UNIVERSIDADE FEDERAL DE UBERLÂNDIA

FACULDADE DE ODONTOLOGIA

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UMA AVALIAÇÃO LABORATORIAL DA VIABILIDADE CELULAR, RADIOPACIDADE E DESCOLORAÇÃO DENTÁRIA INDUZIDA POR MATERIAIS ENDODÔNTICOS REGENERATIVOS

A laboratory evaluation of cell viability, radiopacity and tooth discoloration induced by regenerative endodontic materials

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AVALIAÇÃO DA DESCOLORAÇÃO DO DENTE E RADIOPACIDADE DOS MATERIAIS UTILIZADOS EM PULPOTOMIA

A laboratory evaluation of cell viability, radiopacity and tooth discoloration induced by regenerative endodontic materials

Trabalho de conclusão de curso apresentado a Faculdade de Odontologia da UFU, como requisito parcial para obtenção do título de Graduado em Odontologia

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DEDICATÓRIA

À Deus pois sem tuas graças não conseguiria. Aos meus pais Denison e Neurivânia que nunca mediram esforços para que eu chegasse até aqui. À minhas irmãs Núbia e Geovanna, ao meu namorado Raul, aos meus avós Aneli, José, Maria Aparecida (in memoriam) e Leilson, e a todos os familiares e amigos pelo apoio, carinho e amparo.

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E a todos os *meus pacientes* que me deram a oportunidade de realização profissional e pessoal.

EPÍGRAFE

“O sucesso não consiste em não errar,
mas em não cometer os mesmos
equívocos mais de uma vez.”

George Bernard Shaw

SUMÁRIO

Resumo

Este estudo teve como objetivo, analisar a citotoxicidade, alteração de cor e radiopacidade do MTA Flow (MTA), UltraCal XS (UC) e Bio-C Temp (BT). Para isto, células da polpa dentária humana (hDPCs) estimuladas com lipopolissacarídeo (LPS) foram colocadas em contato com várias diluições de meios de cultura anteriormente exposto aos materiais experimentais e testadas para viabilidade celular usando MTT. 72 incisivos bovinos foram preparados para simular uma fratura extensa da coroa em um dente com rizogênese incompleta. As raízes foram preenchidas com uma mistura de ágar e sangue, e os materiais colocados sobre esta mistura. O grupo controle consistia em dentes preenchidos apenas com ágar e sangue. As avaliações de alteração de cor foram realizadas antes e imediatamente após a inserção do material e repetidas em 30, 45 e 60 dias em espectrofotômetro. A alteração de cor ($D\mathit{E}_{ab}$, $D\mathit{E}_{00}$ e índice de brancura (WI)) foram calculados com base no sistema de cores CIELAB. As radiografias digitais foram adquiridas para análise da radiopacidade. A viabilidade celular foi analisada através de análise de variância (ANOVA) por fator único, as diferenças nos parâmetros de cor ($D\mathit{E}_{ab}$, $D\mathit{E}_{00}$ e WI) foram avaliadas por ANOVA de dois fatores ($\alpha = 0,05$). O teste de Tukey foi usado para comparar os grupos experimentais e o teste de Dunnett foi usado para comparar os grupos experimentais com o grupo controle. MTA, UC e BT tiveram viabilidade celular semelhante ao grupo controle (DMEM) ($P > 0,05$), exceto para o grupo BT nas diluições 1: 1 e 1: 2, que teve viabilidade significativamente menor ($P < 0,001$). Todos os materiais apresentaram valores de alteração de cor maiores do que é considerado ser o limite aceitável e BT resultou em menor alteração de cor comparado ao MTA e similar ao UC. A diminuição da radiopacidade ao longo do tempo foi observada apenas no grupo MTA ($P = 0,007$). Diminuição nos valores de radiopacidade foram observados no grupo BT em comparação com os grupos UC e MTA ($P < 0,001$). O novo material biocerâmico (BT) teve viabilidade celular aceitável, semelhante ao MTA e UC nas maiores diluições, apresentou menor alteração de cor comparado MTA e UC e apesar de menor radiopacidade foi identificado radiograficamente.

Palavras-chave: Citotoxicidade. Pulpotomia. Radiopacidade. Potencial de manchamento. Descoloração dentária.

Abstract

Aim: To analyse the cytotoxicity, colour change and radiopacity of MTA Flow (MTA), UltraCal XS (UC) and Bio-C Temp (BT). Methodology: Human dental pulp cells (hDPCs) stimulated with lipopolysaccharide (LPS) were placed in contact with several dilutions of culture media previously exposed to the experimental materials and tested for cell viability using MTT. Bovine teeth were prepared to simulate an open apex and to mimic extensive crown fracture. The roots were filled with a mixture of agar and blood, and the materials placed over this mixture. The control group consisted of teeth filled only with agar and blood. Colour assessment analyses were performed before and immediately after material insertion and repeated at 30, 45 and 60 days using a spectrophotometer. The total colour change ($D\mathit{E}_{ab}$, $D\mathit{E}_{00}$ and whiteness index (WI)) was calculated based on the CIELAB colour space. Digital radiographs were acquired for radiopacity analysis. Cell viability was analysed by one-way ANOVA, whilst differences in colour parameters ($D\mathit{E}_{ab}$, $D\mathit{E}_{00}$ and WI) were assessed by two-way repeated measures ANOVA ($\alpha = 0.05$). Tukey's test was used to compare the experimental groups, and Dunnett's test was used to compare the experimental groups with the control group. Results: MTA, UC and BT had similar cell viability to that of the control group (DMEM) ($P > 0.05$), except for the BT group at the 1 : 1 and 1 : 2 dilutions, which had significantly lower viability ($P < 0.001$). All materials were associated with discoloration values greater than what is considered to be the acceptable threshold, and BT resulted in less or similar tooth colour change than MTA and UC, respectively. Decreasing radiopacity over time was observed only in the MTA group ($P = 0.007$). Lower values of radiopacity were found in the BT group compared with the UC and MTA groups ($P < 0.001$). Conclusions: The new bioceramic material (BT) had acceptable cell viability, similar to that of MTA and UC at the highest dilutions, and BT resulted in less tooth colour change than MTA and UC. Despite its lower radiopacity, BT was identified radiographically

Keywords: Cytotoxicity. Pulpotomy. Radiopacity. Staining potential. Tooth discoloration.

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A laboratory evaluation of cell viability, radiopacity and tooth discoloration induced by regenerative endodontic materials

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Abstract

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Aim To analyse the cytotoxicity, colour change and radiopacity of MTA Flow (MTA), UltraCal XS (UC) and Bio-C Temp (BT).

Methodology Human dental pulp cells (hDPCs) stimulated with lipopolysaccharide (LPS) were placed in contact with several dilutions of culture media previously exposed to the experimental materials and tested for cell viability using MTT. Bovine teeth were prepared to simulate an open apex and to mimic extensive crown fracture. The roots were filled with a mixture of agar and blood, and the materials placed over this mixture. The control group consisted of teeth filled only with agar and blood. Colour assessment analyses were performed before and immediately after material insertion and repeated at 30, 45 and 60 days using a spectrophotometer. The total colour change (ΔE_{ab}, ΔE_{00} and whiteness index (WI)) was calculated based on the CIELAB colour space. Digital radiographs were acquired for radiopacity analysis. Cell viability was analysed by one-way ANOVA, whilst differences in colour parameters (ΔE_{ab}, ΔE_{00} and WI) were assessed by two-way repeated measures ANOVA ($\alpha = 0.05$). Tukey's test was used to compare the experimental groups, and Dunnett's test was used to compare the experimental groups with the control group.

Results MTA, UC and BT had similar cell viability to that of the control group (DMEM) ($P > 0.05$), except for the BT group at the 1 : 1 and 1 : 2 dilutions, which had significantly lower viability ($P < 0.001$). All materials were associated with discoloration values greater than what is considered to be the acceptable threshold, and BT resulted in less or similar tooth colour change than MTA and UC, respectively. Decreasing radiopacity over time was observed only in the MTA group ($P = 0.007$). Lower values of radiopacity were found in the BT group compared with the UC and MTA groups ($P < 0.001$).

Conclusions The new bioceramic material (BT) had acceptable cell viability, similar to that of MTA and UC at the highest dilutions, and BT resulted in less tooth colour change than MTA and UC. Despite its lower radiopacity, BT was identified radiographically.

Keywords: cytotoxicity, pulpotomy, radiopacity, staining potential, tooth discoloration.

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Introduction

Dental trauma often occurs in young people and may involve teeth and supporting structures (Qudeimat *et al.* 2019). Crown fractures of permanent teeth account for between 26% and 76% of all traumatic injuries (Castro *et al.* 2005). These fractures are often complicated with pulp involvement in teeth with incomplete root formation. In this case, pulpotomy is a viable treatment option because it allows continuous root development and apical closure (Alqaderi *et al.* 2016, Chen *et al.* 2019).

Pulpotomy has an excellent prognosis and comprises surgical amputation of the infected coronal pulp (depth of 1.5–2.0 mm) and placement of a protective material to cover the exposed pulp and in an attempt to preserve its vitality (Tuloglu & Bayrak 2016, Wells *et al.* 2019). For the success of this type of treatment, the materials must have certain essential characteristics, such as radiopacity (Ochoa-Rodríguez *et al.* 2019), biocompatibility and nontoxicity (Lee *et al.* 2017, Parirokh *et al.* 2018, Pedano *et al.* 2018, Cosme-Silva *et al.* 2019), as well as act as a barrier against microorganisms, stimulate tissue healing and not contribute to discoloration (Mozyńska *et al.* 2017). Calcium hydroxide ($Ca(OH)^2$) and mineral aggregate trioxide (MTA) are the most commonly used materials in pulpotomies (Liu *et al.* 2011, Musale *et al.* 2018, Parirokh *et al.* 2018).

$Ca(OH)_2$ has an alkaline pH and can activate alkaline phosphatase and consequently stimulate the production of tertiary dentine. Nevertheless, the long-term use of $Ca(OH)_2$ is associated with poor sealing and high solubility in oral fluids (Gandolfi *et al.* 2015). Another material used in pulpotomy is MTA, which has acceptable physical and chemical properties and excellent biocompatibility (Camilleri 2015, Lee *et al.* 2017, Parirokh *et al.* 2018, Nagendrababu *et al.* 2019, Chen *et al.* 2019). Nonetheless, most studies have concluded that MTA may cause crown discoloration due to the presence of bismuth oxide as a radiopacifier (Yoldas *et al.* 2016, Shokouhinejad *et al.* 2016). In addition, although the material itself may cause discoloration, the presence of blood might intensify this phenomenon (Guimarães *et al.* 2015).

Therefore, new bioceramic materials have emerged to overcome these problems (Beatty & Svec 2015, Marconyak *et al.* 2016, Parirokh *et al.* 2018, Pedano *et al.* 2018, Cosme-Silva *et al.* 2019), such as Sealer Plus (MK Life) and Endosequence BC Sealer (Brasseler), but only a few studies have analysed conditions that mimic pulpotomy in traumatized immature teeth. However, these new materials are expensive, which often makes their use in emerging countries infeasible. Therefore, new, lower cost materials containing bioceramic particles in their composition have been developed as a possible alternative for use in pulpotomies. In this sense, the present study aims to compare the cell viability of human dental pulp cells (hDPCs), radiopacity and crown discoloration produced by a new bioceramic material (Bio-C Temp®; Angelus, Londrina, PR, Brazil) and two materials (MTA Flow and UltraCal XS; Ultradent, South Jordan, UT, USA) traditionally used in pulpotomies. The null hypothesis was that no significant differences would be found in the cell viability, radiopacity or coronal discoloration associated with the evaluated materials.

Materials and methods

Preparation of materials for cell viability test

The materials used were MTA Flow (MTA) (Ultradent), UltraCal XS (UC) (Ultradent) and Bio-C Temp® (BT) (Angelus, Londrina). The components of the root canal filling pastes tested are described in Table 1. The MTA Flow samples were prepared according to the manufacturer's recommendations. Then, 0.22 mL of MTA Flow, UC and BT were inserted in 24-well plates under aseptic conditions in a laminar flow cabinet. Immediately, all materials were covered with 2.5 mL of Dulbecco modified Eagle medium (DMEM) for cell culture (Vitrocell Embriolife, Campinas, SP, Brazil) and incubated in the dark for 24 h at 37 °C (Bin *et al.* 2012). The original extracts (1 : 1) were prepared following the recommendations of the ISO 10993 (2009). After incubation, these original extracts were serially diluted in cell culture medium before testing until the dilution of 1 : 32.

hDPC culture

Primary human dental pulp cell (hDPCs) cultures were donated from the School of Dentistry of the Federal University of Uberlândia (UFU), after signing the informed consent form by the guardians (Ethics Committee protocol number 09016219.1.0000.5152). Two healthy primary teeth nearing (n = 2) were collected, and the pulp was extracted from the pulp chamber using a sterilize sharp excavator. Afterwards,

Table 1 Components of the root canal filling pastes tested

Material	Technical information	Components
UltraCal XS (Ultradent, Indaiatuba, SP, Brasil)	Paste ready for use	Calcium hydroxide, barium sulphate, aqueous matrix of methylcellulose
MTA Flow (Ultradent, Indaiatuba, SP, Brasil)	Powder and gel (two big ends mixed with two drops and inserted with microtips)	Extremely fine inorganic powder of tricalcium and dicalcium silicate with a water-based gel
Bio-C Temp (Angelus, Lindoia, PR, Brasil)	Paste ready for use	Calcium silicate, calcium aluminate, calcium oxide, calcium tungstate in a mixture of ester glycol salicylate and polyethylene glycol and other supplementary agents

the pulp tissue was immersed for 1 h in the following solution: 3 mg mL^{-1} collagenase type I (Sigma-Aldrich, San Louis, MI, USA) and 4 mg mL^{-1} dispase (Sigma-Aldrich). The samples were centrifuged at 250 g (centrifuge 80-2B, Centribio, Curitiba, PR, Brazil) for 2 min and resuspended in basal medium. The cells obtained were plated in 25-cm^2 flasks and incubated for 4 days at 37 °C with 5% CO_2. The culture medium was first replaced after 3 days of incubation; thereafter, it was changed twice a week. The cells were expanded up to the 4th passage and frozen for later experimental use.

hDPCs with lipopolysaccharide-induced stress and exposure to extracts

Cells were cultured in DMEM (Vitrocell) supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and 1% penicillin–streptomycin (Sigma-Aldrich) in a humid atmosphere of 5% CO_2 and 37 °C until confluence. The hDPCs were plated on 96-well plates (2×10^4 cells/well) and allowed to adhere overnight. Then, the cells were incubated with 200 μL of the extracts at pre-determined dilutions (1 : 1, 1 : 2, 1 : 4, 1 : 8, 1 : 16 and 1 : 32) and simultaneously with lipopolysaccharide (LPS) (LPS, Ultra-pure grade, *Escherichia coli* O111:B4, Invitrogen, San Diego, CA, USA) at the concentration of 10 μg mL^{-1}, for a period of 24 h. After the incubation period, the cells were immediately tested for viability by MTT formazan. The control group was maintained in DMEM (not LPS-stimulated). This study was repeated twice using five samples for each group at every moment. Cell viability was evaluated proportionally to absorbance and expressed as the percentage of viable cells. The mean values obtained for the control group were considered as 100% of cell viability, and the values of each sample of the experimental groups were obtained proportionally to the control.

Analysis of viability by MTT formazan

The cell viability was evaluated 24 h after the treatment with the extracts. MTT solution (Sigma-Aldrich; 5 mg mL^{-1}) was added to each well, and the cells were incubated at 37 °C for 4 h. The supernatants were removed, and then, 100 μL dimethyl sulphoxide (DMSO; LGC Biotecnologia, Cotia, SP, Brazil) was added. Afterwards, the optical density (OD) at 570 nm was measured using a microplate reader (Biochrom, Cambridge, UK).

Selection of teeth and sample preparation for colour measurements and radiopacity tests

The sample size calculation was based on data from Yoldas *et al.* (2016). Eighteen teeth per group were required to have a 90% chance of detection as significant at the 5% level (2-sided test), with a minimum detectable difference in means of 5.97 with an expected standard deviation of 4.61 with regard to primary outcome (colour discoloration – ΔE_{ab}) evaluated by a sphere spectrophotometer. The calculation was performed using the statistical software package SigmaStat version 12.5 (Systat Software Inc., San Jose, CA, USA).

Seventy-two central incisors from young cows were obtained from a local abattoir (Real, Uberlândia, MG, Brazil). Each tooth was cleaned and stored in distilled water at 4 °C. Extrinsic stains and calculi were removed with an ultrasonic scaler, followed by polishing with pumice paste and water. To create standardized specimens and to mimic traumatic dental injuries in immature permanent incisors, the apical part of each root was removed with a high-speed disc (12 mm from the amelocemental region to the apical region), and a part of the crown of each tooth was removed (8 mm from the cement–enamel region to the incisal edge). Next, the apical opening of the root canal was treated with 37% phosphoric acid for 15 s and then rinsed. The bonding agent (3M ESPE, St. Paul, MN,

USA) was applied and light cured for 20 s, and then, a composite resin material (3M ESPE Z250, Sumaré, SP, Brazil) was placed and cured for 40 s (Fig. 1a). The specimens were then randomly assigned (n = 18) to three experimental subgroups (BT, MTA and UC) and a control group (agar + blood). Each tooth was included in polystyrene resin, and the preparation was performed with a PM 82 drill (KG Sorensen, Cotia, SP, Brazil) to obtain similar root canals with a large internal diameter. Then, the root canals were rinsed with 2.5% sodium hypochlorite for 20 min followed by 3 mL of 17% EDTA solution and 5 mL of distilled water. To simulate the pulp and inherent difficulties related to the pulpotomy procedure, a mixture of agar (Kasvi, São José dos Pinhais, PR, Brazil) and bovine blood was prepared (Lenherr *et al.* 2012). Agar was weighed and diluted in warm water according to the manufacturer's recommendations. Then, 6 mL of prepared agar was mixed with 100 μL of fresh uncoagulated blood and inserted on the root canal using pipette tips in a volume of approximately 80 μL per tooth.

Colour assessment

A spectrophotometer (Easyshade Compact Advance 4.0; Vita-Zahnfabrik, Bad Sackingen, Germany) was used to assess tooth colour. A silicone index (Precise SX; Dentsply, Petropolis, RJ, Brazil) containing a 6-mm hole for the placement of the spectrophotometer tip was used to standardize the readings and reposition the Easyshade at each time-point (Fig. 1b). Three assessments were performed on each tooth, and the average was recorded. Five sessions of colour measurements were conducted at the following intervals: T0, before application of the root-end filling material (baseline); T1, immediately after application of the root-end filling material; T30, 30 days after; T45, 45 days after; and T60, 60 days after.

The CIE $L^*a^*b^*$ system (L^*: white/black; a^*: red/ green; b^*: yellow/blue) values were noted for each specimen. The mean value of three measurements was calculated at each assessment time/material. The total colour differences (ΔE_{ab}) were calculated using the following equation: $\Delta E_{ab} = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$. In addition, the whitening indexes (WI) were calculated using the following formula: $WI = 0.551^*L - 2.324^*a - 1.1^*b$ (Pérez Mdel *et al.* 2016), and DE_{00} was also calculated using the formula described in a prior study (Sharma *et al.* 2005).

Radiopacity

For radiopacity analysis of the materials (Fig. 2), all teeth in the four groups were radiographed using the VistaScan Mini Plus® photostimulable phosphor (PSP) system (Dürr Dental, Bietigheim-Bissingen, Germany). Each specimen was placed on the centre of a size 2 (3 × 4 cm) PSP plate along with a 10-mm aluminium step wedge. A Timex 70E X-ray unit (Gnatus, Ribeirão Preto, SP, Brazil) was used, operating at 70 kV, 7.0 mA, 0.14-s exposure time and 28 cm focus/film distance. After exposure, the plates were scanned, and the 8-bit images were exported to ImageJ for Windows software (National Institutes of Health, Washington, WA, USA). For each image, one area of the same square format (50 × 50 pixels) was defined as the region of interest (ROI). This ROI was placed in the area of the radiographs that contained the most homogenous part of the restorative material. The mean grey values of the ROIs were determined using the histogram analysis tool of the software. Radiographs and a grey value analysis were performed before, immediately after and thirty days after filling material application.

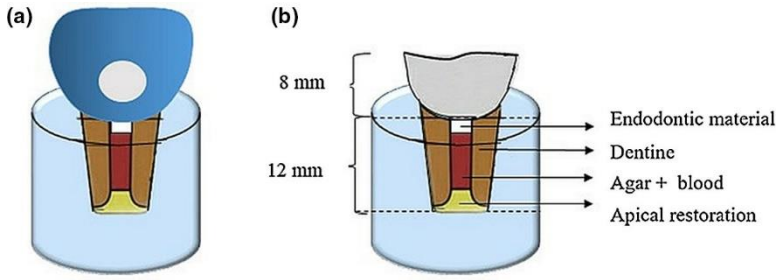

Figure 1 Schematic showing tooth specimen: (a) after coronal and root preparation; (b) after silicone impression material index insertion for colour measurements.

Statistical analysis

Cell viability, colour assessment and radiopacity data were analysed for normality and homoscedasticity using the Shapiro–Wilk and Levene tests. One-way ANOVA followed by Tukey's test was used to compare data of the cell viability intragroup amongst dilutions and amongst the materials at each of the dilutions tested. Two-way repeated measures ANOVA and Tukey's tests were used to compare the radiopacity and colour parameters (*L,*a,*b, ΔE_{ab}, ΔE_{00} and WI), where 'time assessment' was used as a repetition factor. Dunnett's test was used to compare the colour in the experimental groups with the control group. A statistical analysis was performed using SigmaPlot 12.5 statistical software package (Systat Software Inc). The significance level was set at 95% for all data analyses.

Results

Viability by MTT formazan

The cytotoxicity results are presented in Figs 3 and 4. The MTA group was not different amongst the dilu- tions evaluated ($P = 0.09$). Dunnett's test revealed that the MTA-treated cells had greater viability than the control group cells (DMEM) at 1 : 4 dilution ($P = 0.01$).

Significantly lower percentages of viable cells were obtained after the treatment with UC extracts at 1 : 8 dilution, ($P = 0.0364$). All dilutions tested for UC had similar cell viability compared with the control group (DMEM) ($P > 0.05$).

Significant differences were obtained amongst the dilutions in relation to the BT group ($P < 0.0001$). At 1 : 1 and 1 : 2 dilutions, there was a significantly lower viability compared with other dilutions and with the control group (DMEM) ($P < 0.0001$, Fig. 3). Figure 4 shows the comparison between root-end filling materials at the dilutions tested. BT had significantly lower viability than the other materials at the 1 : 1 and 1 : 2 dilutions ($P < 0.0001$). At the 1 : 4 and 1 : 8 dilutions, UC and BT were similar to each other ($P = 0.16$ and $P = 0.97$, respectively), and MTA led to greater viability ($P = 0.001$) at the 1 : 8 dilution. At 1 : 16 and 1 : 32, the three materials had similar values of cell viability.

Colour assessment

Table 2 presents the mean and standard deviation values of colour alteration (ΔE_{ab} and ΔE_{00}) for all groups immediately after application of the root-end filling material (T1) and over time (T30, T45, T60). Two-way repeated measures ANOVA revealed a significant interaction between material and assessment time (ΔE_{ab}: $P = 0.012$ and ΔE_{00}: $P = 0.023$). UC was associated with less colour alteration at 45 days than MTA (ΔE_{ab}: $P < 0.001$ and ΔE_{00}: $P < 0.001$) and BT (ΔE_{ab}: $P < 0.001$ and ΔE_{00}: $P < 0.001$).

At the 60-day measurement, UC and BT had similar tooth discoloration, whereas MTA had significantly more discoloration ($P < 0.001$). The analysis of MTA and UC over time revealed that the colour tended to stay stable over 30–60 days. BT had a maximum discoloration at 45 days followed by a rebound effect at 60 days. When comparing the colour change of the samples from the initial assessment time (T1) to the other experimental time-points (T30; T45; T60), the control group, UC and MTA all had significant discoloration. Dunnett's test immediately after material placement (T1) revealed a significant discoloration between the control group compared

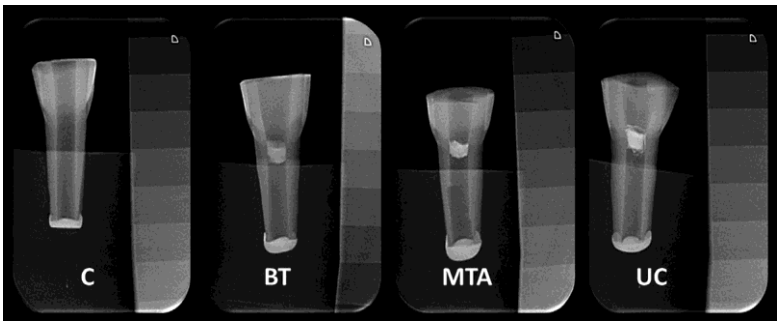

Figure 2 Representative images of radiopacity from each group: C, control (agar + blood); BT, Bio-C Temp; MTA, mineral tri- oxide aggregate (MTA Flow); UC, ultraCal XS.

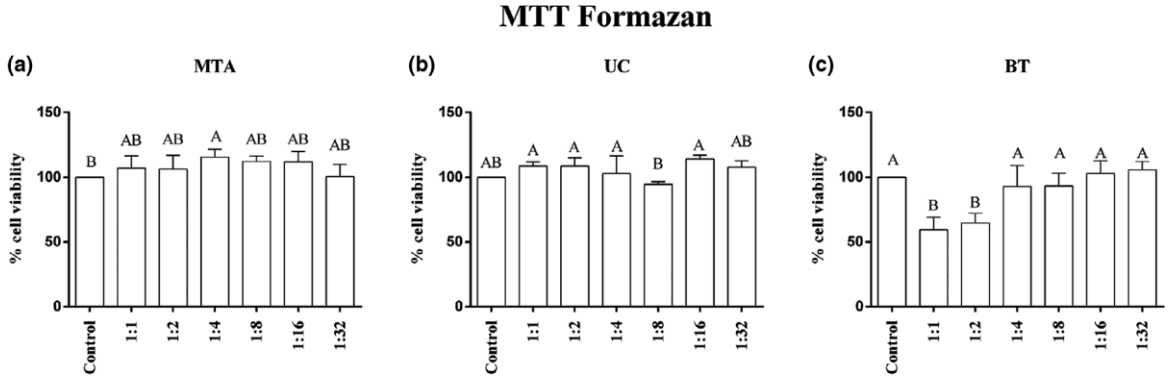

Figure 3 Cell viability percentage of hDPCs after exposure to extracts according to material tested and dilution by the MTT for- mazan method. (a) MTA, mineral trioxide aggregate (MTA Flow) exposure; (b) UC, UltraCal XS exposure; (c) BT, Bio-C Temp exposure. Capital letters indicate comparison amongst different dilutions of extracts and the control group for each material. One-way ANOVA and Tukey's test ($P < 0.05$).

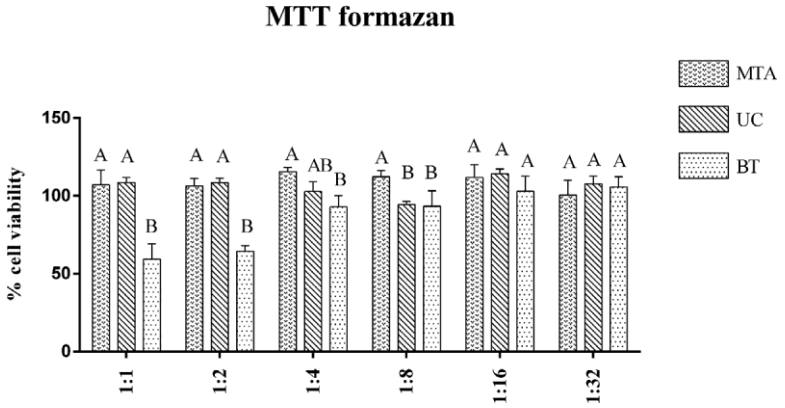

Figure 4 Cell viability percentage of hDPCs after exposure to extracts comparing the materials in the same dilution by the MTT formazan method. MTA, mineral trioxide aggregate (MTA Flow); UC, UltraCal XS; BT, Bio-C Temp. Capital letters indicate comparison amongst different materials at the same dilution. One-way ANOVA and Tukey's test ($P < 0.05$).

Table 2 Mean and standard deviation of discoloration (DE_{ab} and DE_{00}) in the different groups evaluated after root-end filling material and assessment time

	DE_{ab}				DE_{00}			
Groups	T1	T30	T45	T60	T1	T30	T45	T60
Control (agar + blood)	1.9 (2.0)c	9.5 (2.6)a	7.6 (2.3)b	7.4 (2.3)b	1.2 (1.2)b	4.85 (1.5)a	4.27 (1.4)a	4.22 (1.4)a
BT	7.4 (2.4)Ab*	8.1 (1.35)Aab	9.6 (2.6)Aa	7.5 (2.4)Bb	4.27 (1.5)Ab*	5.08 (0.8)Ab	5.9 (1.6)Aa*	4.47 (1.4)Bb
UC	2.8 (2.9)Bb	5.6 (2.4)Ba*	6.0 (2.6)Ba	6.6 (3.2)Ba	1.48 (1.3)Bb	3.25 (1.5)Ba*	3.32 (1.6)Ba	3.4 (1.5)Ba
MTA	9.2 (7.8)Ab*	10.7 (5.9)Aa	14.5 (7.9)Aa*	12.9 (5.2)Aa*	5.35 (4.2)Ab*	5.93 (2.9)Aa	8.63 (4.9)Aa*	7.46 (3)Aa*

T1, after application of root-end filling material; T30, 30 days after; T45, 45 days after; and T60, 60 days after.
Different capital letters in columns indicate significant differences between filling materials in the same assessment time, and dif- ferent lowercase letters in rows indicate significant intragroup differences between the periods analysed (two-way repeated mea- sures ANOVA and Tukey's test - $P < 0.05$).
*Symbol indicates significant differences in columns with the control group (agar + blood) by Dunnett's method, $P < 0.05$.

with BT and MTA ($P < 0.001$). At 30 days, only the UC group differed significantly from the control group, whereas at 60 days, a significant difference from the control group was observed only in the MTA group ($P < 0.001$).

Figure 5 shows the CIELAB parameters. L^* is the parameter that usually represents the major concern from an aesthetic standpoint (darkness to lightness), and it initially presented a similar behaviour between the groups at 30 days. However, at 60 days, the MTA (vs. UC: $P < 0.001$ and vs. BT: $P = 0.031$) and control (vs. UC: $P < 0.001$ and vs. BT: $P = 0.019$) groups had greater darkening than the other groups (low L val- ues). The UC and BT groups had similar behaviour, with a reduction in L values up to 30 days and a tendency of recovery of L values after this period; this was not observed in the control and MTA groups.

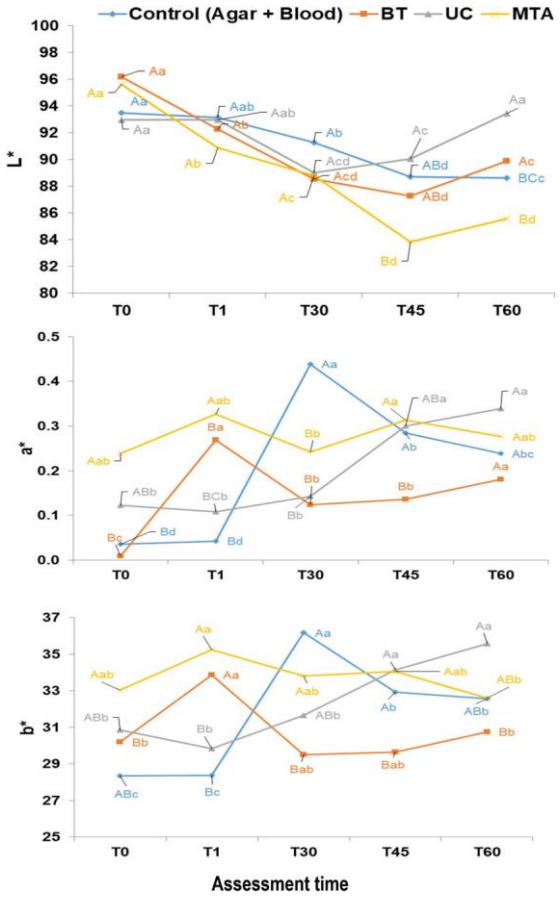

Figure 5 Graphs show the trends in the L^*, a^* and b^* parameters of the materials over time (L^*: white/black; a^*: red/green; b^*: yellow/blue). Different capital letters indicate differences between filling material in the same interval assessment time, and different lowercase letters indicate intragroup differences between the periods analysed.

In relation to the a^* parameter (red-green gradient), there was a fluctuation of mean values in the experimental periods amongst the different materials and the control, with a tendency to equivalence at 60 days. The analysis of the graph (Fig. 5b) reveals that the MTA group had a lower tendency to change in a^* values over time. The BT group had an initial peak after material insertion, a dramatic reduction at 30 days and a subsequent increase at 60 days, whereas the UC group remained stable until 30 days, increasing its a^* values after this period.

The b^* parameter (blue-yellow gradient) was similar amongst groups at 30 and 60 days, except in the BT group. The analysis of the behaviour of each material over time revealed little variation in the mean values of b^* for the BT and MTA groups over the 30- to 60- day period. The UC group had a significant increase in the mean value of b^* at 45 days ($P < 0.001$), and after 60 days, all groups behaved similarly to the con- trol group.

The whiteness index (WI) was significantly influenced by 'material' ($P = 0.044$), 'assessment time' ($P < 0.001$) and the interaction 'material 9 assess- ment time' ($P < 0.001$). These data are presented in Fig. 6. Immediately after material insertion, MTA had significantly lower WI values than UC ($P = 0.009$) and BT ($P = 0.032$), which behaved similar to the control group at T1. At T45, UC ($P = 0.018$) and MTA ($P < 0.001$) demonstrated a reduction in WI compared to BT, and all materials retaining this low index at T60. WI was similar at T60 for all materials. In general, the BT group had the most WI changes compared with control group over time. The alter- ations in colour parameters are illustrated in Fig. 7. To facilitate the visualization of colour changes, the values of L^*, a^* and b^* were converted to an RGB (red, green and blue) system, and coloured rectangles were drawn in RGB using Microsoft® PowerPoint®.

Radiopacity

Table 3 presents the median and standard deviation values for radiopacity expressed as grey values one and thirty days after material insertion. The t-test did not indicate a difference between the initial (104.4 T 8.1) and final (108.5 T 8.6) radiopacities of the control teeth, which did not receive the mate- rial ($P = 0.159$). BT had a significantly lower initial radiopacity than UC and MTA ($P < 0.001$), which

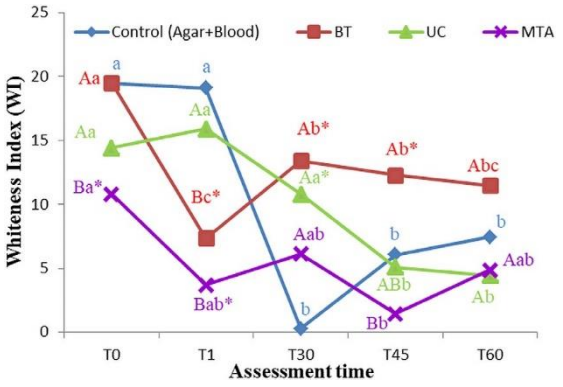

Figure 6 The behaviour of the whiteness index during the entire experiment. T0: baseline, T1: after application of root-end filling material, T30: 30 days after; T45: 45 days after; and T60: 60 days. BT, Bio-C Temp; UC, UltraCal XS; MTA, mineral trioxide aggregate (MTA Flow). Different capital letters indicate significant differences between filling materials in the same assessment time, and different lowercase letters indicate significant intragroup differences between the periods analysed (two-way repeated measures $_{ANOVA}$, $P < 0.05$); *symbol indicates significant differences with the control group (agar + blood) in the same assessment time by Dunnett's method, $P < 0.05$.

had similar radiopacity ($P = 0.97$), at T1. After 30 days, all materials had different radiopacities, with the highest grey values in the UC group (vs. MTA: $P = 0{,}045$ and vs. BT: $P < 0.001$) and the lowest in the BT group (vs MTA: $P < 0.002$). There was an interaction factor, 'assessment time 9 material' ($P = 0.035$), and only MTA had significantly reduced radiopacity after 30 days ($P = 0.007$).

Discussion

The results support the rejection of the null hypothesis tested because significant differences were found between the materials regarding the viability of pulp cells, radiopacity and coronal discoloration in the presence of blood. Previous studies evaluating the cell viability of pulp cells in contact with MTA demonstrated that this material did not affect this parameter in hDPCs (Rodrigues *et al.* 2017, Tomás-Catalá *et al.* 2017, Pedano *et al.* 2018). However, no studies to date have evaluated BT cytotoxicity because this material is new. Biocompatibility is an important property that should be considered when selecting a material for pulpotomies due to its direct contact with vital tissues (Lee *et al.* 2014). Amongst the evaluations that can be performed in this context, the analysis of cytotoxicity and potential adverse effects on cell behaviour is one of the most commonly used. In the present study, the cytotoxicity test selected was MTT formazan. The MTT formazan method is a widely used cytotoxicity test (Pires *et al.* 2016, Collado-González *et al.* 2017) that determines cell viability as a function of their mitochondrial activity through the conversion of tetrazolium salt into formazan crystals by mitochondrial dehydrogenases (Mosmann 1983).

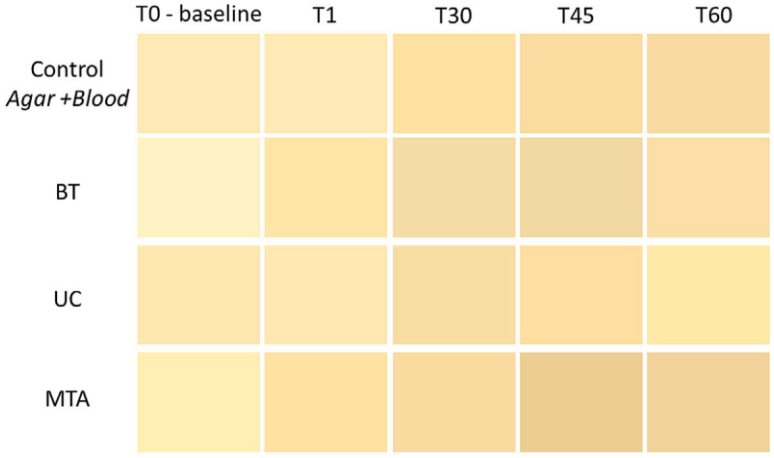

Figure 7 Tooth behaviour illustrated based on data from $L^*a^*b^*$ converted to RGB demonstrating the colour changes of speci- mens during the experiment. T0: baseline, T1: after application of root-end filling material, T30: 30 days after; T45: 45 days after; and T60: 60 days after. BT, Bio-C Temp; UC, UltraCal XS; MTA, mineral trioxide aggregate (MTA Flow). Illustrative cylinder-shaped composite specimens.

Table 3 Mean and standard deviation of radiopacity (grey value) in the different groups evaluated at day 1 and day 30

	Assessment time	
Groups	T1	T30
BT	187.4 (9.7)Ba	186.2 (9.6)Ca
UC	201.2 (11.5)Aa	202.7 (11)Aa
MTA	200.5 (10.6)Aa	196 (11)Bb

T1: immediately after application of root-end filling material and T30: 30 days after.
Different capital letters in columns indicate significant differ- ences between filling material in the same assessment time, and different lowercase letters in rows indicate significant intragroup differences between the periods analysed (two-way repeated measures ANOVA, $P < 0.05$).

The MTT results revealed that MTA and UC were not cytotoxic for hDPCs at all dilutions. MTA has been reported to induce proliferation of hDPCs by elu- tion components such as calcium ions (Takita *et al.* 2006). The high proliferation of hDPCs at the 1 : 4 dilution of extract corroborates previous studies using the MTT assay (Rodrigues *et al.* 2017, Pedano *et al.* 2018). Few researchers have evaluated the cyto- toxicity of $Ca(OH)_2$ paste in the same formulation used in the present study (Althumairy *et al.* 2014). Previously, it was reported that UC extracts caused a significant increase in cell viability (Pires *et al.* 2016), which was not found in the present study. This could be because the referenced study used peripheral blood mononuclear cells, and $Ca(OH)_2$ has the capacity to induce an inflammatory response (Nelson Filho *et al.* 1999). Regarding BT, an increase in cell prolif- eration was expected because its composition includes calcium silicates, calcium aluminate, calcium oxide and calcium tungstate. However, the lowest BT dilu- tions had a cytotoxic effect on hDPCs, decreasing the cell viability to approximately 60% compared with the control. A possible hypothesis for this reduction in viability is the presence of TiO_2 in its composition. This component may interfere with a series of cellular events, including those associated with stimulation of the mitogen-activated protein kinase (MAPK) path- way, with a consequent reduction in cell survival (Yu *et al.* 2019). In addition, previous investigations have demonstrated that TiO_2 induces apoptosis in different cell types including murine leukaemic monocyte macrophages (RAW 246.7 cells) (Dhupal *et al.* 2018), lymphocytes (Wang *et al.* 2007), fibroblasts (Jin *et al.* 2008) and mesenchymal stem cells (Yu *et al.* 2019). However, considering that there are no studies evaluating the cytotoxicity of this component in hDPCs, it is not possible to directly relate to the results of these studies. In addition, it is important to note that at higher dilutions, the behaviour of BT- treated cells was similar to the behaviour of cells trea- ted with the other tested materials, and therefore, the use of BT in pulp cells would not be contraindicated.

In pulpotomies, the material is placed directly into tissue containing blood; therefore, aiming to mimic the clinical situation, all materials of the study were applied directly to a mixture of agar containing blood, as agar has a gelatinous consistency similar to that of pulp tissue. The present results revealed that blood was able to increase the discoloration associated with all materials, including colour changes in the nega- tive control group (agar + blood). The discoloration in the negative control group was greater at 30 days, remaining low in the subsequent periods. Addition- ally, the luminosity and WI of the control group reduced over time, indicating that the presence of blood caused tooth darkening. A possible mechanism explaining the staining caused by blood is related to the accumulation of haemoglobin or other haematin molecules (Marin *et al.* 1997). The haemolysis of these molecules releases haeme groups, which can cause darkening of the tooth structure as they pro- duce black iron sulphide. Therefore, a reduction in lightness values and an increase in redness and yel- lowness values (Fig. 5) following blood exposure to the specimens could be expected.

Beyond the blood, biomaterials are related to tooth staining (Beatty & Svec 2015). Several studies have reported greater staining for grey MTA associated with blood (Lenherr *et al.* 2012, Guimarães *et al.* 2015). Laboratory studies have indicated that MTA is associated with high staining potential as it comprises heavy metal ions and bismuth oxide as a radiopacifier (Marciano *et al.* 2015). Possible explana- tions for MTA-related tooth discoloration are related to the dissociation of oxide bismuth into dark crystals (Yoldas *et al.* 2016, Możyńska *et al.* 2017) or overoxi- dation of this compound due to contact with NaOCl (Camilleri 2014, Marconyak *et al.* 2016), which clini- cally occurs in pulpotomy. Furthermore, this material in contact with blood exacerbates the discoloration process (Guimarães *et al.* 2015, Shokouhinejad *et al.* 2016). White MTA was developed to overcome this issue. However, even white MTA may cause dis- coloration, probably due to oxidation and incorpora- tion of the iron content into the calcium aluminoferrite phase of MTA after setting (Marciano *et al.* 2015).

Particularly in the present study, MTA Flow was selected due to its easier insertion using syringes, which clinically results in a smaller amount of material residues on the dentine walls. This is a relatively new material consisting of a grey powder containing dicalcium and tricalcium silicate, bismuth oxide and a liquid vehicle composed of a water-soluble silicone-based gel that can be manipulated in various consistencies. The manufacturer proposes its use in pulp capping, pulpotomies, sealing perforations and resorptions, retrofillings and teeth with an incomplete root apex (Ultradent, 2011, Ultradent, 2017). The other materials used for comparison (BT and UC) are also injectable and come in a ready-to-use form that also makes them easy to insert. UC is based on a calcium hydroxide paste (calcium hydroxide, barium sulphate and aqueous matrix), and it is known to be used in pulpotomies and direct pulp protection but with high solubility (Pereira *et al.* 2019). BT is a paste recommended for intracanal medication and pulp regeneration by the manufacturer (Angelus 2019). BT colour visually differs from UC, having a more yellowish coloration; additionally, its consistency is slightly different, probably related to the vehicle used. Even though the components of BT medication classify it as a bioceramic material, studies using other bioceramics in pulpotomies have shown less discoloration (Camilleri 2015, Shokouhinejad *et al.* 2016, Yoldas *et al.* 2016). Biodentine (Septodont, France) has shown less discoloration than MTA, possibly due to the use of zirconium oxide as a radiopacifier instead of bismuth oxide (Yoldas *et al.* 2016). BT has a titanium oxide radiopacifier, which is not expected to produce dentine staining. However, BT had ΔE_{ab} and ΔE_{00} values similar to those of MTA and higher than those of UC at 45 days. This could indicate a transient interaction between blood and BT compounds because at 60 days, these values were reduced, making them lower than those of MTA and similar to those of the control. The intense white colour of the UC probably blocks the influence of blood on the initial colour measurement, which could be observed at 30 days, when the UC group presented lower ΔE_{ab} and ΔE_{00} values than the control group. However, this difference was attenuated over time, presenting mean values of discoloration similar to those of the control and BT groups at 60 days. It is important to note that all materials tested had values higher than what is considered the acceptable threshold (ΔE_{ab} = 2.66 and ΔE_{00} = 1.77; Paravina *et al.* 2015).

Associated with the global colour change, the whiteness index (WI), a simple linear formulation obtained using the values of the three CIELAB chromatic coordinates, was used (Pérez *et al.* 2016). It represents a significant step for the assessment of colour change because it correlates with the perception of tooth whiteness. The results of this method are more clinically relevant and provide a clearer interpretation: high positive values of the WI index indicate higher whiteness values. Tooth yellowness may not be a perfect antonym of tooth whiteness, but WI could be used to reflect perceptual yellowness (Sullivan *et al.* 2019). Compared with the classic materials used in pulpotomy, BT had WI values similar or lower than those already established in the literature. Moreover, all materials resulted in a slightly greater difference in tooth whiteness (≥5.69) (Pérez Mdel *et al.* 2016) compared with teeth without any root-end filling material.

For this study, the Vita Easyshade spectrophotometer was used to evaluate colour change. This instrument was applied because of the technique's sensitivity to even slight changes in colour and excellent reproducibility. The same equipment was used in previous studies (Guimarães *et al.* 2015, Marconyak *et al.* 2016, Yoldas *et al.* 2016), which used the CIE $L^*a^*b^*$ space system to evaluate colour change. Regarding radiopacity, the ISO standard was not used, which uses pre-established silicone moulds filled with cement. The reason for inserting the material directly into the tooth was for the study design to approximate clinical practice, where this is the only standard of evaluation by the professional. Bovine teeth have been previously used as a substitute for human in studies of tooth discoloration (Beatty & Svec 2015, Yoldas *et al.* 2016). Considering that the coronal dentine of bovine teeth does not differ significantly from that of human teeth in terms of density or diameter of tubules, bovine mandibular incisors may be used in this kind of study (Lenherr *et al.* 2012, Beatty & Svec 2015, Yoldas *et al.* 2016).

Another parameter evaluated was the radiopacity of the materials, which is an important factor to consider when choosing a material for pulpotomy because it enables the visualization of gaps or absence of material through the X-ray and differences in tooth tissues (Guerreiro-Tanomaru *et al.* 2009, Xuereb *et al.* 2016). In the present study, the higher initial radiopacity values (T1) for MTA and UC groups than for BT were probably related to the differences in radiopacifiers present in each material. Nonetheless, it

is possible to observe that only the MTA group had a reduction in a radiopacity after 30 days, which is in agreement with other studies (Camilleri 2008, Cave- nago *et al.* 2014, Guimaraes *et al.* 2015) and may be caused by the dissociation of bismuth oxide. On the other hand, it is possible that the radiopacity stability provided by the titanium oxide used as a radiopacifier in BT and by the barium sulphate in UC could be related to the smaller colour variations of these mate- rials over time. It is important to observe that despite the lower radiopacity, BT could be a visual differenti- ated from the tooth tissues (Fig. 2), fulfilling this fun- damental requirement for a material used in pulpotomy.

Conclusion

The new bioceramic material BT had acceptable cell viability, similar to that of MTA and UC, at the great- est dilutions, and resulted in less or similar tooth col- our change compared with MTA and UC, respectively. Despite its lower radiopacity, BT was identified radio- graphically.

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Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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