UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

FREDERICO CANATO MARTINHO

ESTUDO MICROBIOLÓGICO E DE ENDOTOXINAS DE CANAIS RADICULARES COM INFECÇÕES ENDODÔNTICAS PRIMÁRIAS E AVALIAÇÃO DA ANTIGENICIDADE DO CONTEÚDO INFECCIOSO CONTRA MACRÓFAGOS NA PRODUÇÃO DE CITOCINAS PRÓ-INFLAMATÓRIAS

Tese apresentada à Faculdade de Odontologia de Piracicaba da UNICAMP para obtenção do título de Doutor em Clínica Odontológica, na Área de Endodontia.

Orientador: Profa. Dra. Brenda Paula Figueiredo de Almeida Gomes

Este exemplar corresponde à versão final da dissertação defendida pelo aluno, e orientada pela Prof Dr Brenda Paula Figueiredo de AlmeidaGomes.

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RESUMO

Bactérias Gram-negativas (BG-) e seu sub-produto [Lipopolisacarídeo (LPS) – endotoxina) são capazes de estimular células a produzirem citocinas pró-inflamatórias envolvidas na destruição tecidual periapical. Os objetivos propostos foram: **1)** analisar os diferentes métodos de LAL para quantificação de endotoxinas, revelando o (s) que melhor (es) se adapta (m) para investigação de endotoxina nas infecções de origem endodôntica **(capítulo 1); 2)** estudar o perfil da microbiota e níveis de endotoxinas nas infecções endodônticas primárias com lesão periapical, determinando a antigenicidade do conteúdo endodôntico contra macrófagos através da produção de IL1- ß e TNF-α **(capítulo 2)**; **3)** investigar a presença de espécies bacterianas Gram-negativas "alvos" e níveis de endotoxinas nas infecções endodônticas primárias com lesão periapical, determinando seu potencial antigênico contra macrófagos através da produção de PGE_2 **(capítulo 3)**; **4)** detectar espécies de Treponema spp e os níveis de endotoxinas em infecções endodônticas primárias e determinar sua antigenicidade contra macrófagos através dos níveis de IL-6 e IL-10, avaliando sua correlação com os achados clínicos e radiográficos **(capítulo 4)**; **5)** avaliar a atividade antigênica de LPS isolado de *P. gingivalis* e *F. nucleatum* encontrados em canais radiculares infectados sobre macrófagos (RAW 264.7) através dos níveis de IL-1β e TNF-α **(capítulo 5)**; **6)** comparar "*in vivo*" a efetividade do preparo químico-mecânico com NaOCl 2.5% e CLX-gel 2% na eliminação de LPS de bactérias orais presentes em dentes com necrose pulpar e presença de lesão periapical **(capítulo 6)**; **7)** avaliar o efeito do preparo químico-mecânico com NaOCl 2.5% + EDTA 17% e limas rotatórias NiTi (Mtwo®) na remoção de endotoxinas de dentes com necrose pulpar e presença de lesão periapical **(capítulo 7)**; **8)** comparar a capacidade de diferentes sequências clínicas do sistema rotatório Mtwo® na remoção de endotoxinas em canais radiculares contaminados. **(capítulo 8)**. **Método:** amostras foram coletadas de canais radiculares com IEPL utilizando cones de papel estéreis/despirogenizados. PCR (16s rDNA) e método LAL foram utilizados. Níveis de citocinas inflamatórias foram quantificados através de ELISA (Duoset-Kit, R&D systems). **Resultados:** Os testes cinéticos (KQCL – *Kinetic Quantitative Chromegenic Limulus* e turbidimétrico) mostraram níveis de endotoxinas inferiores (7,49 EU/mL e 9,19 EU/mL, respectivamente), quando comparados ao teste QCL (*Quantitative Chromegenic*

Limulus) (34,20 EU/mL) ($p<0,05$). *Prevotella nigrescens* (13/21) foi mais frequentemente encontrada. Dente com exsudato foi relacionado com a presença de *F. alocis* ($p<0,05$). Correlações positivas ($p<0,05$) foram encontradas entre: número de BG- e níveis de IL-1 ß, TNF-α e PGE_2; níveis de endotoxinas e de TNF-α ($p<0,05$); IL-1 ß e tamanho de lesão periapical. Endotoxina foi detectada em 100% dos canais radiculares estudados. Maior redução de entodotoxina foi encontrada nos dentes instrumentados com NaOCl 2,5% (57,98%) versus CLX-gel 2% (47,12%) ($p<0,05$) utilizando limas manuais K-file. Após PQM com NaOCl 2,5% e limas rotatórias NiTi endotoxina foi reduzida em 98,06% ($p<0,05$). **Conclusão: 1)** Os testes cinéticos turbidimétrico e cromogênico de LAL apresentaram resultados mais precisos e de melhor reprodutibilidade quando comparados ao QCL **(capítulo 1)**;. **2)** A antigenicidade do conteúdo endodôntico não está relacionada apenas com a quantidade de endotoxinas encontrada nos canais radiculares, mas também com o número de diferentes espécies Gram-negativas presentes na infecção. Maior destruíção óssea periapical foi relacionado com níveis elevados de IL-1ß **(capítulo 2)**; **3)** O número de espécies BG- presentes nas IEPL foi relacionado com diferentes níveis de secreção de PGE_2 via macrófagos. Maior produção de PGE_2 foi relacionada com a presença de sintomatologia clínica **(capítulo 3)**; **4)** espécies de *Treponema* spp. exercem seu papel na patogênese das infecções endodônticas primárias. Além disso, o conteúdo bacteriano e particularmente os níveis de endotoxinas presents nos canais radiculares estimularam a produção de IL-6 e IL-10 por macrófagos **(capítulo 4)**; **5)** LPS isolados de *P. gingivalis* e *F. nucleatum* de canais radiculares infectados estimulam a produção de IL-1β e TNF-α, que são mediadores inflamatórios pleiotrópicos, podendo iniciar a resposta inflamatória e estimular a produção de mediadores secundários envolvidos na destruição tecidual **(capítulo 5)**; **6)** PQM com NaOCl 2,5% ou CLX-gel 2% não foram eficazes na eliminação de endotoxinas presentes na IEPL **(capítulo 6)**; **7)** PQM com NaOCl 2,5% + EDTA 17% e limas rotatórias NiTi (Mtwo®) foi eficaz na remoção de endotoxinas em 98,06% **(capítulo 7)**; **8)** redução significativa de endotoxinas foi obtida utilizando as sequências Mtwo® finalizadas com o preparo apical final #40.04 or #25.07 **(capítulo 8)**.

PALAVRAS-CHAVES: Endodontia, bactéria, inflamação e reação em cadeia da polymerase.

ABSTRACT

Gram-negative bacteria (G-ve) and its by-product [Lipopolysaccharide (LPS) – endotoxin] are capable to stimulate cells to release proinflammatory cytokines that lead to tissue destruction. The aims of this study were: **1)** to determine which of the quantitative methods, namely, chromogenic endpoint, chromogenic kinetic and turbidimetric kinetic ones, best fit for the analysis of primary endodontic infections **(chapter 1); 2)** to investigate the microbial profile and the levels of endotoxin found in primary root canal infection with apical periodontitis (PEIAP), and to determine their antigenicity against macrophages through the levels of IL-1ß and TNF-alpha, evaluating their relationship with clinical and radiographic findings **(chapter 2)**; **3)** to investigate target G-ve bacteria species and endotoxin in PEIAP, determining their antigenicity against macrophages through the levels of PGE_2 and evaluated their relationship with clinical findings **(chapter 3)**; **4)** investigation of Treponema spp. and endotoxin in primary endodontic infection and evaluation of the antigenicity of the infectious content against RAW 264.7 macrophages by the levels of IL-6 and IL-10 production **(chapter 4)**; **5)** to evaluate the antigenic activity of LPS purified from *P. gingivalis* and *F. nulceatum* isolated from infected root canals on macrophages cells (RAW 264.7) by the levels of IL-1β and TNF-α. **(chapter 5)**; **6)** to compare the efficacy of chemomechanical preparation with 2.5% NaOCl and 2% CHX-gel on eliminating oral bacterial LPS in teeth with PEIAP **(chapter 6)**; **7)** to investigate the ability of chemo-mechanical preparation (CMP) with 2.5% NaOCl + 17% EDTA and rotary NiTi system Mtwo® in removing endotoxin from PEIAP **(chapter 7)**; **8)** Comparison of different clinical sequences of NiTi rotary files Mtwo® in the removal of endotoxin from infected root canals **(chapter 8)**. **Methods:** Samples were taken from root canals with PEIAP with paper points. PCR technique (16S rDNA) was used for the detection of the target bacteria. Limulus Amebocyte Lysate (LAL) was used to measure endotoxin. The amounts of IL-1ß, TNF-alpha and PGE_2 in macrophages supernatants were measured by enzyme-linked immunosorbent assay – Duoset-kit (ELISA). **Results:** The KQCL and Turbidimetric -assay yielded a median value of endotoxin of 7.49 EU/mL and 9.19 EU/mL respectively, significantly different from the endpoint-QCL (34.20 EU/mL) ($p<0.05$). *Prevotella*

nigrescens (13/21) was the most frequently Gram-negative bacteria species detected. Tooth with radiolucent area ≥ 2mm was related to *Treponema denticola.* Correlation was found between the number of Gram-negative bacteria and the levels of IL-1ß, TNF-alpha and PGE_2 ($p<0.05$). Increased levels of endotoxin were followed by TNF-alpha release ($p<0.05$). Higher levels of IL-1ß ($p<0.05$) and endotoxin contents were related to the larger size of radiolucent area. Elevated levels of PGE_2 were found in teeth with tenderness to percussion and pain on palpation. Endotoxin was detected in 100% of the root canals investigated. Higher percentage value of endotoxin reduction was found in 2.5% NaOCl (57.98%) when compared to 2% CHX-gel (47.12%) ($p<0.05$) using manual K-files. After chemo-mechanical preparation with 2.5% NaOCl and rotary NI-TI files endotoxin was significantly reduced to 98.06% ($p<0.05$). **Conclusion: 1)** Quantitative kinetic-turbidimetric and kinetic-chromogenic LAL methods are best fitted for analysis of endotoxin in root canal infection, both being more precise and allowing better reproducibility compared to the endpoint-QCL assay; **2)** The antigenicity of the endodontic contents is not related to only the amount of endotoxin found in root canal, but also with the number of different species of Gram-negative bacteria involved in the infection. Moreover larger size (≥ 2mm) of radiolucent area was related to IL-1ß and endotoxin; **3)** Additive effect between the number of G-ve bacterial species involved in endodontic infection regarding the induction of pro-inflammatory cytokine by macrophage cell. Moreover, teeth with clinical symptomatology were related to higher levels of endotoxin and PGE_2 secretion; **4)** a wide variety of Treponema species do play a role in primary endodontic. Moreover, the bacterial endodontic contents, particularly the levels of endotoxin present in root canals, were a potent stimuli for the production IL-6 and IL-10 in macrophages; **5)** LPS of the *P. gingivalis* and *F. nucleatum* isolated from root canal infection is involved in the induction of IL-1 β and TNF-α, which are pleiotropic inflammatory mediators, that can play a role in the initiation of the upregulation of the inflammatory response and can also stimulate the production of secondary mediators involved in tissue destruction; **6)** 2.5% NaOCl and 2% CHX-gel were not effective on eliminating endotoxin from the primarily infected root canals using manual K-files; **7)** CMP with 2.5% NaOCl + 17% EDTA and rotary NiTi files was effective in reducing

98.06% of endotoxin from PEIAP; **8)** substantial reduction of endotoxin contents was achieved by using the Mtwo® sequences finished in APS #40.04 or #25.07.

KEY-WORDS: Endodontics, bacterial infection, inflammation, polymerase chain reaction.

SUMÁRIO

1. INTRODUÇÃO 1
2. CAPÍTULOS..... 13

2.1 **Capítulo 1 –** Comparison of Endotoxin Levels in Previous Studies on Primay Endodontic Infections……... 13

2.2 **Capítulo 2 –** Antigenic Activity of Bacterial Endodontic Contents from Primary Endododontic Infection with Periapical Lesions Against Macrophages in the Release of Interleukin-1ß and Tumor Necrosis factor……..... 31

2.3 **Capítulo 3 –** Antigencity of Primary Endodontic Infection Against Macrophages by the Levels of PGE_2 Production……..... 53

2.4 **Capítulo 4 -** Investigation of Treponema spp. and endotoxin in primary endodontic infection and evaluation of the antigenicity of the infectious content against RAW 264.7 macrophages by the levels of IL-6 and IL-10 production.….....……..... 73

2.5 **Capítulo 5 –** Stimulation of interleukin 1-β and TNF-α production of RAW 264.7 macrophages cells by *Porphyromonas gingivalis* and *Fusobacterium nucleatum* lipopolysaccharide isolated from primary endodontic infection…….. 91

2.6 **Capítulo 6 –** Comparison of 2.5% NaOCl and 2% CHX gel on Oral Bacterial LPS Reduction From Primarily Infected Root Canals…….....……..... 111

2.7 **Capítulo 7 –** Clinical Investigation of the Efficacy of Chemo-Mechanical Preparation With Rotary NiTi Files for Removal of Endotoxin From Primarily Infected Root Canals …….. 127

2.8 **Capítulo 8 –** Comparison of different clinical sequences of NiTi rotary files in the removal of endotoxin from infected root canals.……..……... 143
3. CONSIDERAÇÕES GERAIS..... 159
3.1 Justificativa da pesquisa ……..... 159
3.2 Material e Métodos utilizados ……..... 160
3.3 Complexidade antigênica dos canais radiculares 164
3.4 Lipopolissacarídeo e seu potencial inflamatório …..... 166
3.5 Antigenicidade do conteúdo endodôntico contra macrófagos (RAW 264.7) e sua relação com os achados clínicos e radiográficos …..... 170
3.6 Efeito do preparo químico-mecânico na redução de endotoxinas dos canais radiculares infectados …..... 172
4. CONCLUSÃO..... 175
REFERÊNCIAS..... 177
APÊNDICE I (Material e métodos e ilustrações dos experimentos realizados na pesquisa)..... 189
APÊNDICE II (Manual de dosagem de citocinas pró-inflamatórias (Kit R & D system)..... 229
APÊNDICE III (Dados demográficos, características clínicas e radiográficas do elemento dental presente nos pacientes que participaram da pesquisa)..... 241
APÊNDICE IV (Produção bibliográfica do aluno)..... 243
ANEXO I (Certificado de Comitê de Ética em Pesquisa Humana)..... 247

1. INTRODUÇÃO

Os microrganismos e seus subprodutos tais como endotoxinas desempenham um papel fundamental nas doenças pulpares e dos tecidos perirradiculares. A disseminação bacteriana por toda extensão do canal radicular em direção aos tecidos periapicais decorre num intenso processo inflamatório - via ação direta e/ou indireta do sistema imunológico, caracterizado pela presença de sintomatologia clínica e reabsorção óssea. Se a infecção não for controlada através do tratamento endodôntico, pode ocorrer uma disseminação sistêmica do processo infeccioso.

Ao longo dos últimos 100 anos, endotoxina bacteriana é considerada uma das moléculas mais potentes e de maior interesse encontradas na natureza. Suas peculiaridades estruturais, diversidade química e física e seu amplo espectro de atividade biológica resultaram em uma das linhas de pesquisa mais fomentadas mundialmente. Ainda que sua composição química e estrutural tenha sido amplamente explorada, perguntas surgem quanto ao real papel das endotoxinas nas infecções humanas.

Na endodontia, até o momento, pesquisas foram desenvolvidas focando na identificação/detecção bacteriana ou na quantificação de endotoxinas de canais radiculares, limitando, assim, o conhecimento do real potencial inflamatório do conteúdo endodôntico infeccioso. Nenhum trabalho foi realizado envolvendo simultaneamente técnicas moleculares de identificação bacteriana; quantificação de endotoxinas; e avaliação do potencial antigênico através da expressão gênica de RNAm e produção de diferentes citocinas inflamatórias envolvidas nas alterações periapicais. Além disso, faltam estudos sobre comparação de diferentes protocolos clínicos para redução deste conteúdo infeccioso, visando a instituição de uma conduta terapêutica eficaz.

Desta forma, a imunobiologia nas infecções de origem endodônticas vem contribuir para o entendimento do potencial inflamatório do conteúdo infeccioso presente nos canais radiculares, permitindo melhor interpretação do quadro clínico da

doença e servindo de guia para o tratamento da infecção.

A maioria das bactérias capaz de infectar os canais radiculares é oriunda da cavidade oral (Gomes *et al.*, 1994a); sendo sua sobrevivência intra-radicular e suas propriedades patogênicas influenciadas por uma combinação de fatores, incluindo: interação sinérgica entre espécies, capacidade de interferir e evadir a resposta do hospedeiro, a liberação de endotoxinas e a síntese de enzimas danosas aos tecidos do hospedeiro (Nair, 2004).

As infecções endodônticas primárias são polimicrobianas dominadas por bactérias anaeróbias estritas (Gomes *et al.*, 1994 a,b; Siqueira *et al.*, 2001; Jacinto *et al.* 2003; Gomes *et al.*, 2004). Achados indicam que bacilos Gram-negativos anaeróbios estritos, particularmente espécies pertencentes aos gêneros *Porphyromonas, Prevotella, Fusobacterium* e *Treponema* estão envolvidas com sintomatologia, e exercem um papel significativo na patogênese das lesões inflamatórias periapicais (Griffee *et al.*, 1980; Yoshida *et al.*, 1987; Gomes *et al.*, 1994a, 1996; Baumgartner *et al.*, 1999; Siqueira *et al.*, 2001, Jacinto *et al.,* 2003; Siqueira *et al.*, 2004; Sakamoto *et al.,* 2006, 2009; Montagner *et al.*, 2010).

Bactérias Gram-negativas apresentam como constituinte de membrana externa moléculas de lipopolisacarídeo (LPS), as quais são responsáveis pela organização e estabilidade bacteriana (Vaara & Nikaido, 1984). Aproximadamente 3/4 da superfície bacteriana são formadas por estas moléculas (Petsch & Anspach, 2000); as quais, mesmo em baixas concentrações, são consideradas potentes estimuladores da resposta inata do sistema de defesa do hospedeiro (Beutler, 2000; Hong *et al.*, 2004; Martinho *et al.*, 2010a), podendo gerar danos teciduais e destruição óssea (Darveau, 2000; Hong *et al.*, 2004), exercendo assim sua atividade endotóxica.

Lipopolissacarídeos (Ohno & Morrison 1989), também conhecidos como endotoxinas, consistem de uma porção “Lípide A", Core oligossacarídeo (polissacarídeos) e Antígeno O. A porção “Lípide A" exerce a maior parte das atividades endotóxicas, a qual é referida como princípio endotóxico do LPS (Dixon & Darveau, 2005). A mudança em menor ou maior proporção da porção “Lípide A" pode produzir

efeitos distintos e diversos na modulação da resposta imune (Dixon & Darveau, 2005), podendo apresentar maior ou menor atividade endotóxica contra o organismo.

A porção “Lípide A" pode variar de espécie para espécie, e entre diferentes cepas de mesma espécie, de acordo com o número de grupo de fosfatos, a quantidade e a posição de ácidos graxos na molécula (Bainbridge *et al.*, 2002; Darveau *et al.*, 2004).

Bactérias pertencentes aos gêneros *Prevotella* e *Porphyromonas* parecem apresentar atividade endotóxica mais baixa do que a *Escherichia coli* (*E. coli*) (Duerden, 1994). Por outro lado, LPS do gênero *Fusobacterium* apresenta uma estrutura similar a dos bacilos entéricos Gram-negativos (Bennett & Eley, 1993), contribuindo para a alta virulência destes microrganismos (Duerden, 1994).

Estudos clínicos revelam a presença de LPS bacteriano em 100% dos canais radiculares analisados de dentes com infecções endodônticas primárias e lesão periapical (Jacinto *et al.*, 2005; Vianna *et al.*, 2007; Martinho & Gomes, 2008), sugerindo sua possível participação na destruição óssea periradicular.

A inoculação de LPS da espécie *E. coli* em canais radiculares de animais é capaz de produzir inflamação e reabsorção óssea (Dwyer & Torabinejad, 1981; Dahlén *et al.*, 1981; Pitts, *et al.*, 1982; Mattison *et al.*, 1987). Entretanto, pouco se conhece da atividade endotóxica das bactérias encontradas nos canais radiculares, limitando o entendimento do real papel das endotoxinas nas infecções endodônticas.

A destruição tecidual característica da lesão periapical pode resultar tanto direta quanto indiretamente (via sistema imune) dos efeitos dos produtos bacterianos ou dos componentes bacterianos ou ambos, sob os tecidos periapicais (Mattison *et al.*, 1987).

Infecções endodônticas envolvem diferentes associações bacterianas: entre espécies Gram-negativas – combinando diferentes LPS bacterianos com diferentes potenciais inflamatórios, os quais podem agir sinergicamente aumentando a destruição tecidual (Hong *et al.*, 2004); e ainda entre espécies Gram-negativas e Gram- positivas, onde peptideoglicanos da parede celular das Gram-positivas agem sinergicamente com o

LPS das Gram-negativas (Jiang *et al.*, 2003), tornando ainda mais complexa a antigenicidade do conteúdo endodôntico para a resposta imune.

O acúmulo de componentes bacterianos presentes nas infecções endodônticas primárias, incluindo principalmente endotoxinas e peptideoglicanos, pode estimular a liberação de citocinas pró-inflamatórias por diferentes linhagens celulares (Wilson *et al.*, 1996), através dos receptores de membrana TLR-2 e TLR-4 "TLR- Toll like receptors" (Horiba *et al.*, 1989; Hosoya & Matsushima, 1997; Hong *et al.*, 2004).

Dentre diferentes linhagens celulares envolvidas no processo inflamatório periapical, os macrófagos estão presentes em maior população, sendo considerados a principal fonte de produção de citocinas pró-inflamatórias (Artese *et al.*, 1991; Matsuo *et al.*, 1992; Metzger, 2000), e quase exclusivamente o produtor de TNF-α (Fator de Necrose Tumoral – alfa) na presença de LPS bacteriano (Beutler & Cerami, 1986).

As infecções crônicas envolvem diferentes citocinas multifuncionais que participam no processo de regulação da resposta imunológica e na inflamação, como por exemplo, o TNF-α (Safavi *et al.*, 1991; Ataoglu *et al.*, 2002; Hong *et al.*, 2004), interleucina - 1 (IL-1) e interleucina -6 (IL-6) (Barkhordar *et al.*, 1992; Tani-Ishii *et al.*, 1995; Ataoglu *et al.*, 2002), e prostaglandina – 2 (PGE_2) (Le & Vilcek, 1989; Akira *et al.*, 1990; McNicholas *et al.*, 1991; Shimauchi *et al.*, 1996). As interações sinérgicas entre estas citocinas são reconhecidas em um processo osteoclástico de "feedback" negativo e positivo (Akira *et al.*, 1990; Le & Vilcek, 1989).

IL1-β e TNF-α, produzidas primeiramente pelos macrófagos, são capazes de desenvolver um quadro de pulpite (Tani-Ishii *et al.*, 1995) e iniciar uma cascata da inflamação resultando em destruição tecidual (Hong *et al.*, 2004) culminando em reabsorção óssea periapical (Tani Ishii *et al.*, 1995). Ambas têm sido detectadas nos tecidos periapicais (Barkhordar *et al.*, 1992) em canais radiculares com exsudação e lesão periapical (Safavi & Rossomando, 1991; Matsuo *et al.*, 1992). Particularmente, nível elevado de IL1-β parece estar relacionado com a presença de sinais/sintomas clínicos (Lim *et al.*, 1994; Kuo *et al.*, 1998) e destruição óssea periapical (Matsuo *et al.*, 1992; Ataoglu *et al.*, 2002).

PGE_2 está relacionada com a maioria dos eventos inflamatórios na região periapical, tais como: vasodilatação, aumento da permeabilidade vascular, degradação de colágeno e reabsorção óssea (Offenbacher *et al.*, 1993). Nível elevado de PGE_2 encontrado em dentes com inflamação pulpar, exsudação (Shimauchi *et al.*, 1996) e nos tecidos periapicais (McNicholas *et al.*, 1991) parece estar envolvido no processo sintomático de origem endodôntica (McNicholas *et al.*, 1991; Shimauchi *et al.*, 1996).

Durante a terapia endodôntica várias substâncias químicas têm sido testadas, na eliminação e/ou inativação de endotoxinas (Tanomaru *et al.*, 2003; Niwa *et al.*, 1969; Buttler & Crawford, 1982; Buck *et al.*, 2001; Oliveira *et al.*, 2007; Vianna *et al.*, 2007; Martinho & Gomes 2008). Entretanto, o hipoclorito de sódio (NaOCl) - devido às suas propriedades antimicrobianas e ao seu potencial de dissolução de tecido orgânico (Byström & Sundqvist, 1983; Siqueira *et al.*, 1998), e a clorexidina (CLX) – devido principalmente à sua atividade antimicrobiana (Gomes *et al.*, 2001), baixa toxicidade (Onçag *et al.*, 2003) e substantividade em tecido dentinário (Dametto *et al.*, 2005) - são as substâncias químicas auxiliares mais utilizadas na terapia endodôntica.

Estudos *in vivo* (Vianna *et al.*, 2007; Martinho & Gomes 2008) utilizando limas manuais K-file têm demonstrado a baixa capacidade do preparo químico-mecânico em remover endotoxina dos canais radiculares com necrose pulpar e presença de lesão periapical, sugerindo ainda a possível participação do conteúdo residual de endotoxinas na possível perpetuação de sintomatologia clínica e/ ou insucesso da terapia endodôntica.

Apesar da grande importância das endotoxinas das bactérias Gram-negativas nos processos inflamatórios periapicais existem poucos trabalhos na literatura endodôntica relacionados a LPS das bactérias frequentemente detectadas nos canais radiculares. A maioria destes foi realizada avaliando a atividade de diferentes substâncias químicas auxiliares e uso de medicações intracanais sobre LPS da espécie *E. coli* (bactéria bacilar Gram-negativa, que faz parte da microbiota normal no intestino; não encontrada nas infecções de origem endodônticas).

Schein & Schilder (1975) quantificaram endotoxina através do teste Lisado de Amebócito Limulus (LAL) de amostras coletadas em canais radiculares de 40 dentes,

os quais foram divididos nos seguintes grupos: clinicamente em dentes vitais e assintomáticos (n=10), dentes vitais e sintomáticos (n=10), dentes despolpados e assintomáticos (n=10), e dentes despolpados e sintomáticos (n=10). E radiograficamente em dentes com presença de lesão periapical (n=23) e ausência de lesão periapical (n=17). Os dentes vitais/ assintomáticos apresentaram menor média quando comparados aos dentes vitais/sintomáticos (0,007 e 0,075 µg EU/mL, respectivamente). Os dentes despolpados/ assintomáticos, por sua vez, apresentaram maior concentração de endotoxina (0,192 µg EU/mL) quando comparados com os dentes vitais. Entre os dentes despolpados os sintomáticos apresentaram maior concentração de endotoxina do que os assintomáticos (0,192 e 1,070µg EU/mL, respectivamente). Portanto, os dentes com sintomatologia clínica e presença de lesão periapical apresentaram maior concentração de endotoxina.

Dahlén & Bergenholtz (1980) avaliaram a atividade tóxica das endotoxinas em 13 canais radiculares com polpas necrosadas através do lisado de limulus. Os autores encontraram correlação entre a atividade de endotoxina e o número de bactérias Gram-negativas.

Horiba *et al.* (1990) coletaram amostras de 30 dentes com necrose pulpar com objetivo de verificar possíveis correlações entre a presença de endotoxina e sintomas clínicos ou presença áreas radiolúcidas em canais radiculares infectados. Os autores concluíram que o conteúdo de endotoxina foi maior em dentes sintomáticos. Dentes com áreas radiolúcidas periapicais e dentes com exsudação apresentaram maior concentração de endotoxinas, quando comparado à ausência das mesmas.

Khabbaz *et al.* (2000) investigaram a presença ou ausência de endotoxina, quantificaram endotoxina e associaram a presença de endotoxina com dor aguda de origem pulpar em 24 amostras coletadas da superfície da lesão cariosa em 9 molares e 15 pré-molares com pulpite irreversível e reversível. Os dentes foram divididos em 3 grupos: sintomáticos com presença de lesão cariosa (n=9), assintomáticos com presença de lesão cariosa (n=11), e grupo controle sem cárie (n=4). A quantificação da endotoxina foi realizada através do teste LAL. Os resultados indicaram a presença de endotoxina em

valores mais elevados nos casos sintomáticos, valor médio de 0,15773 ng/mL, quando comparados aos casos assintomáticos, com valor médio de 0,10723 ng/mL. Os dentes do grupo controle estavam livres da presença de endotoxina. Nos dentes sintomáticos (pulpite irreversível) foi encontrada endotoxina na superfície e nas camadas mais profundas de cárie, nos respectivos valores 0,15078 e 0,12111 ng/mL. Nos dentes assintomáticos, as concentrações de endotoxina presentes na superfície e em camadas mais profundas de lesões cariosas foram detectadas nos valores de 0,122091 e 0,07163 ng/mL, respectivamente. Os resultados também demonstraram que a concentração de endotoxina foi maior na superfície de dentes sintomáticos com lesão cariosa quando comparado aos dentes assintomáticos (0,15078 e 0,12091 ng/mL, respectivamente). Similarmente, maior quantidade de endotoxina estava presente em camadas profundas das lesões cariosas de dentes sintomáticos ao comparar com dentes assintomáticos (0,12111 e 0,07163 ng/ mL, respectivamente). Os autores concluíram com o estudo que concentrações de endotoxina estavam presentes em lesões cariosas de dentes sintomáticos e assintomáticos. A quantidade de endotoxina foi significante maior na superfície do que em camadas profundas da dentina cariada. A maior concentração de endotoxina estava presente nos casos sintomáticos, em relação aos assintomáticos.

Khabbaz *et al.* (2001) investigaram a presença ou ausência de endotoxina em polpas de dentes com lesão cariosa, quantificaram endotoxina e associaram sua presença com dor pulpar aguda. As amostras foram coletadas de 28 dentes com lesão cariosa (15 sintomáticos e 13 assintomáticos) e de 5 dentes sem cárie dental. Durante os procedimentos de coleta, as polpas foram pesadas e padronizadas (aproximadamente 8 mg). A extração de endotoxina foi realizada com fenol e água. As quantidades de endotoxinas foram determinadas através do teste LAL. Os resultados mostraram que a concentração de endotoxina nas polpas dos dentes com sintomatologia dolorosa foi em média 0,15773 ng/mL; já nos dentes sem sintomatologia a média foi aproximadamente de 0,10723 ng/mL; e nos dentes sem lesão cariosa, estavam livres de endotoxina. A detecção de endotoxina foi significante maior no grupo de dentes com sintomatologia. Os autoores concluíram que a presença de endotoxina nos tecidos periapicais de dentes com lesão cariosa sugerem um papel importante nas doenças pulpares, visto que há uma quantidade maior de endotoxina nas polpas com sintomatologia dolorosa.

Silva *et al.* (2004) avaliaram histologicamente a efetividade do preparo químico-mecânico em 120 canais radiculares de 6 cães, inoculados com LPS bacteriano de *Escherichia coli* após pulpectomia e, posteriormente, selados com óxido de zinco e eugenol. Os dentes foram divididos em grupos, de acordo com o irrigante utilizado: solução salina; NaOCl 1%; 2,5%; 5%; clorexidina 2%; e grupo controle sem irrigante. Os animais foram mortos em 60 dias. Os dentes seccionados foram fixados e desmineralizados. Subsequentemente foram realizados cortes seriados e corados pela técnica de Brown-Brenn para visualização de contaminação bacteriana. Os resultados demonstraram que o infiltrado inflamatório foi menos intenso nos grupos que continham NaOCl 1% e 2,5% e clorexidina 2%. Entretanto, nenhum dos irrigantes foi capaz de inativar por completo o efeito maléfico do LPS bacteriano. O preparo químico-mecânico associado a diferentes irrigantes não foi capaz de inativar o LPS bacteriano.

Oliveira *et al.* (2007) estudaram, i*n vitro*, a efetividade de irrigantes endodônticos sobre endotoxinas de canais radiculares. Oitenta e oito canais radiculares foram contaminados com endotoxina de *E. coli*, e divididos em 7 grupos de acordo com a substância irrigadora e medicação intra-canal utilizada. G1 (NaOCl 2,5%); G2 (NaOCl 5,25%); G3 (CLX 2%); G4 [$Ca(OH)_2$]; G5 (Polimixina B); G6 – controle positivo (solução salina); e G7 – controle negativo (livre de endotoxinas). As amostras dos canais radiculares foram coletadas imediatamente após instrumentação e após 7 dias. Endotoxina foi quantificada através do método LAL e a atividade endotóxica avaliada através da produção de anticorpo sobre cultura de linfócitos-B. Em ambas as coletas, G4, G5 e G7 apresentaram os melhores resultados quando comparados ao G1, G2, G3 e G6. O $Ca(OH)_2$ e a polimixina B foram capazes de neutralizar a atividade endotóxica do LPS quanto a produção de anticorpos sobre linfócitos B. NaOCl e CLX 2% não foram eficazes da detoxificação de endotoxinas.

Valera *et al.* (2010) avaliaram, *in vitro*, a ação do própolis e diferentes medicações intracanais contra *E. coli* e endotoxina. Quarenta e oito dentes foram instrumentados utilizando própolis. Os dentes foram divididos de acordo com as medicações intracanais testadas: $Ca(OH)_2$, polimixina B e $Ca(OH)_2$ + CLX-gel 2%. Cargas bacterianas e de endotoxinas foram quantificadas através da contagem de

unidades formadoras de colônias (UFC) e método LAL, respectivamente. O uso do própolis durante a instrumentação foi capaz de eliminar *E. coli* dos canais radiculares e reduzir os níveis de endotoxinas. Todas as medicações intracanais testadas contribuíram para a redução de endotoxinas, sendo o $Ca(OH)_2$ mais efetivo.

A partir de 2005, trabalhos clínicos realizados na Endodontia da Faculdade de Odontologia de Piracicaba FOP-UNICAMP procuraram quantificar clinicamente endotoxinas presentes em dentes com infecções endodônticas primárias (Jacinto *et al.*, 2005; Vianna *et al.*, 2007; Martinho & Gomes 2008; Gomes *et al.*, 2009, Endo 2011).

Jacinto *et al.* (2005) investigaram a concentração de endotoxina em dentes com polpa necrosada e avaliaram possível relação entre a concentração de endotoxina e a presença ou ausência de sinais e sintomas. As amostras foram coletadas de 50 canais radiculares em dentes com necrose pulpar. A quantidade de endotoxina foi mensurada através do teste LAL. A quantidade de endotoxina presente nas amostras variaram entre 2,390 e 22,100 EU mL^{-1}. O valor médio da concentração de endotoxina de pacientes com dor espontânea foi 18,540 EU mL^{-1}, enquanto que os casos assintomáticos apresentaram valores inferiores, com média de 12,030 EUmL^{-1}. Foram encontradas associações positivas entre a concentração de endotoxina e a presença de sintomatologia.

Vianna *et al.* (2007) quantificaram a presença de endotoxina em 24 canais radiculares de dentes com necrose pulpar e presença de lesão periapical antes (C1) e após a instrumentação manual com clorexidina gel 2% (C2), após soro fisiológico (C3) e 7 dias após o uso das medicações hidróxido de cálcio, clorexidina gel e sua associação (C4). Endotoxinas foram detectadas em 100% dos canais analisados com média geral equivalente a 151,6 EU/mL (C1), 53,66 EU/mL (C2), 84,93 EU/mL (C3) e 72,75 EU/mL (C4). Após medicação, endotoxina foi reduzida em apenas 1,4% quando comparado a C2. Níveis elevados de endotoxinas foram detectados nos canais radiculares mesmo após preparo químico-mecânico.

Martinho & Gomes (2008) quantificaram a presença de endotoxina em 24 canais radiculares de dentes com necrose pulpar e presença de lesão periapical antes (C1) e após a instrumentação com NaOCl 2,5% (C2). Endotoxina foi quantificada através do

método de LAL. LPS foi detectado em 100% dos canais radiculares estudados em C1 (mediana: 139 EU/mL). Após o preparo químico-mecânico com NaOCl 2,5% e instrumentação manual, endotoxina foi reduzida em 59,99% (mediana: 72,50 EU/mL). Os resultados mostraram que o NaOCl 2,5% foi efetivo na redução de endotoxinas dos canais radiculares. Associação positiva foi encontrada entre níveis elevados de endotoxina e presença de sintomatologia.

Gomes *et al.* (2009) compararam a efetividade do preparo químico-mecânico utilizando o NaOCl 2,5% e clorexidina gel 2% na remoção de endotoxina de canais radiculares de dentes com necrose pulpar e lesão periapical. Endotoxina foi detectada em 100% dos canais radiculares analisados: NaOCl 2,5% (mediana: 272 EU/mL), clorexidina gel 2% (152,46 EU/mL). Níveis de endotoxinas foram reduzidos para 86 EU/mL (NaOCl 2,5%) e 85 EU/mL (clorexidina gel 2%). Percentual de remoção de endotoxinas foi maior com o uso de NaOCl 2,5% ($p<0,05$). Os autores concluíram que nenhuma das substâncias químicas auxiliares testadas foi capaz de eliminar endotoxinas dos canais radiculares.

Endo (2011) avaliou a efetividade do preparo químico-mecânico na redução de endotoxinas de 15 dentes com insucesso da terapia endodôntica. As amostras foram coletadas antes (C1) e após o preparo químico-mecânico (C2) utilizando limas manuais e CLX-gel 2%. Em C1, endotoxina foi detectada em 100% das amostras estudadas (3,96 EU/mL). Endotoxina foi reduzida em 60,6% com o preparo químico-mecânico quando comparado ao conteúdo inicial. Correlação positiva foi encontrada entre maior nível de endotoxina e mairor destruição óssea. Contudo, o preparo químico-mecânico com CLX-gel 2% + EDTA 17% foi eficaz na redução de microrganismos/ endotoxinas dos canais radiculares, mas não foi capaz de eliminar LPS.

Tendo quantificado endotoxinas presentes nos canais radiculares, torna-se importante entender o real potencial inflamatório desta molécula na infecção endodôntica, assim como a determinação do potencial antigênico residual presente nos canais radiculares após preparo químico-mecânico.

O padrão de resposta imune/inflamatória gerada frente aos microrganismos da infecção endodôntica é determinado pela rede de citocinas estabelecidas que, por sua vez, depende da complexidade das vias celulares ativadas diante determinado estímulo externo. Neste sentido, o conhecimento do conteúdo infeccioso presente nos canais radiculares, assim como, a estratégia do tratamento endodôntico em sua eliminação, são importantes para contribuir com o sucesso da terapia endodôntica.

Desta forma o presente estudo utilizou-se de diferentes técnicas moleculares de identificação bacteriana, quantificação de endotoxinas e avaliação do potencial antigênico do conteúdo infeccioso endodôntico para se conhecer o que está presente nos canais. A partir do conhecimento deste conteúdo, foram instituídos diversos protocolos clínicos para a sua redução, visando a instituição de uma conduta terapêutica eficaz.

Esta pesquisa foi dividida em 8 capítulos, tendo como principais objetivos: analisar os diferentes métodos de LAL para quantificação de endotoxinas, revelando o (s) que melhor (es) se adapta (m) para investigação de endotoxina nas infecções de origem endodôntica **(capítulo 1);** estudar o perfil da microbiota e níveis de endotoxinas nas infecções endodônticas primárias com lesão periapical, determinando a antigenicidade do conteúdo endodôntico contra macrófagos através da produção de IL1-ß e TNF-α **(capítulo 2)**; investigar a presença de espécies bacterianas Gram-negativas "alvos" e níveis de endotoxinas nas infecções endodônticas primárias com lesão periapical, determinando seu potencial antigênico contra macrófagos através da produção de PGE_2 **(capítulo 3)**; detectar espécies de Treponema spp e os níveis de endotoxinas em infecções endodônticas primárias e determinar sua antigenicidade contra macrófagos através dos níveis de IL-6 e IL-10, avaliando sua correlação com os achados clínicos e radiográficos **(capítulo 4)**; avaliar a atividade antigênica de LPS isolado de *P. gingivalis* e *F. nucleatum* encontrados em canais radiculares infectados sobre macrófagos (RAW 264.7) através dos níveis de IL-1β e TNF-α **(capítulo 5)**; comparar "*in vivo*" a efetividade do preparo químico-mecânico com NaOCl 2.5% e CLX-gel 2% na eliminação de LPS de bactérias orais presentes em dentes com necrose pulpar e presença de lesão periapical **(capítulo 6)**; avaliar o efeito do preparo químico-mecânico com NaOCl 2.5% + EDTA 17% e limas rotatórias NiTi (Mtwo®) na remoção de endotoxinas

de dentes com necrose pulpar e presença de lesão periapical **(capítulo 7)**; comparar a capacidade de diferentes sequências clínicas do sistema rotatório Mtwo® na remoção de endotoxinas em canais radiculares contaminados. **(capítulo 8)**.

2. CAPÍTULOS

2.1. Capítulo 1 – Comparison of Endotoxin Levels in Previous Studies on Primay Endodontic Infections

ABSTRACT

This study was performed to determine which of the quantitative methods, namely, chromogenic endpoint, chromogenic kinetic and turbidimetric kinetic ones, best fit for the analysis of primary endodontic infections. **Methods:** 21 root canals with apical periodontitis were sampled with paper points. The same sample was analyzed by means of endpoint chromogenic LAL (Limulus Amebocyte Lysate) assay (QCL), quantitative kinetic chromogenic LAL assay (KQCL) and kinetic turbidimetric LAL assay (Turbidimetric). **Results:** All three LAL-methods were effective in the recovery of endotoxin from root canal infection. Regardless of the method tested, endotoxin was detected in 100% of the root canals (21/21). The KQCL-assay yielded a median value of endotoxin of 7.49 EU/mL, close to and not significantly different from those for turbidimetric test (9.19 EU/mL) (both kinetic methods) ($p>0.05$). In contrast, the endpoint-QCL showed a median value of 34.20 EU/mL ($p<0.05$). The comparison of the three methods revealed that both turbidimetric and KQCL methods were more precise, with best reproducibility (the coefficient variation between analysis of the root canal and its duplicate was lower than 10%). The inhibition/enhancement assay indicated a good interaction between the root canal samples with the turbidimetric method. **Conclusion:** This study has revealed that quantitative kinetic-turbidimetric and kinetic-chromogenic LAL methods are best fitted for analysis of endotoxin in root canal infection, both being more precise and allowing better reproducibility compared to the endpoint-QCL assay.

Key words: endotoxin; root canal; LAL methods; endodontic infection

INTRODUCTION

Lipopolysaccharide (LPS, endotoxin) - an outer membrane component of Gram-negative (GN) bacteria predominantly involved in root canal infection (1) – is an important mediator in the pathogenesis of apical periodontitis (2-8).

Over the years, clinical endodontic researchers have not only attempted to investigate LPS in infected root canals by correlating higher endotoxin levels with the presence of clinical signs/ symptoms and radiographic findings (8-13), but also evaluated the effect of root canal procedures on its elimination (8, 14-16) by using the Limulus Amebocyte Lysate (LAL) coagulation system (17).

The LAL assay employs a serine protease catalytic coagulation cascade activated by the presence of Gram-negative bacterial endotoxin (18). Because of its extreme sensitivity to endotoxins (19), LAL is the most widely used assay for analysis of endodontic contents (8, 9, 11-16, 20-23) (Table 1). There are several endotoxin detection methods employing the so-called Limulus reaction using LAL (17, 24, 25), Gel clot (17), turbidimetric (26) and chromogenic (27) tests.

The first studies used a semi-quantitative analysis of endotoxin determined by the endpoint coagulogen assay – detection of endotoxin by the evidence of gelation (Gel clot LAL assay) (12). More recently, endodontic investigations have used quantitative methods such as chromogenic endpoint (QCL-test) (9, 11, 13 - 15) and kinetic chromogenic (KQCL-test) assays (20-22) – both determining the levels of endotoxin by the yellow color intensity (chromogenic LAL assay); and the kinetic turbidimetric assay (8, 16, 23, 28) (turbidimetric test), which is based on the reaction by turbidity (Coagulogen-based LAL assay).

While the endpoint chromogenic method has a limitation regarding the lack of sensitivity (detection limit: 0.1- 1 endotoxin unit/ mL [EU/mL]), the chromogenic kinetic (detection limit: 0.005-50 EU/mL) and turbidimetric kinetic (detection limit: 0.01-100 EU/mL) methods present a higher precision (18). On the other hand, the kinetic

methods have a problem with the duration of the experiment (over 60 min *versus* 16 min in the endpoint chromogenic method) (29).

Overall, due to the different assay performance for endotoxin detection and variability in the sensitivity of LAL methods, this study was conducted in order to determine which of the following quantitative methods – chromogenic endpoint, chromogenic kinetic and turbidimetric kinetic ones – best fit for analysis of primary endodontic infections.

MATERIAL AND METHODS

Patient selection

Twenty-one patients who attended the Piracicaba Dental School, Piracicaba, Brazil, for endodontic treatment were included in this research. The age of the patients ranged from 13 to 73 years. Samples were collected from 21 root canals with pulp necrosis determined by the sensitivity test and showing radiographic evidence of apical periodontitis. The selected teeth showed absence of periodontal pockets deeper than 4 mm. The following clinical/radiographic findings were found: pain on palpation (9/21), tenderness to percussion (8/21), exudation (12/21), radiolucent area $\geq$ 2 mm (11/21) and $<$ 2 mm (10/21). None of the patients reported spontaneous pain.

A detailed dental history was obtained from each patient. Patient who had received antibiotic treatment during the last three months or who had any general disease were excluded. The Human Research Ethics Committee of the Piracicaba Dental School approved the protocol describing specimen collection for this investigation, and all patients signed an informed consent document regarding the study.

Sampling procedures

All materials used in this study were heat-sterilized at 200°C for 4 hours, thus becoming apyrogenic. The method followed for disinfection of the operative field has been previously described (9, 15). Briefly, the teeth were isolated with rubber dam. The crown and the surrounding structures were disinfected with 30% H_2O_2 for 30s followed by 2.5% NaOCl for further 30s. Subsequently, 5% sodium thiosulphate was used to inactivate the disinfectant. Sterility of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it on blood agar plates which were incubated aerobically and anaerobically.

A two-stage access cavity preparation was performed without the use of water spray, but under manual irrigation with sterile/apyrogenic saline solution and by using sterile/apyrogenic high-speed diamond bur. The first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the protocol described above. The sterility of the internal surface of the access cavity was checked as previously described and all procedures were performed aseptically. A new sterile and apyrogenic bur was used under irrigation with sterile apyrogenic water to access the canal. The endotoxin sample was taken by introducing sterile pyrogen-free paper points (size 15; Dentsply-Maillefer, Ballaigues, Switzerland) into the full length of the canal (determined radiographically) and retained in position during 60 seconds. Immediately, the paper point was placed in a pyrogen-free glass and frozen at -80° C for future LAL analysis.

Endotoxin detection assay

Common Test Procedures

Firstly, the endotoxin samples were suspended in 1 mL of LAL water and agitated in vortex for 60 seconds. The LAL water was considered as the blank for all tests. Thereafter, each sample was analyzed by the three different tests using aliquots

from the initial volume according to the test procedure recommended by the manufacturer's instructions as follows. A 96-well microplate (Corning Costar, Cambridge, MA) was used in a heating block at 37°C and maintained at this temperature throughout the assay. The absorbencies of endotoxin were individually measured by using an enzyme-linked immunosorbent assay plate-reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Individual Performance assay

1. **Chromogenic endpoint assay:** The Quantitative Chromogenic LAL-1000 test (QCL-1000) (BioWhittaker, Inc, Walkersville, MD, USA) was used for quantification of endotoxin in root canal samples. Initially, 50 μL of the blank were used according to the standard endotoxin concentrations (i.e. 0.1, 0.25, and 1.0 EU/mL) and 50 μL of the samples were added in duplicate in the 96-well microplate. This was followed by the addition of 50μl LAL to each well, and the microplate was then briefly shaken. Ten minutes later, 100 μl of substrate solution (pre-warmed to 37°C) was added to each well, always maintaining the same sequence. The plate was mixed and incubated at 37°C for 6 minutes. Next, 100 μl of a stop reagent (acetic acid 25% v/v) was added to each well, and the absorbance (405 nm) was read by using an enzyme-linked immune-sorbent assay plate-reader (Ultramark, Bio-Rad Laboratories). Both test procedure and calculation of endotoxin level were performed according to the manufacturer instructions. A color interference assay was performed in the QCL-1000 test (chromogenic endpoint assay), according to the manufacturer's instructions, as recommended if 25% acetic acid is used as stop reagent.

2. **Chromogenic kinetic assay:** The chromogenic kinetic test used for quantification of endotoxin was the Kinetic Quantitative Chromogenic LAL test (KQCL) (BioWhittaker). Firstly, as a parameter for calculation of the amount of endotoxins in root canal samples, a standard curve was plotted by using endotoxins with a known concentration (50 EU/mL) and their dilutions with the following final concentrations,

0.005, 0.05, 0.5, and 5 EU/mL. One hundred μL of the blank were used according to the standard endotoxin concentrations (i.e. 0.005, 0.05, 0.5, 5 EU/mL) and 100 μL of the samples were added in duplicate in the 96-well microplate with respective PPC (Positive Product Control). All reactions were achieved in duplicate in order to validate the test, and the absorbance (405 nm) was read by using an enzyme-linked immune-sorbent assay plate-reader (Ultramark, Bio-Rad Laboratories). Both test procedure and calculation of endotoxin level were performed following the manufacturer's instructions.

3. Turbidimetric kinetic assay: The turbidimetric test - Pyrogent ®5000 (BioWhitaker, Inc., Walkersville, MD, USA) was used to measure endotoxin concentrations in the root canals by using the Limulus Amebocyte Lysate (LAL) technique. Firstly, as a parameter for calculation of the amount of endotoxins in root canal samples, a standard curve was plotted by using endotoxins with a known concentration (100 EU/mL) and their dilutions with the following final concentrations, 0.01, 0.10, 1, and 10 EU/mL. One hundred μL of the blank were used according to standard endotoxin concentrations (i.e. 0.01, 0.10, 1, 10 EU/mL) and 100 μL of the samples were added in 96-well microplate with respective PPC. All reactions were achieved in duplicate to validate the test. Test procedure and calculation of endotoxin level were performed following the manufacturer's instructions. The absorbencies of endotoxin were individually measured by using an enzyme-linked immunosorbent assay plate-reader (Ultramark, Bio-Rad Laboratories) at 340 nm.

Commonly Performance Assay

Inhibition / enhancement assay

Spike procedure / PPC (Positive Product Control)

The spike procedure was performed according to the manufacturer's instructions by the addition of a known concentration value of endotoxin for each LAL

method in order to detect any possible inhibition or enhancement from the samples in relation to the LAL substrate.

Chromogenic endpoint product inhibition control

To verify the lack of product inhibition, an aliquot of test sample (or a dilution of test sample) is spiked with a known amount of endotoxin (0.4 EU/ml). The spiked solution is assayed along with the unspiked samples, and their respective endotoxin concentrations are determined. The difference between these two calculated endotoxin values should be equal to the known concentration of the spike ± 25%.

Kinetically inhibition control

For the Kinetic tests (chromogenic kinetic assay and turbidimetric assay), the WinkQCL® Software (LONZA, Walkersville, MD, USA) was used to calculate the amount of endotoxin recovered in the Positive Product Control (PPC) in the comparison with the known amount of endotoxin spiked. The endotoxin recovered should be equal to the known concentration of the spike or within 50-200% as determined by the pharmacopeia. If positive, the test was considered validated because a good interaction between the samples and LAL substrate was demonstrated without interfering with the recovery of endotoxin.

Performance characteristics

Linearity: The linearity of the standard curve within the concentration range used to determine the endotoxin values were verified for all tests according to the manufacturer's instructions. The absolute value of the correlation coefficient (r value) of the calculated standard curve had to be ≥ 0.980.

Reproducibility: Replicates were run in order to assess technique and coefficient of variation (c.v.). The percentage of C.V. for replicates of a sample had to be less than

10%. Reproducibility between 3-4% was considered the best as indicated by the manufacturer's instructions.

After the measurement of endotoxin, if the levels of endotoxin were out of the standard curve or if any possible interference with LAL method by the root canal samples was detected, serial dilutions were considered and re-assayed.

STATISTICAL ANALYSIS

The endotoxin values were statistically analyzed by using SPSS for WINDOWS, version 12.0 (SPSS Inc, Chicago, IL, USA). The comparison between chromogenic endpoint, chromogenic and turbidimetric kinetic methods was performed by using the Friedman test ($p < 0.05$).

RESULTS

Sterility samples taken from the external and internal surfaces of the crown and its surrounding structures tested before and after entering the pulp chamber showed no microbial growth. A total of 21 root canals with pulp necrosis and apical periodontitis were analyzed by the three different LAL-methods.

Endotoxin detection

All three LAL-methods were effective in the recovery of endotoxin from root canal infection. Regardless of the method tested, endotoxin was detected in 100% of the root canals investigated (21/21). The KQCL-assay yielded a median value of endotoxin of 7.49 EU/mL, which was close to and not significantly different from the Turbidimetric test (9.19 EU/mL) (both kinetic methods) ($p > 0.05$). In contrast, the endpoint-QCL showed a median value of 34.20 EU/mL ($p < 0.05$) (Table 2).

The percentage of PPC values revealed a good interaction between the root canal samples and LAL substrate regarding the Turbidimetric method (% values ranging from 50 to 197) (Table 2). Product inhibition values were found in 2/21 root canal samples analyzed by the KQCL method (PPC value < 50%). The endpoint-QCL revealed product interference in 12/21 root canal samples (values lower than 0.4 EU/mL ± 25%) (Table 2).

The color interference assay performed for the endpoint-QCL method indicated color interference in 11/21 root canal samples, even after a dilution to the 10^{-4}.

Performance characteristics

Linearity assay – The linearity of the standard curve was equally good for all methods (all r =1) (Table 2).

Reproducibility assay – The coefficient of variance (C.V.) for endotoxin concentration was greater than 10% in 17/21 root canal samples analyzed by the endpoint-QCL assay, indicating its low reproducibility (Table 2). In contrast, the KQCL and Turbidimetric kinetic assays revealed as high as 5.50 and 4.46 % values of C.V., respectively (both being precise and with best reproducibility) (Table 2).

DISCUSSION

The LAL tests employ a serine protease catalytic coagulation cascade that is activated by endotoxin (18). Factor C (FC), the first component in the cascade, is a protease zymogen activated by endotoxin binding. Downstream, this pathway activates a pro-clotting enzyme into a clotting enzyme (coagulogen – coagulin) (18). The chromogenic LAL assay (QCL or KQCL) uses the synthetic peptide-pNA substrate,

which is cleaved by the clotting enzyme, imparting a yellow color to the solution. The turbidimetric kinetic assay uses coagulogen by monitoring its conversion into coagulin, which begins to form a gel-clot, increasing the turbidity. The strength of the yellow color (determined at an Optical Density [OD] = 405 nm) resulting from the chromogenic LAL substrate, and the turbidity (determined at an OD = 340 nm) resulting from the coagulogen conversion are correlated with the endotoxin concentration.

The progress of the LAL reaction leading to coagulogen conversion (as measured by optical density [OD]) was monitored in two ways in the current study: using the endpoint and kinetic methods. In the first (QCL test), OD is recorded at single time (≈ 16 min), which compromises its sensitivity (0.1-1 EU/ mL) (18). Conversely, in kinetic assays (KQCL/ Turbidimetric tests), OD is read at multiple time points as the reaction proceeds with no termination step (≈ 60 min), which allows the concentration of endotoxin to be quantified over a wider range sensitivity (0.005-50 EU/mL in the KQCL and 0.01-100 EU/mL in the Turbidimetric methods). Since the levels of endotoxin found in endodontic infection (8, 14, 15) are above the endpoint-QCL sensitivity (1 EU/mL), a higher serial dilution is required for such a method, particularly, in symptomatic teeth (11). Nevertheless, when considering the dilution method, not only the concentration of endotoxin is diluted but the test sensitivity is also affected.

According to the endodontic literature, the present investigation has demonstrated that all three LAL methods tested were sensitive enough for the investigation of endotoxin in primary endodontic infection as endotoxin was detected in 100% of the root canal samples (9, 11, 13-15).

The KQCL- test yielded a median value of endotoxin close to and not significantly different from that of the Turbidimetric kinetic test (7.49 EU/mL *versus* 9.19 EU/mL, respectively). The differences in endotoxin measurement between these two kinetic methods might be related not only to the test principle itself (use of a chromogenic synthetic LAL-substrate in the KQCL *versus* a native substrate (coagulogen) in the Turbidimetric method), but also to unique assay variations, such as time for adding reagent to multiple wells and inability to control the incubation

temperature in the micro-plate readers. These are important factors toward inter-assay comparisons (18, 30). Under these conditions, the inter-assay coefficients of variation between these two kinetic tests were lower than 25% as expected (18).

In contrast to the kinetic tests, the endpoint-QCL method showed a median value of endotoxin approximately 5 times greater than that of both kinetic methods (34.2 EU/mL), suggesting an interference with the LAL substrate by the samples. Such interference with the endpoint-QCL was confirmed by the inhibition/ enhancement assay (spiked values lower than 0.4 EU/mL ± 25%), even after serial dilutions of the clinical samples (up to 10^{-4}). Endodontic investigations (11, 14) using the endpoint-QCL test also reported higher levels of endotoxin.

It is worth pointing out that while the kinetic-QCL uses a single reagent, the endpoint-QCL has two stages: LAL activation, followed by addition of a chromogenic substrate (a chromophore release stage), both critically depending on time and temperature (29). The use of a single-reagent assay seems to improve the precision, speed and accuracy of the tests (27, 29).

Foremost, the inhibition/enhancement assay indicated a good interaction between the root canal samples and both kinetic methods (KQCL and Turbidimetric) by showing most of PPC percentage values within the acceptable range (50-200) as recommended by the U.S. Pharmacopoeia. Additionally, the reproducibility assay (determined by the coefficient of variance [C.V.]) indicated a good technique and low coefficient of variation (all<10%) between the root canal sampling replicates determined by Kinetic LAL- tests, being both more precise and with a better reproducibility than the endpoint-QCL.

The color interference assay indicated possible color interference in more than 50% of the root canal samples analyzed by the endpoint-QCL, even after considering serial dilution method to 10^{-4} – strategy usually attempted to minimize possible sample color interference. In fact, since the endotoxin samples were suspended in a non-colored medium (LAL-water), it can be speculated that the use of 25% acetic acid as a stop reagent might interfered with the assay - due to its capacity to turn yellow -

by increasing the intensity of the yellow color, and consequently overestimating the levels of endotoxin.

Regarding the endotoxin detection, the sample by itself presents critical points that must be considered for an optimal LAL reaction. First of them is the microbiota profile (primary *versus* secondary infection), particularly in secondary endodontic infection in which Gram-positive bacteria (32) are predominantly involved. An unusual reactivity with peptidoglycan from the cell wall of Gram-positive bacteria (≈0.00025%) (33) might account for a positive LAL assay at concentrations 1.000 to 400.000 times higher than the required one because of the alternative glucan pathway (19), requiring specifically blockage with laminarin (34).

The pH variation in the root canals following the use of chemical substances during the treatment also plays an important role in the LAL reaction. In order to get an ideal pH (6.0-8.0) (30,31) for LAL enzyme activation, an adjustment of the pH of the root canal samples might be required, particularly after the use of chemical substances (e.g. sodium hypochlorite, chlorhexidine, ethylenediaminetetraacetic acid).

Moreover, a prior cleaning of the root canal samples by centrifugation or filtration might be necessary, particularly in the analysis of the endotoxin samples after the use of an intracanal medicament (e.g. calcium hydroxide), as the turbidity of the samples might interfere in the endotoxin measurement.

In view of the results, the present study indicated that it is not possible to reconcile the levels of endotoxin determined by the endpoint-QCL with the kinetic LAL methodology. Foremost, future endotoxin comparison studies must take into consideration the method used for the quantification of bacterial LPS before establishing any comparisons of the levels of endotoxin, always comparing endpoint to endpoint-QCL LAL studies, as well as kinetic to kinetic-LAL investigations.

In conclusion, this study has revealed that quantitative kinetic-turbidimetric and kinetic-chromogenic LAL methods are best fitted for analysis of endotoxin in root

canal infection, both being more precise and allowing better reproducibility compared to the endpoint-QCL assay.

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Table 1. Previous investigations of endotoxin in primary endodontic infection using LAL-method.

Samples (n)	Pulp vitality	Spontaneous pain	Necrosis	Periradicular area	LAL-method/ Test	Concentration of endotoxin	Reference number
21	-	-	+	Radiolucency	Turbidimetric (Pyrogent®5000)	Median:7490 pg/mL	8
24	-	-	+	Radiolucency	Endpoint-chromogenic (QCL 1000)	Median: 228 EU/mL	9
31	-	-	+	No alteration	Endpoint-chromogenic (QCL 1000)	Median: 20888.0 EU ml-1	11
19	-	+	+	No alteration		Median: 15145.0 EU ml-1	
10	+	-	-	No alteration	Endpoint-chromogenic (LAL)	Mean: 0.007 µg ET/mL	12
10	+	+	-	No alteration		Mean: 0.075 µg ET/mL	
10	-	-	+	No alteration		Mean: 0.192 µg ET/mL	
10	-	+	+	No alteration		Mean: 1.070 µg ET/mL	
15	+	+	-	No alteration	Endpoint-chromogenic (QCL 1000)	Mean: 0.16 ng/mL	13
13	+	-	-	No alteration		Mean: 0.10 ng/mL	
24	-	-	+	Radiolucency	Endpoint-chromogenic (QCL 1000)	Mean: 151.61 EU/mL	14
27	-	-	+	Radiolucency	Endpoint-chromogenic (QCL 1000)	Median: 57.98 EU/mL	15
24	-	-	+	Radiolucency		Median:47.12 EU/mL	

(+) = presence; (-) = absence

Table 2. Distribution of endotoxin concentration in EU/mL recovered from the 21 root canals with primary endodontic infection and apical periodontitis investigated and the performance characteristic (reproducibility assay) of the LAL methods selected.

LAL-method Test	**Endpoint Chromogenic (QCL-1000)**		**Kinetic Chromogenic (Kinetic QCL test)**		**Kinetic Turbidimetric (Pyrogent 5000)**	
Median values of endotoxin [EU/mL]	34.20		7.49		9.19	
	V Sample (n)	NV Sample (n)	V Sample (n)	NV Sample (n)	V Sample (n)	NV Sample (n)
Inhibition/ enhancement assay	9	12	2	19	21	0
Reproducibility (c.v. value)	4	17	21	0	21	0

(V)= Validate and (NV) = Non-Validate sample according to the inhibition/ enhancement assay and performance characteristic (reproducibility assay).

2.2. Capítulo 2 – Antigenic Activity of Bacterial Endodontic Contents from Primary Endododontic Infection with Periapical Lesions Against Macrophages in the Release of Interleukin-1ß and Tumor Necrosis factor.

ABSTRACT

Introduction: Periradicular tissue chronic stimulation by endotoxin may cause apical periodontitis. This clinical study investigated the microbial profile and the levels of endotoxin found in primary root canal infection with apical periodontitis, and to determine their antigenicity against macrophages through the levels of IL-1ß and TNF-alpha, evaluating their relationship with clinical and radiographic findings. **Methods:** Samples were taken from 21 root canals with primary endodontic infection and apical periodontitis with paper points. PCR technique (16S rDNA) was used for the detection of the target bacteria. Limulus Amebocyte Lysate (LAL) was used to measure endotoxin. The amounts of IL-1ß/ TNF-alpha in macrophages supernatants were measured by enzyme-linked immunosorbent assay – Duoset-kit (ELISA). **Results:** *Prevotella nigrescens* (13/21), *Porphyromonas endodontalis* (6/21) and *Treponema socranskii* (6/21) were the most frequently Gram-negative bacteria species detected. The presence of sinus tract (2/21) was related to the detection of *Filifactor alocis* respectively ($p<0.05$); while tooth with radiolucent area ≥ 2mm with *Treponema denticola.* Correlation was found between the number of Gram-negative bacteria and the levels of IL-1ß/ TNF-alpha ($p<0.05$). Increased levels of endotoxin were followed by TNF-alpha release ($p<0.05$). Higher levels of IL-1ß ($p<0.05$) and endotoxin contents were related to the larger size of radiolucent area. **Conclusion:** The antigenicity of the endodontic contents is not only related to the amount of endotoxin found in root canal, but also with the number of different species of Gram-negative bacteria involved in the infection. Moreover larger size (≥ 2mm) of radiolucent area was related to IL-1ß and endotoxin.

Key-words: bacteria; endotoxin; endodontic; antigenicity; macrophages.

INTRODUCTION

Primary endodontic infection is a polymicrobial infection caused predominantly by Gram-negative anaerobic bacteria (1) that present lipopolysaccharide (LPS) on the outer layers of their cell walls. LPS is released during disintegration, multiplication or bacterial death (2), and is capable to penetrate into the periradicular tissues (3) acting as endotoxin in the host organism (4), leading to periradicular inflammation and bone destruction (5). The lipid A is the bioactive component of LPS, responsible for the majority of the immunoresponse (3).

Accumulation of bacteria components in an infected area, particularly endotoxins (including lipoteichoic acid, peptidoglycan, lipopolysaccharide and others), can stimulate the release of pro-inflammatory cytokines by different cell lines through TLR2 and -4 activation (5-7). The inflammatory tissue present in periradicular lesions is populated predominantly by macrophage (8-9), which is the major source of Interleukin-1 beta (IL-1ß) (10), and almost the exclusive producer of Tumor Necrosis Factor alpha (TNF-alpha) (11) in the presence of bacteria or LPS.

Clinical investigations of primary endodontic infection have elucidated the strong correlation between oral bacteria LPS and the presence of apical periodontitis (6, 12-15). Moreover, higher contents of endotoxins in root canals have been associated with spontaneous pain (6,12,16) and clinical signs/symptoms such as pain on palpation, tenderness to percussion and exudation (6,12,14,16).

Previous *in vitro* investigations (5-7) have shown that oral bacterial LPS extracted from bacteria commonly found in root canal infection induces a potent inflammatory response against different cell line cultures. IL-1ß and TNF-alpha have been detected in periapical tissues (3,9, 17-19) and root canal exudates (20-23) from primary root canal infection in the presence apical periodontitis. Higher contents of IL-1ß have been detected in teeth with clinical signs/symptoms (3, 23) and larger size of radiolucent area

corresponding to bone resorption (21, 22). However, studies correlating all these aspects have not yet been provided in the literature.

Therefore, the aim of this clinical study was to investigate the microbial profile and the levels of endotoxin found in primary root canal infection with apical periodontitis and to determine their antigenicity against macrophages through the levels of IL-1ß/ TNF-alpha, evaluating their relationship with clinical and radiographic findings.

MATERIAL AND METHODS

Patient selection

Twenty-one patients who attended the Piracicaba Dental School, Piracicaba, SP, Brazil, in need of endodontic treatment were included in this research. The age of the patients ranged from 13-73 years. Samples were collected from 21 root canals with pulp necrosis and showing radiographic evidence of apical periodontitis. The selected teeth showed absence of periodontal pockets more than 4 mm. The following clinical/ radiographic features were found: pain on palpation (9/21), tenderness to percussion (8/21), exudation (12/21), radiolucent area $\geq$ 2mm (11/21) and $<$ 2mm (10/21). None of the patients reported spontaneous pain.

A detailed dental history was obtained from each patient. Patient who had received antibiotic treatment during the last three months or who had any general disease were excluded. The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved the protocol describing specimen collection for this investigation, and previously all patients signed an informed consent document.

Sampling procedures

All materials used in this study were heat sterilized at 200°C for 4 hours becoming apyrogenic. The method followed for the disinfection of the operative field had been described previously (14, 15). Briefly, the teeth were isolated with a rubber dam. The crown and the surrounding structures were disinfected with 30% H_2O_2 for 30s followed by 2.5% NaOCl for an additional 30s. Subsequently, 5% sodium thiosulphate was used to inactivate the irrigant. The sterility of the of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it on blood agar plates which were incubated aerobically and anaerobically.

A two-stage access cavity preparation was made without the use of water spray, but under manual irrigation with sterile/apyrogenic saline solution and by using sterile/apyrogenic high-speed diamond bur. A first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected following the protocol described above. The sterility of the internal surface of the access cavity was checked as previously described and all procedures were performed aseptically. A new sterile and apyrogenic bur was used, accomplished by irrigation with sterile apyrogenic water to access the canal. The endotoxin sample was taken introducing sterile pyrogen-free paper points (size 15; Dentsply-Maillefer, Ballaigues, Switzerland) into the full length of the canal (determined radiographically) and retained in position during 60 seconds. Immediately, the paper point was placed in a pyrogen-free glass and frozen at -80° C for future Limulus Amebocyte Lysate assay (LAL) and cell culture stimulation. The procedure was repeated with 5 sterile paper points. The paper points were pooled in a sterile tube containing 1 mL of VMGA III transport medium being immediately processed for DNA extraction for the detection of target bacteria by molecular method (16S rDNA).

Bacterial detection (PCR 16S rDNA)

Reference bacteria strains used in this study were purchased from the American Type Culture Collection and are listed as follow: *Dialister pneumosintes* (ATCC 33048), *Prevotella intermedia* (ATCC 25611), *Prevotella nigrescens* (ATCC 33099), *Aggregatibacter actinomycetecomitans* (ATCC 43718), *Porphyromonas gingivalis* (ATCC 33277), *Filifactor alocis* (ATCC 35896), *Tannerella forsythia* (ATCC 43037), *Prevotella tannerae* (ATCC 51259), *Treponema denticola* (ATCC 35405), *Porphyromonas endodontalis* (ATCC 35406), *Treponema socranskii* (35536), *Parvimonas micra* (ATCC 33270). Bacterial selection criteria were performed based on the most commonly found species in primarily root canal infection.

DNA extraction: Microbial DNA from endodontic samples as well as from ATCC bacteria were extracted and purified with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA concentration (absorbance at 260 nm) was determined using a spectrophotometer (Nanodrop™ 2000, Thermo Scientific, Wilmington, DE, USA).

PCR assay: The PCR reaction was performed in a thermocyler (MyCycler, Bio-Rad, Hercules, CA, USA) thermocycler in a total volume of 25 µl containing 2.5 µl of 10X *Taq* buffer (1X) (MBI Fermentas, Mundolsheim, France), 0.5 µl of dNTP mix (25 µM of each deoxyribonucleoside triphosphate – dATP, dCTP, dGTP and dTTP) (MBI Fermentas, Hanover, MD, USA), 1.25 µl of 25 mM $MgCl_2$, 0.25 µl of forward and reversal universal primers (0.2 µM) (Invitrogen, Eugene, OR, USA), 1.5 µl sample DNA (1 µg/ 50 µl), 1.5 µl Taq DNA polymerase (1 unit) (MBI Fermentas) and 17.25 µl water nuclease free. The primer sequences and PCR cycling parameters were previously optimized (13-15) and listed in Table 1.

Determination of endotoxin concentration (Turbidimetric test - LAL assay)

The turbidimetric test (BioWhitaker, Inc., Walkersville, MD, USA) was used to measure endotoxin concentrations in the root canals using the Limulus Amebocyte Lysate (LAL) technique. First, as a parameter for calculation of the amount of endotoxins in root canal samples, a standard curve was plotted using endotoxins supplied in the kit with a known concentration (100 EU/mL), and its dilutions with the following final concentrations (i.e. 0.01, 0.10, 1, 10 EU/mL) following the manufacturer's instructions.

Test procedure**:** All reactions were accomplished in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA) was used in a heating block at 37°C and maintained at this temperature throughout the assay. First, the endotoxin-samplings were suspended in 1 mL of LAL water supplied on the kit and agitated in vortex for 60 seconds and serial diluted to the 10^{-1}. Immediately, 100 μL of the blank followed the standard endotoxin solutions in concentrations (i.e. 0.01, 0.10, 1, 10 EU/mL) and 100 μL of the samples were added in duplicate in the 96-well microplate. The test procedure was performed following the manufacturer's instructions. The absorbencies of endotoxin were measured individually using an enzyme-linked immunosorbent assay plate-reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 340 nm.

Calculation of endotoxin concentrations: Since the mean absorbance value of the standards was directly proportional to the concentration of endotoxins present, the endotoxin concentration was determined from the standard curve.

Cell culture and cytokines expression

Macrophages (RAW 264.7) were cultured 100mm culture plates in Dulbecco's modified Eagle's minimal essential medium supplemented (DMEM) with 100 IU/ mL of penicillin, 100 lg/mL of streptomycin and 10% heat-inactivated fetal bovine serum, and maintained in a humidified atmosphere at 37°C and 5% CO_2 until 90% confluence. Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Macrophages were released from 100mm plates with 0,25% trypsin, counted in Newbauer chamber and a total of 10^4 macrophages were grown for 48h in each well of six-well plates, de-induced by incubation for 8h in culture medium (DMEM) containing 0.3% fetal bovine serum and stimulated with 60 μL of root canal contents during 24 hours in order to quantify the total amount of protein released in the culture media, IL-1ß and TNF-alpha protein. The supernatants were collected and stored at -80ºC until protein evaluation.

IL-1ß and TNF-alpha mRNA expression

The macrophages cell viability was tested in the present study by its capacity to express IL-1ß and TNF-alpha mRNA after 24 hours of root canal contents stimulation. A total of 10^4 macrophages were grown for 48h in each well of six-well plates, de-induced by incubation for 8h in culture medium (DMEM) containing 0.3% fetal bovine serum and stimulated with 60 uL of primary infection contents for 24 hours for IL-1ß and TNF-alpha mRNA expression. Total RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer's instructions. The quantity and purity of total RNA were determined on a Biomate 3 spectrophotometer (ThermoSpectronic, Rochester, NY, USA). Complementary DNA was synthesized by reverse transcription of 500 ng of total RNA using 2.5 μM Oligo $(dT)_{12-18}$ primers and 1.25 U/uL Moloney murine leukemia virus

reverse transcriptase in the presence of 3 mM $MgCl_2$, 2 mM dNTPs and 0.8 U/µL of RNAse inhibitor, according to the manufacturer's protocol (Improm II, Promega, Madison, WI, USA). The PCR reaction was performed in a MyCycler (Bio-Rad) thermocycler using 2uL of the RT reaction product on a 20 uL total volume PCR reaction mix (GoTaq Flexi, Promega) in the presence of 100 pmol/ul of each gene's primers (50 pmol/ul of sense and antisense primers) for IL-1ß, TNF- alpha and GAPDH genes yielding products of 494, 451 and 418 bp, respectively. The primer pair used for IL-1ß (accession no.: NM031512) was: sense 5'-GACCTGTTCTTTGAGGCTGA-3', antisense 5'-CGTTGCTTGTCTCTCCTTGT-3'; TNF-alpha (accession no.: NM012675) sense 5'-GGAGAACAGCAACTCCAGAA-3', antisense 5'-TCTTTGAGATCCATGCCATT-3'; and GAPDH (accession no.: BC083065) sense 5'-CACCATGGAGAAGGCCGGGG-3'; antisense 5'-GACGGACACATTGGGGTAG- 3'. Optimized cycling conditions used for TNF- alpha and IL-1ß were: initial denaturation at 95°C for 2 min and 35 cycles of: 95°C for 1 min, 58°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 7 min in the presence of 1.5 mM MgCl2 and for GAPDH conditions were as follows: initial denaturation at 95°C for 2 min and 25 cycles of: 95°C for 1 min, 52°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min in the presence of 1.5 mM MgCl2. PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5µg/mL). The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100 – GE Healthcare), using ImageJ 1.32j software (National Institute of Health, USA – http://rsb.info.nih.gov/ij/). The density of the bands corresponding to TNF- alpha and IL-1ß mRNA in each sample was normalized to the quantity of the housekeeping gene GAPDH and expressed as fold change over unstimulated control.

Measurements of total protein levels released to the culture media

The total amount of protein released in the culture media following root canal contents stimulation was measured by Coomassie (Bradford) Protein Assay kit (Rockford, IL, USA). As a parameter for calculation of the amount of protein released to the culture media, a standard curve was plotted using Bovine Serum Albumin (BSA) albumin standard supplied in the kit with a known concentration (2.0 mg/mL), with a series BSA concentration (i.e. 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 μg/mL). The Protein assay was performed following the manufacturer's instructions.

Calculation of protein concentration

The protein standard and sample solutions were measured individually using an enzyme-linked immunosorbent assay plate-reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 595 nm. Since this absorbance value was directly proportional to the concentration of protein, the protein concentration from the samples solutions was determined from the standard curve.

Measurements of IL-1ß and TNF-α levels

The amounts of IL-1ß and TNF-alpha released to the culture media following root canal contents stimulation of macrophages were measured by enzyme-linked immunosorbent assay – Duoset kit (ELISA; R&D, Minneapolis, MN, USA). Medium of unstimulated macrophage culture was used as a negative control. Briefly, standard, control or sample solution was added to ELISA well plate, which had been pre-coated with specific monoclonal capture antibody. After shaking gently for 3h at room temperature, polyclonal

anti-TNF-alpha and IL-1ß antibody, conjugated with horseradish peroxidase, was added to the solution, respectively, and incubated for 1 h at room temperature. Substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 20 min. The levels of cytokines were assessed by a microelisa reader at 450 nm and normalized with the abundance of standard solution. Each densitometric value expressed as mean ± SD was obtained from three independent experiments.

STATISTICAL ANALYSIS

The data collected for each case (clinical features and the bacteria isolated) were typed onto a spreadsheet and statistically analyzed using SPSS for Windows (SPSS, Inc., Chicago, IL). The Pearson chi-square test or the one-sided Fisher's exact test, as appropriate, was chosen to test the null hypothesis that there was no relationship between bacteria species such as endodontic clinical signs/ symptoms and the presence of a specific group of bacteria in the root canal samples. Pearson coefficient was used to correlate the amount of LPS, IL-1ß and TNF-alpha levels each two at a time such as to correlate each one with the size of radiolucent area and the number of Gram-negative bacteria present in root canals with apical periodontitis. Correlation between the presence of clinical/ radiographic findings with the median levels of LPS, IL-1ß and TNF-alpha was analyzed using Student's *t* test or Mann-Whitney. $P<0.05$ was considered statistically significant.

RESULTS

Bacterial detection (16 rDNA)

Bacterial DNA was detected in all root canal samples (21/21). The maximum of 5 species was detected in the root canal samples. At least 1 Gram-negative species was detected in 18/21 root canals (Table 2). *Prevotella nigrescens* (13/21), *Porphyromonas endodontalis* (6/21) and *Treponema socranskii* (6/21) were the three most frequently target

Gram-negative bacterial species detected. A combination of 2 or more Gram-negative target species was detected in 8/21 root canals (Table 2). The *Parvimonas micra* positive samples (6/21) were associated in 100% with at least 1 Gram-negative target bacterial species. Positive associations were found between *P. endodontalis* and *Treponema denticola* (p=0.003, OR= 2.000, CB= 0.899- 4.452); *P. micras* and *Filifactor alocis* (p=0.008, OR= 1.667, CB= 0.815-3.409) in primary root canal infection. Teeth with sinus tract (2/21) was related to the presence of *F. alocis* (p=0.040, OR= 18.000, CB= 0.585-553.586). Radiolucent area ≥ 2 mm was associated with the presence of *T. denticola* (p=0.012, OR= 10.000, CB= 2.685-37.239). Correlation between the number of different Gram-negative bacterial species and the levels of IL-1 ß ($p<0.05$, r Pearson= 0.124) (Figure 1A) and TNF- alpha ($p<0.05$, r Pearson= 0.173) (Figure 1B) was found.

Determination of endotoxin concentration (Turbidimetric test- LAL assay)

The LAL assay (Turbidimetric test) indicated that endotoxin was present in 100% of the root canals samples (21/21). The median value of endotoxin contents was 7490 pg/mL in root canals with periradicular lesions. Higher median values of endotoxin contents was detected in teeth with the presence of radiolucent area ≥ 2 mm - 9190 pg/mL (range: 257-212000 pg/mL) than in teeth with radiolucent area < 2 mm -3480 pg/mL (range: 27-289000 pg/mL). Teeth with exudation presented higher median levels of endotoxin- 9190 pg/mL (range: 355-289000 pg/mL) than teeth with no exudation -2620 pg/mL (range: 27-112000 pg/mL). The median value of endotoxins in the presence of presence of pain on palpation was 5580 pg/mL (range: 27-269000 pg/mL) and in its absence was 35.200 pg/mL (range: 59-289000 pg/mL). Moreover in the presence of tenderness to percussion the median value was 3480 pg/mL (range: 27-289000 pg/mL) and in the absence was 9190 pg/mL (range: 59-232000 pg/mL). Table 3 shows the median concentration of endotoxin according to the clinical findings and size of radiolucent area. A correlation was found between endotoxin contents and the levels of TNF-alpha released in the culture media ($p<0.05$, r Pearson= 0.740) (Figure 1C).

Cell culture and cytokines expression

IL-1ß and TNF-alpha mRNA expression: The macrophages cell viability after 24 hours of root canal contents stimulation was confirmed in the present study tested by its capacity to express IL-1ß and TNF-alpha mRNA.

Measurements of IL-1ß and TNF-alpha levels: IL-1 ß and TNF-alpha were detected in all culture media after stimulation with root canal contents. The median level of IL-1 ß (24.835 pg/mL) was present in almost 90-fold higher than TNF-alpha (0.2830 pg/mL). Higher median level of IL-1 ß was detected when one of the following clinical symptoms/ radiographic finding: pain on palpation (25.528 pg/mL), tenderness to percussion (25.528 pg/mL) or size of radiolucent area ≥ 2mm (25.291 pg/mL) was present (Table 3). Higher level of TNF-alpha was found in teeth with exudation (0.2870 pg/mL) than in its absence (0.2340 pg/mL). Correlation between the levels of IL-1 ß released on the culture media and the size of radiolucent area was found in this study ($p<0.05$, r Pearson= 0.028) (Figure 1D). The median concentration of endotoxin, IL-1 ß and TNF-alpha, according to clinical findings and the size of radiolucent area are shown in Table 3.

DISCUSSION

Data obtained in the present study revealed that a wide variety of Gram-negative bacterial species do play a role in primary root canal infection with apical periodontitis, detecting at least 1/11 Gram-negative target bacterial species in 18/21 root canals investigated, with a predominance of *P. nigrescens, P. endodontalis* and *T. socranskii*.

The high frequency of *P. nigrescens* in primary endodontic infection with apical periodontitis seems to be related to its LPS potential in causing bone resorption (24). The almost exclusive presence of *P. endodontalis* in endodontic infections suggests a specific association with bone resorption and activation of macrophages cells (5, 25).

Moreover, *P. endodontalis* LPS has been detected in a very high percentage in severe endodontic infection (26).

The combination of 2 or more Gram-negative bacterial species found in 8/21 root canals investigated indicates that different bacterial LPS with different toxicity structure (lipid A) (27) can be involved in the root canal infection, enhancing or even inhibiting each other's antigenicity activity over periradicular tissues. For instance, *P. endodontalis*, seems to enhance the *Fusobacterium nucleatum* LPS toxicity (5), while *Porphyromonas* (27) and "*Bacteroides fragilis"* (28) are limited in the presence of *Escherichia coli* LPS.

The association of *Parvimonas micra* (Gram-positive bacteria) with at least 1 Gram-negative target bacteria, e.g. *F. alocis*, found in the present study, turns endodontic contents even more complex and immunogenic to the immune system. Peptidoglycan (PGN), present in a significant amount in the Gram-positive bacterial cells, plays a synergistic effect on LPS antigenic activity when they activate TLR 2 and -4 respectively (29). In this case, more macrophages differentiate into osteoclast-like cells through RANKL: OPG ratio increase (29). Yoshioka *et al.* (30) reported that LPS can bind to *P. micra* cells conferring to this Gram-positive bacteria the capacity to induce a strong TNF-alpha response in macrophage-like cells. Such critical finding stresses the importance of considering Gram-positive non-LPS components when interpreting findings on the expression of pro-inflammatory cytokines.

Previous *in vitro* investigations have attempted to extract LPS in order to determine the antigenic activity of the endodontic contents (5-7). However, the clinical significance of these investigations is unclear considering the complexity of the antigens involved in endodontic infection described above. Moreover, *in vitro* models, using bacterial growth culture media, fail in reproducing the infection environment, particularly regarding the hemin concentration in the infection site (31). Hemin concentration (in the hemoglobin form) varies considerably depending on the inflammatory response and blood

vessels integrity, and it might modulate the lipid A structure in LPS molecule, which is responsible for the majority of IL-1 induction (27).

To address these points, the present study stimulated macrophages cells with material individually isolated from 21 infected root canals presenting primary endodontic infection and apical periodontitis, concomitantly investigating the microbiota and the endotoxin content of each infected root canal. The analysis revealed that the increase in the number of Gram-negative bacteria was significantly followed by an increase in the IL-1 ß and TNF-alpha levels. These findings suggest that the presence of clinical signs/symptoms such as bone destruction involved in apical periodontitis, evoked by the immune system in response to LPS is not only associated with the amount of endotoxin elucidated by previous clinical investigations (12, 14-15), but also with the presence of different number and heterogeneity of Gram-negative bacteria, that acting synergistically, can lead to a stronger immune response in periapical tissues.

P. nigrescens contains a very potent LPS molecule for prostaglandin E_2 (PGE_2) stimulation (24) in inflamed pulp tissue (32) and in acutely inflamed periapical tissue (17). Additionally, IL-1ß and TNF-alpha released from macrophages supernatants treated with infectious material derived from teeth with exudation, are potent stimuli for PGE_2 release (33). Our results agree with Ataoglu *et al.* (22) that reported no association between teeth with exudates and higher levels of IL-1ß and are inconsistent with Kuo *et al.* (23). Furthermore, higher significantly levels of endotoxin were found in root canal exudation in this current study corroborating with previous clinical investigations (6, 12, 16).

Even though higher levels of endotoxins were expected particularly in pain on palpation and tenderness to percussion, it was not found in the present study. This particular data might be explained by the characteristics of the samples investigated, where a greater number of cases in the absence of these clinical features was found, contributing to such findings.

Teeth with larger size of periradicular lesions (≥ 2mm) were related to the detection of *T. denticola* in the root canal positively associated with *P. endodontalis* exhibiting a potent biological activity in cell culture (5) and associated with chronic bone resorption (29). Higher levels of endotoxin were found in those teeth, agreeing with Schein & Schilder (16) who reported that the endotoxin contents of teeth with radiolucent area were five times greater than in teeth without such area.

Larger size of periradicular lesions (≥2 mm) was also correlated with higher levels of IL-1ß in accordance to Tani-Ishii *et al.* (34). This finding might be related to the PGN present in Gram-positive and -negative bacteria, which inhibits the differentiation of monocytes/macrophages into mature cells. Besides this inhibition, PGN promotes cytokine production by undifferentiated precursors (29), consequently increasing localized osteolysis particularly at sites with larger number of macrophages (35).

More studies that analyze the mechanisms involved in the production of cytokines and bone resorption are important for the development of new therapies. The interference in toll-like receptors and interleukin-1 receptor downstream signalling pathways should be considered to control the development and progression of endodontic lesions. In the future the use of RNA interference can reduce the differentiation of macrophages into osteoclasts, the induction of cPLA and beta-defensin2 and consequently the release of TNF-alpha, IL-1ß and IL-6 (36-38). Also, another strategy is the superexpression of specific regulator proteins such as SOCS3, interferon-gamma and interleukin-10 through local delivery of DNA vectors (39).

Overall, the present study suggested that the antigenicity of the endodontic contents against macrophages (IL-alpha and IL-1ß release) is not only associated with the amount of endotoxin, but also with the number of Gram-negative bacteria involved in the infection. Further investigations should be performed in order to assess the effect of antimicrobial agents over different lipid A structures isolated from species commonly found in endodontic infections and their mechanism of action in different cell types.

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Table 1. PCR primer pairs and cycling parameters used for detection of bacteria species in primary root canal infection with apical periodontitis.

Target bacteria	Primer pairs (5'- 3')	Amplicon size	Cycles
Universal (16 rDNA)	Forward: TCC TAC GGG AGG CAG CAG T		Initial denaturation at 95°C for 10min and 40 cycles of: 95°C for 10s, 60°C for 10s
	Reverse: GGA CTA CCA GGG TATCTA ATC CTG TT	466 bp	and a final extension step at 72°C for 25s
Dialister pneumosintes	Forward: TTC TAA GCA TCG CATGGTGC		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 55°C for 1min,
	Reverse: GATTTC GCTTCTCTTTGTTG	1105 bp	72°C for 2min and a final step 72°C for 2min.
Prevotella intermedia	Forward: TTTGTTGGG GAG TAA AGC GGG		Initial denaturation at 95°C for 2mininn and 36 cycles of: 94°C for 30s, 58°C for 1min,
	Reverse: TCA ACA TCTCTG TATCCTGCG T	575 bp	72°C for 2min and a final step 72°C for 10min.
Prevotella nigrescens	Forward: ATG AAA CAA AGG TTTTCC GGTAAG		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 58°C for 1min,
	Reverse: CCC ACG TCTCTG TGG GCTGCG A	804 bp	72°C for 2min and a final step 72°C for 10min.
Aggregatibacter actinomycetecomitans	Forward: AAA CCC ATC TCTGAG TTC TTC TTC		Initial denaturation at 94°C for 30s and 36 cycles of: 95°C for 30s, 55°C for 1min,
	Reverse: ATG CCA ACT TGA CGTTAA AT	557 bp	72°C for 2min and a final step 72°C for 10min.
Porphyromonas gingivalis	Forward: AGG CAG CTTGCC ATA CTG CG		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min,
	Reverse: ACTGTTAGC AAC TAC CGA TGT	404 bp	72°C for 2min and a final step 72°C for 2min.
Filifactor alocis	Forward: CAG GTG GTTTAA CAA GTTAGTGG		Initial denaturation at 95°C for 2min and 26 cycles of: 95°C for 30s, 58°C for 1min,
	Reverse: CTA AGTTGTCCTTAG CTG TCTCG	594 bp	72°C for 1min and a final step 72°C for 2min.
Tannerella forsythia	Forward: GCG TATGTA ACC TGC CCG CA		Initial denaturation at 95°C for 1min and 36 cycles of: 95°C for 30s, 60°C for 1min,
	Reverse: TGC TTC AGTGTC AGTTATACC T	641 bp	72°C for 1min and a final step 72°C for 2min.
Prevotella tannerae	Forward: CTTAGC TTG CTA AGTATG CCG		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 55°C for 1min,
	Reverse: CAG CTG ACTTATACTCCC G	550 bp	72°C for 2min and a final step 72°C for 10min.
Treponema denticola	Forward: TAA TAC CGA ATG TGC TCA TTTACA T		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min,
	Reverse: TCA AAG AAG CATTCC CTC TTC TTC TTA	316 bp	72°C for 2min and a final step 72°C for 10min.
Porphyromonas endodontalis	Forward: GCTGCA GCTCAA CTG TAG TC		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 58°C for 1min,
	Reverse: CCG CTTCATGTC ACC ATG TC	672 bp	72°C for 2min and a final step 72°C for 10min.
Treponema socranskii	Forward: GATCAC TGTATA CGG AAG GTA GAC A		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 56°C for 1min,
	Reverse: TAC ACTTATTCC TCG GAC AG	288 bp	72°C for 2min and a final step 72°C for 10min.
Parvimonas micra	Forward: AGA GTTTGA TCC TGG CTC AG		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min,
	Reverse:ATA TCA TGC GATTCTGTG GTC TC	207 bp	72°C for 2min and a final step 72°C for 10min.

Table 2. PCR detection of bacteria species in 21 root canals with primary root canal infection and apical periodontitis.

(S=samples, + positive detection)

Target bacteria	ATCC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21
Universal (16 rDNA)		+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dialister pneumosintes	+		+																			
Prevotella intermedia	+		+																			
Prevotella nigrescens	+	+		+		+			+	+	+	+	+			+			+	+	+	+
Aggregatibacter actinomycetecomitans	+																					
Porphyromonas gingivalis	+																					
Filifactor alocis	+											+					+					
Tannerella forsythia	+																					
Prevotella tannerae	+																					
Treponema denticola	+	+													+	+						
Porphyromonas endodontalis	+					+		+				+	+	+	+							
Treponema socranskii	+		+			+						+		+	+							+
Parvimonas micra	+											+	+		+		+		+			

Table 3. The median concentration of endotoxin, IL-1 ß and TNF- alpha in pg/mL according to the clinical findings and size of radiolucent area.

	Total Amount	Pain on palpation		Tenderness to percussion		Exudation		Size of radiolucent area	
	n=21	Present n=9	Absent n=12	Present n=8	Absent n=13	Present n=12	Absent n=9	≤ 2 mm n=12	>2 mm n=9
Endotoxin (pg/mL)	7490	5580	35200	3480	9190	9190	2620	22550*	4380*
IL-1 ß (pg/mL)	24.835	25.528	24.835	25.528	24.835	24.835	25.291	24.835	25.528
TNF- alpha (pg/mL)	0.2830	0.2605	0.3150	0.2340	0.2870	0.2870	0.2340	0.3010	0.2575

*Statistically significant difference (p<.005)

2.3. Capítulo 3 – Antigencity of Primary Endodontic Infection Against Macrophages by the Levels of PGE_2 Production.

ABSTRACT

Introduction: Root canal contents are potent stimuli for pro-inflammatory cytokines involved in apical periodontitis. This study investigated target Gram-negative bacterial species and endotoxin in primary endodontic infection with apical periodontitis, determined their antigenicity against macrophages through the levels of PGE_2 and evaluated their relationship with clinical findings. **Methods:** Samples were taken from 21 root canals with primary infection and apical periodontitis by using paper points. PCR (16S rDNA) was used for bacterial detection and LAL-assay for endotoxin measurement. Levels of PGE_2 were measured by ELISA – Duoset-kit. **Results**: *Prevotella nigrescens* (13/21), *Fusobacterium nucleatum* (6/21) and *Porphyromonas endodontalis* (6/21) were the most frequently species. Positive association was found between *F. nucleatum* and *P. endodontalis* ($p<0.05$). A correlation was found between number of Gram-negative bacterial species and levels of endotoxin, such as PGE_2 ($p<0.05$). Higher levels of endotoxin were detected in teeth with exudation, whereas elevated levels of PGE_2 were found in teeth with tenderness to percussion and pain on palpation. **Conclusion:** Our findings imply an additive effect between the number of Gram-negative bacterial species involved in endodontic infection regarding the induction of pro-inflammatory cytokine by macrophage cell. Moreover, teeth with clinical symptomatology were related to higher levels of endotoxin and PGE_2 secretion.

Key-word: endotoxin; PGE_2; root canal; endodontics; bacteria; macrophages.

INTRODUCTION

Primary endodontic infection is a polymicrobial infection caused predominantly by Gram-negative anaerobic bacteria (1). A restricted group of bacteria, especially *Prevotella*, *Porphyromonas* spp and *Fusobacterium nucleatum*, is considered pathogenically important for odontogenic infection (2-6).

Each of the previously described Gram-negative bacterial species possesses lipopolysaccharide (LPS- endotoxin) as a cell wall constituent. LPS released during bacterial disintegration, multiplication and death (1) can egress into periradicular tissue (7), being potent stimuli against different cells (8-12) and leading to periapical inflammatory responses and bone destruction (8).

The inflammatory tissue present in periapical lesions is populated predominantly by macrophage (13). Gram-negative bacterial LPS are one of the mainly potent stimuli for macrophages cells in the release of PGE_2 (14-16). PGE_2 is implicated in most of the inflammatory and destructive changes that occur in apical lesions, such as vasodilatation, increasing vascular permeability, collagen degradation, and bone resorption (17). The possible role of PGE_2 in the pathogenesis of apical periodontitis has been provided in endodontic literature (14, 15, 17-20).

Clinical investigations had elucidated the strong correlation between higher levels of endotoxins in root canals with spontaneous pain (21-23) and clinical symptomatology such as pain on palpation, tenderness to percussion and exudation (22-24, 25). PGE_2 has been reported in inflamed human dental pulp (20), exudation (19) and periapical tissue (18). However, studies correlating all these aspects have not yet been provided in endodontic literature. Therefore, the aim of this clinical study was to investigate the presence of target Gram-negative bacterial species and the levels of endotoxin in primary endodontic infection with apical periodontitis and to determine their antigenicity against macrophages through the levels of PGE_2, evaluating their relationship with clinical findings.

MATERIAL AND METHODS

Patient selection

Twenty-one patients needing endodontic treatment, who attended the Piracicaba Dental School, Brazil, were included in this research. The age of the patients ranged from 13 to 73 years old. Samples were collected from 21 root canals with pulp necrosis, all showing radiographic evidence of apical periodontitis. The selected teeth showed absence of periodontal pockets more than 4 mm in depth.

A detailed dental history was obtained from each patient. Those who had received antibiotic treatment during the last three months or who had any general disease were excluded. The Human Research Ethics Committee of the Piracicaba Dental School approved the protocol describing sample collection for this investigation, and all volunteer patients signed an informed consent document.

Sampling procedures

All materials used in this study were heat sterilized at 200°C for 4 hours, thus becoming apyrogenic. The method followed for disinfection of the operative field had been described previously (14, 15). The teeth were isolated with a rubber dam, with crown and surrounding structures being disinfected with 30% H_2O_2 for 30s followed by 2.5% NaOCl for further 30s. Subsequently, 5% sodium thiosulphate was used to inactivate the irrigant. The sterility of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it on blood agar plates, which were incubated aerobically and anaerobically.

A two-stage access cavity preparation was made without the use of water spray, but under manual irrigation with sterile/apyrogenic saline solution and by using

sterile/apyrogenic high-speed diamond bur. The first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the protocol described above. The sterility of the internal surface of the access cavity was checked as previously described and all procedures were performed aseptically. A new sterile and apyrogenic bur was used, followed by irrigation of the root canal access with sterile apyrogenic water. The endotoxin sample was taken by introducing sterile pyrogen-free paper points (size #15; Dentsply-Maillefer, Ballaigues, Switzerland) into the full length of the canal (determined radiographically) and retained in position during 60 seconds. Immediately, the paper point was placed on a pyrogen-free glass and frozen at -80° C for future Limulus Amebocyte Lysate assay (LAL) and cell culture stimulation. The procedure was repeated with 5 sterile paper points. The paper points were pooled in a sterile tube containing 1 mL of VMGA III transport medium, being immediately processed for DNA extraction to detect target bacteria by molecular method (16S rDNA).

Bacterial detection (PCR 16S rDNA)

The reference bacteria strains used in this study were purchased from the American Type Culture Collection and are listed as follows: *Prevotella intermedia* (ATCC 25611), *Prevotella nigrescens* (ATCC 33563), *Porphyromonas endodontalis* (ATCC 35406), *Porphyromonas gingivalis* (ATCC 33277), and *Fusobacterium nucleatum* (ATCC 25586).

DNA extraction: Microbial DNA was extracted from endodontic samples as well as from ATCC bacteria and purified with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA concentration (absorbance at 260 nm) was determined by using a spectrophotometer (Nanodrop™ 2000, Thermo Scientific, Wilmington, DE, USA).

PCR assay: The PCR reaction was performed in a thermocycler (MyCycler, Bio-Rad, Hercules, CA, USA) at a total volume of 25 µl containing 2.5 µl of 10X *Taq* buffer (1X)

(MBI Fermentas, Mundolsheim, France), 0.5 µl of dNTP mix (25 µM of each deoxyribonucleoside triphosphate – dATP, dCTP, dGTP and dTTP) (MBI Fermentas, Hanover, MD, USA), 1.25 µl of 25 mM $MgCl_2$, 0.25 µl of forward and reversal universal primers (0.2 µM) (Invitrogen, Eugene, OR, USA), 1.5 µl sample DNA (1 µg/ 50 µl), 1.5 µl Taq DNA polymerase (1 unit) (MBI Fermentas) and 17.25 µl nuclease-free water. Primer forward and reverse sequences as well as PCR cycling parameters are listed in Table 1.

Determination of endotoxin concentration (Turbidimetric test - LAL assay)

The turbidimetric test (BioWhitaker, Inc., Walkersville, MD, USA), using Limulus Amebocyte Lysate (LAL) technique, was used to measure endotoxin concentrations within the root canals. First, as a parameter for calculation of the amount of endotoxins in root canal samples, a standard curve was plotted by using endotoxins supplied in the kit with known concentration (100 EU/mL) and their dilutions at the following final concentrations (i.e. 0.01, 0.10, 1, 10 EU/mL) according to the manufacturer's instructions.

Test procedure**:** All reactions were accomplished in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA) was used in a heating block at 37°C and maintained at this temperature throughout the assay. Initially, the endotoxin samples were suspended in 1 mL of LAL water supplied with the kit and agitated in vortex for 60 seconds and serial diluted to 10^{-1}. Immediately, 100 µL of the blank was added according to the standard endotoxin solutions at concentrations of 0.01, 0.10, 1, and 10 EU/mL, with 100 µL of the samples being added to 96-well microplate in duplicate. The test procedure was performed following the manufacturer's instructions. The absorbencies of endotoxin were measured individually by means of enzyme-linked immunosorbent assay plate-reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 340 nm.

Calculation of endotoxin concentrations: Since the mean absorbance value of the standards was directly proportional to the concentration of endotoxins present in the samples, the endotoxin concentration was determined from the standard curve.

Cell culture and cytokines expression

Macrophages (RAW 264.7) were cultured in 100-mm culture plates by using Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 100 IU/ mL of penicillin, 100 lg/mL of streptomycin and 10% heat-inactivated fetal bovine serum, and maintained in a humidified atmosphere at 37°C and 5% CO_2 until 90% confluence. Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Macrophages were released from 100-mm plates with 0.25% trypsin, counted in Newbauer chamber, and a total of 10^4 macrophages were grown for 48h in each well of six-well plates, de-induced by incubation for 8h in culture medium (DMEM) containing 0.3% fetal bovine serum, and stimulated with 60 µL of root canal contents during 24 hours in order to quantify the total amount of PGE_2 released in the culture media. The supernatants were collected and stored at -80ºC until protein evaluation.

mRNA expression of PGE_2

The macrophage cell viability was tested in the present study by its capacity to express PGE_2 mRNA after 24 hours of root canal contents stimulation. A total of 10^4 macrophages were grown and stimulated as described above. Total RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer's instructions. Both quantity and purity of total RNA were determined on a Biomate 3 spectrophotometer (ThermoSpectronic, Rochester, NY, USA). Complementary DNA was synthesized by reverse transcription of 500 ng of total RNA using 2.5 µM Oligo $(dT)_{12-18}$ primers and 1.25 U/uL Moloney murine leukemia virus reverse transcriptase in the presence of 3 mM $MgCl_2$, 2 mM dNTPs and 0.8 U/µL of RNAse inhibitor, according to the manufacturer's

protocol (Improm II, Promega, Madison, WI, USA). The PCR reaction was performed on a MyCycler (Bio-Rad) thermocycler using 2uL of the RT reaction product at a 20 uL total volume PCR reaction mix (GoTaq Flexi, Promega) in the presence of 100 pmol/ul of each gene's primers (50 pmol/ul of sense and antisense primers) for PGE_2 and GAPDH genes, yielding products of 329 and 418 bp, respectively. The primer pair used for PGE_2 was: sense 5'-TGCAACAGCTCAATGACTTCC3', antisense 5'-GCCCCTCACGGACAATGTAGT-3'; and GAPDH sense 5'-CACCATGGAGAAGGCCGGGG-3'; antisense 5'-GACGGACACATTGGGGTAG- 3'.

The optimized cycling conditions used for PGE_2 were: initial denaturation at 95°C for 2 min and 35 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 7 min in the presence of 1.5 mM MgCl2. As for the GAPDH, such conditions were the following: initial denaturation at 95°C for 2 min and 25 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min in the presence of 1.5 mM MgCl2. PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5μg/mL). The amplified DNA bands were analyzed densitometrically by using a digital imaging capture device (Image Quant 100 – GE Healthcare) and ImageJ 1.32j software (National Institute of Health, USA – http://rsb.info.nih.gov/ij/). The density of the bands corresponding to PGE_2 mRNA in each sample was normalized to the quantity of the housekeeping gene GAPDH and expressed as fold change over unstimulated control.

Measurements of total protein levels released into the culture media

The total amount of protein released into the culture media following root canal contents stimulation was measured by Coomassie (Bradford) Protein Assay kit (Rockford, IL, USA). As a parameter for calculation of the amount of protein released into the culture media, a standard curve was plotted by using bovine serum albumin (BSA) supplied with the kit with a known concentration (2.0 mg/mL), with a series BSA concentration (i.e. 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 μg/mL). The Protein assay was performed following the manufacturer's instructions.

Calculation of protein concentration

The protein standard and sample solutions were measured individually by using an enzyme-linked immunosorbent assay (ELISA) plate-reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 595 nm. Since this absorbance value was directly proportional to the concentration of protein, the protein concentration from the samples solutions was determined from the standard curve.

Measurements of PGE_2 levels

The amounts of PGE_2 released into the culture media following root canal contents stimulation of macrophages were measured by enzyme-linked immunosorbent assay – Duoset kit (ELISA; R&D, Minneapolis, MN, USA). Culture medium of unstimulated macrophage was used as negative control. Then, standard, control or sample solution was added to ELISA well plate, which had been pre-coated with specific monoclonal capture antibody. After being shaking gently for 3h at room temperature, the polyclonal anti-PGE_2 antibody, conjugated with horseradish peroxidase, was added to the solution and incubated for 1 hour at room temperature. Substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 20 min. The levels of cytokines were assessed by a micro ELISA reader at 450 nm and normalized with the abundance of standard solution. Each densitometric value expressed as mean ± SD was obtained from three independent experiments.

STATISTICAL ANALYSIS

The data collected for each case (clinical features and bacteria isolated) were typed nto a spreadsheet and statistically analyzed by using SPSS for Windows (SPSS, Inc., Chicago, IL). Pearson's chi-square test and one-sided Fisher's Exact test, as appropriate, were chosen to assess the null hypothesis that there was no relationship between bacteria species. Pearson's coefficient was used to correlate the amount of LPS with the levels of PGE_2, and both with the number of target Gram-negative bacteria

present in root canals with apical periodontitis. Correlation between the presence of clinical findings with the median levels of LPS and PGE_2 was analyzed by using Student's *t* test and Mann-Whitney. $P<0.05$ was considered statistically significant.

RESULTS

All samples were positive for bacterial DNA as determined by the ubiquitous bacterial primers. Individual root canals yielded a maximum of 3 target Gram-negative bacterial species. The following clinical features were found in 21 root canals investigated: pain on palpation (9/21), tenderness to percussion (8/21), and exudation 12/21 (Table2). By using PCR (16S rDNA), the following Gram-negative bacterial species was detected: *Prevotella nigrescens* (13/21), *Fusobacterium nucleatum* (6/21), *Porphyromonas endodontalis* (6/21) and *Prevotella intermedia* (1/21) (Table 2). Ten out of 15 teeth presenting any type of clinical symptomatology accounted for the presence of *P. nigrescens* species. Particularly, teeth with exudation were highly positive for *P. nigrescens* (10/12) (Table2). Almost all *P. endodontalis* infection (5/6) was concomitant with at least one other Gram-negative bacterial species, mostly presenting exudation (n = 4) (Table2). Noteworthy, a positive association was found between *F. nucleatum* and *P. endodontalis* (p=0.031, OR= 13.000, CB= 1.360 – 124.297).

Endotoxin was detected in 100% of the root canals investigated (21/21), with a median value of 7490 pg/mL ranging from 27 pg/mL to 289000 pg/mL (Table 3). A positive correlation was found between number of Gram-negative bacterial species and levels of endotoxin ($p<0.05$). Teeth with exudation presented median level of endotoxin (9190 pg/mL, range from 355 to 289000 pg/mL) higher than that for teeth with no exudation (2620 pg/mL, range from 27 to 112000 pg/mL) (Table 3).

The macrophage cell viability after 24 hours of root canal contents stimulation was confirmed in the present study after testing its capacity to express PGE_2 mRNA.

PGE_2 was detected in all culture media after stimulation with root canal contents (21/21). The median level recorded for PGE_2 was 124.53 pg/mL (range from 53.06 to 217.74 pg/mL) (Table3). A positive correlation was found between number of Gram-negative bacterial species and levels of PGE_2 secretion ($p<0.05$). Higher levels of PGE_2 were found in teeth showing clinical signs/symptoms (126.825 pg/mL) compared to those without them (118.64 pg/mL). Teeth with tenderness to percussion (TTP) presented median level of PGE_2 (131.33 pg/mL, range from 53.06 to 217.74 pg/mL) higher than that for teeth without such a symptomalogy (118.64 pg/mL, range from 63.19 to 195.60 pg/mL) (Table3). Moreover, the median level of PGE_2 was 124.53 (range from 53.06 to 201.94) in the presence of pain on palpation (POP) and 122.99 pg/mL (range from 63.19 to 217.74 pg/mL) in the absence of it (Table 3).

Individual root canal analyses showing the presence of clinical signs and symptoms, bacterial species detection, levels of endotoxin and PGE_2 secretion are all shown in Table 2.

DISCUSSION

Because bacterial stimuli can evoke periapical inflammation, the knowledge of root canal microbiota and its by-products (LPS) such as their antigenicity is important to better understand the immunobiology of apical periodontitis, which may eventually guide the root canal therapy for its remission.

In order to reproduce the complex antigenicity from primarily infected root canal, macrophage cell was stimulated with endodontic contents followed by measurement of PGE_2 by ELISA assay.

The frequent isolation of *Prevotella nigrescens*, *Fusobacterium nucleatum* and *Porphyromonas endodontalis* from endodontic infections suggests a pathogenic role for these species (2-6, 26). Because of their high frequency in root canal infection, special attention is given to their LPS cytotoxicity against different cell line cultures (8-12).

Data obtained in the present study revealed that almost all *P. endodontalis* infections were concomitant with at least one other Gram-negative bacterial species. Although experimental mono-infections by *P. endodontalis* have shown a relatively low pathogenicity, mixed culture containing this species has been demonstrated to cause severe infection (5, 6, 11).

Pathogenicity enhanced by additive or synergistic effects is an important feature of mixed infections and relies on positive relationships between the community members (27). Noteworthy, the Pearson's chi-square test revealed a positive correlation between *P. endodontalis* and *F. nucleatum*. The combination between these two species was previously reported to induce more advanced periapical tissue damage (1). Positive and negative association between endodontic bacterial species had also been reported (26, 28).

The development of both signs and symptoms in endodontic infection seems to depend on the synergy between black-pigmented bacteria and other bacterial species (4). Corroborating this finding, the present study revealed a high frequency of *P. nigrescens* (black-pigmented bacteria) in teeth with clinical symptomatology. According to previous investigations (21, 22), the elevated level of endotoxin was not only related to exudation (positively associated with the number of Gram-negative bacterial species), but also to the presence of *P. nigrescens* bacteria and/or concomitant infection by both *P. endodontalis* and *F. nucleatum*. The high frequency of *P. nigrescens* in endodontic infection seems to be related to its LPS toxicity (29). Teeth with exudation had been previously associated with *F. nucleatum* (3). Of great clinical relevance, the components of *F. nucleatum* LPS are similar to those of *Escherichia coli* (known as the most toxic LPS structure) (30), thereby exhibiting a potent immune response (8, 9, 11).

ELISA assay showed that endodontic contents were potent stimuli for PGE_2 production, with a high median value of 124.53 pg/mL. Conversely, Garrison *et al.* (14) investigated the production of PGE_2 by cell stimulation with individual LPS of oral bacteria and reported a low mean value of 7.01 ng/mL. This can partially explain the

combined bacterial infection present in root canals, which induces a more severe inflammatory response compared to strains individually tested (8-12).

Different authors (14, 15, 17-20) have demonstrated the possible role of PGE_2 in the pathogenesis of apical periodontitis. Higher levels of PGE_2 were found in macrophage supernatants stimulated with infectious material derived from teeth with clinical symptomatology. McNicholas *et al.* (18) have also found higher contents of PGE_2 in periradicular tissue sampled from teeth with clinical symptomatology. Takayama *et al.* (19) observed a positive association between the presence of clinical findings and elevated levels of PGE_2.

A great diversity concerning the influence of combined bacterial infection on inflammation was revealed in the present study by showing a positive correlation between number of Gram-negative species involved in the infection and higher levels of PGE_2. Gram-negative bacteria have the capacity of activating a wide range of immunopathologic mechanisms (31, 32). One of the interactions of LPS with macrophage lineage is mediated by the structure of "lipid A" moiety (33). "Lipid A" is structurally and compositionally different among bacterial species and within bacterial strain (34). Thus, the presence of multiple "lipid A" means a complex interpretation of innate host response. In view of the bacterial positive association reported above, *P. endodontalis* seems to enhance the *F. nucleatum* LPS toxicity against macrophages (11).

The present study partially supports the fact that PGE_2 is directly and indirectly implicated in most of the inflammatory and destructive changes that occur in apical lesions (e.g. vasodilatation), increasing vascular permeability and collagen degradation (17) by detecting higher levels of PGE_2 secretion in teeth with tenderness to percussion or pain on palpation, both clinical indicative of inflammation in periodontal ligament. In addition, *P. nigrescens* recovered from almost all teeth with pain on palpation was shown to possess a very potent LPS molecule for prostaglandin E_2 (PGE_2) stimulation (18, 29).

Peptidoglycan (PGN) from Gram-positive bacterial cells, also present in root canal contents, plays a synergistic role in the LPS antigenic activity when they activate

toll-like receptors (TLRs) - TLR-2 and TLR-4, respectively (35). The recognition of microbial structures through TLRs triggers intracellular signaling pathways that culminate in inflammatory response (36). Both LPS (a toll-like receptor ligand) and macrophages cytokines (e.g. PGE_2, IL1-ß, TNF-α) are involved in the development of apical periodontitis. Altogether in turn, activate fibroblast, amplifying the PGE_2 response (37). PGE_2 synergistically enhances pain response mediated by bradykinin, bone resorptive response caused by IL1-ß and TNF- α, and the collagenase response by IL-1 (19).

Because of the high toxicity of Gram-negative bacterial species and its by-product (endotoxin) on stimulating macrophage cell to release proinflammatory cytokines - PGE_2, its removal from infected root canals during endodontic therapy seems to be important for the healing process of periapical tissues. Foremost, rotary instrumentation (38) had demonstrated to be more effective in the removal of endotoxin from primary root canal infection than conventional technique (24, 25).

Overall, our findings suggest an additive effect on the periapical inflammation process in which the number of Gram-negative bacterial species involved in endodontic infection seems to be related with the induction of PGE_2 pro-inflammatory cytokine by macrophage cell. Further studies should be performed in order to evaluate the effect of root canal therapies on the infection control of bacterial contents through the measurement of cytokines production.

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Table 1. PCR primer pairs and cycling parameters used for detection of bacteria species in primary root canal infection with apical periodontitis

Target bacteria	Primer pairs (5'- 3')	Amplicon size	Cycles
Universal (16srDNA)	Forward: TCC TAC GGG AGG CAG CAG T		Initial denaturation at 95°C for 10min and 40 cycles of: 95°C for 10s, 60°C for 10s
	Reverse: GGA CTA CCA GGG TATCTA ATC CTG TT	466 bp	and a final extension step at 72°C for 25s
Prevotella intermedia	Forward: TTTGTTGGG GAG TAA AGC GGG		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 58°C for 1min,
	Reverse: TCA ACA TCTCTG TATCCTGCG T	575 bp	72°C for 2min and a final step 72°C for 10min.
Prevotella nigrescens	Forward: ATG AAA CAA AGG TTTTCC GGTAAG		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 58°C for 1min,
	Reverse: CCC ACG TCTCTG TGG GCTGCG A	804 bp	72°C for 2min and a final step 72°C for 10min.
Porphyromonas endodontalis	Forward: GCTGCA GCTCAA CTG TAG TC		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 58°C for 1min,
	Reverse: CCG CTTCATGTC ACC ATG TC	672 bp	72°C for 2min and a final step 72°C for 10min.
Porphyromonas gingivalis	Forward: AGG CAG CTTGCC ATA CTG CG		Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min,
	Reverse: ACTGTTAGC AAC TAC CGA TGT	404 bp	72°C for 2min and a final step 72°C for 2min.
Fusobacterium nucleatum	Forward: AGTAGC ACA AGG GAG ATG TATG		Initial denaturation at 94°C for 5 min and 30 cycles of: 94°C for 30s, 50°C for 30seg,
	Reverse:CAA GAA CTA CAA TAG AAC CTG A	645 bp	72°C for 30seg and a final step 72°C for 5min.

Table 2. Bacterial detection, levels of endotoxin and PGE_2 found in 21 root canals with primary root canal infection and apical periodontitis

Samples	(16rDNA)	*P.i.*	*P.n.*	*P.g.*	*P.e.*	*F.n.*	*Gram-ve species*	Exudate	POP	TTP	Endotoxin (pg/mL)	PGE2 (pg/mL)
S1	+		+				1	+	+	+	1310	97.64
S2	+	+					1		+	+	19100	135.32
S3	+		+				1	+	+	+	355	53.06
S4	+						0		+	+	27	129.12
S5	+		+		+		2	+			115000	109.56
S6	+					+	1	+			232000	129.94
S7	+				+	+	2	+	+		7490	113.04
S8	+		+				1	+			35200	63.19
S9	+		+				1	+			4380	63.38
S10	+		+				1				212000	127.34
S11	+		+		+	+	3	+			9190	195.60
S12	+		+		+	+	3				257	115.58
S13	+				+	+	2	+		+	289000	217.74
S14	+				+		1		+	+	5580	133.54
S15	+		+				1				2620	96.22
S16	+						0				112000	142.36
S17	+						0				2360	129.41
S18	+		+				1	+	+	+	3480	201.94
S19	+		+				1	+	+	+	269000	104.84
S20	+		+			+	2	+	+		26000	124.53
S21	+		+				1				59	118.64

P.i.= *P. intermedia*; P.n.= *P. nigrescens*; P.g.= *P. gingivalis*; P.e. = *Porphyromonas endodontalis*; F.n.= *F. nucleatum*; Gram-ve species= Gram-negative species; POP= Pain on palpation; TTP= Tenderness to percussion; "+"= presence.

Table 3. Median values of endotoxin (pg/mL) and PGE2 (pg/mL) according to the clinical findings in 21 root canals with primary root canal infection and apical periodontitis (POP= Pain on palpation; TTP= Tenderness to percussion)

Clinical findings	Endotoxin (pg/mL) Median values	PGE2 (pg/mL) Median values
All teeth (n=21)	7490	124.53
TTP (n=8)	3480	131.33
TTP free (n=13)	9190	118.64
POP (n=12)	5580	124.53
POP free (n=9)	35200	122.99
Exudate (n=12)	9190	111.30
Exudate free (n=9)	2620	129.12

2.4. Capítulo 4 – Investigation of Treponema spp. and endotoxin in primary endodontic infection and evaluation of the antigenicity of the infectious content against RAW 264.7 macrophages by the levels of IL-6 and IL-10 production.

ABSTRACT

Introduction: The aim of this clinical study was to detect the presence of target *Treponema* bacterial species and the levels of endotoxin in PEI with apical periodontitis and to determine their antigenicity against macrophages through the levels of IL-6 and IL-10, evaluating their relationship with clinical and radiographic findings. **Methods:** Nested - PCR technique was used for the detection of the *Treponema* species. Limulus Amebocyte Lysate (LAL) was used to measure endotoxin. The amounts of IL-6 and IL-10 in macrophages supernatants were measured by enzyme-linked immunosorbent assay - Duoset-kit (ELISA). **Results:** *T. medium*, *T. amylovorum* and *T. lecithinolyticum* were the most frequently detected (all in 33%). Positive correlation was found between number of different *Treponem*a species and the level of endotoxin ($p<0.05$). Teeth with larger size of radiolucent area ≥ 2mm presented higher median levels of endotoxin than teeth with <2mm ($p<0.05$). Correlations were found between endotoxin contents and the levels of IL-6 and IL-10 ($p<0.05$) released in the culture media after cell stimulation. **Conclusion:** a wide variety of Treponema species do play a role in primary endodontic. Moreover, the bacterial endodontic contents, particularly the levels of endotoxin present in root canals, were a potent stimuli for the production IL-6 and IL-10 in macrophages.

Key-words: Treponema, Endotoxin, bacteria stimulation, IL-6 and IL-10.

INTORDUCTION

Bacterial infection of the dental pulp result in tissue destruction and ultimately, in periapical bone resorption (1). A proinflammatory cytokine production is induced in response to bacterial infection that is primarily produced, by monocytes/ macrophage lineage (2-6), which plays a central role in cytokine production, and so act to modulate many aspects of the inflammatory response (7).

Primary endodontic infection (PEI) is a polymicrobial infection caused predominantly by gram-negative anaerobic bacteria, specifically, *Prevotella*, *Porphyromonas*, *Treponema* and *Fusobacterium* spp (8-11).

Analysis of the role of the structural constituents of bacteria indicates that many effects of gram-negative bacteria on macrophages is mediated by bacterial LPS and its biologically active lipid moiety lipid A (12). Further, the pattern of the macrophage response varies considerably, depending on the type of bacteria (13) such as the number of different species of Gram-negative bacteria involved in the infection (5-6).

*Treponema*s pp., an oral spirochete, is part of the microbial flora frequently detected in root canals (8, 14-16) that present a strong proteolytic activity. The metabolic products and endotoxins released by *Treponema* cells disrupt the essential functions is host cells, promoting tissue breakdown (17-19). It has been reported that Toll Like Receptor-2 (TLR-2) is critical for macrophages to recognized and respond to *Treponema* cells (19).

The activation of macrophages cells lead to a production of different interleukins (5, 20-22) including IL-6 (20-21) and IL-10 (21-22).

IL-6 act as proinflammatory cytokine during periodontitis and also stimulate osteoclastic differention and bone resorption in chronic inflammatory periodontitis (23-24). This cytokine also induce chemokine secretion during the destruction of tissue of periodontal tissue (25-26). On the other hand, the progressive destruction of tissue caused particularly by IL-6 seem to be suppressed by the production of anti-inflammatory

cytokines such as IL-10 (27-29) which is known to have a protective role against the lethal effects of LPS *in vivo* (30).

In order to attempt to a better understanding of the bacterial infection and the immune-biology involved an endodontic infection, the aim of this clinical study was to detect the presence of target *Treponema* bacterial species and the levels of endotoxin in PEI with apical periodontitis and to determine their antigenicity against macrophages through the levels of IL-6 and IL-10, evaluating their relationship with clinical and radiographic findings.

MATERIAL AND METHODS

Patient selection

Twenty-one patients needing endodontic treatment, who attended the Piracicaba Dental School, Brazil, were included in this research. The age of the patients ranged from 13 to 73 years old. Samples were collected from 21 root canals with pulp necrosis, all showing radiographic evidence of apical periodontitis. The selected teeth showed absence of periodontal pockets more than 4 mm in depth.

A detailed dental history was obtained from each patient. Those who had received antibiotic treatment during the last three months or who had any general disease were excluded. The Human Research Ethics Committee of the Piracicaba Dental School approved the protocol describing sample collection for this investigation, and all volunteer patients signed an informed consent document.

Sampling procedures

All materials used in this study were heat sterilized at 200°C for 4 hours, thus becoming apyrogenic. The method followed for disinfection of the operative field had been described previously. The teeth were isolated with a rubber dam, with crown and surrounding structures being disinfected with 30% H_2O_2 for 30s followed by 2.5% NaOCl for further 30s. Subsequently, 5% sodium thiosulphate was used to inactivate the irrigant. The sterility of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it on blood agar plates, which were incubated aerobically and anaerobically.

A two-stage access cavity preparation was made without the use of water spray, but under manual irrigation with sterile/apyrogenic saline solution and by using sterile/apyrogenic high-speed diamond bur. The first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the protocol described above. The sterility of the internal surface of the access cavity was checked as previously described and all procedures were performed aseptically. A new sterile and apyrogenic bur was used, followed by irrigation of the root canal access with sterile apyrogenic water. The endotoxin sample was taken by introducing sterile pyrogen-free paper points (size #15; Dentsply-Maillefer, Ballaigues, Switzerland) into the full length of the canal (determined radiographically) and retained in position during 60 seconds. Immediately, the paper point was placed on a pyrogen-free glass and frozen at -80° C for future Limulus Amebocyte Lysate assay (LAL) and cell culture stimulation. The procedure was repeated with 5 sterile paper points. The paper points were pooled in a sterile tube containing 1 mL of VMGA III transport medium, being immediately processed for DNA extraction to detect target bacteria by molecular method (Nested PCR-technique).

Bacterial detection (Nested-PCR)

DNA extraction

Microbial DNA from endodontic samples as well as from ATCC bacteria were extracted and purified with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA concentration (absorbance at 260 nm) was determined using a spectrophotometer (Nanodrop™ 2000, Thermo Scientific, Wilmington, DE, USA).

Nested PCR-assay

PCR assay: The PCR reaction was performed in a thermocyler (MyCycler, Bio-Rad, Hercules, CA, USA) thermocycler in a total volume of 25 µl containing 2.5 µl of 10X *Taq* buffer (1X) (MBI Fermentas, Mundolsheim, France), 0.5 µl of dNTP mix (25 µM of each deoxyribonucleoside triphosphate – dATP, dCTP, dGTP and dTTP) (MBI Fermentas, Hanover, MD, USA), 1.25 µl of 25 mM $MgCl_2$, 0.25 µl of forward and reversal universal primers (0.2 µM) (Invitrogen, Eugene, OR, USA), 1.5 µl sample DNA (1 µg/ 50 µl), 1.5 µl Taq DNA polymerase (1 unit) (MBI Fermentas) and 17.25 µl water nuclease free. The samples were subjected to a previous denaturation step at 95C for 2 minutes, followed by 36 cycles of denaturation at 94°C for 30 minutes, annealing at 55°C for 1 minute, primer extension at 72°C for 2 minutes, and a final extension of 72°C for 4 minutes in an automated thermal cycler (Perkin-Elmer Cetus, Scientific support, Inc, Hayward, CA). Positive controls were performed with the using ATCC bacterial strains. Negative controls corresponded to the reaction mixture without DNA.

Treponema species were then identified by a second nested amplification with species-specific 16S primers paired with a universal primer located in the 16S gene. Primer sequences and specific annealing temperatures are shown in Table 1, as previously described by Montagner *et al.* (2010). All primers were synthesized by Invitrogen (Eugene, OR). The PCR reaction conditions were as follows: 36 cycles of 94°C for 30 minutes, specific annealing temperature related to each species for 1 minute (Table 1), and 72°C for 2 minutes. Final extension was performed at 72°C for 4 minutes. PCR products were

analyzed by 1% agarose gel electrophoresis, staining with ethidium bromide, and viewed under ultraviolet transillumination.

A positive or negative detection was based on the presence of clear bands of the expected molecular size by using a 100-kb lambda DNA ladder (Invitrogen Corporation, Carlsbad CA). All assays were conducted twice, and if the results were not in agreement with each other, the would be redone.

Determination of endotoxin concentration (Turbidimetric Test – LAL Assay)

The turbidimetric test (BioWhitaker, Inc., Walkersville, MD, USA) was used to measure endotoxin concentrations in the root canals using the Limulus Amebocyte Lysate (LAL) technique. First, as a parameter for calculation of the amount of endotoxins in root canal samples, a standard curve was plotted using endotoxins supplied in the kit with a known concentration (100 EU/mL), and its dilutions with the following final concentrations (i.e. 0.01, 0.10, 1, 10 EU/mL) following the manufacturer's instructions.

Test procedure: All reactions were accomplished in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA) was used in a heating block at 37°C and maintained at this temperature throughout the assay. First, the endotoxin-samplings were suspended in 1 mL of LAL water supplied on the kit and agitated in vortex for 60 seconds and serial diluted to the 10^{-1}. Immediately, 100 μL of the blank followed the standard endotoxin solutions in concentrations (i.e. 0.01, 0.10, 1, 10 EU/mL) and 100 μL of the samples were added in duplicate in the 96-well microplate. The test procedure was performed following the manufacturer's instructions. The absorbencies of endotoxin were measured individually using an enzyme-linked immunosorbent assay plate-reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 340 nm.

Calculation of endotoxin concentrations: Since the mean absorbance value of the standards was directly proportional to the concentration of endotoxins present, the endotoxin concentration was determined from the standard curve.

Cell culture and Cytokines Expression

Cell culture: Macrophages (RAW 264.7) were cultured 100mm culture plates in Dulbecco's modified Eagle's minimal essential medium supplemented (DMEM) with 100 IU/ mL of penicillin, 100 lg/mL of streptomycin and 10% heat-inactivated fetal bovine serum, and maintained in a humidified atmosphere at 37°C and 5% CO_2 until 90% confluence. Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Macrophages were released from 100mm plates with 0,25% trypsin, counted in Newbauer chamber and a total of 10^4 macrophages were grown for 48h in each well of six-well plates, de-induced by incubation for 8h in culture medium (DMEM) containing 0.3% fetal bovine serum and stimulated with 60 µL of root canal contents during 24 hours in order to quantify the total amount of protein released in the culture media, IL-1ß and TNF-alpha protein. The supernatants were collected and stored at -80ºC until protein evaluation.

IL-6 and IL-10 mRNA expression: The macrophages cell viability was tested in the present study by its capacity to express IL-1ß and TNF-alpha mRNA after 24 hours of root canal contents stimulation. A total of 10^4 macrophages were grown for 48h in each well of six-well plates, de-induced by incubation for 8h in culture medium (DMEM) containing 0.3% fetal bovine serum and stimulated with 60 uL of primary infection contents for 24 hours for IL-6 and IL-10 mRNA expression. Total RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer's instructions. The quantity and purity of total RNA were determined on a Biomate 3 spectrophotometer (ThermoSpectronic, Rochester, NY, USA). Complementary DNA was synthesized by reverse transcription of 500 ng of total RNA using 2.5 µM Oligo $(dT)_{12-18}$ primers and 1.25 U/uL Moloney murine leukemia virus reverse transcriptase in the presence of 3 mM $MgCl_2$, 2 mM dNTPs and 0.8 U/µL of RNAse inhibitor, according to the manufacturer's protocol (Improm II, Promega,

Madison, WI, USA). The PCR reaction was performed in a MyCycler (Bio-Rad) thermocycler using 2uL of the RT reaction product on a 20 uL total volume PCR reaction mix (GoTaq Flexi, Promega) in the presence of 100 pmol/ul of each gene's primers (50 pmol/ul of sense and antisense primers) for IL-6, IL-10 and GAPDH genes yielding products of 408, 354 and 418 bp, respectively. The primer pair used for IL-6 (acession number NM000600) was: sense 5'- AAAGAGGCACTGGCAGAAAA -3', antisense 5'-GAGGTGCCCATGCTACATTT -3'; IL-10 (accession no.: NM012675) sense 5'-GGAGAACAGCAACTCCAGAA-3', antisense 5'-TCTTTGAGATCCATGCCATT-3'; and GAPDH (accession no.: BC083065) sense 5'-CACCATGGAGAAGGCCGGGG-3'; antisense 5'-GACGGACACATTGGGGTAG- 3'. Optimized cycling conditions used for IL-6 and IL-10 were: initial denaturation at 94°C for 3 min and 30 cycles of: 94°C for 1 min, 58°C for 1 min, 72°C for 1 min in the presence of 1.5 mM MgCl2 and for GAPDH conditions were as follows: initial denaturation at 95°C for 2 min and 25 cycles of: 95°C for 1 min, 52°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min in the presence of 1.5 mM MgCl2. PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5µg/mL). The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100 – GE Healthcare), using ImageJ 1.32j software (National Institute of Health, USA – http://rsb.info.nih.gov/ij/). The density of the bands corresponding to TNF- alpha and IL-1ß mRNA in each sample was normalized to the quantity of the housekeeping gene GAPDH and expressed as fold change over unstimulated control.

Measurements of total protein levels released to the culture media - The total amount of protein released in the culture media following root canal contents stimulation was measured by Coomassie (Bradford) Protein Assay kit (Rockford, IL, USA). As a parameter for calculation of the amount of protein released to the culture media, a standard curve was plotted using Bovine Serum Albumin (BSA) albumin standard supplied in the kit with a known concentration (2.0 mg/mL), with a series BSA concentration (i.e. 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/mL). The Protein assay was performed following the manufacturer's instructions.

Calculation of protein concentration -The protein standard and sample solutions were measured individually using an enzyme-linked immunosorbent assay plate-reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 595 nm. Since this absorbance value was directly proportional to the concentration of protein, the protein concentration from the samples solutions was determined from the standard curve.

Measurements of IL-6 and IL-10 levels - The amounts of IL-6 and IL-10 released to the culture media following root canal contents stimulation of macrophages were measured by enzyme-linked immunosorbent assay – Duoset kit (ELISA; R&D, Minneapolis, MN, USA). Medium of unstimulated macrophage culture was used as a negative control. Briefly, standard, control or sample solution was added to ELISA well plate, which had been pre-coated with specific monoclonal capture antibody. After shaking gently for 3h at room temperature, polyclonal anti-IL-6 and IL-10 antibody, conjugated with horseradish peroxidase, was added to the solution, respectively, and incubated for 1 h at room temperature. Substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 20 min. The levels of cytokines were assessed by a microelisa reader at 450 nm and normalized with the abundance of standard solution. Each densitometric value expressed as mean ± SD was obtained from three independent experiments.

STATISTICAL ANALYSIS

The data collected for each case (clinical features and the bacteria isolated) were typed onto a spreadsheet and statistically analyzed using SPSS for Windows (SPSS, Inc., Chicago, IL). The Pearson chi-square test or the one-sided Fisher's exact test, as appropriate, was chosen to test the null hypothesis that there was no relationship between bacteria species such as endodontic clinical signs/ symptoms and the presence of a specific group of bacteria in the root canal samples. Pearson coefficient was used to correlate the amount of LPS, IL-6 and IL-10 levels each two at a time such as to correlate each one with the size of radiolucent area and the number of Gram-negative bacteria present in root canals with apical periodontitis. Correlation between the presence of clinical/ radiographic findings with the

median levels of LPS, IL-6 and IL-10 was analyzed using Student's *t* test or Mann-Whitney. P<0.05 was considered statistically significant.

RESULTS

The following clinical features were observed in the 21 root canals analyzed: pain on palpation (9/21), tenderness to percussion (8/21), presence of exudation (12/21), and radiolucent area ≥2mm (11/21) and < 2mm (10/21). Of the patients presented spontaneous pain.

All samples were positive for bacterial DNA as determined by the use of ubiquitous primer (21/21). No positive results were observed for the presence of bacterial DNA in control samples. Individual root canals yielded a maximum of 6 targets *Treponema* species. *Trepnema* species were detected in 71% of the root canal samples analyzed (15/21). The most frequently detected species were *T. medium*, *T. amylovorum* and *T. lecithinolyticum* (all detected in 33% = 7/21 samples) followed by the detection of *T. socranskii, T. pectinovorum and T. maltophylum* (28.57% = 6/21 samples). Low detection levels were observed for *T. vicentii* (19.04% = 4/21) and *T. denticola* (14,21% = 3/21 samples). A combination of two or more *Treponema* species was detected in 10 out of 21 root canals. Positive associations between different *Treponema* species are shown in Table 3. Statically significant relationship was detected between the presence of *T. lecithinolyticum* and the presence of clean exudate (p=0.041;OR [OddsRatio] = 9.167 and CB [Confidence Bound]= 1.147-73.239).

Endotoxin was detected in 100% of the root canals investigated (21/21), with a median value of 7,490 pg/mL ranging from 27 pg/mL to 289000 pg/mL. Positive correlation was found between number of different *Treponem*a species and the level of endotoxin (p<0.05, Pearson r=0.044). Teeth with larger size of radiolucent area ≥ 2mm (9,190 pg/mL; range 257-212,000 pg/mL) presented higher median levels of endotoxin than teeth with radiolucent area <2mm (3,480 pg/mL; range 27-289,000 pg/mL) (p<0.05).

The cell macrophage viability after 24 hours of root canal contents stimulation was confirmed in the present study in the present study after testing its capacity to express IL-6 and -10 messenger RNA.

IL-6 and IL-10 were detected in all culture media after stimulation with root canal contents. The median levels recorded for IL-6 and IL-10 were 270.151 pg/mL (range, 146.674 – 365.017 pg/mL) and 39.997 pg/mL (range, 17.281-50.111 pgmL) respectively. Relatively higher levels of IL-6 and IL-10 were found in teeth showing radiolucent area ≥2mm (IL-6 = 279.401 pg/mL and IL-10 = 40.992 pg/mL) compared with those without them (IL-6 = 265.418 pg/mL and IL-10=37.427 pg/mL). Correlations were found between endotoxin contents and the levels of IL-6 ($p<0.05$, Pearson r= 0.016) and IL-10 ($p<0.05$, Pearson r= 0.045) released in the culture media after cell stimulation.

DISUCSSION

Analyses of our data demonstrates that the extent of activation macrophages, as assessed by IL-6 and IL-10 expression, depends significantly upon the levels of endotoxin such as different the different sources of this molecule, particularly, different Treponema species.

In the current study, the positive detection of *Treponema* species in primarily infected root canals is agreement to the endodontic literature (8, 14-16)

Because difficulties in isolating and identifying Treponema, different molecular approaches has been used for their detection (5, 14, 16, 31-34). Particularly, the utilization of a nested PCR protocol in this study is justified by the increased sensitivity and specificity of the assay when compared to single PCR (14).

The frequent isolation of *T. medium*, *T. amylovorum*, *T. lecithinolyticum* from primary root canal infection by Nested-PCR supports the pathogenic role for these species suggested by previous investigations (8, 16, 31). The combination of two or more

Treponema species found in 10 out of 21 root canals analyzed, turns endodontic contents even more complex to the immune system, indicating that different bacterial LPS with possibly different toxicity structure (lipid A) (35) can be involved in the root canal infection, enhance or even inhibiting each other's antigenic activity over perirradicular tissues.

Positive correlation was found between larger number of different Treponema species and higher levels of endotoxin. Thus, teeth with larger size of radiolucent area ≥ 2mm presented higher median levels of endotoxin agreeing with Schein and Schildder (1975) (36), who reported that the endotoxin contents of teeth with radiolucent area is 5 times greater than in teeth without. The connection between LPS and the presence of bone destruction is widely discussed in the literature (37, 38).

The host response against bacteria and their toxins is complex and involves the recruitment of inflammatory cells and the participation of an extensive network of immunologic mechanisms. The activation of macrophages cells by Toll like receptors (TLRs) - key pathogen recognition molecules of innate immune – is the first line of defense against bacterial infection (19). Individual TLRs recognize particular surface or intracellular microbial moleculares (39).

Binding of LPS to TLR4 or lipoteichoic acid (LTA) to TLR-2 leads to the induction cytokines production (5, 20, 21, 22,) including IL-6 (20, 21) and IL-10 (21,22).

IL-6 was detected in all culture media after stimulation with root canal contents from teeth with apical peridodontitis. The median levels recorded for IL-6 were 270.151 pg/mL. Bakhordar *et al.* (1999), after obtaining inflamed periapical tissues and diseased pulp tissues from teeth with diagnosed with pulpitis, revealed a mean value of IL-6 of 78.1 pg/mg and 36 pg/mg respectively. The role of IL-6 in the development of a faster and earlier periapical lesion in IL-6 knock-out mice was observed by Huang *et al.* (2001).

ELISA showed that LPS from Gram-negative bacterial species involved in endodontic infection, particularly Treponema spp., is significantly related to the production

of IL-6 and IL-10. Euler G (1998) was able to detect IL-6 transcripts by northern blotting in neutrophils stimulated with different Gram-negative bacterial LPS.

Not only higher production of IL-6 was followed by the stimulation with higher concentration of endotoxin, but also IL-10 was increased. The networks between different cytokines seem to regulates the formation of periapical lesion (23). While IL-6 act as proinflammatory cytokine during periodontitis and also stimulate osteoclastic differention and bone resorption in chronic inflammatory periodontitis (23, 24) IL-10 is actively engaged in the down regulation of the inflammatory response and, thus minimize the damage in response to microbial challenge. In fact, IL-10 inhibits NFk-beta, which consequently results in the reduction of the pro-inflammatory cytokines IL-1 beta, IL-6 and TNF-alpha (27).

Relatively higher levels of IL-6 and IL-10 were found in teeth showing radiolucent area ≥2mm (IL-6 = 279.401 pg/mL and IL-10 = 40.992 pg/mL) compared with those without them (IL-6 = 265.418 pg/mL and IL-10=37.427 pg/mL). IL-6 act as proinflammatory cytokine during periodontitis and also stimulate osteoclastic differention and bone resorption in chronic inflammatory periodontitis (23-24). On the other hand, the progressive destruction of tissue caused particularly by IL-6 seem to be suppressed by the production of anti-inflammatory cytokines such as IL-10 (27-29).

Since macrophages were stimulated with root canal contents from primary root canal infection (a polymicrobial bacterial infection), ubiquitous cell wall components which are common to many microorganisms - not only endotoxin (LPS, Lipopolysccaride) from Gram-negatives, but also proteoglycans from Gram-positives and other cell wall components – must also be considered to analysis of the of the production of IL-6 and IL-10 found in the present study.

A variety of cell surface components have been shown to exert immunobilogical activities (40-42). In particular, *T. medium* contains a glycoconjugate possessing and inhibitory effect on TLR-mediated cell activation through the interaction with LBP (LPS binding protein) and CD14 (41). Additionally, T. *medium* has an ability to inhibit C14/ LPS binding protein function therefore participating in the progression of bacterially destruction.

TLR-2 depend cell activation was observed in response to lipoproteins from *T. pallidum* (40), while glycolipids from *T. maltophilum* exhibits TLR-2 dependence during NF-kB activation and cytokine production (42).

Overall, the present study demonstrates that a wide variety of Treponema species do play a role in primary endodontic infection with apical peridodontitis and the levels of endotoxin involved in endodontic infection demonstrated to be a potent stimuli for the release of pro-inflammatory cytokines, particularly for the stimulation of IL-6 and IL-10 production.

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Table 1. Target sequence primers for the detection of Treponema species in 21 root canals with primary endodontic infection with apical periodontitis

Microrganismos	Primers (5' → 3')	Posição	Fragmento	Anelamento
Universal 16S ribosomal DNA	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	8–1.513	1505 bp	55°C
Treponema denticola	TAATACCGAATGTGCTCATTTACAT TCAAAGAAGCATTCCCTCTTCTTCTTA	193–508	316 bp	60°C
Treponema amylovorum	AGAGTTTGATCCTGGCTCAG CTCACGCCTTTATTCCGTGAG	8–211	193 bp	54°C
Treponema maltophilum	AGAGTTTGATCCTGGCTCAG CCTATTGTGCTTATTCATCAGGC	8–446	438 bp	54°C
Treponema medium	AGAGTTTGATCCTGGCTCAG CCTTATGAAGCACTGAGTGTATTC	8–200	192 bp	54°C
Treponema socranskii	GATCACTGTATACGGAAGGTAGACA TACACTTATTCCTCGGACAG	179–468	285 bp	53°C
Treponema pectinovorum	AGAGTTTGATCCTGGCTCAG ATATATCTCCAACTTATATGACCT	8–205	194 bp	53°C
Treponema vincentii	AGAGTTTGATCCTGGCTCAG AATACTTCTTATGAACATTGAGAC	8–201	193 bp	56°C

2.5. Capítulo 5 – Stimulation of interleukin 1-β and TNF-α production of RAW 264.7 macrophages cells by *Porphyromonas gingivalis* and *Fusobacterium nucleatum* lipopolysaccharide isolated from primary endodontic infection.

ABSTRACT

Introduction: The aim of this study was to evaluate the antigenic activity of LPS purified from *P. gingivalis* and *F. nulceatum* isolated from infected root canals on macrophages cells (Raw 264.7) by the levels of IL-1β and TNF-α. **Methods:** *P. gingivalis* and *F.nucleatum* was isolated and identified by phenotype and molecular method (PCR 16s rDNA). The clinical isolate and respectively ATCC strains were sequenced. LPS from *Escherichia coli* (ATCC 25922) was also extracted. LPS was extracted by Tris-reagent method. Extracted-LPS was purified and stained by the colloidal gold procedure. Macrophages were stimulated with LPS at 100 ng/mL at 12, 24 and 48 hours of incubation. The amounts of IL-1ß/ TNF-alpha were measured by enzyme-linked immunosorbent assay – Duoset-kit (ELISA). **Results:** The production of the IL-1 β and TNF-α significantly increased with the time course of incubation. The secretion of IL-1 β occurred in higher levels than in TNF-α. LPS from *E. coli* was the most toxicity followed by the *F. nucleatum*. LPS from clinical isolate demonstrated to be more toxicity than LPS from ATCC strains for both *P. gingivalis* and *F. nucleatum*. LPS from *P. gingivalis* and *F. nucleatum* is involved in the induction of IL-1 β and TNF-α, which can play a role in the initiation of the upregulation of the inflammatory response and can also stimulate the production of secondary mediators involved in tissue destruction.

Key-words: LPS, *F. nucleatum*, *P. gingivalis,* Root canal, bacteria

INTRODUCTION

The mechanisms of cytokine production and regulation during Gram-negative bacteria infection, such as during the endodontic infection, are currently under extensive investigations (1-5).

Accumulated evidence indicates that the pathology of the endodontic infections is due, in part, to the actions of the host-derived cytokines, induced by lipopolysaccharides (1-7).

LPS molecules, the major constituents of the outer membrane of Gram-negative bacteria, consist of a hydrophobic moiety (lipid A), a core oligosaccharide, and occasionally a chain of repeating units of from 1 to 7 sugars (O chain) (8). The polysaccharide region of LPS shows considerable structural and compositional heterogeneity among different bacterial species and within a bacterial strain (9-10).

Although LPS molecules are known to exert potent stimulatory effects on several cell types *in vivo*, it is now generally recognized that the cell type primarily responsible for its physiologic effects is the macrophage (11-14). Interactions of the lipid A and/or the polysaccharide moiety of LPS with membrane receptors of the monocytes results in a wide range of cellular responses (15-19), including the synthesis of interleukin (IL)-1, tumor necrosis factor (TNF).

IL-1 and TNF cytokines not only amplify the immune response, recruit the immune cells, and activate immune and nonimmune cells, but also may cause significant tissue damage by collagenase induction in fibroblasts and activation of osteoclasts (3, 20-24).

Endodontic pathogens as well as purified LPS have been shown to activate monocytes and induce cytokine secretion (1-3). Furthermore, higher levels of these cytokines have been detected in rat pulpitis (24), teeth with apical periodontitis (26); root canal exudates (27-28), symptomatic teeth (29) and larger size of periapical lesions (24),

suggesting that these cytokines are intimately involved in the pathogenesis of endodontic infection.

Endodontic infection is a polymicrobial infection caused predominantly by gram-negative anaerobic bacteria, specifically, Prevotella, Porphyromonas, Treponema and Fusobacterium spp (30-33). Among the Gram-negative bacteria species, *Fusobacterium nucleatum* and *Porphyromonas gingivialis* are highly detected in root canal infection (34-37). Their LPS, has been suggested as a possible virulence factor, acting by the stimulation of host cells to induce production of pro-inflammatory mediators.

Overall, the aim of this study was to evaluate the antigenic activity of LPS, purified from *P. gingivalis* and F. nulceatum isolated from infected root canals on macrophages cells (Raw 264.7) by the levels of IL-1β and TNF-α.

MATERIAL AND METHODS

Bacterial strains and growth conditions

The clinical isolate from *P. gingivalis* and *F. nucleatum* were obtained from primary endodontic infection with apical periodontitis from patients needing endodontic treatment, who attended the Piracicaba Dental School, Brazil. *P. gingivalis* strains ATCC 33277, *F. nucleatum* ATCC 25586 and *Escherichia coli* ATCC 25922 was obtained from *American Type Culture Collection*. The Clinical strains were examined for purity, primarily identified by Rapid ID 32A (BioMérieux SA, Marcy- l'Etoile, France) and PCR (16 rDNA) and confirmed by sequence analysis. Cultures were immediately processed for LPS extraction in order to avoid freezing them and repetitive subculturing.

Bacterial culture media included both enriched Trypticase soy broth (ETSB) and Trypticase soy broth-yeast extract-hemin-vitamin K (menadione) (TYHK) for the *P.gingivalis* and *F. nucleatum* strains and LB Broth for *E. coli* (Sigma, # L7275-500 TAB) and Trypticase soy broth-yeast extract-hemin-vitamin K (menadione) (TYHK). The ETSB

used was a modification of a previously described medium and consisted of Trypticase soy broth (TSB) (30g/950 ml), yeast extract (Difco) (1 g/950 ml), glucose (1 g/950 ml), and potassium nitrate (0.5 g/950 ml), pH 7.1 to 7.3. This basal medium was then autoclaved, and filter-sterilized supplements were added, which consisted of anhydrous sodium carbonate (0.4 g), cysteine-HCl (Sigma) (0.4 g), hemin (Sigma) stock solution (solution a) (10 ml), vitamin K (Sigma) stock solution (solution b) (0.2 ml), and distilled water (40 ml). Stock solutions were made as follows. Solution a was made by dissolving 50 mg of hemin (Sigma) in 1.0 ml of 1.0 N NaOH, adding 99 ml of distilled water, and storing the solution at 4°C. Solution b was made by dissolving 250 mg of vitamin K (Sigma) in 50 ml of 95% ethanol and storing it at 4°C. These stock solutions were replaced 2 weeks following preparation. The composition of TYHK was Trypticase soy broth (30 g/liter), yeast extract (5g/liter) (Difco), hemin (Sigma) (0.005 g/liter), and vitamin K3 (menadione; Sigma) (0.001 g/liter), pH 7.2, and the medium was subjected to autoclaving. Bacterial growth was monitored by following the optical density at 600 nm, cells were harvested in the stationary phase of growth, and final bacterial yields were determined by wet weight after centrifugation and washing.

Purification and characterization of LPS

LPS was prepared by the Tri-Reagent procedure as previously described (38). Following the final ethanol precipitation, LPS was lyophilized to determine the yield and was resuspended in distilled H2O to 1 mg/ml without the addition of triethanolamine. LPS obtained with Tri-Reagent was further purified by the following steps. One milligram of lyophilized LPS (the last step in the Tri-Reagent procedure) was suspended in 1 ml of cold (stored at 20°C) 0.375 M MgCl2 in 95% ethanol (EtOH) and transferred to a 1.5-ml Eppendorf tube, and after complete mixing, the suspension was centrifuged at 2,300 _ g for 5 min. This step was repeated twice. The second supernatant was decanted, 1 ml of 100% EtOH (room temperature) was added, and the suspension was thoroughly mixed and subjected to centrifugation at 2,300 _ g for 5 min. This process was repeated twice. The final pellet was re-suspended in 0.1 ml of endotoxin-free water. Both the Tri-Reagent and phenol-purified LPS preparations were subjected to sodium dodecyl sulfate-polyacrylamide

gel electrophoresis and stained for protein by the enhanced colloidal gold procedure as described previously (Manthey CL & Vogel 1994). The colloidal gold procedure revealed 0.1% protein contamination in either of the LPS preparations based upon the amount of LPS loaded into the gel and the intensity of the major protein band relative to that of a known bovine serum albumin standard.

Cell culture and cytokines expression

Macrophages (RAW 264.7) were cultured 100mm culture plates in Dulbecco's modified Eagle's minimal essential medium supplemented (DMEM) with 100 IU/ mL of penicillin, 100 lg/mL of streptomycin and 10% heat-inactivated fetal bovine serum, and maintained in a humidified atmosphere at 37°C and 5% CO2 until 90% confluence. Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Macrophages were released from 100mm plates with 0,25% trypsin, counted in Newbauer chamber and a total of 10^4 macrophages were grown for 48h in each well of six-well plates, de-induced by incubation for 8h in culture medium (DMEM) containing 0.3% fetal bovine serum and stimulated with LPS at 100 ng/mL and 1µg/mL at 12, 24 and 48 hours of incubation. in order to quantify the total amount IL-1ß and TNF-alpha protein released in the culture media. The supernatants were collected and stored at -80ºC until protein evaluation.

Reverse transcription-PCR assay of IL-1β and TNF-α mRNA expression

The macrophages cell viability was tested in the present study by its capacity to express IL-1ß and TNF-alpha mRNA after 24 hours of root canal contents stimulation. A total of 104 macrophages were grown for 48h in each well of six-well plates, de-induced by incubation for 8h in culture medium (DMEM) containing 0.3% fetal bovine serum and stimulated with 60 uL of primary infection contents for 24 hours for IL-1ß and TNF-alpha mRNA expression. Total RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer's instructions. The quantity and purity of total RNA were determined on a Biomate 3- spectrophotometer (ThermoSpectronic, Rochester, NY, USA).

Complementary DNA was synthesized by reverse transcription of 500 ng of total RNA using 2.5 μM Oligo (dT)12-18 primers and 1.25 U/uL Moloney murine leukemia virus reverse transcriptase in the presence of 3 mM MgCl2, 2 mM dNTPs and 0.8 U/μL of RNAse inhibitor, according to the manufacturer's protocol (Improm II, Promega, Madison, WI, USA). The PCR reaction was performed in a MyCycler (Bio-Rad) thermocycler using 2uL of the RT reaction product on a 20 uL total volume PCR reaction mix (GoTaq Flexi, Promega) in the presence of 100 pmol/ul of each gene's primers (50 pmol/ul of sense and antisense primers) for IL-1ß, TNF- alpha and GAPDH genes yielding products of 494, 451 and 418 bp, respectively. The primer pair used for IL-1ß (accession no.: NM031512) was: sense 5'-GACCTGTTCTTTGAGGCTGA-3', antisense 5'-CGTTGCTTGTCTCTCCTTGT-3'; TNF-alpha (accession no.: NM012675) sense 5'-GGAGAACAGCAACTCCAGAA-3', antisense 5'-TCTTTGAGATCCATGCCATT-3'; and GAPDH (accession no.: BC083065) sense 5'-CACCATGGAGAAGGCCGGGG-3'; antisense 5'-GACGGACACATTGGGGTAG- 3'. Optimyzed cycling conditions used for TNF- alpha and IL-1ß were: initial denaturation at 95°C for 2 min and 35 cycles of: 95°C for 1 min, 58°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 7 min in the presence of 1.5 mM MgCl2 and for GAPDH conditions were as follows: initial denaturation at 95°C for 2 min and 25 cycles of: 95°C for 1 min, 52°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min in the presence of 1.5 mM MgCl2. PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5μg/mL). The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100 – GE Healthcare), using ImageJ 1.32j software (National Institute of Health, USA – http://rsb.info.nih.gov/ij/). The density of the bands corresponding to TNF- alpha and IL-1ß mRNA in each sample was normalized to the quantity of the housekeeping gene GAPDH and expressed as fold change over unstimulated control.

Measurement of IL-1ß and TNF-alpha levels

The amounts of IL-1ß and TNF-alpha released to the culture media following root canal contents stimulation were measured by enzyme-linked immunosorbent assay – Duoset kit

(ELISA; R&D, Minneapolis, MN, USA). Briefly, standard or sample solution was added to ELISA well plate, which had been pre-coated with specific monoclonal capture antibody. After shaking gently for 3 h at room temperature, polyclonal anti-TNF-alpha and IL-1ß antibody, conjugated with horseradish peroxidase, was added to the solution, respectively, and incubated for 1 h at room temperature. Substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 20 min. The levels of cytokines were assessed by a microelisa reader at 450 nm and normalized with the abundance of standard solution. Each densitometric value expressed as mean ± SD was obtained from three independent experiments.

RESULTS

The bacteria species was confirmed by the sequence analysis – *P. gingivalis* ATCC (accession # AF414809) and *P. gingivalis* clinical isolate (accession # XT3944), *F. nucleatum* ATCC (accession #AE009951) and *F. nucleatum* clinical isolate (accession # DQ440559) and *E. coli* ATCC (accession # AM779083). *The* alignment for *P. ginginvalis*/ *F. nucleatum* with their respectively ATCC are shown in figure 1. The relative expression of mRNA for two different cytokines in macrophages (Raw 264.7) assessed following incubation with media containing 100ng/mL LPS from the five different bacterial strains - *P. gingivalis* ATCC and clinical isolate, *F. nucleatum* ATCC and clinical isolate and *E. coli* ATCC - confirmed the cell viability after 48h of stimulation. After treatment with *P. gingivalis*, *F.nucleatum* and *E. coli* LPS, culture media of Raw 264.7 were collected and analyzed by ELISA for IL-1 β and TNF-α. LPS from all bacteria strains tested stimulated the production of IL-1 β and TNF-α. Figure 2 and 3 shows the time course of the IL-1 β and TNF-α respectively released from macrophages (Raw 264.7) with 100 ng/mL of *P. gingivalis*, *F. nucleatum* and *E. coli* LPS. The production of the IL-1 β and TNF-α significantly increased with the time course of incubation as shown in figure 2 and 3. Regardless the time of incubation, the secretion of IL-1 β occurred in higher levels than in TNF-α (Table 1). LPS from *E. coli* was the most toxicity followed by the *F. nucleatum*. LPS from clinical isolate demonstrated to be more toxicity than LPS from ATCC strains for both *P. gingivalis* and *F. nucleatum* (Figure 3 and 4). The secreted levels of IL-1 β and

TNF-α in the culture media for all LPS strains tested at different times of incubation are shown in table 1.

DISCUSSION

Because LPS stimuli can evoke periapical inflammation, the knowledge of bacterial LPS antigenicity is important to a better understand of the immunobiology of apical periodontitis, which may eventually guide the root canal therapy for its remission. Therefore, the present study investigated the antigenicity of bacteria LPS from *P. gingivalis* and *Fusobacterium nucleatum* over Raw 264.7 macrophages by the levels of IL-1 β and TNF-α.

Many investigators have demonstrated the cytotoxicity of LPS in cell culture (1-3, 39-40); however, there is no investigation in the antigenicity of *P. gingivalis* and *F. nucleatum* (ATCC and clinical isolation) over macrophages Raw 264.7 by the levels of IL-1 β and TNF-α production.

It worth to point out that the antigenicity of LPS varies considerably on the cell line stimulated, and the choice for macrophage stimulation relied upon its predominance in the inflammatory tissue present in periradicular (27).

The selection for the extraction method greatly affects the biological activity of LPS (41). In the present study, the LPS was prepared by the Tris-Reagent procedure as previously described (38). There are different methods for the LPS extraction (38, 42-43) being the triton-Mg^{2+} the one that obtain LPS with the highest activity in terms of cytokine induction (41).

Fusobacterium spp., gram-negative, non-motile, non-spore-forming, obligate anaerobic rods belong to the phylum of Fusobacteria. The frequent isolation of *Fusobacterium* sp. from endodontic infections suggests a pathogenic role for this species (35, 44-45). Gomes *et al.* (2004) suggested that *Fusobacterium* species are more frequently detected in primary than in secondary/persistent infection. Moreover, Siqueira *et al.* (2002)

and reported that these species were present in higher amounts in symptomatic cases. Jacinto *et al.* (2008) reported prevalence in 34% of the samples from symptomatic teeth.

The DNA sequence analyzes for both *F. nucleatum* ATCC and *F. nucleatum* clinical isolation strains revealed a percentage of identification = 99%, confirming the identification disclosed by the phenotype test and PCR assay. However, the DNA alignment of both strains gave out significant difference in between their sequence as shown in figure 1. Foremost, the difference between these two *Fusobacterium* strains was not only restricted to the DNA analyzes but also displayed different antigenic activity over macrophages cells. In fact, the clinical isolate strain showed a higher production of both IL-1 β and TNF-α than in the ATCC strain indicated in table 1. It is not unreasonable to assume that the bacterial DNA might be affected by the interrelationship among bacterial species in a polymicrobial infection (46) such as variation in the bacteria environment (47) might be outlined.

The bacterial environment, particularly the hemin concentrations (47), significantly regulate the cytotoxic activity of the LPS structure, varying the numbers and types of lipid A species found in a single bacterial population. In order to address this point the concentration of hemin was standardize for the *in vitro* growth of all bacteria strains. Nevertheless it is not possible at the site of the infection, once the hemin concentration (in the hemoglobin form) varies considerably depending on the inflammatory response and blood vessels integrity. The increase in the hemin concentrations facilitate the production of lipid A structures that are TLR-4 antagonist (48). The TLR-4 represents a form of immunomodulation (48) that may facilitate survival of the bacterium in different host environments.

Porphyromonas gingivalis (black anaerobic rod) one of the most studied oral pathogens has been demonstrated to produce a large number of potential virulence factors (49). The prevalence of *P. gingivalis* has ranged from 5% to 29% in culture studies and from 9% to 72% in molecular studies.

The DNA sequence analyzes for both for *P. gingivalis* ATCC and *F. nucleatum* clinical isolation revealed a percentage of identification = 99%, confirming the

identification disclosed by the phenotype test and PCR assay. The DNA aligned sequence revealed no significantly changes in the ATCC strain compared with the wild type collected from root canal infection as shown in figure 1. Relative higher levels of IL-1 were found in the supernatants from stimulated cells with the wild type strains compared to the ATCC indicated in table 1.

In the present study the LPS from *E. coli* demonstrated the highest toxicity against macrophages Raw 264.7 cell lines when compared to *P. gingivalis* and *F. nucleatum* strains. Compared to enterobacterial LPS, *P. gingivalis* LPS has a variable potency in stimulating biologic activity depending on the cell type stimulated. Studies demonstrated that for some cell types, such as endothelial cells, the biologic activity of *P. gingivalis* was low compared to that of LPS isolated from enterobacteria. The low endotoxic activity of *P. gingivalis* LPS has been suggested to be due to the unique chemical structure of its lipid A (50). This heterogeneity includes differences in the number of phosphate groups and the amount and position of lipid A fatty acids. Clearly, the presence of multiple lipid A structures has complicated interpretation of innate responses (51). Furthermore, LPS obtained from *P. gingivalis* has the ability to signal through Toll-like receptor 2 (TLR2) or TLR-4 and elicits a different pattern of inflammatory mediators when compared to *E. coli* (43).

Regardless the type of bacterial LPS stimulation, the higher production of IL-1 β was followed by the TNF-α release. Corroborating, Stashenko *et al.* (1991) reported a correlation between IL-1 β and TNF-α levels, suggesting the expression of these two mediators in coordinated. IL-1 β and TNF-α are critical determinants of the progression of periodontitis (52). These two primary cytokines can induce the expression of adhesion molecules and secondary mediators that facilitate and amplify the inflammatory response, matrix metalloproteinase production, and bone resorption (52).

It can be hypothesized combinations of structurally different LPSs could activate various intracellular pathways by interacting with different receptors, such as Toll-like receptors, and lead to the synergistic production of inflammatory mediators. Considering the high diversity of bacterial species found in primary endodontic infection, LPS with

different toxicity structure (lipid A) can be involved in the root canal, enhancing or even inhibiting each other's antigenicity activity over periradicular tissues.

Overall, our data suggest that LPS of the *P. gingivalis* and *F. nucleatum* isolated from root canal infection is involved in the induction of IL-1 β and TNF-α, which are pleiotropic inflammatory mediators, that can play a role in the initiation of the upregulation of the inflammatory response and can also stimulate the production of secondary mediators involved in tissue destruction.

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Table 1. Mean values of TNF–alpha and IL1-beta [pg/mL] production from stimulated macrophages with different bacterial LPS strains [100ng/mL] for each incubation time (12, 24 and 48 hours).

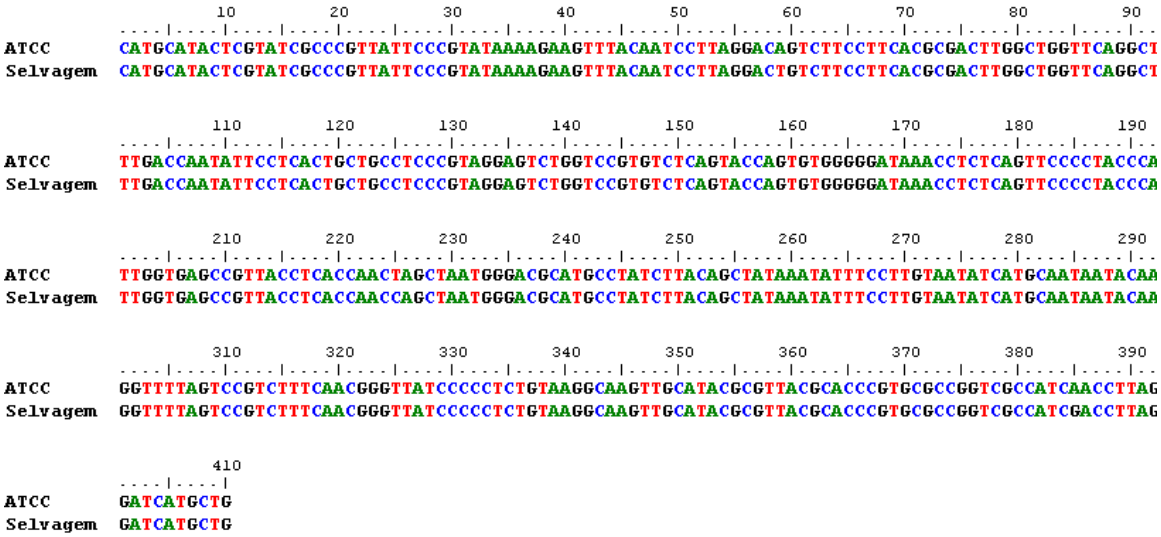
Bacterial strain	Cytokine	12 hours	24 hours	48 hours
	IL-1 beta	69,638	80,530	113,310
E. coli ATCC	TNF-alpha	807	1,269	2,382
	IL-1 beta	33,778	44,496	56,022
P. gingivalis Clinical	TNF-alpha	678	951	1,468
	IL-1 beta	26,972	30,340	51,196
P. gingivalis ATCC	TNF-alpha	665	948	1,409
	IL-1 beta	51,729	53,162	59,670
F. nucleatum Clinical	TNF-alpha	412	704	1,454
	IL-1 beta	38,277	40,215	57,128
F. nucleatum ATCC	TNF-alpha	444	1,016	1,495

Figure 1. Matched DNA sequence from *F. nucleatum* **(a)** and *P. gingivalis* **(b)** clinical isolation with their respectively ATCC strains. (Non-matched sequence is indicated by different colors in the alignment).

a. *Fusobaterium nucleatum*

* Altered-regions: 59, 225, 385 (sequence converage)

b. *Porphyromonas gingivalis*



* Multiple regions altered (sequence coverage)

Figure 2. The effect of incubation time (12, 24 and 48 hours) with LPS from *P. gingivalis* and *F. nucleatum* [100ng/mL] on IL-1β production by macrophage cells (Raw 264.7).

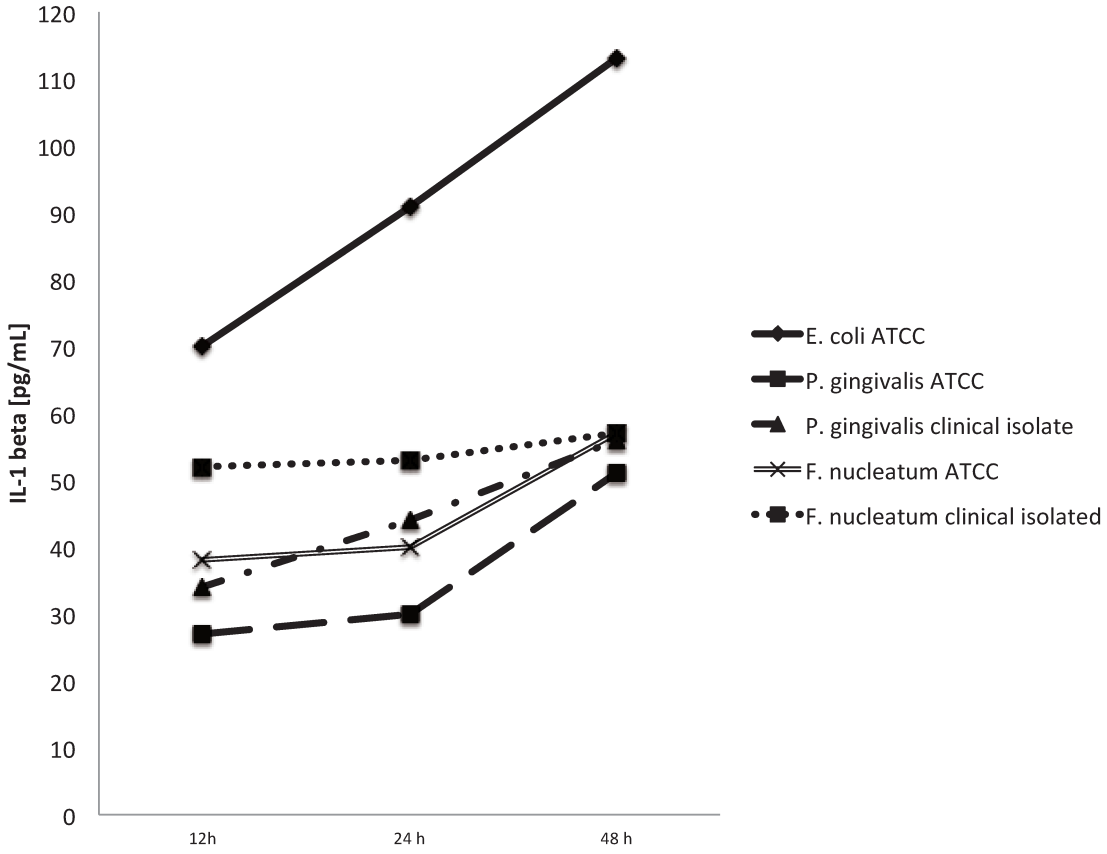
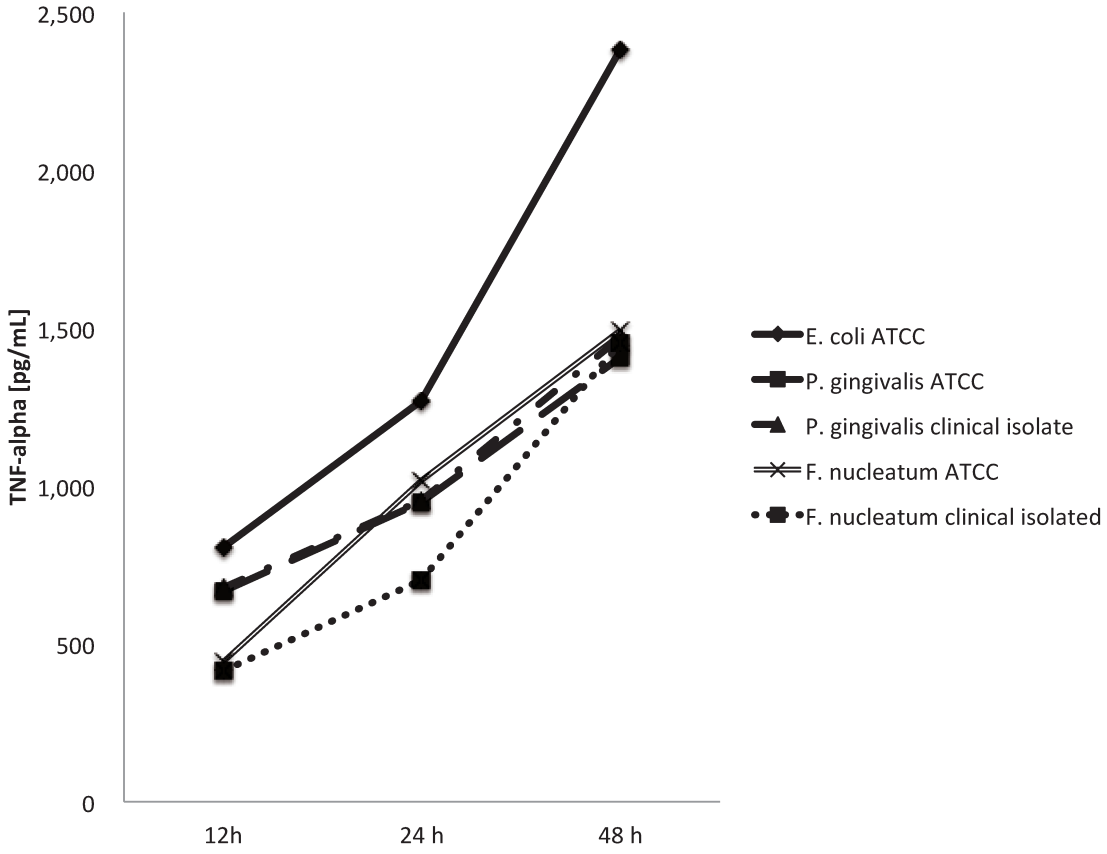


Figure 3. The effect of incubation time (12, 24 and 48 hours) with LPS from *P. gingivalis* and *F. nucleatum* [100ng/mL] on TNF-α production by macrophage cells (Raw 264.7).



2.6. Capítulo 6 – Comparison of 2.5% NaOCl and 2% CHX gel on Oral Bacterial LPS Reduction From Primarily Infected Root Canals

ABSTRACT

Objective This clinical study was conducted to compare the efficacy of chemomechanical preparation with 2.5% NaOCl and 2% CHX-gel on eliminating oral bacterial LPS in teeth with pulp necrosis and apical periodontitis. **Material and methods** Fifty-four root canals were selected. Samples were collect before (s1) and after chemomechanical preparation (s2). Teeth were randomly divided into groups - GI) 2.5% NaOCl (n=27) and GII) CHX-gel (n=27). Limulus Amebocyte Lysate (LAL) assay was used to quantify endotoxins. **Results** Endotoxin was present in 100% of the samples investigated with a median value of 272 EU/mL (GI) and of 152.46 EU/mL (GII). As a result of chemomechanical preparation, LPS content were reduced to a median value of 86 EU/mL (GI) and 85 EU/mL (GII). Higher percentage value of endotoxin reduction was found in GI ($p<0.05$). **Conclusion** 2.5% NaOCl and 2% CHX-gel were not effective on eliminating endotoxin from the primarily infected root canals.

Key-words: bacteria; endotoxin; oral bacterial LPS; root canal; sodium hypochlorite; chlorhexidine.

INTRODUCTION

Endotoxin liberated by Gram-negative bacteria has been detected in primary root canal infections (1-5). High levels of endotoxin have been associated with the development of spontaneous pain (1, 3, 6, 7) and clinical symptoms such as tenderness to percussion (3, 5) and pain on palpation (5).

Because the high toxicity of endotoxin *in vivo* (2, 8, 9), developing periapical inflammation and alveolar bone resorption, and *in vitro* (10-12), stimulating cells to release pro-inflammatory cytokines that lead to tissue destruction (13, 14), its removal/neutralization from infected root canals during endodontic treatment seems to be important for the healing process of periapical tissues.

Therefore in order to achieve an optimally disinfection of the root canal system, endodontic treatment should not rely only upon eliminating microorganisms and substrates (15) but also on inactivating or eliminating LPS.

Several chemical substances have been tested for the inactivation of endotoxin (16-22), particularly sodium hypochlorite (NaOCl) (5, 18-22), the most widely used endodontic irrigant, and chlorhexidine (CHX) (4, 18-20, 22), a potential auxiliary chemical substance applied in endodontic treatment.

Most *in vitro* (23-26) and *in vivo* (27-30) studies evaluating antimicrobial action of NaOCl and CHX assessed their ability on reducing or eliminating bacterial load from infected root canals. However, no clinical study had compared their potential on eliminating LPS from root canal infection.

Thereby, the present clinical study was conducted to compare the efficacy of chemomechanical preparation with either 2.5% NaOCl or 2% CHX-gel on eliminating oral bacterial LPS in teeth with pulp necrosis and apical periodontitis.

MATERIAL AND METHODS

Fifty-four patients who attended the Piracicaba Dental School, Piracicaba, SP, Brazil, and needing endodontic treatment were included in this research. The age of the patients ranged from 18-62 years. Samples were collected from 54 root canals with pulp necrosis and showing evidence of apical periodontitits in radiograph, before and after chemomechanical preparation. The selected teeth (one tooth per patient) were uniradicular, containing a single root canal, their pulp chamber was without visual communication with the oral fluid, they presented with necrotic pulp tissue, and showed radiographic evidence of periapical periodontitis, but an absence of periodontal disease.

None of the patients reported spontaneous pain. A detailed dental history was obtained from each patient. Patient who had received antibiotic treatment during the last three months or who had a general disease were excluded from this research. The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved a protocol describing the specimen collection for this investigation, and all patients signed an informed consent document to participate in this research.

All materials, including the paper points used in this study, were heat sterilized at 200°C for 4 hours becoming apyrogenic.

The method followed for the disinfection of the operative field had been described previously (3-5). Briefly, the teeth were isolated with a rubber dam. The crown and the surrounding structures were disinfected with 30% H_2O_2 [volume/volume (V/V)] for 30 s followed by 2.5% NaOCl for an additional 30s. Subsequently, 5% sodium thiosulphate was used to inactivate the irrigant. The sterility of the of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it on blood agar plates, which were incubated aerobically and anaerobically.

A two-stage access cavity preparation was made without the use of water spray, but under manual irrigation with sterile/ apyrogenic saline solution and by using sterile/ apyrogenic high-speed diamond bur. A first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected following the protocol described above. The sterility of the internal surface of the access cavity was checked as previously described and all procedures were performed aseptically. A new sterile and apyrogenic bur was used, accomplished by irrigation with sterile apyrogenic water, to access the canal. A first endotoxin-sampling were taken (s1), introducing a sterile pyrogen-free paper point (size 35; Dentsply-Maillefer, Ballaigues, Switzerland) into the full length of the canal, determined radiographically, and retained in position during 60 seconds for sampling. Immediately after it was placed in a pyrogen-free glass and frozen at -20° C for the Chromogenic Limulus Amebocyte Lysate (LAL) assay.

After accessing the pulp chamber and subsequent endotoxin sampling (s1), teeth were randomly divided into two groups as follows: GI - 2.5% NaOCl (n=27) and GII 2% CHX-gel (n=27). The CHX-gel (Endogel, Itapetininga, SP, Brazil) consisted of a gel base (1% natrosol) and CHX gluconate at pH 7.0. NaOCl was prepared by Proderma (Farmácia de Manipulação Ltda., Piracicaba, SP, Brazil). The manufacturer diluted NaOCl in sterile water without preservatives. The substances were prepared 24 hours before the beginning of the experiment, always in small portions. The pulp chamber was thoroughly cleaned with substances from each group.

After the first sample procedure, a K-file size 10 or 15 (Dentsply Maillefer, Ballaigues, Switzerland) was placed to the full length of the root canal calculated from the pre-operative radiographs. The coronal two-thirds of each canal was initially prepared using rotary files (GT® rotary files size 20, 0.06 taper - Dentsply Maillefer) at 350 rpm, 4 mm shorter than the estimated length. Gates-Glidden burs sizes 5, 4, 3 and 2 (DYNA-FFDM, Bourges, France) were used in a crown-down technique reaching 6 mm shorter than the working length (1 mm from the radiographic apex). Afterwards, the working length was checked with a radiograph after inserting a file in the canal to the estimated working length, confirmed by the apex locator (Novapex, Forum Technologies, Rishon le-Zion, Israel). The

apical preparation was performed using K-files ranging from size 35-45, followed by a step back instrumentation, which ended after the use of three files larger than the last file used for the apical preparation. The working time of the chemomechanical procedure was established at 20 minutes for all cases.

In NaOCl-group (GI), the use of each instrument was followed by an irrigation of the canal with 5 mL of 2.5% NaOCl solution. After the instrumentation, NaOCl was inactivated with 5 mL of sterile 0.5% sodium thiosulphate during 1-minute period, which was removed with 5 mL of sterile/apyrogenic water.

In the CHX-gel group (GII), root canals were irrigated with a syringe (27-gauge needle) containing 1 mL of the substance before the use of each instrument, being immediately rinsed afterwards with 4 mL of saline solution. After the instrumentation, CHX activity was inactivated with 5 mL of a solution containing 5% Tween 80 and 0.07% (w/v) lecithin during 1-minute period, which was removed with 5 mL of sterile/apyrogenic water.

Afterwards, a second endotoxin sample was taken (s2) as previously described.

To determine the amount of endotoxin present in the samples collected before (s1) and after chemomechanical preparation (s2), the LAL method, a modified Limulus Amebocyte Lysate and a synthetic color-producing substrate to detect endotoxin chromogenically (Quantitative Chromogenic LAL-1000, BioWhittaker, Inc, Walkersville, MD, USA), was used.

A standard curve from *Escherichia coli* was performed, according to the manufacturer's instructions, in order to quantify the endotoxin present in the clinical samples. Briefly, from the endotoxin supplied in the kit (E. coli 0111:B4) with a known concentration (23 EU/mL), four standard endotoxin solutions were prepared with the concentrations of 0.1, 0.25, and 1.0 EU/mL, which were used as positive controls. The absorbencies of endotoxin standard solutions were measured individually with an enzyme-linked immunosorbent assay plate-reader (Ultramark; Bio-Rad Laboratories, Inc, Hercules,

CA, USA) at 405 nm. The standard fulfilled the criteria of linearity ($r \geq 0.980$), as reported by the guide-line on validation of LAL tests.

Initially six samples were randomly selected and diluted in 1/10, 1/100, 1/1000, 1/10,000) to find the ideal concentration within the detection limits of the standard curve. Afterwards, the tests were performed in a 96-well microplate (Corning Costar Corporation, Cambridge, MA, USA) inside heating-block equipment at 37°C throughout the assay. All reactions were performed in duplicate with the mean values used for calculations.

In order to avoid inhibition or enhancement of LAL, the addition of a known concentration of *E. coli* endotoxins was added to it sample, as recommended by the manufacturer (spike procedure). For all tests, the spike recovery was 0.4 EU/mL. This activity was chosen because of its lower position on a logarithmic standard curve (its value was set zero).

For sampling test procedures, serial dilutions were set to 10^{-4}, determined by the standard curve. LAL reagent water (blank) was used as a negative control. All reactions were accomplished in duplicate to validate the test. A 96-well microplate was used in a heating block at 37°C and maintained at this temperature throughout the assay. Initially, 50 µl of the blank was added followed by the standard endotoxin solutions and the samples were consecutively added to the wells. This was followed by the addition of 50µl LAL to each well, and the microplate was then briefly shaken. Ten minutes later, 100 µl of substrate solution (pre-warmed to 37°C) was added to each well, always maintaining the same sequence. The plate was mixed and incubated at 37°C for 6 minutes. Afterwards, 100 µl of a stop reagent (acetic acid 25% v/v) was added to each well, and the absorbance (405 nm) was read by using an enzyme-linked immune-sorbent assay plate-reader.

The calculation of the endotoxin concentrations was performed using the mean absorbance value of the blank, which was subtracted from the mean absorbance value of the standards. This data were subtracted from the value of samples in order to calculate the mean Δ absorbance. Since this absorbance value is in direct proportion to the amount of endotoxins present, the endotoxin concentration can be calculated graphically from the

standard curve. Considering s1 values found as 100% of the endotoxin content and after determining the LPS concentration in s2, the percentage of endotoxin reduction was obtained subtracting s1 from s2.

STATISTICAL ANALYSIS

The results obtained with the Limulus Amebocyte Lysate assay (Kit QLC-1000) were statistically analyzed using SPSS for WINDOWS, version 12.0 (SPSS Inc, Chicago, IL, USA). The one-way ANOVA test was used to compare the percentage of LPS content reduction found in GI and GII.

RESULTS

Sterility samples taken from the external and internal surface of the crown and its surrounding structures tested before and after entering into the pulp chamber showed no microbial growth. Out of 54 root canals selected, three samples were lost during laboratorial procedures, resulting in a total of 51 root canals samples: 27 samples in the 2.5% NaOCl group (GI) and 24 samples in the 2% CHX-gel-group (GII).

In GI, none of the patients reported spontaneous pain, however 11/27 presented any of the following clinical symptoms: tenderness to percussion (n=3), pain on palpation (n=4) and tenderness to percussion/ pain on palpation (n=4). In GII, none of the teeth presented any type of clinical symptoms.

Results of the Limulus Amebocyte Lysate assay (LAL) indicated that endotoxins were initially (s1) present in 100% of the root canal samples, with a median value of 272 EU/mL (range 17-696 EU/mL) in the 2.5% NaOCl-group (GI, n=27) and of 152.46 EU/mL (range 71.92-214.57 EU/mL) in the 2% CHX-gel-group (GII, n=24).

At s2, regardless the auxiliary chemical substance used during chemomechanical preparation, endotoxins were still detected in all root canal samples. As a result of chemomechanical preparation, LPS content was reduced to a median value of 86 EU/mL (range 3 - 881 EU/mL) in the 2.5% NaOCl-group and 85 EU/mL (range 23 – 174 EU/mL) in the 2% CHX-gel group.

There was a wide range of endotoxin content detected in primary root canal infection, as well as a significant difference in the median value of endotoxin detected before (s1) and after chemomechanical preparation (s2) in GI (2.5% NaOCl) and GII (2% CHX-gel). Consequently, a statistical analysis was performed using Mann-Whitney test, taking into consideration the percentage of endotoxin reduction found in each sample.

The median percentage value of endotoxin reduction found in the 2.5% NaOCl-group (GI, n=27) was 57.98 and in the 2% CHX-gel-group (GII, n=24) was 47.126. One out of 24 samples investigated in GII (2% CHX-gel) showed no endotoxin reduction.

Statistical analysis revealed a significant higher percentage value of endotoxin reduction when 2.5% NaOCl was used as an auxiliary chemical substance (Mann-Whitney test; $p<0.05$).

The individual percentage values of endotoxin reduction found in GI (2.5% NaOCl, n=27) and GII (2% CHX-gel, n=24) are shown in Figures 1 and 2.

DISCUSSION

The Limulus Amebocyte Lysate (LAL) assay was chosen to quantify endotoxin levels before and after chemomechanical preparation of primarily infected root canals because of its extreme sensitivity for the detection of minute quantities of endotoxin (31).

At the baseline, in the 2.5% NaOCl-group higher endodotoxin levels were found when compared to 2% CHX-gel-group. The differences in the endotoxin concentrations may be related to the case selection. Some patients in GI group presented

clinical symptoms, such as tenderness to percussion and/or pain on palpation. It is known that symptomatic cases harbor predominantly Gram-negative anaerobic species (3).

Due to the high toxicity of endotoxin demonstrated by *in vivo* (2, 8, 9) and *in vitro* studies (10-12), special attempt has been given to obtain a substance that inactivates bacterial endotoxin, eliminating its biologically toxic potential. Over the years, several substances such as caustic soda (16), polymyxin B (17, 18), NaOCl (5, 18-22), CHX (4, 18-20, 22) and calcium hydroxide (18, 19, 22) have been tested for the inactivation of endotoxin. Recently, ozonated-water has exhibited the ability to directly suppress the biological effects of LPS (32) on a novel odontoblast-like cell line (KN-3) (33).

Particularly, sodium hypochlorite (NaOCl), the most widely used auxiliary chemical substance, had demonstrated low or no efficacy *in vitro* (19, 21) and *in vivo* (18, 22, 34) on inactivating *Escherichia coli* LPS, even at high concentrations (19, 21). In fact, the present study demonstrated that the chemomechanical preparation with 2.5% NaOCl was not able to eliminate oral bacterial LPS from infected root canals with pulp necrosis and periapical lesion.

Chlorhexidine (CHX) has emerged as a safe auxiliary chemical substance to be used during endodontic treatment due to its lower toxicity to periapical tissue when compared to NaOCl (35). Its inability to dissolve pulp tissues (a well-known advantage of NaOCl) (36) is one of its downside. However, CHX gel, a viscous formulation that makes instrumentation easier, thus increasing the mechanical removal of the organic tissues, compensates for its inability to dissolve them (23).

Concerning chlorhexidine detoxifying activity, Buck *et al.* (19) reported its little or no efficacy on inactivating the biologically active portion of the endotoxin - lipid A. Furthermore, previous *in vitro* studies (18, 22, 34) inoculating *E. coli* LPS in the root canals, showed the low effectiveness of CHX in reducing LPS after chemomechanical preparation. The low efficacy of 2% CHX-gel in the present study was assigned to the low median percentage value of endotoxin reduction found after chemomechanical preparation.

Our results indicated a higher percentage value of endotoxin reduction when 2.5% NaOCl was used as an auxiliary chemical substance. However, neither 2.5% NaOCl nor 2% CHX-gel totally eliminated LPS in any of the teeth evaluated, suggesting their low efficacy against oral bacterial LPS in clinical practice.

This clinical study supports the fact that 2.5% NaOCl and 2% CHX have no detoxifying effect on endotoxins. Therefore, it might be argued that the removal of more than 47% of the LPS content from the infected root canals is related to the mechanical action of the instruments in dentin walls accomplished by the flow and back-flow of the irrigants.

Overall, the removal of debris during chemomechanical preparation and the amount of root canal enlargement seem to play an important role on reducing oral bacterial LPS during endodontic treatment. Therefore, the features of the bacteria and endotoxin distribution in the dentinal tissues are important for the establishment of an effective instrumentation protocol.

According to Berkiten *et al* (37), Gram-negative bacteria depth penetration is in a maximum of ~275 μm whilst to 800 μm depth of LPS (38), representing approximately 4 times greater than the bacterial invasion, due to its lower molecule weight. In theory, root canal instrumentation establishing 3 size files enlargement apically might leave behind more than 50% of endotoxin-infected dentin. Such measurement is consistent with the percentage of endotoxin left after chemomechanical preparation in the present study (>47%). An enlargement of more 500 μm would be required to attempt an optimal removal of endotoxin. However, clinically, this procedure might not be compatible with the tooth anatomy in most of the cases.

One clinical implication of the present study is that the auxiliary chemical substances used in endodontic practice (NaOCl and CHX) are not effective on eliminating endotoxin from infected root canals. Moreover, the use of active irrigation (ultrasound) and the placement of an intracanal medication with endotoxin-detoxifying activity might be good strategies against LPS, particularly in those teeth with clinical symptoms (tenderness

to percussion and or pain on palpation), as well as in the presence of exudates, where higher contents of endotoxin can be found.

Further clinical studies involving ultrasound and root canal medication should be performed in order to investigate their efficacy in eliminating LPS from infected root canals.

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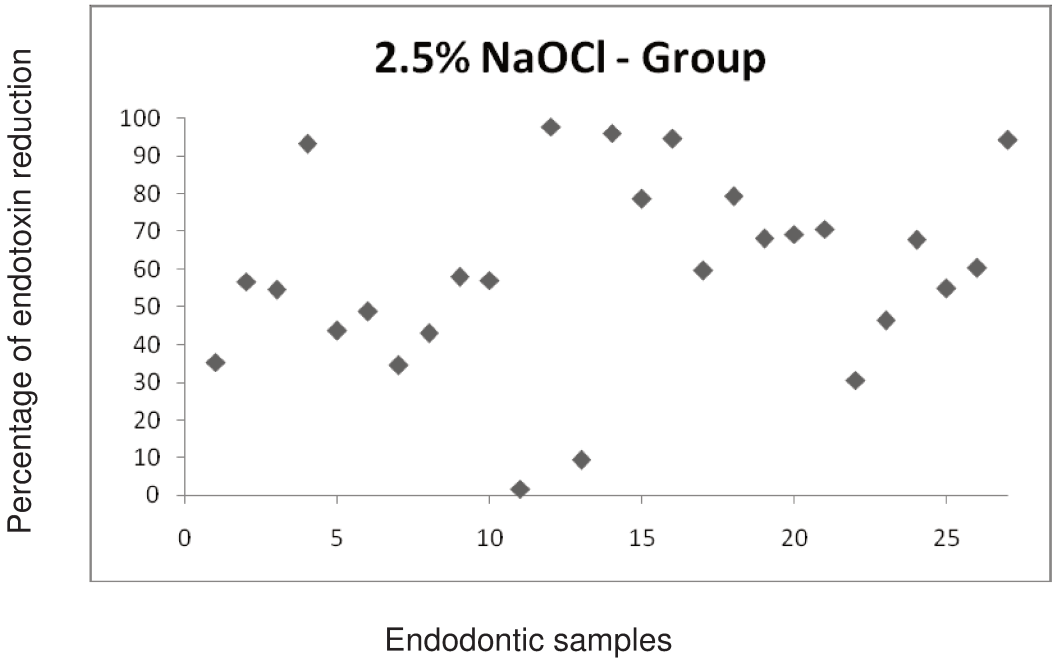

Figure 1. Percentage of LPS reduction for each sample (n=27) when 2.5% NaOCl was used as chemical auxiliary substance to prepare the root canals.

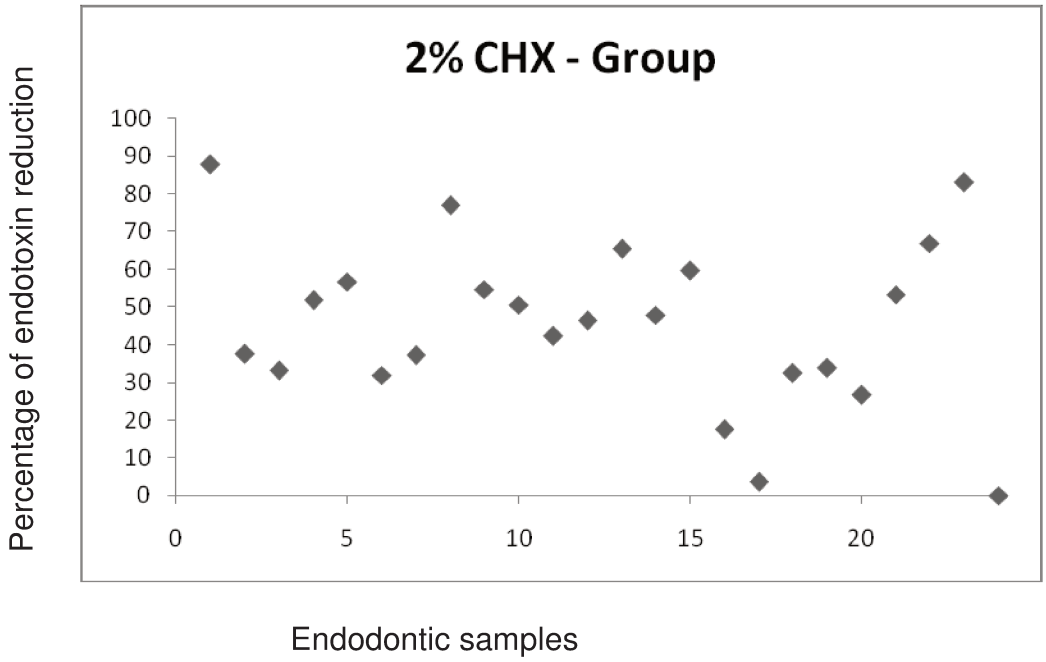

Figure 2. Percentage of LPS reduction for each sample (n=24) when 2% CHX gel was used as chemical auxiliary substance to prepare the root canals.

2.7. Capítulo 7 – Clinical Investigation of the Efficacy of Chemo-Mechanical Preparation With Rotary NiTi Files for Removal of Endotoxin From Primarily Infected Root Canals.

ABSTRACT

This clinical study was conducted to investigate the ability of chemo-mechanical preparation with 2.5% NaOCl + 17% EDTA and rotary NiTi system in removing endotoxin from primary root canal infection with apical periodontitis. **Methods:** Twenty-one root canals with necrotic pulps were selected. Samples were collected before (s1) and after chemo-mechanical preparation (s2). The Limulus amebocyte lysate (LAL) assay was used to quantify endotoxins. **Results**: The LAL assay indicated that endotoxins were present in 100% of the root canals investigated (19/19) before (s1) and after chemo-mechanical preparation (s2). Analyses of the quantitative data revealed that the endotoxin content was significantly reduced at s2 (98.06%) compared to that at s1 ($p < 0.05$). **Conclusion:** Our findings indicate that chemo-mechanical preparation with 2.5% NaOCl + 17% EDTA and rotary NiTi files was effective in reducing endotoxin load in the root canal infection from primarily infected teeth with apical periodontitis.

Key-word: Root canal; Endotoxin; NaOCl; EDTA; Rotary instrumentation; NiTi; Mtwo® system

INTRODUCTION

Endotoxin investigations on primary endodontic infection (1-4) showed strong correlation between oral bacteria LPS and the presence of apical periodontitis. Due to the highly toxic activity of endotoxin to periapical tissues (5), such as its capacity to invade dentin tubules (6) and egress into periradicular tissues (7), special attempt has been made to eliminate/neutralize it within infected root canals during endodontic therapy.

The most commonly used auxiliary chemical substances in endodontic therapy, sodium hypochlorite (NaOCl) and chlorhexidine (CHX-gel), showed no detoxifying effect against endotoxin (8). Therefore, a proper elimination of endotoxin seems to rely on debridement of the root canal system.

Apical patency (9, 10), serial enlargement of the body of root canal (10, 12), and establishment of master apical file size (MAF) (12-14) are important measures to reach an adequate debridement of the root canal system.

Historically, MAF determination using "three sizes up from the first file to bind" rule (15) has been used in modified forms (16). Using this rule combined with the step back technique, our previous investigation (1) showed its inefficiency in eliminating endotoxin from infected root canals. Even increasing the root canal enlargement, more than 50% of endotoxin-infected dentin was left behind, suggesting the need of an extensive enlargement (2).

A greater apical enlargement is beneficial in performing a further debridement of the apical third (14) – known as a "critical zone" (17). However, due to tooth anatomy, this may be better achieved with NiTi rotary instruments instead of the conventional step-back technique using stainless steel files (14, 18).

The superior shaping ability of NiTi rotary files (19), with greater flexibility (20), reduces the incidence of procedural errors (21-24) and consequently yields a higher success rate in root canal treatment compared to conventional technique (23).

The Mtwo® NiTi rotary instruments have a cross-sectional design resembling a 2-blade S-shaped file (22) with its angle almost vertical (21, 24). Its standardized tapers, ranging between 0.04 and 0.07 mm, and great flexibility (20) produce less canal transportation (24) and few changes in canal shape (21), allowing a safe enlargement and removing deeper layers of infected dentin as a result.

Currently, there is no clinical study evaluating the efficiency of rotary instruments for elimination of endotoxin from root canals with primary infection.

Therefore, the aim of this clinical study was to investigate the ability of chemo-mechanical preparation with 2.5% NaOCl + 17% EDTA and rotary NiTi system in removing endotoxin from primary root canal infection with apical periodontitis.

Patient selection

Twenty-one patients who attended the Piracicaba Dental School, Brazil, for endodontic treatment were included in this research. The age of the patients ranged from 13 to 73 years old. Samples were collected from 21 root canals with pulp necrosis, all showing radiographic evidence of apical periodontitis. Fifteen single root teeth and 6 multi-rooted teeth were investigated. Their pulp chamber exhibited no visual communication with the oral fluid. The selected teeth showed absence of periodontal pockets deeper than 4 mm. The following clinical/ radiographic features were found: pain on palpation (9/21), tenderness to percussion (8/21), exudation (12/21), and radiolucent area $\geq$ 2 mm (11/21) and $<$ 2 mm (10/21). None of the patients reported spontaneous pain.

A detailed dental history was obtained from each patient. Patient who had received antibiotic treatment during the last three months or who had a general disease were excluded from this research. The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved a protocol describing the sample collection for this investigation, and all patients signed an informed consent for participation in this research.

Sampling procedures

The method used for disinfecting the operative field had been described previously by the authors (1, 2). Briefly, the teeth were isolated with a rubber dam. The crown and surrounding structures were disinfected with 30% H_2O_2 [volume/volume (V/V)] for 30s followed by 2.5% NaOCl for the same period of time and then inactivated with 5% sodium thiosulphate. The sterility of the samples taken from both external and internal surfaces of the crown and its surrounding structures was checked by taking a swab sample from the crown surface and streaking it on blood agar plates, which were incubated aerobically and anaerobically. NaOCl was provided by Proderma (Farmácia de Manipulação Ltda, Piracicaba, SP, Brazil), being diluted in sterile water without preservatives. The solution was prepared 24 hours before the beginning of the experiment, always in small portions. All materials used in this study were heat sterilized at 200°C for 4 hours, thus becoming apyrogenic.

The root canal sampling method had been previously described by the authors (1, 2). A two-stage access cavity preparation was made without the use of water spray, but under manual irrigation with sterile saline solution and by using sterile/ apyrogenic high-speed diamond bur. The first stage was performed to promote removal of major contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the protocol described above. The sterility of the internal surface of the access cavity was checked as previously described and all procedures were performed aseptically. A new sterile and apyrogenic bur was used during irrigation with sterile apyrogenic saline to access the canal. In each case, even in multi-rooted tooth, a single root canal was sampled in order to confine the endotoxin evaluation to a single ecological environment. The criterion used to choose the canal to be investigated in multi-rooted teeth was the presence of exudation or, in its absence, the largest canal or canal related with apical periodontitis. The first endotoxin sample (s1) was taken by introducing a sterile pyrogen-free paper point (Dentsply-Maillefer, Ballaigues, Switzerland) into the full length of the canal (determined radiographically) and retaining its position during 60 seconds. In those cases where a dry canal was identified, an additional sterile paper point moistened in

sterile/apyrogenic saline was used to ensure the sample acquisition. Immediately after, the sample was placed in a pyrogen-free glass and frozen at -80° C for further Limulus Amebocyte Lysate assay (LAL).

Clinical procedures

After accessing the pulp chamber and subsequent endotoxin sampling (s1), the pulp chamber was thoroughly cleaned with 2.5% NaOCl. A K-file of size 10 or 15 (Dentsply Maillefer, Ballaigues, Switzerland) was placed into the full length of the root canal, calculated from pre-operative radiograph and confirmed by apex locator (Novapex, Forum Technologies, Rishon le-Zion, Israel). Patency of the apical foramina was standardized by inserting a 15 K-file (Dentsply Maillefer, Ballaigues, Switzerland). The root canals were then prepared for Working Length (WL) with Mtwo® instruments (Mtwo®, VDW, Munich, Germany) according to the manufacturer's instructions. The Mtwo® files were set into permanent rotation at a maximum speed of 300 R.P.M. (25). All 5 Mtwo® instruments (10/.04, 15/.05, 20/.06, 25/.06, 30/.05) were used for full length of the canal (WL) in a single-length technique, with gentle in-and-out movement while gradually forcing apically (25). The use of each instrument was followed by irrigation of the canal with 5 mL of 2.5% NaOCl solution, simultaneously removed by suction. All irrigation procedures were performed with a 27-gauge needle. After instrumentation, NaOCl was inactivated with 5 mL of sterile 5% sodium thiosulphate during 1-minute period, which was removed with 5 mL of sterile/apyrogenic saline. Before the second sampling (s2), a continuous rinse with 5 mL of 17% EDTA (ethylenediaminetetra-acetic acid) solution for 3 min followed by a final rinse with 5 mL of sterile/apyrogenic saline solution were performed. Next, a second endotoxin sample was taken (s2) as previously described.

In order to monitor the efficacy of rotary instrumentation in removing endotoxin from infected root canals, 18 primarily infected teeth (10 single root teeth and 8 multi-rooted teeth) with apical periodontitis were prepared with Mtwo® instruments (10/.04, 15/.05, 20/.06, 25/.06, 30/.05) inserted into full length and irrigated with sterile

saline solution (irrigant control). The use of each instrument was followed by irrigation of the canal with 5 mL of sterile/apyrogenic saline, simultaneously removed by suction. All irrigation procedures were performed with a 27-gauge needle. Samples were collected before (s1) and after root canal instrumentation (s2) as described above.

Determination of endotoxin concentration

The turbidimetric test (pyrogent 5000® -BioWhitaker, Inc., Walkersville, MD, USA) was used to measure endotoxin concentrations in the root canals by means of the Limulus Amebocyte Lysate (LAL) technique. It is a kinetic, quantitative assay for detection of endotoxin. Activated LAL converts the coagulogen into coagulagin to form turbidity in the sample. A sample is mixed with the reconstituted LAL reagent, placed in photometer, and automatically monitored over time until appearance of turbidity. The time required before the appearance of turbidity (Reaction Time) is inversely proportional to the amount of endotoxin. That is, in the presence of a large amount of endotoxin the reaction occurs rapidly; in the presence of a smaller amount of endotoxin, the reaction time is increased. The concentration of endotoxin within unknown samples can be calculated from a standard curve. Its wide range detection sensitivity (0.01 – 100 EU/mL) requires small amounts of dilutions of clinical samples, thus optimizing the laboratory time.

Standard Curve

As a parameter for calculation of the amount of endotoxins existing in root canal samples, a standard curve was plotted using the endotoxin of known concentration supplied by the kit (100 EU/mL), with its dilutions reaching the following final concentrations (i.e. 0.01, 0.1, 1, 10 EU/mL) according to the manufacturer's instructions.

Spiking procedure

To avoid inhibition or enhancement of the LAL, a known concentration of *Escherichia coli* endotoxin was added to the clinical samples, as recommended by the manufacturer's instructions (spike procedure). The unspiked samples together with the spiked ones (control) had their endotoxin concentration automatically calculated. The endotoxins recovered were equal to the known concentration of the spike within 50% to 200%.

Test Procedure

Initially, no serial dilution of the clinical samples was considered. Thereafter, if the tested samples were found to be inhibitory to the turbidimetric LAL reaction, serial dilutions of the root canal samples would be made. All reactions were performed in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA, USA) was used in a heating block at 37°C and maintained at this temperature throughout the assay. Firstly, the endotoxin-samplings were suspended in 1 mL of LAL water supplied by the kit and agitated in vortex for 60 seconds. Immediately, 100 μL of the blank, followed by the same volume of the standard endotoxin solutions (i.e. 0.01, 0.1, 1, 10 EU/mL) and 100 μL of the samples, were added in duplicate to the 96-well microplate with their respectively positive controls. The test procedure was performed according to manufacturer's instructions. The absorbance of endotoxins was individually measured using an enzyme-linked immuno-sorbent assay plate-reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Calculation of endotoxin concentration

The microplate reader / WinKQCL® Software (BioWittaker, Cambrex Co., Walkersville, MD, USA) monitors the absorbance at 340 nm of each well of the microplate

continuously throughout the assay. Using the initial absorbance reading of each well as its own blank, the reader determines the time required for absorbance at 0.03 absorbance units. This time is termed ReactionTime. The WinKQCL® Software automatically performs a log/log linear correlation of the Reaction Time of each standard with its corresponding endotoxin concentration,, and the standard curve parameters are printed. If the absolute value of the correlation coefficient (r) is 0.980, a polynomial model can be used to construct a standard curve and in turn predict endotoxin concentrations of test samples. This polynomial curve-fitting model (POWERCURVE) requires the use of the WinKQCL® Software.

STATISTICAL ANALYSIS

The results obtained with the LAL test were statistically analyzed using the SPSS package for WINDOWS, version 12.0 (SPSS Inc, Chicago, IL, USA). The Friedman test was performed to compare the amount of endotoxin before (s1) and after chemo-mechanical preparation (s2). When significant differences were found between the different sampling times, the Wilcoxon test was used subsequently. Significance levels were always set at 5% ($p<0.05$).

RESULTS

Sterility samples taken from the external and internal surfaces of the crown and its surrounding structures tested before and after entering into the pulp chamber showed no microbial growth. Out of 21 root canals selected for instrumentation with 2.5% NaOCl + 17% EDTA, 2 samples were lost during laboratory procedures, resulting in a total of 19 root canal samples analyzed.

The standard curve fulfilled the criteria of linearity (r=0.990). The LAL assay (Pyrogent®-5000) indicated that endotoxins were present in 100% of the root canals investigated (19/19) before (s1) and after chemo-mechanical preparation (s2), with a

median value of 9.19 EU/mL (range, 0.0597 – 289 EU/mL). As a result of chemo-mechanical preparation, LPS content was reduced to a median value of 0.228 EU/mL (range, 0.01 – 4.21 EU/mL). There was a significant difference in the median value of endotoxin detected before (s1) and after chemo-mechanical preparation (s2) ($p<0.05$). Analyses of the quantitative data revealed that the endotoxin content after chemo-mechanical preparation with 2.5% NaOCl + EDTA 17% and rotary NI-TI files (s2) was significantly reduced (98.06%) compared to that at s1 ($p<0.05$).

Root canals irrigated with sterile/apyrogenic saline (irrigant control) revealed a median value of endotoxin of 2.80 EU/mL (range, 1.92 - 124 EU/mL). After root canal instrumentation (s2), the endotoxin level was dropped to a median value of 0.338 EU/mL (range, 0.075 – 0.658 EU/mL), corresponding to 96.27% of endotoxin reduction ($p<0.05$).

DISCUSSION

Endotoxin was recovered in 100% of the root canal samples from primarily infected root canal, which is in agreement with previous investigations (1-4, 7). The primary median levels of endotoxin found in this study endorse the important role both Gram-negative bacteria and their by-products play in the development of the endodontic infection (1-4).

Previous studies (1-2) demonstrated that chemo-mechanical preparation with conventional instrumentation using 2.5% NaOCl was able to reduce LPS content by only ≈ 59%. However, until now no clinical study had evaluated the efficiency of rotary NiTi instruments in the elimination of endotoxin from primary endodontic infected root canals with apical periodontitis. Considering the widespread use of rotary instrumentation in clinical practice, the present work aimed to investigate its ability in the removal of endotoxins, demonstrating a reduction of 98.06% using rotary instrumentation associated with 2.5% NaOCl + 17% EDTA.

It is reasonable to assume that due to the similar irrigant protocol (2.5% NaOCl) and the lack of detoxifying effect of this auxiliary chemical substance against endotoxin (8), difference in percentage values of endotoxin reduction can be attributed to the mechanical performance of the instruments (manual K-files vs Mtwo® rotary NiTi-files). This was confirmed by a reduction of 96.27% in the endotoxin level when the root canals were irrigated with inert solution.

The best performance of the rotary instrumentation might be attributed to a better-centered root canal instrumentation (22), with uniformly round space preparation and disinfection, compared to hand instrumentation. Additionally, it requires a continuous activation of the irrigant, which might contribute to a better debridement.

The direct physical contact between NiTi file and canal walls throughout rotary instrumentation might generate frictional heat, enhancing chemical reactivity of the NaOCl and consequently its disinfecting potential. A moderate increase of the temperature of any solution inside the root canal is considered, in principle, desirable (28). In particular, the positive angle of the Mtwo® files seems to cut dentin more effectively (21, 22) and the flute/cross-sectional design of the instrument allows a better removal of infected debris during shaping (29).

Unlike other NiTi systems, the Mtwo® reverts to the standardized technique, as all instruments are taken to the full WL from the beginning (22, 24). The manufacturer calls this procedure the "single length technique". Concerns exist about it (21, 22, 24), however Schafer *et al* (22, 24) found that it maintained the original canal curvature better than other systems. Additionally, Sonntag *et al* (21) reported that taking these files to full working length had no negative effect on fracture behavior. The establishment of the WL into the full extension of the canal rather than 1 mm from the apical foramen (1-3) might have ensured a better debridement, comparing the present findings in endotoxin reduction to previous investigations (1, 2).

The sequence of Mtwo®-files used in the present study, according to Foschi *et al.* (25), had an outcome similar to that using a Master Apical File (MAF) size of #30. It has been reported that using #30 (26) or greater (11, 27) apical file for canal enlargement is

important for improving the access of the auxiliary chemical substances to the apical third of the canal (12, 26). Regarding endotoxin reduction, our previous studies revealed no significant improvement compared to a # 30 file (1), with tip ranging from 35-45 (2) at 1 mm short of the WL using manual K-files.

The apical third of the root canal may harbor a critical count of microorganisms, and their by-products are able to maintain a periradicular host response (7). This region is known as a "critical zone" for management of the infection (17). Even though it can be theorized that the step-back-technique (three sizes up from the MAF) used in conventional instrumentation (K-file, taper .02) can compensate the rotary standardized instrumentation using taper of 0.05 along the 4-mm of apical root, difference in the percentage value of endotoxin reduction is not reflected in the clinical practice when comparing the actual 98.06% reduction to the 58% found in our previous study (2).

In clinical practice, adding 17% EDTA to NaOCl is routinely used for an effective removal of the smear layer (30). The smear layer produced during the root canal preparation can promote adhesion and colonization of Gram-negative bacteria to dentin matrix (31). Thereby, the use of EDTA is essential in the disinfection protocol. Even though previous investigations (1-3) had not provided data after this clinical step, the following evidence (32-34) elucidates the possible contribution of 17% EDTA in the removal/neutralization of endotoxins. EDTA acts on the deeper layers (≈ 130μm) of the infected dentin not enclosed by the chemo-mechanical preparation (32). Also, it is a strong chelating agent (33) that might react with the Ca^{++} present in the Lipid A molecule (the bioactive center of endotoxin) (34), thus affecting its structure.

Theoretically, the residual LPS found in the present study (1.94%), if allowed access to the periradicular tissues after obturation, would only temporarily modulate host defenses. Moreover, in the absence of living Gram-negative cells to maintain LPS levels, these effects would be only transient and not expected to influence treatment outcome. Therefore, regarding the endotoxin reduction, one can speculate whether the root canal is dry and without periapical symptomatology before obturation in a single session.

Further clinical investigation should be performed to verify the antigenic activity of the post-instrumentation residual amount of endotoxin found in root canals in order to determine its inflammatory potential.

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2.8. Capítulo 8 – Comparison of different clinical sequences of NiTi rotary files in the removal of endotoxin from infected root canals.

ABSTRACT

Introduction: The aim of this study was to compare the ability of different clinical sequences of Mtwo® rotary files for removal of endotoxin from infected root canals. **Methods:** Seventy mandibular teeth were used. *Escherichia coli* endotoxin (055: B55) was inoculated into the root canals. After the incubation period, teeth were randomly divided into groups according to instrumentation sequences for different apical preparation sizes (APS) as follows: GI (# 25/.06) (10/10), GII (# 30/.05) (10/10); GIII (# 35/.04) (10/10); GIV (40/.04) (10/10), and GV (25/.07) (10/10). Ten teeth served as sterilization control group and other 10 teeth as contamination control group. Samples were collected from the root canals by using sterile/apyrogenic paper points after the use of each instrument in the sequence tested. Limulus amebocyte lysate (LAL) was used to measure endotoxin. **Results:** A gradual improvement in the endotoxin removal was achieved by increasing the APS: APS # 25.06 (324.28 EU/mL) (GI), APS #30/.05 (337.92 EU/mL) (GII), APS #35/.04 (363.21 EU/mL) (GIII), APS #40/.04 (374.00 EU/mL) (GIV), and APS#25.07 (379.87 EU/mL). Statistically significant differences were found comparing GI to GIII, GVI, and GV ($p < 0.05$). Substantial reduction of LPS contents was achieved by using the Mtwo® sequences finished in either APS #40.04 or #25.07 ($p<0.05$). **Conclusion:** Substantial reduction of endotoxin contents was achieved by using the Mtwo® sequences finished in APS #40.04 or #25.07.

INTRODUCTION

Clinical investigations of endodontic infection have elucidated the strong correlation between oral bacterial lipopolysaccharide (LPS, known as endotoxin), present in the outer layers of Gram-negative bacterial cell walls (1), and the presence of apical periodontitis (2-7).

Because of the high endotoxic activity of bacterial LPS, which stimulates cells to release pro-inflammatory cytokines involved in apical tissue destruction (4,6-8) and its correlation with the development of clinical symptomatology (2-3,9), special attention has been given for removal/neutralization of this endotoxin from infected root canals during endodontic therapy (5, 10).

The gradual enlargement of the root canal body and the establishment of apical preparation size (APS) are important measures to reach an adequate debridement of the root canal system (11-12). Wider instrumentation is recommended to remove more infected dentin and to allow deeper irrigation and better placement of intracanal dressing (13-16). However, there still remains some controversy over how wide to shape a given canal to achieve antimicrobial efficiency without causing overly anatomic limitations (11, 15, 17-21).

A wider apical enlargement and root canal disinfection seem to be better achieved with nickel-titanium (NiTi) rotary instruments instead of the conventional instrumentation using stainless steel files (10, 12).

Among the various NiTi rotary systems developed, the Mtwo® files (VDW, Munich, Germany) stands out as a new generation of rotary instruments (22). The Mtwo® system comprises 8 instruments with constant tapers raging between 0.04 - 0.07, including 7 tip sizes (10-40). Due to the number of files available in this system, different clinical sequences finishing in different APS are proposed for root canal instrumentation, namely, #25/.06 (23), #30/.05 (10, 24, 25, 26), #35/04 (22, 27), #40/.04 (28-29), and #25/.07 (30-31).

Previous investigations have evaluated the performance of Mtwo® rotary files for root canal instrumentation in terms of cleaning and shaping ability (22, 27-28), transportation of debris into the apex (25), bacterial removal (26), preservation of the original geometry of the root canal (30), and fracture risk (31). However, no previous study had compared different clinical sequences of Mtwo® rotary files finishing in different apical preparation sizes for removal of endotoxin from infected root canals.

Therefore, the aim of this study was to compare the ability of different clinical sequences of Mtwo® rotary files for removal of endotoxin from infected root canals.

MATERIAL AND METHODS

Specimen selection and preparation

Seventy extracted human teeth (single rooted and straight single-canal mandibular pre-molars) were used in the study. The teeth were collected from a general dental practice and had been extracted for pulpal or periodontal reasons. All teeth were stored in 0.9% saline solution. The study protocol was approved by the local research ethics committee.

Specimen selection was made on the basis of relative dimensions and similarity in root morphology, determined both visually and radiografically. All teeth had mature root apex with no evidence of external resorption, none had received prior root canal treatment.

Debris, calculus, and soft tissue remnants on the root surfaces were cleaned by using periodontal curettes (Millennium, São Paulo, SP, Brazil). Crowns were transversely sectioned at the level of cementum-enamel junction by using a water-cooled diamond disk (KG-Sorensen, Barueri, SP, Brazil). The root length was standardized to 15 mm and the canals were negotiated to patency with size 8 and 10 K-files (Dentsply- Maillefer, Ballaigues, Switzerland). Root canals were instrumented at the apical foramen in alternated rotation motions, under copious irrigation with 5 mL of saline solution after each file use. Smear layer was removed with 5.25% NaOCl for 10 minutes under constant agitation

(Agitador-Aquecedor Fanem, São Paulo, SP, Brazil), followed by 17% EDTA. Teeth were then washed thoroughly with distilled water for 1 hour (32), and then the canals were dried by using paper points (Dentsply-Maillefer, Ballaigues, Switzerland). The apical region was sealed with light-cured resin composites (3M Dental Products, St Paul, MN, USA) to prevent any extrusion or leakage of endotoxin. Next, the outer surfaces of the specimens were covered with 2 layers of epoxy resin (Araldite; Brascola, São Paulo, SP, Brazil), except the cervical opening (26).

Specimen sterilization - All specimens were sterilized with autoclave (Fanem, São Paulo, SP, Brazil) at 121°C for 20 minutes in distilled water and then seeded into 24-well cell culture plates (Corning Costar, Cambridge, MA). To make both handling and instrumentation easier, teeth were vertically fixed to the cervical region with chemically activated acrylic resin (Artigos Odontológicos Clássico, São Paulo, Brazil) inside the plate well.

Endotoxin Decontamination - Plates, instruments and all materials used in the experiment were sterilized by gamut radiation with cobalt 60 (20 KGy for 6 hours) for degradation of preexisting endotoxins (33).

Specimen contaminated with endotoxin - 30 mL of a solution containing *Escherichia coli* 055: B55 endotoxin (1.000.000 EU/mL-1) was inoculated into the root canals by using a micropipette (Axygen Scientific Inc, California, USA). The plates were sealed and incubated for 24 hours at 37°C in a humidified atmosphere.

Contamination control group - 10 teeth were inoculated with 30 mL of the endotoxin solution and incubated for 24hours at 37°C in a humidified atmosphere. Next, root canals were sampled and the presence of endotoxin was then validated by Limulus amebocyte lysate assay (LAL)

Sterilization control group - 10 teeth were inoculated with 30 mL of LAL water and incubated for 24hours at 37°C in a humidified atmosphere. Next, sterilization of the specimens was validated by LAL-assay.

Experimental groups - Fifty root canals were randomly divided into experimental groups according to different Mtwo® rotary sequences finishing in different apical preparation sizes as follows: GI (10/.04; 15/.05; 20/.06; 25/.06) (n=10) (basic series recommended by the manufacturer´s instructions), GII (10/.04, 15/.05, 20/.06, 25/.06, 30/.05) (n=10); GIII (10/.04, 15/.05, 20/.06, 25/.06, 30/.05, 35/.04) (n=10); GIV (10/.04, 15/.05, 20/.06, 25/.06, 30/.05, 35/.04, 40/.04) (n=10), and GV (10/.04, 15/.05, 20/.06, 25/.06, 30/.05, 35/.04, 40/.04, 25/.07) (n=10).

Rotary instruments were set into permanent rotation with a 6:1 reduction handpiece (Sirona, VDW, Munich, Germany) powered by a torque-limited electric motor (VDW-Silver, VDW, Munich, Germany). Individual torque limit and rotational speed for each file were set in the file library of the VDW motor. The Mtwo® files were used in permanent rotation at a maximum speed of 300 rpm (24). Root canal instrumentation was carried out according to the manufacturer's instructions towards a single-length technique. Specimens had their root canals fully instrumented by using a gentle in-and-out motion. Once the instrument had achieved the end of the canal and rotated freely, it was removed (24, 26).

In order to avoid possible cutting interference of the Mtwo® files after their first use in the root canal instrumentation, each rotary file was used at a single time.

Sampling procedure - To evaluate the cutting effectiveness of an individual file within the sequence tested, the root canal was sampled every time after the use of each instrument by introducing a sterile/apyrogenic paper point into the fully extension of the canal. The paper point remained in position for 60 seconds and then placed in a pyrogen-free glass and frozen at 80° C for further quantification of endotoxin. Before the use of each instrument, the root canal was soaked with 10 µL of LAL water by using pyrogen-free pipettes tips

(Axygen Scientific Inc., California, CA, USA), which was renewed after every single sampling.

Determination of Endotoxin Concentration - The turbidimetric test (Pyrogent 5000®; BioWhitaker, Inc, Walkersville, MD) was used to measure endotoxin concentrations in the root canals by means of the LAL technique, previously published by the authors (6,7,10). It is a kinetic, quantitative assay for detection of endotoxin in which the activated LAL converts the coagulogen into coagulin to form turbidity in the sample. A sample was mixed with the reconstituted LAL reagent, placed in photometer, and automatically monitored over time until appearance of turbidity. The time required before the appearance of turbidity (reaction time) is inversely proportional to the amount of endotoxin. In the presence of a large amount of endotoxin, the reaction occurs rapidly; in the presence of a small amount of endotoxin, the reaction time is increased. The concentration of endotoxin within unknown samples can be calculated from a standard curve. Its wide-range sensitivity [0.01–100 endotoxin units (EU/mL)] requires small amounts of dilutions of clinical samples, thus optimizing the laboratory time.

Standard Curve - As a parameter for calculation of the amount of endotoxins existing in root canal samples, a standard curve was plotted by using the endotoxin of known concentration supplied by the kit (100 EU/mL), with its dilutions reaching the final concentrations (0.01, 0.1, 1, 10 EU/mL) according to the manufacturer's instructions.

Spiking Procedure - To avoid inhibition or enhancement of LAL, a known concentration of *Escherichia coli* endotoxin was added to the clinical samples, as recommended by the manufacturer's instructions (spike procedure). Both unspiked and spiked samples (control) had their endotoxin concentrations automatically calculated. The endotoxins recovered were equal to the known spike concentration ranging from 50% to 200%.

Test Procedure - Initially, no serial dilution of the clinical samples was considered. Therefore, if the tested samples were found to inhibit the turbidimetric LAL reaction, serial dilutions of the root canal samples would be made. All reactions were performed in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA,

USA) was used in a heating block at 37°C and maintained at this temperature throughout the assay. First, the endotoxin samples were suspended in 1 mL of LAL water supplied by the kit and agitated in vortex for 60 seconds. Immediately after, 100 mL of the blank, followed by the same volume of the standard endotoxin solutions (0.01, 0.1, 1, 10 EU/mL) and 100 mL of the samples, were added in duplicate to the 96-well microplate with their respectively positive controls. The test procedure was performed according to the manufacturer's instructions. The absorbance of endotoxins was individually measured by using an enzyme-linked immunosorbent assay plate reader (Ultramark; Bio-Rad Laboratories, Inc, Hercules, CA).

Calculation of Endotoxin Concentration - The microplate reader, using the WinKQCL Software (BioWittaker, Cambrex Co, Walkersville, MD, USA), monitors the absorbance of each well of the microplate continuously at 340 nm throughout the assay. By using the initial absorbance reading of each well as its own blank, the reader determines the time required for absorbance at 0.03 absorbance units. This time is termed reaction time. The WinKQCL Software automatically performs a log/log linear correlation of the reaction time of each standard curve with its corresponding endotoxin concentration, including printing the standard curve parameters. If the absolute value of the correlation coeficiente (r) is 0.980, a polynomial model can be used to construct a standard curve and in turn predict endotoxin concentrations of the test samples. This polynomial curve-fitting model (POWERCURVE) requires the use of the WinKQCL Software.

STATISTICAL ANALYSIS

The data obtained with LAL assay were statistically analyzed by using the SPSS for Windows, version 12.0 (SPSS Inc, Chicago, IL, USA). The Shapiro-Wilk test was used to verify normal data distribution. Both mean and standard deviation regarding the endotoxin removed in each sequence tested were determined. One-way repeated measures analysis of variance was applied. When significant differences were found between the different clinical sequences, Tukey's test was used subsequently. Significance levels were always set at 5% ($p<0.05$).

RESULTS

The Shapiro-Wilk test showed data normality ($p>0.266$). Sterility of the specimens was confirmed with sterilization control group showing absence of endotoxin in all root canals (10/10). The endotoxin contamination protocol was validated by the contamination control group in which endotoxin was detected in 100% of the specimen (10/10). For validation of LAL assay, the standard curve fulfilled the criteria of linearity for all running assays ($r > 0.099$) as reported by the guidelines. The mean values of endotoxin removal, such as standard deviation, for all the sequences tested are shown in Table 1. A gradual improvement in the endotoxin removal was achieved by increasing the apical preparation sizes, as follows: APS # 25.06 (324.28 EU/mL) (GI), APS #30/.05 (337.92 EU/mL) (GII), APS #35/.04 (363.21 EU/mL) (GIII), APS #40/.04 (374.00 EU/mL) (GIV), and APS#25.07 (379.87) (Figure 1). No statistically significant difference was found between the mean values of endotoxin removal in GI (APS #25/.06) – 324.28 EU/mL (10/10) compared to GII (APS #30/.05) – 337.92 EU/mL (10/10) ($p>0.05$) (Table 1). In contrast, statistically significant difference was found in the mean values of endotoxin removal found in GI (APS # 25.06, 324.28 EU/mL) compared to GIII (APS #35/.04, 363.21 EU/mL) (10/10), GIV (APS #40/.04, 374.00 EU/mL), and GV (#APS 25.07, 379.87) ($p< 0.05$), as shown in Table 1. Substantial reduction of LPS contents was achieved by using the Mtwo® sequences finished in either APS #40.04 or #25.07 ($p<0.05$) (Figure 1).

DISCUSSION

The LA assay has been widely used in Endodontics to investigate/quantify endotoxin in root canal infection and to evaluate the effect of root canal procedures on its removal (5,6,7,10). Among the LAL methods, the turbidimetric method (Pyrogent-5000®) used in the present study proved to be most effective in recovering endotoxin from endodontic samples (7).

The cutting efficiency of rotary NiTi instruments is a complex interrelationship of different parameters, including cross-sectional design and helical and rake angle, such as metallurgical properties (22). Particularly, the Mtwo® files have an S-shaped cross-section with two cutting blades, positive rake angle, no radial lands and a progressive blade chamber (pitch) in the apical-coronal direction (34).

In this study, the wider instrumentation (finishing in APS #25.07) yielded the best disinfection of the root canal systems agreeing with previous investigations (13, 15-16).

Of clinical importance, the most commonly Mtwo® sequence used by endodontic practitioners – the basic series kit comprised by the first four files (10/.04, 15/.05, 20/.06, 25/.06) – presented the lowest value of endotoxin removal (mean value: 324.28 EU/mL) when compared to all other sequences tested. It might be argued that the wider taper of 0.06 did not compensate the largest APS #25 achieved by the sequence, resulting in a removal of endotoxin contents by only 32.5%. This, in fact, corroborates the studies reporting that a wider tapper cannot compensate for a smaller apical preparation size from a disinfection perspective (11, 18).

It is worth to point out that the increase in APS, rather than the taper size, seemed to be mandatory for a gradually improvement in the removal of endotoxin – APS #30/.05 (337.92 EU/mL), APS#35/.04 (363.21 EU/mL), and #40/.04 (mean value: 374.00 EU/mL).

Although an improvement in the endotoxin removal was achieved when the basic series was accomplished by the file #30/.05 (mean removal: 337.92 EU/mL, 59.85%), it was not significant ($p>0.05$). Noteworthy, the choice for adding the file #30.05 to the Mtwo® basic series, as reported in the literature (24-26), does not seem to be a good strategy to significantly improve the disinfection of the root canal system. Additionally, instrumentation wider than ISO #30 is recommended to remove more infected dentin as well as to allow deeper irrigation (11, 19, 21, 35).

When instrumentation was finished in APS larger than #30/.05 (35/.04, 40/.04 or 25/.07), significantly higher levels of endotoxin removal were achieved in comparison to the basic series (ending APS #25/.06). In particular, no statistically differences were found

by comparing the sequences concluded for apical size #35/.04 (mean removal: 363.21 EU/mL) (22, 27) to #40/.04 - (mean removal: 374.00 EU/mL) (28-29). In contrast, root canal instrumentation using file size up to #25/.07 (30, 31) yielded the best value of endotoxin removal (379.81 EU/mL).

From an irrigation perspective, controversy exists over how large enough the APS should be for a better penetration of the endodontic irrigant into the apical third of the canal (11, 19, 21, 35). Some authors (19, 35) have recommended instrumentation to #35, whereas others (11, 21) even larger as #40. Usman *et al.* (2004) reported that root canals prepared to size #20, even with taper of 0.06, showed significantly more apical debris than those prepared to size #40.

Our findings certainly encourage the use of wider APS in order to achieve an optimal removal of endotoxin from the root canal system, which possibly contributes to enclose canal cross-sections, accessory canals, and apical deltas, mostly inaccessible to smaller apical preparation. Nevertheless, the clinician has to carefully decide on how wide to shape a given canal to achieve antimicrobial efficiency without causing overly anatomic limitations (17, 19-21). Therefore, one should take into account the root canal wall thickness as well as the presence of severe curvatures that increase the risk of transportation (21).

The choice among these clinical sequences for root canal preparation should also take into account the possible clinical effects of larger instrumentation, such as compromised restorability, fracture susceptibility, apical laceration or ledging and canal path alterations (36).

The enlargement of single rooted and straight single-canal mandibular pre-molars to an APS #40 (wider APS achieved by sequences finishing in the either #40/.04 or #25/.07 file) is in agreement with the existing literature (15-16).

Overall, regarding the optimal clinical sequences for removal of endotoxin, substantial reduction of LPS contents was achieved by using the sequences finished in APS

#40.04 or #25.07. However, it should be point out that none of these sequences were able to eliminate endotoxin from infected root canals.

Further clinical studies should be performed in order to evaluate other teeth, because it is likely that certain teeth have a complex root canal anatomy, which makes them poor candidates for this type of treatment.

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Table1 – Mean and standard deviation (± SD) values of the amount of endotoxin removed by different clinical sequence of Mtwo® rotary files tested.

Sequence of instrumentation	Endotoxin removal (EU/mL)
	Mean value ± SD
GI (APS #25/.06)	324,38 (± 136.42) **C**
GII (APS #30/.05)	337,92 (± 131.09) **BC**
GIII(APS #35/.04)	363,21 (± 139.07) **AB**
GIV (APS #40/.04)	374,00 (± 141.19) **A**
GV(APS #25/.07)	379,87 (± 139.33) **A**

Mean values followed by different letters indicate statistical significant difference among the sequences tested ($p<0.05$).

3. CONSIDERAÇÕES GERAIS

3.1 Justificativa da pesquisa

O conhecimento da microbiota envolvida numa infecção polimicrobiana, particularmente de origem endodôntica, é importante para o melhor entendimento da ativação antigênica causada ao sistema imune, a qual desencadeará um processo inflamatório, responsável pela destruição dos tecidos periapicais, manifestações clínicas de sintomatologia e possivelmente em um processo de reabsorção óssea perapical.

A literatura endodôntica revela que a infecção endodôntica primária é causada predominantemente por microrganismos Gram-negativos anaérobios estritos e achados indicam que bacilos Gram-negativos estão envolvidos com sintomatologia clínica, e exercem papel significativo na patogênese das lesões inflamatórias periapicais (Griffee *et al.*, 1980; Yoshida *et al.*, 1987; Gomes *et al.*, 1994a, 1996; Baumgartner *et al.*, 1999; Siqueira *et al.*, 2001, Jacinto *et al.,* 2003; Siqueira *et al.*, 2004; Sakamoto *et al.,* 2006; 2009; Martinho *et al.*, 2010a; Montagner *et al.*, 2010).

Diante do predomínio de bactérias Gram-negativas, e considerando lipopolissacarídeo como seu principal constituinte inflamatório, deu-se início na Endodontia a era da quantificação de endotoxinas em canais radiculares (Schein & Schilder, 1975; Horiba *et al.*, 1991; Khabbaz *et al.*, 2001; Jacinto *et al.*, 2005; Vianna *et al.*, 2007; Oliveira *et al.*, 2007; Martinho & Gomes, 2008; Gomes *et al.*, 2009; Valera *et al.*, 2010; Martinho *et al.*, 2010 a,b; 2011; Endo, 2011).

Ao longo dos últimos 100 anos, endotoxina bacteriana é considerada uma das moléculas mais potentes e de maior interesse encontrada na natureza (Petsch & Anspach, 2000). Suas peculiaridades estruturais, diversidade química e física e seu amplo espectro de atividade biológica resultaram em uma das linhas de pesquisa mais fomentadas mundialmente. Ainda que sua composição química e estrutural tenha sido amplamente

explorada (Raetz, 1990), perguntas surgem quanto ao real papel das endotoxinas nas infecções humanas (Petsch & Anspach, 2000).

Para melhor conhecimento da atividade endotóxica das bactérias orais, estudos têm sido realizados com enfoque na linha de extração do LPS e estimulação celular (Horiba *et al.*, 1989; Hosoya *et al.*, 1997; Hong *et al.*, 2004). Entretanto, devido a limitação em reproduzir *in vitro* o micro-ambiente inflamatório, particularmente a determinação da concentração de hemina – crucial para a toxicidade do LPS a ser expresso - a qual varia dependendo do *status* inflamatório (sob a forma de hemoglobina); e principalmente, desconsiderando a complexidade da infecção polimicrobiana, limitadas conclusões surgem para o entendimento da antigenicidade envolvida nas infecções.

Contudo, para melhor representar a complexidade antigênica presente nas infecções endodônticas e melhor entendimento da resposta celular, particularmente sobre os macrófagos (primeiras células de defesa do hospedeiro a desencadear o processo inflamatório), o presente estudo utilizou diretamente o conteúdo infeccioso coletado individualmente de 21 canais radiculares na etapa de estimulação celular, correlacionando os dados microbiológicos, níveis de endotoxinas e de citocinas pró-inflamatórias, com os achados clínicos diagnosticados. Avaliou também diferentes protocolos de instrumentação (manual e rotatório) utilizando substâncias químicas auxiliares com diferentes propriedades antimicrobianas (NaOCl 2.5% e CLX-gel 2%) na redução do conteúdo endotóxico dos canais radiculares.

3.2 Material e Métodos utilizados

A presente pesquisa foi submetida ao Comitê de Ética em Pesquisa da Faculdadede Odontologia de Piracicana-UNICAMP, cujo certificado de aprovação encontra-se no Anexo I.

Neste estudo, foram avaliados microbiologicamente amostras de infecções endodônticas primárias obtidas de 21 canais radiculares com lesão periapical.

A desinfecção do campo operatório foi realizada de acordo com Gomes *et al.* (2004). As coletas da superfície externa da coroa e da face interna da cavidade de acesso resultaram em cultura negativa, cofirmando sua desinfecção. A esterelidade da substância química auxiliar (NaOCl), Clorexidina gel 2% (CLX-gel) e do soro fisiológico estéril/ apirogênico usados no preparo químico-mecânico foi testada anteriormente para que não houvesse contaminação externa do canal radicular.

Todos os testes microbiológicos das substâncias químicas auxiliares e soro fisiológico resultaram em culturas negativas indicando ausência de possível contaminação microbiológica.

O hipoclorito de sódio 2.5% utilizado como substância química auxiliar no presente estudo foi manipulado pela Drogal Farmácia de Manipulação Ltda (Piracicaba, SP). A diluição do hipoclorito foi realizada com água estéril e sem conservantes. Devido à instabilidade do NaOCl, o mesmo foi manipulado em pequenas porporções, 24 horas antes da intervenção endodôntica.

A clorexidina gel (Endogel; Itapetininga, SP, Brasil) utilizada no presente estudo consistiu de base de gel natrosol 1% e gluconato de clorexidina 2% pH 7,0.

A técnica utilizada para realização das coletas microbiológicas foi descrita previamente em detalhes por Gomes *et al.* (1994a,b; 1996; 2004) e detalhada no Apêndice I.

O meio de transporte VMGAIII (Viability Medium Götemborg Agar) foi descrito inicialmente por Möller (1966) e por Dahlén *et al.* (1993) e é preparado para suportar microrganismos anaeróbios estritos e facultativos sem favorecer crescimento microbiano. É aplicado para amostras pequenas, como as coletadas por pontas de papel absorvente. Sua consistência é semi-sólida em temperatura ambiente e semi-fluida acima de 30°C (Apêndice II).

Dentre os diferentes patógenos endodônticos investigados, muitas das espécies Gram-negativas, tais como os bacilos produtores de pigmento negro (*Prevotella tannerae*, *Porphyromonas gingivalis, Porphyromonas endodontalis Prevotella intermedia*, *Prevotella nigrescens*), os fastidiosos (*Filifactor alocis*, *Tannerella forsythia*), *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* e os *Treponemas* spp. (*Treponema denticola*, *Treponema socranskii*) investigadas no presente estudo, são de difícil cultivo microbiano, porém frequentemente detectadas através dos diferentes métodos moleculares (Rôças *et al.*, 2003 Foschi *et al.*, 2005; Gomes *et al.*, 2005; Gomes *et al.*, 2006; Gomes *et al.*, 2007; Montagner *et al.*, 2010).

A seleção dos "primers" para a detecção bacterina foi realizada baseada na literatura. Apesar de utilizarmos, na maioria dos casos, oligonucleotídeos já descritos na literatura, os ciclos de termociclagem e as temperaturas foram totalmente adaptados à estrutura do laboratório de Microbiologia Endodôntica da FOP-UNICAMP (Apêndice I). Fato este, devido principalmente a pouca informação contida nos artigos científicos, pois pequenas diferenças de temperatura, quantidade de ciclos, proporções e quantidade de reagentes podem interferir nos resultados.

Limulus amebocyte lysate (LAL), método de quantificação de endotoxinas utilizado no presente estudo, é o método mais sensível e específico disponível para a detecção e quantificação de endotoxina provenientes das bactérias Gram-negativas (Novitsky, 1985). O princípio do método baseia-se na reação entre a endotoxina e um componente protéico do LAL.

Particularidades dos diferentes testes de quantificação de endotoxinas utilizados na presente pesquisa são discutidos e apresentados no Capítulo 1.

Devido à sensibilidade do LAL a outros materiais biológicos além da endotoxina, quando presentes em altas concentrações (Dahlén & Bergenholtz, 1980), tais como proteínas, ácidos nucléicos e peptideoglicanos de bactérias Gram-positivas e presença de exsudato do tipo inflamatório (Sullivan & Watson, 1974), foram realizadas diluições seriadas, incubação a 70°C (Friberger *et al.*, 1982) assim como a realização do procedimento de *spike* (adição de uma concentração conhecida de endotoxina de

Escherichia coli à amostras para verificar possíveis interferências na quantificação de endotoxinas).

Para não haver risco de contaminação, a coleta das amostras de endotoxinas antes do preparo químico-mecânico foi realizada previamente à coleta para detecção de microrganismos. Tal coleta foi feita com um cone de papel, previamente esterilizado a 200°C, por 4 horas em estufa, tornando-o apirogênico, como recomendado pelo fabricante.

Para a realização do preparo mecânico dos canais radiculares (Capítulo 7 e 8), foi escolhido o sistema rotatório Mtwo® por apresentar boa capacidade de corte, atribuída ao design da secção tranversal do instrumento, resultando em lâminas de corte agressivo e ângulo de corte positivo (Vahid *et al.*, 2009), além de preservar a geometria do canal radicular com baixo risco de desvios (Sonntag *et al.*, 2007). Também tem excelente capacidade de remoção de debris dentinários (Foschi *et al.*, 2004; Schafer *et al.*, 2006).

Devido ao número de limas apresentadas no sistema rotatório Mtwo®, diferentes sequências clínicas são apresentadas na literatura (Foschi *et al.*, 2004; Kim *et al.*, 2009; Uroz-Torres *et al.*, 2009; Bonaccorso *et al.*, 2009; Machado *et al.*, 2010). A sequência de limas utilizadas no presente estudo foi de acordo com Foschi *et al.* (2004), utilizando 4 instrumentos (10.04, 15.05, 20.06, 25.06, 30.05), resultando num diâmetro apical de #30. Esta ampliação anatômica do canal radicular até #30, parece promover ampliação suficente para chegada da substância química auxiliar no 1/3 apical (Dummer *et al.*, 1998; Usman *et al.*, 2004).

Dentre diferentes linhagens celulares envolvidas no processo inflamatório periapical, o presente estudo utilizou macrófagos murinos da linhagem RAW 264.7. Macrófagos estão presentes em maior população no tecido periapical, os quais são considerados a principal fonte de produção de citocinas inflamatórias (Metzger, 2000; Artese *et al.*, 1991; Matsuo *et al.*, 1992), e quase exclusivamente o produtor de TNF-α na presença de LPS bacteriano (Beutler & Cerami, 1986).

Citocinas inflamatórias foram dosadas através do método ELISA de sobrenadantes obtidos da estimulação de macrófagos (RAW 264.7) com conteúdo endodôntico coletados dos canais radiculares após 24 horas (Apêndice I).

A seleção dos pacientes incluindo critérios de inclusão/exclusão, a técnica passo a passo da coleta microbiológica e detalhamento dos materiais métodos, processamento laboratoria utilizados na presente pesquisa, encontram-se detalhado no Apêndice I.

3.3 Complexidade antigênica dos canais radiculares

Neste estudo observou-se que as infecções endodônticas primárias apresentam grande complexidade antigênica, a qual é justificada principalmente pelo seu conteúdo microbiano infeccioso, destacando a diversidade de espécies bacterianas Gram-positivas e Gram-negativas, número de espécies presente na infecção – principalmente de Gram-negativos (diversidade/quantidade de LPS com diferentes atividades endotóxicas), e suas inter-relações de diferentes espécies bacterianas - comensais ou antagônicas, potencializando ou reduzindo a toxicidade deste conteúdo infeccioso.

Dentre 12 espécies de bactérias Gram-negativas investigadas as mais frequentemente detectadas foram *Prevotella nigrescens*, *Porphyromonas endodontalis*, *Fusobacterium nucleatum* e *Treponema socranskii*.

A alta prevalência da espécie *Prevotella nigrescens* nas infecções endodônticas com lesão periapical parece estar relacionada com o potencial inflamatório de seu LPS (Chung *et al.*, 2006). Uma das diversas funções do LPS é a estimulação de reabsorção óssea *in vivo* (Umezu *et al.*, 1989; Chung *et al.*, 2006). LPS é um potente estimulador de osteoblastos na secreção das citocinas pró-inflamatórias – IL1, IL6, TNF e PGE_2 (Nair *et al.*, 1996). Particularmente, IL1 e TNF-α induzem indiretamente a formação de células osteoclásitcas através da ativação de células osteoblásticas (Bertolini *et al.*, 1986; Thomson

et al., 1987).

As inter-relações entre os microrganismos sejam elas comensais ou antagonistas durante a evolução da infecção pulpar estão baseadas principalmente na demanda de nutrientes (Sundqvist *et al.*, 1992; Gomes *et al.*, 1996), podendo influenciar na prevalência de espécies Gram-positivas e Gram-negativas.

No presente estudo, a ocorrência da espécie *Parvimonas micra* (Gram-positiva) foi de 100% com pelo menos 1 das 12 espécies Gram-negativas "alvo". Peptideoglicano, componente estrutural da parede celular de bactérias Gram-positivas, exerce papel sinérgico à atividade antigênica do LPS de bactérias Gram-negativas (Jiang *et al.*, 2003). Yoshioka *et al.* (2005) reportaram que a molécula LPS pode ser ligar particularmente à célula bacteriana de *P. micra*, potencializando a resposta inflamatória via macrófagos principalmente na produção TNF-α .

As espécies bacterianas frequentemente detectadas nos canais radiculares estudados têm a capacidade de ativar diferentes mecanismos imunopatológicos (Holt *et al.*, 1999; 2005). Dentre eles, através da interação do LPS bacteriano com macrófagos, mediada por uma parte da molécula do LPS denominada "Lipide A" – a qual exerce a maior parte das atividades endotóxicas, sendo chamada de princípio endotóxico do LPS.

A porção "Lipide A" varia estruturalmente, e em sua composição entre diferentes espécies bacterianas e entre diferentes cepas da mesma espécie (Reife *et al.*, 2006; Dixon & Darveau, 2005). Desta forma, a ocorrência de diferentes LPS com diferentes toxicidades nas infecções pode potencializar ou inibir a atividade antigênica entre eles sobre os tecidos periapicais (Magnuson *et al.*, 1989; Hong *et al.*, 2004; Dixon & Darveau, 2005).

Para determinar associações entre espécies, Sundqvist *et al.* (1992); Gomes *et al.* (1994a,b) e Peters *et al.* (2002) utilizaram teste de odds ratio verificando a presença de uma determinada espécie, na presença ou ausência de demais espécies bacterianas identificadas. O mesmo foi realizado no presente estudo, considerando associação positiva entre uma determinada espécie na presença de outra espécie, quando o odds ratio foi maior

do que 2; enquanto duas espécies foram consideradas negativamente associadas, quando o odds ratio em detectar uma espécie na presença de outra fosse menor 0,5.

Diferentes associações microbianas entre espécies "alvo" investigadas foram encontradas neste estudo, particularmente: *P. endodontalis*/ *T. denticola*; *P. micra*/ *F. alocis* e *F. nucleatum*/ *P. endodontalis*.

Porphyromonas endodontalis, segunda espécie mais frequente nos canais radiculares estudados, foi detectada quase exclusivamente com pelo menos outra espécie Gram-negativa. Associação positiva foi encontrada entre P. *endodotalis* e *F. nucleatum*. Apesar do LPS da espécie *P. endodontalis* apresentar baixo potencial inflamatório isoladamente, sua presença numa infecção polimicrobiana está associada a quadros infecciosos mais severos (van Winkelhoff *et al.*, 1992; Siqueira *et al.*, 1998; Hong *et al.*, 2004; Montagner *et al.*, 2010). Particularmente, a toxicidade do LPS da espécie *F. nucleatum* é potencializada na presença da *Porphyromonas endodontalis* (Hong *et al.*, 2004), enquanto, a toxicidade do LPS das espécies de *Porphyromona*s é limitada na presença de *Escherichia coli* (Dixon & Darveau, 2005).

3.4 Lipopolissacarídeo e seu potencial inflamatório

Lipopolisacarídeo/ organização arquitetônica

Lipopolissacarídeos, fator determinante de virulência nas espécies patogênicas, chamado de endotoxina, são moléculas presentes na superficie da membrana das bactérias Gram-negativas, as quais são secretadas em vesículas durante a fase de crescimento bacteriano ou liberadas durante a morte celular (Rietschel *et al.*, 1992).

As bactérias produzem LPS para se reproduzir, se proteger de agressões externas, para servir de arcabouço e até para diminuir a permeabilidade da membrana que pode ser a causa de maior resistência a determinados antibióticos (Rietschel & Brade, 1992).

LPS representa a principal superfície antigênica das bactérias Gram-negativas, apresentando significância microbiológica e imunológica. Apesar da existência de algumas exceções, muitas espécies possuem formas ou arquiteturas constituídas de componentes específicos: uma porção medial- lípide A, uma porção distal ou externa- Antígeno O, e um núcleo- o "core" (Rietschel *et al.*, 1992).

Antígeno O é a cadeia mais longa e a porção mais variável da molécula entre as espécies bacterianas, capaz de induzir uma resposta imunológica específica, com a produção de anticorpos específicos. O "core" tem constituição muito pouco variável, porém também pode induzir a formação de anticorpos específicos. Este é constituído de uma parte de açúcares e uma parte chamada KDO (2- keto-3-deoxioctano), que se liga ao lípide A, podendo inclusive, interferir na sua bioatividade (Rietschel *et al.*, 1992).

Lípide A é a porção efetivamente tóxica da molécula, causando respostas imunológicas específicas. É a porção menos variável do LPS entre as espécies bacterianas, constituída de duas moléculas de açúcares (glicosamina) modificada por fosfato (PO_4) e um número variável de cadeias de ácidos graxos, com 14 átomos de carbono (Rietschel *et al.*, 1992).

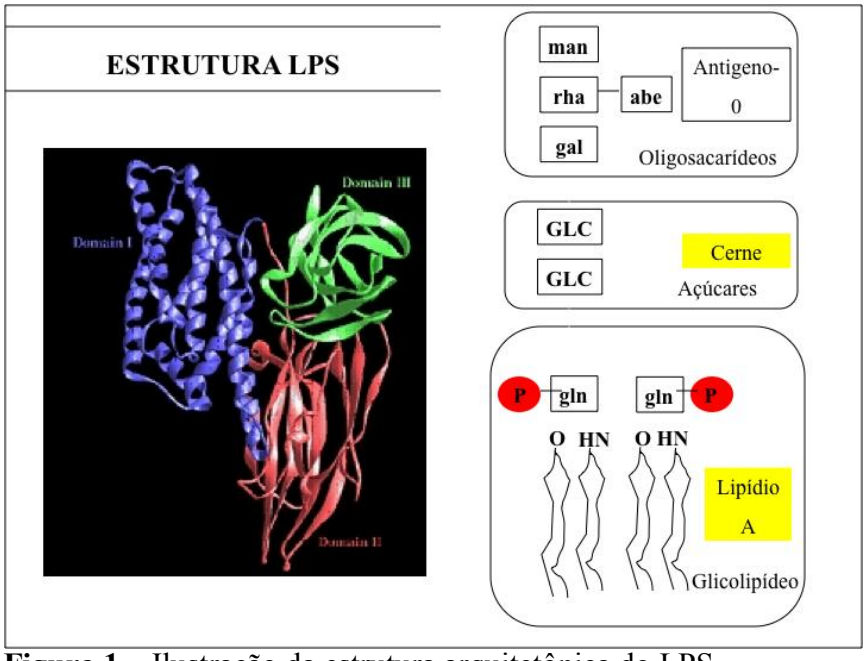

Figura 1 – Ilustração da estrutura arquitetônica do LPS.

Antigenicidade do LPS bacteriano contra macrófagos – ativação celular/ nuclear e liberação de citocinas inflamatórias

O efeito tóxico das endotoxinas é dependente da resposta do hospedeiro, podendo interagir com vários sistemas celulares e humorais, causando febre (ação no hipotálamo), neutrofilia (ação na medula óssea), proliferação de colágeno (estímulo de fibroblastos), liberação de aminoácidos nos músculos, produção de interleucina e a produção de anticorpos (Rietschel & Brade 1992).

O tecido inflamatório presente na lesão perirradicular é composto em sua maioria por macrófagos (Artese *et al.*, 1991; Matsuo *et al.*, 1992).

O LPS participa da resposta immune imediata a partir da fagocitose de microrganismos Gram-negativos e a liberação de endotoxinas no interior dos macrófagos decorrendo na produção de mediadores químicos, como fator de necrose tumoral, interleucinas -1, 6 e 8, lipídios e radicais livres, altamente reativos.

Para que as respostas imunológicas/ inflamatórias ocorram, as células do hospedeiro possuem múltiplos receptores, como por exemplo, CD14, TLRs (Toll Like Receptors) e proteínas ligantes de LPS (LBP), os quais reconhecem o LPS de forma específica e única para cada bactéria.

Existe receptor nos macrófagos (CD14), ao qual a endotoxina se liga através da porção do "lípide A", estimulando as funções dos receptores toll (TLR). O TLR4 faz com que o macrófago sintetise mediadores químicos, chamados citocinas (interleucinas -1, 6 e 8, TNF e fator de agregação plaquetária - PAF). As citocinas, por sua vez, se ligam aos receptores de citocinas presentes nas células – linfócitos T, macrófagos, fibroblastos, osteoblastos, células endoteliais e epiteliais.

A ativação de NF-kB (nuclear factor kappa B), por exemplo, está associada a diversas condições inflamatórias crônicas, como aterosclerose, artrite reumatóide e as doenças periodontais (Nichols *et al.*, 2001). Basicamente esta ativação (dependente de um estímulo externo que é internalizado e transmitido por alterações conformacionais de proteínas citoplasmáticas ativadas em direção ao núcleo), é decorrente da dissociação entre

a subunidade reguladora IB (inhibitor of kappa B) e NF-kB (promovida por fosforilação) e permite a translocação deste fator de transcrição para o núcleo, seguida da interação com as sequências de DNA apropriadas no promotor do gene alvo, iniciando a transcrição. Importantes mediadores inflamatórios requerem a ativação de NF-kB para sua expressão gênica, como as citocinas: IL-1, IL-6 e TNF (Vicenti *et al.*, 2001).

As citocinas, por sua vez, são polipeptídeos produzidos e secretados frente à presença do microrganismo ou outros antígenos, desencadeando respostas celulares diversas que mediam e regulam processos fisiológicos importantes, como o desenvolvimento de células hematopoiéticas, das reações imunes e inflamatórias. No entanto, quando sintetizadas em níveis elevados, as citocinas modificam o padrão de resposta celular, participando substancialmente no desenvolvimento de patologias de caráter inflamatório crônico (Graves, 2003). Tanto o LPS das bactérias Gram-negativas (ligado aos receptores TLRs) quanto as citocinas resultantes da ativação do sistema imune (IL-1, TNF, PGE_2) estão envolvidos no desenvolvimento das alterações inflamatórias periapicais.

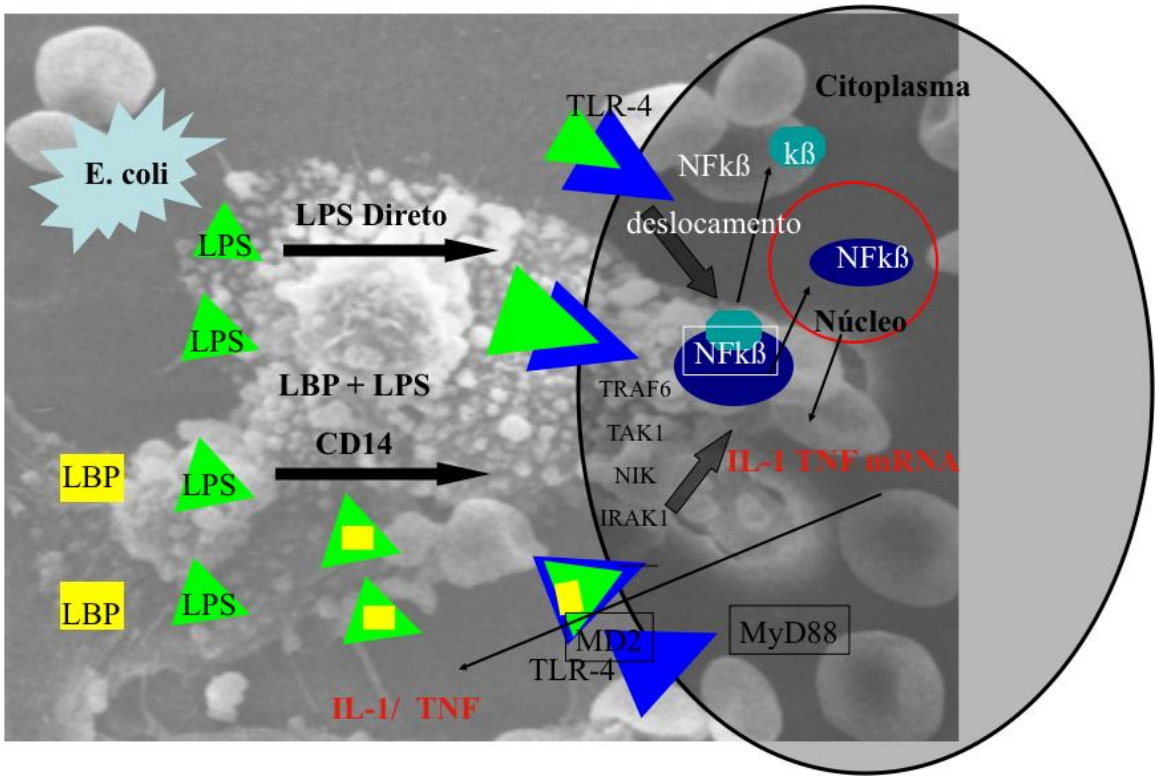

Figura 2 – Antigenicidade do LPS bacteriano contra macrófagos via TLR4.

3.5 Antigenicidade do conteúdo endodôntico contra macrófagos (RAW 264.7) e sua relação com os achados clínicos e radiográficos

No presente estudo observou-se que a antigenicidade do conteúdo endodôntico (produção de IL1-beta, TNF-α e PGE_2) não está relacionada apenas com a quantidade de endotoxina conforme proposto anteriormente na literatura (Jacinto *et al.*, 2005; Vianna *et al.*, 2007); mas também com a diversidade de espécies Gram-negativas envolvidas na infecção – diferentes LPS. Tal fato deve-se as correlações positivas encontradas entre o número de espécies de Gram-negativo e níveis de IL1-beta, TNF-α e PGE_2.

Estudos microbiológicos têm relacionado a simples presença de espécies bacterianas, principalmente os BPPN (Siqueira *et al.*, 2001; Rôças *et al.*, 2003; Foschi *et al.*, 2005; Gomes *et al.*, 2005; Gomes *et al.*, 2006a; Gomes *et al.*, 2007; Montagner *et al.*, 2010) com o desenvolvimento de sintomatologia e destruição óssea. Entretanto, devemos considerar que nenhuma espécie bacteriana individualmente é responsável pela infecção de origem endodôntica.

Bacilos anaeróbios produtores de pigmento negro, *Parvimonas micra* e espiroquetas do gênero *Treponema* spp. são importantes patógenos humanos e já foram relacionados aos sinais e sintomas de origem endodôntica (Siqueira *et al.*, 2001a; Rôças *et al.*, 2003; Foschi *et al.*, 2005; Gomes *et al.*, 2005; Gomes *et al.*, 2006; Gomes *et al.*, 2007).

Não apenas correlações positivas foram encontradas entre espécies bacterianas e a presença de sinais/ sintomas clínicos (*Filifactor alocis* e *Treponema denticola* com a presença fistula e lesão ≥ 2mm respectivamente); como também, associações positivas entre estas espécies com outros patógenos identificados (*Filifactor alocis* e *Parvimonas micra*; *Treponema denticola* e *Porphyromonas endodontalis*).

Associação microbiana entre *Parvimonas micra* (bacteria Gram-positiva) com pelo menos 1 espécie Gram-negativa "alvo" (*Filifactor alocis*) foi encontrada nos canais radiculares estudados. Contudo, numa infecção polimicrobiana, mais macrófagos se

diferenciam em células osteoclásticas através do receptor NF-kB, contribuindo possivelmente para maior destruição óssea, mediado por intensa produção de citocinas (Jiang *et al.*, 2003).

Níveis elevados de endotoxina foram detectados em dentes com exsudação concordando com a literatura endodôntica (Schein & Schilder, 1975; Horiba *et al.*, 1989; Jacinto *et al.*, 2005).

Devido a alta citotoxicidade do LPS contra tecidos periapicais via ativação do sistema immune na produção de citocinas pro-inflamatórias, surgiu a hipótese da maior produção de citocinas pro-inflamatórias – IL-1 ß, TNF-α e PGE_2 estar diretamente relacionada com níveis mais elevados de endotoxinas. Todavia, a destruição dos tecidos periapicais mediada por TNF-α , parece estar diretamente relacionada com níveis elevados de endotoxinas presente nos canais radiculares, uma vez que, correlação positiva foi encontrada entre níveis de TNF-α e endotoxinas.

Dente com lesão periapical ≥ 2mm foi relacionado com a deteção de *Treponema denticola*. A presença de *T. denticola*, por sua vez, esteve associada à identificação da *P. endodontalis*, a qual apresenta potente atividade biológica em cultura celular (Hong *et al.*, 2004) relacionada ao processo crônico de reabsorção óssea (Jiang *et al.*, 2003). Níveis elevados de endotoxinas foram encontrados em dentes com maior destruição óssea (≥ 2mm), corroborando com os achados Schein & Schilder (1975) ao relatarem que o nível de endotoxinas em dentes com lesão periapical é cinco vezes maior do que sua ausência.

Além disso, maior tamanho de lesão periapical (≥ 2mm) foi correlacionado com níveis elevados de IL-1ß no presente estudo, concordando com os achados de Tani-Ishii *et al.* (1995). Vale ressaltar que a presença de petideoglicano nas bactérias Gram-positivas induz a produção de citocinas inflamatórias por pre-cursores indiferenciados (Jiang *et al.*, 2003), consequentemente aumentando a lise óssea regional, particularmente em sítios com maior número de macrófagos (van Winkelhoff *et al.*, 1992).

Niveis elevados de PGE_2 foram detectados em dentes com dor à percussão e/ou

dor à palpação, ambos indicativos de inflamação do ligamento periodontal. Este achado suporta o fato da participação direta e indireta da PGE_2 nos processos inflamatórios que ocorrem na região periapical, tais como: vasodilatação, aumento da permeabilidade vascular e degradação do colágeno (Offenbacher *et al.*, 1993).

3.6. Efeito do preparo químico-mecânico na redução de endotoxinas dos canais radiculares infectados

Devido à alta toxicidade do LPS de bactérias orais em estimular a produção de diferentes citocinas inflamatórias, sua remoção e/ou eliminação dos canais radiculares durante a terapia endodôntica pode contribuir para o processo de reparo dos tecidos periapicais.

Ao longo dos anos, estudos têm sido realizados em busca de substâncias químicas auxiliares ou medicamentos que sejam capazes de inativar moléculas de LPS presente nos canais radiculares (Niwa *et al.*, 1969; Buttler & Crawford, 1982; Porro *et al.*, 1998; Buck *et al.*, 2001; de Oliveira *et al.*, 2007; Vianna *et al.*, 2007; Martinho *et al.*, 2008).

No presente estudo, ao comparar as duas substâncias químicas auxiliares mais utilizadas na Endodontia - NaOCl 2,5% e CLX-gel 2% - na remoção de endotoxinas de canais radiculares com infecções endodônticas primárias e lesão periapical, verificamos que o preparo químico-mecânico com NaOCl 2,5% apresentou maior percentual de redução (57,98%) quando comparado à CLX-gel 2% (47,12%); entretanto, nenhuma das substâncias testadas foi capaz de eliminá-las.

Diante da baixa atividade do NaOCl (Buttler & Crawford, 1982; Tanomaru *et al.*, 2003; Silva *et al.*, 2004; de Oliveira *et al.*, 2007; Martinho *et al.*, 2008) e da CLX (Tanomaru *et al.*, 2003; Silva *et al.*, 2004; de Oliveira *et al.*, 2007; Vianna *et al.*, 2007) sob LPS bacteriano, e ao fato do mesmo se aderir irreversivelmente ao tecido mineralisado (Barthel *et al.*, 1997), a redução de endotoxinas em mais de 47% do conteúdo inicial

presente nos canais radiculares parece não estar relacionada apenas com a substância química auxiliar utilizada, mas principalmente pela ação mecânica dos instrumentos endodônticos conjuntamente com o fluxo e refluxo da solução irrigadora.

Apesar de estudos prévios (Martinho & Gomes 2008; Gomes *et al.*, 2009) demonstrarem que o preparo químico-mecânio dos canais radiculares com NaOCl 2,5% foi capaz de reduzir em ≈ 59% do conteúdo de endotoxina inicial utilizando técnica de instrumentação convencional, no presente estudo, com o advento das limas rotatórias Mtwo®, foi possível atingir nível médio de redução de 99,91%. Considerando o mesmo protocolo de irrigação utilizados nestes estudos, e a baixa ou nenhuma ação do NaOCl sobre endotoxinas, a diferença no percentual encontrada entre os diferentes estudos (59% x 98,06%) deve ser atribuída principalmente ao desempenho da instrumentação endodôntica utilizando limas rotatórias sobre limas manuais – confirmado pelo grupo controle utilizando solução inerte (96,27%).

O melhor desempenho das limas rotatórias Mtwo® na remoção de endotoxina dos canais radiculares deve-se possivelmente à uma instrumentação mais centralizada dos canais radiculares (Schäfer *et al.*, 2006), e mais uniforme quando comparada ao sistema manual. Particularmente, as limas Mtwo® apresentam ângulo de ataque "positivo" e guia radial amplo (Schäfer *et al.*, 2006; Sonntag *et al.*, 2007) que permitem a expulsão de debris dos canais radiculares mais facilmente durante a instrumentação, consequentemente contribuindo para melhor limpeza (Gambarini *et al.*, 2009).

Durante a instrumentação rotatória, o contato físico da lima com a parede do canal, provavelmente gera um aquecimento da substância química auxiliar causado pela fricção do instrumento contra a dentina acionado em baixo torque, consequentemente potencializando a desinfenção promovida pelo NaOCl 2,5%; um aumento limitado aumento da temperatura do NaOCl, à príncipio, é desejado (Woodmansey, 2005).

Diferente dos demais sistemas rotatórios, as limas Mtwo® são preconizadas pelo fabricante para atuarem em toda extensão do canal radicular (Schäfer *et al.*, 2006). Este procedimento é denominado pelo fabricante como "single lenght technique". Questionamentos surgem por diferentes autores (Schäfer *et al.*, 2006; Sonntag *et al.*, 2007)

quanto a esta técnica. Entretanto, Schäfer *et al.*, (2006) observaram que a curvatura do canal era mantida quando comparada aos demais sistemas rotatórios.

Possivelmente a limpeza do canal radicular em toda sua extensão utilizando limas do sistema Mtwo® *versus* 1 mm aquém utilizado nos estudos prévios (Martinho & Gomes 2008; Gomes *et al.*, 2009) pode ter contribuído ainda mais para a melhor desinfecção dos canais radiculares (≈59% x 99,91%).

O padrão de resposta immune/inflamatória gerada frente aos microrganismos investigados na infecção endodôntica e seus subprodutos foram determinados pela rede de citocinas analisadas no presente estudo – IL1-beta, TNF-α e PGE_2 - que, por sua vez, revelou complexa atividade antigênica do conteúdo presente nos canais radiculares infectados. A terapia endodôntica instituída, utilizando limas rotatórias de NiTi-flex, foi capaz de reduzir o conteúdo infeccioso dos canais radiculares de forma significante. Contudo, com o melhor entendimento da cinética de ativação celular e papel das vias de sinalização intracelular na doença endodôntica, pesquisas futuras são de fundamental importância para instituíção de terapia endodôntica mais eficaz no controle da inflamação dos tecidos periapicais e no processo de reabsorção óssea.

4. CONCLUSÃO

De acordo com a metologias propostas no presente estudo, foi possível concluir que nas infecções endodônticas primárias com lesão periapical:

1. Os testes cinéticos turbidimétrico e cromogênico de LAL (*Limulus Ameobcyte Lysate*) apresentaram resultados mais precisos e de melhor reprodutibilidade quando comparados ao QCL (*Quantitative Chromegenic Limulus*). **(capítulo 1)**
2. A antigenicidade do conteúdo endodôntico não está relacionada apenas com a quantidade de endotoxinas encontrada nos canais radiculares, mas também com o número de diferentes espécies Gram-negativas presentes na infecção. Maior destruição óssea periapical foi relacionada com níveis elevados de IL-1ß. **(capítulo 2)**
3. O número de espécies bactérianas Gram-negativas foi relacionado com diferentes níveis de secreção de PGE_2 via macrofagos. Maior produção de PGE_2 foi relacionada com a presença de sintomatologia clínica. **(capítulo 3)**
4. Espécies de *Treponema* spp. exercem seu papel na patogênese das infecções endodônticas primárias. Além disso, o conteúdo bacteriano e particularmente os níveis de endotoxinas presents nos canais radiculares estimularam a produção de IL-6 e IL-10 por macrófagos. **(capítulo 4)**
5. LPS isolados de *P. gingivalis* e *F. nucleatum* de canais radiculares infectados estimulam a produção de IL-1β e TNF-α, que são mediadores inflamatórios pleiotrópicos, podendo iniciar a resposta inflamatória e estimular a produção de mediadores secundários envolvidos na destruição tecidual **(capítulo 5)**
6. O preparo químico-mecânico com NaOCl 2,5% ou CLX-gel 2% não foi eficaz na eliminação de endotoxinas de canais radiculares. **(capítulo 6)**

7. O preparo químico-mecânico com NaOCl 2.5% + EDTA 17% e limas rotatórias NiTi (Mtwo®) foi eficaz na remoção de endotoxinas em 98,06%. **(capítulo 7)**
8. Redução significativa de endotoxinas foi obtida utilizando as sequências Mtwo® finalizadas com o preparo apical final #40.04 or #25.07. **(capítulo 8)**

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APÊNDICE I – Material e métodos e ilustrações dos experimentos realizado na pesquisa.

SELEÇÃO DAS AMOSTRAS

Foram selecionados pacientes que compareceram à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, São Paulo-Brasil, os quais possuíam dentes indicados para a realização de tratamento endodôntico devido a necrose pulpar e presença de lesão periapical. Foram selecionados pacientes que: não fizeram uso de antibiótico num período mínimo de 3 meses, para não interferir com a microbiota dos canais radiculares; não apresentavam dor espontânea; e cujos dentes apresentavam sondagem periodontal menor que 3 mm. Antes de participar da pesquisa, os pacientes assinaram um Termo de Consentimento Livre e Esclarecido elaborado de acordo com as normas do Comitê de Ética em Pesquisa da Faculdade de Odontologia de Piracicaba – UNICAMP. A seguir, foi realizada a avaliação do estado geral do paciente, exame clínico, radiográfico e teste de sensibilidade pulpar (frio). Antes da intervenção endodôntica foram realizados testes clínicos para avaliação do estado perirradicular do dente (Teste de percussão e palpação).

PROCEDIMENTOS CLÍNICOS

Foram realizados bochechos com enxaguatório bucal - clorexidina 0,12%. Em seguida, os pacientes foram anestesiados localmente para posterior remoção de contaminantes coronários, tecido cariado e restaurações. O dente envolvido recebeu polimento coronário com pedra-pomes seguido de isolamento absoluto. A seguir, foi realizado o vedamento da interface coroa/lençol com cianocrilato (Super Bonder; Loctite, SP) para evitar infiltração de saliva. A anti-sepsia do campo operatório (superfície externa da coroa, grampo, lençol de borracha, arco e cianocrilato) foi realizada com *swabs* estéreis umedecidos primeiramente em H_2O_2 a 30% (v/v) e, depois, em NaOCl 5,25%, por 30 segundos cada, subsequentemente neutralizado com solução estéril de tiossulfato de sódio a 5%. A esterelidade do campo operatório, após o protocolo de desinfecção, foi checada por

coletas realizadas da coroa dental antes da abertura coronária. A fase de acesso coronário foi realizada em duas etapas operatórias. A água proveniente do equipo foi cessada, sendo a irrigação realizada manualmente com solução salina estéril e apirogênica (livre de endotoxinas). Brocas de alta rotação diamantadas estéreis/ apirogênicas (esterelizadas através de calor seco, em estufa por um período de tempo de 4 horas a 200ºC) foram utilizadas.

Na primeira etapa operatória foi realizada a remoção dos contaminantes coronários (restaurações, tecido cariado e microrganismos). Na segunda etapa, realizada para a confecção da cavidade de acesso, nova broca estéril/ apirogênica foi utilizada. Após a confecção da cavidade de acesso, nova desinfecção foi realizada, e sua face interna foi checada quanto à esterilidade. Prosseguindo, com o completo acesso ao canal radicular com uma nova broca esférica diamantada estéril/ apirogênica.

Coleta dos canais radiculares

Em dentes multiradiculares, apenas o canal mais amplo diretamente relacionado com a presença da lesão periapical foi coletado (canal palatino dos molares superiores e canal distal dos molares inferiores).

Coleta de endotoxina

As coletas foram realizadas, anteriormente à coleta microbiológica. Foram coletadas amostras de endotoxinas provenientes de canais radiculares de dentes com necrose pulpar e presença de lesão periapical avaliados. Para a coleta, foi introduzido um único cone de papel apirogênico (#15; Dentsply-Mailefer, Ballaigues, Switzerland) próximo ao comprimento aparente do dente, permanecendo em posição por um minuto, em seguida o mesmo foi transferido para um frasco de vidro livre de endotoxinas, previamente submetido ao protocolo de esterilização em estufa, como descrito a seguir, e armazenado à -80ºC, para futura análise de endotoxina pelo método LAL e estimulação celular.

De acordo com o manual da Lonza (Walkersville, MD, EUA), os materiais utilizados

para a realização da coleta de endotoxinas foram esterelizados a 200ºC através de calor seco (estufa), por um período de 4 horas. As pinças clínicas, uma para cada coleta (antes do preparo químico-mecânico-C1 e após o preparo químico-mecânico-C2), foram embaladas em papel alumínio. Frascos de vidro, para armazenamento do cone de papel, foram selados com papel alumínio, para evitar contaminação proveniente das tampas dos frascos, que não podem ser submetidas a altas temperaturas por longo período de tempo, por serem de plástico. Os cones de papéis estéreis (Dentsply-Mailefer, Ballaigues, Switzerland) foram aposicionados em frascos de vidros, selados com gaze e, em seguida, esterelizados utilizando calor seco, como descrito acima.

Coleta microbiológica

As amostras dos canais radiculares para detecção bacteriana foram realizadas utilizando 5 cones de papéis absorventes estéreis e livres de endotoxinas, previamente submetidos ao protocolo de esterilização previamente descrito. Cone de papel estéril foi introduzido próximo ao comprimento total do canal radicular (determinado pela radiografia pré-operatória), permanecendo nesta posição por 60 segundos.

Nos casos onde os canais estavam secos, o mesmo foi umedecido com água de Lisado de Amebócito *Limulus* (LAL) (Lonza, Walkersiville, MD, EUA) para assegurar uma amostra viável. O cone de papel absorvente, ao ser retirado do canal, foi imediatamente introduzido em microtubos plásticos de 1,5 mL do tipo "eppendorf" contendo 1,0 mL de meio de transporte VMGAIII, procedendo a coleta com mais 2 cones de papel da mesma forma.

Coleta após preparo químico-mecânico dos canais radiculares

Após o uso do último instrumento endodôntico da sequência, os canais radiculares intrumentados com NaOCl 2,5%, foram irrigados com 5 mL de tiossulfato de sódio (5%) durante 1 minuto, para neutralização do hipoclorito de sódio (2,5%) (Proderma, Farmácia de Manipulação-Piracicaba-SP), sendo posteriormente lavados com 5mL de água de LAL estéril/ apirogênica. Já os canais instrumentados com CLX-gel 2% foram neutralizados com

Tween 80 a 0,5% (Sigma Chemical Com, Saint Louis, MO, EUA) + Lecitina de soja a 0,07% (Proderma Farmácia de Manipulação, Piracicaba, SP).

Em seguida foi realizada a coleta de endotoxina Segundo a coleta de microrganismos. Após o término das coletas, os canais foram secos com cones de papel estéreis e obturados se estivessem sem exsudato.

PROCESSAMENTO LABORATORIAL

3.1. Manual de preparo doVMGA (Viability Medium Götemborg Agar)

1. Preparo do meio

1.1. Componentes necessários

a) Acetato de fenilmercúrio (Sigma, St. Louis, EUA), Ref. P5554 – 25 g;

b) Glicerofosfato de sódio (Merck, Alemanha) Ref. 4168 – 25 g

c) CaCl anidro ou CaCl2.6H2O (Ecibra, Santo Amaro – SP, Brasil), Ref. 0490 – 500 g;

d) KCl (Synth, Diadema – SP, Brasil), Ref. C1058.01.AG – 500 g;

e) MgSO4.7H2O (Sigma, St. Louis, EUA), Ref. M1880 – 500 g;

f) NaOH (Dinâmica, Brasil) Ref. CAS1310-73-2 cod. 1702-1 – 500 g

g) Agar bacteriológico (Acumedia, EUA), Ref. 7178A – 500 g

h) Gelatina (DIFCO, Detroit, EUA), Ref. 214340 – 500 g

i) Triptose (DIFCO, Detroit, EUA), (Sigma, St. Louis, EUA) Ref. T4532 – 100 g

j) Thiotona ou Peptona (BBL, Cockeyville, EUA), (Biobrás, MG, Brasil) Ref. 177-2 – 500 g

k) L-cisteína-dihidroclorito (Sigma, St. Louis, EUA), Ref. C4820 - 25 g

l) Ácido tioglicólico (Sigma, St. Louis, EUA), Ref. T3758 – 500 mL

1.2 Autoclavar previamente:

a) Frascos de Eppendorf (1,5 mL) com 3 bolinhas de vidro (Glass beads).

A quantidade de Eppendorfs é de acordo com o volume que será preparado do meio. Neste caso, se for preparar 250 mL de VMGA III, autoclavar cerca de 130 frascos.

b) Proveta de 50mL

c) 2 frascos vazios de 500mL

d) 2 frascos vazios de 1000mL

e) 4 funis

f) 4 tubos de ensaio

g) 4 espátulas

h) 1 frasco de Becker pequeno

i) 2 provetas de 200mL

j) 30 pedaços de papel alumínio

k) 1 vidro de 500mL

1.3. Preparo dos volumes líquidos

Para os volumes de VMGA III, autoclavar os respectivos volumes de água bidestilada.

	500 mL	250 mL	125 mL
1 frasco	275 mL	137,5 mL	68,75 mL
1 frasco	150 mL*	75 mL*	37,5 mL*
1 frasco	600 mL	300 mL	150 mL
1 frasco	5 mL	2,5 mL	1,25 mL

*Colocar um peixinho para ajudar a dissolver a gelatina

Para os volumes de VMGA III, autoclavar os respectivos volumes de **solução salina**.

	500 mL	250 mL	125 mL
1 frasco	50 mL	25 mL	12,5 mL

1.4. Solução de Sais de Estoque IV

Verificar se tem a solução de sais de estoque IV (Cor azul) e autoclavar, caso não tenha, autoclavar:

1 frasco	60 mL de água bidestilada (1)
1 frasco	40 mL de água bidestilada (2)
1 frasco	60 mL de água bidestilada (3)
1 frasco	40 L de água bidestilada (4)

Verificar se tem a solução de NaOH e KOH (8M), caso não tenha, preparar.

Volume final de 200 mL. Modo de preparo: Pesar 64g de NaOH e 89,76g de KOH. Dissolver em 140 mL de água bidestilada. Depois de dissolvido, acrescentar água bidestilada q.s.p 200 mL. Autoclavar por 20 minutos.

No dia anterior, preparar as soluções:

a) SOLUÇÃO 1 – Água bidestilada estéril 60mL + Acetato de fenilmercúrio 0,1g (não pesar com espátula de metal). Dissolver em banho-maria e deixar overnight a 56°C.

b) SOLUÇÃO 2 – Água bidestilada estéril 40mL + Glicerofosfato de sódio 20g.

Dissolver aquecendo levemente na manta e deixar overnight.

No dia do preparo do VMGA III, para preparar a solução azul:

c) SOLUÇÃO 3 – Água bidestilada estéril 60mL + CaCl anidro 0,24g ou CaCl2.6H2O 0,32g + KCl 0,84g + MgSO.7HO 0,2g.

Misturar bem as SOLUÇÕES 1, 2 e 3 e resfriar em uma proveta graduada. Adicionar 40mL de água bidestilada já estéril para completar 200 mL. Ao final, adicionar 0,006g de azul de metileno e acondicionar na geladeira.

2.4. Mistura das soluções e preparo do VMGA III

a) Solução A:

SOLUÇÃO A	1000 mL	250 mL	125 mL
Água bidestilada	550 mL	137,5 mL	68,75 mL
Triptose	0,5 g	0,125 g	0,0625 g
Thitona e Peptona	0,5 g	0,125 g	0,0625 g

O frasco com água bidestilada deve estar estéril no dia anterior. Dissolver com agitação e aquecimento na manta ou no microondas por 10 segundos.

b) Solução B:

SOLUÇÃO B	1000 mL	250 mL	125 mL
Água bidestilada	300 mL	75 mL	37,5 mL
Gelatina	50 g	12,5 g	6,25 g

A água bidestilada deve estar pré-aquecida. O frasco com água destilada e o peixinho já devem estar estéreis no dia anterior. Aquecer no microondas por 10 segundos + 10 segundos + 10 segundos (total de 30 segundos), observando atentamente se derrete a gelatina. Deixar em banho-maria caso não derreta tudo. Essa solução não pode mais ser autoclavada.

c) Solução C:

SOLUÇÃO C	1000 mL	250 mL	125 mL
Água bidestilada	50 mL	12,5 mL	6,25 mL
Agar	2 g	0,5 g	0,25 g

Autoclavar a 121°C por 20 minutos, manter em banho-maria.

d) Solução D:

SOLUÇÃO D	1000 mL	250 mL	125 mL
Água bidestilada	10 mL	2,5 mL	1,25 mL
L-cisteína	0,05 g	0,125 g	0,0625 g

Autoclavar a 121°C por 20 minutos, manter em banho-maria.

e) Solução E:

SOLUÇÃO E	1000 mL	250 mL	125 mL
Solução salina	100 mL	25 mL	12,5 mL
Ácido Tioglicólico	0,5 mL	0,125 mL	0,0625 mL

A solução salina deve estar estéril no dia anterior. O ácido tioglicólico encontra-se no freezer, e deve ser manipulado com muito cuidado, empregando luvas e máscara.

Misturar as SOLUÇÕES A + B + C em frasco de 500 mL na manta. Resfriar a 45-50°C. Adicionar 25mL da solução de sais de estoque azul IV. Adicionar a solução de ácido tioglicólico (SOLUÇÃO E). Ferver a solução por 5 minutos até que a cor azul desapareça (fica amarelo). Resfriar em água morna sob fluxo de N2. Levar para a câmara de anaerobiose. Adicionar a solução de cisteína (SOLUÇÃO D).

Ajustar o pH 7,2 a 7,4 com a solução de NaOH + KOH, adicionando de 10 em 10 microlitros. Adicionar aos poucos, pois não tem volta. A solução de NaOH + KOH vai diminuir o pH.

3.2. Investigação bacteriana através do método molecular de PCR simples (16S rDNA).

Extração do DNA bacteriano

Extração de DNA foi realizada das 21 amostras coletadas dos canais radiculares. *Dialister pneumosintes* (ATCC 33048), *Prevotella intermedia* (ATCC 25611), *Prevotella nigrescens* (ATCC 33099), *Aggregatibacter actinomycetecomitans* (ATCC 43718), *Porphyromonas gingivalis* (ATCC 33277), *Filifactor alocis* (ATCC 35896), *Tannerella forsythia* (ATCC 43037), *Prevotella tannerae* (ATCC 51259), *Treponema denticola* (ATCC 35405), *Porphyromonas endodontalis* (ATCC 35406), *Treponema socranskii* (35536), *Parvimonas micra* (ATCC 33270) e *Fusobacterium nucleatum* (ATCC 15033) foram utilizados como controle das reações de PCR.

A extração de DNA foi realizada com o QIA amp DNA kit (QYAGEN, Valencia, Califórnia, EUA, Ref. 51306 – 250 reações) de acordo com as instruções do fabricante:

1. Remover 300µL da amostra e adicionar a um eppendorf de 1,5mL.
2. Adicionar 180 µL de ATL e 20 µL de Proteinase K.
3. Agitar e incubar a 56°C por 2 horas.
4. Adicionar 200 µL de AL.
5. Agitar e incubar a 70°C por 10 min em banho seco.
6. Adicionar 200 µL de etanol puro.
7. Agitar e transferir para os tubos com filtros/colunas.
8. Centrifugar a 8000 rpm por 1 min.
9. Transferir o filtro para o outro tubo vazio do kit.
10. Adicionar 500 µL de AW1.
11. Centrifugar a 8000 rpm por 1 min.
12. Transferir a coluna para outro tubo.
13. Adicionar 500 µL do AW2.
14. Centrifugar a 13000 rpm por 3 min.
15. Transferir o filtro para um eppendorf normal de 1,5mL com tampa.

16. Adicionar 100 µL de AE e aguardar 3 minutos.

17. Centrifugar a 8000 rpm por 1 minuto.

18. Armazenar o DNA extraído a -20°C.

Após extração, a leitura da concentracão de DNA presente nas amostras coletadas dos canais radiculares e ATCCs foi realizada a 260 nm através de espectrofotometria (Nanodrop 2000; Thermo Scientific, Wilmington, DE, EUA).

Reação de PCR

A reação de PCR foi processada na quantidade de 25 µL de uma mistura de reagentes (Master Mix) contendo as quantidades especificadas no quadro 1 para 1,5 µL do DNA da amostra.

Quadro 1. Proporções dos reagentes no Master Mix.

Reagentes	Quantidade (µL)
Tampão *(*10 x Reaction *buffer* Invitrogen ® - Life Technology do Brasil)	2,5 µL
*DNTPs (*Invitrogen ® - Life Technology do Brasil)*:*	0,5 µL
*$MgCl_2$ (*Invitrogen ® - Life Technology do Brasil)	1,25 µL
H_2O MiliQ	17,625 µL
Primer Forward 100µM (Invitrogen ® - Life Technology do Brasil)	0,75 µL
Primer Reverse 100µM *(*Invitrogen ® - Life Technology do Brasil)	0,75 µL
*Taq Platinum (*Invitrogen ® - Life Technology do Brasil)	0,125 µL

A Taq DNA Polimerase escolhida foi a Platinum®Taq DNA Polymerase (Invitrogen®, São Paulo, SP, Brasil) que não se degrada com o aumento gradativo da temperatura, podendo ser acrescentada diretamente na mistura (Mix) da reação evitando a necessidade de *hot start.*

 # PREPARO DE SOLUÇÕES PARA PCR

 # PREPARO DNTPs (Invitrogen®, São Paulo, SP, Brasil):

Acrescentar 10 uL de cada (A, T, C, G) em 360 uL de água MiliQ estéril

(100mM DNTPs Set, PCR Grade / Invitrogen, Cat Nº 10297117)

 # PREPARO DOS PRIMERS (Invitrogen®, São Paulo, SP, Brasil):

Quando o primer chega liofilizado, acrescentar água Mili-Q (conforme exemplo abaixo). Esse volume dependerá de cada síntese. Deve-se verificar no rótulo do tubo do primer, ou naquele documento que vem junto, o valor indicativo da quantidade de nm (canto direito abaixo da OD). Esse valor será a quantidade de água que deverá será utilizada para diluição do primer e assim vai ficar uma concentração de 1000 pMol (essa é a solução estoque). Para preparar a solução de uso, cuja concentração requerida é de 25 pMol, faz-se necessário uma diluição 1:40, ou seja, acrescentar 1 µl de primer estoque a 39 µl de água ultra pura/miliQ estéril.

→EXEMPLO:

Solução Estoque (1000 pMol)

- Se estiver escrito no rótulo do primer "28,31 nm"
- Acrescenta "28,31 uL" de água Mili-Q estéril, agita bem, centrifuga rapidamente apenas para concentrar o líquido no fundo do tubo e armazena-se em -20ºC.

Solução de Uso (25 pMol)

- Diluir 1:40 = 1ul do estoque + 39 ul de água Mili-Q estéril

C1 x V1 = C2 x V2

1000 x 1ul = 25 x ?

? = 40 (quer dizer que pegando 1 ul da solução estoque, para ter a solução final de uso a 25pMol, o volume total deve ser 40ul, então colocar 39ul de água)

- Se quiser fazer uma quantidade maior de primer, é só aumentar proporcionalmente.

Ex: 5 ul de estoque + 195 ul de água

C1 x V1 = C2 x V2

1000 x 5 = 25 x ?

? = 200 (para uma solução de uso de 200 ul, deve pegar 5 ul do estoque + 195 ul de água)

 # DESENHO DE PRIMERS – "OLIGO PERFECT" (Invitrogen ®)

1) Entrar no site da pubmed.gov
2) No **Search** colocar **Nucleotide**
3) Logo embaixo, escrever o nome da bactéria, por exemplo: ***F. necrophorum***
4) Clicar em Buscar
5) Vários artigos vão aparecer. Pegamos o que tem o seqüenciamento do gene 16S da bactéria escolhida na forma (ATCC/16S)
6) Vai aparecer a sequência da bactéria (**devemos copiar**)
7) Agora, abrir o site do google.com.br
8) Digitar **Oligoperfect** e dentre os resultados no Google, selecionar o primeiro para abrir o programa (na página da invitrogen)
9) Na região **Sequence name** colocar novamente o nome da bactéria que estamos procurando
10) Na região **Applicancion** colocar **PCR detection**
11) Na região **Research name** colocar por exemplo Brenda
12) Agora, colar a sequencia escolhida no numero 6. A sequencia vem com números e devemos tirá-los (apagar).
13) Clicar em Submit
14) Agora vai aparecer as especificações:

(**Primer Size**: 12-20-27 / **Primer TM °C** 57-60-63 / **Primer GC** 40-50-60 / **Product Size** 300-500 / **Experimental conditions** 50-50 / **Region of analysis** 1-746 / Max number of primer to returns 5.

15) Clicar em **Submit**
16) A ordem que aparece é a que ele acha melhor (sempre aparece duas sequencias que será o foward e o reverse)
17) Entrar novamente no pubmed e na parte da esquerda colocar **Blast (sequence analysis)**
18) Clicar em **Nucleotides blast**
19) Clicar em **Others**
20) Copiar o primer que foi encontrado no programa da invitrogen e colar.

21) Clicar em Show results
22) Testar o Forward e o Reverse (um pode ser + e o outro -)
23) Preferencialmente deve aparecer apenas a bactéria em questão no resultado do BLAST.

As vezes aparecem outras, mas se a nossa bactéria estiver com identidade de 100% e as outras com menor porcentagem também é aceitável. Mas se estiver aparecendo muitas espécies diferentes (e que sejam encontrada em canal), com alta porcentagem ou se a porcentagem de identidade com a nossa bactéria for baixa, devemos desconsiderar a sequencia e testar outra.

Para determinar a temperatura de anelamento ideal, reações de PCR contendo primers espécie-específicos (Quadro 2) foram realizadas em um aparelho termociclador convencional e submetidas a vários gradientes de temperatura (MJ96G, Biocycler, termocicladores, Curitiba, SC, Brasil) utilizando amostras ATCCs correspondentes e baseadas na literatura de suporte.

Quadro 2. Dados sobre as reações e "primers" espécie - específicos utilizados para cada microrganismo investigado.

Bactéria alvo	Primer (5'- 3')	Amplicon	Ciclo
Universal (16srDNA)	TCC TAC GGG AGG CAG CAG T		95°C 10min; 40 ciclos: 95°C 10s, 60°C 10s
	GGA CTA CCA GGG TAT CTA ATC CTG TT	466 bp	e extensão final: 72°C 25s
Dialister pneumosintes	TTC TAA GCA TCG CAT GGT GC		95°C 2min; 36 ciclos: 94°C 30s, 55°C 1min,
	GAT TTC GCT TCT CTT TGT TG	1105 bp	72°C 2min e extensão final 72°C 2min.
Prevotella intermedia	TTT GTT GGG GAG TAA AGC GGG		95°C 2min; 36 ciclos: 94°C 30s, 58°C 1min,
	TCA ACA TCT CTG TAT CCT GCG T	575 bp	72°C 2min; e extensão final 72°C 10min.
Prevotella nigrescens	ATG AAA CAA AGG TTT TCC GGT AAG		95°C 2min; 36 ciclos: 94°C 30s, 58°C 1min,
	CCC ACG TCT CTG TGG GCT GCG A	804 bp	72°C 2min; e extensão final 72°C por 10min.
Aggregatibacter	AAA CCC ATC TCT GAG TTC TTC TTC		94°C 30s; 36 ciclos: 95°C 30s, 55°C 1min,
actinomycetemcomitans	ATG CCA ACT TGA CGT TAA AT	557 bp	72°C 2min; entensão final 72°C 10min.
Porphyromonas gingivalis	AGG CAG CTT GCC ATA CTG CG		95°C 2min; 36 ciclos: 94°C 30s, 60°C 1min,
	ACT GTT AGC AAC TAC CGA TGT	404 bp	72°C 2min; e extensão final 72°C 2min.
Filifactor alocis	CAG GTG GTT TAA CAA GTT AGT GG		95°C 2min; 26 ciclos: 95°C 30s, 58°C 1min,
	CTA AGT TGT CCT TAG CTG TCT CG	594 bp	72°C 1min; e extensão final 72°C 2min.
Tannerella forsythia	GCG TAT GTA ACC TGC CCG CA		95°C 1min; 36 ciclos: 95°C 30s, 60°C 1min,
	TGC TTC AGT GTC AGT TAT ACC T	641 bp	72°C 1min; e entensão final 72°C 2min.
Prevotella tannerae	CTT AGC TTG CTA AGT ATG CCG		95°C 2min; 36 ciclos: 94°C 30s, 55°C 1min,
	CAG CTG ACT TAT ACT CCC G	550 bp	72°C 2min; e extensão final 72°C 10min.
Treponema denticola	TAA TAC CGA ATG TGC TCA TTT ACA T		95°C 2min; 36 ciclos: 94°C 30s, 60°C 1min,
	TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp	72°C 2min; e extensão final 72°C 10min.
Porphyromonas endodontalis	GCT GCA GCT CAA CTG TAG TC		95°C 2min; 36 ciclos: 94°C 30s, 58°C 1min,
	CCG CTT CAT GTC ACC ATG TC	672 bp	72°C 2min; e extensão final 72°C 10min.
Treponema socranskii	GAT CAC TGT ATA CGG AAG GTA GAC A		95°C 2min; 36 ciclos: 94°C 30s, 56°C 1min,
	TAC ACT TAT TCC TCG GAC AG	288 bp	72°C 2min; e extensão final 72°C 10min.
Parvimonas micra	AGA GTT TGA TCC TGG CTC AG		95°C 2min; 36 ciclos: 94°C 30s, 60°C 1min,
	ATA TCA TGC GAT TCT GTG GTC TC	207 bp	72°C 2min; e extensão final 72°C 10min.
Fusobacterium nucleatum	AGT AGC ACA AGG GAG ATG TAT G		95°C 5 min; 30 ciclos: 94°C 30s, 40°C 1min,
	CAA GAA CTA CAA TAG AAC CTG A	645 BP	72°C 2min; e extensão final 72°C 10min.

Além das amostras e dos controles positivos o DNA genômico purificado dos microrganismos ATCC, foi utilizado como controle negativo água MiliQ esterilizada.

Eletroforese

As amostras após a reação de PCR (produtos da amplificação) foram analisadas imediatamente por eletroforese. Foi utilizado gel de agarose a 1% (Invitrogen® - Life Technology do Brasil) em tampão de Tris-borato EDTA (pH 8,0) (TBE) e corado com brometo de etídio (5ug/mL-Invitrogen®, São Paulo, SP, Brasil).

Foi incluído em cada gel, um padrão de peso molecular de 100 bp (DNA ladder, Invitrogen® - Life Technology do Brasil). Após o término de cada corrida (60 volts por 40 min), as bandas foram observadas com auxílio de um transiluminador de luz ultravioleta. A documentação fotográfica dos géis foi obtida com o sistema Image Master-VDS (Pharmacia Biotech, Cambridge, England) e a captura das imagens foi realizada pelo programa LISCAP Image Capture software.

 # PREPARO DE SOLUÇÕES PARA ELETROFORESE

- LADDER (100bp ou 1 kb - (Invitrogen®) Diuir 1:15

- Brometo de etídio (10mg/mL) (Invitrogen®) Dissolver 0,2 g de brometo de etídio em 20 mL de água destilada/MiliQ**;** Manter em temperatura ambiente e proteger da luz. Invitrogen já vende solução pronta.

- Syber Safe concentrado 10000x (Invitrogen®) É uma alternativa ao Brometo. Não é cancerígeno, porém bem mais caro.

- Taq Platinum + Buffer + MgCl2 Já vem pronto para usar, e são vendidos os 3 juntos

- **Loading Dye** (Invitrogen®) Também já vem pronto para uso.

- **EZ-Vision** (Invitrogen®) É semelhante ao loading, mas apresenta um component responsável pela fluorescência do DNA. Quando usamos na eletroforese não é necessário usar brometo ou Syber Safe no gel de agarose.

- **TBE 10X BUFFER** (Invitrogen®) Pegar 100 mL de TBE 10x acrescentar 900 mL de H_2O destilada.

 # REALIZAÇÃO DA ELTROFORESE

*** Gel Pequeno (Cuba de eletrofororese 12X12CM):**

0,63 g de agarose (0,7%) / 0,9 g agarose (1%) / 1,8g agarose (2%)

90 mL de TAE 1X ou TBE 1X

2 µl de solução de brometo de etídio (10mg/mL) ou se for usar Syber Safe vai colocar 9 µl (?) para 90 mL de TAE

OBS.: No gel pequeno utiliza-se 2 pentes de 20 wells, considerando o primeiro well de cada pente para o ladder, e o segundo para o controle do DNA bacteriano (ATCC), é possível analisar 36 amostras.

*** Gel Grande (Cuba de eletroforese 25X20CM):**

1 – Pesar (no copinho de café descartável) na balança de precisão de acordo com a concentração do gel (0,7%, 1% ou 2%)

2 – Misturar a agarose com 90 mL do tampão (TAE ou TBE) em Becker e aquecer em microondas até ficar transparente (+- 1min: deixa um pouco, tira para mexer e voltar p microondas, até desaparecer partículas)

3 – Passar conteúdo para o Becker contaminado com brometo e acrescentar 2uL do brometo de etídio.

(bancada de baixo é contaminada. Na de cima não deve ter nada que ficou em contato com brometo)

4 – Misturar e verter na bandeja.

Importante: Encaixar bem as borrachas para vedar as extremidades e colocar o pente.

5 –Observar se tem bolhas para remover e esperar esfriar/endurecer (+-30 min)

6 – Remover as borrachas e pentes.

7 – Levar a bandeja com o gel para dentro da cuba e acrescentar tampão se necessário, até cobrir todo gel.

Importante: o tampão da cuba deve ser o mesmo usado no gel (TAE ou TBE)

8 – Aplicar 4 ul da amostra + 2ul do corante (loading dye) em cada poço.

Essa mistura é feita em um pedaço de parafilm, e não dentro do eppendorf.

9 – Fechar cuba e Ligar fonte de eletroforese

+- 90 V / +-40 minutos. Observar no visual a posição do corante pra ter noção de onde a banda está! (se quiser acelerar corrida aumenta voltagem)

Obs: Quando vai fazer purificação a partir da banda, deve fazer receita dobrada (brometo tb é 2x) para os poços ficarem maior. No gel 2% o brometo não dobra.

OBS.: No gel Grande utiliza-se 3 pentes de 40 wells, considerando o primeiro well de cada pente para o ladder, e o segundo para o controle do DNA bacteriano (ATCC), é possível analisar 114 amostras.

Figura 1. **PCR – A.** Kit de extração de DNA (QIA amp); **B.** Filtro de DNA (QIA amp); **C.** Par de primers (Invitrogen); **D.** Preparo da reação de PCR; **E.** Amostras em Termociclador (Biocycler); **F**. Inoculação do produto da reação de PCR em gel de agarose a 1% em tampão de Tris-borato EDTA; **G.** Fonte de eletroforese (PWSys® PW300); **H.** Análise das bandas em gel de agarose em transiluminador de luz ultravioleta.

3.3. Quantificação de endotoxina através do método Limulus Amobocyte Lysate (LAL) – Teste Turbidimétrico.

Para quantificação de endotoxinas presentes em 21 dentes com infecção endodôntica primária e lesão periapical foi utilizado o método turbidimétrico: teste Pyrogent® -5000 (Lonza, Walkersville, MD, EUA) - uma preparação de Lisado de Amebócito *Limulus* (LAL).

Extração de endotoxinas do cone de papel

As amostras de endotoxina coletadas dos canais radiculares foram reconstituídas em 1 mL de água de LAL e em seguida extraídas sob agitação mecânica em vortex por 60 seg.

TESTE TURBIDIMÉTRICO CINÉTICO PARA QUANTIFICAÇÃO DE ENDOTOXINAS LAL (PYROGENT-5000, LONZA WALKERSVILLE, MD, EUA)

Pyrogent-5000 é um ensaio cinético quantitativo para detecção de endotoxina de bactérias Gram-negativas.

A amostra é colocada com o reagente LAL reconstituído, em seguida incubada em um leitor de microplacas e monitorada automaticamente através de um software até o desenvolvimento de uma aparência de turvação. O tempo necessário antes da aparição da turvação (Tempo de reação) é inversamente proporcional a quantidade de endotoxina presente na amostra.

O kit Pyrogent-5000 apresenta: 1) Pyrogent-5000 LAL Reagente; 2) Endotoxina de *Escherichia Coli* 055:B5; 3) Tampão de reconstituição Pyrogent-5000.

Para a realização do teste foi utilizado: 1) Água Reagente LAL (Lonza, Walkersiville, MD, EUA); 2) Hidróxido de sódio 0.1 N ou Ácido clorídrico 0.1N, dissolvido em água reagente LAL, para ajuste do pH da amostra, se necessário; 3) Tubo de vidro descartável para diluição, isentos de endotoxina; 4) Ponteira estéreil descartáveis de 10 μL, 200 μL e 1000 μL; 5) Microplaca estéril descartável (Corning Costar, Cambridge, MA, UK); 6) Multipipetador de 8 canais; 7) Reservatório de reagente

(Lonza, Walkersiville, MD); 8) Leitor de microplacas (Ultramark, Bio-Rad Laboratories); 9) Software WinKQCL® (Lonza, Walkersiville, MD); 10) Cronômetro e agitador vortex.

Todo material utilizado, proveniente da CAMBREX (WALKERSVILLE, MD, EUA) era apirogênico (livre de endotoxinas).

Padronização da curva padrão

O estabelecimento de uma curva padrão com quantidade de endotoxinas conhecidas é necessário para determinar a concentração de endotoxinas com quantidades desconhecidas (Figura 2).

Esta curva foi preparada utilizando a solução concentrada 100 EU/mL e suas diluíções - 10 EU/mL, 1 EU/mL, 0,10 EU/mL, 0,01 EU/mL (Tabela 1).

Tabela 1. Estabelecimento das diluíções da curva padrão nas concentracões 10 EU/mL, 1 EU/mL, 0,10 EU/mL, 0,01 EU/mL.

Concentração de Endotoxina (EU/mL)	Volume de Água Reagente LAL	Volume de Solução de Endotoxina adicionado à Água para Reagente LAL
10	0,9mL	0,1mL de 100 EU/mL solução
1	0,9mL	0,1mL de 10 EU/mL solução
0,10	0,9mL	0,1mL de 1 EU/mL solução
0,01	0,9mL	0,1mL de 0,10 EU/mL solução

1. Preparar uma solução contendo 10 EU/mL de endotoxina por adição de 0,1mL de 100 EU/mL endotoxina estoque em 0,9mL de água para reagente LAL. Essa solução deve ser vigorosamente agitada em vortex por pelo menos 1 minuto antes do procedimento.
2. Transferir 0,1mL da solução de endotoxina 10 EU/mL em 0,9mL de água para reagente LAL para um recipiente adequado e rotular 1 EU/mL. A solução deve ser

vigorosamente agitada em Vortex por pelo menos 1 minuto antes do início do procedimento;

3. Transferir 0.1mL da solução de endotoxina 1 EU/mL em 0,9mL de água para reagente LAL para um recipiente adequado e rotular 0,10 EU/mL. Essa solução deve ser vigorosamente agitada em Vortex por pelo menos 1 minuto antes do procedimento.

4. Transferir 0,1 mL da solução de endotoxina 0,10EU/mL em 0,9mL de água para reagente LAL para um recipiente adequado e rotular 0,01 EU/mL. Essa solução deve ser vigorosamente agitada em Vortex por pelo menos 1 minuto antes do início do procedimento.

A eficiência da curva padrão deve ser maior ou igual à 0,98, valor este denominado de r ($r \geq 0,98$).

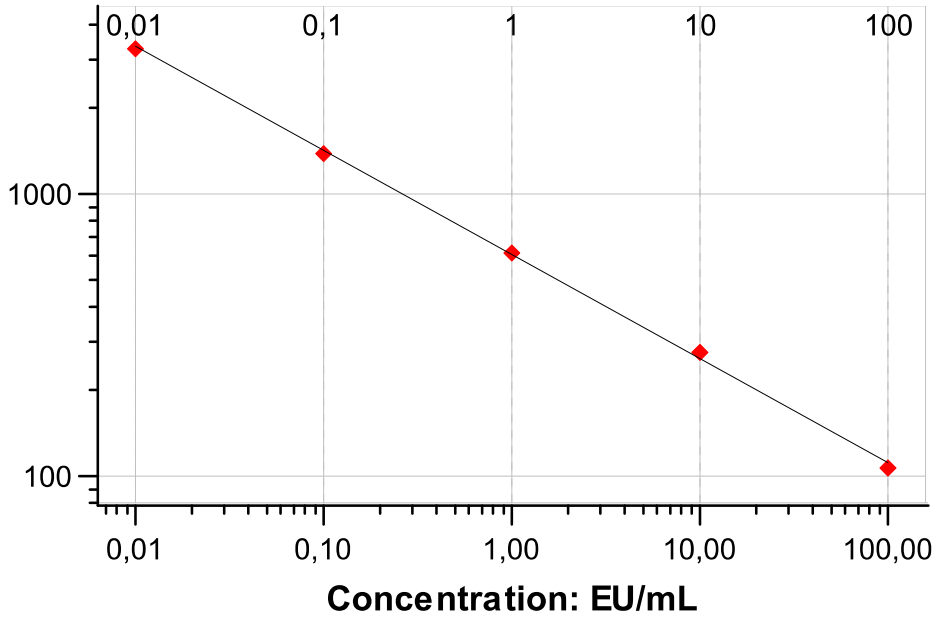


Figura 2. Curva padrão (Método Turbidimétrico).

Ensaio de inibição/ desenvolvimento

O teste LAL pode ser influenciado por muitos fatores. Fatores da inibição devem ser evitados, para isso foi realizada a adição de uma quantidade conhecida de endotoxina

(0,1 EU/mL), este procedimento é denominado "Spike". A adição de 0,1 EU/mL de endotoxina foi realizada na amostra mãe e em 4 diluições (1:10, 1:100, 1:1000 e 1:10000) de 4 amostras coletadas dos canais radiculares. Em seguida, os testes foram realizados em duplicata, sendo da mesma amostra - 2 poços contendo 100 µL da amostra mãe ou suas diluições; e 2 poços contendo 100 µL amostra + 0.1 EU/mL de endotoxina (spike). O teste foi realizado como descrito a seguir. Para comprovar que não houve qualquer tipo de interferência (inibição ou desenvolvimento) da amostra com o reagente de LAL, a concentracão de endotoxina recuperada dos poços contaminados, calculada pelo software, deve ter sido entre 50% - 200%, valor este denominado controle positivo do produto (PPC). A amostra mãe e sua diluição 10^{-1} foram as que apresentaram maiores números dentro do padrão do PPC, então a diluição 10^{-1} foi selecionada para a realização dos testes de todas as amostras através do método turbidimétrico.

Procedimento para realização do teste Pyrogent-5000

Inicialmente, 100 µL do "blank" (água de LAL) foi inoculdo em duplicata na placa de 96 poços (Corning Costar, Cambridge, MA) (Figura 3). Em seguida, 100 µL de cada ponto da curva padrão nas diferentes concentracões (100 EU/mL, 10 EU/mL, 1 EU/mL, 0,10 EU/mL e 0,01 EU/mL) foi distribuído em duplicata (Figura 3). Após, 100 µL das amostras e seus respectivos controles (PPC) - ambos em duplicata foram inoculados com reagente de LAL (Figura 3). Para cada poço do controle positivo foi adicionado 10 µL de 0,1 EU/mL de endotoxina de *E. coli* (spike). A placa já posicionada no leitor de ELISA (Ultramark, Bio-Rad Laboratories) com Software WinKQCL® e programado para - 340 nm, 37 º C e 100 leituras - 100 µL do reagente de LAL foi adicionado em todos os poços. Em seguida, a leitura foi iniciada, levando aproxidamente 60 min para o término do teste.

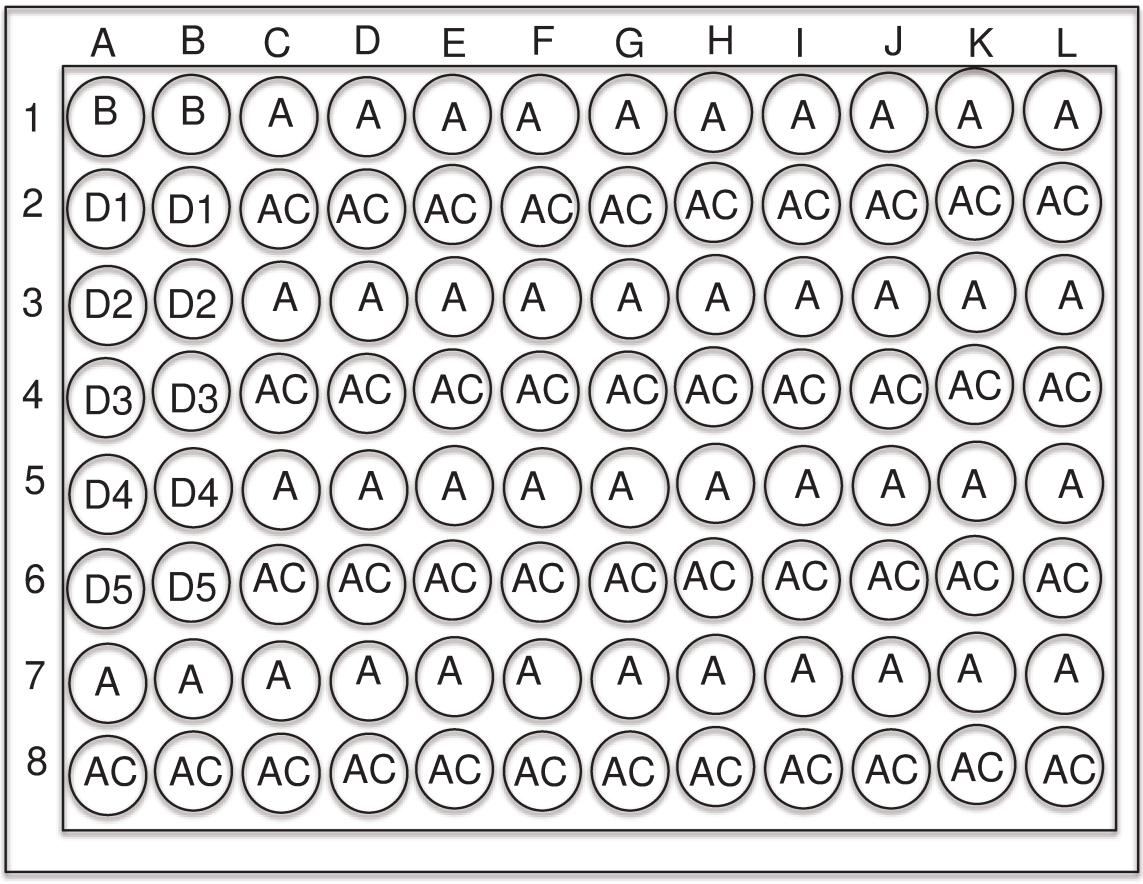


Figura 3. Distribuição das amostras : B= blank; D1= 0,01 EU/mL, D2= 0,10 EU/mL; D3= 1 EU/mL; D4= 10 EU/mL; D5= 100 EU/mL; A= Amostra; AC= Amostra Controle.

Cálculo da concentração de endotoxina

De forma contínua durante todo o ensaio, o leitor de microplacas / software Kinetic-QCL é monitorado na absorbância de 340nm de cada orifício da microplaca. Usando a leitura de absorbância inicial de cada orifício como seu próprio branco, o leitor determina o tempo necessário para que a absorbância aumente a 0.03 unidades. Este tempo é denominado Tempo de reação. O software WinKQCL executa automaticamente uma correlação linear log/log do Tempo de Reação de cada padrão com a concentração de endotoxina correspondente.

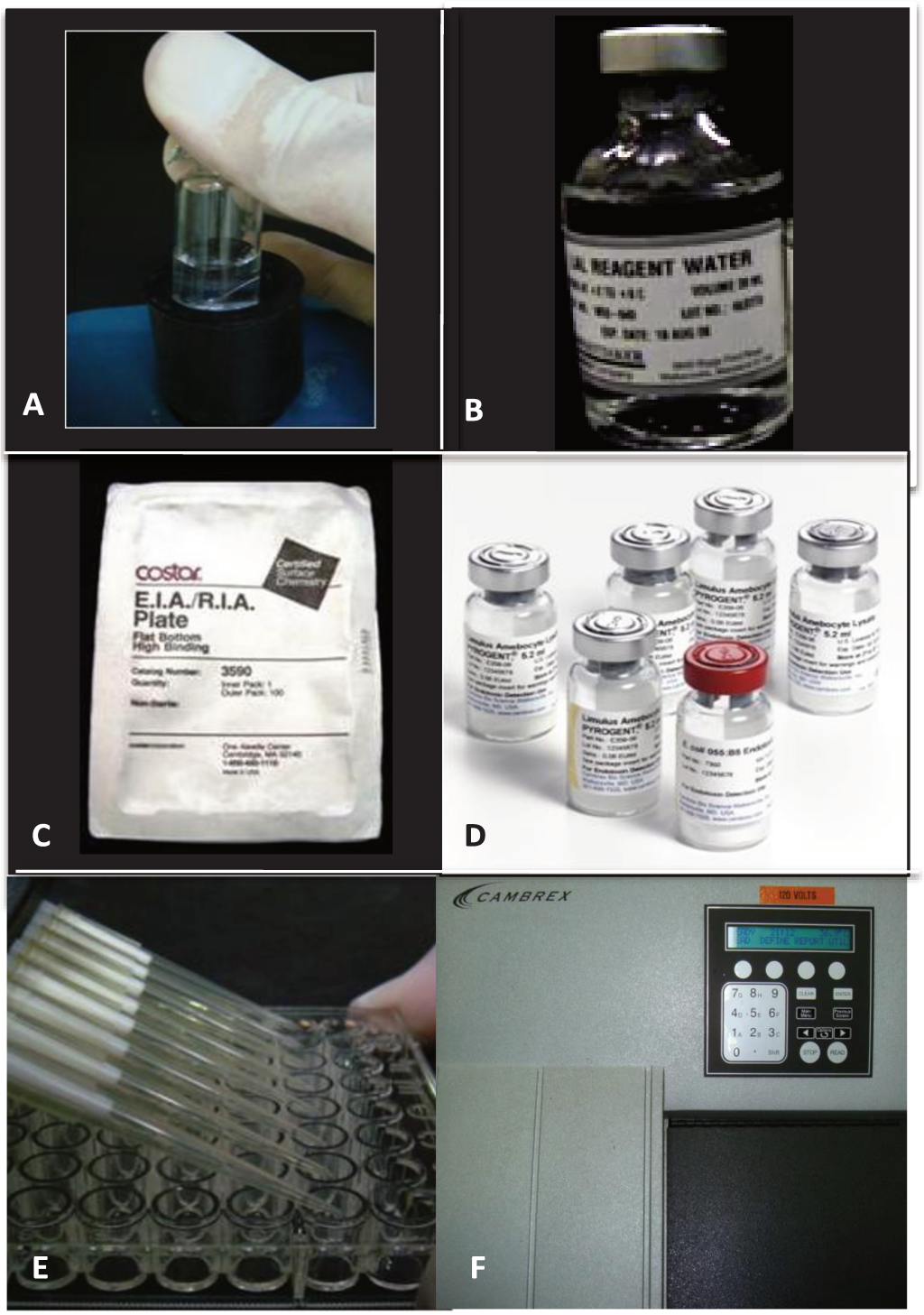


Figura 4. A. Extração de endotoxina em vortex por 60 seg; **B.** Água de LAL; **C.** Placas de 96 poços (Costar); **D.** Reagentes Kit pyrogent-5000; **E.** Inoculação das amostras, padrões, blank e substrato de LAL; **F.** Leitor de microplacas.

3.4. Avaliação dos efeitos citotóxicos do conteúdo do canal radicular – quantificação de citocinas inflamatórias

3.4.1. *Cultura Celular*

Para avaliação do potencial inflamatório endodôntico coletado dos canais radiculares foi utilizada a linhagem de macrófagos murinos imortalizada (RAW264.7) do Laboratório de Biologia Celular e Molecular da Faculdade de Odontologia de Araraquara – UNESP. As células foram mantidas em meio DMEM – Dulbecco's modified Eagle minimal essential medium supplemented (GIBCO) - enriquecido com 10% de soro fetal bovino inativado em calor, 100U/mL de penicilina e 100U/mL de estreptomicina (Invitrogen, Carlsbad, CA, EUA) em placa de polistireno de 100 mm (Corning Costar, Cambridge, MA). O meio de cultura foi trocado a cada 4 dias e as células mantidas em estufa a 37°C com 5% de CO_2, até confluência de 90%.

Em seguida as células foram suspensas em solução de tripsina-EDTA 0.25% (Invitrogen, Carlsbad, CA). A contagem de células viáveis foi realizada através da câmara de Neubauer, utilizando o teste de exclusão de azul de tripan.

3.3.2. *Estimulação celular*

Um total de 10^4 macrófagos viáveis foram colocados em cada poço da placa de poliestireno de 6 poços (Corning Costar), acrescentando meio para cultura DMEM enriquecido com 10% de soro fetal bovino e 100U/mL de antibiótico até obter o volume final de 1000 μL. As placas foram mantidas em estufa a 37°C com 5% de CO_2 durante 48 horas. Previamente a estimulação com o conteúdo do canal, as células foram desinduzidas por 8h em meio de cultura (DMEM) contendo 0.3% de soro fetal bovino, com finalidade de sincronizar o ciclo celular e reduzir a influência dos componentes do soro fetal nas células. Em seguida, os macrófagos foram estimulados com 60 microlitros do conteúdo endodôntico coletado dos canais radiculares e mantidas em estufa a 37°C com 5% de CO_2 . Todos experimentos foram realizados em duplicata.

Após 24 horas, os sobrenandantes foram coletados utilizando pipeta de 1000 μL

com ponteiras plásticas estéreis e armazenados em tubos plásticos estéreis de 1.5 mL, congelados a -80°C para futura quantificação de proteínas e citocinas inflamatórias (IL-1 ß, TNF-α e PGE_2).

Macrófagos aderidos no fundo dos poços foram removidos quimicamente com solução de Trizol® *reagent* (Invitrogen, Carlsbad, CA) juntamente com fluxo e refluxo utilizando ponteira de 1000 µL estéril. A determinação da viabilidade celular após o período de 24 horas de estimulação com o conteúdo endodôntico foi através da expressão de RNA-mensageiro (RNAm) para as citocinas inflamatórias - IL-1 ß, TNF-α e PGE_2 conforme descrito à seguir.

Figura 5 – <u>Crescimento/ estimulação celular</u> - A. Células congeladas (macrofagos RAW 264.7); **B.** Inoculação em meio DMEM + penicilina + Streptomicina + Soro fetal bovino; **C.** Incubação em estufa 5% CO_2 a 37° C; **D.** Verificação do crescimento celular até confluência de 90%; **E.** Distribuição celular em placa de 6 poços; **F.** Crescimento celular durante 48 horas (10^4 macrófagos) em estufa de CO_2 a 37° C. **G.** Contagem celular em câmara de Newbauer; **H.** Agitação em vortex do conteúdo endodôntico; **I.** Estimulação celular com 60 µL conteúdo endodôntico; **J.** Incubação em estufa de CO_2 a 37° C durante 24 horas; **K.** Coleta do sobrenadante; **L.** armazenamento em tubo plástico.

3.4.3. *Extração de RNA total, transcrição reversa e PCR*

RNA total das células foi extraído com o reagente Trizol, segundo o protocolo do fornecedor (Invitrogen Corp.). A quantidade e pureza do RNA foram determinadas em espectrofotômetro de luz UV (Biomate 3 - Thermo Electron Corporation) por meio da avaliação das absorbâncias a 260 nm e da relação entre as absorbâncias a 260/280 nm, respectivamente.

 # PROTOCOLO DE EXTRAÇÃO DE RNA E DOSAGEM EM ESPECTROFOTÔMETRO

Obs.: - antes de começar, ligar centrífuga e colocar a 4ºC e o banho seco a 55ºC

1. Aspirar meio de cultura da placa e lavar com 10 mL de PBS 1x
2. Adicionar 1mL de trizol, agitar por 5 minutos e coletar em eppendorf
3. Adicionar 200µL $CHCl_3$(clorofórmio) para cada 1mL de trizol agitar vigorosamente
4. Aguardar 2min
5. Centrifugar 14.000 rpm por 15 min a 4ºC
6. Transferir sobrenadante (líquido incolor-clorofórmio RNA e DNA) para outro eppendorf

 # Protocolo de RT-PCR:

1. Remover as amostras do freezer -80 graus e deixar descongelar em bancada dentro do gelo.
2. Para 900 mL (Trizol + Célula), acrescentar 180 mL de clorofórmio (CHCL3 – Qhemis, Catálogo: QHC028 1L).
3. Após acrescentar CHCL3, colocar os eppendorfs em rack e agitar vertendo forte contra outro rack.
4. Aguardar em bancada por 02 minutos.
5. Centrifugar 12.000 rpm durante 15 minutos à 4 graus.
6. Após esta etapa, formam-se 03 fases, cuidado na hora de remover a fase límpida

(fase intermediária).

Importante: cuidado para não puxar o possível anel rosa que forma logo acima da camada à ser retirada. E ainda, cuidado para não puxar alíquota esbranquiçada logo abaixo da fase intermediária.

7. No novo eppendorf com a fase límpida, acrescentar 250 mL de Isopropanol (Qhemis, Catálogo: QHA011 – 1L).
8. Agitar o ependorf, apenas vertendo lentamente 4 vezes.
9. Incubar na bancada em temperatura ambiente por 10 minutos.
10. Centrifugar 12.000 rpm durante 15 minutos à 4 graus.
11. Descartar o sobrenadante, vertendo o eppendorf, em seguida, verter o eppendorf em papel de filtro por 5 minutos e deixar na própria bancada.
12. Acrescentar 500 mL de Etanol 75% (não precisa ser álcool DNAse e RNAse free); agitar em vortex 2 segundos.
13. Centrifugar 12.000 rpm durante 15 minutos à 4 graus.
14. Verter o álcool e deixar o eppendorf secar na posição vertida em papel filtro na capela durante 1 hora.
15. Após secagem, acrescentar 20 mL de H2O (DNAse e RNAse free – GIBCO, Catálogo: 10977); incubar à 56 graus durante 5 minutos (em banho seco).
16. Centrifugar 12.000 rpm durante 15 segundos.
17. Em caso de armazenar, guardar em freezer à -80 C
18. Para leitura da concentração de RNA extraído em Nano Drop, utilizar 2mL;
- Para um RNA puro a leitura de A260/A280 deve estar em torno de 1.5 a 1.9;
- Programar o Nano Drop para nanograma/ microlitro.

Após a dosagem da concentração de RNA de cada amostra, deve-se realizar o cálculo abaixo, para uitilizar o kit RNAaqueous – 4 PCR (Catálogo: 1914-4).

19. O Kit de CDNA aceita até 2 microgramas de RNA, como concentração final. Mas na presente pesquisa, utilizamos 1 micrograma de RNA.

Para o cálculo, devemos realizar : C1V1= C2V2.

Exemplo: se o nanodrop acusar 500ng/microlitros, e a concentração de uso for determinada em 1 micrograma de RNA; então, se 1000 ng é = 1 micrograma, tem que utilizar 2 microlitros do eppendorf contendo o RNA para a reação.

Exemplo: 468 ng/microlitros ou seja 468 ng e, 1 microlitro.

20. 10 mL é o volume final a ser utilizado na reação de cDNA.

Exemplo: se a conta no item 19 foi de 2 mL do RNA, deve-se então acrescentar no 8 mL de H_2O para a realização da reação final no eppendorf de 10 mL. (10-2=8).

Importante: Nos casos em que a amostra apresentar dosagem muito baixa de RNA e com o cálculo não conseguir chegar na concentração de 1 micrograma para fazer o cDNA; deve-se excluir o volume da água da reação (não colocar 3,2 microlitros na reação de H2O); e na reação final acrescentar: 6,8 mL do mix + 13,2 mL de amostra de RNA.

Preparo do mix para reação:

Se for preparar o mix para analisar 32 amostras, preparar no eppendorf volume correspondente para 35 amostras.

Volume do mix para cada reação (para 35 amostras tem que multiplicar por 35 os respectivos volumes abaixo)

Mix:

- Buffer - 2 mL
- DNTP - 0,8 mL
- Randon - 2 mL
- Enzima multiscribe - `1 mL
- Inibidor – 1 mL
- H_2O – 3,2 mL

Importante: até adicionar a enzima pode agitar em vortex; manter todos os reagentes no Neb Cool durante o preparo do mix.

21. Após o preparo do mix, pegar eppendorf de 20 mL e enumerar de acordo com as amostras a serem analisadas.

22. Acrescentar em cada eppendorf o volume de H_2O calculado no item 20, em cada eppendorf.

23. Acrescentar em cada eppendorf o volume equivalente ao calculo do volume de RNA a ser utilizado (item 19)

23. Distribuir 10 mL do mix em cada um dos eppendorfs contendo 10 mL de H_2O + RNA – totalizando 20 mL de volume.

24. Programar no termociclador (MyCycler, Bio-Rad) o seguinte ciclo: 25 C – 10', 27 C 120', 85 C 5' e 4 C ∞ (Hold).

(Correr gel de agarose a 1,5% e corado com bormeto de etídeo, conforme PCR para DNA).

25. Pares de primers específicos para IL-1 ß, TNF-α, PGE_2 e para o gene gliceraldeído-3-fosfato desidrogenase (GAPDH) com seus respectivos ciclos estão apresentados na tabela 2. GAPDH foi utilizado como controle endógeno do RT-PCR por ser um gene constitutivo, cuja expressão não se altera.

26. A reação de PCR foi realizada em termociclador (MyCycler - Bio-Rad), com um volume total de 2 □µL, utilizando 2 µL do produto da reação de transcriptase reversa na presença de 100 pmol/□L de primers de cada gene (50 pmol/□L de cada primer, sense e antisense), além de uma concentração de $MgCl_2$ de 1,5 mM para GAPDH e 2,5 mM para IL-1 ß, TNF-α, PGE_2.

27. Os produtos da reação de PCR foram analisados por meio de eletroforese em gel de agarose a 1.5% (Invitrogen Corp.) e corados com brometo de etídeo (0.5 □g/mL – Invitrogen Corp.). Para documentação e análise, foram obtidas imagens digitalizadas destes géis (ImageQuant 100 – GE Healthcare, Sunnyvale, CA, EUA). A expressão do gene - alvo foi normalizada para a expressão do *housekeeping gene* (GAPDH).

Tabela 2. Seqüência dos primers e ciclos da reação de PCR (Camundongo).

Gene	Primers (5'- 3')	Ciclo	Amplicon
GADPH	CACCATGGAGAAGGCCGGGG GACGGACACATTGGGGTAG	95°C - 2 min e 25 ciclos de : 95°C 1 min, 52°C 1 min, 72°C 1 min extensão final: 72°C for 10 min.	418 bp
IL-1ß	GACCTGTTCTTTGAGGCTGA CGTTGCTTGTCTCTCCTTGT	95°C - 2 min e 35 ciclos de : 95°C 1 min, 58°C 1 min, 72°C 2 min extensão final: 72°C for 7 min.	494 bp
TNF-α	GGAGAACAGCAACTCCAGAA TCTTTGAGATCCATGCCATT	95°C - 2 min e 35 ciclos de : 95°C 1 min, 58°C 1 min, 72°C 2 min extensão final: 72°C for 7 min.	451 bp
PGE_2	TGCAACAGCTCAATGACTTCC GCCCCTCACGGACAATGTAGT	95°C - 2 min e 35 ciclos de : 95°C 1 min, 58°C 1 min, 72°C 2 min extensão final: 72°C for 7 min.	329 bp

3.4.4. Quantificação de proteína total – Coomassie (Bradford) kit.

Para quantificação total de proteína presente nos sobrendantes dos macrófagos após estimulação por conteúdo endodôntico foi utilizado método colorimétrico quantitativo – Coomassie (Bradford) (Pierce, Rockford, IL, EUA).

O kit Coomassie (Bradford) possui: 1) 1 frasco do Reagente Coomassie (Bradford) e 2) 10 Ampolas de albumina bovina padrão (2mg/mL) (BSA).

Inicalmente, 5 µL da curva padrão (quantidade de proteínas conhecidas), do blank (meio de cultura DMEM) e das amostras (sobrenadantes das células após estimulação com conteúdo endodôntico) foram distribuídos em placa de microtitulação de 96 poços (Corning Costar, Cambridge, MA, UK). Após, foi aplicado 250 µL do reagente Coomassie em cada poço, seguido de agitação, por 30 segundos. Em seguida, as placas foram incubadas em temperatura ambiente durante 10 minutos e as densidades ópticas (DO) lidas no leitor de microplacas (marca) com comprimento de onda de 595 nm. Todos as análises foram realizadas em duplicata.

PADRONIZAÇÃO DA CURVA PADRÃO

O estabelecimento de uma curva padrão com quantidade de proteínas conhecidas é importante e necessário para determinar a concentracão de proteínas com quantidades desconhecidas (Figura 6).

Esta curva foi preparada utilizando soluções concentradas de 2,000 µg/mL, 1,500 µg/mL, 1,000 µg/mL 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 25 µg/mL de BSA (albumina bovina).

Tabela 3. Estabelecimento da curva padrão nas diferentes concentricões (SE=Solução de estoque)

Tubos	Volume do diluente	Volume de BSA	Concentração final de BSA
A	0	300 µl da SE	2,000 µg/mL
B	125 µl	375 µl da SE	1,500 µg/mL
C	325 µl	325 µl da SE	1,000 µg/mL
D	175 µl	175 µl do tubo B	750 µg/mL
E	325 µl	325 µl do tubo C	500 µg/mL
F	325 µl	325 µl do tubo E	250 µg/mL
G	325 µl	325 µl do tubo F	125 µg/mL
H	400 µl	100 do tubo G	25 µg/mL

Para determinação da concentração final de proteínas presente em cada uma das amostras estudadas, o valor do *Blank* foi automaticamente subtraído de cada uma das amostras analisadas.

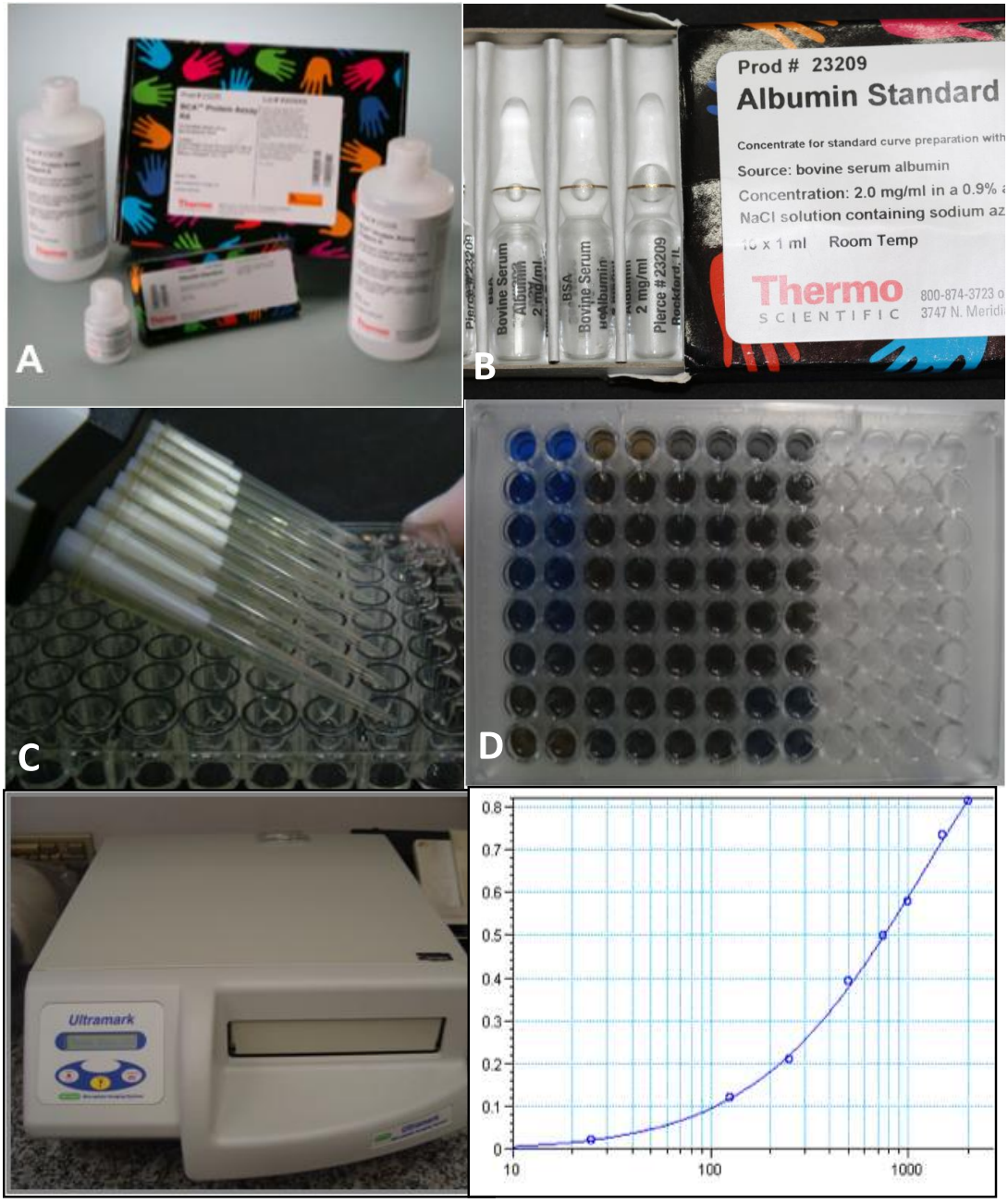


Figura 6. **<u>Quatificação de Proteína total</u>** – **A.** Kit Duoset IL1-ß; **B.** Albumina do kit para realização da curva padrão; **C.** Distribuíção e incubação dos padrões e amostras em da placa de 96 poços; **D.** Revelação da concentracão de proteína; **E.** Leitor de ELISA; **F.** Curva padrão.

3.4.5. *Quantificação de citocinas inflamatórias* IL-1 *β, TNF-α, PGE_2 (Teste imunoenzimático – ELISA)*

Análise da quantidade das citocinas inflamatórias - IL-1β, TNF-α e PGE_2 - liberadas em meio de cultura após estimulação por conteúdo endodôntico foi realizada através do teste imunoenzimático (ELISA), utilizando Duoset kits (R & D systems, Mineapolis MN, EUA).

TESTE IMUNOENZIMÁTICO (ELISA)- TÉCNICA SANDUÍCHE

Principo do teste (ELISA sanduíche)

Nesse método, o anticorpo de um antígeno particular é, inicialmente, adsorvido no poço da placa. Depois, o antígeno é adicionado e se liga ao anticorpo (AC). Em seguida, um segundo e diferente anticorpo ligado à enzima é adicionado (anticorpo biotinilado). Na sequência, é adicionado estreptovidina conjugada a peroxidase, onde a estreptovidina irá se ligar na biotina do AC secundário e a porção peroxidase ficará exposta para reagir com o substrato cromogênico. É então, adicionado o substrato cromogênico [(solução A (H_2O_2) + Solução B (Tetrametilbenzodina – TMB)]. No qual, a H_2O_2 sera quebrada pela peroxidase em H_2O + ½ O_2. Por sua vez, o O_2 irá agir sobre o TMB, deixando de incolor para azul. E finalmente é adicionado uma solução de parada "stop solution" (2 N H2SO4), tornando a reação amarela, e permitindo a mensuração da quantidade de antígeno presente na amostra, de acordo com a intensidade da cor amarela lida em espectofotômetro, numa densidade óptica (DO) de 450 nm.

Figura 7. Esquema ilustrativo da técnica sanduíche - ELISA.

Kit Duo-set (R&D System, Minneapolis, EUA)

O kit Duo-set apresenta: 1) Anticorpo de captura; 2) Anticorpo detecção biotinilado; 3) Padrão; 4) Streptovidina conjugada com peroxidase (*Streptavidin-Horseradish-peroxidase*) (Tabela 4).

Tabela 4. Anticorpo de captura, detecção e padrão dos kits Duo-set.

Citocina inflamatória	AC captura	AC detecção	Padrão
IL1- beta	goat anti-rat IL-1 beta	goat anti-rat IL1 beta biotinylated	recombinant rat Il1-beta
TNF-alpha	mouse anti-rat TNF-alpha	goat anti-rat TNF-alpha beta biotinylated	recombinant rat TNF-alpha
PGE2	goat anti-mouse PGE2	mouse anti-rat PGE2 biotinylated	recombiant rat PGE2

Padronização da curva padrão

O estabelecimento de uma curva padrão com quantidade de citocinas inflamatórias conhecidas foi necessário para determinar a concentração de citocinas inflamatórias de amostras com quantidades desconhecidas.

- Curva padrão para IL1- ß ou TNF-α: esta curva foi realizada utilizando a solução concentrada do padrão (recombiante de IL1-□ß) 4000 pg/mL e suas diluíções – 2000 pg/mL(picogramas/mL) , 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL e 62,5 pg/mL.

- Curva padrão para PGE_2: esta curva foi realizada utilizando a solução concentrada do padrão (recombiante de PGE_2) 25,000 pg/mL e suas diluíções – 25,000 pg/mL, 2500 pg/mL, 1250 pg/mL, 625 pg/mL, 313 pg/mL, 156 pg/mL, 78 pg/mL e 39 pg/mL.

Procedimento laboratorial

O teste imunoenzimático ELISA foi realizado no Laboratório de Endodontia da Faculdade de Odontologia de Piracicaba, São Paulo, Brasil.

Placas de microtitulação de 96 poços (*Corning Cell Culture Cluster*, Corning, EUA) foram sensibilizadas com anticorpo de captura anti- IL-1 ß, anti- TNF-α ou anti- PGE_2 de camundongo (100 µL/ poço) de acordo com a recomendação do fabricante.

As placas foram mantidas *overnight* em temperatura ambiente, em seguida, foram lavadas 3 vezes com solução tampão de lavagem (PBS 0.05% de Tween 20) e bloqueados com 300 µL de solução tampão de bloqueio (PBS com 1% de soro albumina bovina, BSA) por 1 hora em temperatura ambiente. Após, as placas foram novamente lavadas (3 vezes) com solução tampão de lavagem e incubadas com 100 µL dos padrões de citocinas inflamatórias com concentrações conhecidas (curva padrão) e 100 µL dos sobrenadantes da cultura de célula estimulados com conteúdo endodôntico durante 2 horas em temperatura ambiente. Todos realizados em triplicata.

Em seguida, as placas foram lavadas 3 vezes com sulução tampão de lavagem e incubadas com 100 µL de anticorpos de detecção anti- IL-1 ß, anti- TNF-α ou anti- PGE_2

marcados com biotina. As placas foram mantidas por 2 horas em temperatura ambiente e novamente lavadas. Após a lavagem, foi acrescentado 100 µL de streptavidina em cada poço e protegidas contra luz utilizando papel alumínio durante 20 minutos.

Uma nova lavagem foi realizada, e a reação revelada utilizando 100 µL de solução contendo substrato cromogênico e peróxido de hidrogênio em cada poço, e incubado por 20 minutos em temperatura ambiente, enrolado em papel alumínio. Em seguida, foi adicionado 50 µL do reagente de parada "Stop solution" em cada poço – ácido sulfúrico 2N. A densidade óptica (DO) de cada poço foi imediatamente determinada no leitor de microplacas com comprimento de onda de 405 nm.

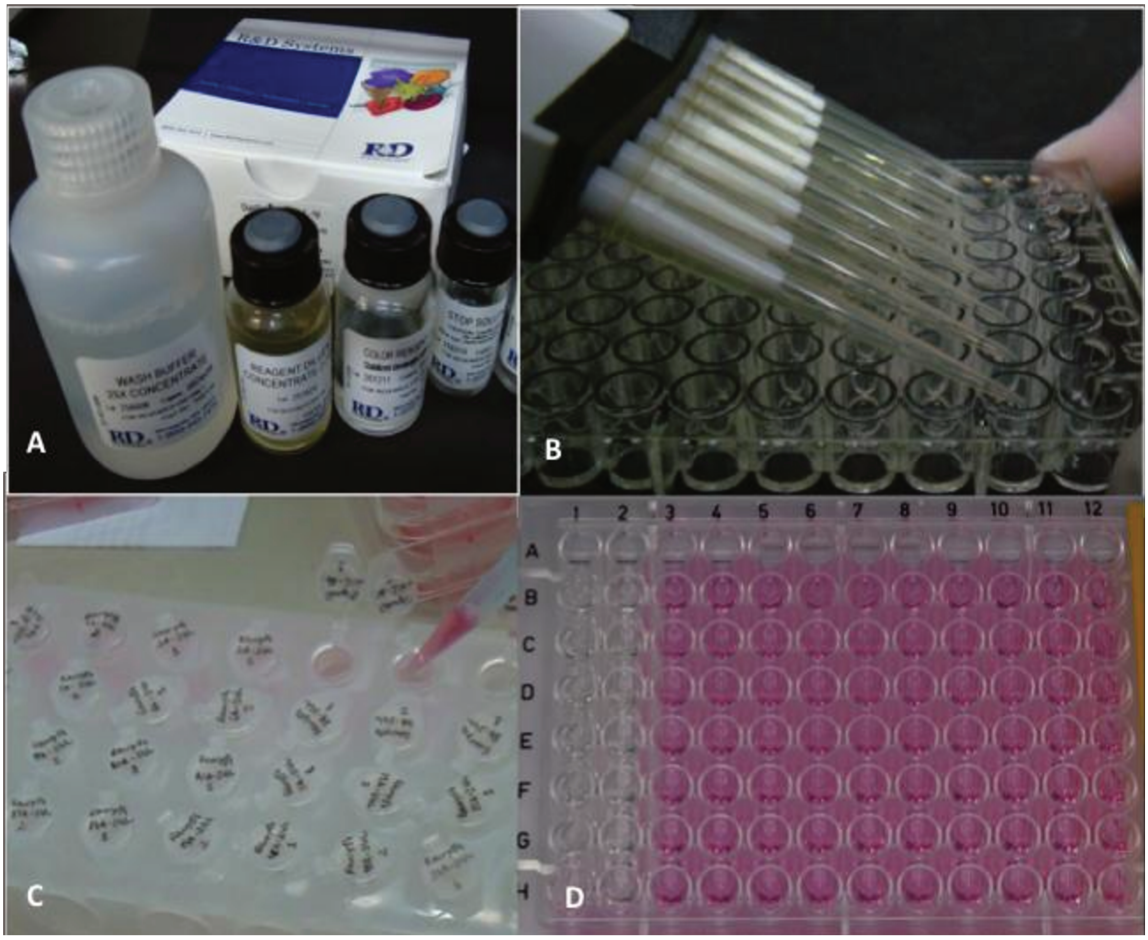


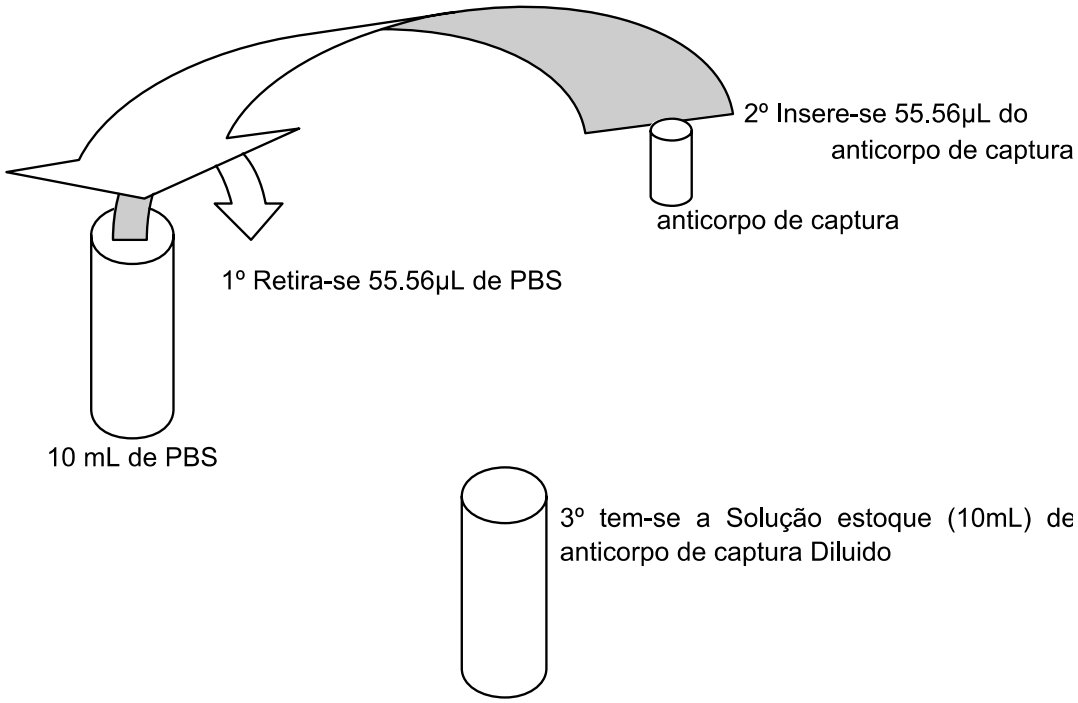
Figura 8. **Kit Duo-set ELISA** – **A.** Kit Duoset IL1-ß; **B.** Sensibilização da placa com anticorpo de captura; **C-D.** Distribuição e incubação da placa com padrões e amostras.

APÊNDICE II – Manual de dosagem de citocinas pró-inflamatórias (Kit R&D System)

*** DuoSet ELISA rat IL-β**

#Enquanto as amostras descongelam, fazer:
#Preparo das amostras
Placa de 96 wells
Preparo dos reagentes
-todos os reagentes devem estar em temperatura ambiente.

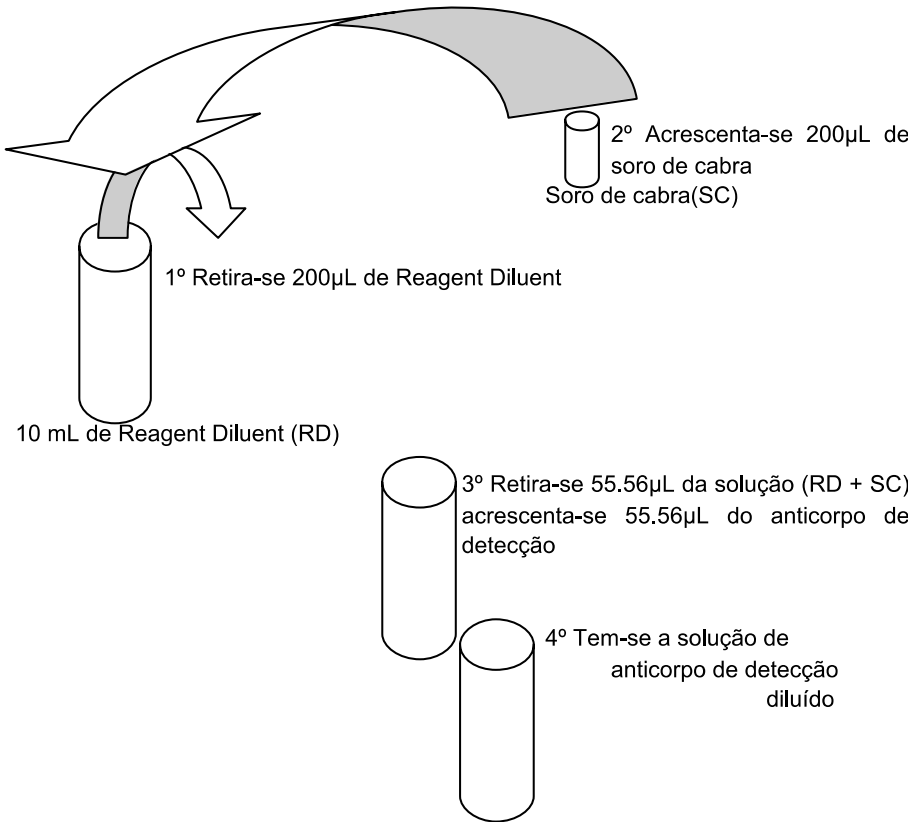
Anticorpo de Captura (Part 840414, 1 vial)-144µg/mL de anti-rat IL-1β que deve ser reconstituído com 1.0mL de PBS. Após reconstituição, estocar a 2-8°C por máximo de 60 dias ou alíquota e estoque de -20°C a -70°C em freezer por máximo de 6 meses. Diluir a concentração de trabalho de 0.8µg/mL em PBS.
Uso:
C1.V1=C2.V2
144.V1=0.8.10.000µL (p/ 1 placa)
V1=55,56µL do anticorpo de captura
Com um tubo contendo 10 mL de PBS, retira-se o volume de 55.56 µL de PBS, ou seja,10 .000 µL – 55,56 µL;
Em seguida, insere-se, neste mesmo tubo, 55.56 µL do anticorpo de captura e tem-se a **solução estoque do anticorpo de captura diluído** (q.s.p).
Faz 17 alíquotas de 55,56µL de anticorpo de captura.



Esquema 1. preparo do anticorpo de captura.

Anticorpo de Detecção (Part 840415,1vial)- 63µg/mL de anti-rat IL-1β biotinilado de cabra que deve ser reconstituído com 1.0mL do Reagent Diluent (ver soluções

necessárias). Após reconstituição, estocar a 2-8°C por máximo 60 dias ou alíquota e estocar de -20°C a -70°C em um freezer por no máximo 6 meses. Diluir uma concentração de trabalho de 350ng/mL em Reagent Diluent (R&D Systems).
Uso:
C1.V1=C2.V2
63.000.V1=350.10.000 (p/ 1 placa)
V1=55,56µL do anticorpo de detecção
200µL de soro de cabra
Com um tubo contendo 10 mL de Reagent Diluent , retira-se o volume de 200 µL (referente ao soro de cabra que será colocado) do Reagent Diluent, ou seja,10 .000 µL – 200 µL;Em seguida, acresecenta-se os 200µLde soro de cabra. Em seguida, neste mesmo tubo, retira-se o volume de 55.56 µL (referente ao anticorpo de detecção que será adicionado) e acrescenta-se o volume de 55.56µL do anticorpo de detecção. Aí tem-se a **solução estoque de anticorpo de detecção diluído** (q.s.p).
Faz 17 alíquotas de 55,56µL de anticorpo de detecção.



Esquema 2. preparo do anticorpo de detecção.

Standard (Part 840416, 1 vial)- 63µg/mL de recombinante rat IL-1β que deve ser reconstituído com 0.5mL do Reagent Diluent (500µL RD) (ver soluções necessárias).

Permitir a padronização do local por um mínimo de 15 minutos agitando gentilmente antes de fazer as diluições. Estocar standard reconstituído a 2-8°C por no máximo 60 dias ou alíquota e estocar a -70°C em freeezer por no máximo 6 meses. Uma curva standard de sete pontos usa-se 2 diluições seriadas em Reagent Diluent, e um alto Standard de 4000pg/mL é recomendado.

Uso:
C1.V1=C2.V2
100.000.V1=4000.600
V1=24 µL do Standard
Com um tubo contendo 600 µL de Reagent Diluent , retira-se o volume de 24 µL do Reagent Diluent, ou seja, 600 µL – 24 µL;Em seguida, acrescenta-se, neste mesmo tubo, 24µL do Standard e tem-se a **solução estoque Standard diluído** (q.s.p). Pegar o valor EXATO de 24µL.
Faz 15 alíquotas de 30µL do Standard.

Streptavidin-HRP (Part 890803, 1 vial)- 1.0mL de streptavidin conjugado a horsedish-peroxidase. Estocar a 2-8°C por no máximo 6 meses após início de uso. NÃO COLOCAR NO FREEZER! Diluir a concentração de trabalho especificado no rótulo do frasco usando 200µL de Reagent Diluent (ver soluções necessárias).
1µL de streptavidin - 200µL Reagent Diluent vol. Final
X - 12000µL vol. Reagent Diluent
X= 60µL de strepta
Com um tubo contendo 12mL de Reagent Diluent , retira-se o volume de 60µL do Reagent Diluent;
Em seguida, acrescenta-se, neste mesmo tubo, 60µL do streptavidin e tem-se a **solução estoque de streptavidin diluído** (q.s.p). (p/1 placa)

Soluções necessárias-
PBS- 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2 - 7.4, 0.2 µm filtrado.

8,02g NaCl
0,201g KCl
2,9g de Na2HPO4.12H2O (2,9g)
0,21g KH2PO4
q.s.p. 1 litro de água destilada – pH 7,2-7,4
Preparar 1 dia antes no máximo) e manter sempre em geladeira. O PBS deve ser sempre NOVO, pois pode interferir no resultado da leitura!

Wash Buffer (Tampão de Lavagem) (WB) diluido- 0.05% Tween® 20 em PBS, pH 7.2 - 7.4 (R&D Systems Catalog # WA126).
20mL de WB +480mL de água destilada

Reagent Diluent (RD) diluido - 1% BSA em PBS, pH 7.2 - 7.4, 0.2 µm filtrado (R&D Systems Catalog # DY995). Qualidade do Bovine Serum Albumin (BSA) é critica, sendo necessário utilizar de alta qualidade pois é crucial para o bom desenvolvimento do teste DuoSet ELISA.

Diluir 10x em água destilada: 5mL de Reagent Diluent concentrado em 45mL de água destilada. (p/ 1 placa)

Substrate Solution - 1:1 mistura do Color Reagent A (H2O2) e Color Reagent B (Tetramethylbenzidine) (R&D Systems Catalog # DY999).
Misturar V:V Solução A+ Solução B
5.5mL de solução A + 5.5mL de solução B
Fazer no dia do uso.

$$H_2O_2 \xrightarrow{\text{peroxidase}} H2O + 1/2O_2$$

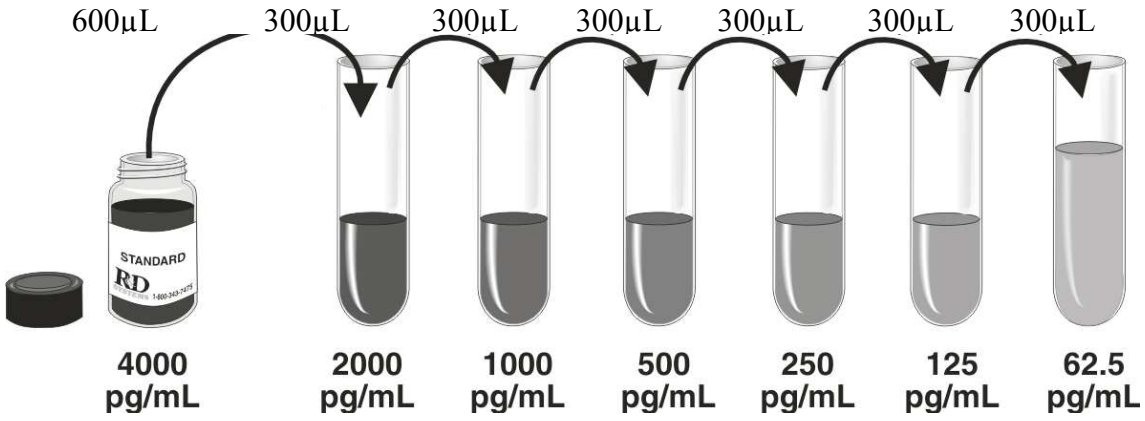
TMB (incolor) ⟶ TMB (azul)

Reação diretamente proporcional aproximadamente 20 a 30 minutos de incubação.

Stop Solution - 2 N H2SO4 (R&D Systems Catalog # DY994). Cuidado é ácido proteja-se! Usa-se como está! (A reação ficará amarela)

Solução de Streptavidin diluido- 60µL de streptavidin em 11,40mL de Reagent Diluent. Fazer no dia do uso.

Diluição da Curva Padrão com o Standard



Esquema 3. diluição da curva padrão.

Protocolo Geral Elisa

Preparo da placa

Fase de Sensibilização

1. Adicionar 100 µL do Anticorpo Capture diluido, agitar gentilmente (virando de cima para baixo o eppendorf antes de inserir no well) em cada well. Selar a placa e incubar overnight em temperatura ambiente (25°C).
2. Aspirar cada well e descartar. Após, lavar com Wash Buffer (WB) diluído, repetindo o processo com um total de 3 lavagens. Para o descarte deve-se virar a placa com força, ou seja fazendo movimento de saque de volei! Lavar com 300µL (400 transborda) de WB em cada vez e em cada well. Realizar a remoção do total do líquido. Após a última lavagem remover todo resíduo do WB por aspiração ou invertendo a placa e batendo (com muita força!) contra um papel toalha (absorvente) limpo na bancada. Não pode ficar nenhum liquido na placa e nenhuma bolha! VERIFICAR SE HOUVE FORMAÇÃO DE BOLHAS nos wells após o procedimento de secagem, onde houver, remove-las com a ponta de uma ponteira de pipeta com muito cuidado, (NÃO TOCAR NO FUNDO DA PLACA), utilizando uma ponteira para cada well! Não repetir a ponteira, UTILIZAR UMA PONTEIRA EM CADA WELL! Usou, descarta!;

Fase de Bloqueio

3. Adicionar 300µL do Reagent Diluent diluído em cada well, agitar gentilmente o RD (virando de cima para baixo o eppendorf) antes de inserir no well. Após inserir, incubar em temperatura ambiente por no mínimo de 1 hora.(Albumina bloqueando) Enquanto ocorre o bloqueio fazer a Diluição da Curva padrão (Standard).
4. Repetir a aspiração/lavagem (3x) como no item 2. As placas serão lidas com a adição da amostra.

Procedimento do teste

1. Adicionar 100µL de amostras (devem ser agitadas gentilmente virando de cima para baixo, antes de inserir nos wells) e Standards diluido (agitar no vortex) (Ver mapa**), em cada well. Cobrir com a fita adesiva e incubar por 2 horas em temperatura ambiente. Após as 2 horas descarta vertendo a placa ou aspirando.
   Ex de Mapa a seguir**:

Faça o seu Layout da Placa para auxiliar na colocação das amostras (para não se perder):
**mapa da placa

	1	2	3	4	5	6	7	8	9	10	11	12
A	4000	4000	Control	Control	8ª	8A	16A	16ª				
B	2000	2000	1Amostra	1Amostra	9ª	9	17	17				
C	1000	1000	2Amostra	2	10A	10	18	18				
D	500	500	3Amostra	3	11A	11	19	19				
E	250	250	4Amostra	4	12	12	20	20				
F	125	125	5Amostra	5	13	13	...	....				
G	62.5	62.5	6Amostra	6	14	14	....	....				
H	0	0	7Amostra	7	15A	15						

4000, 2000, 1000, 500, 250, 125, 62,5 são a curva Padrão. As amostras são colocadas em duplicata para validar o teste (confiabilidade)

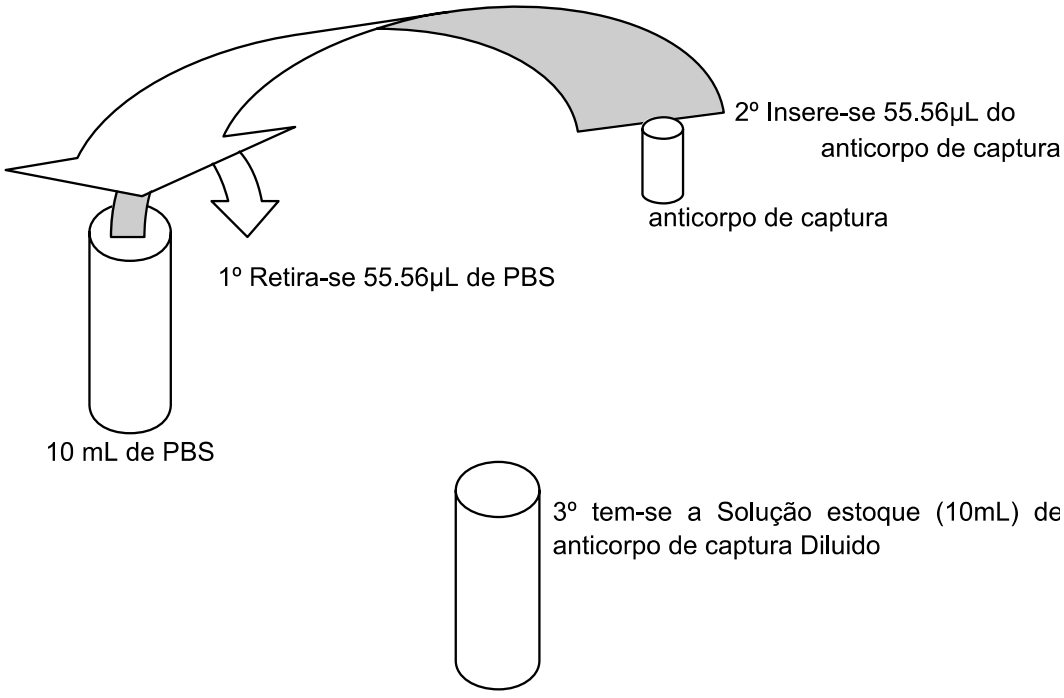
2. Repetir a aspiração/lavagem (3x) como no item 2 do preparo da placa.
3. Adicionar 100µL do anticorpo de Detecção diluido em cada well. Cobrir com uma nova fita adesiva e incubar por 2 horas em temperatura ambiente. Após as 2 horas verter a placa ou aspirar.
4. Repetir a aspiração/lavagem (3x) como no item 2 do preparo da placa.
5. Adicionar 100µL da solução de Streptavidin-HRPdiluida em cada well. Cobrir a placa e incubar por 20 minutos em temperatura ambiente. Evitar que a placa fique em contato direto com a luz. Embalar a placa em papel aluminio! Após os 20 min. verter a placa.
6. Repetir a aspiração/lavagem (3x) como no item 2 do preparo da placa.
7. Adicionar 100µL de Substrate solution (A+B) em cada well. Incubar por 20 minutos em temperatura ambiente. Evitar que a placa fique em contato direto com a luz. Embalar a placa em papel aluminio! De incolor ficará azul durante o período do processo de revelação. Após os 20 min. descarta.
8. Adicionar 50µL de Stop solution (cuidado é um acido se proteja!) em cada well. Misturar gentilmente batendo na mesa a placa. A reação de azul ficará amarela imediatamente, variando de acordo com a concentração amarelo forte para mais claro. Está pronto para a leitura. Os wells das amostras devem ficar com a coloração amarela mais clara que os wells do Standard (da curva padrão). Pois as amostras devem ficar com valores dentro da curva padrão na hora da leitura Elisa.
9. Determinar a densidade ótica de cada well imediatamente, usando o leitor com **450**nm.
   A correção é feita em 540ou 570nm.

Não deve ser usado com a data de validade vencida;
Recomenda-se o uso do R&D systems Reagent Diluent ou o uso de Milipore BSA livre de protease;
Realizar o teste em duplicata para os Standards e amostras;
Evitar contaminação microbiana de reagentes e liquido de lavagem. Isto pode interferir na sensibilidade do teste.
Buffers contendo grand quantidade de proteína devem ser feitos sob condições estéreis e estocados a 2-8°C ou preparados diariamente conforme o uso.
O Grafico produzido no leitor Elisa deve ser realizado em 4 parâmetros.

*** DuoSet ELISA rat TNF-α**

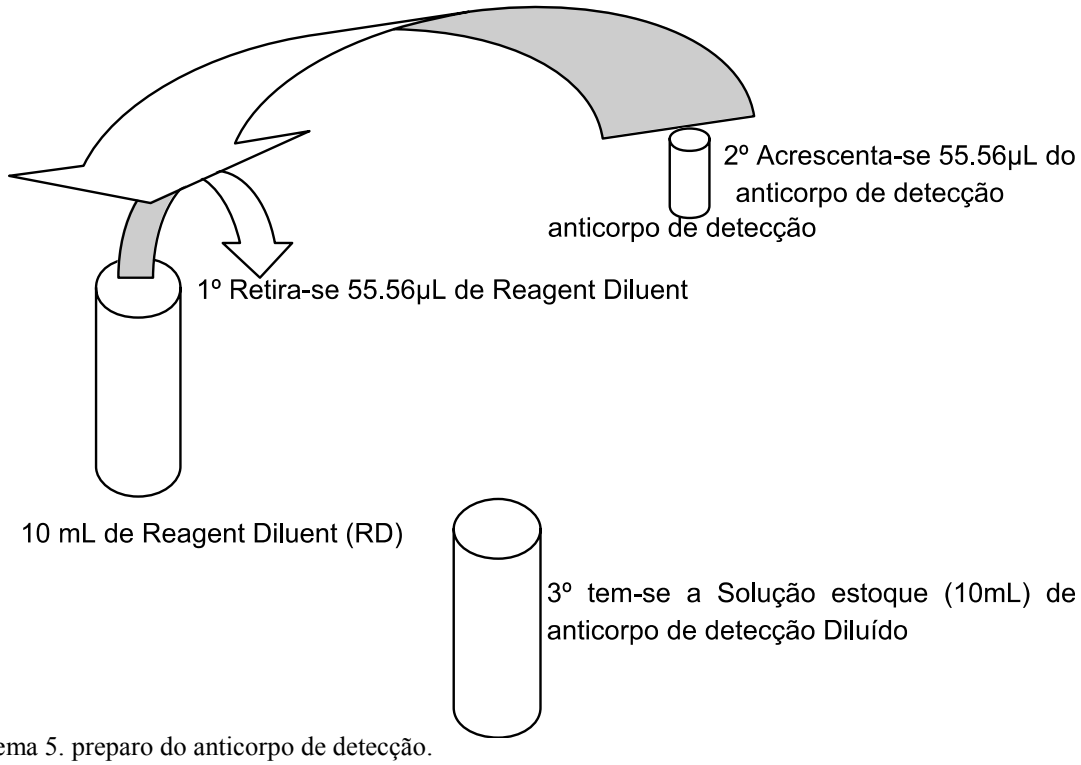
#Enquanto as amostras descongelam, fazer:
#Preparo das amostras
Placa de 96 wells
Preparo dos reagentes
-todos os reagentes devem estar em temperatura ambiente.

Anticorpo de Captura (Part 840175, 1 vial)-720µg/mL de anti-rat TNF-α de rato que deve ser reconstituído com 1.0mL de PBS. Após reconstituição, estocar a 2-8°C por máximo de 60 dias ou alíquota e estoque de -20°C a -70°C em freezer por máximo de 6 meses. Diluir a concentração de trabalho de 4.0µg/mL em PBS.
Uso:
C1.V1=C2.V2
720.V1=4.10.000 (p/ 1 placa)
V1=55,56µL do anticorpo de captura
Com um tubo contendo 10 mL de PBS, retira-se o volume de 55.56 µL de PBS, ou seja,10 .000 µL – 55,56 µL;
Em seguida, acrescenta-se, neste mesmo tubo, 55.56 µL do anticorpo de captura e tem-se a **solução estoque do anticorpo de captura diluído** (q.s.p).
Faz 17 alíquotas de 55,56µL de anticorpo de captura.



Esquema 4. preparo do anticorpo de captura.

Anticorpo de Detecção (Part 840175,1vial)- 18µg/mL de anti-rat TNF-α biotinilado de cabra que deve ser reconstituído com 1.0mL do Reagent Diluent (ver soluções necessárias). Após reconstituição, estocar a 2-8°C por máximo 60 dias ou alíquota e estocar de -20°C a -70°C em um freezer por no máximo 6 meses. Diluir uma concentração de trabalho de 100ng/mL em Reagent Diluent concentrado (R&D Systems).
Uso:
C1.V1=C2.V2
18.000.V1=100.10.000 (p/ 1 placa)
V1=55,56µL do anticorpo de detecção
Com um tubo contendo 10 mL de Reagent Diluent , retira-se o volume de 55.56 µL do Reagent Diluent, ou seja,10 .000 µL – 55,56 µL;
Em seguida, acrescenta-se, neste mesmo tubo, 55.56 µL do anticorpo de detecção e tem-se a **solução estoque de anticorpo de detecção diluído** (q.s.p).
Faz 17 alíquotas de 55,56µL de anticorpo de detecção.



Esquema 5. preparo do anticorpo de detecção.

Standard (Part 840176, 1 vial)- 140ng/mL de recombinante rat TNF-α que deve ser reconstituído com 0.5mL do Reagent Diluent (500µL RD) (ver soluções necessárias). Permitir a padronização do local por um mínimo de 15 minutos agitando gentilmente antes de fazer as diluições. Estocar standard reconstituído a 2-8°C por no máximo 60 dias ou alíquota e estocar a -70°C em freeezer por no máximo 6 meses. Uma curva standard de sete pontos usa-se 2 diluições seriadas em Reagent Diluent, e um alto Standard de 4000pg/mL é recomendado.
Uso:
C1.V1=C2.V2
140.000.V1=4000.600
V1=17,14 µL do Standard
Com um tubo contendo 600 µL de Reagent Diluent , retira-se o volume de 17.14 µL do Reagent Diluent, ou seja, 600 µL – 17.14 µL;
Em seguida, acrescenta-se, neste mesmo tubo, 17.14µL do Standard e tem-se a **solução estoque Standard diluído** (q.s.p). Pegar o valor EXATO de 17.14µL.
Faz 25 alíquotas de 55,56µL do Standard.

Streptavidin-HRP (Part 890803, 1 vial)- 1.0mL de streptavidin conjugado a horsedish-peroxidase. Estocar a 2-8°C por no máximo 6 meses após início de uso.
1µL de streptavidin - 200µL Reagent Diluent vol. Final
X - 12000µL vol. Reagent Diluent
X= 60µL de strepta
Com um tubo contendo 12mL de Reagent Diluent , retira-se o volume de 60µL do Reagent Diluent;
Em seguida, acrescenta-se, neste mesmo tubo, 60µL do streptavidin e tem-se a **solução estoque de streptavidin diluído** (q.s.p). (p/1 placa)

Soluções necessárias-
PBS- 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2 - 7.4, 0.2 µm filtrado.

8,02g NaCl
0,201g KCl
2,9g de Na2HPO4.12H2O (2,9g)
0,21g KH2PO4
q.s.p. 1 litro de água destilada – pH 7,2-7,4
Preparar 1 dia antes no máximo) e manter sempre em geladeira. O PBS deve ser sempre NOVO, pois pode interferir no resultado da leitura!

Wash Buffer (Tampão de Lavagem) (WB) diluido- 0.05% Tween® 20 em PBS, pH 7.2 - 7.4 (R&D Systems Catalog # WA126).
20mL de WB +480mL de água destilada

Reagent Diluent (RD) diluido - 1% BSA em PBS, pH 7.2 - 7.4, 0.2 µm filtrado (R&D Systems Catalog # DY995). Qualidade do Bovine Serum Albumin (BSA) é critica, sendo

necessário utilizar de alta qualidade pois é crucial para o bom desenvolvimento do teste DuoSet ELISA.

Diluir 10x em água destilada: 5mL de Reagent Diluent concentrado em 45mL de água destilada. (p/ 1 placa)

Substrate Solution - 1:1 mistura do Color Reagent A (H2O2) e Color Reagent B (Tetramethylbenzidine) (R&D Systems Catalog # DY999).
Misturar V:V Solução A+ Solução B
5.5mL de solução A + 5.5mL de solução B
Fazer no dia do uso.

$$H_2O_2 \xrightarrow{\text{peroxidase}} H2O + 1/2O_2$$

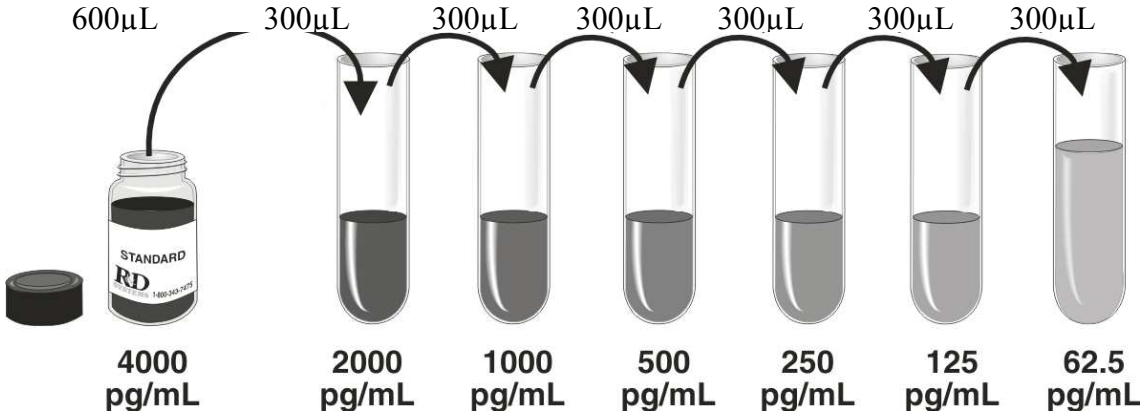
TMB (incolor) ⟶ TMB (azul)

Reação diretamente proporcional aproximadamente 20 a 30 minutos de incubação.

Stop Solution - 2 N H2SO4 (R&D Systems Catalog # DY994). Cuidado é ácido proteja-se! Usa-se como está! (A reação ficará amarela)

Solução de Streptavidin diluido- 60µL de streptavidin em 11,40mL de Reagent Diluent. Fazer no dia do uso.

Diluição da Curva Padrão com o Standard



Esquema 6. diluição da curva padrão.

APÊNDICE III – Dados demográficos, características clínicas e radiográficas do elemento dental presente nos pacientes que participaram da pesquisa.

					Presença de dor							
Amostra	Caso	idade	dente	Gênero	Dor à percussão	Pdor à alpação	Percussão/Palpação	Dor previa	Tamanho da lesão	Canal	Fístula	Espécies bacterianas "alvo" detectadas PCR
1	1	73	44	Masculino	Sim	Sim	Sim	1	1.5	Molhado	Ausente	*Prevotella nigrescens*
												Treponema denticola
2	2	38	46	Masculino	Sim	Sim	Sim	0	3	Seco	Ausente	*Dialister pneumosintes*
												Prevotella intermedia
												Treponema socranskii
3	3	40	31	Masculino	Sim	Sim	Sim	1	3	Molhado	Ausente	*Prevotella nigrescens*
4	4	39	14	Masculino	Sim	Sim	Sim	0	1.5	Seco	Ausente	*Não detectado*
5	5	37	22	Feminino	Não	Não	Não	0	4	Molhado	Ausente	*Prevotella nigrescens*
												Porphyromonas endodontalis
												Treponema socranskii
6	6	13	36	Masculino	Não	Não	Não	1	1.5	Molhado	Presente	*Fusobacterium nucleatum*
7	7	52	23	Feminino	Não	Sim	Não	0	0.5	Molhado	Ausente	*Porphyromonas endodontalis*
												Fusobacterium nucleatum
8	8	22	36	Feminino	Não	Não	Não	1	4	Molhado	Ausente	*Prevotella nigrescens*
9	9	50	44	Feminino	Não	Não	Não	0	4	Molhado	Ausente	*Prevotella nigrescens*
10	10	28	22	Feminino	Não	Não	Não	1	3	Seco	Ausente	*Prevotella nigrescens*
11	11	13	46	Feminino	Não	Não	Não	1	4	Molhado	Presente	*Prevotella nigrescens*
												Filifactor alocis
												Treponema socranskii
												Parvimonas micra
												Fusobacterium nucleatum
												Porphyromonas endodontalis
12	12	39	26	Masculino	Não	Não	Não	0	3	Seco	Ausente	*Prevotella nigrescens*
												Porphyromonas endodontalis
												Parvimonas micra
												Fusobacterium nucleatum
13	13	14	46	Feminino	Sim	Não	Não	0	1.5	Molhado	Ausente	*Porphyromonas endodontalis*
												Treponema socranskii
												Fusobacterium nucleatum
14	14	43	12	Feminino	Sim	Sim	Sim	0	10	Seco	Ausente	*Treponema denticola*
												Porphyromonas endodontalis
												Treponema socranskii
												Parvimonas micra
15	16	30	35	Masculino	Não	Não	Não	0	4	Seco	Ausente	*Prevotella nigrescens*
												Treponema denticola
16	17	51	34	Feminino	Não	Não	Não	0	4.5	Seco	Ausente	*Filifactor alocis*
												Parvimonas micra
17	18	56	42	Masculino	Não	Não	Não	0	1.5	Seco	Ausente	*Não detectado*
18	19	47	45	Feminino	Sim	Sim	Sim	0	1.5	Molhado	Ausente	*Prevotella nigrescens*
												Parvimonas micra
19	20	20	14	Masculino	Sim	Sim	Sim	1	1	Molhado	Ausente	*Prevotella nigrescens*
20	21	52	46	Masculino	Não	Sim	Não	0	1	Molhado	Ausente	*Prevotella nigrescens*
												Fusobacterium nucleatum
21	22	41	25	Masculino	Não	Não	Não	0	1.5	Seco	Ausente	*Prevotella nigrescens*
												Treponema socranskii

APÊNDICE IV – Produção bibliográfica do aluno

1. Martinho FC, Gomes BPFA. Quantification of endotoxins and cultivable bacteria in root canal infection before and after chemomechanical preparation with 2.5% sodium hypochlorite.J Endod. 2008;34:268-72

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7. Martinho FC, Chiesa WMM, Leite FRM, Cirelli JA, Gomes BPFA. Investigation of Treponema spp. and endotoxin in primary endodontic infection and evaluation of the antigenivity of the infectious contente against Raw 264.7 macrophages by the

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10. Martinho FC, Chiesa WMM, Leite FRM, Cirelli JA, Gomes BPFA. Investigation of Endotoxin in primary root canal infection and its correlation with the levels of IL-1 beta, TNF-alpha, PGE2, IL-6 produced by Raw macrophages 264.7 cells. (Submmited to Oral medicine, Oral pathogology, Oral radiology and Endodontics).

11. Martinho FC, Chiesa WMM, Leite FRM, Cirelli JA, Gomes BPFA. Comparison of the residual citotoxic activity of endodontic contents against macrophages (Raw 264.7) after chemomechanical preparation with NaOCl and CHX-gel. (Submitted to Journal of endodontics).

12. Martinho FC, Chiesa WMM, Leite FRM, Cirelli JA, Gomes BPFA. Detection of IL-12 p70 in primary endodontic infection by Cytometric Bead Array. (Submitted to jornal of endodontics).

13. Endo MS, Martinho FC, Gomes BP. Quantification of cultivable bacteria and endotoxin in postreatment apical periodontitis before and after chemomechanical preparation (Submmited to International Endodontic jornal).

14. Nobrega LM, Delboni MG, Martinho FC, Gomes BP. Detection of diferente oral

Treponema species in endodontic failure (Submmited to Oral medicine, Oral pathogology, Oral radiology and Endodontics).

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

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Análise microbiológica e estudo de endotoxinas de canais radiculares infectados"**, protocolo nº 103/2008, dos pesquisadores Brenda Paula Figueiredo de Almeida Gomes, Ariane Cássia Salustiano Marinho, Frederico Canato Martinho, Letícia Maria Menezes Nóbrega, Thais Mageste Duque e Wanderson Miguel Maia Chiesa, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 16/12/2010.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Microbiological analysis and investigation of endotoxin in infected root canals"**, register number 103/2008, of Brenda Paula Figueiredo de Almeida Gomes, Ariane Cássia Salustiano Marinho, Frederico Canato Martinho, Letícia Maria Menezes Nóbrega, Thais Mageste Duque and Wanderson Miguel Maia Chiesa, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 12/16/2010.

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

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