

JULIA MOURÃO BRAGA DINIZ

EFEITO DO MTA FILLAPEX SOBRE A ATIVIDADE
DE MACRÓFAGOS PERITONEAIS

FACULDADE DE ODONTOLOGIA
UNIVERSIDADE FEDERAL DE MINAS GERAIS
BELO HORIZONTE
2013

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EFEITO DO MTA FILLAPEX SOBRE A ATIVIDADE DE MACRÓFAGOS PERITONEAIS

Dissertação apresentada ao Colegiado do programa de Pós-Graduação
da Faculdade de Odontologia da Universidade Federal de
Minas Gerais, como requisito parcial para a obtenção
do grau de Mestre em Odontologia - área de
concentração em Endodontia

Orientador: Prof. Dr. Antônio Paulino Ribeiro Sobrinho
Co-Orientador(a): Prof(a). Dr(a). Leda Quercia Vieira

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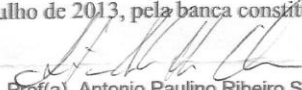
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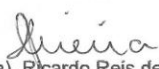
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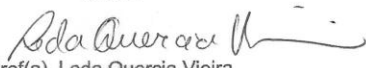
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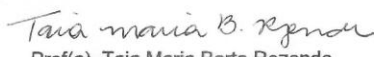
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Aprovada em 10 de julho de 2013, pela banca constituída pelos membros:


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Universidade Católica de Brasília

Belo Horizonte, 10 de julho de 2013.



UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA



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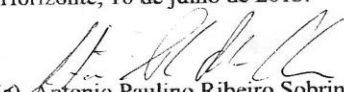
Realizou-se, no dia 10 de julho de 2013, às 14:30 horas, Faculdade de odontologia, da Universidade Federal de Minas Gerais, a defesa de dissertação, intitulada *O efeito do Mineral Trioxide Aggregate e do MTA Fillapex sobre a atividade de macrófagos M1 e M2.*, apresentada por JULIA MOURÃO BRAGA DINIZ, graduada no curso de ODONTOLOGIA, como requisito parcial para a obtenção do grau de Mestre em ODONTOLOGIA, à seguinte Comissão Examinadora: Prof(a). Antonio Paulino Ribeiro Sobrinho - Orientador (UFMG), Prof(a). Ricardo Reis de Oliveira (UFMG), Prof(a). Leda Quercia Vieira (UFMG), Prof(a). Taia Maria Berto Rezende (Universidade Católica de Brasília).

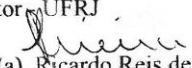
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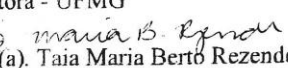
Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.

Belo Horizonte, 10 de julho de 2013.


Prof(a). Antonio Paulino Ribeiro Sobrinho
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DEDICATÓRIA

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RESUMO

A obturação do sistema de canais radiculares após a limpeza e modelagem é fundamental para o sucesso do tratamento endodôntico. Os materiais obturadores devem ter propriedades físicas e químicas favoráveis. Além disso, é altamente desejável que sejam biocompatíveis, porque, muitas vezes, são colocados em contacto íntimo com os tecidos periapicais, através do forame e das comunicações acessórias apicais. O Agregado Trióxido Mineral (MTA) é conhecido por sua excelente biocompatibilidade e capacidade de vedação. No entanto, apesar das suas características favoráveis, o MTA não apresenta as propriedades físicas necessárias para ser usado como cimento obturador. Em uma tentativa de combinar as propriedades físico-químicas de um cimento endodôntico com as propriedades biológicas do MTA, um cimento obturador à base de MTA (MTA Fillapex®-Angelus, Londrina, Paraná, Brasil), foi introduzido no mercado. Neste estudo, testou-se o efeito do MTA e do MTA Fillapex (FLPX) sobre a atividade de macrófagos inflamatórios peritoneais M1 (provenientes de camundongos C57BL/6) e M2 (provenientes de camundongos Balb/c). Foram avaliadas: a viabilidade e aderência celular, a fagocitose de *Saccharomyces Boulardii*, a produção de ROIs, quando os macrófagos foram ou não estimulados com zymosan, e a produção de TNF, IL-12, IL-10 e NO, quando estimulados ou não com o *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius*, na presença ou ausência do IFN- γ . O FLPX afetou a viabilidade celular, a aderência e a atividade fagocitária dos macrófagos M1 e M2. Ao contrário, o MTA não afetou a viabilidade celular, a capacidade de aderência e a atividade fagocitária dos macrófagos peritoneais M1 e M2. O FLPX diminuiu a produção de ROS e também a produção de NO, em culturas com estímulo bacteriano, pelos macrófagos M1 e M2. O MTA e o FLPX não diminuíram a expressão de IL-10

pelos M1 e M2. Os macrófagos M2 não produziram IL-12. O FLPX diminuiu a expressão de TNF- α por macrófagos M2 estimulados com *F.nucleatum*. Podemos concluir que o MTA Fillapex inibiu atividades importantes dos macrófagos peritoneais M1 e M2.

Palavras-chave: macrófagos, MTA, MTA Fillapex, sistema imunológico.

ABSTRACT

Root canal filling after cleaning and shaping is paramount for a successful endodontic treatment. Filling materials should have favorable physical and chemical properties. Furthermore, is highly desirable for them to be biocompatible, because, often, they are in close contact with periapical tissues through foramen and accessory periapical communications. The Mineral Trioxide Aggregate (MTA) is known by its excellent biocompatibility and sealing aptitude. Although its favorable characteristics, MTA do not have the physical properties needed to be used as sealing cement. In a attempt to combine endodontic sealers physical and chemical properties with MTA biological properties, a filling cement based on MTA (MTA Fillapex[®] - Angelus, Londrina, PR, Brazil), was introduced in the market. In this study, MTA and MTA Fillapex (FLPX) effect were tested over M1 (C57BL/6 mice provided) and M2 (Balb/c mice provided) inflammatory macrophages. Cellular viability, cellular adherence, phagocytosis of *Saccharomyces Boulardii*, production of reactive oxygen intermediates (ROIs), nitric oxide (NO), TNF, IL-12 and IL-10 were assessed . Production of ROIs was stimulated by zymozan. Production of cytokines was stimulated with *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius* and IFN- γ . FLPX impaired cellular viability, cellular adherence and phagocytosis activity of M1 and M2 macrophages. On the other hand, MTA did not affected cellular viability, cellular adherence and phagocytic activity of M1 and M2 macrophages. FLPX reduced NO and ROS production by M1 and M2 macrophages, in bacteria stimulated cultures. MTA and FLPX did not impair IL-10 expression by M1 and M2 macrophages. FLPX decreased TNF- α expression in *F.nucleatum*-stimulated cultures by M2 macrophages. In conclusion, FLPX impairs important activities of M1 and M2 macrophages.

Keywords: macrophages, MTA, MTA Fillapex , immunologic system.

LISTA DE ABREVIATURAS E SIGLAS

1. ELISA: *Enzyme-linked immunoabsorbent assay*
2. FLPX: MTA Fillapex
3. IFN: interferon
4. IL: interleucina
5. LPS: lipopolissacáride
6. M1: macrófago do tipo 1
7. M2: macrófago do tipo 2
8. MTA: *mineral trioxide aggregate*
9. NO: óxido nítrico
10. ROIS: radicais livres de oxigênio
11. SCR: sistema de canais radiculares
12. TNF: fator de necrose tumoral

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INTRODUÇÃO E RELEVÂNCIA

1 Introdução e Relevância

O tratamento endodôntico consiste na limpeza mecânico-química e formatação do sistema de canais radiculares, seguidos por uma obturação hermética (Brkanic *et al.*, 2005). A obturação do sistema de canais radiculares (SRC) é considerada uma das etapas de importância singular dentro da terapia endodôntica. Tem como objetivo vedar tridimensionalmente o SRC, impedindo a percolação de fluidos dos tecidos perirradiculares e a contaminação pelos microrganismos presentes na cavidade oral. A obturação deve impedir, também, que qualquer microrganismo que não tenha sido removido durante os procedimentos de limpeza e formatação recolonize os canais radiculares, além de criar um ambiente biológico favorável para que se processe a cicatrização dos tecidos periapicais (Nguyen, 1994).

Tradicionalmente, para a confecção da obturação dos sistemas de canais radiculares utilizam-se materiais semi-sólidos, como a gutta percha, em combinação com cimentos ou pastas obturadoras. Estes devem apresentar propriedades físico-químicas favoráveis (Grossman, 1988), desejando-se também que apresentem biocompatibilidade, por serem colocados em íntimo contato com os tecidos periapicais via forame apical, ramificações e canais acessórios (Bernath & Szabo 2003). Em contato com os tecidos, os cimentos obturadores podem desencadear processos inflamatórios de diversas intensidades, afetando o processo de reparo (Langeland 1974, Giardino et al. 2006, Gomes-Filho et al. 2008, Batur & Erserv 2008).

Durante muitos anos, o selamento de perfurações ou rasgos, acidentes que podem ocorrer nos procedimentos operatórios, era um complicador da terapia endodôntica. Os materiais utilizados na obturação dos SCRs não eram capazes de selar adequadamente estas cavidades. Nos anos 90, um novo e promissor cimento, indicado para tratar estes acidentes, o agregado de trióxido mineral (MTA), foi apresentado e

imediatamente incorporado pela comunidade odontológica. Desde então, o MTA vem sendo largamente utilizado na terapia endodôntica devido a sua biocompatibilidade e excelente capacidade de selamento (Torabinejad & Chivian 1999, Rezende et al. 2007, Yasuda et al. 2008, Scarparo et al. 2010, Parirokh & Torabinejad 2010, Batur et al 2013, Guven et al. 2013, Hirschberg et al. 2013).

O MTA é composto de partículas de silicato tricálcico hidrofílicas, aluminato tricálcico, óxido tricálcico, óxido de silicato e outros óxidos minerais (Lee et al. 1993, Torabinejad et al. 1993, Torabinejad & Chivian 1999). O MTA foi inicialmente indicado como material retrobturador nas cirurgias periapicais e no selamento de comunicações entre o sistema de canal radicular e os tecidos adjacentes. O MTA quando aplicado junto ao osso permite a deposição de cimento e a formação óssea, facilitando a regeneração do ligamento periodontal (Schwartz et al. 1999). Atualmente, vem sendo utilizado na indução da apexificação em dentes com rizogênese incompleta e, também, no capeamento pulpar direto (Saidon et al. 2003). Entretanto, apesar de suas propriedades desejáveis, que o credencia como o material de escolha em vários procedimentos endodônticos, o MTA não apresenta propriedades compatíveis para ser utilizado como cimento endodôntico. Dentre elas, destacam-se: a dificuldade de manipulação, reduzido tempo de presa e de trabalho (Roberts et al. 2008).

Várias composições de cimentos endodônticos foram desenvolvidas com o objetivo de ser biocompatível aos tecidos periapicais, respeitando-se suas propriedades físico-químicas (Al-Hiyasat et al., 2010). Neste contexto surgiu o MTA Fillapex®- (Angelus; Londrina, Paraná, Brazil), na tentativa de combinar as propriedades físico-químicas de um cimento endodôntico com as propriedades biológicas do MTA.

O MTA fillapex (Angelus Soluções Odontologicas, Londrina, Brazil) é um cimento endodôntico à base de silicato de cálcio e resina dissalicilato. Segundo o

fabricante, o MTA fillapex, que possui 13% de MTA, apresenta como características: ser biocompatível, apresentar efeitos antimicrobianos, induzir o reparo biológico graças à liberação de íons cálcio, promover selamento adequado, e radiopacidade elevada em virtude da presença do óxido de bismuto (Nagas et al.2012, Assmann et al. 2012).

Nos tecidos perirradiculares inflamados, células imunocompetentes estarão presentes, havendo um predomínio de macrófagos (Van Dyke 2008). Os macrófagos desempenham um importante papel na patogênese do processo inflamatório, constituindo uma das primeiras células que entram em contato com partículas estranhas. Desempenham um papel regulatório direcionando o processo inflamatório (Hasturk et al.2012).

As principais funções dos macrófagos são: eliminar antígenos invasores, recrutar células de defesa para o sítio da infecção, sintetizar citocinas e quimiocinas, promover o *clearance* de neutrófilos e ativar a resposta imune adaptativa mediada por linfócitos (Hasturk et al.2012). Os macrófagos inflamatórios diferem dos macrófagos residentes especialmente pelo aumento de sua capacidade fagocítica e pela habilidade aumentada de gerar metabólitos tóxicos de oxigênio e nitrogênio (Fujiwara & Kobayashi, 2005). As citocinas e produtos microbianos afetam diferentemente a função destas células ativando ou desativando-as (Mantovani *et al.*, 2004).

Os macrófagos foram divididos em dois subtipos, M1 e M2, de acordo com sua capacidade de produzir diferentes tipos de respostas (Mills et al. 2000, Bastos et al. 2002, Mantovani et al. 2002, Mosser 2003, Rezende et al.,2005). Macrófagos M1 são ativados pela via clássica , na presença de IL-12 , por IFN- γ , produzindo óxido nítrico (NO) e intermediários do oxigênio (ROI) em resposta à presença de microrganismos (Mosser 2003). Na presença de oxigênio, o NO gera intermediários reativos do nitrogênio que causam danos às células e agentes infecciosos (Nathan & Shiloh 2000).

ROI são produtos de redução intermediária do oxigênio, como o superóxido, peróxido de hidrogênio, e radicais hidroxíla, bem como os produtos reativos dos mesmos (Nathan & Shiloh 2000). Por outro lado, os macrófagos M2 convertem, preferivelmente, a arginina em uréia e ornitina, o que resulta na produção de colágeno e proliferação celular.

A polarização M1/M2 é diferenciada pelos níveis constitutivos de IL-12/IL-10 (Bastos *et al.*, 2002; Mosser, 2003; Mantovani *et al.*, 2004). Macrófagos M1 produzem tipicamente altas concentrações de IL-12 e baixas de IL-10, enquanto macrófagos M2 secretam altas concentrações de IL-10 e baixas de IL-12 (Verreck, *et al.*, 2004; Edwards *et al.*, 2006).

Tendo em vista que a biocompatibilidade é uma propriedade biológica desejável a um material destinado ao uso biológico, alguns estudos avaliaram a citotoxicidade do MTA Fillapex. Bin *et al.* (2012), Yoshino *et al.* (2013), e Silva *et al.* (2013) estudaram o efeito do MTA Fillapex sobre a viabilidade celular de fibroblastos. Estes autores demonstraram que o MTA Fillapex reduziu significativamente a viabilidade dessas células. Salles *et al.* (2012) também observaram uma redução na viabilidade de osteoblastos após a exposição a diferentes cimentos endodônticos, dentre eles o FLPX.

Zmener *et al.* (2012) e Tavares *et al.* (2013) analisaram a resposta do tecido subcutâneo de ratos à cimentos endodônticos. Zmener *et al.* (2012) concluíram que o FLPX não apresentou nenhuma vantagem em relação à biocompatibilidade tecidual quando comparado ao cimento de Grossman. Tavares *et al.* (2013) observaram uma maior ocorrência de abscessos nos grupos tratados com o FLPX e Endofill quando comparado ao AH Plus e ao grupo controle não tratado.

A atividade antimicrobiana e a solubilidade de diferentes cimentos endodônticos foi avaliada por Faria-Júnior *et al.* (2013) que concluíram que o Sealapex e o FLPX

reduziam no número de unidades formadoras de colônia em biofilmes induzidos em dentina bovina. Estes cimentos também apresentaram uma maior solubilidade quando comparados ao AH Plus, Sealer 26, Epiphany SE, Activ GP e a um cimento à base de MTA.

Tendo em vista que a adesão é uma propriedade físico-química desejável à um cimento endodôntico, Sagsen *et al.* (2011) compararam a adesividade às paredes do canal promovida pelo FLPX àquela de outros materiais obturadores. O FLPX apresentou menor capacidade de adesão quando comparado ao I Root SP e ao AH Plus.

As citocinas são importantes mediadores envolvidos na patogênese da reabsorção óssea e do processo inflamatório. Gomes-Filho *et al.* (2009) investigaram os efeitos de um cimento endodôntico a base de MTA, o Endo CPM sealer (CPM Sealer; EGEO SRL, Buenos Aires, Argentina), do Sealapex e do MTA Ângelus sobre a expressão da IL1- β e a IL-6 em culturas de fibroblastos. Observaram que nenhum dos cimentos testados alterou os índices de viabilidade celular e que todos aumentaram a expressão da IL-6. A expressão da IL-1 β foi maior no grupo tratado pelo MTA. Não se observou diferença estatística entre os outros grupos experimentais em relação ao grupo controle.

Tendo em vista a importância dos macrófagos na patogênese dos processos inflamatórios e cicatriciais que ocorrem nos tecidos perirradiculares, é relevante conhecer a atividade de macrófagos M1 e M2 na presença do MTA Fillapex. Para tal, este estudo avaliou a viabilidade celular, a aderência, o processo de fagocitose, a produção das citocinas (TNF, IL-12, IL-10 e a produção de ROI e NO) em culturas de macrófagos M1 e M2 murinos.

OBJETIVOS

2. Objetivos

Objetivo geral:

Avaliar o efeito dos cimentos MTA fillapex (Ângelus) e do MTA (Ângelus) nas respostas dos macrófagos M1 e M2 murinos *in vitro*.

Objetivos específicos:

- Avaliar a viabilidade celular de macrófagos M1 e M2, em contato com o MTA Fillapex (Ângelus) e com o MTA (Ângelus).
- Avaliar a capacidade de aderência dos macrófagos M1 e M2, após contato com o MTA Fillapex (Ângelus) e com o MTA (Ângelus).
- Avaliar a capacidade de fagocitose dos macrófagos M1 e M2, após contato com MTA Fillapex (Ângelus) e com o MTA (Ângelus).
- Avaliar a produção de TNF, IL-12, IL-10, ROIs e NO pelos macrófagos M1 e M2, após contato com o MTA Fillapex e com o MTA (Ângelus).

TRABALHOS CIENTÍFICOS

Assessment of the cytotoxicity of a mineral trioxide aggregate-based sealer with respect to macrophage activity.

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Abstract

Aim

To assess the influence of co-culture with mineral trioxide aggregate (MTA) and MTA Fillapex (FLPX) on the viability, adherence and phagocytosis activity of M1 and M2 peritoneal macrophages.

Methodology

Cellular viability, adherence and phagocytosis of *Saccharomyces boulardii* were assayed in the presence of capillaries containing MTA and MTA Fillapex. The data were analyzed using parametric (Student's t) and nonparametric (Mann–Whitney) tests.

Results

FLPX was severely cytotoxic and decreased cell viability, adherence and phagocytic activity of both macrophage subtypes. Cells that were treated with MTA Fillapex remained viable ($\square$ 80%) for only 4 hours after stimulation. M1 macrophages presented higher adherence ability and higher phagocytic activity compared with M2 macrophages.

Conclusion

FLPX was severely cytotoxic and remarkably decreased M1 and M2 macrophage viability. FLPX also impaired cell adhesion properties and the phagocytic ability of both macrophage subtypes. Conversely, MTA did not interfere with the viability, adherence or phagocytic activity of M1 and M2 macrophages.

Keywords: macrophage, mineral trioxide aggregate, MTA Fillapex, viability, adherence, phagocytosis.

Introduction

A complete sealing of the root canal system after cleaning and shaping is critical for a successful endodontic treatment. Root canal are traditionally filled with gutta-percha cones and a sealer. It is highly desirable for sealers to be biocompatible because they can be placed in intimate contact with periapical tissues through the apical foramen and accessory communications.¹ Moreover, sealers may release substances that may generate periapical inflammatory reactions.

Mineral trioxide aggregate (MTA) has been extensively studied and widely accepted for its biocompatibility and excellent sealing capacity.²⁻⁴ In an attempt to combine the physicochemical properties of the root canal sealer with the biological properties of MTA, an MTA-based sealer (MTA Fillapex®-Angelus; Londrina, Paraná, Brazil) was introduced to the market.

Macrophages are key cells involved in inflammation during chronic or healing processes.⁵ According to their ability to elicit different responses, macrophages have been divided into two subtypes: M1 and M2.⁶⁻¹⁰ M1 macrophages mediate resistance to pathogens,¹¹ while M2 macrophages are involved in healing process.^{6,7,9}

Few studies have analyzed the cytotoxicity of MTA Fillapex, and no one has looked for its effects on macrophage viability and activity. This study assessed the influence of MTA and MTA Fillapex on viability, adherence and phagocytic activity of M1 and M2 macrophages.

Materials and methods

Mice

Male and female 4- to 8-week-old C57BL/6 and Balb/c mice were obtained from CEBIO (UFMG, Belo Horizonte, Brazil) and kept in a conventional animal house with barriers as well as temperature and light control. Food and water were offered *ad libitum*.

Isolation of macrophages

Cells were isolated from the peritoneal cavity of C57BL/6 (M1 macrophages) and Balb/c (M2 macrophage) mice 5 days after 2 mL of 3% thioglycolate medium (Biobrás S.A., Montes Claros, MG, Brazil) was injected into the peritoneum. The cells were resuspended in complete medium (RPMI 1640; Sigma Chemical Co., St Louis, MO, USA), supplemented with 10% of fetal calf serum (Nutricell, Campinas, SP, Brazil), 0.1% β -mercaptoethanol (0.05 mol L^{-1}) (Sigma Chemical Co.), 0.2% penicillin (100 U mL^{-1})/streptomycin (0.1 mg mL^{-1}) and 200 mmol L^{-1} L-glutamine.¹²

MTA and MTA fillapex manipulation

MTA and MTA Fillapex[®] (FLPX) were prepared according to the manufacturer's instructions under sterile conditions. Soon after preparation, MTA and FLPX were inserted into the tips of previously sectioned sterilized capillary tubes (test group), to ensure that their contact with the cell suspension was standardized.¹² Empty capillary tubes were used in control cultures.

Cell viability

The viability of cells was determined using two methods: the trypan blue exclusion assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell viability was assayed by trypan blue exclusion in 24-well culture plates (2×10^5 cells/mL for 2, 4, 6, 8, 10 and 24 hours) and in propylene tubes (1×10^6 cells/mL for 24 hours), as described previously.^{3,12} Briefly, cells were incubated in the presence of capillary tubes in 1 ml of RPMI (Sigma Chemical Co.) containing 10% fetal calf serum (Nutricell, Campinas, SP, Brazil), 2mM of L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C and 5%CO₂ humidified atmosphere containing 5% CO₂. After the incubation periods, 100 µl of 0.25% trypan blue (Sigma Chemical Co.) in saline were added, and the cultures were examined under an inverted microscope. At least 300 cells were counted per culture, and the cultures were performed in triplicate. The results are expressed as percentage of viability. The experiment was repeated three times.

Additionally, cell viability was also determined using MTT assay (Sigma-Aldrich, St Louis, MO). The viability of cells in the presence of capillary tubes was tested by culturing 1×10^6 cells in 96-well culture plates for 24 hours. A total of 1×10^6 cells plus capillary tubes in 100 µL of RPMI-1640 medium (supplemented with 10% fetal bovine serum, penicillin and streptomycin) were seeded into each well of a 96-well plate. The plate was then incubated for 24 hours at 37°C. After 24 hours, the culture medium was removed and the cells were gently washed with phosphate-buffered saline (PBS). A 100 µL aliquot of MTT-succinate solution (1mg/mL) was added to each well, and cells were incubated for an additional 4 hours. The resulting formazan crystals were

dissolved when removing the culture medium and adding 100 μ L of dimethyl sulfoxide solvent (Sigma-Aldrich) to each well.

The absorbance was measured at 540 nm using a microplate reader (PowerWave HT, BioTek, USA). The formazan content of each well was computed as a percentage of the control group.¹³ The results were expressed as the percentage viability. The experiments were repeated 3 times in triplicate.

Cell adherence

Polypropylene tubes containing macrophages (1×10^6 cells/mL) were incubated for 2 h at 37°C with capillaries (MTA, FLPX, and control groups) in a humidified atmosphere containing 5% CO₂. Tubes were agitated in a vortex agitator for 15 s at low speed. An aliquot (20 μ L) of the cellular suspension was removed, placed into a Newbauer chamber and incubated for 1 h at 37°C as described above. The percentages of adherent and nonadherent macrophages were then established by counting under an optical microscope.¹⁴ Spread cells were counted as adhered cells. At least 300 cells were counted per sample.

Phagocytosis assay

Macrophages (1×10^6 cells/mL) were incubated for 2 h in 24- well culture plates (Nunclon, Nalge Nunc International, Miami, FL, USA), on round glass coverslips (Glasst cnica S o Paulo, SP, Brazil) in an incubator as described above. Nonadherent cells were removed by washing with warm complete medium. Subsequently, 10^7 CFU of *Saccharomyces boulardii* (Floratil; Merck S.A., Rio de Janeiro, RJ, Brazil) and capillaries with MTA and MTA fillapex were added to the medium and plates were incubated for 1 h. An empty capillary tube was added to control cultures. Unbound yeast cells were removed by washing with complete medium and the coverslips were

covered for 1 min with 1 mL of 1% tannic acid (Merk) so that the distinction could be made between extracellular and intracellular yeast cells. One drop of fetal calf serum was applied onto each coverslip. The dried coverslips were stained with Panótico Rápido (Laborclin Ltd, Pinhais, PR, Brazil) and glued to glass microscope slides with Entellan (Merck). The slides were observed under an optical microscope at 1000X magnification using an oil immersion objective.¹⁵ The cells were counted until 200 macrophages with phagocytosed yeast were found. The results are expressed as percentages.

Statistical Analysis

The data were analyzed using parametric (Student's *t*) and nonparametric (Mann–Whitney) tests ($P < 0.05$). Analyses were performed using the Statistical Package for Social Sciences (version 18.0; SPSS Inc., Chicago, IL, USA).

Results

Cell viability

Cell viability is represented in Figure 1. Macrophages of both subtypes in the control and MTA exposure groups remained viable for 24 h in the 24-well culture plates (Figure 1a, b), in the propylene tubes assayed by trypan blue exclusion and in the 96-well culture plates assayed by MTT (data not shown). On the other hand, FLPX was severely cytotoxic and remarkably decreased the viability of both macrophages when the control group was compared to the test group ($p < 0.05$) (Figure 1). However, the M2 subtype survived better than M1 in the presence of FLPX ($p = 0.05$). Figure 1C shows the viability curve of both macrophage signatures: Cells treated with MTA Fillapex remained viable ($\square$ 80%) for only 4 h after stimulation. Similarly, the MTT

viability assay showed that more than 90% of the cells in the FLPX group died 24 hours after capillary exposure (data not shown). Interestingly, MTA-treated macrophages of both subtypes showed greater viability compared with control macrophages of both subtypes ($p \leq 0.021$) when cell viability was assayed by MTT (data not shown).

Cell adherence

M1 macrophages in both control and MTA groups presented higher adherence ability compared with M2 macrophages ($p = 0.02$) (Figure 2). However, the adherence of both macrophage subtypes was impaired by FLPX ($p < 0.05$) when compared with MTA and control groups.

Phagocytic activity

The capacity of mouse peritoneal cells to uptake yeast cells was assayed in this study (Figure 3). Under the control conditions, the M1 macrophages showed statistically higher phagocytic ability compared with the M2 macrophages ($P = 0.033$). However, both macrophage subtypes showed significant impairment in phagocytosis of *S. boulardii* in the presence of FLPX ($p < 0.05$), and M1 cells exhibited enhanced phagocytic activity compared with M2 cells ($p < 0.05$).

Discussion

Successful root canal treatment depends on the elimination of intracanal infection followed by effective and biocompatible root canal filling to avoid reinfection and irritation of the periradicular tissue.¹⁶ Thus, the biocompatibility of the endodontic root canal sealer is an important requirement for achieving this goal.^{17,18}

Macrophages, along with lymphocytes and plasma cells, prevail in inflammatory infiltrates in periapical inflamed tissues.¹⁹ The major functions of macrophages include

elimination of invading bacteria, recruitment of other cells to the site of infection, clearance of the excess neutrophils, production of cytokines and chemokines and activation of the lymphocyte-mediated adaptive immune response.⁵ Macrophages, together with neutrophils, are responsible for phagocytosis and digestion of microorganisms and foreign substances.²⁰

Using a well-tested methodology,^{3,12,21} the present study evaluated and compared the macrophage immune response to MTA Fillapex and MTA. The viability, adherence and phagocytic activity of M1 and M2 macrophages were assayed.

FLPX remarkably decreased cell viability under all conditions tested. Accordingly, other studies have reported that FLPX reduced cell survival rates.²²⁻²⁴ It is important to note that FLPX contains toxic components, such as salicylate resin, which may explain its negative effects on cell viability. Previous studies have evaluated the effect of salicylate resin on a human fibrosarcoma cell line (HT-1080) and observed that it induces apoptosis.^{25,26}

In this study, MTA promoted enhanced viability of M1 and M2 macrophages as previously described.³ MTA was also shown to exert similar effects on fibroblasts,^{27,28} osteoblasts^{29,30} and macrophages.^{3,10,31} Conversely, in this study, the cells treated with FLPX remained viable (> 80%) for only 4 h (Figure 1C). This brief period of cell viability determined our next experiments with macrophage adherence and phagocytosis. In these experiments, the cells were incubated for 2 h to ensure substantial viability of macrophages treated with FLPX.

Adherence is the first step of the phagocytic process.³² Several studies have assayed macrophage adherence by examining macrophage spreading onto coverslips with the aim of evaluating the biocompatibility of endodontic materials.^{3,12,33} Here, MTA Fillapex decreased the ability of M1 and M2 macrophages to adhere to glass,

clearly showing that this sealer is able to impair immune system responses. Accordingly, lower cell adherence in the presence of ZOE sealers ^{12,21,34} and in the presence of other endodontic materials ³⁵⁻³⁷ has been shown. On the other hand, MTA did not interfere with macrophage ³, osteoblast ²⁹ or osteosarcoma cell line adherence ³³. Interestingly, when M1 and M2 control groups were compared, M1 macrophages showed significantly higher adherence compared with M2 macrophages.

Additionally, the ability of macrophages to phagocytose *S. boulardii* in the presence of MTA and FLPX sealers was analyzed. This yeast was selected because of its size, which makes counting easier and therefore allows greater data precision.³ The M1 macrophages showed significantly greater phagocytic ability than M2 macrophages in all tested groups. Consistent with this result, Oliveira Mendes et al. ²¹ have reported that M2 macrophages treated with two different zinc oxide eugenol-based materials also showed a decreased capacity for phagocytosis of *S. boulardii*.

In conclusion, our study shows that MTA Fillapex was severely cytotoxic and remarkably decreased the viability of M1 and M2 macrophages. Moreover, FLPX also impaired the adhesion properties and the phagocytic ability of both macrophage subtypes. These outcomes suggest that the composition of MTA Fillapex requires some modifications to ensure its safety.

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Figure Legends

Figure 1 – The percentage of living macrophages (a) from C57BL/6 mice and macrophages (b) from BALB/c mice, after incubation of the culture plates with capillaries containing MTA or MTA Fillapex. The controls were cultured with empty capillaries. The cultures were maintained for 24 h as described in Materials and Methods. Bars represent the mean of three experiments; lines represent the standard error of the means. (c) The percentage of living macrophages from C57BL/6 and from BALB/c treated with MTA Fillapex at different time-points. —* indicates a statistically significant difference between groups ($p < 0.05$).

Figure 2 – The percentage of living macrophages (a) from C57BL/6 mice (a) and macrophages from BALB/c mice (b), after MTT assay with capillaries containing MTA or MTA Fillapex. The controls were cultured with empty capillaries. The cultures were maintained for 24 h as described in Materials and Methods. Bars represent the mean of three experiments; lines represent the standard error of the means. —* indicates a statistically significant difference between groups ($p < 0.05$).

Figure 3- The percentage of adherent macrophages from C57BL/6 mice (a) and macrophages from BALB/c mice (b) after incubation in polypropylene tubes with capillaries containing MTA and MTA Fillapex. The controls were cultured with empty capillaries. The cultures were performed as described in Materials and Methods. Bars represent the mean results of four experiments performed in duplicate. Lines indicate the standard error of the means. —* indicates a statistically significant difference

between groups ($p < 0.05$). #, Φ and Δ indicate significant differences between macrophage sources ($p < 0.05$).

Figure 4- The percentage of macrophages from C57BL/6 mice (a) and macrophages from BALB/c mice (b) that were observed to phagocytose yeast. The controls were cultured with empty capillaries. The cultures were performed as described in Materials and Methods. Bars represent the mean results of two experiments performed in triplicate. Lines indicate the standard error of the means. ^{*} indicates a statistically significant difference between groups ($p < 0.05$). #, Φ and Δ indicate significant differences between macrophage sources ($p < 0.05$).

Figure 1

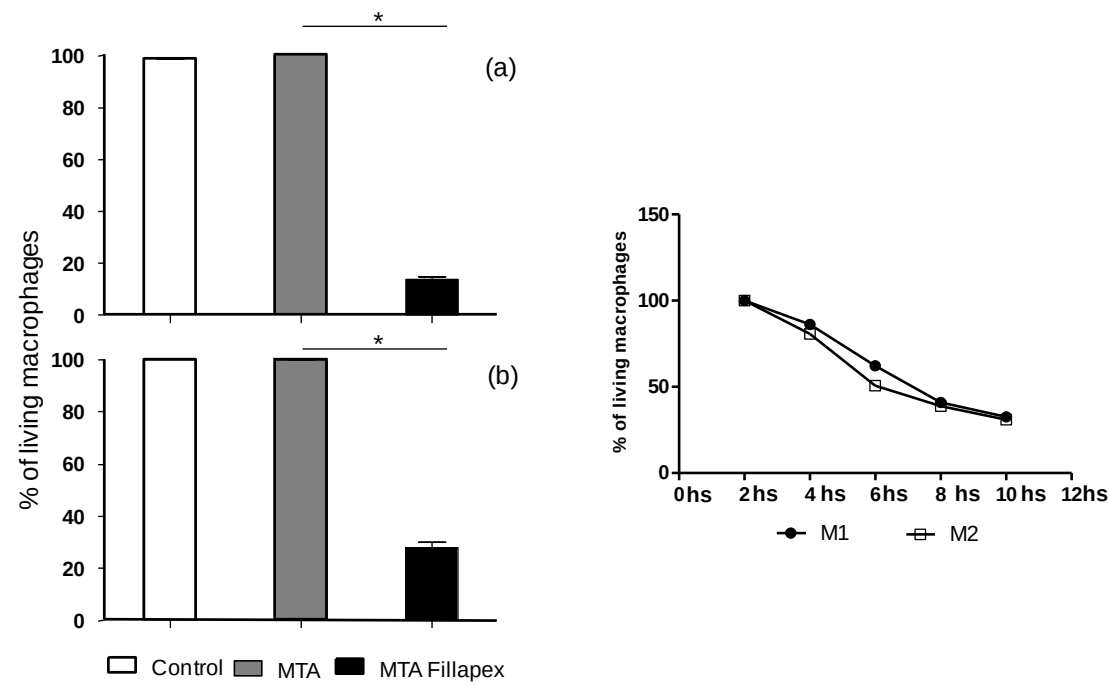


Figure 2

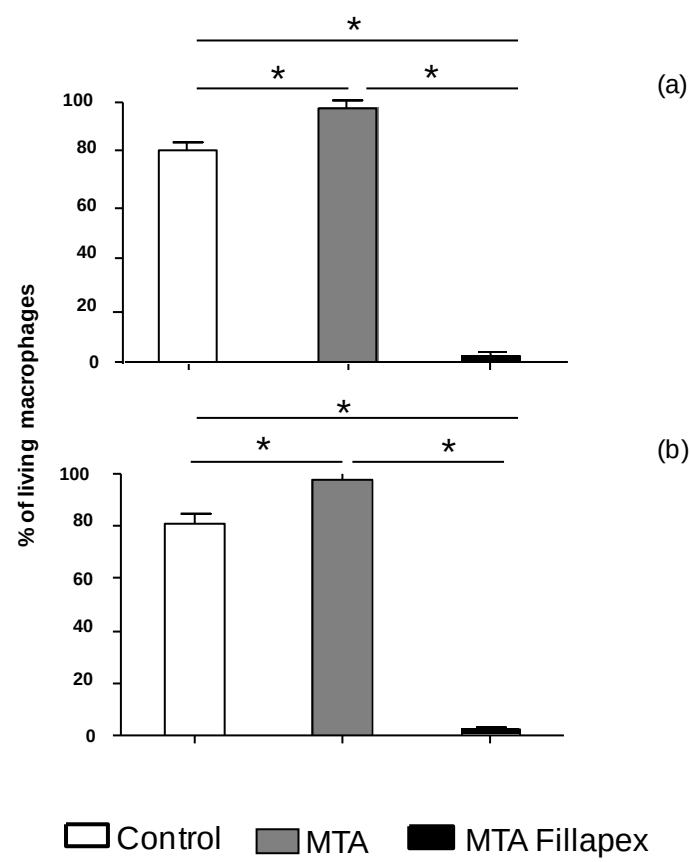


Figure 3

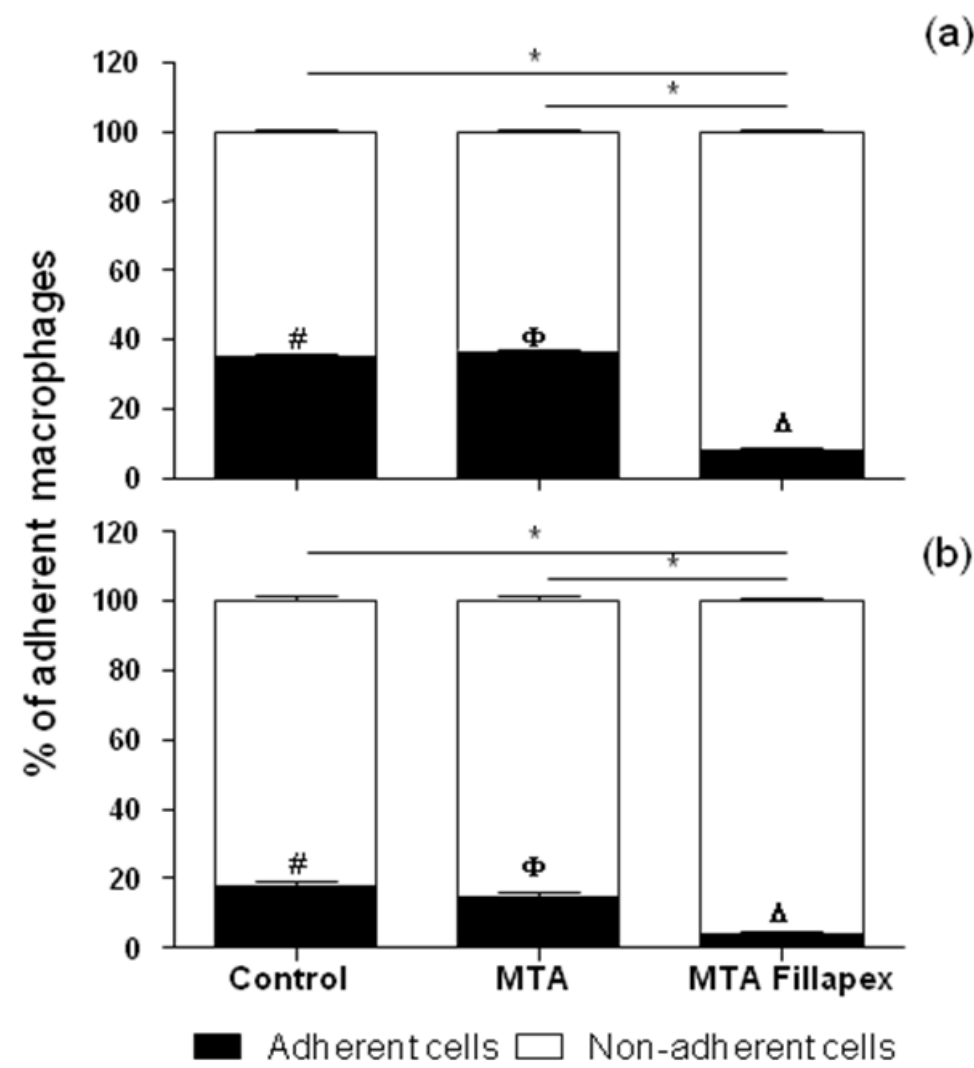
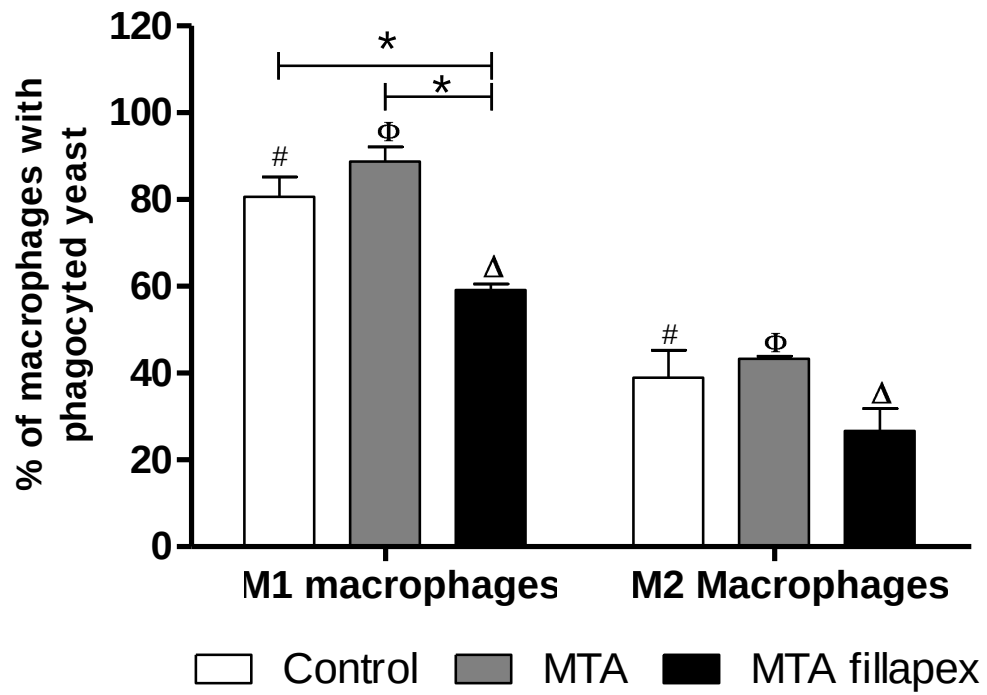


Figure 4



The effect of a mineral trioxide aggregate- based sealer on the production of reactive oxygen species, nitrogen species and cytokine by two macrophage subtypes.

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Abstract

Aim

To test the effects of a mineral trioxide aggregate-based sealer (MTA Fillapex®) and MTA (MTA-Ângelus®) on viability and on the production of cytokines, reactive oxygen species (ROS) and nitrogen species (NO) by M1 and M2 inflammatory macrophages.

Methodology

M1 (from C57BL/6 mice) and M2 (from BALB/c mice) peritoneal inflammatory macrophages were obtained and cultured in vitro in the presence of original and diluted extracts of MTA and MTA Fillapex (FLPX). The cellular viability, ROS production and the production of tumour necrosis factor- α , interleukin (IL)-12, IL-10 and NO in response to stimulation with interferon- γ and *Fusobacterium nucleatum* or *Peptostreptococcus anaerobius* were evaluated. The data were analysed using the Mann–Whitney test and Student's t-test.

Results

FLPX was extremely cytotoxic at the highest concentrations and significantly decreased the viability of both macrophage types. MTA did not interfere with cellular viability. FLPX inhibited the production of ROS and decreased NO production in *F.nucleatum* and *P. anaerobius* -stimulated M1 and M2 macrophages. *F. nucleatum*–stimulated M2 macrophage cultures produced lower levels of TNF- α when FLPX was added. M2 macrophages produced higher levels of IL-10 than M1 macrophages. Only M1 macrophage cultures produced IL-12p70.

Conclusions

MTA treatment resulted in had excellent superior macrophage viability and cytokine production. FLPX impaired important effector responses to bacteria in both macrophage types.

Keywords: biocompatibility, reactive oxygen, nitrogen species, cytokines, macrophages, MTA and MTA Fillapex sealer.

Introduction

Successful endodontic therapy consists of cleaning and shaping procedures followed by tridimensional filling of the root canal with gutta-percha and sealer. Independent of the physicochemical properties of the sealers, if these materials are not biocompatible, it can cause degeneration of the periapical tissue and delay wound healing (Murray *et al.* 2007, Sousa *et al.* 2009). Recently, MTA Fillapex (Angelus, Londrina, PR, Brazil), an MTA-based sealer, was introduced to the market in an attempt to combine the physicochemical properties of a root canal sealer with the biological properties of Mineral trioxide aggregate (MTA) (Torabinejad & Chivian 1999, Rezende *et al.* 2007, Scarparo *et al.* 2010).

Frequently, root canal sealers extrude or contact periapical tissues beyond the apical foramen, and these sealers can occasionally cause tissue inflammation. In this latter condition, the recruitment of inflammatory cells and the release of pro-inflammatory and immune regulatory cytokines have occurred (Stashenko *et al.*, 1998, Brito *et al.*, 2011). Macrophages predominated among the cells that were recruited to this area (Van Dyke 2008, Hasturk *et al.* 2012). The main functions of macrophages include the elimination of invading bacteria, recruitment of other cells to the site of infection, clearance of excess neutrophils, production of cytokines and chemokines, and activation of the lymphocyte-mediated adaptive immune response (Taylor *et al.* 2005, Hasturk *et al.* 2012).

Macrophages were divided into the M1 and M2 subtypes based on their receptor relationship, effector function, and cytokine production (Mills *et al.* 2000, Bastos *et al.* 2002, Mantovani *et al.* 2002, Mosser 2003, Rezende *et al.* 2005, Oliveira Mendes *et al.* 2010). The M1 macrophage subtype typically produces high concentrations of interleukin (IL)-12 and low concentrations of IL-10, whereas M2 cells secrete high

concentrations of IL-10 and low levels of IL-12 (Mantovani *et al.* 2002, Bastos *et al.* 2002, Edwards *et al.* 2006, Verreck *et al.* 2004). Moreover, in pro-inflammatory M1 cells, high levels of inductive nitric oxide synthase (iNOS) result in citrulline and nitric oxide (NO) production. In M2 cells, arginine metabolism leads to ornithine and urea production, which culminates in collagen synthesis and cell proliferation (Mosser 2003, Bogdan *et al.* 1991, Bronte & Zanovello 2005).

The aim of this study was to determine the effects of MTA Fillapex (Angelus, Londrina, PR, Brazil) and white MTA (Angelus, Londrina, PR, Brazil) on M1 and M2 macrophage viabilities. Additionally, the effects of these materials on cytokine production and the release of reactive oxygen species and nitrogen species by both macrophage types were assessed.

Materials and Methods

Mice

Male and female C57BL/6 and BALB/c mice between 4 to 8 weeks of age were obtained from CEBIO (UFMG, Belo Horizonte, Brazil) and kept in a conventional animal house with barriers, temperature and light control. Food and water were offered ad libitum.

Isolation of macrophages

Cells were isolated from the peritoneal cavity of C57BL/6 (M1 macrophages) and Balb/c (M2 macrophage) mice 5 days after peritoneal injection of 2 mL of 3% thioglycolate medium (Biobrás S.A., Montes Claros, MG, Brazil). Cells were resuspended in complete medium: RPMI 1640 (Sigma Chemicals Co., St. Louis, MO, USA), supplemented with 10% foetal calf serum (Nutricell, Campinas, SP, Brazil), 0.1% of 0.05 mol L⁻¹ β-mercaptoethanol (Sigma Chemicals Co.), 0.2% penicillin (100 U mL⁻¹)/streptomycin (0.1 mg mL⁻¹) and 200 mmol L⁻¹ L-glutamine (Oliveira Mendes *et al.* 2003).

Preparation of Extracts

MTA and MTA Fillapex[®] (FLPX) were prepared in sterile conditions in accordance with manufacturer's instructions. The sealer samples were prepared in 24-well plates (16.2 mm in diameter, 2 mm high), and they were incubated at 37°C for 24 hours immediately after mixing. Specimens were layered with 2.5 mL RPMI 1640 (Sigma Chemicals Co., St. Louis, MO, USA) supplemented with 10% foetal bovine serum, penicillin, and streptomycin and incubated for 24 hours at 37°C. After incubation, these

original extracts (1:1) were then serially diluted (1:2, 1:4, 1:8, 1:16 and 1:32) in cell culture medium before testing.

Cytotoxicity Testing

The viability of cells in the presence of the original extracts and in serial dilutions was tested by culturing 1×10^6 cells in 96-well culture plates for 24 and 72 hours. One hundred microliters of RPMI 1640 supplemented with 10% foetal bovine serum, penicillin, and streptomycin containing 1×10^6 cells/well were seeded in 96-well plates and incubated for 24 hours at 37°C. The cells were then exposed to 200 µL of the original extracts and their serial dilutions. After 24 hours, cell survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO) (Van de Loosdrecht *et al.* 1991). The results were expressed as the percentage of viable cells. Experiments were reproduced in triplicate.

Microorganism preparation

To induce tumour necrosis factor (TNF), reactive oxygen species (ROS), IL-12, IL-10 and NO, root canal pathogens were selected: *Fusobacterium nucleatum* (ATCC 10953), a gram-negative bacterium, and *Peptostreptococcus anaerobius* (ATCC 27337), a gram-positive bacterium. Zymosan A from *Saccharomyces cerevisiae* (Sigma Chemical Co., St. Louis, MO) was used as a positive control for the ROS assay (Rezende *et al.* 2007).

The microorganisms were grown in brain heart infusion broth (BHI; Difco, Detroit, MI), supplemented and prereduced (BHI-PRAS) in an anaerobic chamber (Forma Scientific, Marietta, OH) containing an atmosphere composed of 85% N₂, 10% H₂, and 5% CO₂ for 48 hours at 37°C. The samples were adjusted in phosphate-buffered saline (PBS) to a concentration of 8×10^7 CFU/mL. To kill the bacteria, the

methodology proposed by Ribeiro Sobrinho *et al.* (2002) was used. Zymosan A was diluted in PBS (10^9 particles/mL) (Trusk *et al.* 1978).

Reactive oxygen intermediates assay

Reactive oxygen species were assayed as described by Trusk *et al.* (1978) with slight modifications. Macrophages (1×10^6 cells/well) were transferred to a C96 White Maxisorp (Nalgene, Rochester, New York, USA) plate in a total volume of 200 μ L that contained: 0.05 mmol/L luminol, diluted extract (1:4) and 10^7 zymosan particles (Sigma Chemical Co.) in RPMI 1640 without phenol red. Diluted extract was not used in control groups. The plates were read in a luminometer every 2 minutes for a total of 118 minutes (LumiCount Packard Instrument Company, Downers Grove, IL). The results were expressed as the area under each of the curves.

Cell culture and cytokine assays

Fusobacterium nucleatum and *Peptostreptococcus anaerobius* (10^7 UFC mL⁻¹) preparations were added to 24-well plates (Nunc) containing a peritoneal cell suspension (2×10^6 cells/well) and the diluted extract (1:4) in the presence or absence of 10 U mL⁻¹ of recombinant murine interferon (IFN)- γ (Pharmingem, San Diego, CA, USA), in a final volume of 1 mL. Plates were incubated for 24 hours to assay for TNF and IL-12 production, and for 72 hours to assay for IL-10 and NO production. Supernatants were harvested, and cytokine readings were performed.

Statistical analysis

The data were analysed using parametric (Student's t-test) and nonparametric (Mann–Whitney) tests ($P < 0.05$). Analyses were made using the SPSS 18.0 Inc. (Statistical Package for Social Sciences, Chicago, IL, USA) software.

Results

Cell Viability

Cell viability using MTT is demonstrated in Fig. 1. When the control group was compared to the test group, FLPX sealer was extremely cytotoxic at the highest concentrations (1:1, 1:2) and significantly decreased the viability of both macrophage types at 24 h and 72 h after stimulation ($p < 0.05$). However, cells treated with diluted FLPX extracts (1:4, 1:8) remained viable ($\geq 80\%$) for 24 h and 72 h. (Fig. 1a, b). Macrophages of both subtypes remained viable ($\geq 80\%$) after stimulation with diluted MTA extracts (1:1, 1:2, 1:4 and 1:8) (Fig. 1c, d). No differences were found between the viabilities of M1 and M2 macrophages.

ROS assay

Analysis of the area under each ROS production curve showed that zymosan stimulated significantly higher respiratory bursts for M2 macrophages compared to M1 macrophages (Fig. 2). Figure 2 demonstrates that FLPX sealer inhibited the production of ROS by M1 and M2 macrophages ($p \leq 0.05$). Additionally, FLPX sealer significantly decreased ROS production in zymosan-stimulated cultures ($p < 0.0001$). MTA treatment did not affect ROS production by M2 macrophages; however, lower levels of ROS were observed in zymosan-stimulated M1 macrophages compared to the control group ($p < 0.004$).

NO production

M1 and M2 macrophages produced similar levels of NO in all of the conditions examined. The only exception was observed in *Peptostreptococcus anaerobius*-stimulated M2 macrophages, where high amounts of NO were observed ($p \leq 0.046$). NO production by M1 and M2 cells was increased when IFN- γ was added to the cultures (Fig. 3). The addition of *Fusobacterium nucleatum*-stimulated cells induced NO production, and these levels were higher when IFN- γ was added to the culture. MTA decreased NO production in M1 *F.nucleatum*-stimulated cultures with IFN- γ stimuli ($p \leq 0.001$). As observed for ROS, FLPX sealer decreased NO production in *F. nucleatum* and *P. anaerobius* -stimulated M1 and M2 macrophages ($p < 0.05$).

TNF- α

M2 macrophages produced higher levels of TNF- α compared to M1 macrophages in all of the conditions tested ($p \leq 0.05$) (Fig. 4). FLPX sealer induced TNF- α production in *P. anaerobius*-stimulated M1 macrophage cultures in the presence of IFN- γ ($p \leq 0.05$) (Fig. 5a). TNF- α production by *F. nucleatum*-stimulated M1 cells was not influenced by the presence of FLPX sealer. However, FLPX sealer significantly decreased TNF- α production in the presence or absence of IFN- γ in *F. nucleatum*-stimulated M2 macrophages ($p \leq 0.05$) (Fig. 4b).

IL-12p70

IL-12p70 production was detected only in M1 macrophages. FLPX sealer did not interfere with IL-12p70 production in any condition as compared to the control groups. An exception was observed in *P. anaerobius*-stimulated cells, which increased IL-12p70 production in the presence of IFN- γ ($p < 0.05$). MTA significantly stimulated IL-

12p70 production by M1 macrophages in all conditions ($p < 0.05$) except for *F. nucleatum*-stimulated cultures (Fig. 5).

IL-10

Figure 4 shows that M2 macrophages produced higher levels of IL-10 than M1 macrophages in all conditions tested ($p < 0.05$). FLPX sealer stimulated the production of IL-10 by M1 and M2 macrophages in *P. anaerobius*-stimulated macrophages cultured in the presence or absence of IFN- γ . MTA also stimulated IL-10 production by M2 macrophages in *P. anaerobius* and *F. nucleatum*-stimulated cultures (Fig. 6). M2 macrophages that were pre-incubated with MTA extract and subsequently treated with *F. nucleatum* antigen in the absence of IFN- γ produced the highest levels of IL-10 ($p < 0.05$) (Fig. 6b).

Discussion

Studies to assay the effects of root canal sealers on macrophage function bring important knowledge about the innate and adaptive immune responses in inflamed periapical tissues surrounding teeth subjected to endodontic treatments. Professional antigen-presenting cells (APCs) such as macrophages ingest foreign particles, including infectious agents and cellular debris (Kopitar *et al.* 2006, Hasturk *et al.* 2012). Several studies have shown that endodontic materials can impair the phagocytic activities of periapical APCs (Lee *et al.* 2007, Sousa *et al.* 2009, de Oliveira Mendes *et al.* 2010, Brackett *et al.* 2011).

In this study, we attempted to analyse the innate immune response (M1 and M2 macrophage responses) to MTA Fillapex, a new root canal sealer, and MTA. Moreover, we assayed this response in the presence of root canal pathogens. Using the experimental design explored extensively by our group, we evaluated the macrophage

response to sealers during the inflammation (M1 macrophages) and healing (M2 macrophages) processes.

The first step is to analyse the cell viability in the presence of sealers. As previously shown (Rezende *et al.* 2005), MTA did not affect M1 or M2 macrophage viability. However, FLPX sealer significantly decreased the cell viability of both macrophage subtypes after stimulation with high extract concentrations (1:1, 1:2). Accordingly, Bin *et al.* (2012) observed similar results when using original and serially diluted extracts of these sealers. Several other groups have confirmed these findings (Scelza *et al.* 2011, Silva *et al.* 2013, Salles 2012). It is possible that toxic components in FLPX, such as salicylate resin, could be responsible for the negative outcomes observed here (Stark *et al.* 2001, Mahdi *et al.* 2006, Faria-Júnior *et al.* 2013). After analysing these findings, we determined that the best concentration of FLPX and MTA was 1 to 4 (1:4) because this ratio did not interfere with macrophage viability.

The secretory activity of stimulated macrophages was investigated using this sealer concentration. After being phagocytised, microorganisms are killed via ROS and NO production (Garcia & Stein 2006, de Oliveira Mendes *et al.* 2010). Therefore, we tried to analyse whether sealers would interfere with this response in the presence or absence of endodontic pathogens or zymosan (yeast positive control for ROS).

Both macrophages subtypes produced significant amounts of ROS in response to zymosan, although M2 macrophages produced greater levels than M1 cells ($p < 0.05$). FLPX sealer inhibited the production of ROS by M1 and M2 macrophages. In contrast, Camargo *et al.* (2009) found that the ROS production was increased in response to other sealers such as Epiphany, Acroseal and AH Plus. MTA did not affect ROS production by M2 macrophages. However, zymosan-stimulated M1 cells produced lower levels of ROS with MTA treatment compared to the control group. This interesting result was

previously observed for M1 macrophages treated with EWT Pulp Canal sealer (de Oliveira Mendes *et al.*, 2010), even though others have shown that MTA treatment does not affect ROS production (Rezende *et al.*, 2007; Camargo *et al.*, 2009).

The FLPX sealer decreases NO production by M1 and M2 macrophages in *F. nucleatum* and *P. anaerobius*-treated cells. Yoshino *et al.* (2013) reported low levels of NO in fibroblast culture supernatants after FLPX extracts were added. In contrast, NO production by bacteria-treated M1 and M2 macrophages increased when IFN- γ was added to the cultures, as described elsewhere (Rezende *et al.* 2007; de Oliveira Mendes *et al.* 2010). As described previously, there was no difference in NO production with MTA treatment when compared with the control group (Rezende *et al.* 2007). Taken together, these results show that FLPX sealer impairs the immune response against endodontic pathogens and decreases NO and ROS production.

Pro-inflammatory mediators, such as cytokines, and microbial challenge, are responsible for the changes that occur in inflamed periapical tissues (Stashenko *et al.*, 1998). Macrophages secrete a number of cytokines that perpetuate inflammation or direct healing processes (Sjogren *et al.* 1998, Brackett *et al.* 2011). Therefore, we were interested in whether FLPX sealer interferes with TNF- α , IL-12 and IL-10 production by both macrophage subtypes. These cytokines are involved in the onset of the inflammatory processes (TNF- α), in the interconnection of the innate and adaptive immune responses (IL-12), or in immune regulation (IL-10). Heat-killed bacteria and IFN- γ stimuli were added to cell cultures to reproduce clinical conditions because anaerobic bacteria and other cytokines are frequently found in compromised periapical tissues (Rezende *et al.*, 2005).

The major physiological functions of TNF- α are to induce neutrophil and macrophage migration to the site of the infection and activate these cells to kill

microorganisms (Locksley *et al.* 2001, Xanthoulea *et al.* 2004); it also indirectly induces osteoclast activation (Stashenko 1990, Wang *et al.*, 1997, Maciel *et al.*, 2011). In contrast to a previous study, M2 macrophages produced higher levels of TNF- α than M1 macrophages (Resende *et al.*, 2005). FLPX sealer induced TNF- α production in *P. anaerobius*-stimulated M1 macrophages cultured in the presence of IFN- γ ($p \leq 0.05$). However, TNF- α production by *F. nucleatum*-stimulated M1 cells was not influenced by the presence of FLPX sealer. The opposite was observed in M2 cells: FLPX sealer significantly decreased TNF- α production in *F. nucleatum*-stimulated cells cultured in the presence or absence of IFN- γ . Other studies have also found that TNF- α production by macrophages was impaired by sealers (Perassi *et al.* 2004, Sousa *et al.* 2009, Brackett *et al.* 2009, de Oliveira Mendes *et al.* 2010). Moreover, MTA did not have negative effects on TNF- α production by M1 and M2 macrophages, as demonstrated by others (Rezende *et al.*, 2005, 2007). These results suggest that in the absence of residual infection, MTA and FLPX sealers do not interfere with the production of TNF- α by either macrophage subtype.

Interleukin-12 is a pro-inflammatory cytokine that promotes cell-mediated immune responses in inflammatory and infectious disorders. Additionally, it functions as a link between the innate and the adaptive antigen-specific responses (Trinchieri & Scott 1995). As predicted, M2 did not produce IL-12p70 cytokine in this study. The gram-negative bacteria, *F. nucleatum*, stimulated M1 macrophages to produce higher levels of IL-12p70. FLPX sealer did not interfere with IL-12p70 production, although this sealer increased IL-12p70 production in *P. anaerobius*-stimulated cells in the presence of IFN- γ . Conversely, it was shown that a resin-based sealer (AH-Plus) decreased IL-12 production by LPS-stimulated monocytic cells (Brackett *et al.*, 2011). Furthermore, MTA significantly stimulated IL-12p70 production by M1 macrophages

in control cells and in *P. anaerobius*-stimulated cells, as demonstrated elsewhere (Resende *et al.*, 2005).

The M2 profile is involved in the progression of infectious diseases as well as in the healing process (Benoit *et al.*; 2008; de Oliveira Mendes *et al.*, 2010). Collagen synthesis and cell proliferation is associated with IL-10 production (Kawashima & Stashenko, 1999). Interleukin-10 induces tissue homeostasis, which leads to the inhibition of pro-inflammatory cytokine production by activated T cells and macrophages (Kawashima *et al.* 1996, Stashenko *et al.* 1998, Gerber & Mosser 2001, Borish & Steinke 2003, Brito *et al.*, 2012). As expected, M2 macrophages produced higher levels of IL-10 than did M1 macrophages. FLPX sealer stimulated the production of IL-10 by M1 and M2 macrophages in *P. anaerobius*- stimulated cells cultured in the presence or absence of IFN- γ . Moreover, M2 macrophages that were pre-incubated with MTA extract and subsequently treated with *F. nucleatum* antigen in the absence of IFN- γ produced the highest levels of IL-10 ($p < 0.05$) (Fig. 4b). Previously, it was demonstrated that M2 macrophages treated with *F. nucleatum* in the absence of the sealers produced higher levels of IL-10 than did M1 macrophages under the same conditions (de Oliveira Mendes *et al.*, 2010).

In conclusion, the aim of associating a root canal sealer with MTA to improve its biological properties was not achieved for the FLPX sealer; these results show that it impaired effector immune responses during inflammation (M1 macrophages), as well as during healing (M2 macrophages) responses.

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Figure legends

Figure 1- Percentage of living M1 macrophages (a and c) from C57BL/6 mice and M2 macrophages (b and d) from BALB/c mice after exposure to extracts and organised according to type of dilution. Original extracts (1:1) were serially diluted in fresh medium as indicated. Cell cultures were exposed to original extracts and to serially diluted extracts for 24 hours □ and for 72 hours ■. Columns represent the mean cell viability expressed as percentages. The bars represent the means of 3 experiments performed independently in triplicate. Black stars (★) indicate significant differences between treatment with original and diluted extracts.

Figure 2- Production of reactive oxygen intermediates (ROS) by M1 macrophages (a) from C57BL/6 mice and M2 macrophages (b) from BALB/c mice. Representative kinetics of ROS production by zymosan-stimulated and unstimulated M2 macrophages in the presence or absence of sealers are shown in (c). Cells were cultured with diluted extracts of MTA and FLPX sealer (1:4) and stimulated with zymosan as described in materials and methods. Bars represent the mean results of three experiments performed in triplicate; lines represent the standard error of the mean. —*— indicates a statistically significant difference between groups ($p < 0.05$). Black stars (★) indicate significant differences between control and stimulated groups, and white circles (⊙) indicate significant differences between macrophage sources ($p < 0.05$).

Figure 3- Nitric oxide (NO) production by M1 macrophages (a) from C57BL/6 mice and M2 macrophages (b) from BALB/c mice. Cells were cultured with diluted extracts

of MTA and FLPX (1:4) and stimulated with *F. nucleatum* (F) or *P. anaerobius* (P), and IFN-gamma (I) as indicated. Bars represent the mean results of three experiments performed in triplicate; lines represent the standard error of the mean. —* indicates a statistically significant difference between groups ($p < 0.05$). Black stars (★) indicate significant differences between control and stimulated groups, and white circles (⊙) indicate significant differences between macrophage sources ($p < 0.05$).

Figure 4- Production of TNF by M1 (a) and M2 (b) macrophages cultured in the absence (control) or presence of sealer extracts (1:4) 24 hours after incubation. The cells were cultured in the medium alone (M - control), or with *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P) preparations. Interferon- γ (I) was added where indicated at 10 U/mL (1 μ g = 8,430 U). The bars represent the means of 2 experiments performed independently in quadruplicate, and the lines represent the standard deviation of the means. —* indicates a statistically significant difference between groups ($p < 0.05$). Black stars (★) indicate significant differences between control and stimulated groups, and white circles (⊙) indicate significant differences between macrophage sources ($p < 0.05$).

Figure 5- Production of IL-12 by M1 (a) macrophages cultured in the absence or presence of diluted sealer extracts (1:4) 24 hours after incubation. The cells were cultured in medium alone (M - control), or with *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P) preparations. Interferon- γ (I) was added where indicated at 10 U/mL (1 μ g=8,430 U). The bars represent the means of 2 experiments performed independently in quadruplicate, and the lines represent the standard deviation

of the means. M2 macrophages did not produce IL-12. Moreover, ^{*} indicates a statistically significant difference between groups ($p < 0.05$). Black stars (★) indicate significant differences between control and stimulated groups ($p < 0.05$).

Figure 6- Production of IL-10 by M1 (a) and M2 (b) macrophages cultured in the absence (control) or presence of diluted sealer extracts (1:4) 72 hours after incubation. The cells were cultured in medium alone (M - control) or with *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P) preparations. Interferon- γ (I) was added where indicated at 10 U/mL (1 μ g = 8,430 U). The bars represent the means of 2 experiments performed independently in quadruplicate, and the lines represent the standard deviation of the means. Moreover, ^{*} indicates a statistically significant difference between groups ($p < 0.05$). Black stars (★) indicate significant differences between control and stimulated groups, and white circles (⊙) indicate significant differences between macrophage sources ($p < 0.05$).

Figure 1

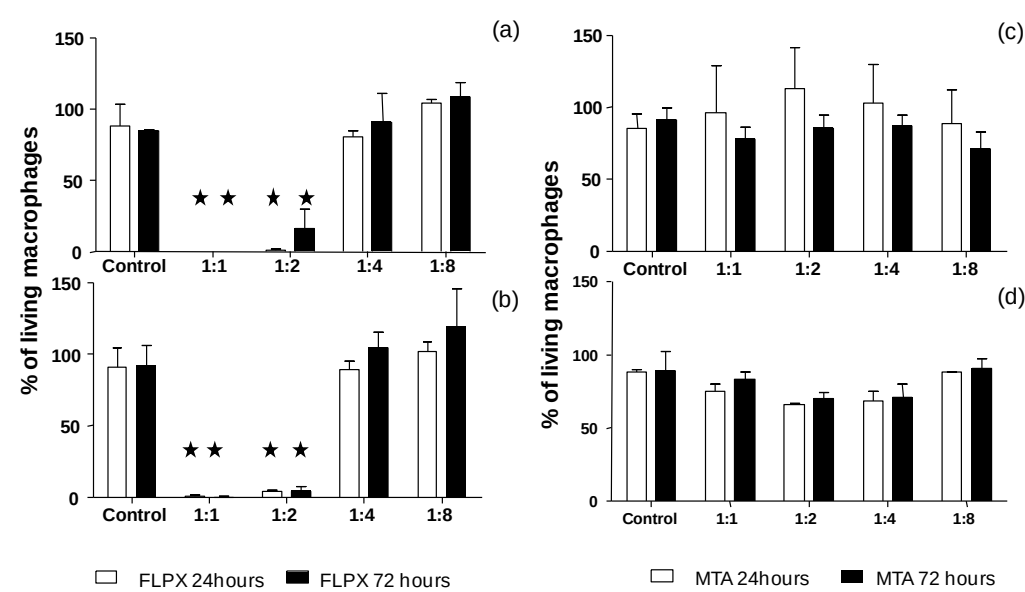


Figure 2

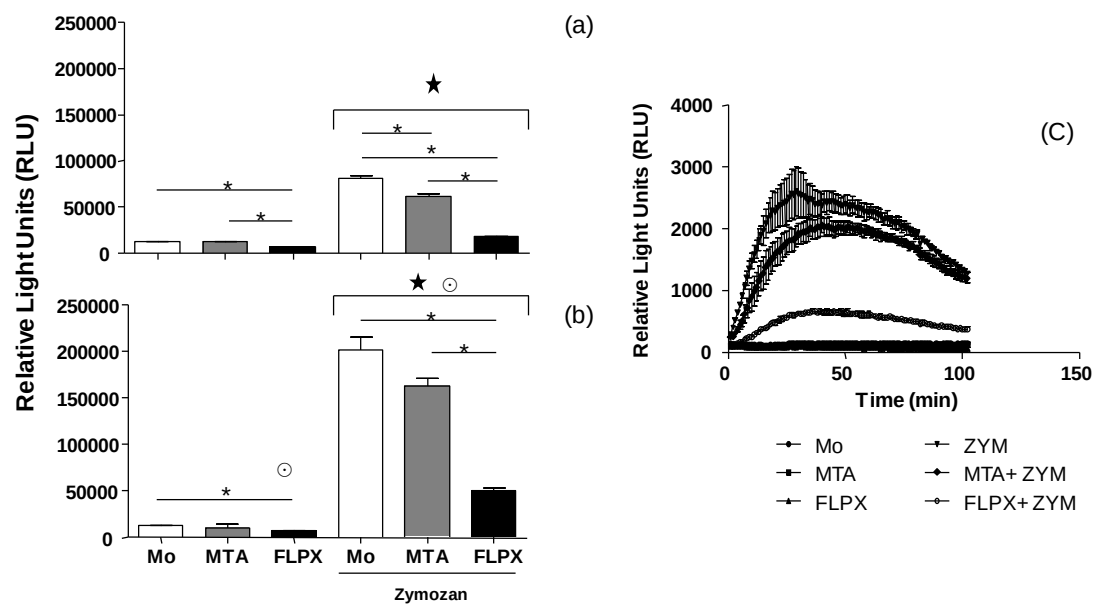


Figure 3

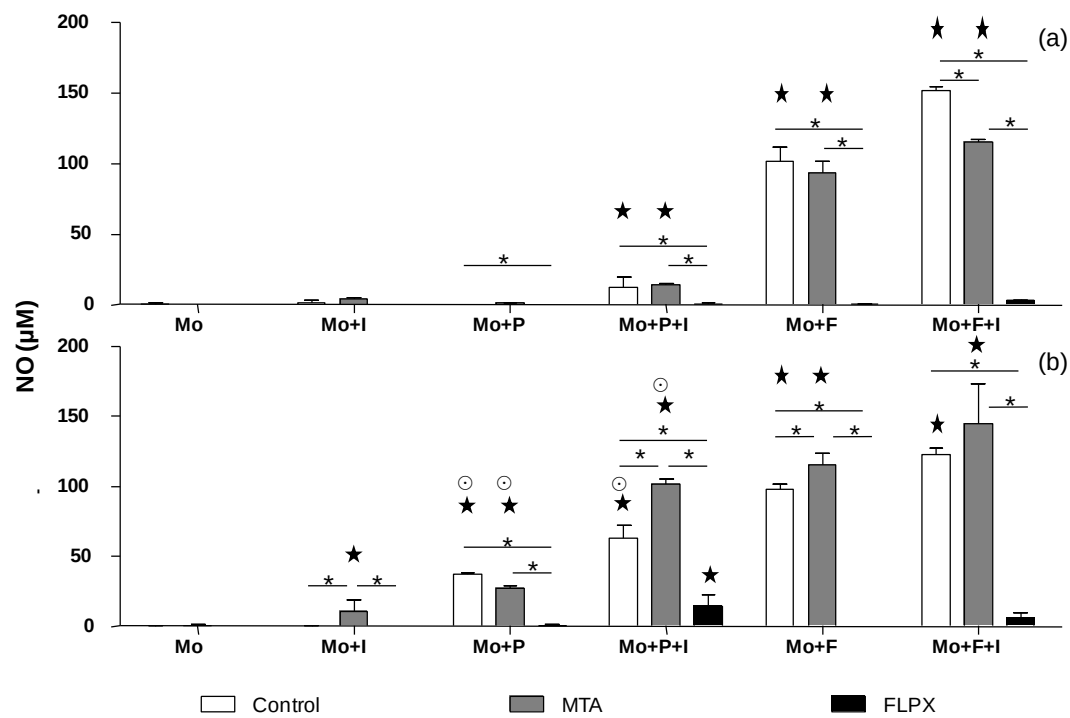


Figure 4

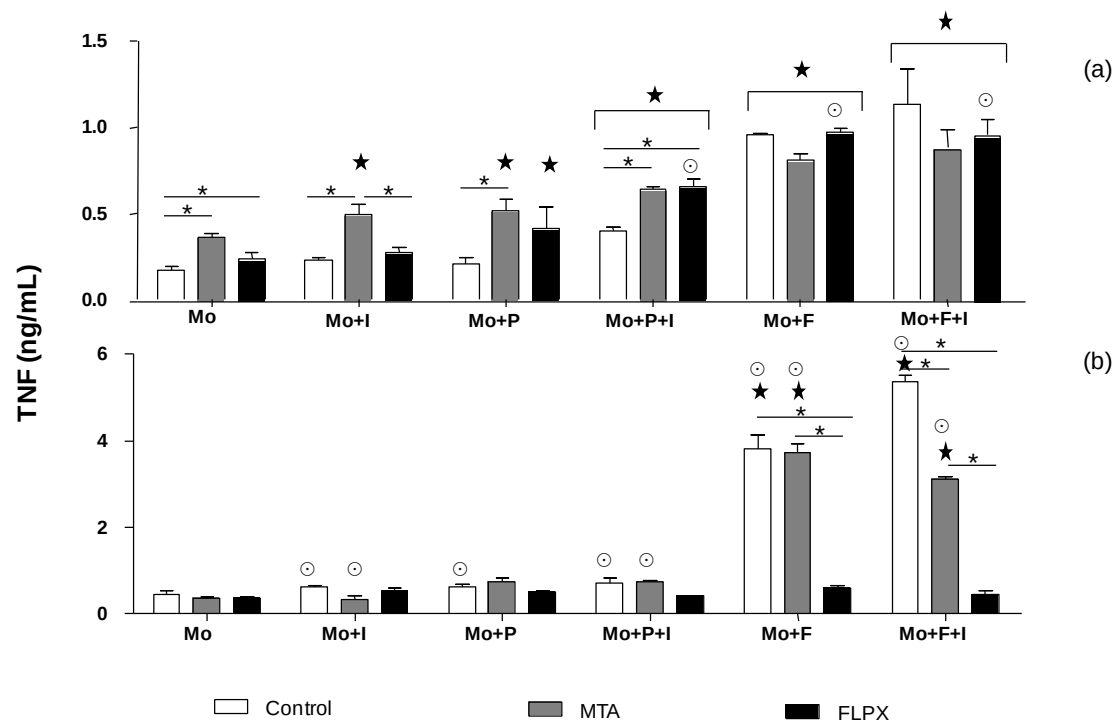


Figure 5

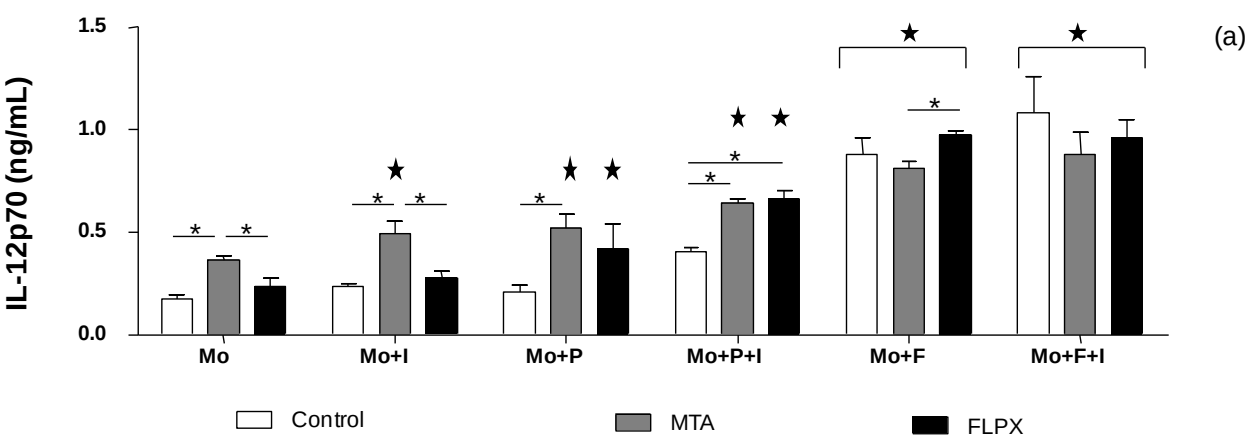
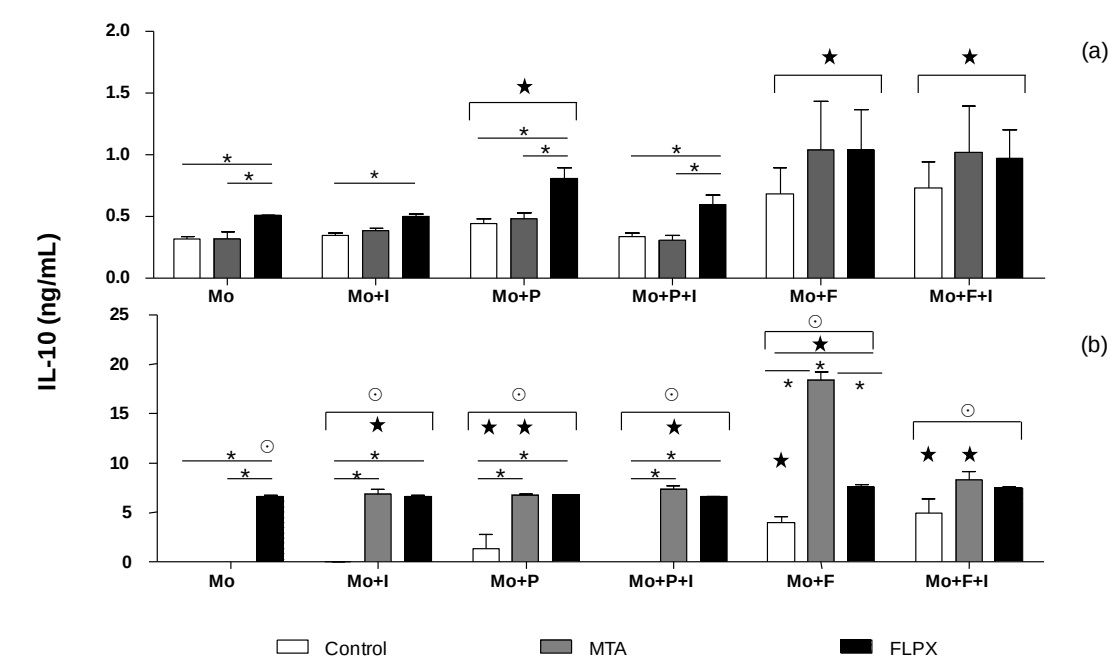


Figure 6



CONCLUSÃO

Conclusões

Pôde observar-se que:

- O FLPX interferiu na viabilidade celular de macrófagos M1 e M2. Estes se mantiveram viáveis por somente 4 horas, em placa de 24 poços, após a incubação com capilares contendo este cimento. Além disso, a viabilidade celular dos macrófagos M1 e M2 foi severamente afetada quando estes foram colocados em contato com altas concentrações de extratos de FLPX (1:1, 1:2).
- O MTA não afetou negativamente a viabilidade celular de macrófagos M1 e M2 em nenhum ensaio realizado. Após o ensaio de MTT observou-se que ambos os macrófagos apresentavam índices de viabilidade maior que o grupo controle.
- Macrófagos M1 aderiram melhor ao substrato que os macrófagos M2. O FLPX diminuiu a capacidade de aderência de ambos os macrófagos.
- Macrófagos M1 apresentaram maior capacidade de fagocitar leveduras *S.boulardii* que macrófagos M2. O MTA não alterou a capacidade de fagocitose de ambos os macrófagos. No entanto, o FLPX diminuiu a capacidade dos macrófagos M1 de realizarem a fagocitose.
- A produção de ROS foi maior pelos macrófagos M2 do que pelos macrófagos M1, quando estes foram estimulados com zymosan. O FLPX diminuiu a produção de ROS por ambos os macrófagos.
- O FLPX diminuiu a produção de NO pelos macrófagos M1 e M2 em culturas estimuladas com *F.nucleatum* e com *P.anaerobius*.
- A produção de IL-10 foi maior por macrófagos M2. O MTA e o FLPX não afetaram negativamente a produção de IL-10.

- A produção de IL-12p70 só foi observada por macrófagos M1. O MTA e o FLPX não afetaram negativamente a produção de IL-12p70 .
- A produção de TNF- α pelos macrófagos M2 estimulados com *F.nucleatum* foi diminuída na presença de FLPX.

Assim, concluiu-se que o FLPX interferiu negativamente em importantes respostas efectoras dos macrófagos. Sugere-se então, que a extrusão do cimento para os tecidos perirradiculares, no momento da obturação, deve ser evitada.

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