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In vitro evaluation of cell viability, radiopacity and tooth discoloration induced by regenerative endodontic materials

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Abstract

Aim: Different materials have been used for pulpotomy of teeth with incomplete rhizogenesis. This study aimed to analyze the cytotoxicity, color 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 different dilutions of culture media previously exposed to such materials and tested for cell viability using MTT. Bovine teeth were prepared to simulate open apex and to mimic extensive crown fracture. The roots were filled with a mixture of agar and blood (control) or experimental groups on which the studied materials were placed over. Color assessment analyses were performed before and immediately after material insertion and repeated at 30, 45, and 60 days using a spectrophotometer. The total color change (ΔEab, ΔE_{00} and whiteness index (WI)) were calculated based on the CIELab color space. colDigital radiographs were acquired for radiopacity analysis. Cell viability was analyzed by one-way ANOVA, while differences in color parameters (ΔE_{ab}, ΔE_{00} and WI) were assessed by two-way repeated measures ANOVA (α= 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 showed cell viability similar to that of the control group (DMEM) ($P > 0.05$), except for the BT group at the 1:1 and 1:2 dilutions, which presented lower viability ($P < 0.001$). All materials promoted discoloration values higher than what is considered the acceptable threshold, and BT resulted in less or similar tooth color 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) has acceptable cell viability, similar to that of MTA and UC at the highest dilutions, and BT resulted in less tooth color change than MTA and UC. Moreover, despite its lower radiopacity, BT may still be radiographically identified; therefore, it can be used in pulpotomies.

Introduction

Dental trauma often occurs in young people, and it may result in different traumatic injuries involving teeth and supporting structures (Qudeimat *et al*. 2019). Crown fractures of permanent teeth represent between 26% and 76% of all traumatic injuries (Castro *et al*. 2005). These fractures are often complicated and deserve special attention as they may have 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 (Chen *et al.* 2019, Alqaderi *et al.* 2016).

Pulpotomy has an excellent prognosis and comprises surgical amputation of the infected coronal pulp (depth of 1.5 to 2.0 mm) and placement of protective material to seal the exposed pulp and preservation of tooth vitality (Tuloglu *et al*. 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 (Parirokh *et al.* 2018, Lee *et al.* 2017, Cosme-Silva *et al.* 2019, Pedano *et al.*2018), as well as act as a barrier against microorganisms, stimulate tissue healing and not contribute to discoloration (Możyńska *et al*. 2017). Calcium hydroxide (Ca (OH)²) and mineral aggregate trioxide (MTA) are the most commonly used materials in pulpotomies (Parirokh *et al.* 2018, Liu *et al*. 2011, Musale *et al*. 2018).

$Ca(OH)_2$ has been used in pulpotomy for years due to the alkaline pH and ability to activate alkaline phosphatase and consequently stimulate the production of tertiary dentin. Nevertheless, the long-term use of $Ca(OH)_2$ presents poor sealing and high solubility in oral fluids (Gandolfi *et al.* 2015). Another material used in pulpotomy is MTA, which presents great physical and chemical properties and excellent biocompatibility (Camilleri *et al*. 2015, Lee *et al.* 2017, Parirokh *et al.* 2018, Nagendrababu *et al*. 2019, Chen *et al.* 2019). Nonetheless, most studies have shown that MTA may cause crown discoloration due to the presence of bismuth oxide as a radiopacifier (Yoldas *et al*. 2016, Shokouhinejad *et al*. 2016). 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, Parirokh *et al.* 2018, Marconyak *et al.* 2016, Cosme-Silva *et al.* 2019, Pedano *et al.* 2018), such

as Sealer Plus (MK Life), Endosequence BC Sealer (Brasseler) and ProRootMTA (Dentsply Maillefer), but only a few studies have analyzed conditions that mimic pulpotomy in traumatized immature teeth. However, these new materials are expensive, which often makes their use in emerging countries unfeasible. 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 coronal 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 promoted by the evaluated materials.

Materials and methods

Preparation of Extracts

The materials used were MTA Flow (MTA) (Ultradent, South Jordan, UT, USA), UltraCal XS (UC) (Ultradent), and Bio-C Temp® (BT) (Angelus, Londrina, PR, Brazil). 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 under aseptic conditions in a laminar flow in 24-well plates. 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. After incubation, these original extracts were serially diluted in cell culture medium before testing until the dilution of 1:32.

hDPCs cell culture

Primary human dental pulp cells (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 deciduous teeth

nearing the time of exfoliation (n=2) were collected, and the pulp was extracted from the pulp chamber using a sterilized sharp dentine spoon. Afterward, the pulp tissue was immersed for 1 h in the following solution: 3 mg/mL collagenase type I (Sigma-Aldrich, San Luis, MI, USA) and 4 mg/mL dispase (Sigma-Aldrich). The samples were centrifuged at 1,200 rpm for 2 minutes and resuspended in basal medium. The obtained cells were plated in 25-cm² 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 fetal 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 predetermined 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 hours 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.

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) was added to each well, and the cells were incubated at 37ºC for 4 h. The supernatants were removed, and then 100 µl dimethyl sulfoxide (DMSO) (LGC Biotecnologia, Cotia, SP, Brazil) was added. Afterward, the optical density (OD) at 570 nm was measured using a microplate reader (Biochrom, Cambridge, UK). The mean values obtained for the control group were considered as 100% of cell viability. Cell viability was evaluated proportionally to absorbance and expressed as the percentage of viable cells.

Selection of teeth and Sample Preparation for color 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 regards to primary outcome (color 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 bovines were extracted. 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 to its long axis 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 incisor). Next, the apical opening of the root canal was treated with 37% phosphoric acid for 15 seconds 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 40s (Figure 1A). The specimens were then randomly assigned (n=18) to 3 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 minutes 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 tips in a volume of approximately 80 µl per tooth.

Color Assessment

A spectrophotometer (Easyshade Compact Advance 4.0, Vita-Zahnfabrik, Bad Sackingen, Germany) was used to assess tooth color. 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 (Figure 1-B). Three assessments were performed on each tooth, and the average was recorded. Five sessions of color 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 3 measurements was calculated at each assessment time/material. The total color differences (ΔE_{ab}) were calculated using the following equation: $\Delta E_{ab}=[(\Delta L)^2 + (\Delta a)^2+ (\Delta b)^2]$ ½. In addition, the whitening indexes (WI) were calculated using the following formula: WI= 0.551*L-2.324*a-1.1*b (Peres *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 (Figure 2), all teeth of 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 center of a size 2 (3x4 cm) PSP plate along with a 10-mm aluminum step wedge. A Timex 70E X-ray unit (Gnatus, Ribeirão Preto, SP, Brazil) was used, operating at 70 kV, 7.0 mA, 0.14-second 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 x 50 pixels) was defined as the region of interest (ROI). This ROI was placed in the area of the radiography that contains the restorative material in the most homogenous part. The mean gray values of the ROIs were determined using the histogram analysis tool of the software. Radiographs and a gray value analysis were performed before, immediately after and thirty days after filling material application.

Statistical Analysis

Cell viability, color assessment, and radiopacity data were analyzed 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 among dilutions and among the materials at each of the dilutions tested. Two-way repeated measures ANOVA and Tukey's tests were used to compare the radiopacity and color parameters (*L,*a,*b, $\Delta E_{ab,}$ ΔE_{00} and WI), where 'time assessment' was used as a repetition factor. Dunnett's test was used to compare the color 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 Figures 3 and 4. The MTA group showed no differences among the dilutions evaluated (P=0.09). Dunnett's test showed that the MTA-treated cells had higher viability than the control group cells (DMEM) at 1:4 dilution (P=0.01).

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 presented similar cell viability compared with the control group (DMEM) (P>0.05).

In relation to the BT group, differences were obtained among the dilutions (P<.0001). At the 1: 1 and 1: 2 dilutions presented lower viability compared with other dilutions (P<0.05) and with the control group (DMEM) (P<.0001, Figure 3). Figure 4 shows the comparison between root-end filling materials at the dilutions tested. BT showed lower viability than the other materials at the 1: 1 and 1: 2 dilutions (P<.0001). At the 1:4 and 1:8 dilutions, UC and BT were similar to each other (P=0.16 and P=0.97, respectively39), and MTA was the material that led to highest viability (P=<0.005) at the 1:8 dilution. At 1:16 and 1:32, the three materials presented similar values of cell viability. (P>0.05).

Color Assessment

Table 2 presents the mean and standard deviation values of color 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 showed a significant interaction between material and assessment time (ΔE_{ab}: P = 0.012 and ΔE_{00}: P= 0.023). UC promoted less color alteration at 45 days than MTA and BT.

At the 60-day measurement UC and BT presented similar tooth discoloration, whereas MTA presented the highest significant discoloration (P<.001). The analysis of MTA and UC over time showed that the color tended to stay stable over 30 to 60 days. BT presented a maximum discoloration at 45 days followed by a rebound effect at 60 days. When comparing the color 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 showed significant discoloration. Dunnett's test immediately after material placement (T1) showed significant discoloration between the control group compared with BT and MTA (P<.001). At 30 days, only the UC group differed significantly from the control group, whereas at 60 days, a difference from the control group was observed only in the MTA group (P<.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 behavior between the groups at 30 days. However, at 60 days, the MTA and control groups presented greater darkening than the other groups (low L values). The UC and BT groups showed similar behavior, 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.

In relation to the a* parameter (red-green gradient), there was a fluctuation of mean values in the experimental periods among the different materials and the control, with a tendency to equivalence at 60 days. The analysis of the graph (Figure 5B) shows that the MTA group showed less tendency to change in a* values over time. The BT group showed 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 among groups at 30 and 60 days, except in the BT group. The analysis of the behavior of each material over time shows little oscillation in the mean values of b* for the BT and MTA group over the 30- to 60-day period. The UC group showed a significant increase in the mean value of b* at 45 days, and after 60 days, all groups behaved similarly to the control group.

The whiteness index (WI) was influenced by "material" (P= 0.044), "assessment time" (P<.001) and the interaction "material x assessment time" (P<.001). These data are presented in Figure 6. Immediately after material insertion, BT and MTA showed significantly lower WI values than UC, which behaved similar to the control group at T1. At T45, all materials demonstrated a reduction in WI, retaining this low index at T60. WI was similar at T60 for all materials. In general, the BT group was the one that presented the most WI differences compared with the control group, showing higher values over time. The alterations in color parameters are illustrated in Figure 7. To facilitate the visualization of color changes, the values of L*, a*, and b* were converted to an RGB (red, green and blue) system, and colored rectangles were drawn in RGB using Microsoft® PowerPoint®.

Radiopacity

Table 3 presents the median and standard deviation radiopacity values expressed as gray values one and thirty days after material insertion. The t test did not indicate a difference between the initial (104.4 ± 8.1) and final (108.5 ± 8.6) radiopacities of the control teeth, which did not receive the material (P = 0.159). BT presented lower initial radiopacity than UC and MTA (P <0.001), which presented similar radiopacity (P = 0.97), at T1. After 30 days, all materials presented different radiopacities, with the highest gray values in the UC group and the lowest in the BT group. There was an interaction factor, "assessment time x material" (P = 0.035), and only MTA presented reduced radiopacity after 30 days (P = 0.007).

Discussion

The results found in this study 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 to the market. 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). Among the evaluations that can be performed in this context, the analysis of cytotoxicity and potential adverse effects on cell behavior is one of the most commonly used. In the present study, the cytotoxicity test selected was MTT formazan. The MTT formazan method is a cytotoxicity test widely used in the literature (Pires *et al.* 2016, Collado-Gonzalez *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).

The MTT results showed that MTA and UC were not cytotoxic for hDPCs at all dilutions. MTA has been reported to induce proliferation of hDPCs by elution 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 cytotoxicity 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 our 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 proliferation was expected because its composition includes calcium silicates, calcium aluminate, calcium oxide and calcium tungstate. However, the lowest BT dilutions showed 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) pathway, 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 leukemic 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 behavior of BT-treated cells was similar to the behavior of cells treated 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 presents 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 color changes in the negative control group (agar + blood). The discoloration in the negative control group was greater at 30 days, remaining low in the subsequent periods. Additionally, the luminosity and WI of the control group presented a reduction over time, indicating that the presence of blood caused tooth darkness. A possible mechanism explaining the staining caused by blood is related to the accumulation of hemoglobin or other hematin molecules (Marin *et al.* 1997). The hemolysis of these molecules releases heme groups, which can cause darkening of the tooth structure as they produce black iron sulfide. Therefore, a reduction in lightness values and an increase in redness and yellowness values (Figure 5) following blood exposure to the specimens could be expected.

Beyond the blood, biomaterials are related to tooth staining (Beatty & Svec 2015). Some studies have shown greater staining for gray MTA associated with blood (Lenherr *et al*. 2012, Guimarães *et al*. 2015). *In vitro* studies have indicated that MTA provides high staining potential as it comprises heavy metal ions and bismuth oxide as a radiopacifier (Marciano *et al*. 2015). Possible explanations for MTA-related tooth discoloration are related to the dissociation of oxide bismuth into dark crystals (Możyńska *et al*. 2017, Yoldas *et al*. 2016) or overoxidation of this compound due to contact with NaOCl (Camilleri

2014, Marconyak *et al*. 2016), which clinically 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 discoloration, probably due to oxidation and incorporation of the iron content into the calcium aluminoferrite phase of MTA after setting (Marciano *et al*. 2015).

Particularly in the present study, the use of MTA Flow was selected due to its easier insertion using syringes, which clinically results in a smaller amount of material residues in the dentin walls. This is a relatively new material consisting of a gray 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, 2011,2017). 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 sulfate, 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 color 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 classifies 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 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 dentin staining. However, BT showed 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 color of the UC probably blocks the influence of blood on the initial color 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 color change, the whiteness index (WI), a simple linear formulation obtained using the values of the three CIELab chromatic coordinates, was used (Perez *et al.* 2016). It represents a significant step for the assessment of color change because it correlates with the perception of tooth whiteness. The results of this method are more clinically relevant and have 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 presented WI values similar or lower than those already established in the literature. Moreover, all materials resulted in a barely greater than acceptable difference in tooth whiteness (≥5.69) (Pérez *et al.* 2016) compared with teeth without any root-end filling material. Thus, the use of BT is not contraindicated in pulpotomies of anterior teeth.

For this research, the Vita Easyshade spectrophotometer was used to evaluate color change. This instrument was applied because of the technique's sensitivity to even slight changes in color 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 color change. Regarding radiopacity, we chose not to use the ISO standard, which uses pre-established silicone molds 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. Regarding the use of bovine teeth, 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 dentin 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 presented a radiopacity reduction after 30 days, which is in agreement with other studies (Camilleri 2008, Cavenago *et al*. 2014, Guimarães *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 sulfate in UC could be related to the smaller color variations of these materials over time. It is important to observe that despite the lower radiopacity, BT shows a visual difference from the dental tissues (Figure 2), fulfilling this fundamental requirement for a material used in pulpotomy.

Conclusion

The new bioceramic material BT has acceptable cell viability, similar to that of MTA and UC, at the highest dilutions, and it results in less or similar tooth color change compared with MTA and UC, respectively. Moreover, despite its lower radiopacity, it can be radiographically identified, and therefore it can be used in pulpotomies.

Conflict of Interest

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Tables

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 Hydoxide, Barium Sulphate, Aqueous Matrix of Methycellulose
MTA Flow (Ultradent, Indaiatuba, SP, Brasil)	Powder and Gel (2 big ends mixed with 2 drops, and inserted with micro tips)	Extremely fine inorganic poder 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 agentes.

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

Groups	ΔE_{ab}				ΔE_{00}			
	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 different lowercase letters in rows indicate significant intragroup differences between the periods analyzed (two-way repeated measures ANOVA and Tukey's test - $P < 0.05$). * symbol indicates significant differences in columns with the control group (agar + blood) by Dunnett's method, $P < 0.05$.*

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

Groups	Assessment time	
	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 differences between filling material in the same assessment time, and different lowercase letters in rows indicate significant intragroup differences between the periods analyzed (two-way repeated measures ANOVA, $P < 0.05$).

Figure Legends

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

Figure 2. Representative images of radiopacity from each group: C: Control (agar + blood); BT: Bio-C Temp; MTA: Mineral Trioxide 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 formazan method. a) MTA: Mineral Trioxide Aggregate (MTA Flow) exposure; b) UC: UltraCal XS exposure; c) BT: Bio-C Temp exposure. Capital letters indicate comparison among 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 among different materials at the same dilution. One-way ANOVA and Tukey´s test ($P < 0.05$)

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 analyzed.

Figure 6. The behavior 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 analyzed (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, $P < 0.05$.

Figure 7. Tooth behavior illustrated based on data from L*a*b* converted to RGB demonstrating the color changes of specimens 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.

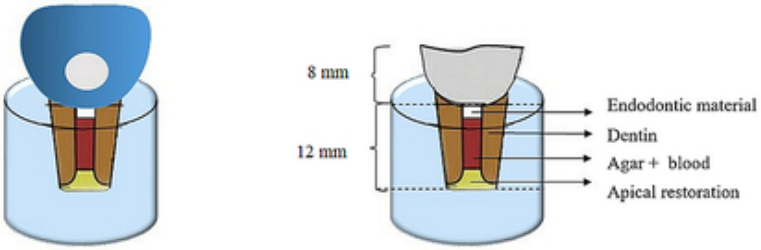

Figure 1. Schematic showing tooth specimen: a) after coronal and root preparation; b) after silicone impression material index insertion for color measurements

45x31mm (300 x 300 DPI)

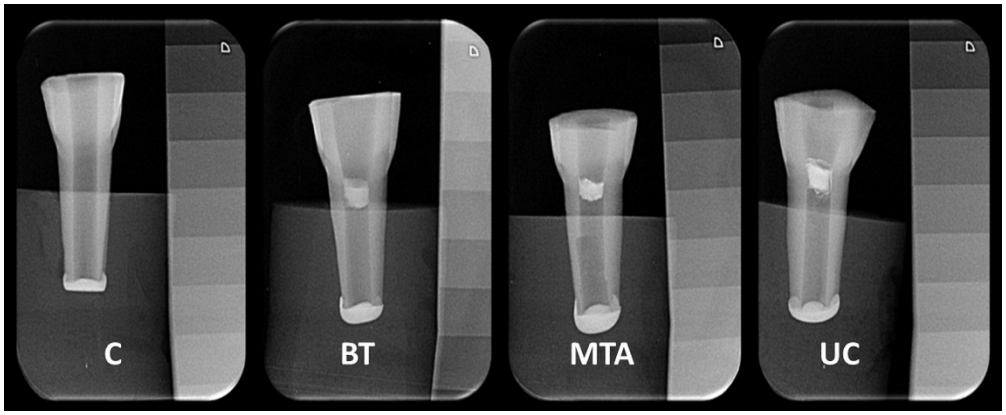

Figure 2. Representative images of radiopacity from each group: C: Control (agar + blood); BT: Bio-C Temp; MTA: Mineral Trioxide Aggregate (MTA Flow); UC: UltraCal XS

76x31mm (600 x 600 DPI)

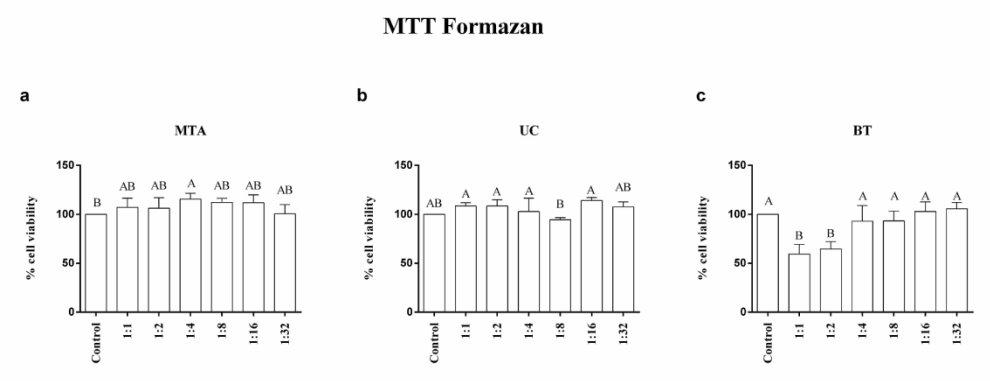

Figure 3. Cell viability percentage of hDPCs after exposure to extracts according to material tested and dilution by the MTT formazan method. a) MTA: Mineral Trioxide Aggregate (MTA Flow) exposure; b) UC: UltraCal XS exposure; c) BT: Bio-C Temp exposure. Capital letters indicate comparison among different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

76x29mm (600 x 600 DPI)

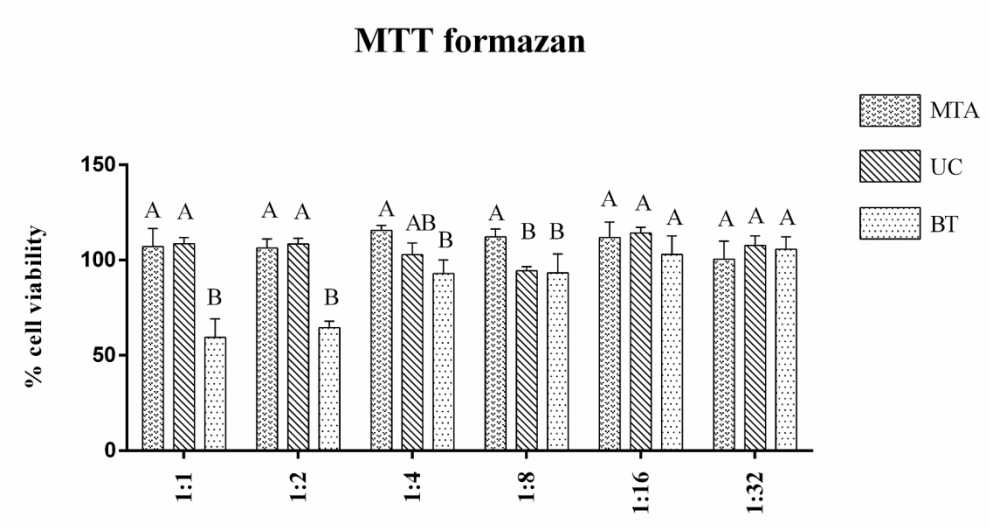

Figure 4. Cell viability percentage of hDPCs after exposure to extracts comparing the materials in the same dilution by the MTT formazan method. MTA: Mineral Trioxide Aggregate (MTA Flow); UC: UltraCal XS; BT: Bio-C Temp. Capital letters indicate comparison among different materials at the same dilution.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

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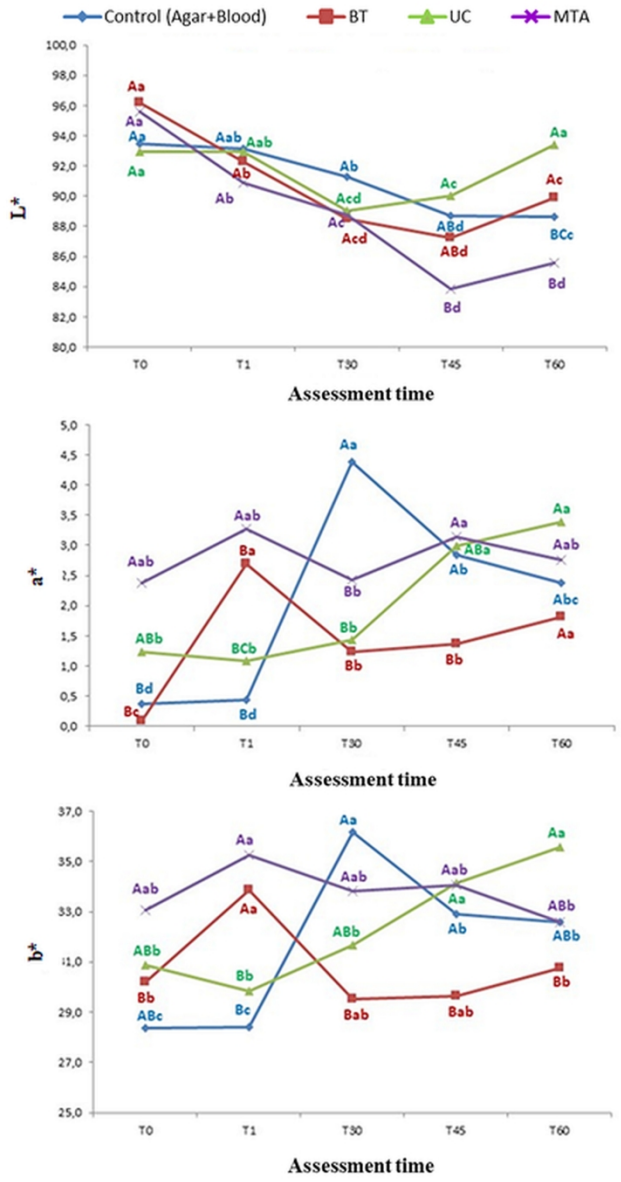

Figure 5. Graphs show the trends in the L*, a*, and b * parameters of the materials over time (L*: white/black; a*: red/green; b*: yellow/blue). Different capital letters indicate differences between filling material in the same interval assessment time, and different lowercase letters indicate intragroup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

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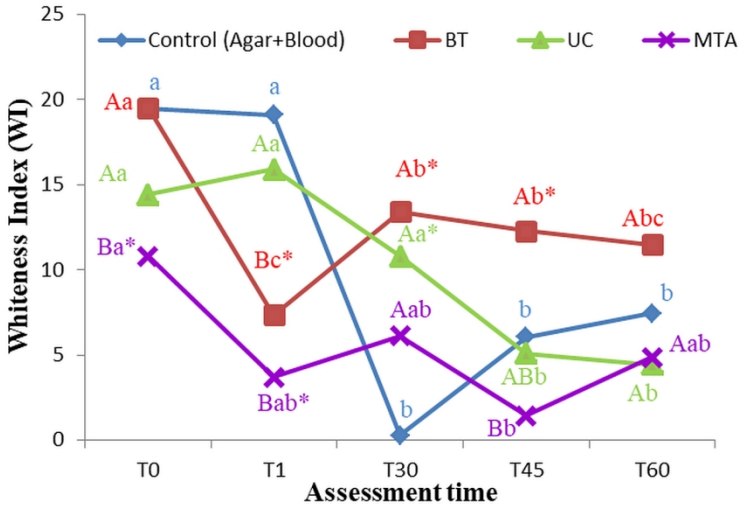

Figure 6. The behavior of the whiteness index during the entire experiment. T0: baseline, T1: after application of root-end filling material, T30: 30 days after; T45: 45 days after and T60: 60 days. BT: Bio-C Temp; UC: UltraCal XS; MTA: Mineral Trioxide Aggregate (MTA Flow). Different capital letters indicate significant differences between filling materials in the same assessment time, and different lowercase letters indicate significant intragroup differences between the periods analyzed (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, $P < 0.05$.

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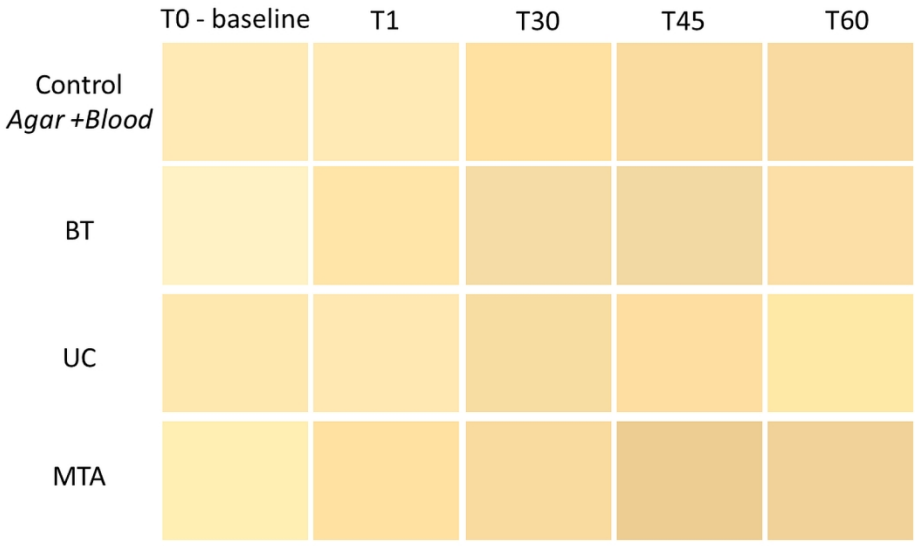

Figure 7. Tooth behavior illustrated based on data from L*a*b* converted to RGB demonstrating the color changes of specimens during the experiment. T0: baseline, T1: after application of root-end filling material, T30: 30 days after; T45: 45 days after and T60: 60 days after. BT: Bio-C Temp, UC: UltraCal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

76x45mm (600 x 600 DPI)