (fast element: time interval t1 = 0.01s to 0.5s), E2 (intermediate: t2 = 0.5s to 3s) and E3 (slow: t3 = 3s to 100s) were monitored for 100s. The samples were submitted to optical coherence tomography (OCT) analysis, where the biofilm thickness was measured before and after treatment. The data were submitted to ANOVA. No statistical difference was observed in the stress relaxation values between the groups. There was also no statistically difference between the elements and the biofilm thickness before and after treatment, regardless of the time exposed ($P > .05$). We concluded that NaOCl solutions with a higher alkaline capacity were not able to alter the biofilm viscoelastic properties. The second study evaluated the interaction between 3% NaOCl and 2% chlorhexidine (CHX) on the viscoelastic properties described above. The same experimental biofilm was used, differing in the experimental groups as follows (n = 05): 3% NaOCl group (control 1); 2% CHX group (control 2); 2% CHX + 3% NaOCl group and 3% NaOCl + 2% CHX group. In the combining groups, a neutralizing solution was used between each exchange. 20 μl was applied. A time of 60s was evaluated. The samples were analyzed by LLCT and OCT with the same variables being evaluated (stress relaxation, elements, biofilm thickness). No statistical difference was found between the groups ($P > .05$). The combined use of NaOCl with CHX was not able to effectively alter the structure of the biofilm. Considering the results presented, it is possible to argue that either the isolated use of NaOCl, even with stable alkaline pH, or its combination with CHX, was not able to modify the viscoelastic characteristics of a dual-species biofilm.

Keywords: Biofilms. Chlorhexidine. Endodontics. Extracellular Matrix. Root Canal Preparation. Sodium Hypochlorite.

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LISTA DE ABREVIATURAS E SIGLAS

BHI	*Brain Heart Infusion* (Caldo BHI)
CDFF	*Constant depth film fermenter*
CHX	*Chlorhexidine Digluconate* (Digluconato de Clorexidina)
E_1	*Fast Element* (Elemento Rápido)
E_2	*Intermediate Element* (Elemento Intermediário)
E_3	*Slow Element* (Elemento Lento)
e.g.	*Exempli Gratia* (Por Exemplo)
EPS	*Matrix of Extracellular Polymeric Substance* (Matriz Extracelular de Substância Polimérica)
HA	*Hydroxyapatite* (Hidroxiapatita)
HOCl	*Hypochlorous Acid* (Ácido Hipocloroso)
h	*Hour(s)* (Hora(s))
OCl^-	Hypochlorite Ion (Íon Hipoclorito)
pH	Potencial Hidrogeniônico
pK_a	Logaritmo Negativo da Constante de Dissociação do Ácido
LLCT	*Low-Load Compression Testing* (Teste de Compressão de Baixa Carga)
Min	*Minute(s)* (Minuto(s))
mol/L	*Molarity* (Molaridade ou Concetração Molar)
n=	Número de Amostras
NaOCl	*Sodium Hypochlorite* (Solução de Hipoclorito de Sódio)
NaOH	*Sodium Hydroxide* (Hidróxido de Sódio)
OCT	*Optical Coherence Tomography* (Tomografia de Coerência Ótica)
τ	*Time* (Tempo)
s	*Second(s)* (Segundo(s))

LISTA DE SÍMBOLOS

°C	Grau Celsius
%	*Percentage* (Porcentagem)
µl	*Microliter* (Microlitro(s))
µm	*Micrometer* (Micrometro(s))
g/L	*Gramme per Liter* (Grama por litro)
>	Maior que
<	Menor que
=	Igual
mL	*Millilter* (Mililitro(s))
mL/h	*Milliter per Hour* (Mililitro por hora)
mM	*Millimolar* (Milimol(s))
W	Watt

SUMÁRIO

1 INTRODUÇÃO

A terapia endodôntica, desde o estabelecimento do correto diagnóstico até a proservação e sucesso do caso clínico, constitui-se de etapas operatórias que estão interligadas e são diretamente dependentes umas às outras, não podendo qualquer destas etapas serem omitidas ou negligenciadas (COHEN e BURNS, 2000). Dentre estas fases cirúrgicas, o preparo biomecânico possui como objetivo principal a limpeza e conformação do canal radicular. Isto é obtido por meio da remoção do tecido pulpar infectado, de restos de tecido necrótico e de microrganismos e seus subprodutos, rompendo assim, o equilíbrio da microbiota presente no interior do sistema de canais e, também, pela modelagem do conduto radicular em uma forma cônica de sentido ápico-cervical, resultando em um espaço propício para a obturação, preservação da saúde apical ou promoção de um meio favorável ao reparo periapical nos casos em que a lesão perirradicular está presente (SCHILDER, 1974).

Essa fase operatória se dá pela ação conjunta entre os instrumentos endodônticos e as propriedades físico-químicas das soluções irrigadoras visando eliminar/reduzir os agentes etiológicos das patologias pulpares e periapicais (LEONARDO, 2005). Embora os instrumentos endodônticos desempenhem um papel essencial, sua ação mecânica não é suficiente para completa limpeza do sistema de canais, pois tendo em vista a complexidade anatômica desse sistema, o processo de antissepsia do instrumento fica restrito à luz do canal principal (PAQUÉ et al., 2010; PETERS, ARIAS e PAQUÉ, 2015; GUIMARÃES et al., 2017). Peters et al (2001) demostraram que cerca de 35% do canal não foi tocado quando a instrumentação rotatória e manual foram realizadas. À vista disso, a limpeza/desinfecção desse complexo sistema de canais depende também do emprego de substâncias auxiliares, que através de suas propriedades químicas, físicas e biológicas auxiliarão no completo preparo químico-mecânico (EL KARIM, KENNEDY e HUSSEY, 2007; LOPES e SIQUEIRA, 2010; STOJICIC et al., 2010).

Mesmo seguindo os preceitos do preparo biomecânico, algumas espécies de microrganismos podem sobreviver a este procedimento de saneamento e se adaptar ao novo ambiente escasso de nutrientes e substratos (SUNDQVIST et al., 1998). Isso pode ser explicado pelo elevado grau de organização bacteriana (VAN DER MEI et al., 2006). Esses microrganismos alocados no sistema de canais radiculares exercem um importante papel no desenvolvimento e perpetuação de patologias periapicais, sendo a eliminação destes um dos propósitos da endodontia, especialmente quando essas bactérias estão estruturadas em uma forma organizada como o biofilme (RICUCCI et al., 2009).

A formação desse biofilme, através da adesão bacteriana a um substrato sólido mediado por moléculas de condicionamento adsorvido à uma superfície (e.g. saliva), é responsável pela proteção bacteriana contra a resposta de defesa do organismo, agentes químicos e tensões mecânicas. Isto ocorre devido a diferença fisiológica e estrutural desse biofilme em relação a microrganismos que crescem em um ambiente livre numa forma planctônica (KOLENBRANDER e LONDON, 1993; FLEMMING et al., 2007; STEWART e FRANKLIN, 2008). Na cavidade oral, já foram identificados cerca de 700 a 1000 tipos de espécies de bactérias presentes no biofilme oral, conhecido também como placa dental (FRIAZ-LOPES e DURAN-PINEDO, 2012; DARRENE e CECILE, 2016).

Muitas vezes, a patogenicidade do biofilme oral é resultado de uma mudança na composição bacteriana devido às alterações ambientais, como por exemplo, uma alteração de pH da cavidade oral ou uma mudança na dieta do hospedeiro. Um desequilíbrio da composição do biofilme oral pode causar gengivite, periodontite e cárie. Bactérias, liberadas a partir do biofilme oral e transmitidas pelo sangue para diferentes áreas do corpo humano, podem causar, por bacteremia, doenças como a aterosclerose (REYES et al., 2013), a endocardite (NAGATA et al., 2005), infecções relacionadas com biomateriais (implantes) (MOMBELLI e DECAILLET, 2011) entre outros.

Em canais radiculares infectados, esse biofilme adere as paredes do conduto (RICUCCI et al., 2009) e são, particularmente, mais problemáticos em morfologias acessórias, tais como canais laterais e istmos, pois, como já supracitado, essas áreas não são tocadas durante a instrumentação do canal radicular (PETERS et al., 2001; RICUCCI et al., 2013).

Biofilmes são caracterizados por densos aglomerados de células bacterianas separados por canais e estruturados por uma matriz extracelular de substância polimérica (EPS), que compreende até 90% do mesmo. Esta matriz EPS, secretada pelas próprias células bacterianas, contém polissacarídeos, proteínas e DNA extracelular (RUPP et al., 2005) e fornece à esta comunidade bacteriana funções como: adesão, agregação de microrganismos, coesão do biofilme, retenção de água, atividade enzimática, propriedades viscoelásticas, facilita a nutrição, além de atuar como uma barreira protetora contra ataques químicos e mecânicos provenientes dos procedimentos de limpeza dentária e desinfetantes (STEWART e FRANKLIN, 2008; FLEMMING e WINGENDER, 2010).

Klapper et al (2002) têm mostrado que o biofilme se comporta como um fluido viscoelástico polimérico. Em resposta à uma tensão de curto prazo, o mesmo comporta-se como um estado sólido-elástico, já quando submetido a uma tensão de longo prazo seu

comportamento dimensional apresenta-se como um fluido-viscoso (RUPP et al., 2005). Esta característica "bipolar" atua como um mecanismo de proteção para sobreviver a uma tensão mecânica externa, como por exemplo, o da escovação. A característica elástica amortece a energia de estresse durante a deformação e o recurso viscoso dissipa e resiste ao estresse transitório (RUPP et al., 2005; FLEMMING e WINGENDER, 2010).

As características viscoelásticas do biofilme oral dependem do grau de compactação durante a formação, da ausência ou presença de fluxo durante o crescimento e da arquitetura e composição da microbiota (PARAMONOVA et al., 2009). As propriedades viscoelásticas desses biofilmes podem ser determinadas pela avaliação do relaxamento após a deformação durante um carga externa. O relaxamento durante a deformação é um processo tempo-dependente e pode ser separado em várias respostas, cada uma com uma constante de tempo característica. Esse relaxamento pode envolver um número de processos, como exclusão de água ou matriz EPS, mudanças na conformação da estrutura superficial bacteriana e rearranjo da posição ou compactação bacteriana (HE et al., 2013).

Sabe-se que as propriedades viscoelásticas determinam a capacidade do biofilme de retardar a difusão dos desinfetantes (HE et al., 2013). Porém, poucos estudos até agora, tem focado como os agentes químicos podem mudar as propriedades viscoelásticas do biofilme, interferindo a sua difusão através dessa matriz EPS.

Em endodontia, esses químicos deveriam, idealmente, apresentar características como: poder bactericida com amplo espectro de ação, capacidade dissolvente, inativar endotoxinas, evitar a formação ou promover a remoção de *smear layer*, ser lubrificante e não ser tóxico e cáustico aos tecidos periapicais (HARRISON, 1984; RETAMOZO et al., 2010). Diante disto, diversos agentes químicos têm sido propostos e utilizados como soluções irrigadoras durante o tratamento endodôntico, tais como: compostos halogenados (soluções de hipoclorito de sódio em diferentes concentrações de cloro ativo e clorexidina); compostos tensoativos (Texapon, Biosept, Zefirol, Tween 80); substâncias quelantes (ácido etilenodiaminotetracético – EDTA); peróxidos (peróxido de hidrogênio e de uréia); associações (detergente + hipoclorito de sódio, detergente + EDTA, RC Prep, hipoclorito de sódio + ácido cítrico,...) entre outros, como água destilada, soro fisiológico e solução de hidróxido de cálcio (LEONARDO, 2005).

Perante o exposto, a solução de hipoclorito de sódio (NaOCl) – que consiste em um líquido claro, com forte odor, miscível em água e que sofre decomposição na presença da luz (FACHIN et al., 1994) – tem sido amplamente utilizada como irrigante principal do sistema de canais radiculares (DUTNER et al., 2012) devido a sua capacidade de ação contra

microrganismos (MCDONNELL e RUSSEL, 1999; ZEHNDER, 2006), biofilme (SPRATT, PRATTEN, WILSON e GULABIVALA, 2001; ARIAS-MOLIZ et al., 2009) e inativação de endotoxinas (SILVA et al., 2004), além de sua capacidade única de dissolver tecido pulpar e necrótico (NAENNI, THOMA e ZEHNDER, 2004; SIRTES et al., 2005) bem como componentes orgânicos da *smear layer* (BAUMGARTNER e MADER, 1987).

A ação do NaOCl se dá através do "cloro livre disponível", que consiste na forma de apresentação de todo cloro presente nessa solução. A reação do NaOCl com componentes do sistema radicular (incluindo a parede do canal) reduz a disposição desse cloro livre (MACEDO et al., 2010) e essa taxa de reação e/ou a capacidade de dissolução de tecido do NaOCl são significantemente influenciadas por fatores como: concentração da solução, tempo de exposição ao substrato, energia laser/ultrassônica aplicada (MACEDO et al., 2010), área de contato (ROSENFELD et al., 1978; MOORER e WESSELINK, 1982), temperatura (SIRTES et al., 2005), interação com outras substâncias químicas (SHIOZAWA, 2000; ZEHNDER et al., 2002; ZEHNDER et al., 2005) e pH (JUNGBLUTH et al., 2011).

O pH da solução determina o equilíbrio do cloro disponível na solução de hipoclorito de sódio, representados pelo ácido hipocloroso (HOCl) e pelo íon hipoclorito (OCl^-)(BAKER, 1947). A atividade biológica do NaOCl, que pode ser definido como a capacidade de dissolução tecidual e o efeito antimicrobiano, é diretamente influenciado por este equilíbrio. Em soluções alcalinas (pH > 7,5), íons hipoclorito prevalecem em maior quantidade, sendo assim, estas soluções apresentam uma capacidade de dissolução tecidual maior do que soluções ácidas (CHRISTENSEN, MCNEAL e ELEAZER, 2008). Por outro lado, quando o HOCl prevalece, em soluções ácidas (3 < pH < 7,5), esse composto químico apresenta um poderoso efeito bactericida e uma capacidade dissolvente menor (MORRIS, 1966). A ação germicida dessa solução ácida se deve, provavelmente, pelo fato de o HOCl ser uma pequena molécula sem carga, o que poderia, de maneira relativamente fácil, penetrar na membrana bacteriana e interferir no metabolismo bacteriano através da degradação proteica (WINTER et al., 2008). No entanto, o NaOCl quando na forma ácida apresenta-se como uma solução instável, tendo seu efeito antimicrobiano útil apenas por um curto período de tempo.

A solução de hipoclorito de sódio com pH básico ao entrar em contato com matéria orgânica sofre um decréscimo nessa alcalinidade (MOORER e WESSELINK, 1982), sendo assim, seria interessante, em teoria, adicionar um composto alcalino como o hidróxido de sódio (NaOH) ou uma solução tampão junto ao NaOCl para evitar que tal diminuição ocorresse, fazendo com que a quantidade de íons OCl^- e, consequentemente, sua capacidade dissolvente permanecessem altos (JUNGBLUTH et al., 2011).

Contudo, a solução de hipoclorito de sódio apresenta algumas desvantagens como citotoxicidade aos tecidos perirradiculares (HÜLSMANN E HAHN, 2000) e redução da resistência flexural e do módulo de elasticidade da dentina, quando usado em altas concentrações (SIM et al., 2001).

Desta forma, diferentes soluções irrigadoras têm sido sugeridas para substituir o NaOCl durante a desinfecção dos canais radiculares, sendo o digluconato de clorexidina (CHX) uma destas sugestões (OKINO et al., 2004; RÔÇAS e SIQUEIRA, 2011). CHX é uma base forte (grupo biguanida) e estável na forma de sal e possui como características principais: ação antimicrobiana (LEONARDO et al., 1999; DORNELLES-MORGENTAL et al., 2011); substantividade, o que prolonga seu efeito bactericida (CARRILHO, CARVALHO e SOUSA, 2010); e boa tolerância aos tecidos periapicais (YESILSOY et al., 1995). Todavia, essa substância não possui capacidade dissolvente de tecido pulpar/necrótico (NAENNI, THOMA e ZEHNDER, 2004) e é menos eficaz contra bactérias Gram-negativas (EMILSON, 1977). Essas desvantagens a impedem de ser a solução irrigadora de eleição na terapia endodôntica, considerando que para um adequado tratamento é necessário a remoção de toda matéria orgânica do interior dos canais radiculares e que em infecções primárias, apesar de esta ser de origem polimicrobiana, bactérias anaeróbias Gram-negativas predominam (SUNDQVIST, 1994). Assim, a CHX tem sido usada como medicação intracanal (HAMIDI et al., 2012) ou em associação com o NaOCl como irrigante final no tratamento endodôntico (SPRATT et al., 2001).

Diante dos pressupostos teóricos apresentados acima e tendo-se que poucos estudos tem objetivado conhecer como as soluções químicas auxiliares podem interferir e modificar as propriedades viscoelásticas do biofilme, a presente Tese teve como objetivos:

- Estudo 1: avaliar o efeito da soluções de hipoclorito de sódio 3% com pH alcalino estável nas propriedades viscoelásticas de um biofilme dupla-espécie.

- Estudo 2: avaliar o efeito da interação da solução de hipoclorito de sódio 3% e do digluconato de clorexidina 2% nas propriedades viscoelásticas de um biofilme dupla-espécie.

2 ARTIGO 1 – EFFECT OF SODIUM HYPOCHLORITE AT HIGH pH ON BIOFILM VISCOELASTIC PROPERTIES: OPTICAL COHERENCE TOMOGRAPHY AND LOW LOAD COMPRESSION TESTING ANALYSIS

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Title Page

Title: Effect of sodium hypochlorite at high pH on biofilm viscoelastic properties: optical coherence tomography and low-load compression testing analysis.

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The authors deny any conflicts of interest related to this study.

Abstract

Objectives: To evaluate the influence of a standard 3% NaOCl solution and the same NaOCl concentration that was stabilized at a high pH level on dual-species biofilm viscoelastic properties.

Methods: A dual-species biofilm (*S. oralis* and *A. naeslundii*) was grown on hydroxyapatite discs, using CDFF biofilm model, for 96h at 37°C. The HA discs containing biofilm were exposed for 60s and 300s to experimental solutions (n=05): 3% NaOCl diluted in Milli-Q water (standard group), 6% NaOCl mixed (1:1) with 2 mol/L NaOH (stable group); 6% NaOCl diluted (1:1) with Na_2HPO_4 buffer solution (buffered group); 1 mol/L NaOH (control group) and one group was left untreated. The samples were evaluated by LLCT with 20% and 50% of induced deformation applied in 1s. The percentage of biofilm relaxation and the biofilm elements (E_1, E_2, E_3) were monitored for 100s and the data were adjusted using Maxwell model. The biofilm thickness was recorded before and after treatment by means of optical coherence tomography. The data were compared using one-way ANOVA (α= .05).

Results: The stress-relaxation percentage of the biofilm from treated groups compared to the untreated group showed no statistical difference (P >.05). For the Maxwell elements, when compared to each other, also no showed difference. The biofilm thickness for NaOCl groups (standard, stable and buffered) increased for the 60s evaluated time, however, a thickness decreasing was observed when 300s was applied, but no statistically significant (P >.05). The time of 60s and 300s did not show to influence on the measures of variables. The pH and the chlorine concentration did not differ over time.

Conclusions: Sodium hypochlorite solutions with a higher alkaline capacity were not able to affect the viscoelastic properties of the experimental biofilm regardless of the exposure time.

Key words: Root Canal Irrigants; Sodium Hypochlorite; Biofilms; Microbial Viability

Introduction

Microbial infection has been described as the major causative factor of pulpal and periapical pathologies (1, 2). Failure to effectively eradicate the microorganisms and their by-products from root canal surfaces might maintain a chronic apical periodontitis and, consequently, an impaired healing (3). It can be explained because bacteria are organized as biofilms present in areas unreachable by instrumentation process during the root canal therapy such as lateral canals, fins, deltas and isthmus (3, 4).

Biofilm can be defined as microbial communities consisting of bacterial cells attached to a surface and surrounded by a matrix of extracellular polymeric substance (EPS), which encompasses up to 90% of this structure. Bacteria that compose biofilms show 10 to 1000 times more resistance to antimicrobial compounds when compared with bacteria in a planktonic shape (5) because the EPS works as a physical barrier (6). In addition, the EPS matrix provides to the biofilm: viscoelastic properties, supports the nutrition and plays an important role in protecting from chemical and mechanical attacks imposed by external dental cleaning procedures and oral disinfectants (7). It is knwon that the viscoelastic properties determine the ability of biofilm to slowdown disinfectants diffusion (8).

The viscoelastic properties from oral biofilms are dependent of some factors as follow: the absence or presence of flow over the biofilm expansion, the compression degree during its formation, its arrangement and microbial composition (9). These viscoelastic features from biofilms can be evaluated through their stress-relaxation process after deformation during an external loading. The stress-relaxation when a deformation is applied over biofilm is a time-dependent process and can be discriminate in a number of responses, each one with a characteristic time constant. Stress-relaxation may involve a number of processes, like conformational changes of bacterial cell surface structures, elimination of water or EPS matrix and re-arrangement of bacterial positions within the biofilm or compaction (8).

Few studies have thus far focused on how chemicals change the viscoelastic properties of biofilm, interfering with its diffusion through the EPS matrix and in endodontics this topic has yet to be addressed.

Several substances have been proposed as endodontic irrigants in an attempt to eliminate or reduce the amount of bacteria from inside the root canals. Sodium hypochlorite (NaOCl) is currently the most popular irrigating solution due to its unpaired action against microorganisms (10) and biofilm (11) and unique property to dissolve pulp tissue and organic components of the smear layer (12,13). The active compound in NaOCl is the chlorine. All

presentation of chlorine in hypochlorite solutions is collectively named “free available chlorine” and the reactions with root canal components reduce its concentration (14). The reaction rate and/or tissue dissolution capacity of NaOCl are significantly influenced by the concentration, time of contact, type of energy applied (ultrasonic/laser) (14), contact area (15), temperature (12), interaction with other substances (16, 17) and pH (18). The pH of the solution determines the equilibrium of the free available chlorine and the NaOCl can present it in the forms of hypochlorous acid (HOCl) and hypochlorite ion (OCl^-). Its predominance depends if the solution is rendered acidic or alkaline, respectively (14). It is noteworthy that in a pH of 7.5 (pK_a value of HOCl = 7.5), equal amounts of HOCl and OCl^- are in the solution.

Sodium hypochlorite solutions have a pH in the range of 11.5 to 12.5 depending on their concentration, thus the OCl^- prevails in an unaltered chemical, which has a higher tissue dissolving capacity than HOCl. On the other hand, HOCl prevails in acidic solutions ($3 < pH < 7.5$). It has a powerful bactericidal effect and considerably lowest soft tissue dissolving capacity than unaltered counterparts (19, 20). Additionally, in contact with tissues and/or microorganisms, the pH drops so that HOCl predominates in solution, for this reason, it is necessary to add alkali (sodium hydroxide (NaOH) or buffer solution) to stabilize the OCl^- in a high prevalence, which would provide to the solution a stronger tissue-dissolving effect (18) and its effect on biofilm properties has not been described.

The aim of this investigation was to assess the influence of a standard 3% NaOCl solution and the same NaOCl concentration that was stabilized at a high pH level on biofilm properties by LLCT and OCT analysis.

Materials and Methods

Bacterial Strains and Growth Conditions

Streptococcus oralis J22 and *Actinomyces naeslundii* T14V-J1 grown on blood agar plates, were used to inoculate 10 mL modified Brain Heart Infusion broth (BHI, Oxid Ltd., Basingstoke, Hampshire, UK)(37.0 g/L yeast extract, 0.4 g/L NaOH, 1.0 g/L hemin, 0.04 g/L vitamin K_1, 0.5 g/L L-cysteine, pH 7.3) and were cultured for 24h in ambient air for *S. oralis* J22 and 48h in anaerobically environment for *A. naeslundii* T14V-J1, both at 37°C (pre-culture). From these pre-cultures, 2.5 mL were used to inoculate 50 mL modified BHI (main culture), where each bacteria sample grown in the same conditions aforementioned. Bacteria were harvested by centrifugation at 870 g, 10°C for 5 minutes and washed twice in sterile

adhesion buffer (50 mM potassium chloride, 2 mM potassium phosphate, 1 mM calcium chloride, pH 6.8). The bacterial pellet was suspended in 10 mL adhesion buffer and sonicated intermenttently in an ice water bath for 3x 10s at 30 W (Vibra cell model 375, Sonics and Materials Inc., Newtown, CT, USA) to break bacterial chains and agglomerates. Next, bacteria were resuspended in adhesion buffer. A concentration of 6 x 10^8 bacteria/mL for *S. oralis* J22 and 2 x 10^8 bacteria/mL for *A. Naeslundii* T14V-J1 was used and mixed to make 200 mL of dual strain bacterial suspension.

Biofilm Formation

In the CDFF (21, 22), 200 mL bacterial suspension was introduced droplet by droplet in an interval of 01h, while the plate containing the sample holders rotated in a speed of 0.8 rpm. The CDFF system stayed stopped for 30 min to allow bacteria to adhere previously that growth medium was introduced. The dual species biofilm was grown for 96h at 37°C under uninterrupted supply of modified BHI with a rate of 80 mL/h supported by a peristaltic pump (Gilson Inc., Middleton, USA). The CDFF device was equipped with 15 sample holders and each sample holder contained 05 sterile hydroxyapatite (HA) discs with 05 mm in diameter (Clarkson Chromatography Products, South Williamsport, PA), recessed to a depth of 250 μm. Before addition of the bacterial suspension, the HA discs were covered with a salivary conditioning film from reconstituted human saliva for 14h at 4°C under static conditions. Freeze dried whole saliva from at least 20 healthy volunteers of both genders were dissolved in adhesion buffer (1.5 g/L) to prepare the salivary film. All volunteers gave their informed consent to saliva donation, in agreement with the guidelines set out by the Medical Ethical Committee at UMG, Groningen, The Netherlands (letter 06-02-2009). The purpose of salivary conditioning film was to allow the bacterial adhesion on HA discs surface (8).

Desinfectant Solutions and Their Characterization

A standard 10-15% sodium hypochlorite solution (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was used. Its content of available chlorine was measured by a standard iodine/thiosulfate titration assay (23). The solution was then diluted to 6% NaOCl using Milli-Q water (Millipore Corporation, Billerica, MA, USA). Four solutions were prepared/used for the present study: (1) a mixture 1:1 of 6% NaOCl with Milli-Q water

(standard group); (2) a ratio 1:1 of 6% NaOCl with 2 mol/L NaOH (stable group); (3) a 1:1 mixture of 6% NaOCl with sodium phosphate buffer (24) (buffered group); (4) a 1:1 mixture of 2 mol/L NaOH with Milli-Q water (NaOH group) and one group was left untreated (control group). The solutions were prepared freshly before the experiment. The pH and concentration from sodium hypochlorite solutions were measured before starting each test and 07 days later, using a Sentron pH meter (SENTRON Europe B.V., Roden, The Netherlands) and a standard titration test, respectively.

Sampling from the CDFF

Sample pans were taken out from CDFF system using a custom tool. The sample holders containing 05 HA discs covered with biofilm (n= 05 per group) received the disinfectant solution treatment in a fixed solution volume of 20 μL, assisted by a pipette, for two different times: 60 seconds and 300 seconds. After this time, 20 μL of 5% sodium thiosulfate (The Science Company, Lakewood, USA) was applied during 1-minute period to neutralize the NaOCl (25). Then, the HA discs were removed from holders and dipped in 10 mL of adhesion buffer inside of a Petri dish to withdraw unattached cells and to maintain the cellular viability throughout the experiment.

Low Load Compression Testing (LLCT)

The viscoelastic properties of biofilms were evaluated by the LLCT after compressed to an induced deformation of 20% and 50% from biofilm thickness in an interval of 01s and this deformation was sustained for 100s. The biofilm relaxation was monitored over time and the percentage change that occurred in the biofilm during 100s under compression loading was called the percentage stress relaxation (26). Measured relaxation curves for each sample were modeled using a Maxwell model expressed by

$$E_{(t)} = E_1 e^{-t/\tau 1} + E_2 e^{-t/\tau 2} + E_3 e^{-t/\tau 3}$$

in which $E_{(t)}$ is the total stress relaxation of the biofilm given as the sum of three Maxwell elements (E_1, E_2, E_3). The elements were called “fast”, “intermediate” and “slow” based on their τ values: τ_1 (0.01 to 0.5s), τ_2 (0.5 to 3s) and τ_3 (3 to 100s). The full explanation of the Maxwell model applied to the LLCT was already described (8). The data acquisition for each element and the total stress relaxation were supported using LabVIEW 7.1 software (National

Instruments Corporation, Austin, TX, USA) and it was registered in a text file for analysis by Microsoft Excel (Microsoft Corporation, Albuquerque, USA).

Optical Coherence Tomography (OCT) Evaluation

The OCT scans were performed by using a Thorlabs Ganymede-II SD-OCT system (Thorlabs, Dachau, Germany) from the shelf equipped with an OCT-LK3-BB objective lens kit. The settings for device were a high swept rate (5.5 kHz to 36 kHz), continuous wavelength scanning laser centered near 930 nm, an imaging depth of 2.2 mm at water and 4.5 μm of axial depth resolution. The biofilm thickness from each sample was recorded before and after treatment with the irrigant solution for comparison. The cross-sectional OCT images from biofilms were acquired by ThorImage® OCT software (Thorlabs) and the biofilm thickness data were evaluated using ImageJ/Fiji package (National Institutes of Health, USA). The threshold of gray level for picture processing was determined by Otsu method (27).

Results

The values pertaining to stress-relaxation, Maxwell elements and biofilm thickness were evenly distributed. For this reason, parametric statistics were applied. The alpha-type error was set at .05. A one-way ANOVA showed no statistical difference among the stress-relaxation values, regardless of exposure time ($P > .05$). In addition, no difference was observed in elements data. Table 1 and Table 2 summarize the percentage of biofilm stress-relaxation and the percentage of each Maxwell element inside the overall relaxation data for 20% and 50% of deformation, respectively. The average biofilm thickness (μm) is expressed before and after the irrigants treatment. When 60s was assessed, the biofilm thickness, for all experimental solutions, increased regarding the untreated samples, for 300s exposure time, the opposite happened, the thickness decreased from the original samples, however, no statistical difference was found for it ($P > .05$)(Figure 1 and Figure 2). The *t*-student test showed no statistical difference in the pH and concentration values for fresh solutions and 01 week following ($P > .05$)(Table 3).

Discussion

Biofilms are conditioned to self-protecting from external agents (6). Mechanical removal has remained the preferred way to avoid biofilm formation, however, in endodontics field it is limited particularly due to great anatomical variation of root canal system (2). The use of chemical irrigants in root canal therapy is to aid and improve this biofilm removal. NaOCl is known to act on microrganisms by its available chlorine, which is presented in HOCl and OCl^- form (14). In alkaline solutions, OCl^- prevails, rendering to solution more proteolytic effect, notwithstanding, in contact with organic matter the active chlorine is consumed and the pH drops, decreasing such effect (18). Several studies have evaluated the the influence of stabilized alkaline sodium hypochlorite solutions for different outcomes (16,18, 28, 29) . In the current study, we assessed whether adding an alkali or a buffer solution in a standard sodium hypochlorite would modify the viscoelastic properties of a biofilm.

Our result showed to stabilize the pH of sodium hypochlorite in a high alkaline medium was not able to affect efficiently the viscoelasticity of biofilm. Viscoelastic property can be measured by stress-relaxation analysis and isolated in fast, intermediate and a slow response of a biofilm to an induced compression, corresponding the presence of water, the influence of EPS matrix and re-arrangement of the bacteria respectively as described by He et al (8). Involvement of fast response suggests that the water could dilute the NaOCl solution and hence decreases its pH to levels that would not promote a dissolving effect. When 20% of induced deformation was applied, the E_1 percentage was higher for all groups comparing with untreated group, however, it was not statistically significant (Table 1). Preliminary study (8) reported the influence of penetration of an antimicrobial into biofilms, where increasing importance of the slow Maxwell element and decreasing importance of the fast response, the solution penetration was increased. In our study, when 50% of deformation was induced, the 300s buffered group showed higher importance of the E_3 within the total sum of the components, the same was found for 300s standard group in 20% of deformation. The implication of this slow component suggests that biofilm composition allowing large bacterial re-arrangement after deformation is more susceptible for better OCl^- effect.

A plausible explanation for non-influence of sodium hypochlorite and sodium hydroxide in the results for stress-relaxation process and Maxwell components is the type of biofilm. We worked with CDFF, which is a dynamic biofilm model that allows the control of environmental factors such as the substratum, the temperature, the biofilm height and nutrient source (21). The principal advantage of CDFF is to reproduce a dense biofilm due to rotary scraper blades that compacting the biofilm during its formation, mimicking *in vivo* conditions

(22). In this uniform compact carpet-like biofilm, the irrigating solutions may not reach the microrganisms as a result of diffusional resistance of EPS matrix or neutralization of irrigant inside the matrix (30), which is in accordance with other study (8). Still concerning to the biofilm model used in this study, dual-species biofilms composed of streptococci and actinomyces grown under low shear forces have a behavior similar to full dental plaques (9) and better antimicrobial resistance than mono-specie biofilm (8). Considering that dual-species biofilms are easier to replicate than multi-species biofilms, in vitro irrigating solution experiments might take advantage from the use of dual-species biofilms, without loss of relevance (8, 9).

The logical behind our study was that in standard NaOCl solutions, the pH goes down while the chlorine is consumed by its interaction with biofilm, however, in our methodology was not possible to measure the pH in contact with the sample, as previously done (18), due to small amount of solutions applied. For this reason, to minimize any instability of the NaOCl tested solutions, the pH and the concentration were checked on the experimental day and one week after (Table 3).

Macedo et al (14) investigated the effect of exposure time of NaOCl solutions. Their results showed that longer exposure time enhanced the chemical efficacy of sodium hypochlorite. It is in disagreement with results presented, where the time did not seem to influence the stress-relaxation values, the elements and the biofilm thickness. Low load compression testing (LLCT) is a method for measuring biofilm thickness and viscoelastic properties and the major advantage is the relatively low cost of the system compared to other used, such as scanning electron microscopy, magnetic resonance imaging or confocal laser scanning microscopy (CLSM)(9). From the data obtained in the LLCT, it is possible to perform mathematical determination of stress-relaxation values as aforementioned using a Maxwell model. In spite of this, it is only a mathematical description and does not provide a microbiological description of effect of the irrigating solutions on biofilm (32).

OCT is an imaging technique and its remarkable features are non-destructive method, fast acquisition, no sample preparation and multi-dimensional images acquisition (35). Cross-sectional images were obtained from all samples before and after treatment, and the biofilm thickness was measured by ImageJ/Fiji (27). In spite of no significant difference between original biofilm thickness and after treated, it is possible to observe in a descriptive analysis a difference in the biofilm architecture. In untreated samples, a compact and dense biofilm was present whilst in treated samples, particularly NaOCl groups, a “fluffy” and non-uniform biofilm was observed (Figures 1 and 2). It suggests that the irrigants promotes some biofilm

removal effect. The HA discs were immersed in adhesion buffer solution during OCT analysis, thus some aggregates of biofilm got into suspension after detachment. It may clarify why in some cases the thickness increased after the treatment. Another highlight is in the treated groups, a compact biofilm was observed only in deeper layers whereas in outer layers the biofilm had a non-dense aspect.

Being aware that is a laboratory study, there are limitations, and extrapolations to the clinical situation should be carefully conducted. The current study aimed to gain some basic insight into the effect of mixing alkali or buffer solution to NaOCl solutions to apply on biofilm. Based in our findings and knowing the caustic effect of sodium hypochlorite on periapical tissue and dentin matrix (14, 28), the adding alkali may aggravate these untoward effects. For this reason, an alkalinization does not seem to be necessary. Another limitation, within the methodology employed, was that OCT analysis does not allow to verify the microrganisms viability. CLSM has been reported for such purpose (8, 31) in spite of it has limited dye penetration rate (35).

Conclusion

Under the conditions of this study, we concluded that sodium hypochlorite solutions with a higher alkaline capacity were not able to affect the viscoelastic properties of the experimental biofilm regardless of the exposure time.

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TABLE 1. Data related to percentage of stress-relaxation and percentage of Maxwell elements in **20%** of induced deformation after 60s and 300s of exposure time solution (LLCT analysis). Values are expressed as mean and standard deviation

GROUPS	Relaxation (%) E_{total}	Maxwell Elements (%)			
		E_1	E_2	E_3	Exposure Time
Untreated[a]	72.2 ± 6.6	46.1 ± 8.9	17.3 ± 7.4	5.0 ± 1.4	*
Standard 3%NaOCl	76.7 ± 8.6	61.5 ± 13.4	11.5 ± 7.5	4.6 ± 2.1	60s
Stable 3% NaOCl	70.7 ± 18.1	58.3 ± 21.2	9.8 ± 4.7	4.2 ± 2.4	60s
Buffered 3% NaOCl	71.5 ± 16.4	57.8 ± 15.7	10.1 ± 10.1	4.1 ± 1.0	60s
1 mol/L NaOH	70.1 ± 6.6	55.1 ± 21.6	10.8 ± 3.2	5.1 ± 4.2	60s
Standard 3% NaOCl	78.6 ± 7.3	54.5 ± 15.8	14.7 ± 9.4	9.7 ± 4.9	300s
Stable 3% NaOCl	75.1 ± 16.4	53.1 ± 13.3	15.9 ± 9.4	6.7 ± 5.6	300s
Buffered 3% NaOCl	75.6 ± 19.2	50.5 ± 16.1	12.9 ± 7.3	7.5 ± 1.6	300s
1 mol/L NaOH	69.4 ± 20.3	48.5 ± 17.2	16.8 ± 5.4	4.8 ± 2.8	300s

[a]Control group was left untreated; the samples were immersed in buffer solution. *No time was applied for control group.

TABLE 2. Data related to percentage of stress-relaxation and percentage of Maxwell elements in **50%** of induced deformation after 60s and 300s of exposure time solution (LLCT analysis). Values are expressed as mean and standard deviation

GROUPS	Relaxation (%) E_{total}	Maxwell Elements (%)			
		E_1	E_2	E_3	Exposure Time
Untreated[a]	80.8 ± 5.6	68.6 ± 8.6	10.5 ± 4.4	2.1 ± 0.3	*
Standard 3% NaOCl	73.4 ± 7.6	61.8 ± 4.3	10.8 ± 6.9	2.3 ± 0.7	60s
Stable 3% NaOCl	71.9 ± 7.3	61.2 ± 7.9	7.9 ± 4.5	2.1 ± 0.8	60s
Buffered 3% NaOCl	80.1 ± 6.8	61.7 ± 5.8	13.2 ± 5.6	2.7 ± 1.0	60s
1 mol/L NaOH	77.7 ± 2.3	64.1 ± 6.5	11.9 ± 4.9	2.5 ± 0.4	60s
Standard 3% NaOCl	79.9 ± 3.9	66.5 ± 8.8	11.5 ± 5.1	2.7 ± 1.1	300s
Stable 3% NaOCl	70.4 ± 8.1	59.5 ± 6.5	9.3 ± 3.1	3.2 ± 0.7	300s
Buffered 3% NaOCl	82.1 ± 8.6	61.5 ± 12.4	14.5 ± 11.1	7.3 ± 5.0	300s
1 mol/L NaOH	81.0 ± 7.2	67.6 ± 6.6	12.8 ± 6.3	3.5 ± 1.0	300s

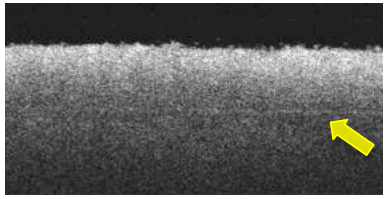
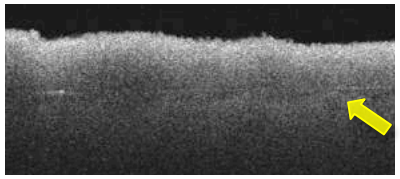
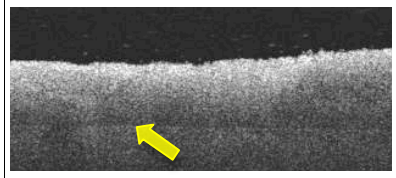
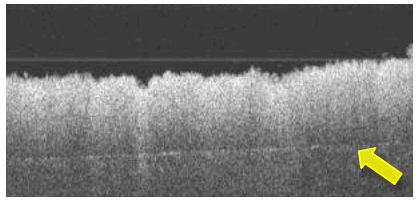
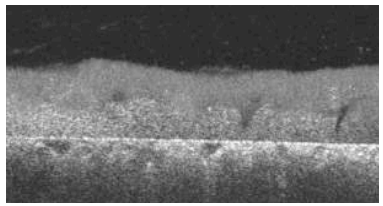
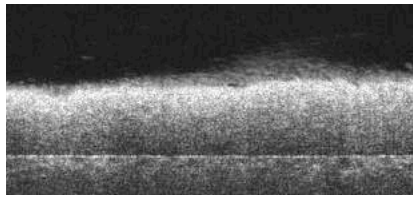
[a]Control group was left untreated; the samples were immersed in buffer solution. *No time was applied for control group.

TABLE 3. pH and concentration of NaOCl over time (mean ± standard deviation)

	pH		Concentration (%)	
NaOCl Groups	**Experimental day**	**07 days**	**Experimental day**	**07 days**
3% NaOCl standard	11.3 ± 0.05[a]	11.2 ± 0.1[a]	3.0 ± 0.03[b]	3.0 ± 0.05[b]
3% NaOCl stable	12.2 ± 0.05[a]	12.3 ± 0.05[a]	3.1 ± 0.02[b]	3.0 ± 0.1[b]
3% NaOCl buffered	12.4 ± 0.02[a]	12.4 ± 0.05[a]	3.0 ± 0.05[b]	3.0 ± 0.03[b]

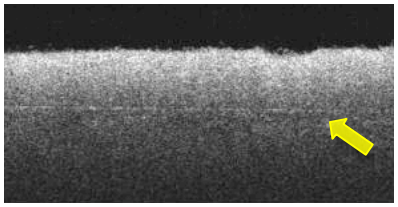
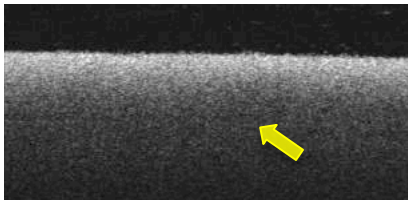
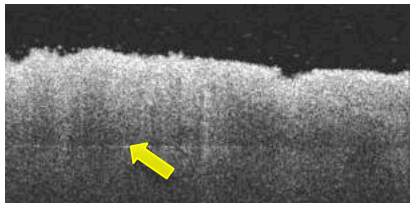
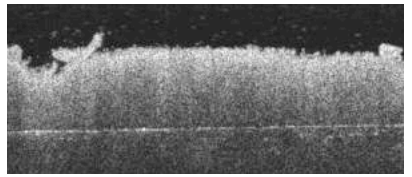
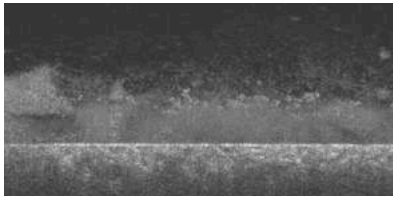
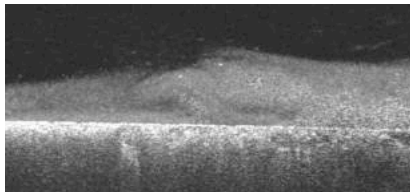
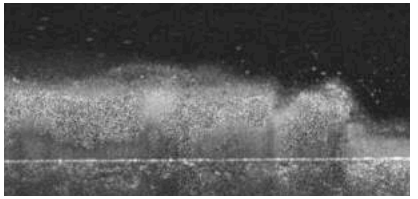
Same superscript letters in the columm are not statiscally significant ($P > .05$).

Figure 1. OCT images, before and after treatment, for the different experimental groups (**60s** exposure time). The mean and standard deviation of the biofilm thickness (μm) are presented below each image.

	3% NaOCl standard	**3% NaOCl stable**	**3% NaOCl buffered**	**NaOH group**
BEFOE TREATMENT				
	219.6 ± 45.5	223.4 ± 75.9	229.6 ± 67.7	227.6 ± 67.5
AFTER TREATMENT				
	232.3 ± 92.6	280.2 ± 155.1	286.5 ± 120.9	263.6 ± 121.6

Arrows indicate the limit of substrate surface (thin white line) from where the biofilm grows, for untreated samples. Below the line is represented the HA disc and above the line is shown the biofilm structure.

Figure 2. OCT images, before and after treatment, for the different experimental groups (**300s** exposure time). The mean and standard deviation of the biofilm thickness (μm) are presented below each image.

	3% NaOCl standard	**3% NaOCl stable**	**3% NaOCl buffered**	**NaOH group**
BEFOE TREATMENT				
	209.6 ± 57.5	239.8 ± 50.5	229.5 ± 67.7	235.9 ± 69.9
AFTER TREATMENT				
	193.7 ± 49.1	219.2 ± 151.5	194.7 ± 104.9	221.7 ± 70.7

Arrows indicate the limit of substrate surface (thin white line) from where the biofilm grows, for untreated samples. Below the line is represented the HA disc and above the line is shown the biofilm structure.

3 ARTIGO 2 – IMPACT OF INTERACTION BETWEEN SODIUM HYPOCHLORITE AND CHLORHEXIDINE ON BIOFILM VISCOELASTIC PROPERTIES

Este artigo será submetido à publicação no periódico *Journal of Endodontics*, Elsevier, ISSN: 0099 - 2399, Fator de impacto = 2.788; Qualis A1. As normas para publicação estão descritas no Anexo B

Title Page

Title: Impact of interaction between sodium hypochlorite and chlorhexidine on biofilm viscoelastic properties.

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The authors deny any conflicts of interest related to this study.

Abstract

Objectives: To investigate the influence of 3% sodium hypochlorite and 2% chlorhexidine, in a combining use, on viscoelastic properties of an experimental biofilm.

Methods: A dual-species biofilm (*S. oralis* and *A. naeslundii*) was grown on hydroxyapatite (HA) discs, using CDFF biofilm model, for 96h at 37°C. The HA discs, recessed 250 μm, were exposed to 20 μL for 60s to experimental solutions (n=05 per group): 3% NaOCl (control group); 2% CHX (control group 2); 2% CHX + 3% NaOCl and 3% NaOCl + 2% CHX in that order. Between each solution changing, a neutralizing solution was applied. The samples were evaluated by LLCT with 20% and 50% of induced deformation applied in an interval of 1s. The percentages of stress-relaxation and of the biofilm elements (E_1, E_2, E_3) were monitored during 100s and the data were adjusted using Maxwell model. The biofilms thicknesses were recorded before and after treatment by means of OCT analysis. The data were compared using one-way analysis of variance (α= .05).

Results: No statistical difference was found in the stress-relaxation percentage of the biofilms (P >.05). For the Maxwell elements, when compared to each other, also no showed difference. The biofilm thickness after the treatment remained approximately the same values that untreated samples (P > .05).

Conclusions: The combining use of 3% NaOCl and 2% CHX, regardless its sequence of application, does not seem to improve the effect of these irrigating solutions on viscoelastic characteristic of a dual-species biofilm.

Key words: Root Canal Irrigants; Biofilms; Sodium Hypochlorite; Chlorhexidine

Introduction

The successful of endodontic treatment is directly dependent on the elimination of microorganisms and their by-products from the necrotic root canal system. These bacteria, which colonized the root canal space, are present either as free-floating single cells or as biofilm (1). Biofilms are communities of bacteria that attach to solid surfaces and synthesize their self-extracellular hydrated polymeric matrix (EPS) forming a sessile complex organization (2). EPS matrix consists of a conglomeration of polysaccharides, proteins, nucleic acids and lipids. It provides to the biofilm several functions as follows: adhesion to a solid surface, aggregation of bacterial cells, sorption of organic compounds, nutrient source, enzymatic activity, export of cell components, three-dimensional architecture, retention of water and a protective barrier from chemical and mechanical attacks imposed by external dental cleaning procedures and oral disinfectants (3,4).

The chemomechanical procedures play an important role in attempt to reduce/eliminate the biofilm from the root canal system. This endodontic step is performed by the root canal instrumentation with endodontic files associated with antimicrobial irrigating solutions. Moreover, a well-done root canal instrumentation allows a proper placement root canal filling, thereby preventing a bacterial re-colonization (5). One of the main roles of root canal irrigation is to improve the bacteria removal from areas of the root canal system that the instruments could not to reach (6). Several endodontic irrigants have been proposed to optimize the root canal disinfection and they ideally should have a broad antimicrobial spectrum, the capacity to inactivate endotoxin, avert the smear layer formation, dissolve necrotic tissue remnants and not be toxic to the periapical tissues (7).

Sodium hypochlorite (NaOCl) has been the most popular used endodontic irrigant, because it encompasses the majority of the requirements aforementioned. NaOCl is related to its strong antibacterial effect (8, 9), tissue-dissolving capacity (10) and ability to kill microorganisms organized in biofilms (8). These current evidences are in favor of NaOCl as main choice for endodontic irrigant. However, NaOCl shows some disadvantages, including tissue toxicity with serious complications when it was inadvertently extruded into the periapical space (11). In addition, NaOCl at high concentration showed to decrease the flexural strength and the elastic modulus of dentin from human teeth (12).

Therefore, one alternative approach was to search for different irriganting solutions to replace NaOCl during root canal disinfection, such as chlorhexidine digluconate (CHX). CHX is a cationic agent (from biguanide group) and its antibacterial capacity has been widely

reported (13-15). As main properties, CHX exhibits a broad-spectrum antimicrobial action besides it possesses substantivity property to dentin, which may prolong the bactericidal effects (16). The ability of CHX to be less irritating to vital tissues also has been reported (17). The main limitation is its incapacity to dissolve organic matter (18).

It stands out that each irrigant used during endodontic therapy mentioned before has own specificity against microorganisms. A protocol using 2.5% sodium hypochlorite during instrumentation in combination with 0.2% CHX shown to have better antimicrobial effect than that of either component used singly, as reported by Kuruvilla et al (19). For this reason, irrigation regimens combining NaOCl for instrumentation and a final flush of root canals using CHX have been proposed (7). Currently, no information how these chemicals in combination could change the viscoelastic properties of biofilm are available.

In front of the above, the current study aimed to evaluate the influence of 3% NaOCl and 2% CHX in combination on the viscoelastic properties of a biofilm dual species by low load compression testing (LLCT) and optical coherence tomography (OCT) analisys.

Materials and Methods

Bacterial Strains and Growth Conditions

Streptococcus oralis J22 and *Actinomyces naeslundii* T14V-J1 grown on blood agar plates, were used to inoculate 10 mL modified Brain Heart Infusion broth (BHI, Oxid Ltd., Basingstoke, Hampshire, UK) (37.0 g/L yeast extract, 0.4 g/L NaOH, 1.0 g/L hemin, 0.04 g/L vitamin K_1, 0.5 g/L L-cysteine, pH 7.3) and were cultured for 24h in ambient air for *S. oralis* J22 and 48h in anaerobically environment for *A. naeslundii* T14V-J1, both at 37°C (pre-culture). From these pre-cultures, 2.5 mL were used to inoculate 50 mL modified BHI (main culture), where each bacteria sample grown in the same conditions aforementioned. Bacteria were harvested by centrifugation at 870 g, 10°C for 5 minutes and washed twice in sterile adhesion buffer (50 mM potassium chloride, 2 mM potassium phosphate, 1 mM calcium chloride, pH 6.8). The bacterial pellet was suspended in 10 mL adhesion buffer and sonicated intermenttently in an ice water bath for 3x 10s at 30 W (Vibra cell model 375, Sonics and Materials Inc., Newtown, CT, USA) to break bacterial chains and agglomerates. Next, bacteria were resuspended in adhesion buffer. A concentration of 6 x 10^8 bacteria/mL for *S. oralis* J22 and 2 x 10^8 bacteria/mL for *A. Naeslundii* T14V-J1 was used and mixed to make 200 mL of dual strain bacterial suspension.

Biofilm Formation

In the CDFF (20), 200 mL bacterial suspension was introduced droplet by droplet in an interval of 01h, while the plate containing the sample holders rotated in a speed of 0.8 rpm. The CDFF system stayed stopped for 30 min to allow bacteria to adhere previously that growth medium was introduced. The dual species biofilm was grown for 96h at 37°C under uninterrupted supply of modified BHI with a rate of 80 mL/h supported by a peristaltic pump (Gilson Inc., Middleton, USA). The CDFF device was equipped with 15 sterile sample holders. Each sample holder contained 05 hydroxyapatite (HA) discs (0.5 mm in diameter; Clarkson Chromatography Products, South Williamsport, PA), recessed to a depth of 250 μm, which were used as biofilm substrate. Prior to the addition of the bacterial suspension, the HA discs were coated with a salivary conditioning film from reconstituted human saliva for 14h at 4°C under static conditions. Freeze dried whole saliva from at least 20 healthy volunteers of both genders were dissolved in adhesion buffer (1.5 g/L) to prepare the salivary film. All volunteers gave their informed consent to saliva donation, in agreement with the guidelines set out by the Medical Ethical Committee at UMG, Groningen, The Netherlands (letter 06-02-2009). The purpose of salivary conditioning film was to allow the bacterial adhesion on HA discs surface (21).

Sampling from the CDFF and Irrigation Protocols

A standard 10-15% sodium hypochlorite solution (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was used. Its content of available chlorine was measured by a standard iodine/thiosulfate titration assay (22). The solution was then diluted to 3% NaOCl using Milli-Q water (Millipore Corporation, Billerica, MA, USA). For CHX usage, 20% chlorhexidine digluconate solution (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was diluted to obtain a 2% concentration solution.

Sample pans were taken out from CDFF system using a custom tool. The sample holders containing the HA discs covered with biofilm were divided in 04 groups with 05 samples per group (n=05) and received the irrigation protocols as follows: 1) only 3% NaOCl (control group 1); 2) only 2% CHX (control group 2); 3) 2% CHX + 3% NaOCl and 4) 3% NaOCl + 2% CHX. The solution volume was fixed in 20 μL, delivered by a pipette. Each solution covering the biofilm was left for a 60 seconds-period. After each use of NaOCl, 20

μL of 5% sodium thiosulfate (The Science Company, Lakewood, USA) was applied during 1-minute period to neutralize the NaOCl (23). In the CHX group, 20 μL of adhesion buffer was used to remove this solution. For the interaction groups (2% CHX + 3% NaOCl and 3% NaOCl + 2% CHX), the same individual irrigation protocol was performed in a combining way. Then, the HA discs were removed from holders and dipped in 10 mL of adhesion buffer inside of a Petri dish to withdraw unattached cells and to maintain the cellular viability throughout the experiment.

Low Load Compression Testing (LLCT)

The viscoelastic properties of biofilms were evaluated by the LLCT after compressed to a deformation of 20% and 50% from the biofilm thickness previously measured by device. The compression was applied in an interval of 01s and the deformation was sustained for 100s. The biofilm stress-relaxation was monitored over time and the percentage change that occured in the biofilm during 100s was called the percentage stress-relaxation (E_{total})(21). The three Maxweel elements to know: E_1 - "fast element" (range time value: 0.01 to 0.5s); E_2 – "intermediate element" (range time value: 0.5 to 3s); and E_3 – "slow element" (range time value: 3 to 100s), also were evaluated. The percentage of each element within of E_{total} was assessed using a Maxwell model. The full explanation of the Maxwell model applied to the LLCT was already described (21). The data acquisition for each element and the total stress-relaxation were supported using LabVIEW 7.1 software (National Instruments Corporation, Austin, TX, USA) and it was recorded in a text file for analysis by Microsoft Excel (Microsoft Corporation, Albuquerque, USA).

Optical Coherence Tomography (OCT) Evaluation

The OCT scans were performed by using a Thorlabs Ganymede-II SD-OCT system (Thorlabs, Dachau, Germany) from the shelf equipped with an OCT-LK3-BB objective lens kit. The settings for device were a high swept rate (5.5 kHz to 36 kHz) continuous wavelength scanning laser centered near 930 nm, an imaging depth of 2.2 mm at water and 4.5 μm of axial depth resolution. The biofilm thickness from each sample was recorded before and after treatment with the irrigating protocols for comparison. The cross-sectional OCT images were acquired by ThorImage® OCT software (Thorlabs) and the biofilm thickness data were

evaluated using ImageJ/Fiji package (National Institutes of Health, USA). The threshold of gray level for picture processing was determined by Otsu method (24).

Data analysis

The stress-relaxation data, the percentage of each Maxwell elements and the biofilm thickness previous and after treatment were statistically analyzed with the SPSS software (Version 21, IBM, Chicago, IL, USA). The normality was verified, and the data were subjected to one-way analysis of variance (ANOVA) with significance level at α=0.05.

Results

Table 1 summarizes the percentage of biofilm stress-relaxation and the percentage of each Maxwell element inside the overall relaxation data for 20% and 50% of deformation, respectively. A one-way ANOVA showed no statistical difference among the stress-relaxation values (P >.05). In addition, no difference was observed in Maxwell responses data. The averages biofilms thicknesses (μm) from OCT images are expressed before and after the irrigants treatment (Figure 1). No significant difference was found for treated and untreated biofilms regarding its height (P >.05).

Discussion

Bacteria organized as biofilms has been documented as the etiology of pulpar and periapical diseases (1). Growing within a competitive environment, bacterial cells shown more resistance to external agents than in planktonic form (2). In endodontic therapy, the irrigation step plays an important role in aiding the biomechanical preparation, for this reason the choice of an adequate chemical substance must always consider aspects as antimicrobial, dissolving tissue capacity and biocompatibility (7). NaOCl has been the main irrigating solution for root canal treatment, however, it also cytotoxic effects (11). Chlorhexidine has show promise as endodontic irrigant (15). CHX has long-term antimicrobial properties because of its substantivity (16). Several studies have demonstrated the ability of 2% CHX to eliminate bacteria in root canals and dentinal tubules (17, 23). The combining use of NaOCl and CHX has been advocated to improve their antimicrobial effects, and a protocol using

CHX as final rinse has been suggested (7). Thus, the present study aimed to check the influence of 3% NaOCl and 2% CHX on dual-species biofilmviscoelastic properties.

The results of this study indicate that 3% NaOCl, 2% CHX and the use of this solutions in combination regardless of delivery sequence were not able to affect the biofilm structure. This confirms results of previous studies that also demonstrated which using the same biofilm model (CDFF), the chemical substance penetration was not efficient to alter the viscoelastic characteristics of a biofilm (21).

Viscoelastic property is measured by total stress-relaxation and subsequently isolated in fast, intermediate and slow components after an induced compression over biofilm (25). In the current study these elements were named E_1, E_2 and E_3, corresponding the presence of water, the influence of EPS matrix and re-arrangement of the bacteria respectively as described before (21). The involvement of E_1 suggests the outflow of water and it may dilute the substance decreasing its antimicrobial activity. In our results, when 20% of induced deformation was applied, the E_1 was less influential for 2% CHX and 2% CHX + 3% NaOCl groups comparing with others group. It may indicate lower bactericidal effect of CHX into biofilm. However, it was not statistically significant. The bacteria in a biofilm constitute the heaviest masses, and their re-arrangement upon a deformation will be slow, which associates the influence of the slow Maxwell element (E_3) with the bacterial re-arrangement inside biofilm (25). In our findings, the combining groups, after 20% of induced deformation, showed higher E_3 percentage that could indicate a better penetration rate of irrigants solutions.

The total stress-relaxation percentage presented in Table 1 showed no significant difference regardless the deformation applied. A reasonable explanation is the biofilm model tested. CDFF is a dynamic biofilm model that allows the control of some environmental factors and produces a compact and uniform biofilm due to rotating scraper blades and the continuous supply of nutrients (20). Bacteria are protected by a highly hydrated extracellular matrix presented in a compact biofilm form (3), it may retard the irrigants penetration into the biofilm. It is in agreement with previous study that has been reported that the penetration of chlorine was detected mainly depending on the structure of the biofilm and the chlorine concentration was reduced by EPS matrix (26). In addition, the results of this study are in agreement with previous study (21) that reported no influence of stress-relaxation process of the antimicrobial solutions in a CDFF biofilm.

The combination of sodium hypochlorite and chlorhexidine has been advocated to enhance their antimicrobial properties, therefore, it seemed reasonable to investigate their combination. NaOCl can react with CHX forming a precipitate named parachloroaniline

(PCA)(19), when NaOCl and CHX are the CHX molecules may become hydrolyzed into smaller fragments, each forming a by-product, resulting in the formation of PCA (27). PCA and its degradation product are toxic and carcinogenic, and this precipitate may hinder the antimicrobial diffusion through the biofilm, which might explain our results. In this study, the hypothesis of alterning the application sequence of the irrigating solution could influence the viscoelastic behavior of the biofilm, however, our results showed that it was not plausible.

Optical coherence tomography is an interferometric imaging technique and it has been reported as a suitable imaging tool for biofilms at the mesoscale (28). The biofilm thickness for untreated and treated samples was measured by OCT technique. In spite of no significant difference was found, the thickness for treated samples regardless of the group, showed an increase in its height (Figure 1). Based on a structural classification developed by Wimpenny and Colasanti (29), biofilms can be divided into two layers: a base film and surface film. In base film cells are presented in a more compact and adhered structure whereas in surface film the biofilm architecture is more “fluffy”. In our OCT images this outermost layer shown more detached in the treated groups, it may clarify the increase of biofilm height and also hypothesize that cells in the upper layers were more affected by endodontic irrigants than those in deeper layers.

Chlorhexidine has a broad spectrum of antimicrobial activity, mainly over bacteria gram-positive (7). In this present study, the biofilm were composed of *S. oralis* and *A. naeslundii*, both Gram-positive bacteria and a confocal laser scanning microscopy analysis could provide additional information relative the cellular viability as reported before (21), being a limitation of study.

Conclusion

Being aware that the current study was a basic research and showed limitations, we concluded that the interaction of 3% NaOCl with 2% CHX regardless of the sequence of utilization was not able to effectively alter the viscoelastic properties of an experimental biofilm.

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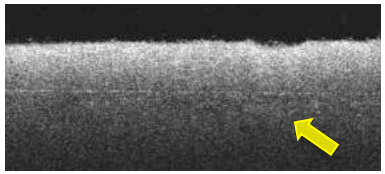
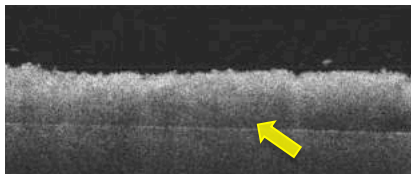
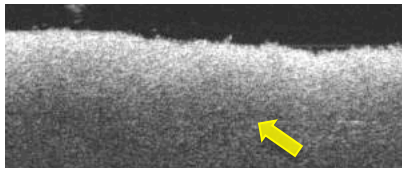
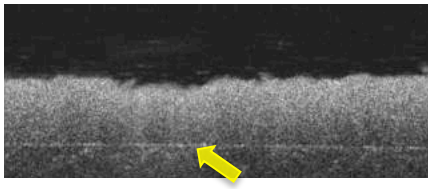
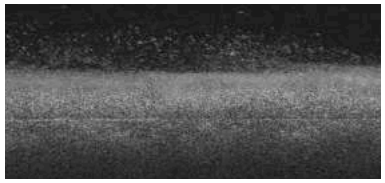
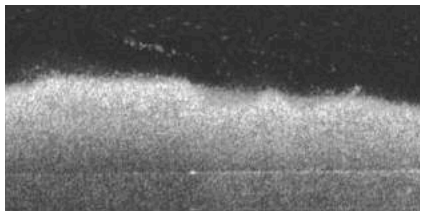
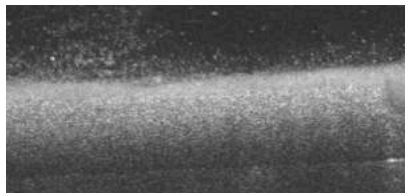
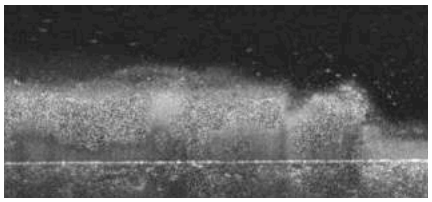
TABLE 1. Data related to percentage of stress-relaxation and percentage of Maxwell elements in **20%** and **50%** induced deformation (LLCT data). Values are expressed as mean and standard deviation

GROUPS	Relaxation (%) E_{total}	Maxwell Elements (%)			
		E_1	E_2	E_3	Deformation (%)
2% CHX	70.7 ± 16.1	47.4 ± 14.1	18.3 ± 6.2	5.9 ± 3.8	20
3% NaOCl	76.7 ± 8.6	61.5 ± 13.4	11.5 ± 7.5	4.6 ± 2.1	20
2% CHX + 3% NaOCl	64.4 ± 17.5	40.1 ± 19.5	13.2 ± 4.9	10.8 ± 5.2	20
3% NaOCl + 2% CHX	71.2 ± 13.5	51.6 ± 19.8	10.8 ± 5.9	9.2 ± 5.0	20
2% CHX	82.9 ± 8.7	70.8 ± 5.8	10.0 ± 2.8	2.9 ± 0.6	50
3% NaOCl	73.4 ± 7.6	61.8 ± 4.3	10.8 ± 6.9	2.3 ± 0.7	50
2% CHX + 3% NaOCl	77.4 ± 12.1	60.4 ± 4.4	7.3 ± 3.5	3.3 ± 0.8	50
3% NaOCl + 2% CHX	70.7 ± 12.9	56.6 ± 17.6	9.3 ± 2.8	5.7 ± 2.8	50

Figure 1. OCT images, before and after treatment, for the different experimental groups. The mean and standard deviation of the biofilm thickness (μm) are presented below each image.

	3% NaOCl	2% CHX	2% CHX + 3% NaOCl	3% NaOCl + 2% CHX
BEFOE TREATMENT				
	219.6 ± 45.5	209.8 ± 44.5	209.9 ± 37.2	193.8 ± 41.4
AFTER TREATMENT				
	232.3 ± 92.6	242.8 ± 81.5	260.7 ± 62.2	273.1 ± 125.3

Arrows indicate the limit of substrate surface (thin white line) from where the biofilm grows, for untreated samples. Below the line is represented the HA disc and above the line is shown the biofilm structure.

4 DISCUSSÃO

A presente tese teve como objetivo elucidar alguns aspectos em relação as características de viscoelasticidade de um biofilme experimental.

A presença de microrganismos no interior do sistema de canais radiculares tem sido o principal agente etiológico causador das doenças pulpares e periapicais (RICUCCI e SIQUEIRA, 2010). Estas bactérias são encontradas sob a forma de biofilmes (COSTERTON, 1999). Biofilmes podem ser descritos como comunidades bacterianas que estão aderidas à uma superfície e estão circundadas por uma matriz altamente hidratada de substâncias poliméricas (WIMPENNY e COLASANTI, 1997) além da presença de canais de água. Essa matriz, composta por polissacarídeos, proteínas, enzimas e material genético, é responsável por fornecer ao biofilme funções como adesão, agregação de células bacterianas, coesão interna, retenção de água, atua como uma barreira protetora, além de fonte de nutrição (FLEMMING e WINGENDER, 2010). As propriedades viscoelásticas do biofilme fornecem a este uma proteção contra agentes externos, sejam eles de origem mecânica ou química (RUPP et al., 2005). Poucos estudos tem demostrado como esses agentes externos afetam na viscoelasticidade do biofilme (HE et al., 2013; PETERSON et al., 2014; PETERSON et al., 2015) e em endodontia, esse tema ainda não foi abordado.

Na terapia endodôntica, a remoção do biofilme é dado por meio da ação mecânica dos instrumentos endodônticos associados ao efeito químico-físico das soluções irrigadoras (LEONARDO, 2005). Somente a ação mecânica dos instrumentos não é capaz de promover uma adequada limpeza, uma vez que os instrumentos não conseguem atuar sobre todo o sistema de canais radiculares (PETERS et al., 2015). Desse modo, a escolha da solução irrigante deve fazer parte da rotina clínica do endodontista. Soluções de hipoclorito de sódio tornaram-se as soluções mais utilizadas no tratamento endodôntico devido as suas duas principais propriedades: efeito antimicrobiano e capacidade de dissolução tecidual (COHENS e BURNS, 2000). Outros fatores relacionados à solução de NaOCl, como toxicidade tecidual (HÜLSMANN e HAHN, 2000) e efeitos sobre a dentina (SIM et al., 2001) também tem sido relatados. A capacidade dissolvente de matéria orgânica e o poder bactericida do hipoclorito de sódio está diretamente relacionado com a presença do cloro livre e a forma que este cloro se encontra nessa solução (BAKER, 1947). Em soluções alcalinas, o cloro se apresenta na forma de ânion hipoclorito (OCl^-), proporcionado a estas soluções uma maior capacidade de dissolução, já em soluções ácidas, o ácido hipocloroso (HOCl) predomina como forma de cloro livre (MACEDO et al., 2010), dando à solução um alto efeito antimicrobiano.

Jungbluth et al. (2011) avaliaram o efeito da alcalinização e estabilização do hipoclorito de sódio em um pH alto em relação a capacidade dissolvente de tecido palatino de porcos e o efeito desta solução na matriz dentinária, sendo seus resultados favoráveis ao efeito dissolvente, porém, não vantajosos em relação a dentina radicular. Este estudo norteou o artigo 1 da presente tese, uma vez que o efeito do hipoclorito de sódio com alto poder alcalino sobre a viscoelasticidade do biofilme não foi reportado até o presente momento.

As propriedades viscoelásticas, como já descritas na introdução deste trabalho, podem ser determinadas pela avaliação do relaxamento, que foi nomeado em ambos os artigos como "stress-relaxation percentage", uma vez que, após tabulação esses dados são apresentados em valores de porcentagem. Relaxamento é um processo tempo-dependente e pode ser separado em várias respostas, cada uma com uma constante de tempo característica, nomeadas nos artigos como Elemento 1 (E_1), Elemento 2 (E_2) e Elemento 3 (E_3), sendo estes relacionados com exclusão de água ou matriz EPS, mudanças na conformação da estrutura superficial bacteriana e rearranjo da posição ou compactação bacteriana, respectivamente (HE et al., 2013). Os resultados do artigo 1 mostraram não haver diferença estatisticamente significante entre as soluções de hipoclorito de sódio com pH alcalino estável seja pela adição de um álcali ou pela mistura com uma solução tampão. Não houve também, diferença entre os elementos de Maxwell nos diferentes grupos, independentemente do tempo de exposição dos irrigantes sobre o biofilme. Um explicação plausível está no modelo de biofilme utilizado para os dois artigos. CDFF (*constant depth film fermenter*), em uma tradução literal – fermentador de filme de profundidade constante – é um modelo de biofilme dinâmico que possui como vantagens o controle de algumas variáveis como a temperatura, a introdução contínua do meio de cultura, ambiente anaeróbio entre outros (HOPE e WILSON, 2006; PRATTEN, 2007).

Em endodontia, muitos estudos têm avaliado a capacidade de desinfecção das substâncias químicas aplicando-as em bactérias de culturas planctônicas, entretanto, é fato notório que a alta eficácia desses agentes desinfetantes sobre bactérias livres em um ambiente planctônico não reflete bem o efeito desses mesmos agentes em microrganismos numa forma estruturada como o biofilme (D'ARCANGELO, VARVARA e DE FAZIO, 1999; PORTENIER et al., 2005). Ceri et al (1999) demonstraram que bactérias arranjadas dentro de uma estrutura organizada e envolta por uma matriz polimérica podem ser de 100 a 1000 vezes mais resistentes aos agentes antimicrobianos que suas contrapartes na forma planctônica. Esses achados justificam o uso do modelo de biofilme no presente trabalho. Um denso e compacto biofilme pode retardar/dificultar a penetração da solução irrigante nessa estrutura,

fazendo assim com que os valores de relaxamento não fossem influenciados. Esses resultados estão de acordo com os achados de He et al (2013). Neste estudo, os autores avaliaram a taxa de penetração de um antimicrobiano em relação as características viscoelásticas do biofilme produzido em dois modelos de biofilme. Para o biofilme originário do CDFF os valores de relaxamento e suas respostas não foram influenciados pela solução, corroborando nossos achados.

Outro fator importante em relação ao biofilme utilizado nesta tese é que biofilmes de dupla-espécies, compostos por estreptococos e actinomyces cultivados sob contínua compactação, possuem características mecânicas semelhantes ao de placas orais completas, conforme reportado por Paramonova et al (2009). Considerando que este tipo de biofilme é mais fácil de reproduzir laboratorialmente do que os biofilmes multi-espécies, estudos que avaliam propriedades mecânicas podem se beneficiar (VAN DER MEI et al., 2007), sem perda de relevância.

A ação do cloro a partir das soluções de hipoclorito de sódio é tempo-dependente (MACEDO et al., 2010; ALVES et al., 2011), por este motivo, o artigo 1 avaliou o efeito das soluções alcalinizadas em dois tempos diferentes (60 segundos e 300 segundos). Contrariando os achados dos autores supracitados, nossos resultados não foram influenciados pelos tempos aplicados.

Tomografia de coerência ótica (OCT) é uma técnica de imagem interferométrica que utiliza a reflexão e dispersão de luz sobre a amostra como princípio de funcionamento (WAGNER e HORN, 2017). Esse método apresenta vantagens como não ser destrutivo, reprodução em tempo real e a confecção de imagens bi e tri-dimensionais das amostras. Nos dois artigos, uma análise por meio de OCT foi realizada. A espessura dos biofilmes foram calculadas antes e após o tratamento a partir das imagens obtidas desse dispositivo. As imagens também forneceram condições para uma análise descritiva. Em ambos os artigos foi possível visualizar uma diferença de densidade (tons de cinza) nas amostras do grupo controle e dos grupos tratados. Wimpenny e Colasanti (1997) classificaram estruturalmente o biofilme em camada base e camada superficial. A camada base encontra-se mais compactada e aderida a superfície enquanto a camada superficial é menos aderida e com aspecto mais *"soft"*. A partir das imagens tomográficas nos dois artigos foi possível perceber que a camada superficial foi modificada após a aplicação das soluções irrigadoras e isto poderia estar relacionado com o poder de penetração de cada irrigante e o desprendimento dessa camada explicaria os valores de aumento da espessura do biofilme em alguns grupos.

O segundo artigo da presente tese objetivou avaliar a interação do digluconato de clorexidina 2% (CHX) com a solução de hipoclorito de sódio 3% sobre as mesmas características do biofilme descritas acima. CHX tem sido mostrado promissor como um agente antimicrobiano oral e como um irrigante endodôntico (GOMES, MARTINHO E VIANNA, 2009). Como vantagens, a CHX possui ação antimicrobiana de amplo espectro, especialmente contra bactérias Gram-positivas, além de sua capacidade única de substantividade (CARRILHO et al., 2010). Muitos estudos demonstraram a capacidade de CHX 2% em eliminar bactérias de canais radiculares e túbulos dentinários (YESILSOY et al., 1995; LEONARDO et al., 1999). Como desvantagem, a CHX não possui capacidade dissolvente de tecido (NAENNI et al., 2004). O uso combinado de NaOCl e CHX tem sido recomendado para melhorar suas propriedades antimicrobianas. Um protocolo usando NaOCl durante o preparo biomecânico dos canais radiculares e uma irrigação final com CHX foi sugerido na literatura endodôntica (ZEHNDER, 2006). Os resultados deste estudo mostraram que a tensão de relaxamento e seus elementos não foram influenciados pelos protocolos de irrigação aplicados. Assim como no primeiro estudo, uma possível explicação de tais resultados pode ser atribuído ao biofilme utilizado. Os achados de He et al (2013) corroboram com os nossos, onde a penetração da CHX não influenciou os valores de relaxamento e suas respostas quando um biofilme denso e compacto foi utilizado. Outra possível explicação para a baixa penetração das soluções utilizadas seria a formação do precipitado amarronzado quando NaOCl e CHX se misturam. Esse precipitado, conhecido como paracloroanilina, poderia retardar a difusão das soluções irrigadoras (KOLOSOWSKI et al., 2014).

Por se tratar de um trabalho *in vitro*, algumas limitações são inerentes e merecem um apontamento. Nos dois artigos apresentados o número de amostras foi de 05 por grupo. Essa pequena quantidade de amostras poderia limitar a análise estatística. Outra limitação presente foi a quantidade de solução aplicada, 20 μl poderiam não ser suficientes para o efeito desejado, entretanto, em um estudo piloto um maior volume de solução foi aplicado e nenhuma diferença foi encontrada (dados não publicados), além disso o porta-amostras que continham os discos de hidroxiapatita não permitiam a aplicação de um volume maior que 40 μl. De modo final, a viabilidade bacteriana não foi avaliada, o que poderia ter sido feito através de análise por microscopia confocal de varredura à laser como já reportado previamente (HE et al., 2013; PETERSON et al., 2014; SHEN et al., 2009).

A presente tese teve por objetivo compreender mais sobre a influência das soluções irrigadoras utilizadas na terapia endodôntica sobre as propriedades de viscosidade e elasticidade do biofilme. Clinicamente, estes achados poderiam ser extrapolados para uma

melhor compreensão do efeito dessas soluções nos casos de necrose pulpar e ratificar que o preparo biomecânico deve ser realizado através de um conjunto de ações e que somente a etapa da irrigação não é suficiente para a remoção do biofilme.

5 CONCLUSÃO

Diante dos dados apresentandos nos artigos desta tese é possível concluir:

- Artigo 1: a solução de hipoclorito de sódio, mesmo apresentando uma maior capacidade alcalina não foi capaz de afetar eficazmente as características viscoelásticas de um biofilme de dupla-espécie.

- Artigo 2: O uso combinado da solução de hipoclorito de sódio 3% com digluconato de clorexidina 2% ou o uso destes isoladamente, não foi capaz de modificar as propriedades viscoelásticas de um biofilme experimental.

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ANEXO A – PARECER DO ORIENTADOR ESTRANGEIRO

P.O. Box 196, 9700 AD Groningen, The Netherlands

University Medical Center Groningen

Center for Dentistry and Oral Hygiene
Head a.i. mw. S. van der Ploeg, MBA

To
To whom it may concern

Phone +3150 363 2654
E-mail l.w.m.van.der.sluis@umcg.nl
Internal ZIP code FB21
www.ctm.umcg.nl

Enclosure(s)
Ref. NPD15.0247

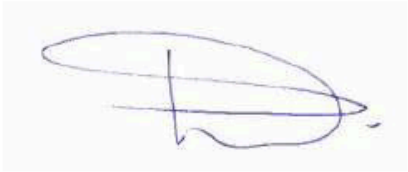
Date November, 2015
Re. Dr. Rafael Pillar

Dear Sir/Lady

I am writing to inform you that Dr. Rafael Pillar successfully finished his Sandwich Doctorate Training at the Laboratory of the Dept of Dentistry and Oral Hygiene, University of Groningen, University Medical Center Groningen, the Netherlands from January to September 2015. He performed two studies entitled *"Influence of high pH of Sodium Hypochlorite in the biofilm viscoelastic properties: OCT, LLCT and Confocal analysis"* and *"Interaction between Sodium Hypochlorite 3% and Chlorhexidine 2% in the biofilm viscoelastic properties: LLCT analysis"*. We were happy to work with Raphael and he was a dedicated researcher.

If you require any further information, please do not hesitate to contact me.

Sincerely,



Luc van der Sluis DDS, MS, PhD.
Associate Professor, PI Investigator,
Head of the Department of Conservative Dentistry
Faculty of Medical Sciences
University of Groningen

The Center is a cooperation between
UMCG and Hanze University Groningen

umcg

ANEXO B – NORMAS PARA PUBLICAÇÃO NO PERIÓDICO *JOURNAL OF ENDODONTICS*

Guidelines for Publishing Papers in the JOE

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**

a. 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*:

b. 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.

c. 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.

d. 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.

e. 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.)

f. 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.

g. 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."

h. 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.

i. 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.*

a. **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.

b. **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.

c. **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.

d. **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.

e. **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).

f. **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.

g. **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

h. 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).

i. **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.

j. **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.

k. **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:

A. 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*.

B. 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*.

C. 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*.

D. 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*.

E. 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 *.

F. Case Report/Clinical Technique (*e.g.*, report of an unusual clinical case or the use of cutting-edge technology in a clinical case). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or tables*.

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

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

Available Resources: Strunk W, White EB. The Elements of Style. Allyn & Bacon, 4th ed, 2000, ISBN 020530902X. Day R. How to Write and Publish a Scientific Paper. Oryx Press, 5th ed. 1998. ISBN 1-57356-164-9. Woods G. English Grammar for Dummies. Hungry Minds: NY, 2001 (an entertaining review of grammar). Alley M. The Craft of Scientific Writing. Springer, 3rd edition 1996 SBN 0-387-94766-3. Alley M. The Craft of Editing. Springer, 2000 SBN 0-387-98964-1