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NATÁLIA MARTINS JOAQUIM

Análise da Citotoxicidade de Materiais Obturadores de Dentes Decíduos

Cytotoxicity analyses of filling materials for primary teeth

Piracicaba

2014

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

NATÁLIA MARTINS JOAQUIM

Análise da Citotoxicidade de Materiais Obturadores de Dentes Decíduos

Cytotoxicity analyses of filling materials for primary teeth

Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do Título de Mestra em Odontologia, na área de Odontopediatria.

Dissertation presents to the Piracicaba Dental School of the University of Campinas for the degree of Master Dentistry, in Pediatric Dentistry Area.

Orientadora: Profa. Dra. Fernanda Miori Pascon

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RESUMO

Endodontia em dentes decíduos é um procedimento de suma importância para manter a integridade e saúde dos dentes e tecidos de suporte. Sendo assim, o uso de materiais obturadores de canais radiculares que apresente o máximo de propriedades desejáveis é indispensável. O objetivo do presente estudo foi analisar a citotoxicidade de diferentes materiais obturadores em fibroblastos do ligamento periodontal humano (PDL) e em células osteoblásticas de osteossarcoma humano (Saos-2). Os fibroblastos foram cultivados em meio de cultura (meio Eagle modificado por Dulbeco - DMEM) suplementado com 1% soro fetal bovino (FBS) e antibióticos. Os osteoblastos foram cultivados em meio de cultura McCoy's suplementado com 15% FBS e antibióticos. Próximos de atingir a confluência, as células foram plaqueadas na concentração de $7x10^3$ células por poço e foram expostas aos seguintes materiais, conforme os grupos: G1- Meio de cultura sem material obturador (controle negativo), G2- Dimetiilsulfóxido (DMSO) (controle positivo), G3- Calen®, G4- Calen® associada ao Óxido de Zinco, G5- Calen® associada ao Iodofórmio, G6- Óxido de zinco e eugenol (OZE), G7- Vitapex® e G8- UltraCal®XS. A manipulação dos materiais foi realizada em condições assépticas. A citotoxicidade dos materiais obturadores foi analisada em intervalos de tempos (8, 24 e 48 horas) pelo método de redução MTS e classificado como não citotóxico, citotoxicidade leve, moderada e grave. O grupo controle negativo foi composto apenas por células, sem o uso de material obturador. A análise morfológica das células foi realizada por meio da microscopia de fluorescência. Os dados obtidos foram submetidos à análise de variância dois fatores e ao teste Tukey para comparação entre os grupos, com nível de significância 5%. As imagens obtidas por meio da microscopia de fluorescência foram analisadas de forma descritiva. Os resultados mostraram que para os fibroblastos, Calen®(85,91±10,01), Calen® associada ao Óxido de Zinco (85,91±8,16) e Calen® associada ao Iodofórmio (83,96±13,95) diferiram do controle negativo (100±0) e positivo (19.72±5,70) após 8 horas de exposição. Para os osteoblastos, Calen® associada ao Óxido de Zinco (75,87±19,16), Calen® associada ao Iodofórmio (75,5±12,40) e o OZE (68,71±22,19) foram os únicos grupos que em 8 horas diferiram do controle negativo (100±0) e positivo (22,18±6,77). Pode-se concluir que todos os materiais avaliados, para fibroblastos do ligamento periodontal humano, não foram citotóxicos ao longo do tempo.

No entanto, Calen® associada ao Iodofórmio apresentou toxicidade leve em 48 horas para os osteoblastos. Vitapex® foi o material que apresentou menor toxicidade celular nos osteoblastos em 8 e 48 horas, comparando-se os outros materiais avaliados. Calen® associado ao Óxido de zinco, Calen® associado ao Iodofórmio e OZE foram capazes de modificar a morfologia dos fibroblastos, mas para os osteoblastos não foram observadas alterações morfológicas.

Palavras-Chave: Dente Decíduo. Endodontia. Materiais Restauradores do Canal Radicular. Forma celular. Técnicas de cultura de células. Sobrevivência celular.

ABSTRACT

Endodontics in primary teeth is an important procedure to maintain the integrity and health of the teeth and supporting tissues. Using a root canal filling material that shows desirable properties is indispensable. The aim of the present study was to evaluate the root filling materials cytotoxicity on periodontal ligament fibroblasts (PDL) and osteoblastic human osteosarcoma cells (Saos-2). Fibroblasts were cultured in culture medium (Dulbeco modified Eagle medium - DMEM) supplemented with 1% fetal bovine serum (FBS) and antibiotics. The osteoblasts were cultured in McCoy's culture medium supplemented with 15% FBS and antibiotics. Next to reach confluence, the cells were plated at a concentration of $7x10^3$ cells per well and were exposed to materials, according to the groups: G1 - culture medium without filling material (negative control); G2- Dimethyl sulfoxide (DMSO) (positive control); G3 – Calen®; G4 - Calen® associated with Zinc Oxide; G5 - Calen® associated with Iodoform; G6 – Zinc Oxide and eugenol (ZOE); G7 - Vitapex®; G8 - UltraCal® XS. The materials were prepared under aseptic conditions. Cytotoxicity was evaluated by cell viability at time intervals (8, 24 and 48 h) by MTS assay and rated as non-cytotoxic, mild, moderate and severe cytotoxicity. The negative control group was composed only of cells without the use of filling material. Cells morphological were observed by fluorescence microscopy. Data were submitted to two-way analysis of variance with post-hoc comparisons base on Tukey's multiple comparisons, with the significance level fixed at 5%. The images obtained at fluorescence microscopy were evaluated using descriptive analysis. The results showed that for fibroblasts, Calen®(85.91±10.01), Calen® associated with Zinc Oxide (85.91±8.16) e Calen® associated with Iodoform (83.96±13.95) was differ from the negative control (100±0) and positive (19.72±5.70), at 8 h. For osteoblasts, Calen® associated with Zinc Oxide (75.87±19.16), Calen® associated with Iodoform (75.5±12.40) and ZOE (68.71±22.19) differed from negative control (100±0) and positive (22.18±6.77) in 8 h. It can be concluded that all materials were non- cytotoxic to human fibroblasts cells over time. However, Calen® + Iodoform showed higher cytotoxicity to osteoblasts at 48 h. Vitapex® was the material that showed the less cell cytotoxicity in osteoblasts at 8 and 48 h, compared to the other materials tested. Calen® associated with

Zinc Oxide, Calen® associated with Iodoform and ZOE was able to modify the morphology of fibroblasts, but osteoblasts but no morphologic alterations were observed.

Keywords: Tooth, Deciduous. Endodontics. Root Canal Filling Materials. Cell Shape. Cell Culture Techniques. Cell Survival.

SUMÁRIO

DEDICATÓRIA	xiii
AGRADECIMENTOS	xv
INTRODUÇÃO	1
CAPÍTULO 1	5
Cytotoxicity analyses of filling materials for primary teeth	5
CONCLUSÕES GERAIS	30
REFERÊNCIAS	31
ANEXOS	33
ANEXO 1	34
ANEXO 2	35
ANEXO 3	36

DEDICATÓRIA

Dedico esta conquista….

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INTRODUÇÃO

A rápida progressão da cárie dental nos dentes decíduos pode produzir danos pulpares devido à contaminação do tecido pulpar por bactérias e toxinas produzidas pelas mesmas (Camp, 1994; Carrote, 2005). Sendo assim, dentes decíduos com envolvimento pulpar devem ser tratados, seja por meio da terapia pulpar ou por extração do elemento dental (Carrote, 2005). Entretanto, a perda prematura desses elementos pode causar deficiências na função mastigatória, hábitos linguais deletérios, alterações na fala, movimentos indesejáveis dos dentes decíduos ou permanentes e ainda ocasionar perda do comprimento do arco dentário. Ainda, a manutenção do comprimento é importante para guiar a futura erupção da dentição permanente com desenvolvimento satisfatório da oclusão (Carrote, 2005; Trairatvorakul & Chunlasikaiwan, 2008; Barcelos *et al*., 2011).

A pulpectomia é uma das terapias pulpares realizadas em dentes decíduos. Este tratamento foi preconizado em 1932 como sendo um método de manutenção dos dentes decíduos no arco dentário para evitar a perda do mesmo (Mortazavi & Mesbahi, 2004). Segundo a Academia Americana de Odontopediatria (AAPD) (2014), a pulpectomia tem como objetivo principal a manutenção do dente, a integridade e saúde dos dentes e dos tecidos de suporte, sendo realizado quando o tecido pulpar apresenta inflamação irreversível ou necrose (AAPD, 2014; Barcelos *et al*., 2011). O procedimento consiste em realizar a limpeza, o alargamento, a desinfecção e a obturação do sistema de canais radiculares com um material obturador adequado, que considere as diferenças existentes entre os dentes decíduos e permanentes, principalmente o processo de reabsorção radicular (AAPD, 2014; Barcelos *et al*., 2011). Como os materiais obturadores estão em íntimo contato com os tecidos periapicais, sua biocompatibilidade é um fator extremamente importante (Cotti *et al*., 2014). Entretanto, devido à ausência de um material obturador adequado, o sucesso do tratamento pode ser comprometido (Barcelos *et al*., 2011; Barja-Fidalgo *et al*., 2011) .

Sendo assim, a Odontopediatria busca um material obturador que apresente o máximo de propriedades desejáveis: reabsorção concomitante com o processo de rizólise, biocompatibilidade com os tecidos periapicais e germe do dente permanente, atividade antimicrobiana, adequado preenchimento e aderência às paredes dos canais, facilidade de inserção e remoção, se necessário, ser reabsorvível e não solúvel em água (Leal, 1998;

Mortazavi & Mesbahi, 2004; Brackett *et al*., 2010; Sarigol *et al*., 2010; Yoshino *et al*., 2013).

Segundo a AAPD (2014), os materiais obturadores de dentes decíduos mais utilizados são à base de OZE, hidróxido de cálcio e iodofórmio. Sendo que para obter melhores resultados quanto ao processo de reabsorção, materiais à base de hidróxido de cálcio podem ser associados ao óxido de zinco (Silva *et al.*, 2010).

Materiais à base de óxido de zinco e eugenol foram recomendados por Sweet em 1930 (Mortazavi & Mesbahi, 2004) e ainda são utilizados. Apesar do poder antimicrobiano, não são considerados histologicamente biocompatíveis, produzindo reação inflamatória tecidual e reabsorção apical mais lenta, podendo prejudicar a formação e erupção dos dentes permanentes sucessores (Mortazavi & Mesbahi, 2004; Trairatvorakul & Chunlasikaiwan, 2008; Silva *et al*., 2010; Barcelos *et al.*, 2011). Portanto, estes materiais vêm sendo substituídos por materiais mais adequados como os materiais à base de hidróxido de cálcio (Mortazavi & Mesbahi, 2004; Trairatvorakul & Chunlasikaiwan, 2008; Silva *et al*., 2010; Barcelos *et al.*, 2011). O hidróxido de cálcio apresenta atividade antibacteriana, induz formação de tecido mineralizado e apresenta excelentes propriedades biológicas. Entretanto, possui algumas propriedades físico-químicas desfavoráveis, como permeabilidade tecidual, reabsorção na região periapical e solubilidade no canal radicular, além de baixa radiopacidade (Faria *et al.*, 2005; Queiroz *et al.*, 2009; Silva *et al.*, 2010).

A Calen® é um material presente no mercado à base de hidróxido de cálcio, o qual apresenta como veículo o polietilenoglicol 400, um veículo hidrossolúvel que mantém o hidróxido de cálcio por mais tempo na área desejada, prolongando a ação, diminuindo a solubilização e aumentando a penetrabilidade na dentina radicular (Cerqueira, 2009). Outro material utilizado é a UltraCal®XS, o qual apresenta em sua composição um veículo aquoso. Estudos mostraram altos valores de pH e atividade antimicrobiana para esse material. Entretanto, até o presente momento, poucos estudos mostram a biocompatibilidade do mesmo (Andolfatto *et al.*, 2012). Além disso, associações de materiais, como hidróxido de cálcio e óxido de zinco, tem sido estudadas com o intuito de se observar biocompatibilidade da associação (Silva *et al.*, 2010), entretanto, mais estudos precisam ser conduzidos.

A AAPD também indica um material à base de hidróxido de cálcio associado ao iodofórmio. Comercialmente há um material com essa composição chamado Vitapex®. Este apresenta óleo de silicone como excipiente/veículo e o iodofórmio como agente bacteriostático. Estudos demonstraram como características desse material, a ausência de efeitos tóxicos e deletérios ao sucessor permanente, reabsorção semelhante ao dente decíduo e radiopacidade adequada (Kubota *et al.*, 1992; Cerqueira, 2009).

Biocompatibilidade de materiais utilizados em endodontia pode ser caracterizada por diversos parâmetros como genotoxicidade, mutagenicidade, carcinogenicidade, histocompatibilidade ou citotoxicidade (Keresztesi & Kellner, 1966) e pode ser avaliada por diferentes métodos. De acordo com Costa & Souza (2005) a citotoxicidade pode apresentar amplas consequências, desde as mais simples, como morte celular, até aberrações metabólicas, nas quais não ocorre morte celular, mas ocorrem alterações funcionais em alguma via específica.

Dessa forma, estudos com cultura de células tem sido realizados para investigar reações citotóxicas (Rappaport *et al.*, 1964) podendo ser utilizadas células osteoblásticas de osteosarcoma (U2OS, Saos-2) e fibroblastos (L929, 3T3) , fibroblastos da polpa, gengiva e ligamento periodontal (Huang *et al.*, 2002; Camps, 2003; Brackett *et al*., 2010; Karapınar-Kazandag *et al.*, 2011; Silva *et al.*, 2010; Scelza *et al.*, 2012; Yoshino *et al.*, 2013; Cotti *et al*., 2014). Entretanto, poucos estudos avaliaram a citotoxicidade de materiais obturadores indicados para dentes decíduos sobre células osteoblásticas e fibroblastos do ligamento periodontal (Samara *et al.*, 2011), o que seria relevante, uma vez que os fibroblastos são responsáveis pela formação e manutenção das fibras do ligamento, assim como, pela reparação, remodelação e regeneração do osso alveolar adjacente ao cemento, e as células osteoblásticas são responsáveis pela síntese dos compostos orgânicos da matriz óssea, como o colágeno, proteoglicanos e glicoproteínas (Gandolfi *et al*., 2008).

Além disso, o emprego de testes *in vitro* oferece a possibilidade de estudar os efeitos da liberação dos componentes dos materiais nos sistemas celulares (Oztan *et al.*, 2003), sendo relativamente simples, reprodutíveis, com boa relação custo-benefício e adequados para a avaliação de aspectos biológicos básicos em relação à biocompatibilidade (De-Deus *et al.*, 2009). Um destes testes avalia quantitativamente a viabilidade celular após os tratamentos e é chamado de teste de MTS. O MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) é um sal de tetrazólio solúvel em água que é reduzido por células viáveis em um produto violeta, insolúvel em solução aquosa. O número de células viáveis se correlaciona com a intensidade da cor, determinado por colorimetria após a dissolução do formazano em álcool (Guven *et al.,* 2013). Ainda, a microscopia de fluorescência também é uma ferramenta utilizada para verificar a morfologia das células em contato com os materiais obturadores.

Em virtude do exposto e conhecendo-se a importância da avaliação das propriedades citotóxicas de materiais obturadores utilizados na endodontia de dentes decíduos, para auxiliar na seleção e aplicabilidade clínica dos mesmos, justifica-se a realização desse estudo. Os objetivos da presente dissertação foram: (1) Analisar a citotoxicidade de materiais obturadores sobre fibroblastos do ligamento periodontal (PDL) e células osteoblásticas de osteossarcoma humano (Saos-2), por meio de ensaio de viabilidade celular; e (2) Analisar a morfologia celular de fibroblastos e osteoblastos humanos, frente à exposição aos materiais obturadores por meio de microscopia de fluorescência.

Para alcançar esses objetivos, esta Dissertação foi apresentada em um capítulo.[1]

[1] * Esta dissertação está baseada na resolução CCPG/002/06, a qual dispõe a respeito do formato das teses de mestrado e doutorado aprovados pela UNICAMP

CAPÍTULO 1

Cytotoxicity analyses of filling materials for primary teeth

Short title: Cytotoxicity of filing materials

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Cytotoxicity analyses of filling materials for primary teeth

ABSTRACT

Objectives: To evaluate cytotoxicity of filling materials used in primary teeth on human periodontal ligament fibroblasts and osteoblastic osteosarcoma cells by MTS assay and to evaluate cell morphology by fluorescence microscopy.

Methods: Cultured cells ($7x10^3$ cells/well) were exposed to different media, as follows: G1-Culture medium (negative control); G2-Dimethyl sulfoxide (positive control); G3-Calen®; G4-Calen®+Zinc Oxide; G5-Calen®+Iodoform; G6-Zinc Oxide and Eugenol (ZOE); G7-Vitapex®; G8-UltraCal®XS. Cytotoxicity was evaluated by cell viability at time intervals (8, 24 and 48 h), and rated as non-cytotoxic, mild, moderate and severe cytotoxicity. Data were submitted to two-way ANOVA and Tukey tests ($p>0.05$) and to descriptive analysis.

Results: At 8 h, for fibroblasts, G3 (85.91±10.01), G4 (85.91±8.16) and G5 (83.96±13.95) was statistically different from G1 (100±0) and G2 (19.72±5.70). For osteoblasts, G4 (75.87±19.16), G5 (75.5±12.40) and G6 (68.71±22.19) differed from G1 (100±0) and G2 (22.18±6.77). At 48 h, G5 (73.98±7.89) and G7 (139.88±38.67) differed from G1 (100±0). When the materials were compared, G7 showed the highest cell viability at 48 h for osteoblasts, differing from all groups ($p<0.05$). As regards the percentage of toxicity, groups that showed cytotoxicity were rated as having a mild degree. Fibroblasts exposed to G4, G5 and G6 showed loss of morphological features and for osteoblasts none of groups showed significant alterations.

Conclusions: All materials tested showed a mild degree of cytotoxicity at different times, for both cells, except Vitapex®, which was non-cytotoxic for osteoblasts cells at all times of exposure. Morphological alterations were observed in fibroblasts for Calen®+Zinc Oxide, Calen®+Iodoform and ZOE.

Clinical Significance: Pediatric Dentists should be aware that some chemical components of filling materials used in primary teeth may cause long-term local irritation to the permanent successor. Based on this aspect, it is important to know the cytotoxic effects of these materials to help with the selection of materials and clinical application.

1. Introduction

Pulpectomy therapy consists of cleaning, widening, disinfecting and obturating the root canal system with a suitable filling material, when the pulp tissue shows irreversible inflammation or necrosis and the aim is to preserve the integrity and health of the teeth and their supporting tissues. [1,2]

Considering the differences between primary and permanent teeth, mainly as regards the root resorption process, studies have been conducted on root canal filling materials that present the most desirable properties. These include being resorbable along with the roots, biocompatibility with the periapical tissues and permanent tooth germ, antimicrobial activity, adequate filling adherence to the root canal walls and being easily inserted and removed if necessary.[3-8]

Materials based on zinc oxide, eugenol and calcium hydroxide are commonly used in primary teeth.[1] Historically zinc oxide and eugenol (ZOE) were first recommended by Sweet in 1930, for primary tooth pulpectomy.[9] Eugenol, the main constituent of clove oil, is weakly ionized and has a dimeric structure with both hydrogen bonds.[10] It is the component responsible for the antimicrobial activity.[11] However, studies have reported slow resorption of this material, and the non-reabsorbed particles can make the formation and eruption of permanent tooth successors difficult.[2,4,12,13] Thus, ZOE-based material have been replaced by those based on calcium hydroxide.[4,12] Furthermore, to achieve the best results as regards the resorption process, calcium hydroxide-based material may be associated with zinc oxide and iodoform.[4,13]

Calcium hydroxide is a white odorless powder, chemically classified as a strong base, which dissociates into calcium (Ca^{2+}) and hydroxyl ions (OH^-) in aqueous fluids. It has low solubility in water and the pure powder has a high pH.[14] The important actions of calcium hydroxide come from the ionic dissociation of Ca^{2+} and OH^- ions and their effect on vital tissues, generating induction of hard-tissue deposition and being antibacterial. In addition to the capacity of calcium hydroxide to induce pulp/periapical tissues conservation and repair, it is permeable to tissue fluids and has antimicrobial activity.[2,13-16]

A calcium hydroxide-based material, commercially known as Calen® can be used in Pediatric Dentistry. It is composed of calcium hydroxide, polyethylene glycol 400 and a water-soluble vehicle. The vehicle maintains the calcium hydroxide in the desired area over

time, prolonging its action and decreasing is solubility. This material has antibacterial activity and biological properties, but it has some unfavorable physicochemical properties such as tissue permeability, solubility in the root canal and low radiopacity.[13,17-19] UltraCal®XS is also a calcium hydroxide-based material with an aqueous vehicle. Studies have shown high pH values and antimicrobial activity. However few studies have demonstrated the biocompatibility of this material.[20]

Vitapex® is also recommended by the AAPD for filling root canals of primary teeth. This material is composed of calcium hydroxide, silicone oil and iodoform. The presence of calcium hydroxide and iodoform provides the antibacterial properties of this material and iodoform is also used to increase radiopacity.[2] Studies have shown that this material has good radiopacity, absence of toxicity and resorption similar to tooth resorption.[4,6,15]

Knowing that endodontic treatment creates a unique and complex biomaterial-tissue interface at the tooth root apex, and that canal filling materials are in intimate, long-term contact with multiple cell types in the periradicular tissues, it is relevant to analyze the biocompatibility of materials.[5,21] Studies with cultured cells have been conducted to investigate cytotoxic reactions[22] and osteosarcoma, osteoblast and fibroblast cells may be used.[3,5,8,13,23-25] Many studies have evaluated the properties of these materials.[18,19,26,27] However, no studies have evaluated the cytotoxicity of endodontic materials indicated for primary teeth on periodontal ligament fibroblast and osteoblast cells. Periodontal ligament fibroblasts are responsible for the formation and maintenance of the ligament cell fibers, as well as the repair, remodeling and regeneration of the adjacent alveolar bone and cementum. Osteoblast-like cells are responsible for the synthesis of organic compounds in the bone matrix, such as collagen, proteoglycans and glycoproteins.[28] Thus, knowing the importance of the cytotoxic properties, the present study was conducted to evaluate the filling materials used in primary teeth, in order to support their clinical selection and application.

The aim of this paper is to evaluate the *in vitro* cytotoxicity of filling materials frequently used in endodontics of primary teeth on human periodontal ligament fibroblasts and osteosarcoma osteoblastic cells lines (Saos-2) and cell morphology after exposure to filling materials. The tested hypothesis was that there would be a difference in cytotoxicity

and cell morphology when different filling materials were used for different exposure times.

2. Materials and methods

2.1. Sample selection and cell cultures

This study was conducted after approval of Ethical Committee and was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The sample consisted of periodontal ligament fibroblasts (PDL) obtained from four human premolars extracted for specific clinical reasons (pre-orthodontic treatment) and human Saos-2 osteoblast-like cells. The extracted teeth were immediately stored in Dulbecco Modified Eagle medium (DMEM) (Gbico, Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Gbico, Life Technologies, Carlsbad, CA) and antibiotics (ATB) (Gbico, Life Technologies, Carlsbad, CA). The PDL cells were carefully removed circumferentially from the middle third of the root surface and the tissue was processed with DMEM (Gbico, Life Technologies, Carlsbad, CA) supplemented with 10% FBS (Gbico, Life Technologies, Carlsbad, CA) and ATB. Cells were cultivated under standard conditions in a conventional incubator at 37°C in a humidified atmosphere with 95% air and 5% CO_2.

The PDL and Saos-2 osteoblast-like cell growth was monitored daily and when cells were close to reaching confluence (80-90% subconfluent), the subculture of these cells was performed, to obtain an optimal number for the entire experiment. DMEM + ATB + 10% FBS medium was used for PDL subculture and McCoy's 5A + ATB + 15% FBS for Saos-2 osteoblast-like cells. For routine cell counting, a cell counter (Countess Automated Cell Counter – Invitrogen) was used. After obtaining the optimal number of cells for the entire experiment, cell monolayers were trypsinized, and harvested cells were used for cytotoxicity experiments.

The cells were placed in a culture flask at a density of $30x10^5$cells/cm^2 and incubated at 37ºC in a humidified 5% CO_2 atmosphere for 24 h. After this period, the cells were trypsinized, as described above, and placed in a 96-well tissue culture plate, at a density of $7x10^3$per well and incubated at 37ºC in a humidified 5% CO_2 for 24 h. The PDL cells used for this study were between the third and tenth passages.[6]

2.2. Filling materials preparation

Controls and filling materials used in this study were divided into 8 groups: G1 - Culture medium without filling material (negative control), G2 - Dimethyl sulfoxide (DMSO) (positive control); G3 – Calen®; G4 - Calen® associated with Zinc Oxide; G5 - Calen® associated with Iodoform; G6 - ZOE; G7 - Vitapex® ; G8 - UltraCal®XS. The compositions and their manufacturers are listed in Table 1.

The concentration used for preparation of the conditioned medium was 0.1g of crude extract for filling material in 2ml of culture medium.[15] The materials were prepared under aseptic conditions according to the manufacturers' instructions. Calen® associated with Zinc Oxide and Calen® associated with iodoform, were manipulated in the ratio 1:1. ZOE was manipulated with 1g of powder to 10 liquid droplets.[13] The materials were weighed in an analytical precision balance. After preparation of the filling materials, they were incubated in rotation at 4ºC, for 16 h. After this period, they were centrifuged at 500g for 5 minutes at room temperature and the supernatant was removed and filtered with a 5ml syringe and syringe filters (0.22µm) in a laminar flow chamber (Thermo Heraeus, Berlin, Germany). Serial dilutions of the filtered supernatant were performed up to 10x concentration.

2.3. Exposure of cells to materials

After overnight cell attachment, the culture medium was aspirated and 100µl of the previously prepared materials were added per well. For positive control, 10% DMSO was used, and as negative control only cells without filling material were used. After filling materials and controls had been placed in 96-well tissue culture plates they were incubated at 37ºC in a humidified 5% CO_2 for time intervals of 8, 24 and 48 h. All materials and control group cultures were performed in quintuplicate to ensure reproducibility.

2.4. Cytotoxicity Assays

The cytotoxicity of filling materials was evaluated using the MTS (CellTiter 96® Aqueous One Solution Cell Proliferation Assay) test according to the manufacturer's instructions. MTS is a measure of cell viability based on the amount of formazan released into the surrounding medium, and is proportional to mitochondrial succinic dehydrogenase

activity of viable cells.[29] In addition, this assay has one less step, and produces water-soluble sorely colored formazans facilitating reading in the spectrophotometer (absorption maximum).[30]

Four hours before the time of completion of each group, 20µl MTS per well was added in 96 well tissue culture plates and incubated at 37ºC in a humidified 5% CO_2. After 4 h the absorbance was measured at a wavelength of 490 nm using an ELISA plate reader (Titrek; Multiskan plus EFIAB, Helsinki, Finland). Culture medium without cells was used as a blank. Percentage of cell viability was calculated using the following formula:

$$\% \text{ of cell viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

After this, the cytotoxicity of filling materials was rated, based on cell viability relative to control group, as: non cytotoxic (> 90%), mild (60 - 90%), moderate (30 - 59%) and severe (< 30%) cytotoxicity.[31-33]

2.5. Fluorescence microscopy analysis

For the fluorescence microscopy the cell growth was monitored daily up until an optimal number was obtained for the entire study. The cells were trypsinized and placed in a culture flask at a density of 30×10^5cells/cm^2 and incubated at 37ºC in a humidified 5% CO_2 atmosphere for 24 h. After this period, the cells were trypsinized and placed in a Lab TekII Chamber Slide (Sarstedt), 8 wells, at a density of 5×10^4cells per well for fibroblasts and 1×10^5cells per well for osteoblasts, and incubated at 37ºC in a humidified 5% CO_2 atmosphere for 24 h. After this period, the culture medium was aspirated and 500µL per well of filtered filling material (10x dilution) was added to the plate at 8 h. The plates always were incubated at 37ºC in a humidified 5% CO_2.

After a specific time, the materials were removed, and 120µl of 10% neutral formol per well was added, and the plates were stored in a humid atmosphere containing 95% air and 5% CO_2 at 37°C for 1hour. Then, the formol was removed; the cells were washed 1x with PBS and fixed with 500µl 4% paraformaldehyde for 1hour. After this, they were washed again with PBS (3x), permeabilized with 500µl 0.5% Triton X-100 for 10 minutes and washed 3x with PBS. Cells were stained with Alexa Fluor® 488 phalloidin (1:500)

(300μl/well) (Molecular Probes, Eugene, OR, USA) to label the F-actin filaments, for 1hour. The samples were washed again with PBS (3x), 3 drops of Viectashield® (Vector Laboratories, Inc. Burlingame CA94010) were added, and a coverslip was placed for microscopy (24x60mm) (Glasscyto).

The fluorescent images were obtained using fluorescence microscopy (Nikon Eclipse Ti) with appropriate filters, at 100x magnification.

2.6. *Statistical analysis*

The cell viability data were submitted to the Shapiro-Wilk test in order to confirm a normal distribution (BioEstat 5.0, Mamirauá, Belém, PA, Brazil). The data were submitted to two-way analysis of variance with post-hoc multiple comparisons using the Tukey test, with the significance level fixed at 5%. Statistical analysis was performed by using the Assistat 7.7 beta software program (Campina Grande, PB, Brazil).

The fluorescence microscopy images obtained were evaluated using descriptive analysis.

3. Results

3.1. *Cell viability Assays*

The cytotoxic effect of each material on cultured human periodontal ligament fibroblasts was compared in 3 different time intervals (8, 24 and 48 h). As regards the exposure times, G3, G5, G6 and G7 showed significant difference between 8 and 48 h and between 24 and 48 h, but showed no difference between 8 and 24 h. It was observed that G4 differed significantly between 8 and 24 h and between 8 and 48 h, but showed no difference between 24 and 48 h. G8 showed significantly lower level of cell viability at 8h, but no difference at 8 and 24 h and between 24 and 48 h. With regard to the percentage of toxicity, the groups G3 (85.91±10.01), G4 (85.91±8.16), G5 (83.96±13.95), G7 (89.04±11.67) and G8 (89.89± 11.20) showed a mild cytotoxic effect in 8 h, and the group G6 (89.36±13.60) showed mild cytotoxic in 24 h. Group G2 showed severe cytotoxicity for the 3 periods (19.72±5.70; 14.85±4.73; 14.43±3.47, respectively) (Figure 1).

Figure 2 shows the percentage of viability of human Saos-2 osteoblast-like cells after exposure to filling materials and control groups at 8, 24 and 48 h. As regards the

exposure times G3 and G8 showed no significant difference between exposure times. G4 showed significant difference between 8 and 24 h and between 8 and 48 h, but showed no difference between 24 and 48 h. G5 showed significant difference between 8 and 24 h and between 24 and 48 h, but showed no difference between 8 and 48 h. G6 showed a significantly lower level of cell viability between 8 and 48 h, but no difference at 8 and 24 h and between 24 and 48 h. G7 showed no difference between 8 and 24 h but both times showed differences for 48 h (Figure 2). As regards the percentage of toxicity, the materials of the groups G3 (81.77±12.98; 81.61±9.94) and G5 (75.5±12.40; 73.98±7.89) showed mild cytotoxic effect in 8 and 48 h. G4 (75.87±19.16) and G8 (86.63±11.36) showed mild cytotoxic effect only in 8 h. G6 showed mild cytotoxicity in 8 (68.71±22.19) and 24 h (81.49±11.25). G7 (110.72±22.37; 104.42±16.33; 139.88±38.67) showed non cytotoxicity and the G2 (22.18±6.77; 22.44±3.54; 15.48±9.71) showed severe cytotoxicity for the periods.

Each material was compared with the negative and positive control (cells with culture medium without filling material) and comparison was made between the materials at different exposure times. For PDL cell viability it could be observed that G3 (85.91±10.01), G4 (85.91±8.16) and G5 (83.96±13.95) showed a significantly lower level of cell viability than the negative control (100±0) at 8 h. At 24 and 48 h there was no difference between the materials and negative control. In 24 h G4 (101.53±7.69) showed a higher level of cell viability than G6 (89.36±13.60), but both did not differ from the other materials. In 48 h there was no difference between the materials (Figure 3). However, for viability of Saos-2 osteoblast-like cells in 8 h G4 (75.87±19.16), G5 (75.5±12.40) and G6 (68.71±22.19) showed a significantly lower level of cell viability than the negative control (100±0) ($p>0.05$). All materials showed no significant difference to negative control at 24 h ($p<0.05$) but in 48 h G7 showed a significantly higher level of cell viability than the negative control ($p>0.05$). Comparing the materials, in 8 h (110.72±22.37) and 48 h (139.88±38.67) G7 showed a higher level of cell viability differing from that of the other materials. In 24 h, only G6 (81.49±11.25) differed from G7 (104.42±16.33) (Figure 4).

3.2. Cell morphology analysis

Figures 5 and 6 show representative images of human PDL and Saos-2 osteoblast-like cells, respectively, after 8 h of exposure to the filling materials evaluated. The presence of nuclei stained blue and the plasma membrane stained green could be observed. For fibroblasts cells, membrane extensions and large, ovoid nuclei could be observed. For osteoblasts cells, a polygonal shape, typical feature of the osteosarcoma Saos-2 culture, more mature osteoblast cell profile could be observed.

For fibroblasts cells, Calen® associated with Zinc Oxide, Calen® associated with Iodoform and ZOE showed loss of morphological features, such as starry profile. Others materials showed no difference in morphological characteristics compared with the negative control group. For osteoblasts none of the materials evaluated showed significant morphological features.

4. Discussion

In the present study, the cytotoxicity of six filling materials frequently used in primary teeth on a human cell line was evaluated. The results showed that there was difference in cytotoxicity of the studied materials at different exposure times and morphological alteration in fibroblast cells. Therefore, the tested hypothesis was partially accepted.

Root canal filling materials are in intimate contact with periapical tissues. Thus, the biocompatibility of these materials is essential for successful endodontic therapy[11] and a filling material requires certain physical-chemical and biological properties. However, to our knowledge, there is no material that meets all required criteria and there is little information about the cytotoxicity of filling materials for primary teeth. Therefore, this *in vitro* study was conducted to compare the cytotoxic effects of calcium hydroxide based-materials, when associated with other substances or not, and ZOE-based material on human fibroblasts and osteoblast-like cells.

Several materials have been proposed for filling primary root canals, such as ZOE. This material has been widely used, nevertheless, there have been reports of undesirable properties of this material. The adverse effects include irritation of periapical tissues and low resorption capacity. The irritating potential of ZOE can be attributed to eugenol.[13] In addition, cell density and number of U2OS cells decreased to almost zero when Super EBA

(reinforced zinc oxide cement based on a mixture of 32% eugenol and 68% ethoxy benzoic acid) and Life (calcium hydroxide based material) on bone cell viability were evaluated.[34] In the present study, ZOE material showed a lower level of viability only for Saos-2 osteoblast-like cells, compared with the control group at 8 h. In a previous study, a clinical success rate of 78.5% was observed for filling root canals with OZE; a value that was statistically lower in comparison with filling with Vitapex® (100% clinical success).[4]

Calcium hydroxide based-material is one of the types most frequently used in Pediatric Dentistry, since shows high antimicrobial potential, biocompatibility, tissue permeability and induction of mineralized tissue formation.[2,13-16,18,19] The results of the present study showed that Calen® (a calcium hydroxide-based material and zinc oxide powder), when pure or associated, showed mild cytotoxic effect in 8 hours, differing from the negative control group. As regards osteoblastic cells, Calen® associated with Zinc Oxide, and Calen® associated with Iodoform also showed a mild cytotoxic effect and differed from the negative control in 8 h and 48 h. In 24 h for both cells studied, none of materials differed from the negative control group. Our results were similar to those found in the study of Sarigol et al. (2010)[6] and different from those of Yilmaz et al. (2012)[11]. Sarigol et al. (2010)[6] also assessed the cytotoxicity of five filling materials used in primary teeth and showed a lower level of cell viability but did not differ from the negative control after 24 h. Furthermore, Yilmaz et al. (2012)[11] evaluated the cytotoxic effects of commercial root canal sealers in L929 fibroblasts and showed that calcium hydroxide based-material led to the highest cell viability. The mild cytotoxicity of calcium hydroxide-based materials observed at 8 h may be due to the superficial necrosis and inflammatory reaction which occurs reversibly in tissues exposed to materials based on calcium hydroxide.[35,36] However, there was maximum mild cytotoxicity at 8 h and it decreased over the period of time.

As regards the Iodoform-based materials, it has been reported that these filling materials caused considerable tissue necrosis and a higher cytotoxicity,[13,37] but this was not observed in the present study. Vitapex® increased cell viability for Saos-2 osteoblast-like cells and for fibroblasts the viability was similar to that of the control group at 8 and 24 h (Figures 3 and 4). At 48 h there was a higher level of cell viability than that of the negative control (Figure 4). In addition, the highest level of cell survival (L929) was observed when

other Iodoform based-materials (Kri 1 paste® and Diapex®) were evaluated. The former is composed of 80% of Iodoform and the latter of calcium hydroxide and Iodoform.[6] Clinically, Vitapex® has shown better results for treating furcation pathology at a faster rate than ZOE.[12] However, at 12 months both materials yielded similar results; in 6 months Vitapex® demonstrated clinical and radiographic success at a faster rate than ZOE (78% vs. 48%). In addition, it could be observed that direct and indirect exposure to low concentrations of filling materials that contain Iodoform induced cell proliferation, while high concentrations showed a cytotoxic effect on macrophages and epithelial cells (RAW 264.7 and RKO, respectively).[38]

In this study, the difference in the results obtained for calcium hydroxide and Iodoform based materials (Calen® associated with Iodoform and Vitapex®) could be explained by the vehicle present in the materials. Calen® associated with Iodoform presents an aqueous vehicle and Vitapex® is oily. In general, three types of vehicles are used: aqueous, viscous or oily. When calcium hydroxide is mixed with an aqueous vehicle the substances (Ca^2 and OH^-) are rapidly released and promote a high degree of solubility, when the material remains in direct contact with the tissue and tissue fluids for longer periods. This allows a greater degree of diffusion and consequently greater action through contact, causing the material to be rapidly solubilized and reabsorbed. From a clinical standpoint the root canal may become empty in a short period. Materials containing oily vehicles promote the lowest solubility and diffusion of the materials within the tissues, promoting a slower ionic dissociation.[39]

A fundamental issue to consider during *in vitro* biocompatibility studies is the cell type.[8] There are some types of cell usually used in such experiments, such as mouse fibroblasts (L929), osteoblasts (MC3T3.E; ROS 17/2.8; Saos-2) and human fibroblasts. Human fibroblasts and osteoblasts were selected based on their relevance to clinical conditions. Human periodontal ligament fibroblasts have the advantage of reducing bias as regards the origin of species and non-tissue specific cell lines beyond the close relationship between filling materials and periapical tissues.[8] Saos-2 are bone forming cells, characterized by a phenotype of mature osteoblasts and are used in studies of cell proliferation, differentiation and metabolism.[28]

Alterations at the cell surface or the cellular cytoskeleton can be observed by morphological assays using fluorescence microscopy analysis. In the present study, analysis of cell morphology was performed only at 8 h since the decrease in toxicity to both cells was observed by the MTS assay after this exposure time. Calen® associated with Zinc Oxide, Calen® associated with Iodoform and ZOE showed loss of morphological features, such as a starry profile for fibroblast cells (Figure 5). Moreover, in a study using a human osteosarcoma cell line (U2OS), the loss of intercellular connection and appearance of cell body contraction was observed when the cells were exposed to a ZOE-based material (Super EBA) and calcium hydroxide + zinc oxide (Life).[34] This same result was also found in PDL fibroblasts when in contact with the Pulp Canal Sealer (ZOE), in which cells partially altered in shape, with a round appearance and no visible cytoplasmic structures were observed.[40]

Long-term *in vivo* studies are a gold standard for determining clinical performance. However, the use of *in vitro* tests allows for the evaluation of biocompatibility, due to the advantages of using standardized growth medium compositions, a defined incubator milieu, and sterile working conditions. Furthermore, *in vitro* tests allow for an accurate quantitative and qualitative evaluation of the results and offer the possibility of studying the effect of the material components on cell systems, and materials that show improving trends in *in vitro* tests pose fewer risks *in vivo*.[5,6,11,25]

Due to fewer studies being conducted with filling materials used in primary teeth and different methodologies used, experimental variations such as cell type, method of cell-material contact and exposure time, it is difficult compare the results. However, cell viability is an important parameter that should be evaluated in *in vitro* tests. The results of the present study show the response of two important cell lines when they were in contact with different filling materials used for primary teeth. Thus they may contribute to further studies being conducted and based on scientific evidence, in order to reach a consensus on which material is more suitable for filling endodontically treated primary teeth.

5. Conclusion

Within the conditions of the present study, it could be concluded that all materials tested showed a mild degree of cytotoxicity at different times, for both cells, except Vitapex®, which was non-cytotoxic for osteoblasts cells at all times of exposure. Morphological alterations were observed in fibroblasts for Calen®+Zinc Oxide, Calen®+Iodoform and ZOE.

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Table 1. Filling materials used in the study, according to the material composition and their respective manufacturers.

MATERIALS	COMPOSITION	MANUFACTURER
Calen®	2.5g calcium hydroxide, 0.5g zinc oxide, 0.05g colophony and 1.75mL polyethylene glycol 400	S.S. White
Zinc Oxide	99 to 100.5% of zinc oxide	Biodinâmica
Eugenol	99 to 100.5% of eugenol	Biodinâmica
Iodoform	99 to 100.5% of iodoform	Biodinâmica
Vitapex®	30% calcium hydroxide; 40.4% iodoform; 22.4% silicone oil and 6.9% inert substances	Neo Dental
UltraCal® XS	35% calcium hydroxide; aqueous vehicle	UltraDent

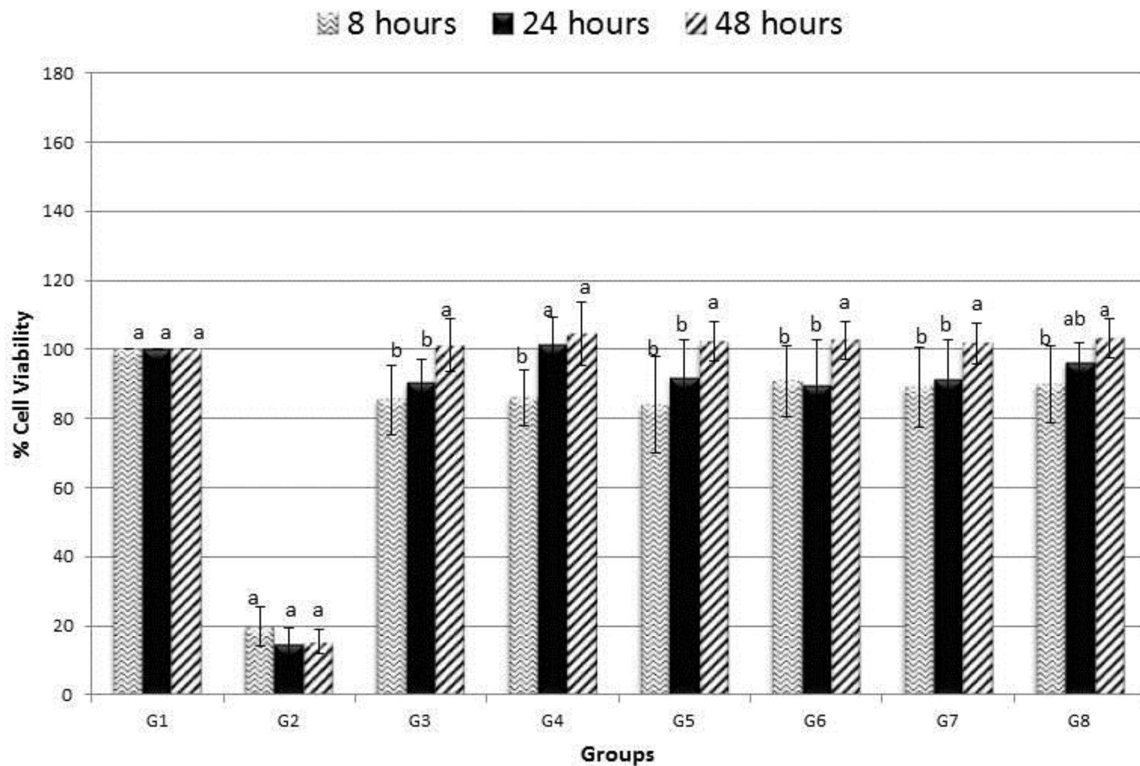

Figure 1. Percentage of cell viability of cultured human periodontal ligament fibroblasts after exposure to filling materials and control at 8, 24 and 48 h. G1=culture medium (negative control); G2= dimethyl sulfoxide (positive control); G3=Calen®; G4=Calen® associated with Zinc Oxide; G5=Calen® associated with Iodoform; G6=ZOE; G7=Vitapex®; G8=UltraCal®XS.

Similar lower case letters mean no statistically difference between each material and exposure time by the Tukey test ($p>0.05$).

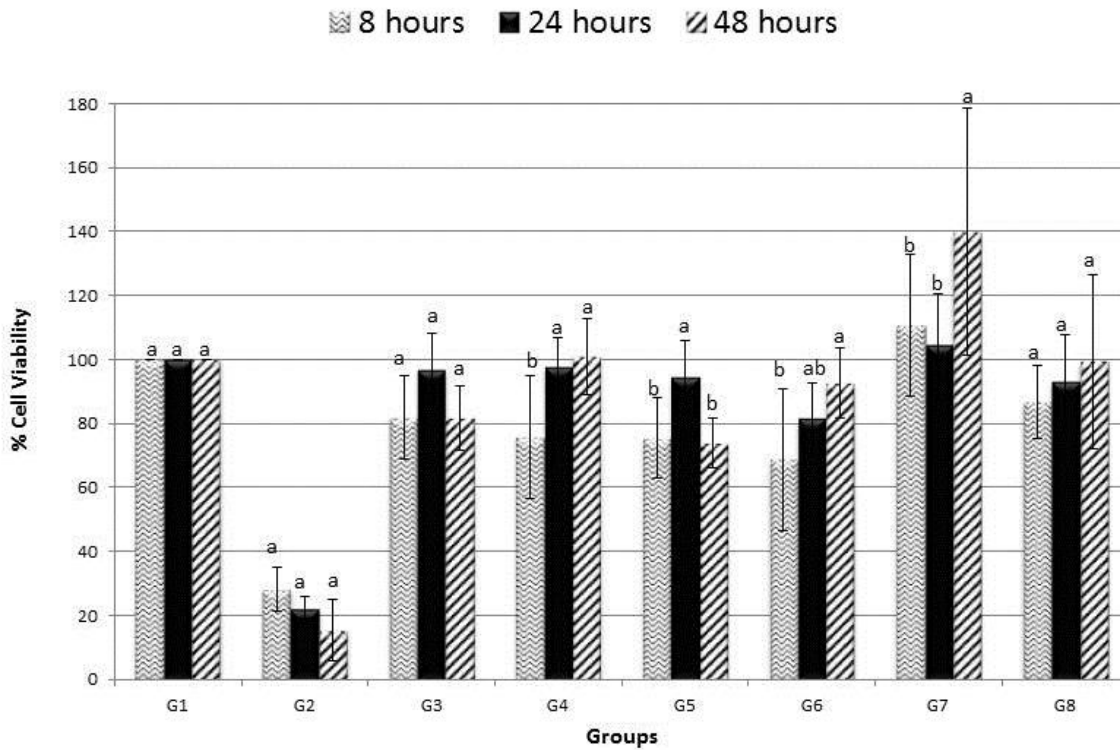

Figure 2. Percentage cell viability of human Saos-2 osteoblast-like cells after exposure to filling materials at 8, 24 and 48 h. G1=culture medium (negative control); G2= dimethyl sulfoxide (positive control); G3=Calen®; G4=Calen® associated with Zinc Oxide; G5=Calen® associated with Iodoform; G6=ZOE; G7=Vitapex®; G8=UltraCal®XS.

Similar lower case letters mean no statistically difference between each material and exposure time by the Tukey test (p>0.05).

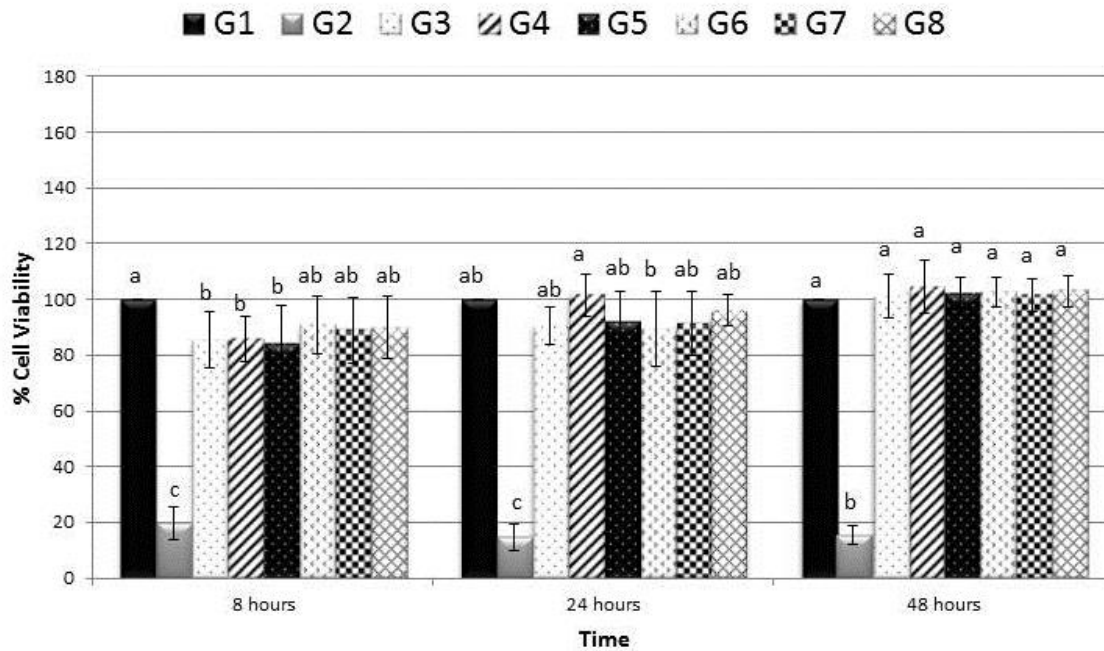

Figure 3. Percentage of cell viability of cultured periodontal ligament fibroblasts after exposure to filling materials at 8, 24 and 48 h. G1=culture medium (negative control); G2= dimethyl sulfoxide (positive control); G3=Calen®; G4=Calen® associated with Zinc Oxide; G5=Calen® associated with Iodoform; G6=ZOE; G7=Vitapex®; G8=UltraCal®XS.

Similar lower case letters mean no statistically difference between filling materials at each exposure time by the Tukey test ($p<0.05$).

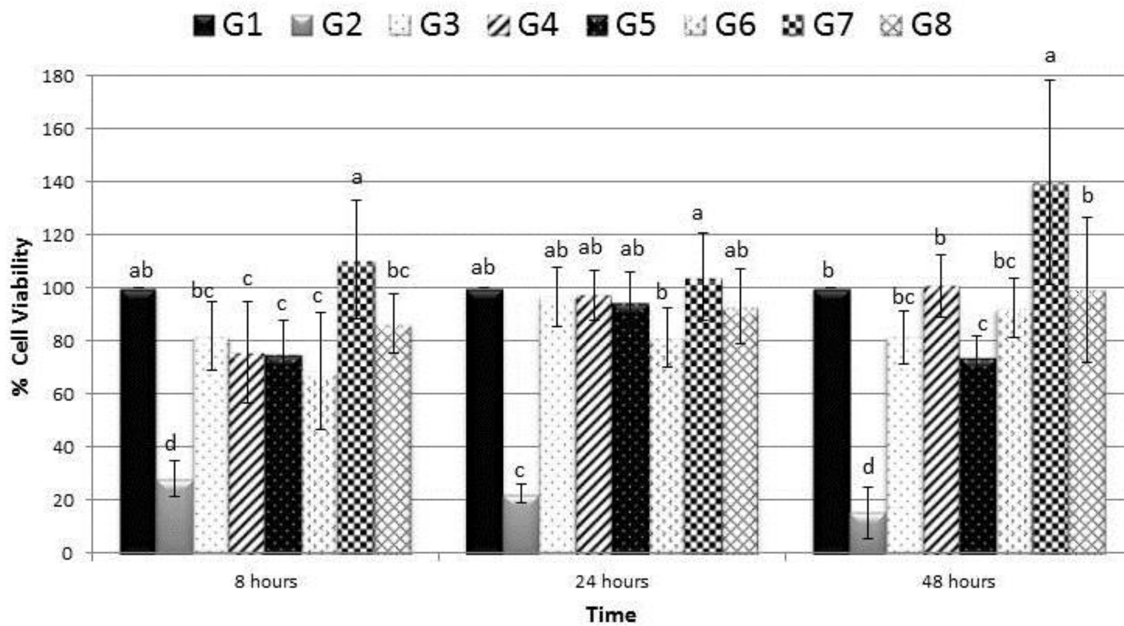

Figure 4. Percentage of cell viability of human Saos-2 osteoblast-like cells after exposure to filling materials at 8, 24 and 48 h. G1=culture medium (negative control); G2= dimethyl sulfoxide (positive control); G3=Calen®; G4=Calen® associated with Zinc Oxide; G5=Calen® associated with Iodoform; G6=ZOE; G7=Vitapex®; G8=UltraCal®XS.

Similar lower case letters mean no statistically difference between filling materials at each exposure time by the Tukey test ($p<0.05$).

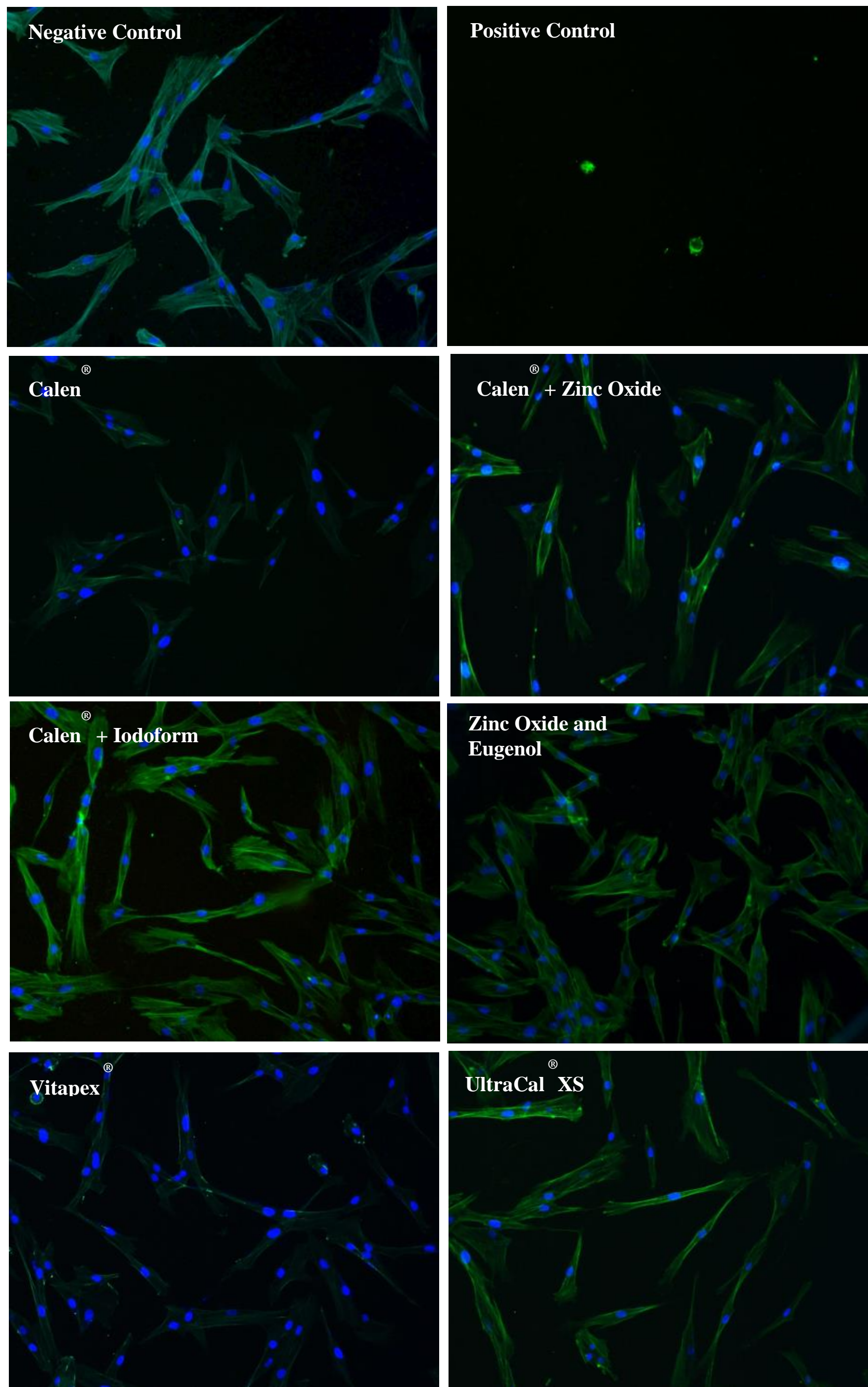

Figure 5. Representative images obtained by fluorescence microscopy (100x) of human periodontal ligament fibroblasts after exposure to filling materials and controls at 8 h. The presence of nuclei stained blue and the plasma membrane stained green could be observed. Fibroblasts were observed in the membrane with extensions and large, ovoid nuclei.

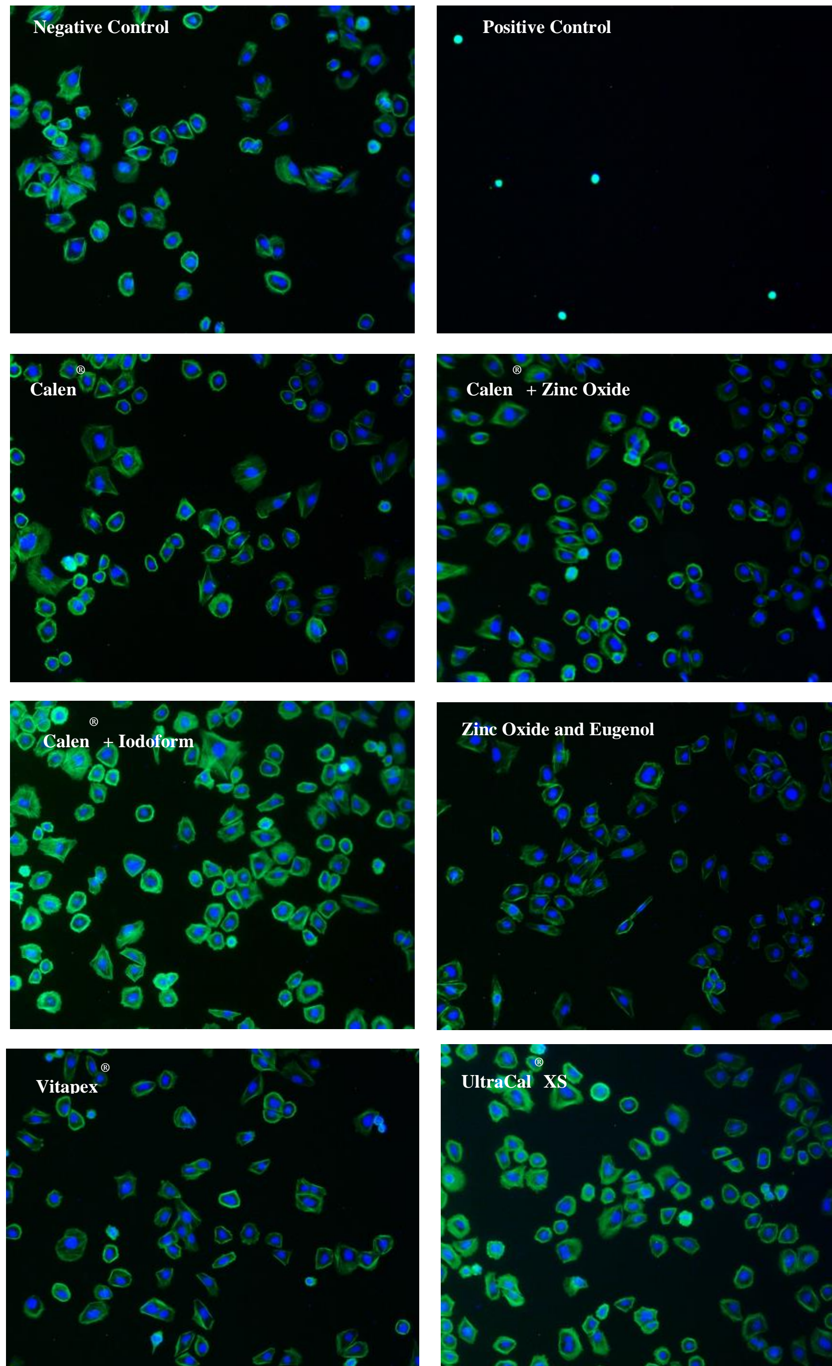

Figure 6. Representative images obtained by fluorescence microscopy (100x) of Saos-2 osteoblast-like cells after exposure to filling materials and controls at 8 h. The presence of nuclei stained blue and the plasma membrane stained green could be observed. The polygonal shape of osteoblasts cells, a typical feature of the culture of osteosarcoma Saos-2, more mature osteoblast cell profile could be observed. None of materials evaluated showed significant morphological features.

CONCLUSÕES GERAIS

De acordo com os objetivos propostos e das condições do presente estudo, pode-se concluir que:

(1) Todos os materiais avaliados apresentaram grau leve de citotoxicidade em diferentes tempos, para as células do ligamento periodontal humano e as osteobláticas, exceto Vitapex®, que não se mostrou não-citotóxica para células osteoblásticas em todos os tempos de exposição.

(2) Morfologicamente foram observadas alterações nos fibroblastos para Calen® + Óxido de Zinco, Calen® + Iodofórmio e OZE.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors. Abreviatura dos periódicos em conformidade com o Medline.

ANEXOS

ANEXO 1

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Análise da citotoxicidade de materiais obturadores de dentes decíduos"**, protocolo nº 073/2013, dos pesquisadores Fernanda Miori Pascon e Natalia Martins Joaquim, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 14/08/2013.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Analysis of cytotoxicity of filling materials of primary teeth"**, register number 073/2013, of Fernanda Miori Pascon and Natalia Martins Joaquim, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Aug 14, 2013.

Prof. Dr. Felippe Bevilacqua Prado
Secretário
CEP/FOP/UNICAMP

Lívia M. A. Tenuta
Profa. Dra. Lívia Maria Andaló Tenuta
Coordenadora
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 2

Assunto:	Submission Confirmation for Journal of Dentistry
De:	Journal of Dentistry (ees.jjod.0.2ad9fb.b85a6a50@eesmail.elsevier.com)
Para:	fmpascon@fop.unicamp.br; fmpascon@yahoo.com;

Dear Prof. Pascon,

Your submission entitled "Cytotoxicity analyses of filling materials for primary teeth" has been received by the Journal of Dentistry.

You will be able to check on the progress of your paper by logging on to Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/jjod/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

Matt Walmsley
Journal Manager
Journal of Dentistry

ANEXO 3

DECLARAÇÃO

A cópia do artigo da minha autoria, já submetido para publicação em revista científica, que consta na minha Dissertação de Mestrado, intitulada “Análise da Citotoxicidade de Materiais Obturadores de Dentes Decíduos”, não infringe os dispositivos da Lei nº. 0.610/98, nem o direito autoral de qualquer editora.

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