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Cirurgião-Dentista

Ação de diferentes cimentos endodônticos sobre a citotoxicidade e a produção de gelatinases em culturas de fibroblastos

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para a obtenção do Título de Mestre em Clínica Odontológica – Área de Endodontia.

Orientador: Prof. Dr. Alexandre Augusto Zaia

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RESUMO

Os cimentos endodônticos podem entrar em contato com os tecidos periapicais no momento da obturação, gerando uma inflamação transitória. Esta inflamação pode estar associada a uma degradação das proteínas da matriz extracelular pelas metaloproteinases da matriz (MMPs). Dessa forma, o objetivo deste estudo foi avaliar os efeitos de exposição de cimentos endodônticos sobre a atividade gelatinolítica das MMP-2 e -9, produzidas por fibroblastos humanos. Fibroblastos da linhagem MRC5 ($3x10^5$ células/poço) foram incubados diretamente ou indiretamente com os cimentos AH Plus, Endomethasone N, Pulp Canal Sealer EWT e Sealapex nos períodos de 1/2h, 1h, 4h e 24h. A citotoxicidade dos cimentos foi determinada pela contagem de células viáveis, utilizando para isso o teste do azul de tripan. Sobrenadantes da cultura de células incubadas com os cimentos endodônticos, nas duas formas testadas, foram coletadas após cada período de exposição, com o objetivo de determinar os níveis de atividade gelatinolítica de MMP-2 e -9, pela técnica da zimografia. Os dados foram submetidos à análise de variância (ANOVA) e avaliados estatisticamente através do teste t ($p<0,05$). Os resultados mostraram haver uma maior atividade gelatinolítica de MMP-2 após os períodos de 4 e 24 horas, sem haver diferença entre os cimentos testados. Uma maior atividade gelatinolítica pode ser observada nas células que foram expostas ao cimento de forma direta, quando comparadas com aquelas que de receberam o contato indireto com o cimento ($p<0,05$). Nos períodos de tempo testados nenhuma atividade gelatinolítica pode ser observada no grupo controle, que não recebeu contato com os cimentos. Os resultados de citotoxicidade mostraram que os cimentos testados foram citotóxicos em ambas as formas de contato sendo que o Sealapex apresentou menor citotoxicidade e que o AH Plus foi o cimento mais citotóxico. Pode-se concluir que todos os cimentos endodônticos podem induzir a expressão de MMP-2 em fibroblastos MRC5 e que apesar de o AH Plus possuir a maior citotoxicidade, todos os cimentos testados apresentaram efeitos citotóxicos.

Palavras-chave: Cimentos endodônticos; Citotoxicidade; Fibroblastos; Gelatinases

ABSTRACT

Root canal sealers might be into contact with periapical tissues during root canal filling. This inflammation can be associated with extracellular matrix proteins degradation by matrix metalloproteinases (MMPs). The aim of this study was to investigate the effects of root canal sealers on the gelatinolytic acitivity of MMP-2 and -9 produced by human fibroblast cells. Human fibroblast cells MRC5 ($3x10^5$ cells/well) were incubated directly or indirectly with AH Plus, Endomethasone N, Pulp Canal Sealer EWT or Sealapex for 1/2h, 1h, 4h or 24h (timepoints). The cytotoxicity of all root canal sealers was determined by counting viable cells using the trypan blue assay. Supernatants of cell cultures incubated with root sealers, directly or indirectly, were collected after each time point to determine the levels of MMP-2 and MMP-9 gelatinolytic activity by gelatin zymography. Data were analyzed using ANOVA and t tests ($p<0.05$). The results showed that the cells secreted MMP-2 after the periods of 4 and 24 hours. However, there were no statistical differences between the sealers. Secretion of gelatinases was found to be elevated by the sealers in direct contact with the cell monolayer, when compared to the indirect contact ($p<0.05$). In the timepoints tested no MMP activity could be detected in the control group without the sealers. The cytotoxicity results showed that all the sealers were cytotoxic in both contact forms. These results indicated that Sealapex had a lower cytotoxicity while AH Plus was the most citotoxic endodontic sealer. In conclusion all root canal sealers can induce the expression of MMP-2 in MRC5 fibroblast cells. AH Plus presented the highest cytotoxicity among the tested sealers, but all tested sealers presents citotoxic effects.

Keywords: Cytotoxicity; Fibroblasts; Gelatinases; Root canal sealer

SUMÁRIO

1. INTRODUÇÃO

O propósito do tratamento endodôntico é a remoção do tecido pulpar, a eliminação da infecção no canal radicular e o adequado selamento do canal. A obturação do canal radicular é a etapa do tratamento endodôntico que objetiva o total preenchimento do sistema de canais radiculares recém descontaminado, a fim de impedir a microinfiltração bacteriana do meio oral, dos tecidos apicais e periapicais para o interior dos mesmos (Ray & Trope, 1995; Cohen & Burns, 2000; Barthel *et al.*, 2001). Esse preenchimento é considerado uma das chaves do sucesso da terapia endodôntica (Schilder, 1967; Saunders & Saunders, 1994).

O material obturador, por poder permanecer em contato com os tecidos periapicais adjacentes por tempo prolongado, deve ser biocompatível, não causando reação adversa ao paciente, nem provocando inflamação ou danos aos tecidos circunjacentes (Huang *et al.,* 2002; Bernarth & Szabo, 2003; Lodiene *et al.,* 2008). A maioria dos tratamentos endodônticos utiliza-se da guta percha em combinação com algum cimento endodôntico. A principal função do cimento é preencher espaços existentes entre a guta-percha e as paredes do canal radicular. Atualmente os cimentos endodônticos são comercialmente disponíveis em diversas fórmulas tais como cimentos a base de óxido de zinco e eugenol; cimentos ionoméricos; cimentos contendo hidróxido de cálcio e cimentos resinosos, dentre outros (Silva *et al.,* 2008). Quando o comportamento dos diferentes cimentos endodônticos que buscam aliar propriedades físicas, químicas e biológicas foi avaliado, verificou-se que todos apresentam significantes limitações, mostrando vantagens e desvantagens (Bouillaguet *et al*., 2004).

Os esforços em se desenvolver materiais obturadores mais eficazes, aliados ao aperfeiçoamento das técnicas de obturação, fazem com que sejam desenvolvidos materiais com propriedades favoráveis, tais com plasticidade, estabilidade dimensional, facilidade de inserção e remoção, radiopacidade e, principalmente biocompatibilidade (Siqueira-Jr. & Lopes, 2009). Os diversos cimentos endodônticos comercialmente disponíveis

demonstraram potencial para induzir reação inflamatória de moderada a severa nos tecidos periapicais (Holland & de Souza, 1985; Lambjerg-Hansen, 1987; Tagger & Tagger, 1989; Tepel *et al*., 1994). Assim como outras inflamações, a inflamação periapical esta relacionada à degeneração tecidual (Huang *et al.,* 2008). Estudos recentes mostraram que a ativação da cicloxigenase-2, interleucina-6 e inteleucina-8, podem ter papéis importantes na participação dos cimentos endodônticos em tal inflamação periapical (Chang *et al.,* 2001; Huang *et al.,* 2003; Huang & Chang, 2005, Huang *et al*., 2005). Entretanto, existe pouca informação sobre a presença de enzimas proteolíticas, que participam na degradação da matriz extracelular (MEC), nas reações periapicais induzidas por cimentos endodônticos.

A MEC compreende uma intrincada rede de componentes fibrosos embebidos em gel de polissacarídeos hidratados, glicosaminoglicanas e proteoglicanas. Além de servir como suporte para as células, a matriz extracelular exerce influência no desenvolvimento, migração, proliferação, forma e funções metabólicas das células (Alberts *et al.*, 1989). A degeneração das proteínas da MEC ocorre na presença de inflamação e o *turnover* dessa matriz extracelular depende da atividade de diferentes proteinases que atuam sobre diversas proteínas. Embora diversos tipos de proteinases possam participar do *turnover* da MEC, as metaloproteinases da matriz (MMPs) são o principal grupo de enzimas que atuam sobre esse substrato. (Huang *et al*., 2003; Huang & Chang, 2005; Huang *et al*., 2005).

As MMPs são uma família de endopeptidases zinco-dependentes, representando a maior classe de enzimas envolvidas no processo de remodelação da matriz extracelular através da degradação de macromoléculas do tecido conjuntivo, incluindo colágeno, lâminina, fibronectina e core protéico das proteoglicanas. (Salo *et al*., 1994; Souza & Line, 2002). A primeira publicação sobre metaloproteinases da matriz foi realizada em 1962 por Gross & Lapière. Eles observaram que as MMPs atuavam como enzimas, participando no processo de metamorfose da rã, na reabsorção da cauda, atuando na tripa hélice de colágeno. Desde então, mais de 66 MMPs foram clonadas e seqüenciadas, sendo 25 MMPs em vertebrados e 23 membros homólogos em humanos. Além destas, é descrita uma série de MMPs em seres não vertebrados e na maioria dos seres vivos, inclusive bactérias, mostrando haver processo evolutivo de algumas MMPs primordiais ao longo de bilhões de

anos (Sternlicht & Werb, 2001; Souza & Line, 2002). MMPs são divididas de acordo com a especificidade do substrato. Assim, subdividem-se em colagenases (MMP-1/colagenase de fibroblastos, MMP-8/colagenase de neutrófilos, MMP-13 e MMP-18); estromelisinas (MMP-3, MMP-10 e MMP-11); gelatinases (MMP-2/A e MMP-9/B); matrisilina (MMP-7 e MMP-26), metaloproteinases tipo membrana (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24 e MMP-25) e outros (MMP-20/Enamelisina, MMP-12/elastase de macrófagos, etc.) (Souza & Line, 2002; Hannas *et al.* 2007; Birkedal-Hansen *et al.,* 2008).

As MMPs são expressas por diversas células, incluindo células epiteliais, fibroblastos, osteoblastos, osteoclastos e células endoteliais, em respostas a estímulos, como também, pela maioria das células inflamatórias que invadem o tecido durante eventos de remodelamento in vivo (Birkedal-Hansen, 1993). O níveis constitutivos de expressão dos genes de MMPs são normalmente baixos e o adequado equilíbrio dos componentes da MEC é essencial a diversos processos fisiológicos e patológicos (Nagase & Woessber, 1999; Curran & Murray, 1999; Sternlicht & Werb, 2001). Dessa forma, em situações de saúde as MMPs estão envolvidas em diversos eventos fisiológicos, como remodelação e reparação tecidual, reações imunológicas, regulação das respostas inflamatórias, desenvolvimento embriogênico e morfogênese tecidual. Já em situações patológicas as MMPs foram associadas a diversas condições inflamatórias envolvendo injúria tecidual, tais como em doenças do pulmão, artrite, tumores e outras condições patológicas (Tsai *et al*., 2005; Palosaari *et al*., 2003). Também há evidências que as MMPs exercem um papel importante durante o desenvolvimento e remodelamento dos tecidos orais (Hannas *et al.*, 2007).

As gelatinases são MMPs envolvidas na proteólise e rompimento de membranas basais, bem como na degradação de colágenos tipo IV, V, colágenos desnaturados (gelatinas), fibronectina e elastina (Thomaz *et al.*, 1999; Kahari & Saarialho-Kere, 1999), sendo classificadas como MMP-2 (72-kDa, gelatinase A) (Collier *et al.*, 1988) e MMP-9 (92-kDa, gelatinase B) (Wilhelm *et al.*, 1989).

As MMPs são sintetizadas como zimogênios inativos que requerem ativação (Harper *et al*., 1971). Existem ainda MMPs com perfil próprio de expressão, localização na superfície celular, ativação, inibição e degradação, bem como espectro de substratos preferenciais (Howard *et al*., 2001). A multiplicidade das MMPs com funções distintas e às vezes sobrepostas, provavelmente atua como uma proteção contra qualquer perda de controle regulatório. Apesar dos diversos estudos direcionados a ativação das MMPs, este processo permanece incompletamente elucidado. Diversos fatores estimulatórios são capazes de determinar padrões variados da expressão das MMPs em diferentes tipos teciduais, bem como, efeitos diversos em membros distintos da família das MMPs, dificultando a compreensão da regulação das MMPs em condições fisiológicas e patológicas (Sternlicht & Werb, 2001).

As diversas MMPs têm um papel fundamental no desenvolvimento e remodelação dos diversos componentes da cavidade bucal. As principais MMPs encontradas na cavidade bucal são: MMP-8 (Colagenase), MMP-2 e -9 (Gelatinases) e a MMP-20 (Estromelisinas). A literatura relata que as MMPs participam ativamente no desenvolvimento do esmalte dental, no estabelecimento da fluorose, na destruição do tecido periodontal e na destruição de dentina por lesões de cárie. (Birkedal-Hansen, 1993; Hannas *et al*., 2007). Fibroblastos gengivais, queratinócitos, macrófagos, leucócitos polimorfonucleares são capazes de expressar MMPs -1, -2, -3, -8, -9, citocinas inflamatórias e fatores de crescimento que regulam a transcrição das MMPs. Altos níveis de MMPs nos tecidos periodontais provocam um desequilíbrio entre produção e degradação do colágeno, causando perda de inserção dental (Birkedal-Hansen, 1993).

As MMPs também são necessárias para a remoção das proteínas da matriz de esmalte durante a sua maturação, resultando em um tecido altamente mineralizado. Várias MMPs são expressas nos tecidos dentais em formação e participam da biomineralização da dentina e do esmalte. A expressão de MMP-2 tem sido detectada na camada de odontoblastos da papila dental e no epitélio do esmalte de germe dentário humano principalmente na fase tardia de campânula do desenvolvimento do germe dentário (Fanchon *et al*., 2004)

Da mesma forma, foi demonstrada a participação das MMPs-2 e -9 na destruição da dentina por lesões de cárie, principalmente nos casos de lesão da superfície radicular, evidenciando a necessidade de MMPs para a remoção da matriz orgânica (Souza *et al*., 2003).

Em endodontia, as MMPs também têm ganhado atenção e tem sido objeto de diversos estudos. As MMPs são estimuladas durante o processo inflamatório pulpar intenso como em qualquer outro processo inflamatório. Com relação ao papel das MMPs na polpa, já é comprovado que durante o estágio crônico da inflamação pulpar, as células pulpares têm a capacidade de aumentar a expressão de MMPs, contribuindo dessa forma para a degradação da MEC presente neste tecido (Chang *et al*., 2001). Diversos estudos comprovam o papel da MMP-9 na degradação do tecido pulpar inflamado. (Gusman *et al*., 2002; Hannas *et al*., 2007).

Apesar de existirem trabalhos na literatura avaliando a citotoxicidade dos diferentes tipos de cimentos endodônticos (Geurtsen *et al*., 1998; Schwarze *et al*., 2002; Huang *et al*., 2002; Key *et al.* 2002; Kin *et al*., 2003; Bouillaguet *et al*., 2004; Miletìc *et al*., 2005), pouco se sabe sobre o correlacionamento desses cimentos, com as alterações na expressão de metaloproteinases. Portanto o objetivo deste estudo foi analisar o potencial de diferentes cimentos endodônticos (AH Plus, Endomethasone N, Pulp Canal Sealer EWT e Sealapex) para alterar a expressão das MMP-2 e MMP-9.

2. PROPOSIÇÃO

Os objetivos específicos do presente estudo foram:

Capítulo 1

- Investigar o efeito da exposição de diferentes cimentos endodônticos (AH Plus, Endomethasone N, Pulp Canal Sealer EWT e Sealapex), de forma direta e indireta, por diferentes períodos de tempo, na alteração da atividade gelatinolítica de MMP-2 e -9 em culturas de fibroblastos humanos MRC5.
- Além disso, verificou-se a citotoxicidade desses cimentos endodônticos, nas mesmas condições expressas anteriormente.

3. CAPÍTULOS

Esta dissertação está baseada a resolução CCPG/02/06 UNICAMP que regulamenta o formato alternativo para teses de Mestrado e Doutorado. Um capítulo contendo artigo científico compõe este estudo, conforme descrito abaixo:

Capítulo 1

Cytotoxicity evaluation and up-regulation of gelatinases by four root canal sealers in human fibroblast cells

- Artigo submetido à publicação no periódico **International Endodontic Journal**.

CAPÍTULO 1

CYTOTOXIC EVALUATION AND UP-REGULATION OF GELATINASES BY FOUR ROOT CANAL SEALERS IN HUMAN FIBROBLAST CELLS

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ABSTRACT

Aim: The aim of this study was to investigate the effects of root canal sealers on cytotoxicity and gelatinolytic activity of matrix metalloproteinases (MMPs) on human fibroblast cells. **Methods:** Human fibroblast cells MRC5 ($3x10^5$ cells/well) were incubated directly or indirectly with AH Plus, Endomethasone N, Pulp Canal Sealer EWT or Sealapex for 1/2h, 1h, 4h or 24h (timepoints). The cytotoxicity of all root canal sealers was determined by counting viable cells using the trypan blue exclusion assay. Supernatants of cell cultures incubated with root sealers directly or indirectly were collected after each time point to determine the levels of MMP-2 and MMP-9 gelatinolytic activity by gelatin zymography. Data were analyzed using ANOVA and t tests. **Results:** The results showed that the cells secreted MMP-2 after the periods of 4 and 24 hours. However, there were no statistical differences between the sealers. Secretion of gelatinases was found to be elevated by root canal sealers in direct contact with the cell monolayer when compared to the indirect contact ($p<0.05$). In the timepoints tested no gelatinolytic activity could be detected in the control group without the sealers. The cytotoxicity results showed that all the sealers were cytotoxic in both contact forms. These results indicated that Sealapex had a lower cytotoxicity and that AH Plus was the most cytotoxicity endodontic sealer. **Conclusions:** All root canal sealers can induce the expression of MMP-2 in MRC5 fibroblast cells. AH Plus presented the highest cytotoxicity among the tested sealers, but all tested sealers presents citotoxic effects.

Keywords: Cytotoxicity; Fibroblasts; Gelatinases; Root canal sealer

INTRODUCTION

A complete sealing of the root canal system after cleaning and shaping is critical to successful endodontic therapy (Schilder, 1967). In endodontic treatment, most root canals are filled with gutta-percha points in combination with a root canal sealer. Currently, root canal sealers are available based on various formulas such as epoxy resin, calcium hydroxide and zinc oxide-eugenol. Although endodontic sealers are designed to be used only within the root canal during endodontic therapy, sometimes they can extrude though the apical constriction (Ricucci & Langeland, 1998). Indeed they are often placed in intimate contact with the periapical tissues for extended period of time (Huang *et al*., 2002; Bernath & Szabo, 2003; Lodiene *et al.,* 2008). It is generally accepted that the biocompatibility of endodontic sealers is critical to clinical success of endodontic therapy (Bratel *et al.,* 1998). A sealer should neither prevent nor hinder tissue repair, but aid or stimulate the reorganization of injured structures.

The irritative effects of root canal sealers have been evaluated by histopathological examinations of the tissue response. Several methods have been used to evaluate tissue responses to endodontic materials and unfortunately most studies have shown that root canal sealers can induce some inflammatory alteration within apical tissues (Holland & De Souza, 1985; Lambjerg-Hansen, 1987; Tagger & Tagger, 1989). Degeneration of matrix proteins is thought to occur in periapical inflammation, and matrix turnover requires the activity of many different endopeptidases. Recently, one study has shown that endodontic sealers can activate matrix metalloproteinases, playing an important role in the pathogenesis of root canal sealer-induced periapical inflammation (Huang *et al.,* 2008).

Matrix metalloproteinases (MMPs) are an important group of zinc enzymes responsible for degradation of extracellular matrix components such as collagen and gelatin. They are involved in many normal remolding processes such as embryonic development, postpartum involution of the uterus, bone and growth plate remodeling, wound healing and some important disease processes such as joint destruction in rheumatoid and osteoarthritis, tumor invasion and periodontitis (Souza & Line, 2002; Hannas *et al.,* 2007). MMP-2 and MMP-9, sometimes referred to as gelatinases, are of particular interest because some studies suggest that these MMPs play an important role in the pathogenesis of chronic inflammatory process and in pulp, periodontal and periapical tissue destruction (Shin *et al.,* 2002; Tsai *et al.,* 2005).

Cell culture techniques are useful for evaluation of the biocompatibility of medical devices and materials and also have some advantages like being an inexpensive and quick way of screening a large number of materials (Schwarze *et al.,* 2002). Cytotoxicity tests can be determined with reliability and reproducibility (Beltes *et al.,* 1995). Although there are some studies in literature evaluating fibroblasts cytotoxicity to different endodontic sealers, to date, the interactions of root canal sealers and fibroblasts, as well as MMPs expression in this condition, are still not fully understood. Thus, the aim of this study was to investigate the gelatinolytic activity of MMP-2 and -9 produced by human fibroblasts cells after stimulation with commonly used endodontic sealers, as well as their cytotoxicity potential.

MATERIAL AND METHODS

Cell Culture

Human fibroblast cells (lineage MRC5) were obtained from the American Type Culture Collection and were cultured in Dulbecco Modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 µg/ml of streptomycin, 100 mg/ml of penicillin at 37°C in humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cells were detached with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid (Gibco, Grand Island, NY, USA) for 5 min, and aliquots were subcultured. For the experimental set cells were plated at a concentration of $3x10^5$ cells in each well of a 6-well plate and allowed to achieve confluence. Cells were cultured for 24 hours, at which time culture medium was replaced with fresh DMEM without serum, and the cells were exposed to the root canal sealers directly and indirectly, as described in the sample preparation topic, for the periods of ½, 1, 4 and 24 h. The control group was not exposed to the sealers. After the timepoints the cell culture supernatants were collected to be used in the zymography assay and the citotoxicity was determined using the trypan blue exclusion assay.

Sample Preparation

Four root canal sealers were evaluated: AH Plus, Endomethasone N, Pulp Canal Sealer EWT and Sealapex. The tested materials, product names, manufacturers and components are listed in Table 1. Under aseptic conditions the sealers were mixed according to the manufacturer`s instructions and then were weighed in a precision scale so

that each sample contained 300mg. Immediately after weighing, each specimen was placed in the bottom of each well of a six-well plate so that they were in direct contact with the cell monolayer. A second set was prepared as described above and placed in inserts (Millipore Corp., Bedford, MA, USA), with 0.4 µm pores, separating the sealer and establishing an indirect contact with the sealers and the cell monolayer.

Table 1. Composition of the materials and their manufactures

Endodontic Sealers	Components	
AH Plus, Dentsply Germany	Paste A: Epoxy resins, calcium tungstate, Zirconium oxide, Silica, Iron oxide pigments, Aerosil	Paste B: Adamantane amine, N,N-Dibenzyl-5-oxanonane, TCD-Diamine, Calcium tungstate, Zirconium oxide, Aerosil
Endomethasone N, Septodont France	Powder: Hydrocortisone acetate, Thymol iodide, Barium sulphate, Zinc oxide, Magnesium stearate	Liquid: Eugenol
Pulp Canal Sealer EWT, SybronEndo USA	Powder: Silver powder, Zinc oxide, Thymol iodide, Dimeric acid resin	Liquid: Clove oil, Canada Balsam
Sealapex, SybronEndo USA	Paste A: Isobutyl salicylate resin, Silicon dioxide, Bismuth trioxide, Titanium dioxide pigment	Paste B: N-ethyl toluene solfanamide resin, Silicon dioxide, Zinc oxide, Calcium oxide

Zymography

The activities of MMP-2 and MMP-9 of the cell culture supernatants were measured by gelatin zymogram protease assay as previously described (17). Aliquots of supernatants (20 µl) were mixed with sample buffer (2% SDS; 125 mM Tris-Hcl pH 6,8, 10% glycerol and 0.001% bromophenol blue) and loaded on the gel. Then, prepared samples were

subjected to electrophoresis with 8% SDS polyacrylamide gels containing 0.1% gelatin. Following electrophoresis the gels were washed twice in 2.0% Triton X-100 for 30 min at room temperature to remove SDS. The gels were then incubated at 37°C for 18 h in substrate buffer containing 50 mM Tris-HCL and 10 mM $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid. Proteins standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. Enzyme activity was assayed by densitometry using a Kodak Electrophoresis Documentation and Analysis System (Kodak, Rochester, NY, USA), and the intensities of digitalized bands were normalized with regard to an internal standard (FBS) to allow intergel analysis and comparation, as previously described (Gerlach *et al.,* 2007).

Cytotoxicity Assay

To verify if the fresh sealers were cytotoxic, MRC5 fibroblasts cells were exposed to the sealers and cultured for 1/2, 1, 4 and 24h in the two contact forms. After the predetermined time period, the tested material and medium were removed and 1 ml trypsin was added to remove the cell from the bottom of the wells. Cytotoxicity test was performed using trypan blue stain. Briefly, a freshly prepared solution of 10µl trypan blue (0.05%) in distilled water was mixed with 10µl of each cellular suspension for 5 min, spread onto a microscope hemocytometer, covered with a coverslip and counted with a light microscope at 100X. Dead cells allow the stain to enter their membranes, coloring their cytoplasm blue.

The live cells excluded the stain, remaining clear. At least 200 cells were counted per treatment (Zeferino *et al.,* 2000).

Statistical Analysis

Triplicate experiments were performed throughout this study. All assays were repeated 3 times to ensure reproducibility. Data from assays are presented as means ± standard deviation (SD). The results were subjected to one way analysis of variance (ANOVA) and statistical differences among the groups were analyzed using the Student´s *t*-test at a significance level of 5%. Data were analyzed using statistical software SPSS® (IBM, USA).

RESULTS

Zymography

Specific characterization of MMPs in the cell culture supernatants by gelatin zymography demonstrated that MMP-2 (72kd) was released by MRC5 cells, when the cells were in booth contact forms with de sealers. However, the cell culture supernatants did not express detectable gelatinolytic activity of MMP-9 (92kd). In the control group no MMP-2 and -9 activities could be detected (Fig. 1a). The induction of MMP-2 activity by MRC5 cells was similar to all tested root canal sealers. Gelatinolytic activity was found to be elevated by root canal sealers in direct contact with the cell monolayer (Fig. 1b), when compared to the indirect contact (Fig. 1b) ($p<0.05$). In the periods of 1/2h and 1 h, the gelatin zymogram did not express any detectable levels of MMP-2, but at the periods of 4h

and 24h, the MMP-2 gelatinolytic activity was detected for booth contact forms. There were no statistical differences in these two periods of contact. The quantitative measurements by the Kodak Electrophoresis Documentation and Analysis System are shown in Table 2.

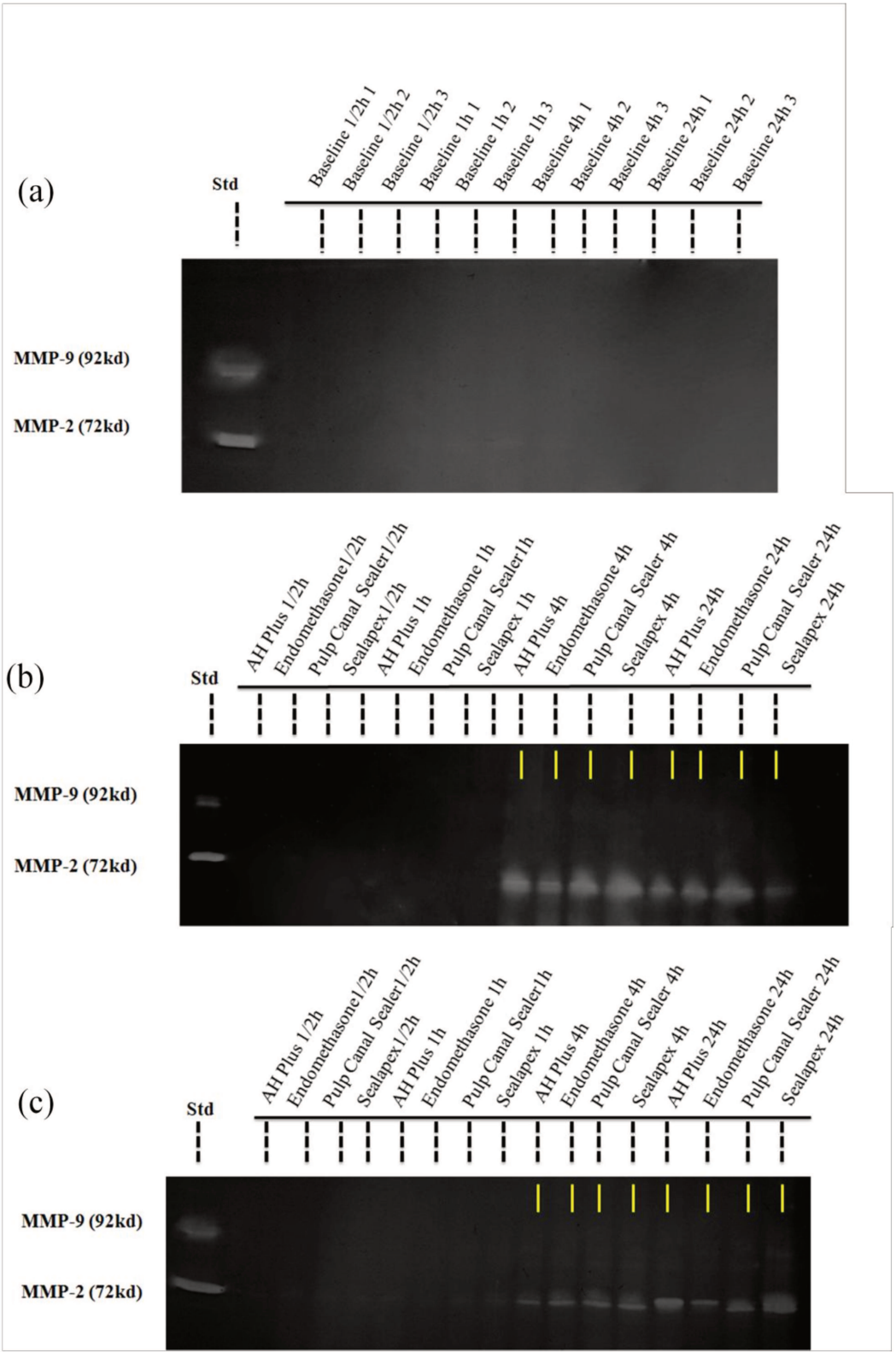
(a)
Std
Baseline 1/2h 1
Baseline 1/2h 2
Baseline 1/2h 3
Baseline 1h 1
Baseline 1h 2
Baseline 1h 3
Baseline 4h 1
Baseline 4h 2
Baseline 4h 3
Baseline 24h 1
Baseline 24h 2
Baseline 24h 3
MMP-9 (92kd)
MMP-2 (72kd)
(b)
Std
AH Plus 1/2h
Endomethasone1/2h
Pulp Canal Sealer1/2h
Sealapex1/2h
AH Plus 1h
Endomethasone 1h
Pulp Canal Sealer1h
Sealapex 1h
AH Plus 4h
Endomethasone 4h
Pulp Canal Sealer 4h
Sealapex 4h
AH Plus 24h
Endomethasone 24h
Pulp Canal Sealer 24h
Sealapex 24h
MMP-9 (92kd)
MMP-2 (72kd)
(c)
Std
AH Plus 1/2h
Endomethasone1/2h
Pulp Canal Sealer1/2h
Sealapex1/2h
AH Plus 1h
Endomethasone 1h
Pulp Canal Sealer1h
Sealapex 1h
AH Plus 4h
Endomethasone 4h
Pulp Canal Sealer 4h
Sealapex 4h
AH Plus 24h
Endomethasone 24h
Pulp Canal Sealer 24h
Sealapex 24h
MMP-9 (92kd)
MMP-2 (72kd)

Figure 1. (a) Gelatin zymogram of medium from MRC5 cells without any treatment (Control Group) (b) Gelatin zymogram of conditioned medium from MRC5 cells treated with AH Plus, Endomethasone, Pulp Canal Sealer and Sealapex in different times with a direct contact. (c) Gelatin zymogram of conditioned medium from MRC5 cells treated with AH Plus, Endomethasone, Pulp Canal Sealer and Sealapex in different times with an indirect contact. STD shows the fetal bovine serum, which was used as a standard to normalize the data from all the gels, thus allowing comparisons among.

Table 2. Levels of MMP-2 from conditioned medium treated with different endodontic sealers and different contact forms.

Endodontic Sealer	Contact	4h	24h
AH Plus	Direct	1.76 ± 0.377^{A}	1.68 ± 0.400^{A}
	Indirect	1.02 ± 0.230^{B}	1.08 ± 0.188^{B}
Endomethasone N	Direct	1.37 ± 0.290^{A}	1.36 ± 0.167^{A}
	Indirect	0.94 ± 0.230^{B}	0.84 ± 0.262^{B}
Pulp Canal Sealer EWT	Direct	1.64 ± 0.355^{A}	1.61 ± 0.351^{A}
	Indirect	0.96 ± 0.230^{B}	1.07 ± 0.212^{B}
Sealapex	Direct	1.48 ± 0.306^{A}	1.34 ± 0.386^{A}
	Indirect	0.84 ± 0.184^{B}	0.92 ± 0.247^{B}

Table 2- Levels of MMP-2 from conditioned medium were calculated from their gelatinolytic activity, as measured by Kodak Digital Science. Values are means and standard deviations of optical density from triplicate experiments. Different letters represents significant differences between the groups ($p<0.05$).

Cytotoxicity assay

The cytotoxicity of endodontic sealers was measured in MRC5 fibroblasts by trypan blue exclusion assay. All root canal sealers tested were cytotoxic, but the toxicity depended on the materials tested and the contact form used. Figures 2a and 2b shows the cytotoxic effects of different sealers, in different times of exposure and in the direct and indirect contact, respectively.

Among the materials tested with a direct contact after mixing, Sealapex exhibited the lowest cytotoxicity. The AH Plus and the Pulp Canal Sealer EWT were statistically more cytotoxic that the Sealapex at all time points. Endomethasone also demonstrated cytotoxic effects, but the cytotoxicity was lower than the AH Plus and Pulp Canal Sealer EWT in the periods of 1/2h and 1h.

AH Plus also caused the greatest cytotoxic effects when used in indirect contact. In this contact form do not appear to be any difference in the cytotoxic effects of the other tested sealers, although all the materials showed some cytotoxic effects. In the two contact forms cytotoxic effects varied with time, causing greater toxicity with increased sealer exposure time.

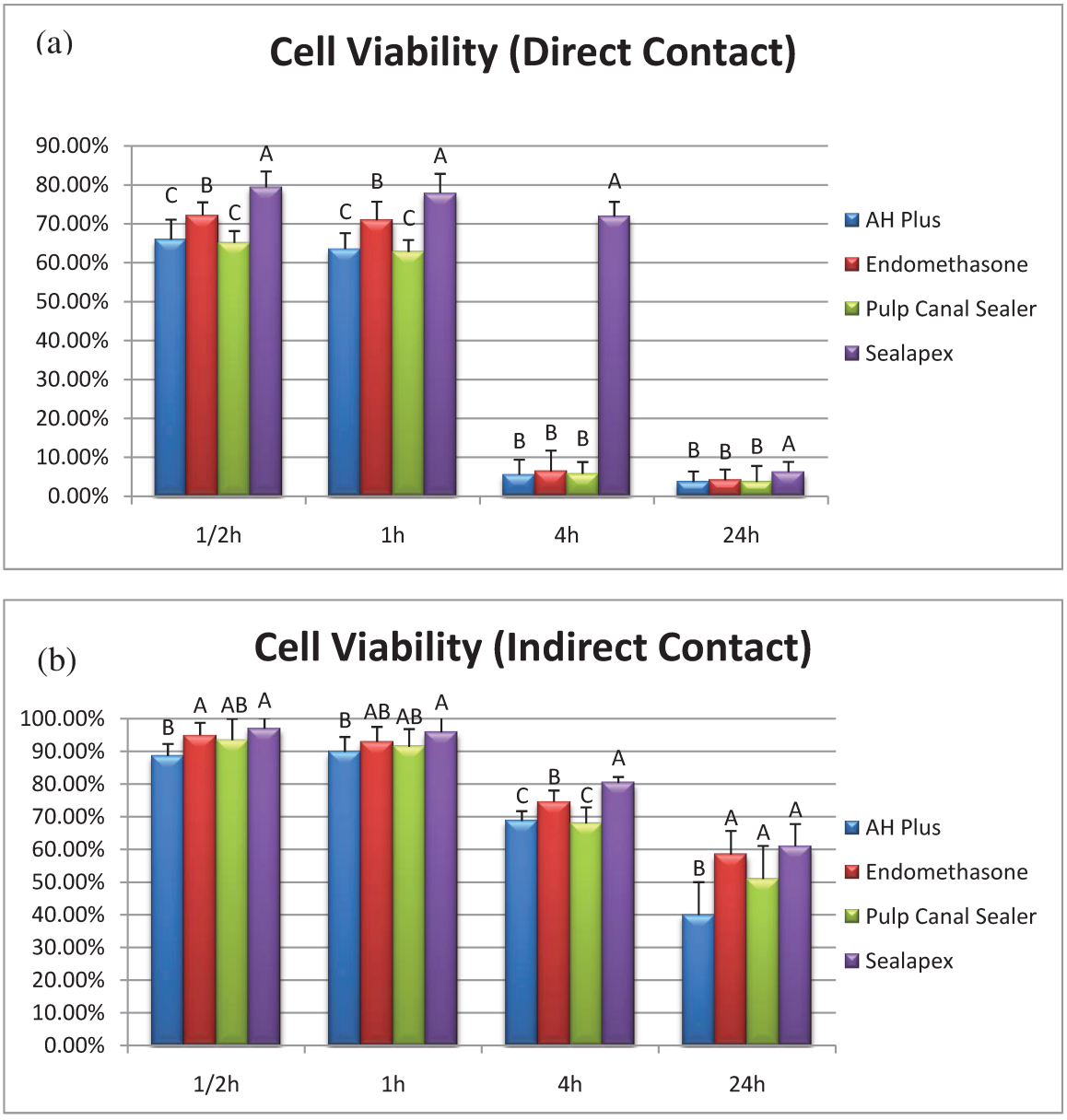

Fig 2- (a) Citotoxic effects following direct exposure to different endodontic sealers in MRC5 fibroblast cells. Results are expressed as mean and standard deviation. Different letter shows statistical difference between the groups in the exposure time ($p<0.05$). (b) Citotoxic effects following indirect exposure to different endodontic sealers in MRC5 fibroblast cells. Results are expressed as mean and standard deviation. Different letter shows statistical difference between the groups in the exposure time ($p<0.05$).

DISCUSSION

In this study, the citotoxicity of endodontic sealers and effect of these sealers on the gelatinolytic activity in human MRC5 fibroblast cells were investigated. Fibroblasts are the major constituents of connective tissue and are the most important collagen producers in this tissue. Human fibroblasts secrete MMPs capable of initiating the degradation of Extra Cellular Matrix macromolecules, and it seems to be a key event for the progression of the inflammatory process. MMP-2 and MMP-9 are of particular interest because they are synthesized by fibroblasts and pulp cells and have been implicated in the pathogenesis of periodontitis and pulpal inflammation (Chang *et al.,* 2001; Shin *et al.,* 2002; Tsai *et al.,* 2005). In this study MRC5 cells have been demonstrated to produce MMP-2, after exposure of endodontic sealers. Although the cell culture conditions and the endodontic sealers used in this study differed from those used in a previous report (Huang *et al.,* 2008), booth results suggests endodontic sealers can induce the production of MMP-2. MMP-9 gelatinolytic activity could not be detected by Kodak Digital Science software. Although some authors demonstrated that fibroblasts do not secret MMP-9 (Ghahary *et al.,* 2004; Sawicki *et al.,* 2005), previous studies have showed MMP-9 could be expressed in small amounts when compared with MMP-2 (Chang *et al.,* 2001; Chang *et al.,* 2002; Shin *et al.,* 2002; Tsai *et al.,* 2005; Huang *et al.,* 2008). The absence of gelatinolytic activity in this experiment could be attributed to the non detectable activity by Kodak Digital Science software. The control group did not produce MMP-2 or –9 in the tested periods.

Several *in vivo* studies have evaluated the biocompatibility of endodontic sealers, and indicated that toxic components present in these materials could produce irritation or

even degeneration, especially when accidentally extruded into the periradicular tissues (Bernath & Szabo, 2003; Kao *et al.,* 2006). The sealers exposed in the direct contact induced a major expression of MMP-2, showing statistical differences when compared to the indirect contact ($p<0.05$). This result is in agreement to a previous study which reveals that endodontic sealers confined to the canal is an important factor in reducing periapical inflammation (Bouillaguet *et al.,* 2004).

The citotoxicity of the four root canal sealers evaluated using the trypan blue exclusion assay in human MRC5 fibroblast cells, was dependent on time of exposure, contact form and material. The results of the current study showed that all classes of currently available endodontic sealers had cytotoxic effects in the freshly mixed condition. The freshly mixed condition is relevant to clinical use because the sealers are placed into the canal unset and must set *in situ* (Bouillaguet *et al.,* 2004; Bouillaguet *et al.,* 2006). The freshly mixed materials in both contact forms were severely cytotoxic increasing the cytotoxicity with time. Our results agree with previous reports demonstrating that all tested materials were cytotoxic (Huang *et al*., 2002; Schwarze *et al*., 2002; Bernath & Szabo, 2003; Bouillaguet *et al.,* 2004; Bouillaguet *et al.,* 2006; Huang *et al.,* 2008; Lodiene *et al.,* 2008). The sealers were moderately toxic initially (1/2 h and 1 h exposure), but the toxicity increased with time (4 h and 24 h exposure) and this result is in agreement with previous *in vitro* investigations (Bouillaguet *et al.,* 2004; Bouillaguet *et al.,* 2006)

AH Plus is a 'formaldehyde free' material according to the manufacturer. However a previous *in vitro* study reported that this new formulation could also release minimal but existent formaldehyde release from AH Plus (Leonardo *et al.,* 1999). In the present study

AH Plus showed marked cytotoxic effects in the MRC5 fibroblast cells. It can be attributed to a release of small amounts of formaldehyde or amine and epoxy resins components of the sealer. The results of the current study correlate with the severe toxicity of AH Plus confirmed in previous reports (Huang *et al.,* 2002, Huang *et al.,* 2004; Miletic *et al.,* 2005). Pulp Canal Sealer EWT and Endomethasone N are both zinc oxide-eugenol sealers, and a moderate cytotoxicity was observed for both endodontic sealers. This toxicity can be attributed to free eugenol liberated from the set material. Released eugenol may participate in the development of periapical inflammation or the continuation of a pre-existing periapical lesion (Ho *et al.,* 2006). A previous study also has shown that Endomethasone can release formaldehyde after setting (Leonardo *et al.,* 1999), but in the present study was used Endomethasone N, a new formulation that is 'formaldehyde free' according to the manufacturer. The groups treated with Sealapex, a calcium hydroxide-based sealer, appeared to have the highest cell viability. Several *in vitro* studies using calcium-hydroxide based sealers showed a similarly good biocompatibility (Beltes *et al.,* 1995; Miletic *et al.,* 2005; Willershausen *et al.,* 2006). Other studies showed different results, appointing Sealapex as a high cytotoxic sealer (Beltes *et al.,* 1995; Leonardo *et al.,* 2000; Huang *et al.,* 2004). However, it is difficult to compare the results from different cell culture experiments because of the main variations in experimental conditions such as the cell type, the cell material contact method, the applied methodology of cytotoxicity test and the exposure time (Spangberg, 1981).

CONCLUSION

Although the relevance of *in vitro* tests to clinical conditions has been frequently questioned, data from our *in vitro* experiments showed that all root canal sealers can induce the expression of MMP-2 in MRC5 fibroblast cells. It can also be concluded that AH Plus presented the highest cytotoxicity among the tested sealers, but all tested sealers presents citotoxic effects on the cells culture. Despite the transitory irritability that sealer in contact with periapical tissues may cause, endodontist should evaluate the advantages and disadvantages of this sealer extrusion, since the remaining areas not sealed in the apical region may serve as microorganism niches, initiating or perpetuating an endodontic failure.

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4. CONCLUSÃO

Dentro da metodologia empregada e de acordo com os resultados apresentados pode-se concluir que:

1. A atividade gelatinolítica de MMP-2 mostrou um aumento após 4h e 24h de exposição com os cimentos endodônticos, sem haver diferença entre esses dois períodos de tempo.
2. Não houve diferença na atividade gelatinolítica de MMP-2 entre os cimentos testados.
3. A atividade gelatinolítica de MMP-2 foi maior quando os cimentos eram expostos à cultura celular de forma direta, do que quando comparados a exposição de forma indireta.
4. Não houve atividade gelatinolítica de MMP-9 em nenhuma das condições testadas.
5. O Sealapex foi o material menos citotóxico enquanto o AH Plus apresentou os maiores níveis de citotoxicidade.
6. A citotoxidade aumentou proporcionalmente com o aumento do tempo de exposição ao cimento até o período de 24h.

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6. APÊNDICE

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