Original Research
Dental Materials

Physicochemical properties and osteoclastogenesis for three premixed calcium silicate-based sealers post set

Camila Maria Peres de ROSATTO[a]
Gabriela Leite de SOUZA[a]
Danilo Cassiano FERRAZ[b]
Marcelo José Barbosa SILVA[c]
Mario TANOMARU-FILHO[b]
Camilla Christian Gomes MOURA[a]

[a]Universidade Federal de Uberlândia – UFU, School of Dentistry, Department of Endodontics, Uberlândia, MG, Brazil.

[b]Universidade Estadual Paulista – Unesp, School of Dentistry, Department of Restorative Dentistry, Araraquara, SP, Brazil.

[c]Universidade Federal de Uberlândia – UFU, Institute of Biomedical Sciences, Department of Immunology, Uberlândia, MG, Brazil.

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

Corresponding Author:
Camilla Christian Gomes Moura
E-mail: camillamoura@ufu.br

https://doi.org/10.1590/1807-3107bor-2022.vol36.0065

Submitted: April 19, 2021
Accepted for publication: December 14, 2021
Last revision: February 9, 2022

Abstract: Solubility, pH, ion release, cytotoxicity, and osteoclastogenesis inhibition in bone marrow-derived monocyte macrophages (BMMs) were evaluated in EndoSequence BC Sealer (END), Bio-C Sealer (BC), and Sealer Plus BC (SPBC). pH was determined after immersion of the sealers in deionized water (DW) and Minimum Essential Medium Alpha (α-MEM). Solubility was obtained by mass loss. Ion release was measured by using X-ray fluorescence spectroscopy (XRF). Cytotoxicity was evaluated by MTT assay. Inhibition of osteoclastogenesis was evaluated by tartrate-resistant acid phosphatase (TRAP). Data were analyzed using the t-test, ANOVA and Tukey/Dunnett's post-hoc tests ($\alpha = 0.05$). END had the highest pH in DW ($p < 0.05$), and BC, in α-MEM ($p < 0.05$). Solubility in DW was the lowest for SPBC ($p < 0.005$). The highest calcium release was observed for BC in DW at 12 h ($p < 0.05$), and in α-MEM at 12 and 24 h ($p < 0.05$). The lowest toxicity was detected for END ($p < 0.05$). BC had the highest inhibitory effect on osteoclasts ($p < 0.05$). Overall, the highest solubility and pH values were found in DW. However, the calcium silicate-based sealer showed higher solubility than the ISO standards. Calcium release was the highest for BC. END showed the highest cell viability, and BC, the highest osteoclast inhibition.

Keywords: Endodontics; Materials Testing; Osteogenesis; Root Canal Filling Materials.

Introduction

Root canal sealers may extrude through the foramen, and leachable sealer compounds may come into contact with periradicular tissues and adversely influence the healing process.[1] Teeth with periapical periodontitis may have local immunoinflammatory reactions that lead to alveolar bone resorption adjacent to the root apex and dentin.[1,2] The reciprocal coordination between osteoblasts and osteoclasts is essential to provide the repair of periapical periodontitis.[3] Particularly, the upregulation of osteoclastic activity has been associated with the control of lesion expansion.[4] In recent years, research has evaluated the effect of calcium silicate-based materials on the modulation of osteoclastogenesis.[5-8]

Calcium silicate-based sealer, a new class of endodontic sealer characterized as a bioceramic,[9,10] has been described as bioactive and osteoconductive, and as having the potential to be used as an adjuvant

in periapical remineralization, hence favoring regeneration.[11,12] Bio-C Sealer (Angelus, Londrina, Brazil) and Sealer Plus BC (MK Life, Porto Alegre, Brazil) are premixed, ready-to-use calcium silicate-based endodontic sealers, which have recently been evaluated to determine their physiochemical[13,14] and biological properties.[15-18]

New calcium silicate materials are commonly evaluated to determine porosity,[13] water resorption,[13,14] solubility,[13,14,19] pH,[14,19,20] and ability to release calcium ions,[19,20] which depends on particles of natural material and on the network structure. The combination of free calcium ions and basic pH is associated with the formation of calcium phosphate deposits, and probably facilitates the biomineralization process.[20,21] Silicon apparently acts as a favorable site for nucleation and crystallization of apatite, and involves an interesting process of periapical repair.[22] However, there is no evidence of the ions leached by Bio-C Sealer or Sealer Plus BC in solutions, or the effects of this leaching on osteoclastic differentiation, in comparison with EndoSequence BC Sealer (Brasseler, Savannah, USA), which has been the subject of the largest number of research investigations.[9,12,20,23]

Hence, the aim of this study was to evaluate the effect of the premixed EndoSequence BC Sealer, Sealer Plus BC and Bio-C Sealer calcium silicate-based endodontic sealers on bone marrow-derived monocyte-macrophages (BMMs), cytotoxicity, inhibition of osteoclastogenesis, pH, solubility, and calcium and silicon ion release. The null hypothesis was that there would be no difference among the sealers regarding all the evaluated parameters.

Methodology

Preparation of sealer discs and extracts

The following endodontic sealers were tested: EndoSequence BC Sealer (Brasseler) (END), Bio-C Sealer (Angelus) (BC), and Sealer Plus BC (MK Life) (SPBC). The sample size was calculated using the BioEstat 5.3 (Mamirauá Institute, Tefé, Brazil) statistical software program.[13,14] Six samples per group were required to detect a 90% chance of finding significant differences at the 5% level (2-sided test).

Acrylic plates with six perforations, each measuring 7.75 mm diameter x 1.5 mm thick,[24] were molded with silicone. The molds were filled with the sealers and kept in a humidified chamber at 37°C for 24 h, according to methodology proposed by Tanomaru-Filho et al.[25] for the analysis of pH, solubility and chemical compounds using X-ray fluorescence spectroscopy.

Sealer extracts were prepared according to the ISO standard (#10993–5/2009)[26]. An aliquot of 0.3 mL of each sealer was placed at the bottom of each well of a 24-well plate with an insulin syringe, and each was filled with 1.5 mL of Minimum Essential Medium Alpha (α-MEM) (LGC Biotecnologia, Cotia, Brazil), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (LGC Biotecnologia) and 1% penicillin-streptomycin (10% α-MEM) (Sigma-Aldrich, Saint Louis, USA). The plates were kept for an additional 24 h in a humidified incubator at 37°C (5% CO_2/air) before extract preparation.[27] The tested sealers were manipulated according to the respective manufacturer's directions, shown in Table 1.

Sealer pH, solubility and calcium/silicon release

The same samples were used for the three methodologies. The sealer discs were removed from the molds, kept in a desiccator, and then weighed several times on an analytical balance (Shimadzu Corporation, Kyoto, Kansai, Japan), until the initial mass was stabilized. Six discs from each sealer were placed inside plastic tubes containing 10 mL deionized water (DW), and another six in tubes containing 10 mL α-MEM. These samples were kept in a humidified incubator at 37°C for a total of 48 h. The pH values were determined after 12, 24 and 48 h of immersion in DW or α-MEM solutions, using a previously calibrated digital pH meter (MicroNal B 374, São Paulo, Brazil). The controls comprised both of these solutions, but no sealer were immersed in them. The mean pH of each sample in each experimental period was calculated for DW and α-MEM after three measurements. After each measurement, DW and α-MEM were carefully moved to new tubes for X-ray fluorescence spectroscopy measurement, and were replaced by fresh solutions. After 48 h, the discs were removed from the tubes, placed in a desiccator,

Table 1. The calcium silicate-based sealer, manufacturers, compositions and proportions used.

Sealer	Manufacturer	Composition	Proportion
END	Brasseler, Savannah, USA	Zirconium oxide, dicalcium silicate, tricalcium silicate, calcium phosphate monobasic, calcium hydroxide, filler, thickening agents	Premixed
BC	Angelus, Londrina, Brazil	Calcium silicates, calcium aluminate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide and dispersing agent	Premixed
SPBC	MK Life, Porto Alegre, Brazil	Zirconium oxide, tricalcium silicate, dicalcium silicate, calcium hydroxide and propylene glycol	Premixed

and then reweighed, as described by Torres et al.[13] The solubility of the samples stored in DW and α-MEM was obtained by calculating a percentage of the original mass at the initial and final time points.

The percentage of calcium/silicon released by the sealers in DW and α-MEM was obtained by the fluorescence spectra of the liquid samples collected at each time point, using X-ray fluorescence spectroscopy (XRF; model S8 Tiger Series 2, Bruker, Kontich, Flanders, Belgium). Trace elements can be analyzed in a concentration of parts per million (ppm). A 10 mL aliquot of liquid from each sample to be analyzed was transferred to sample cups of 40 mm (model SC-4340, PremierLab Supply, Port St. Lucie, USA), and weighed on an analytical balance for further normalization of data. Quantitative analysis of the components was performed by Quant Express for fast and reliable analysis of unknown liquid samples. The data acquisition time for each sample measurement was 420 s, and the spectra were processed using Spectra Plus software, which automatically determines the X-ray peak intensities for the elements, and quantifies their concentration.

Bone-marrow derived monocyte-macrophage (BMM) culture and exposure to the sealer extracts

Animal procedures were performed with the approval of the ethics committee on animal research at the Federal University of Uberlândia (protocol number 003/2019). Bone marrow cells (BMCs) were isolated from 6-week-old C57BL/6 mouse femurs, as previously described.[28] The BMCs were seeded in 6 well/plates containing 10% α-MEM and 30 ng/mL of recombinant murine monocyte colony stimulating factor (M-CSF) (PeproTech, London, United Kingdom). After 3 days, the BMMs were plated in 96-well plates (2×10^4 cells/well) in 10% α-MEM, and allowed to adhere overnight. The cultures were then exposed to the sealer extracts at all the dilutions (1:20, 1:100, 1:500, and 1:2500), with M-CSF at 30 ng/mL for 12 h, 24 h and 48 h. After the incubation periods, the cells were immediately tested for cytotoxicity using the MTT assay. The control group was maintained in α-MEM with M-CSF.

MTT assay

Cytotoxicity of sealer extracts in BMMs was evaluated at 12 h, 24 h and 48 h. MTT solution (Sigma-Aldrich) (5 mg/mL) was added to each well, and the cells were incubated at 37°C for 4 h. Supernatants were removed, and 100 μL of dimethyl sulfoxide (LGC Biotecnologia) was added. The optical density at 570 nm was measured using a microplate reader (Biochrom, Cambridge, EN, United Kingdom).

Differentiation of BMMs into osteoclasts (osteoclastogenesis), exposure to the sealer extracts, and tartrate-resistant acid phosphatase (TRAP) stain

BMMs were exposed simultaneously to the sealer extracts, 50 ng/mL of recombinant murine RANK Ligand (RANKL) (PeproTech), and 30 ng/mL of M-CSF (PeproTech) during a 5-day period. The extracts and reagents were replaced on day 3. The positive control group contained cells kept in 10% α-MEM stimulated with M-CSF and RANKL, and the negative control group contained cells maintained only in 10% α-MEM and M-CSF (not induced to undergo differentiation). At day 5, the cells were stained for TRAP using a commercially available staining kit (Sigma-Aldrich), according to the manufacturer's protocol. TRAP-

positive multinucleated (> 3 nuclei) cells were counted as osteoclasts, and expressed as a percentage. Images were obtained at 10x magnification using a Leica DM IRB-inverted microscope coupled to a DFC490 camera (Leica, Wetzlar, HE, Germany).

Statistical analysis

All the data were collected and tabulated on Microsoft Excel sheets (Microsoft Corporation, Redmond, USA), and then analyzed for normal distribution using the Shapiro-Wilk test ($p > 0.05$), and homoscedasticity, using the Levene test. Two-way ANOVA, Tukey's and Dunnett's tests were used to determine pH and osteoclastic differentiation (TRAP stain). Two-way ANOVA and Tukey's tests were used to evaluate ion release (XRF). Two-way and three-way ANOVA were used for viability. One-way ANOVA and Tukey's tests for multiple comparisons were used to determine the solubility rates among the sealers in each solution, and the unpaired t-test was used to compare the same sealer in different solutions. Statistical analyses were performed with GraphPad Prism software v8.2.0 (San Diego, USA), and the significance level was set at $\alpha = 0.05$.

Results

Solubility, pH and calcium/silicon release

Solubility values were significantly lower for SPBC than END and BC ($p < 0.0001$) after DW immersion. After α-MEM immersion, BC presented significantly higher values than END and SPBC ($p < 0.0001$). The results are shown in Table 2. Figure 1 shows the mean pH values for the three sealers evaluated, and respective controls in DW and α-MEM. DW analysis revealed that BC and SPBC groups were associated with pH reduction at the evaluation time points ($p < 0.0001$) (Figure 1A); in contrast, END presented high pH values at 48 h ($p < 0.0001$). Comparisons among sealers in DW showed the lowest pH values for SPBC at the three experimental periods ($p < 0.0001$). Samples stored in α-MEM showed no differences for the END and SPBC groups ($p = 0.1748$) at the evaluated time points, while the lowest pH values were found for BC at 48 h ($p < 0.0095$) (Figure 1B). Comparisons among sealers in α-MEM showed the highest pH values for BC at 12 h ($p < 0.0087$) and 24 h ($p = 0.0025$). In contrast, the highest pH values were observed for SPBC at 48 h ($p = 0.0009$) (Table 3). Overall, the values for solubility and pH after α-MEM immersion were significantly lower than after DW immersion ($p < 0.0001$).

The results for ion release are shown in Figure 2. BC leached calcium ions in DW, reaching a peak at 12 h, with an expressive reduction over time ($p = 0.0073$) (Figure 2A). On the other hand, END exhibited low leaching of calcium in the early stages of hydration, with a substantial reduction at 48 h ($p < 0.0001$) in both soaking solutions (Figure 2A,B). Overall, calcium levels were similar for BC and END, except for α-MEM at 12 h ($p < 0.0001$), in which BC presented the highest amount of this element (Figure 2B). Calcium levels were practically undetectable for SPBC in DW solution at all time points evaluated, with significantly reduced results in comparison with those for END and BC in DW at 12 h ($p < 0.0001$) and 24 h ($p < 0.0001$). In α-MEM, only BC was significantly different from SPBC at both periods ($p < 0.0001$ and $p = 0.0145$, respectively). The silicon ion release was extremely low for all sealers in both soaking solutions ($p > 0.9999$), expressed in ppm (Figure 2C,D) (Table 3).

Viability

The dilution and time-dependent effects of the extracts on cell viability for each sealer are shown in Figure 3. Two-way ANOVA demonstrated that the END group at different extract dilutions, and at different experimental time points, showed similar viability, except 1:20-1:2500 at 48 h ($p = 0.035$)

Table 2. Mean solubility values and standard deviation revealed in calcium silicate-based sealer after storage in DW and α-MEM.

Solubility (% mass loss)	Calcium silicate-based sealer		
	END	BC	SPBC
DW	6.82 (0.41) Aa	6.67 (1.21) Aa	4.56 (1.88) Ab
α-MEM	3.84 (1.04) Bb	5.38 (0.87) Ba	3.66 (0.96) Bb

Different uppercase letters in the same column represent significant differences for the same sealer after immersion in different solutions, calculated by using unpaired t-test ($p < 0.05$); different lowercase letters on the same line represent significant differences in the sealers, calculated by using One-Way ANOVA and Tukey's test ($p < 0.05$).

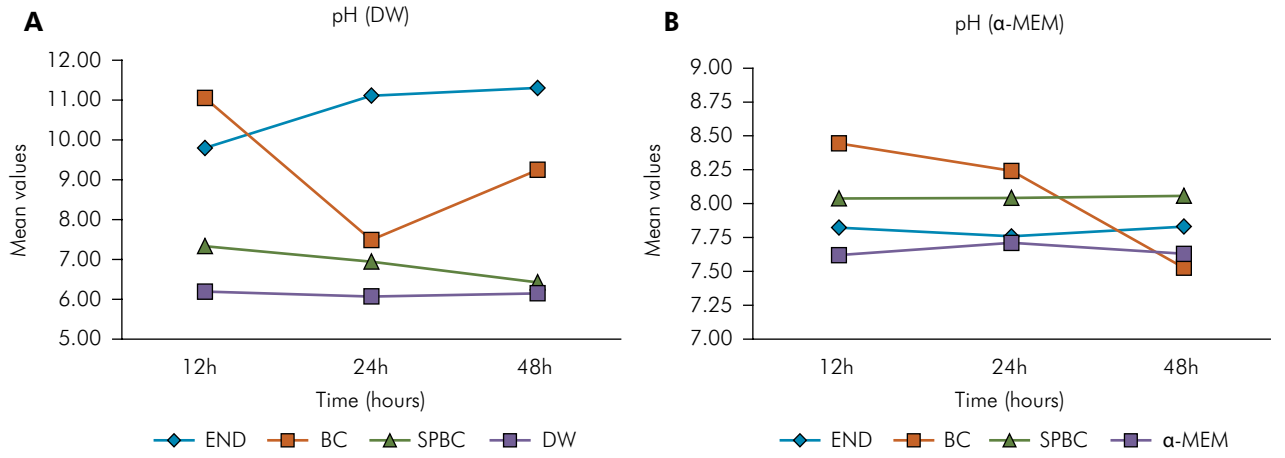


Figure 1. Graphic representation of mean pH curves for all calcium silicate-based sealers, compared with the control soaking solutions at the different experimental time periods. (A) After immersion in DW; and (B) after immersion in α-MEM.

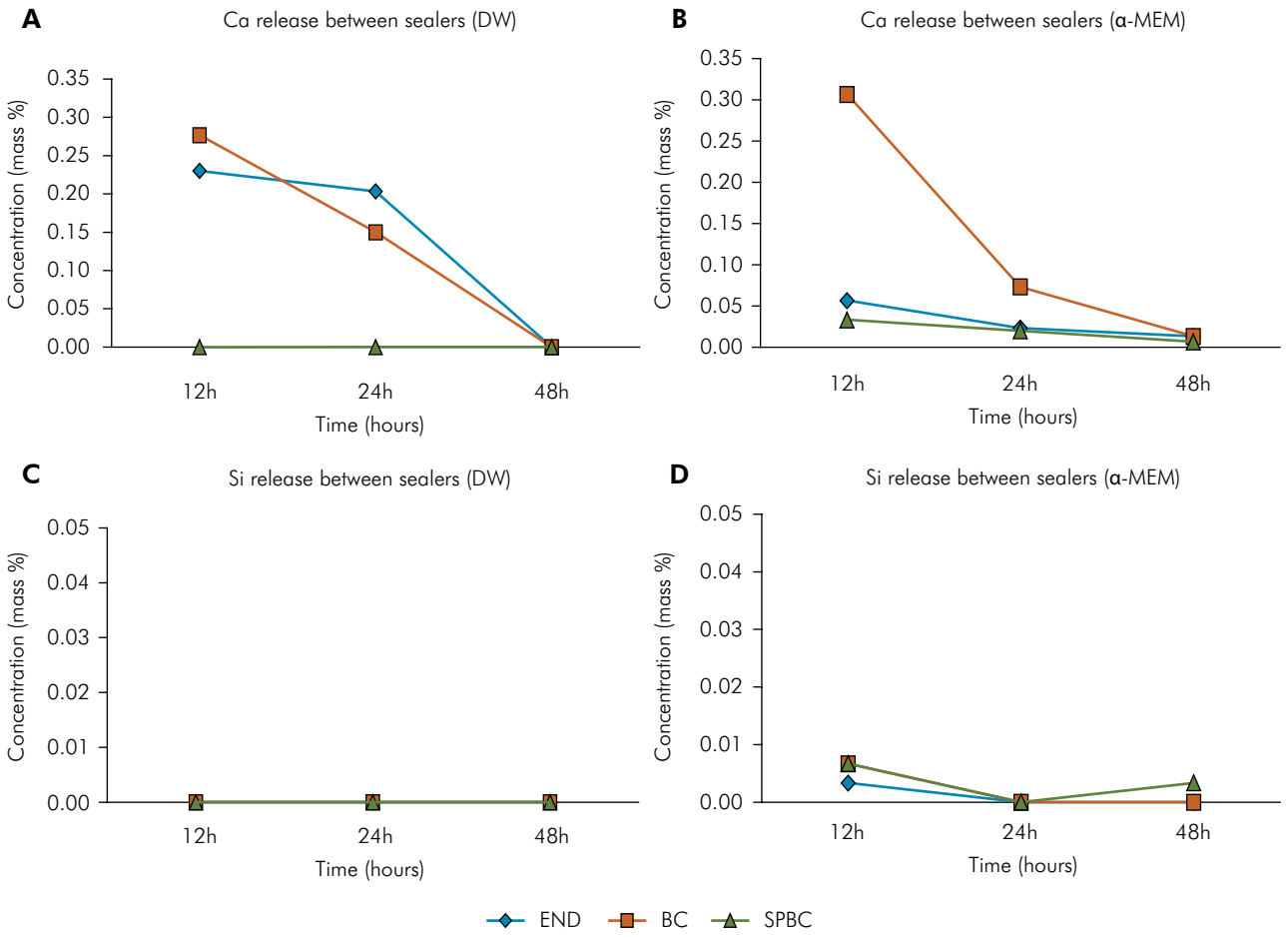


Figure 2. Concentration of ion release among the calcium silicate-based sealers for each soaking solution at the experimental time periods. (A) Calcium release after immersion in DW; (B) calcium release after immersion in α-MEM; (C) silicon release after immersion in DW; and (D) silicon release after immersion in α-MEM.

Table 3. Mean and standard deviation values for pH and calcium/silicon released (ppm) by the calcium silicate-based sealers in soaking solutions and at the different experimental time points.

Parameter	END	BC	SPBC
(DW) pH – 12 h	9.80 (0.09)	11.05 (0.08)	7.34 (0.08)
(DW) pH – 24 h	11.11 (0.37)	7.49 (0.04)	6.95 (0.20)
(DW) pH – 48 h	11.31 (0.18)	9.25 (1.01)	6.43 (0.14)
(α-MEM) pH – 12 h	7.82 (0.10)	8.44 (0.15)	8.04 (0.03)
(α-MEM) pH – 24 h	7.76 (0.16)	8.24 (0.03)	8.04 (0.27)
(α-MEM) pH – 48 h	7.83 (0.19)	7.53 (0.06)	8.06 (0.17)
(DW) Ca ion release – 12 h	0.23 (0.13)	0.28 (0.03)	0.00 (0.00)
(DW) Ca ion release – 24 h	0.20 (0.14)	0.15 (0.07)	0.00 (0.00)
(DW) Ca ion release – 48 h	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
(DW) Si ion release – 12 h	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
(DW) Si ion release – 24 h	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
(DW) Si ion release – 48 h	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
(α-MEM) Ca ion release – 12 h	0.06 (0.05)	0.31 (0.02)	0.03 (0.02)
(α-MEM) Ca ion release – 24 h	0.02 (0.01)	0.07 (0.03)	0.02 (0.01)
(α-MEM) Ca ion release – 48 h	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)
(α-MEM) Si ion release – 12 h	0.00 (0.00)	0.01 (0.01)	0.01 (0.01)
(α-MEM) Si ion release – 24 h	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
(α-MEM) Si ion release – 48 h	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)

(Figure 3A). BC (Figure 3B) and SPBC (Figure 3C) groups presented a significant reduction in the percentage of cell viability at 48 h for all dilutions; however, no significant differences were observed for the interaction of dilutions versus time ($p = 0.560$ and $p = 0.661$, respectively). A significant reduction in the cell viability was observed for all extract dilutions at the different experimental time points for END, BC and SPBC sealers, compared with the control ($p = 0.031$, $p < 0.001$, and $p < 0.001$ respectively) (Figure 3D-F). There were significant differences in the three-way ANOVA and Tukey's post-tests comparing the sealers ($p < 0.001$), dilutions ($p = 0.002$) and evaluation time points ($p < 0.001$). END showed the highest viability ($p < 0.001$). The viability percentage for SPBC was similar to that of BC ($p = 0.156$).

Effects of the sealer extracts on osteoclastic differentiation and function

The dose-dependent effects of the sealer extracts on TRAP stain are shown in Figure 4. The unstimulated cells (negative control) did not show TRAP stain, whereas cultures treated with M-CSF and RANKL (positive control) exhibited significantly higher values (Figure 4A,B). END inhibited TRAP stain in a dose-dependent manner ($p < 0.001$), except between dilutions 1:500-1:2500 ($p = 0.078$). Cultures exposed to SPBC presented similar values of TRAP-positive cells at dilutions of 1:20 and 1:100 ($p = 0.464$), with lower inhibition in more diluted extracts ($p < 0.001$). BC presented higher inhibitory effects at the extract concentration of 1:20 ($p < 0.001$), trending toward stabilization in more diluted samples.

Comparisons among the sealers in each extract dilution did not present significant differences for inhibitory effects at 1:20 dilution ($p < 0.001$). At 1:100 dilution, the highest number of TRAP-positive multinucleated cells was found in END ($p < 0.003$). Similar TRAP stain was detected in BC and SPBC ($p = 0.215$). At dilutions of 1:500 and 1:2500, there were no significant differences for SPBC-END ($p = 0.860$ and $p = 0.139$, respectively), and BC presented the highest inhibitory values ($p < 0.001$). The number of TRAP-positive multinucleated cells in the positive control was higher than in all the sealers and dilutions tested ($p < 0.001$).

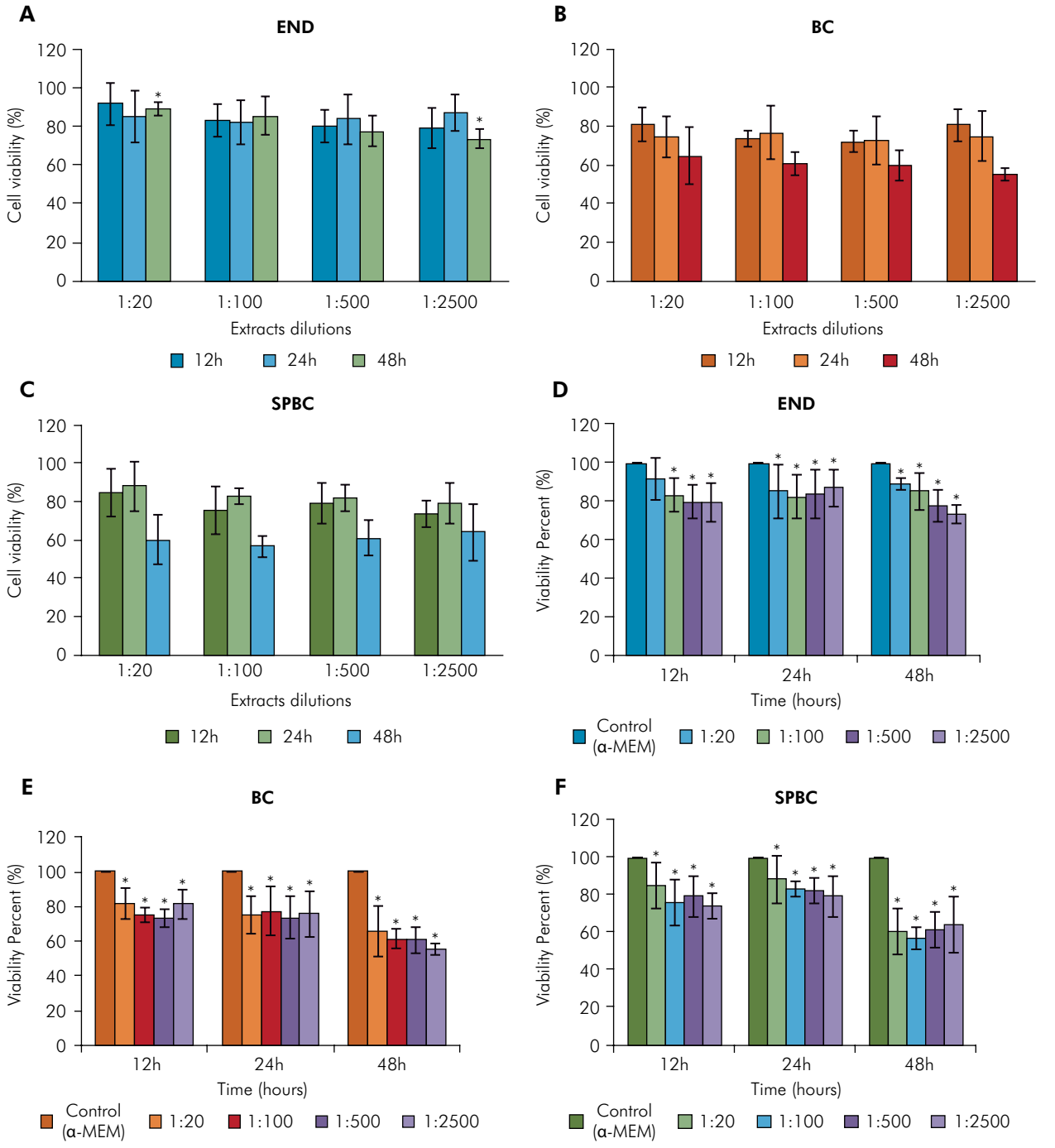
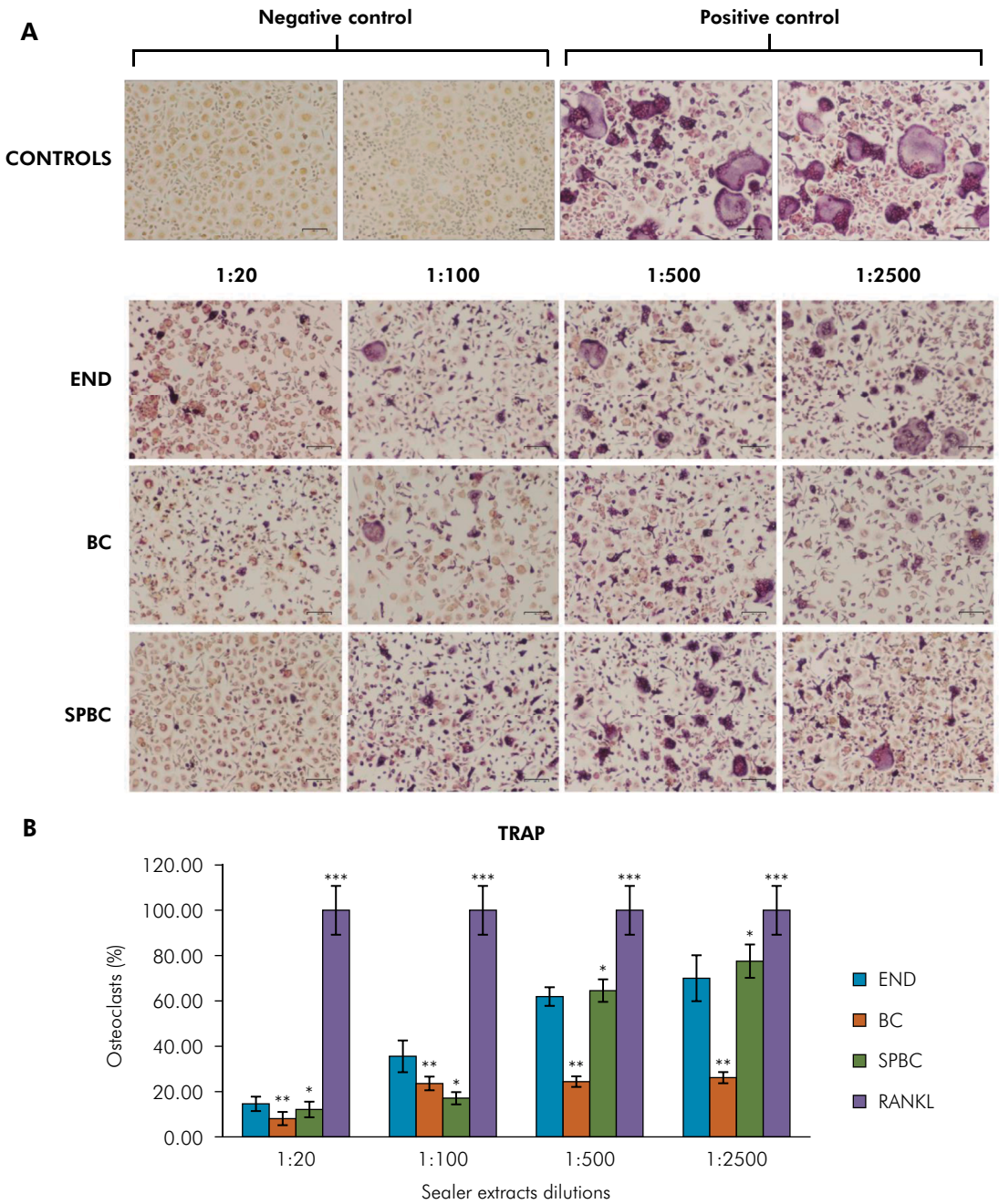


Figure 3. Viable cells (%) by MTT assay of the sealer extracts in several dilutions at different experimental time points. (A) END dilutions; (B) BC dilutions; and (C) SPBC dilutions. Time-dependent effects of (D) END; (E) BC; and (F) SPBC extracts. A 100% cell viability was considered for the control group (α-MEM). Asterisk indicates a significant difference ($p < 0.05$).

Discussion

The null hypothesis was rejected, since differences were detected among the premixed calcium silicate-based sealers regarding the physicochemical and biological parameters evaluated. Although pH, solubility and release of calcium ions promoted by END, BC and SPBC have been previously



*p < 0.05 compared each sealer at the different dilutions; **p < 0.05 compared the sealers among one another; ***p < 0.05 compared each sealer with the positive control group.

Figure 4. TRAP stain. (A) Osteoclastic differentiation by TRAP-positive multinucleated cells compared with the positive control (RANKL) and the negative control groups (α-MEM); (B)

evaluated,[13,14,19,20,23,29] the analysis periods were different. In the present study, these parameters were evaluated at periods corresponding to the cytotoxicity analysis of the BMM culture. Moreover, no studies have compared BC or SPBC with END, which has been investigated in a relevant number of publications.[9,12,20,23]

Solubility and pH were evaluated in DW – as recommended by the American National Institute/ American Dental Association and the International

Organization for Standardization tests (ANSI/ADA 57/2008; and ISO 6876/2012)[30,31] – and also in the cell culture medium, which is the condition used for cell treatment. The calcium silicate-based sealer in contact with the soaking solutions tested presented different solubility patterns. This may be attributed to differences in the sealer composition,[9-11] characteristics of the soaking solution,[13] and the methodology used for analysis.[14,25] Although the sealers have some similar components, such as calcium silicate and zirconium oxide, the component proportions are variable, as are the dispersing agents of each sealer.[13,14,19] END is a calcium phosphate silicate–based sealer,[10,29] whereas BC and SPBC are a combination of calcium silicates.[14,15] In addition, different environments have been shown to affect the material chemistry, and also change other material properties.[32] The fluid used for immersion may interact with the sealer, thus influencing the results obtained in the solubility tests, as previously reported in studies using phosphate buffered saline (PBS),[13] and Dulbecco's Modified Eagle Medium (DMEM).[32]

It has been argued that the immersion of endodontic sealers in simulated body fluids (SBF) better mimics clinical conditions.[9,13,21,32,33] Previous studies in agreement with the present results have demonstrated reduced solubility of calcium silicate-based materials when immersed in SBF[18,21] or PBS.[13,33] It has been speculated that calcium ions under immersion combine with phosphate to promote the formation of superficial hydroxyapatite,[34] which may counteract the solubility.[33] In general, the mean solubility values of the present study were slightly divergent from those of the current literature for the three sealers, owing to differences in the size of the samples, setting period, immersion solutions, and the evaluation time points.[12,13,19,29] Nevertheless, the present results confirm that BC has higher solubility than SPBC, despite the storage solution.[13] As previously reported, the solubility of calcium the silicate-based sealers remained above the minimum level recommended by ISO 6876.[10,13,14,19,31,32] This kind of solubility test shows limitations when it is used to evaluate biomaterials that present water absorption.[11,13,25] Calcium silicate–based sealers may absorb water, because of their hydrophilic particles,[13,14,19] and require moisture to set.[9-11] In this sense, the results obtained using conventional solubility tests could have been overestimated, since the water not incorporated during hydration of the samples in the dissector may have evaporated.[14] Great dryness may have occurred, thus interfering in the result observed for the real mass loss. Therefore, the association of methodologies like micro-CT imaging may complement the evaluation of calcium silicate–based sealer solubility,[13,14,25] and allow better insight into the behavior of these materials.[25]

Solubility has been straightforwardly related to ion release and pH values,[9,12,32] especially calcium ions, which may promote an alkaline pH.[12] SPBC stored in DW showed the lowest pH values in the three experimental periods. According to Mendes et al.,[19] the alkalizing effect of SPBC remains stable for 7 days, ranging from 9.09 to 10.05. In the current study, SPBC maintained a constant pH over time, but only when stored in α-MEM. The exact reason why the culture medium and the DW results showed different pH patterns cannot be explained, but it is clear that the type of soaking medium affects the material properties.[32]

Calcium ion release has often been evaluated in analyzing tri-calcium silicate-based materials,[9,12,32,34] because of its relationship with biomineralization and repair. In the present study, the XRF methodology allowed simultaneous multi-element analysis, although only calcium and silicon levels were subjected to quantitative analysis, because of their presence in END, BC, and SPBC.[17,19,20] In contrast to Mendes et al.,[19] the calcium levels for SPBC in DW in our study were almost undetectable, and did not increase over time, seemingly related to low calcium solubility.[13] On the other hand, the highest calcium levels in BC are probably related to the high solubility of this material, as previously described.[13,14] In general, the leaching of calcium in DW was higher than in α-MEM, most likely related to material solubility and the soaking solution.[32] The silicon levels (in ppm) were close to zero in DW, and slightly higher in the culture medium than in DW, but this result is different from that found in the literature,[17] reportedly due to the method of analysis. It is important to emphasize that other

methodologies have been used to determine the pH and calcium release levels, particularly by filling polyethylene tubes with fresh sealers and then soaking the sealers immediately in DW.[19] The setting of sealer discs before soaking in DW or α-MEM may have influenced the amount of calcium and silicon released, as well as the pH values.

The components leached by the endodontic sealers may have influenced not only the other physicochemical parameters evaluated, but also cell behavior.[27,35] It was postulated that the components eluted by fresh sealers on extracts could affect BMM viability and osteoclastic differentiation. In fact, sealer extracts caused a dose-dependent reduction in the cell viability, except for END. The lower toxicity of END confirms the data obtained from the two systematic reviews,[11,12] even though this is a parameter determined by the type of cell evaluated.[35] BC and SPBC showed similar viability, close to 80% within the first 24 h of contact; nevertheless, this did not classify them as initially cytotoxic. The biocompatibility of BC and SPBC[15,16] and their cytotoxicity in distinct cell models[16-18] were previously evaluated, and showed that both were biocompatible, and that cytotoxicity was reduced in more diluted extracts. The increased cytotoxicity levels in BMMs at 48 h probably reflect the cumulative effect of toxic compounds on BC and SPBC.

Regarding osteoclastic differentiation, the inhibitory effect of BC on RANKL-induced BMMs was evident when compared to that of the other sealers. RANKL-stimulated BMMs are often used for investigation into osteoclastogenesis.[7,28] Bias caused by the differences in the number of viable cells was avoided by counting the same number of cells per field. The suppression of osteoclastic differentiation obtained by using the TRAP stain, seems to be an interesting property of BC, compared with the other sealers. Although molecular analyses are often used to confirm osteoclastic differentiation,[5,6,27,35] assessments of TRAP-positive cells is an appropriate method for preliminary studies. An increased pH in the inflamed periapical region[33] may neutralize the acid environment promoted by osteoclasts, or reduce its differentiation,[12,36] thus contributing to hard tissue deposition in resorbed areas.

Despite the several methodologies used in the current research, the fact that it was completely performed *in vitro* is a limitation, since it does not reflect the real conditions observed *in vivo*. However, the parameters chosen for analysis are of paramount importance, since the solubility of endodontic sealers may influence treatment success, and compromise the sealing of the root canal.[13] Furthermore, parameters such as pH and calcium ion release are relevant when evaluating sealers, whose main advantage is their ability to induce the mineralization process.[11,12] Specifically in relation to clastic inhibition, materials with this ability may be extremely useful in cases of root resorption resulting from trauma or an infectious process.[7] On the other hand, professionals should be aware of their high solubility, which is higher than those recommended by current regulations.

Taken together, the present results indicate that the material properties and the composition were affected by the soaking medium, probably influenced by the testing methodology used. BC and SPBC showed reduced cytotoxicity in higher dilutions, while the cytotoxicity of END not only was reduced, but also remained constant over time. Although the present methodology precludes knowing the mechanisms of osteoclastic inhibition, BC seems to be superior and SPBC, inferior to the other sealers.

Conclusions

The biological and physicochemical properties of the calcium silicate-based sealers evaluated in this *in vitro* study were affected by the soaking solutions. All the sealers demonstrated higher solubility than the ISO standards. The calcium level was higher in BC, while the silicon ions were extremely low in all the sealers. END showed the highest cell viability, and BC, the highest inhibitory effects of osteoclastogenesis; both END and BC can be considered more favorable for periapical tissue repair.

Acknowledgments

The authors are grateful to FAPEMIG, CNPq, and CAPES for their financial support (Funding Code 001), and also to Isadora Akemi Uehara and Rafael

Ramos Heilbuth for their technical support. This study was carried out at the CPBio (Biomechanics, Biomaterials and Cell Biology Research Center of the Federal University of Uberlândia - UFU, Minas Gerais, Brazil), at the Institute of Biomedical Sciences (UFU), and at the School of Chemical Engineering at UFU.

References

1. Holland R, Gomes JE, Cintra LT, Queiroz iO, Estrela C. Factors affecting the periapical healing process of endodontically treated teeth. J Appl Oral Sci. 2017 Sep-Oct;25(5):465-76. https://doi.org/10.1590/1678-7757-2016-0464
2. Ricucci D, Rôças IN, Alves FR, Loghin S, Siqueira Junior JF. Apically extruded sealers: fate and influence on treatment outcome. J Endod. 2016 Feb;42(2):243-9. https://doi.org/10.1016/j.joen.2015.11.020
3. Braz-Silva PH, Bergamini ML, Mardegan AP, De Rosa CS, Hasseus B, Jonasson P. Inflammatory profile of chronic apical periodontitis: a literature review. Acta Odontol Scand. 2019 Apr;77(3):173-80. https://doi.org/10.1080/00016357.2018.1521005
4. Ricucci D, Siqueira Junior JF, Loghin S, Lin LM. Repair of extensive apical root resorption associated with apical periodontitis: radiographic and histologic observations after 25 years. J Endod. 2014 Aug;40(8):1268-74. https://doi.org/10.1016/j.joen.2014.01.008
5. Cheng X, Zhu L, Zhang J, Yu J, Liu S, Lv F, et al. Anti-osteoclastogenesis of Mineral Trioxide Aggregate through Inhibition of the Autophagic Pathway. J Endod. 2017 May;43(5):766-73. https://doi.org/10.1016/j.joen.2016.12.013
6. Tian J, Qi W, Zhang Y, Glogauer M, Wang Y, Lai Z, et al. Bioaggregate Inhibits Osteoclast Differentiation, Fusion, and Bone Resorption In Vitro. J Endod. 2015 Sep;41(9):1500-6. https://doi.org/10.1016/j.joen.2015.05.018
7. Zhang J, Zhu L, Peng B. Effect of BioAggregate on osteoclast differentiation and inflammatory bone resorption in vivo. Int Endod J. 2015 Nov;48(11):1077-85. https://doi.org/10.1111/iej.12405
8. Zhang J, Zhu L, Yan P, Peng B. Effect of bioaggregate on receptor activator of nuclear factor-Kappa B ligand-induced osteoclastogenesis from murine macrophage cell line in vitro. J Endod. 2015 Aug;41(8):1265-71. https://doi.org/10.1016/j.joen.2015.03.021
9. Donnermeyer D, Bürklein S, Dammaschke T, Schäfer E. Endodontic sealers based on calcium silicates: a systematic review. Odontology. 2019 Oct;107(4):421-36. https://doi.org/10.1007/s10266-018-0400-3
10. Jafari F, Jafari S. Composition and physicochemical properties of calcium silicate based sealers: A review article. J Clin Exp Dent. 2017 Oct;9(10):e1249-55. https://doi.org/10.4317/jced.54103
11. Lim M, Jung C, Shin DH, Cho YB, Song M. Calcium silicate-based root canal sealers: a literature review. Restor Dent Endod. 2020 Jun;45(3):e35. https://doi.org/10.5395/rde.2020.45.e35
12. Almeida LHS, Moraes RR, Morgental RD, Pappen FG. Are premixed calcium silicate-based endodontic sealers comparable to conventional materials? A systematic review of in vitro studies. J Endod. 2017 Apr;43(4):527-35. https://doi.org/10.1016/j.joen.2016.11.019
13. Torres FF, Zordan-Bronzel CL, Guerreiro-Tanomaru JM, Chávez-Andrade GM, Pinto JC, Tanomaru-Filho M. Effect of immersion in distilled water or phosphate-buffered saline on the solubility, volumetric change and presence of voids within new calcium silicate-based root canal sealers. Int Endod J. 2020 Mar;53(3):385-91. https://doi.org/10.1111/iej.13225
14. Zordan-Bronzel CL, Esteves Torres FF, Tanomaru-Filho M, Chávez-Andrade GM, Bosso-Martelo R, Guerreiro-Tanomaru JM. Evaluation of physicochemical properties of a new calcium silicate-based sealer, Bio-C Sealer. J Endod. 2019 Oct;45(10):1248-52. https://doi.org/10.1016/j.joen.2019.07.006
15. Alves Silva EC, Tanomaru-Filho M, Silva GF, Delfino MM, Cerri PS, Guerreiro-Tanomaru JM. Biocompatibility and bioactive potential of new calcium silicate-based endodontic sealers: bio-c sealer and sealer plus BC. J Endod. 2020 Oct;46(10):1470-7. https://doi.org/10.1016/j.joen.2020.07.011
16. Benetti F, Queiroz IOA, Oliveira PH, Conti LC, Azuma MM, Oliveira SH, et al. Cytotoxicity and biocompatibility of a new bioceramic endodontic sealer containing calcium hydroxide. Braz Oral Res. 2019;33:e042. https://doi.org/10.1590/1807-3107bor-2019.vol33.0042
17. López-García S, Lozano A, García-Bernal D, Forner L, Llena C, Guerrero-Gironés J, et al. Biological Effects of New Hydraulic Materials on Human Periodontal Ligament Stem Cells. J Clin Med. 2019 Aug;8(8):E1216. https://doi.org/10.3390/jcm8081216
18. López-García S, Pecci-Lloret MR, Guerrero-Gironés J, Pecci-Lloret MP, Lozano A, Llena C, et al. Comparative Cytocompatibility and Mineralization Potential of Bio-C Sealer and TotalFill BC Sealer. Materials (Basel). 2019 Sep;12(19):E3087. https://doi.org/10.3390/ma12193087
19. Mendes AT, Silva PB, Só BB, Hashizume LN, Vivan RR, Rosa RA, et al. Evaluation of Physicochemical Properties of New Calcium Silicate-Based Sealer. Braz Dent J. 2018 Nov-Dec;29(6):536-40. https://doi.org/10.1590/0103-6440201802088

20. Candeiro GT, Correia FC, Duarte MA, Ribeiro-Siqueira DC, Gavini G. Evaluation of radiopacity, pH, release of calcium ions, and flow of a bioceramic root canal sealer. J Endod. 2012 Jun;38(6):842-5. https://doi.org/10.1016/j.joen.2012.02.029
21. Carmo SS, Néspoli FF, Bachmann L, Miranda CE, Castro-Raucci LM, Oliveira IR, et al. Influence of early mineral deposits of silicate- and aluminate-based cements on push-out bond strength to root dentine. Int Endod J. 2018 Jan;51(1):92-101. https://doi.org/10.1111/iej.12791
22. Zhao W, Wang J, Zhai W, Wang Z, Chang J. The self-setting properties and in vitro bioactivity of tricalcium silicate. Biomaterials. 2005 Nov;26(31):6113-21. https://doi.org/10.1016/j.biomaterials.2005.04.025
23. Rodríguez-Lozano FJ, López-García S, García-Bernal D, Tomás-Catalá CJ, Santos JM, Llena C, et al. Chemical composition and bioactivity potential of the new Endosequence BC Sealer formulation HiFlow. Int Endod J. 2020 Sep;53(9):1216-28. https://doi.org/10.1111/iej.13327
24. Carvalho-Junior JR, Correr-Sobrinho L, Correr AB, Sinhoreti MA, Consani S, Sousa-Neto MD. Solubility and dimensional change after setting of root canal sealers: a proposal for smaller dimensions of test samples. J Endod. 2007 Sep;33(9):1110-6. https://doi.org/10.1016/j.joen.2007.06.004
25. Tanomaru-Filho M, Torres FF, Chávez-Andrade GM, Almeida M, Navarro LG, Steier L, et al. Physicochemical properties and volumetric change of silicone/bioactive glass and calcium silicate-based endodontic sealers. J Endod. 2017 Dec;43(12):2097-101. https://doi.org/10.1016/j.joen.2017.07.005
26. International Standards Organization. ISO 10993-52009. Biological evaluation of medical devices. Part 5: Tests for in vitro cytotoxicity. London: British Standards Institution; 2009.
27. Rodrigues C, Costa-Rodrigues J, Capelas JA, Fernandes MH. Behaviour of co-cultured human osteoclastic and osteoblastic cells exposed to endodontic sealers' extracts. Clin Oral Investig. 2014;18(2):479-88. https://doi.org/10.1007/s00784-013-0983-6
28. Soares MP, Silva DP, Uehara IA, Ramos Junior ES, Alabarse PV, Fukada SY, et al. The use of apocynin inhibits osteoclastogenesis. Cell Biol Int. 2019 May;43(5):466-75. https://doi.org/10.1002/cbin.11110
29. Zhou HM, Shen Y, Zheng W, Li L, Zheng YF, Haapasalo M. Physical properties of 5 root canal sealers. J Endod. 2013 Oct;39(10):1281-6. https://doi.org/10.1016/j.joen.2013.06.012
30. American Dental AssociationANSI/ADA Standard No. 57: Endodontic sealing material: 2000 (reaffirmed 2012) [cited 2017 Feb 4]. Avaiable from: http://www.ada.org/en/science-research/dental-standards/dental-products/products-standards-technical-specificationsand-technical-reports
31. International Standards Organization. ISO 68762012. Dentistry — Root canal sealing materials. London: British Standards Institution; 2012.
32. Kebudi Benezra M, Schembri Wismayer P, Camilleri J. Influence of environment on testing of hydraulic sealers. Sci Rep. 2017 Dec;7(1):17927. https://doi.org/10.1038/s41598-017-17280-7
33. Urban K, Neuhaus J, Donnermeyer D, Schäfer E, Dammaschke T. Solubility and pH value of 3 different root canal sealers: a long-term investigation. J Endod. 2018 Nov;44(11):1736-40. https://doi.org/10.1016/j.joen.2018.07.026
34. Abdalla MM, Lung CY, Neelakantan P, Matinlinna JP. A novel, doped calcium silicate bioceramic synthesized by sol-gel method: investigation of setting time and biological properties. J Biomed Mater Res B Appl Biomater. 2020 Jan;108(1):56-66. https://doi.org/10.1002/jbm.b.34365
35. Rodrigues C, Costa-Rodrigues J, Capelas JA, Fernandes MH. Long-term dose- and time-dependent effects of endodontic sealers in human in vitro osteoclastogenesis. J Endod. 2013 Jun;39(6):833-8. https://doi.org/10.1016/j.joen.2012.11.001
36. Lee JK, Kwak SW, Ha JH, Lee W, Kim HC. Physicochemical properties of epoxy resin-based and bioceramic-based root canal sealers. Bioinorg Chem Appl. 2017;2017:2582849. https://doi.org/10.1155/2017/2582849