UNESP - Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

Marcela Borsatto Queiroz

Physicochemical and biological properties of tricalcium silicate-based reparative materials with alternative radiopacifiers and Biosilicate

Características físico-químicas e biológicas de materiais reparadores à base de silicato tricálcico associados à radiopacificadores alternativos e Biosilicato

Araraquara

2018

UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

Marcela Borsatto Queiroz

Physicochemical and biological properties of tricalcium silicate-based reparative materials with alternative radiopacifiers and Biosilicate

Características físico-químicas e biológicas de materiais reparadores à base de silicato tricálcico associados à radiopacificadores alternativos e Biosilicato

Dissertação apresentado ao programa de Pós-Graduação em Odontologia Área de Endodontia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista para Obtenção do Título de Mestre em Endodontia.

Orientador: Prof. Dr. Mário Tanomaru Filho

Araraquara

2018

Marcela Borsatto Queiroz

Physicochemical and biological properties of tricalcium silicate-based reparative materials with alternative radiopacifiers and Biosilicate

Características físico-químicas e biológicas de materiais reparadores à base de silicato tricálcico associados à radiopacificadores alternativos e biosilicato

Comissão julgadora

Dissertação para obtenção do grau de Mestre

Presidente e orientador: Prof. Dr. Mário Tanomaru Filho

2º Examinador: Profa. Dra. Josette Camilleri

3º Examinador: Profa. Dra. Roberta Bosso Martelo

Araraquara, 16 de março de 2018

Queiroz, Marcela Borsatto
Physicochemical and biological properties of tricalcium silicate-based reparative materials with alternative radiopacifiers and Biosilicate / Marcela Borsatto Queiroz. -- Araraquara: [s.n.], 2018
75 f. ; 30 cm.

Dissertação (Mestrado em Odontologia) – Universidade Estadual Paulista, Faculdade de Odontologia
Orientador: Prof. Dr. Mario Tanomaru Filho

1. Calcium silicate 2. Physical properties 3. Chemical properties 4. Cell culture techniques I. Título

CURRICULAR DATA

Marcela Borsatto Queiroz

Date of birth: July 31, 1990 - Araraquara – SP

Parents: Rudney da Conceição Queiroz

Elaine Maria Borsatto Queiroz

2010-2013 Graduated in Dentistry from the Sacred Heart University – (Universidade do Sagrado Coração – USC) - Bauru, S.P. Brazil.

2014 Course in Oral Surgery at São Paulo Dental Surgeons' association - "Associação Paulista dos Cirurgiões Dentistas - APCD", Araraquara

2014-2016 Specialization in Endodontics at the Hospital for rehabilitation of craniofacial anomalies - "Hospital de Reabilitação de Anomalias Craniofaciais - HRAC" - University of São Paulo - (USP), Bauru, SP, Brazil.

2016-2018 Master's Program in Dentistry, area of Endodontics, at Araraquara Dental School - UNESP, SP, Brazil.

Dedico esse trabalho primeiramente aos meus pais (Rudney e Elaine), por todo amor, esforço e apoio. Ao meu namorado por toda paciência e amor em todos esses 12 anos ao meu lado. Aos meus avós: Aurora, José (*in memoria*), Hilda (*in memoria*) e Fernando (*in memoria*). As minhas amigas queridas (Jéssica, Pitty, Flávia e Luiza), que mesmo com a distância sempre estão em meus pensamentos. E por fim, dedico também a professora Lidiane, uma amiga muito querida e que sempre acredito no meu potencial, me incentivando para que eu pudesse chegar aqui hoje. E a meu grande amigo Tulio por todo conhecimento, conversas e momentos compartilhados.

AGRADECIMENTOS

Agradeço primeiramente à Faculdade de Odontologia de Araraquara-UNESP, onde eu tive a oportunidade de realizar meu trabalho.

Agradeço principalmente e especialmente ao meu orientador Prof. Dr. Mario Tanomaru Filho, pelos ensinamentos em Endodontia, pela oportunidade a pós-graduação, por todo apoio, paciência e compreensão comigo.

Agradeço a Prof. Dra. Juliane M. Guerreiro Tanomaru, por todo conhecimento transmitido e paciência comigo. Agradeço a todos os professores do departamento de Endodontia da FOAr-UNESP.

Ao Prof. Dr. Renato T. Leonardo pela oportunidade de conhecer o programa de pós-graduação da faculdade.

Agradeço imensamente as alunas da pós-graduação: Fernanda, Kênnia, Gissele e Elisandra por todas as correções, sugestões, ajuda, conhecimentos compartilhados e paciência que tiveram comigo. Agradeço também a professora da Universidade Federal da Bahia, Dra. Roberta Bosso Martelo por toda ajuda, considerações, correções, apoio, sugestões e paciência que teve comigo.

Agradeço à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) protocolo número 2016/11036-4, pelo apoio a minha pesquisa, financiamento e pela oportunidade de bolsa.

Agradeço também aos meus professores de Endodontia da graduação: Prof. Dr Rodrigo R. Vivan, Prof. Dr. José Carlos Yamashita, Prof. Dr. Silvio Fraga e a Profa. Dra. Daniela Colaço. Aos meus grandes mestres na especialização de endodontia: Profa. Dra. Lidiane de Castro Pinto, ao Dr. Tulio L. Olano Dextre, ao MSc. J. F. Mateo Castilho, ao Prof. Dr. Celso Kenji Nishiyama e a Profa. Dra. Cláudia. Sem vocês eu não teria todo esse amor pela endodontia e conhecimento que vocês puderam compartilhar.

Agradeço por fim, aos meus pais por todo esforço e apoio para que eu estivesse aqui hoje. E também ao meu namorado por todo amor, carinho e compreensão comigo.

Queiroz MB. Physicochemical and biological properties of tricalcium silicate-based cements associated with alternative radiopacifiers and Biosilicate [Dissertação de Mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2018.

ABSTRACT

Tricalcium silicate cements associated with radiopacifiers are used as repair materials. **Publication 1:** Evaluation of tricalcium silicate-based cements (TCS) associated with zirconium oxide (ZrO_2), calcium tungstate ($CaWO_4$) or niobium oxide (Nb_2O_5) radiopacifiers compared to MTA Repair HP (MTA HP). **Publication 2:** Evaluation of tricalcium silicate-based cements (TCS) associated with zirconium oxide (ZrO_2) radiopacifier with 10% or 20% of Biosilicate (TCS ZrO_2 + 10% Biosilicate and TCS ZrO_2 + 20% Biosilicate) compared to Biodentine. Setting Time (ST) and radiopacity were evaluated based on ISO 6876/2002 standard. Solubility was evaluated according to the method proposed by Carvalho-Júnior *et al.* (2007) modified. pH was measured at 3, 12 and 24 hours and 7, 14 and 21 days after immersion in distilled water. Cellular cytotoxicity and bioactivity were evaluated by methyltetrazolium (MTT), neutral red (NR), alkaline phosphatase (ALP), alizarin red (ARS) and real time PCR (qPCR) (Publication 1) assays in different periods of contact with eluates of the materials in Saos-2 cells. Antibacterial activity was evaluated by direct contact on *Enterococcus faecalis* in the planktonic form. For the physico-chemical and ARS tests, the data were submitted to ANOVA and Tukey tests; for MTT, NR and ALP tests the data were analyzed by the Two-Way ANOVA and Bonferroni tests; the antibacterial activity, were submitted to Kruskall-Wallis and Dunn tests ($\alpha = 0.05$). **Publication 1:** TCS + $CaWO_4$ presented the highest setting time and MTA HP the lowest ($p<0.05$). Except for TCS, all materials presented radiopacity above 3 mm Al. The materials presented solubility in accordance with ISO 6876/2002. The cements evaluated presented alkaline pH values in all periods. The materials were cytocompatible at the dilution of 1:8. The highest ALP activity occurred in 14 days for all the cements, especially TCS, TCS + ZrO_2 e TCS + $CaWO_4$ when compared with the negative control (NC). TCS + Nb_2O_5 presented higher formation of mineralization nodules in comparison with the NC ($p<0.05$). After 7 days, there was no statistically significant difference ($p>0.05$) in mRNA expression for ALP, when compared to NC. However, after 14 days there was na overexpressed ALP mRNA, especially TCS + Nb_2O_5 ($p<0.05$), in relation to the CN. All the materials TCS presented antimicrobial action against *E. faecalis*. **Publication 2**: TCS presented the highest setting time ($p<0.05$) and the other materials presented no statistical difference ($p<0.05$). TCS ZrO_2 + 10% Biosilicate and TCS ZrO_2 + 20% Biosilicate showed radiopacity and solubility in accordance with ISO 6876/2002. All materials presented alkaline pH in the different periods. In the MTT and NR assays, the materials presented no cytotoxic effects, except for Biodentine that presented lower cell viability compared with the NC at the lower dilutions (1:1 and 1:2). The highest ALP activity was observed in the period of 14 days, with emphasis on TCS cements and Biodentine. All the materials TCS and Biosilicate presented antimicrobial action against *E. faecalis*. It could be concluded that tricalcium silicate-based cement associated with different radiopacifiers presented proper physicochemical properties, bioactive potential and was non-cytotoxic in Saos-2, suggesting a potential use as a repair material (Publication 1). Biosilicate in two proportions, associated with TCS and ZrO_2, presented proper physicochemical properties and no cytotoxic effect. Therefore, this material showed perspectives for clinical application (Publication 2).

Key-words: Calcium silicate. Physical properties. Chemical properties. Cell culture techniques.

Queiroz MB. Características físico-químicas e biológicas de materiais reparadores à base de silicato tricálcico associados à radiopacificadores alternativos e Biosilicato [Dissertação de Mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2018.

RESUMO

Cimentos de silicato tricálcico com radiopacificadores são utilizados como materiais reparadores. **Publicação 1:** Avaliação de cimento à base de silicato tricálcico (STC) associado aos radiopacificadores óxido de zircônio (ZrO_2), tungstato de cálcio ($CaWO_4$) ou óxido de nióbio (Nb_2O_5) em comparação ao MTA Repair HP (MTA HP). **Publicação 2:** Avaliação de material à base de silicato tricálcico (STC) e radiopacificador óxido de zircônio (ZrO_2) e 10% ou 20% de Biosilicato (STC ZrO_2 + 10% de Biosilicato e STC ZrO_2 + 20% de Biosilicato) em comparação ao Biodentine. Tempo de presa e a radiopacidade foram avaliados seguindo ISO 6876/2002. A solubilidade foi avaliada de acordo com o método proposto por Carvalho-Júnior *et al.* (2007) modificado. pH foi avaliado 3, 12 e 24 horas, 7, 14 e 21 dias após imersão em água destilada. A citotoxidade e bioatividade celular foram avaliadas pelos testes metiltetrazólio (MTT), vermelho neutro (VN), atividade de fosfatase alcalina (ALP), ensaio de vermelho de alizarina (ARS) e PCR em tempo real (qPCR) (Publicação1), em diferentes períodos de contato com eluídos dos materiais em células Saos-2. Atividade antimicrobiana dos materiais foi avaliada por meio do teste de contato direto com *Enterococcus faecalis* na forma planctônica. Para os testes físico-químicos e ARS, os dados foram submetidos aos testes ANOVA e Tukey; para os ensaios do MTT, VN e ALP e qPCR os dados foram analisados aos testes Two Way ANOVA e Bonferroni; os dados da atividade antimicrobiana foram submetidos aos testes Kruskall-Wallis e Dunn. (α=0.05). **Publicação 1:** STC + $CaWO_4$ apresentou o maior tempo de presa e MTA HP o menor ($p<0.05$). Com exceção do STC, todos os materiais apresentaram radiopacidade acima de 3 mm Al. Os materiais apresentaram solubilidade de acordo com a ISO 6876/2002. Os cimentos avaliados apresentaram valores de pH alcalino em todos os períodos. Os materiais foram citocompatíveis na diluição 1:8. A maior atividade de ALP ocorreu em 14 dias para todos os cimentos, com destaque para STC, STC + ZrO_2 e STC + $CaWO_4$ quando comparados ao controle negativo (CN) ($p<0.05$). Após 7 dias, não houve diferença estatística ($p>0.05$) na expressão gênica (mRNA) para ALP, quando comparados ao CN. Entretanto, após 14 dias houve um aumento do transcrito ALP, especialmente STC + Nb_2O_5 ($p<0.05$), em relação ao CN. Todos os materiais de STC apresentaram ação antimicrobiana contra *E faecalis***. Publicação 2**: STC apresentou o maior valor de tempo de presa ($p<0.05$) e os demais materiais não apresentaram diferença estatística ($p>0.05$). STC ZrO_2 + 10% de Biosilicato e STC ZrO_2 + 20% de Biosilicato apresentaram valores de radiopacidade e solubilidade de acordo com a norma ISO 6876/2002. Todos os materiais, nos diferentes períodos apresentaram valores de pH alcalino. Nos ensaios MTT e VN os materiais não apresentaram efeitos citotóxicos, com exceção do Biodentine que apresentou menor viabilidade celular que o CN nas menores diluições (1:1 e 1:2). A maior atividade de ALP foi observada no período de 14 dias, com destaque para os cimentos STC e Biodentine. Todos os materiais de STC e Biosilicato apresentaram ação antimicrobiana contra *E faecalis***.** Conclui-se que cimento de silicato tricálcico associado aos diferentes radiopacificadores apresentam propriedades físico-químicas adequadas, potencial bioativo e citocompatibilidade que sugere potencial para uso como material reparador (Publicação 1). O Biosilicato nas duas proporções, associado ao STC e ZrO_2,

apresentou adequadas propriedades físico-químicas e não demonstrou efeitos citotóxicos. Portanto, este material mostrou perspectivas para aplicação clínica (Publicação 2).

Palavras-chave: Calcarea silicata. Propriedades físicas. Propriedades químicas. Técnicas de cultura de célula.

SUMMARY

1 INTRODUCTION

Mineral Trioxide Aggregate (MTA) is a hydraulic calcium silicate-based material[1] developed for sealing communication between the pulp cavity and external tooth surface[2]. MTA is biocompatible and has sealing ability and bioactivity[3]. MTA is composed of 53.1% tricalcium silicate, 22.5% dicalcium silicate, 21.6% bismuth oxide (Bi_2O_3) and traces of calcium sulphate[4-6].

Tricalcium silicate-based cements have been proposed as alternative materials to MTA[7,8], due the tricalcium silicate phase that is responsible for the bioactive potential of this material. Tricalcium silicate hydration after reaction with water forms hydrated hydraulic calcium silicate and calcium hydroxide[9].

The presence of bismuth oxide Bi_2O_3 as radiopacifier in MTA reduces the release of calcium hydroxide, increases the solubility and harms the dimensional stability of the material[10]. Moreover, this radiopacifier increases the porosity of Portland Cement (PC)[11], and diminishes its compressive strength[11,5]. The presence of bismuth oxide[12] affects the color of MTA Angelus when it comes into contact with the dental structures. Therefore, the use of alternative radiopacifiers such as zirconium oxide (ZrO_2), niobium oxide (Nb_2O_5) or calcium tungstate ($CaWO_4$) have been indicated as potential substitutes for Bi_2O_3.

ZrO_2 has been shown to be inert[10], without changing the properties of MTA[13,14]. The association of tricalcium silicate with ZrO_2 leads to calcium ion release[15], promotes alkalinization and bioactive potential[16]. Furthermore, this radiopacifier does not undergo leaching when immersed in water or HBSS[15]. When hydraulic calcium silicate is associated with the radiopacifier ZrO_2, it has a setting time similar to that of MTA Angelus; low solubility; radiopacity higher than 3 mm Al, and alkaline pH[17]. Hydraulic calcium silicate cement with ZrO_2 and MTA present better bacterial sealing capacity when compared with zinc oxide and eugenol[18].

Biodentine (Septodont, Saint Maur des Fossés, France) is a hydraulic calcium silicate-based biomaterial with indications similar to those of MTA[19-23], and has a better consistency[24]. Biodentine consists of a powder that contains tricalcium and dicalcium silicate ($3CaO\ SiO_2$ and $2CaO\ SiO_2$), calcium carbonate ($CaCO_3$) and ZrO_2 as radiopacifier. The liquid is made up of calcium chloride ($CaCl_2\ 2H_2O$) and a water reducing agent in an aqueous solution with a mixture of polycarboxylate (a super plasticizing agent)[20,22]. MTA hydration is slower than that of Biodentine and tricalcium silicate associated with 20% ZrO_2, due to the smaller quantity of tricalcium silicate present in the

material[7]. Biodentine has a shorter initial setting time than that of MTA Angelus, and radiopacity below the value of the ISO Standard 6876/2002[25]. MTA and Biodentine are biocompatible and show bioactivity in Saos-2 cell cultures transfected with the gene that codifies bone morphogenetic protein 2 (BMP-2)[26].

Nb_2O_5 has been used in bone implants due to its biocompatibility, resistance to corrosion and disintegration[27]. Nb_2O_5 used as micro and nano particulate radiopacifier in hydraulic calcium silicate cement in the proportion of 30% promoted radiopacity in accordance with the ANSI/ADA specifications[28,29]. The addition of Nb_2O_5 to hydraulic calcium silicate-based cement promoted a biocompatible material with adequate radiopacity and setting time[30].

Nb_2O_5 provides radiopacity and improves the biological properties of materials, well as their biocompatibility and bioactivity[31]. Nb_2O_5 and ZrO_2 associated with hydraulic calcium silicate cement in endodontic cement formulations promoted the deposition of hydroxyapatite crystals[32]. Nb_2O_5 associated with hydraulic calcium silicate cement shows cytocompatibility in Saos-2 cells, stimulates alkaline phosphatase activity[33], in addition to showing cellularbioactivity[34].

When $CaWO_4$ is associated with Portland cement (PC), it promotes higher pH and calcium ion release values similar to those of MTA[35], increases the compressive strength and diminishes the solubility of PC[35,36]. Moreover, it does not change its mechanical properties and final setting time[36]. When $CaWO_4$ is associated with PC it presents antimicrobial action[37] and is not cytotoxic[38]. When associated with PC in the proportion of 20%, it presents radiopacity equivalent to 3.11 mm Al[39]. $CaWO_4$ associated with hydraulic calcium silicate cement shows bioactive potential similar to that of MTA Angelus[40]; low solubility, and alkaline pH[17].

Experimental cements with ZrO_2 and $CaWO_4$ as radiopacifiers have adequate hydration properties, high pH and calcium ion release values[41]. MTA HP is a cement similar to MTA developed by Angelus (Brazil), which has $CaWO_4$ as radiopacifier agent, in addition to a plasticizing agent in the liquid. Evaluation of the cytotoxicity, biocompatibility and biomineralization capacity has demonstrated that MTA HP has similar biocompatibility and biomineralization to that of White MTA (Angelus)[42].

Different materials, including metals, polymers, ceramics and composites have been used to repair or reconstruct parts of the injured musculo-skeletal system. According to Hench and Wilson[43], biomaterials are classified according to their type of interaction with live tissues. The concept of bioactive material was introduced by Hench: ‘‘A bioactive material generates a specific biological response at the interface, resulting in the formation

of a bond between the tissue and material"[44]. More specifically, bioactive materials are materials which, when in contact with live tissue, produce a hydroxycarbonate apatite (HCA) in the superficial layer, thus promoting an extremely strong chemical bond between the tissue and implant[43,44].

Some glass-ceramic biomaterials with high bioactive potential have been developed. Thus, a completely crystallized bioactive glass-ceramic denominated Biosilicate (Federal University of São Carlos, São Carlos) has been developed by controlled heat treatment, for use in the medical and dental areas[45]. Biosilicate (Biosilicate, Vitrovita, São Carlos, SP, Brazil) has demonstrated bioactivity[46,47]. Biosilicate has positive characteristics for bone tissue regeneration: it is highly bioactive, osteoconductive, osteoinductive, non-cyctotoxic, non-genotoxic, and has antibacterial properties[48]. Martins *et al.*[49] observed that Biosilicate is a broad-spectrum antimicrobial agent, including anaerobic bacteria. The antimicrobial effect of Biosilicate may be explained by its highly crystalline bioactive phase, elevated superficial area and ultrafine particles (between 0.1 and 20 μm). Biosilicate is capable of increasing the pH of aqueous solutions[50,51]. The basic pH of the solution makes the bioglass more soluble due to the presence of OH^- that has the capacity to cleave the chains of silica[46,47,52].

Biosilicate has a stimulatory effect on bone cell metabolism[46]. Complete crystallization of bioactive glasses in Biosilicate can promote an increase in the formation of tissue similar to that of bone tissue, in vitro, in an osteogenic cell culture system[46]. Biosilicate promotes the bone regenerative process of surgically created defects in rat tibias[53]. Bone repair adjacent to conventional titanium implants placed in dental alveoli of dogs has demonstrated that filling the alveolus with Biosilicate particles preserved the alveolar bone height and allowed osseointegration[47]. Biosilicate shows osteogenic activity, accelerating bone repair, shown by the activating of immunomarkers, such as COX-2, BMP-9 and RANKL, related to tissue repair[54]. Biosilicate does not induce damage to DNA in fibroblast and osteoblast cell lineages[55]. Therefore, Biosilicate is used as a synthetic bone graft for replacing bone in various types of surgery[56]. Biosilicate has shown osteogenic activity and the capacity to accelerate bone matrix deposition in experimental models of bone defects[57]. However, the properties of manipulation and low dissolution of the material represent a disadvantage to the biological properties of Biosilicate[53].

The aim of the present study was to develop and evaluate tricalcium silicate cement associated with ZrO_2, Nb_2O_5 and $CaWO_4$ radiopacifiers, and with Biosilicate.

2 PROPOSITION

2.1 General Proposition: Evaluation of physicochemical and biological properties of tricalcium silicate-based and Biosilicate reparative materials.

2.2 Specific Propositions:

Publication 1 – To evaluate tricalcium silicate-based materials associated with zirconium oxide, calcium tungstate or niobium oxide radiopacifiers, and compare them with MTA HP with regard to the physicochemical properties (setting time, solubility, radiopacity and pH), in addition to cytocompatibility, antimicrobial ability and potential to induce mineralization.

Publication 2 - To evaluate a new tricalcium silicate-based material associated with the zirconium oxide radiopacifier and 10% and 20% Biosilicate, and compare it with Biodentine with regard to the physicochemical properties (setting time, solubility, radiopacity and pH), cytocompatibility, antimicrobial ability and potential to induce mineralization.

3 PUBLICATION 1*

Physicochemical, biological, and antibacterial evaluation of tricalcium silicate-based reparative cements with different radiopacifiers

ABSTRACT

Aim To evaluate the physicochemical, biological, and antibacterial properties of tricalcium silicate-based (TCS) experimental materials, associated with zirconium oxide (ZrO_2), calcium tungstate ($CaWO_4$) or niobium oxide (Nb_2O_5) radiopacifiers, in comparison with MTA Repair HP (MTA HP).

Methodology Physicochemical tests: setting time, radiopacity, pH and solubility. In Vitro Assays: Cytotoxicity (MTT and Neutral Red - NR) and cell bioactivity: alkaline phosphatase activity (ALP), Alzarin red staining (ARS) and real time PCR (qPCR). Antibacterial activity was evaluated by direct contact on *Enterococcus faecalis* in the planktonic form. Physicochemical and ARS data were submitted to ANOVA and Tukey tests; antibacterial activity, to Kruskall-Wallis and Dunn tests; MTT, NR, ALP and qPCR were analyzed by the Two-Way ANOVA and Bonferroni tests (α=0.05).

Results TCS + $CaWO_4$ presented the longest setting time and MTA HP, the shortest ($P < 0.05$). Except for TCS, all the materials presented radiopacity over 3 mm Al. The cements presented alkaline pH, antibacterial activity, low solubility and presented no cytotoxic effects in the MTT and NR assays. The highest ALP activity occurred in 14 days, especially to the TCS, TCS + ZrO_2 and TCS + $CaWO_4$ ($P < 0.05$) cements. TCS + ZrO_2, TCS + Nb_2O_5 and MTA HP presented higher mineralized nodule formation ($P < 0.05$) than those of the negative control. After 7 days, there was no statistically significant difference ($p>0.05$) in mRNA expression for ALP, when compared to NC. However, after 14 days there was na overexpressed ALP mRNA, especially TCS + Nb_2O_5 ($p<0.05$), in relation to the CN. All the materials TCS presented antimicrobial action against *E. faecalis*.

* article in the norms of the International Endodontic Journal

Conclusions The tricalcium silicate-based cements associated with ZrO_2, $CaWO_4$ or Nb_2O_5 radiopacifiers presented proper physicochemical properties, antibacterial activity, cytocompatibility and induced mineralization in Saos-2, indicating their use as reparative materials.

Key Words: physical properties; chemical properties; calcium silicate; cytotoxicity.

INTRODUCTION

Mineral Trioxide Aggregate (MTA) is a reparative material composed of Portland Cement associated with bismuth oxide (Bi_2O_3) (Lee *et al.* 1993). Portland cement is composed of 53.1% tricalcium silicate and 22.5% dicalcium silicate (Camilleri 2007). Tricalcium silicate is used to formulate biomaterials due its biological and physicochemical properties, in addition to bioactivity (Camilleri 2011c, Camilleri *et al.* 2013, Grech *et al.* 2013, Gomes-Cornélio *et al.* 2017).

The Bi_2O_3 used as radiopacifier in the composition of MTA jeopardize the physicochemical properties and may cause tooth discoloration (Coomaraswamky *et al.* 2007, Camilleri 2008, Marciano *et al.* 2014). Therefore, alternative radiopacifiers have been proposed, as zirconium oxide (ZrO_2), niobium oxide (Nb_2O_5) or calcium tungstate ($CaWO_4$).

The association of tricalcium silicate and ZrO_2 as radiopacifying agent presents proper physicochemical properties, alkaline pH, calcium ion release and induces the formation of hydroxyapatite, suggesting that it has excellent bioactive potential (Camilleri 2011c, Camilleri *et al.* 2011b, Camilleri *et al.* 2013, Camilleri *et al.* 2014). It also presents ability to induce cell growth and differentiation (Gomes-Cornélio *et al.* 2017).

Nb_2O_5 is used to improve the mechanical properties in the titanium alloys of osseointegrated implants due its resistance to corrosion, biocompatibility, and hydroxyapatite deposition (Denry *et al.* 2005). The use of Nb_2O_5 as radiopacifier in hydraulic calcium silicate- based cements provides radiopacity in accordance with the ANSI/ADA specifications (Wang *et al.* 2010, Guerreiro-Tanomaru *et al.* 2014, Guerreiro-Tanomaru *et al.* 2016), in addition to improving the biological properties of the materials, such as biocompatibility and biomineralization. (Wang *et al.* 2010, Mestieri *et al.* 2014, Mestieri *et al.* 2017). Viapiana *et al*. (2014), demonstrated the biomineralization for Nb_2O_5 and ZrO_2 used in the composition of hydraulic calcium silicate- based filling materials, showing deposition of hydroxyapatite crystals.

$CaWO_4$, also used as radiopacifying agent in endodontic cements, promotes alkaline pH (Húngaro-Duarte *et al.* 2012), antibacterial activity (Guerreiro-Tanomaru *et al.* 2012), and

biocompatibility (Gomes-Cornélio *et al.* 2011). When associated with hydraulic calcium silicate-based cements, it presents biomineralization and has a composition similar to that of MTA Angelus (Bosso-Martelo *et al.* 2015). It maintains the setting time, low solubility, alkaline pH and calcium ion release similar to those of MTA (Bosso-Martelo *et al.* 2016). Experimental hydraulic calcium silicate-based cements with ZrO_2 and $CaWO_4$ as radiopacifiers present proper radiopacity, high pH and calcium ion release values (Marciano *et al.* 2016).

MTA Repair HP (MTA HP) was developed to improve the physicochemical properties of MTA and avoid tooth discoloration and has $CaWO_4$ as radiopacifying agent. MTA HP demonstrated biocompatibility and biomineralization similar to White MTA (Angelus) after subcutaneous implants in rats (Cintra *et al.* 2017).

The aim of this study was to evaluate a new tricalcium silicate-based material associated with the ZrO_2, Nb_2O_5 or $CaWO_4$ radiopacifiers, in comparison with MTA HP. The null hypothesis was that there is no difference among the properties of the materials evaluated.

MATERIALS AND METHODS

The materials evaluated were pure tricalcium silicate (TCS), tricalcium silicate associated with ZrO_2 (TCS+ZrO_2), $CaWO_4$ (TCS+$CaWO_4$) or Nb_2O_5 (TCS+Nb_2O_5) and MTA Repair HP (MTA HP). Their respective manufacturers and proportions used are described in Table 1.

Setting Time

The setting time was evaluated based on ISO Standard 6876/2002. The cements were mixed and placed into stainless steel rings measuring 10 mm in diameter and 1 mm high (n=6). A Gilmore needle having 100 ± 0.5g and a tip diameter of 2 ± 0.1 mm was used, and the materials were kept in an oven (37°C and 95% humidity) throughout the analysis. The setting time was the mean time elapsing from the time the materials were mixed up to when the needle did not leave any indentation on the specimen surface.

Radiopacity

The radiopacity test was performed based on ISO Standard 6876/2002. Stainless steel rings measuring 10 mm in diameter and 1 mm high were used to fabricate 6 test specimens of each material, which was kept in an oven at 37°C and 95% humidity for 24 hours. The specimens and an aluminum scale were placed on an occlusal film (Insight – Kodak Comp, Rochester, NY, USA) to take the radiograph (X-ray appliance -X GE 1000 - General Electric, Milwaukee, WI, USA). The parameters used were 60 kV, 7 mA, 0.32 pulses per second and focal distance of 33 cm. The exposed films were processed, digitized, and evaluated by using Image J for Windows software, to determine the radiopacity equivalence of the cements in millimeters of aluminum (mm Al) (Tanomaru-Filho *et al.* 2007).

Solubility

Solubility was evaluated based on Carvalho-Junior *et al.* (2007) modified. Circular plastic molds measuring 1.5 mm high and 7.75 mm in internal diameter were fabricated. and placed on a glass plate covered with cellophane paper film and filled with the materials (n=6). A Nylon thread was included in the cement mass and another glass plate also covered by cellophane was placed on the mold. This unit was kept in an oven at 37°C for 24 hours. The test specimens were removed from the molds and-weighed on a precision balance (HM-200 (A & D Engineering, Inc., Bradford, MA, USA). Then, they were suspended and fixed by means of nylon threads inside plastic flasks containing 7.5 mL of distilled, and kept in an oven at 37°C for 7days. The test specimens were removed from the distilled water, dried with absorbent paper, and placed in a dehumidifying chamber. The mass was measured before and after the immersion of the samples in distilled water, and every 24 h thereafter, until the mass was stabilized. The loss of mass was expressed as a percentage of the original mass.

pH

For the pH tests, polyethylene tubes 10 mm long and 1 mm in diameter were filled with each material (n=10). Each tube was immersed in 10 mL distilled water and kept in the-oven during the experimental period. At each period, the tubes were removed from the flasks and put into a new flask with 10 mL distilled water. Flasks containing only distilled water were used as control. The experimental time intervals were 3, 12, and 24 hours, 7, 14 and 21 days after manipulation of the materials. Immediately after the end of each experimental time interval, the pH of the solutions was measured with a previously calibrated digital pH meter (Digimed Analítica Ltda. Grupo Digicrom Analítica. São Paulo, Brazil.)

Preparation of material extracts

This was carried out in accordance with the ISO Standard 10993-5/2005. Of each material, 0.5 g was measured on a precision balance Adventurer ^{TM} (Ohaus; Barueri, São Paulo, Brazil), manipulated in the due proportions on sterile glass, then placed on the bottoms of 12-well culture plates (Corning, New York, NY, USA) and hydrated with humidified gauze. The culture plates were kept at 37 °C at 95% humidity, 5% CO_2, for 24 hours to allow complete setting of the materials. After this period, the plates with the cements were exposed to U.V. light for 30 minutes to prevent contamination (Katara *et al.* 2008). Then, 5mL DMEM culture medium (Sigma/Aldrich) serum-free (without the presence of fetal bovine serum - FBS) were placed in each well of the plates, in which the material was accommodated, and maintained for 24 h (37 °C, 95% humidity and 5% CO2) to create the extract of each material. The extracts were collected and diluted in DMEM without FBS, thereby obtaining the 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions for performing the cytotoxicity tests. As negative control, DMEM culture medium serum-free; and as positive control 20% DMSO were used.

Viability and Biomineralization Assays

MTT

Human osteoblastic cells (Saos-2) were plated ($1x10^5$ cells/mL) in 96-well plates (Corning) and kept at 37 °C at 95% humidity, 5% CO_2, for 24 hours. After this period, the cells were exposed to the cement extracts in cell culture for 24 hours. For the MTT assay, cement extracts were replaced with 100 µl of a 5 mg/mL MTT solution (Sigma-Aldrich), and cells were incubated at 37 °C at 95% humidity, 5% CO_2, for 3 hours. After this period, each well was washed with 100 µL phosphate buffer solution (PBS) and 100 µl isopropyl alcohol (acidified 0.04 N HCl) was added to solubilize the formazan. The optical density was measured at 570 nm in an automatic microplate reader (ELx800; Instruments Bio-Tek, Winooski, VT, USA). Three independent experiments were performed in triplicate for each experimental group and outcome.

Neutral Red

The cells ($1x10^5$ cells/mL) were plated in 96-well plates (Corning) in DMEM medium supplemented with 10% FBS and kept at 37 °C at 95% humidity, 5% CO_2, for 24 hours. After the cells had remained in contact with the cement extracts in their different concentrations for 24 hours, the extracts were replaced by 100 µL DMEM serum free, containing 50 µg NR/mL (Sigma-Aldrich), followed by incubation at 37º C, 95% humidity and 5% CO_2 for 3 hours. The coloring agent was removed and the colorimetric product was solubilized in 100 µL of 50% ethanol and 1% acetic acid solution (Sigma-Aldrich). The optical density was measured in the plate reader at 570 nm (Asys-UVM 340, Biochrom – MikroWin 2000, USA). Three independent experiments were performed in triplicate for each experimental group and outcome.

Alkaline Phosphatase

Alkaline phosphatase activity was determined by using the commercial Labtest kit (Labtest, Lagoa Santa, MG, Brazil). After plating for 24 hours, ($7x10^4$ cells/mL in 96-well plates), the Saos-2

cells were exposed to the cement extracts for 7 and 14 days. The extracts were renewed every two days throughout the exposure period. After each experimental period, the cells were washed with 200 µL of PBS 1X, followed by the addition of 200 µL of a sodium lauryl sulphate solution (1% in distilled water, Sigma-Aldrich) to each well. The samples remained at rest at room temperature for 30 minutes, and then 12.5 µL of each sample was transferred to Eppendorf tubes with reagents of the commercial Alkaline Phosphatase kit, according to the manufacturer's instructions (Labtest).

The optical density was evaluated in an automatic miniplate reader (ELx800, Bio-Tek Instruments, Winooski, VT, USA) at 590 nm. The data were expressed as ALP activity normalized by the number of viable cells determined in the MTT assay, in the respective culture period. Three independent experiments were performed in sextuplicate for each experimental group and outcome.

Quantitative Real Time PCR (qPCR)

For gene expression study, RNA extraction was performed from cells using Trizol (Life Technologies – Invitrogen, Carlsbad, CA, USA) according to the manufacturer's Kit instructions (PureLink ^{TM}, RNA Mini Kit, Carlsbad, CA, USA). The transcripts were synthesized from total Saos-2 RNA (0,5µg/µL) by reverse transcriptase reaction using the ImProm-II Reverse Transcription System (PROMEGA, Mandison, WI, USA) kit according to the manufacturer instructions. The gene expression was analysed by qPCR (StepOne, Applied Biosystems, Life Technologies, Grand Island, NY, USA) using TaqMan chemistry and pre-designed primers and probe sets alkaline phosphatase (ALP) (Hs01029144_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (Hs02758991_g1) (Applied Biosystems, Life Technologies). The levels of target gene expression for each sample group were calculated with the ΔΔCt method (fold expression = $2^{-(\Delta\Delta Ct \pm stdev)}$) compared to control (serum-free DMEM medium).

Alizarin Red

With the purpose of evaluating the calcium deposition induced by the materials, Saos-2 cells were plated ($1x10^4$ cells/mL) in 24-well culture plates (Corning, New York, NY, USA) in DMEM medium supplemented with 10% FBS. For 21 days, the cement extracts prepared with osteogenic DMEM culture medium (DMEM 10% SFB; 100 IU/mL penicillin; 100 mg/mL streptomycin; 0.023 g/mL β-Glycerophosphate; 0.055 mg/mL ascorbic acid – Sigma Chemicals St Louis MO, USA) were renewed every 2 days. The medium was then aspirated, the wells washed with PBS 1X, and the cells fixed with 70% ethanol (Sigma/Aldrich) at 4^0C, for 1 hour. The cells were washed twice with distilled water before the addition of 300 μL Alizarin Red S (at 2% and pH 4.1). The cells were incubated at room temperature for 2 minutes. After this they were washed 4 times with 1 mL distilled water/well for 5 minutes. For quantitative analysis of mineralization nodule formation, the nodules were solubilized in 0.5 mL Cetylpyridinium chloride (Sigma-Aldrich) under agitation for 15 minutes. After homogenization, three aliquots of 100 μL from each well were transferred to a 96-well plate. Mineral nodule formation was analyzed in accordance with the absorbance determined at 562 nm in a plate reader (ELx800, Bio-Tek Instruments). Three independent experiments were performed in triplicate for each experimental group and outcome.

Test of direct contact on planktonic *E. faecalis* cells

Microplates with 96 wells were used, in which the experimental materials were manipulated and inserted on the lateral wall of the well in a standardized manner. The materials were submitted to decontamination under ultraviolet light for 30 minutes. After this, 10 μL of each inoculum was deposited on the materials and on the positive control, to which only the inoculum was added. The plates were kept in an oven at 37°C for 1 hour and 30 minutes. Afterwards, 250 μL of *brain heart infusion* (BHI) were added to each contaminated well, serial decimal dilution was performed, and the plates containing *Tryptic soy agar* (TSa) were seeded. After incubating the plates in the oven at 37°C for 48 hours, cells were counted in CFU mL^{-1}.

Statistical Analysis

For the physicochemical tests the data obtained were submitted to a normality test, and afterwards to the ANOVA and Tukey tests, with 5% level of significance. For the cell viability and bioactivity, the data were analyzed by using Two-way ANOVA and Bonferroni tests (α=0.05). Alizarin red staining (ARS) was compared by ANOVA one-way and Tukey tests. For the antibacterial test, the Kruskall-Wallis and Dunn multiple comparison tests were performed, with 5% significance, after logarithmic transformation (log 10).

RESULTS

The radiopacity, setting time and solubility results are described in Table 2. All the cements presented values higher than 3 mm aluminum, except for pure TCS ($P<0.05$). TCS + $CaWO_4$ presented the longest setting time ($P<0.05$), while MTA HP presented the shortest setting time ($P<0.05$). Low solubility values were observed for the materials, with the lowest values for TCS + $CaWO_4$.

The results of the pH test are described in Table 3. All the showed an alkaline pH, in the different time intervals evaluated.

Cell Viability

In the MTT assay, at the 1:1 dilution, the pure TCS, TCS + ZrO_2, TCS + Nb_2O_5 and MTA HP cements presented higher or equal cell viability the negative control ($P<0.05$). At the 1:2 dilution, the TCS + Nb_2O_5 cement presented cell viability similar to the negative control ($P>0.05$). At the 1:4 dilution, all the cements presented higher cell viability than the negative control ($P<0.05$), with the exception of TCS + $CaWO_4$ that presented no statistically significant difference when compared to the control ($P>0.05$). At the 1:8, 1:16 and 1:32 dilutions, all the cements presented cell viability similar ($P>0.05$) or higher than ($P<0.05$) the negative control (Figure 1).

In the neutral red assay, the cements showed no statistically significant difference ($P>0.05$) in the dilutions tested when compared with the negative control (Figure 2).

ALP Activity

All the groups presented lower cell viability than the control group ($P<0.05$) in the time interval of seven days. At 14 days, the cements presented cell viability similar (MTA HP and TCS + $CaWO_4$) ($P>0.05$) or lower (TCS, TCS + ZrO_2 and TCS + Nb_2O_5) ($P<0.05$) than the control (Figure 3a).

In the time of 7 days exposure to the cements, an increase in ALP activity was observed in the TCS + ZrO_2 and TCS + $CaWO_4$ groups of cement ($P<0.05$). The highest ALP activity was detected in the period of 14 days, especially to the TCS, TCS + ZrO_2, TCS + $CaWO_4$ cements, in comparison with the control ($P<0.05$) (Figure 3b).

Quantitative Real Time PCR (qPCR)

On day seven, there was no statistically significant difference ($P>0.05$) in mRNA expression for ALP in all material groups when compared to control. However, at day 14, all materials overexpressed ALP mRNA, especially TCS + Nb_2O_5 and TCS + $CaWO_4$ ($P<0.05$) in relation to the control (Figure 4)

Alizarin Red

In the Alizarin Red assay, the TCS + ZrO_2, TSC + Nb_2O_5 and MTA HP cements presented higher mineralized nodule formation ($P<0.05$) in comparison with those of the negative control (Figure 5).

Test of direct contact on planktonic *E. faecalis* cells

For the test of direct contact on planktonic *E. faecalis* cells, the materials presented antibacterial activity, differing from the control group. Although the materials presented no statistical difference among them ($P>0.05$), TCS + Nb_2O_5 and TCS + $CaWO_4$ presented the lowest bacterial count ($P<0.0001$), followed by TCS ($P<0.001$), TCS + ZrO_2 ($P<0.01$) and MTA HP ($P<0.05$), when compared with the control. The data are presented in Table 4.

DISCUSSION

The main component of MTA, tricalcium silicate cement (Camilleri 2008, Taddei *et al.* 2011, Gandolfi *et al.* 2013) has been evaluated as a dental biomaterial (Camilleri 2011c). The association of tricalcium silicate-based materials with ZrO_2 promoted calcium ion release and resulted in a material with bioactive potential (Camilleri *et al.* 2013). ZrO_2 has been demonstrated to be an inert agent in the cement hydration reaction (Camilleri *et al.* 2013) and presented greater stability than the association of Bi_2O_3 with PC (Camilleri *et al.* 2011).

The radiopacity observed for the cements evaluated in the present study is in accordance with the ISO Standard 6876/2002, with values above 3 mm aluminum, except for pure TCS, as demonstrated by previous studies (Hungaro-Duarte *et al*. 2009, Bosso-Martelo *et al*. 2016). Adequate radiopacity has been reported for hydraulic calcium silicate-based cements associated with ZrO_2 radiopacifiers (Hungaro-Duarte et al. 2009; Cutajar *et al.* 2013, Grech *et al.* 2013b; Bosso-Martelo *et al.* 2016), $CaWO_4$ (Hungaro-Duarte *et al.* 2009) or Nb_2O_5 (Mestieri *et al.* 2014, Silva *et al*. 2015, Bosso-Martelo *et al*. 2016).

In this study, the highest radiopacity value was observed for TCS + $CaWO_4$ with values higher than 4 mm Al, in agreement with Bosso-Martelo *et al.* (2016). Although MTA HP also presented $CaWO_4$ as radiopacifier, its radiopacity was lower, probably because of the proportion of radiopacifier in the composition of this material.

MTA HP is composed of tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, calcium carbonate, calcium tungstate as radiopacifier and a liquid containing water

and a plasticizing agent (Silva *et al.* 2016). The addition of calcium carbonate to tricalcium silicate improved the hydration of the material, accelerating its setting time (Camilleri *et al.* 2013). In this study, MTA HP presented the shortest setting time among the materials evaluated. Thus, a possible explanation for its shorter setting time could be the presence of calcium carbonate in its composition.

Furthermore, the results of this study showed that the addition of Nb_2O_5 decreased the setting time of tricalcium silicate, in agreement with the study of Bosso-Martelo *et al*. (2016). However, the addition of $CaWO_4$ increased the setting time of this material, corroborating the findings in the study of Hungaro-Duarte *et al.* (2012), for its association with Portland Cement. Based on a study of hydraulic calcium silicate cement hydration (Camilleri *et al.* 2013), it may be suggested that the addition of the Nb_2O_5 and $CaWO_4$ radiopacifiers interfered in the hydration process of tricalcium silicate, accelerating and retarding the setting, respectively. Another factor that could be associated with the setting process is related to the powder/liquid ratio (Cutajar *et al.* 2011), which was established for each material according to the consistency for clinical applicability.

The solubility was perfomed by a modified method proposed by Carvalho-Júnior (2007). Thereby, we evaluated the solubility of the materials in 7 days of immersion in distillated water. Low solubility was observed for all the cements evaluated, similar to those observed in previous studies that also evaluated hydraulic calcium silicate-based cements with ZrO_2 or $CaWO_4$ radiopacifiers (Bosso-Martelo *et al.* 2016, Espir *et al.* 2016, Húngaro-Duarte *et al.* 2012). MTA HP and TCS + Nb_2O_5 presented a water absorption, indicating that a mass increased occurred after 7 days of immersion in distilled water. A possible explanation for the water absorption would be the quantity of hydraulic calcium silicates in the materials, since Silva *et al.* (2017) affirmed that hydraulic calcium silicate-based cements had greater capacity for fluid absorption and sorption.

The reparative cements of the present study presented an alkaline pH, in agreement with Camilleri (2010), who studied associations of Portland Cement with different radiopacifiers. Bosso-

Martelo *et al.* (2016) observed an alkaline pH for hydraulic calcium silicate-based cement associated with ZrO_2, NbO_2 or $CaWO_4$ radiopacifiers.

The MTT assay is the most usual method for determining cytotoxicity, evaluated by means of cell metabolism, with the use of a reaction catalyzed by the dehydrogenase enzyme from the mitochondria of viable cells (Jung *et al.* 2015). The Neutral Red assay is based on the uptake and accumulation of the neutral red supravital dye in the cells in which the lysosomes present intact membranes (Scelza *et al.* 2012). All the cements evaluated presented cytocompatibility and induced cell proliferation. Cintra *et al.* 2017 observed significant increase in cell viability of MTA HP when compared with the negative control. This response could be related to the replacement of Bi_2O_5 by $CaWO_4$ (Chiang & Ding 2010, Min *et al.* 2007).

The alkaline phosphatase enzyme is a known osteogenic marker, based on thymolphthalein used for staining the cells that are associated with the process of mineralization (Lee *et al.* 2011). In this study, after 7 days exposure to the cements, an increase in alkaline phosphatase enzyme activity was observed in the TCS + ZrO_2 and TCS + $CaWO_4$ cements. After 14 days, the TCS, TCS + ZrO_2 and TCS + $CaWO_4$ cements presented higher alkaline phosphatase enzyme activity values when compared with those of the negative control. Tricalcium silicate cements have demonstrated the ability to increase ALP activity and osteogenic differentiation of stem cells (Gandolfi *et al*. 2011, Eid *et al*. 2014, Lee *et al*. 2014). All tested materials overexpressed ALP mRNA at day 14, especially TSC + Nb_2O_5 and TCS + $CaWO_4$ when compared to the control group. These results corroborate with previous study which revealed using the qPCR assay that hydraulic calcium silicate experimental cement associated with niobium (CSCR Nb_2O_5) oxide as radiopacifier increased the ALP transcript at day 3 of cell exposure. $CaWO_4$ contributed to higher calcium ion release values, helping with the process of biomineralization (Huffman *et al.* 2009).

Alizarin Red is a derivative of anthraquinone, which may be used to identify calcium deposits in cell cultures (Gregory *et al.* 2004). Higher production of mineralization nodules was observed for the TCS+ZrO_2, TCS+Nb_2O_5 and MTA HP cements. Saos-2 osteoblast cells exposed to

the calcium-based cement associated with Nb_2O_5 showed a larger mineralized area than the hydraulic calcium silicate materials with different radiopacifiers (Gomes-Cornélio *et al.* 2017). Tanomaru-Filho et al. 2017 evaluated the biocompatibility and mineralization of Saos-2 cells in contact with the tricalcium silicate cement and the tantalum oxide radiopacifier, and observed that the tricalcium silicate cements presented cytocompatibility and induced the production of mineralization nodules essential for the endodontic repair process.

All the materials evaluated were shown to be effective against *E. faecalis,* in agreement with previous studies with hydraulic calcium silicate-based cements associated with different radiopacifiers, such as Portland Cement with the addition of ZrO_2 (Guerreiro-Tanomaru *et al.* 2012, Guerreiro-Tanomaru *et al.* 2014a,b, Vazquez-Garcia *et al*. 2016), $CaWO_4$ (Guerreiro-Tanomaru *et al.* 2012, Elashiry *et al.* 2017) and Nb_2O_5 (Guerreiro-Tanomaru *et al.* 2014b).

CONCLUSION

The tricalcium silicate cement associated with different radiopacifiers, as well as MTA HP presented adequate setting time, radiopacity, solubility and pH. Regarding the *in vitro* assays, the materials presented cytocompatibility, bioactive potential in Saos-2 cells, and antibacterial activity.

ACKNOWLEDGMENTS

The authors thank the São Paulo Research Foundation (FAPESP 2015/03437-6, 2016/11036-4 and 2016/00321-0) and the scholarship from CAPES (2016/1604159) for supporting the study.

CONFLICT OF INTEREST

The authors deny any conflicts of interest related to this study.

REFERENCES

American international standards institute/American dental association (ANSI/ADA). Specification no. 57 ADA (2000) *Laboratory testing methods: endodontic filling and sealing materials. Endodontic sealing materials*. Chicago, USA.

Bosso-Martelo R, Guerreiro-Tanomaru JM, Viapiana R, Berbert FL, Duarte MA, Tanomaru-Filho M (2016) Physicochemical properties of calcium silicate cements associated with microparticulate and nanoparticulateradiopacifiers *Clinical Oral Investigation* **20**, 83-90.

Bosso-Martelo R, Guerreiro-Tanomaru JM, Viapiana R, Berbert FL, Tanomaru-Filho M (2015) Calcium silicate-based cements associated with micro- and nanoparticle radiopacifiers: physicochemical properties and bioactivity. *International Scholarly Research Notices* **23**, 874283.

Camileri J (2007) Hydration mechanisms of mineral trioxide aggregate. *International Endodontic Journal* **40**,462-70.

Camilleri J (2008) Characterization of hydration products of mineral trioxide aggregate. *International Endodontic Journal* **41**, 408–17.

Camilleri J (2010) Evaluation of the physical properties of an endodontic Portland cement incorporating alternative radiopacifiers used as root-end filling material. *International Endodontic Journal* **43**, 231–40.

Camilleri J (2011) Evaluation of the effect of intrinsic material properties and ambient conditions on the dimensional stability of white mineral trioxide aggregate and Portland cement. *Journal of Endodontic* **37,** 239-45.

Camilleri J, Cutajar A, Mallia B (2011c) Hydration characteristics of zirconium oxide replaced Portland cement for use as a root-end filling material. *Dental Materials* **27**, 845–854.

Camilleri J, Grech L, Galea K *et al.* (2014) Porosity and root dentine to material interface assessment of calcium silicate-based root-end filling materials. *Clinical Oral Investigation* **18**, 1437-46.

Camilleri J, Mallia B (2011b) Evaluation of the dimensional changes of mineral trioxide aggregate sealer. *International Endodontics Journal* **44**, 416-24.

Camilleri J, Sorrentino F, Damidot D (2013) Investigation of the hydration and bioactivity of radiopacified tricalcium silicate cement, Biodentine and MTA Angelus. *Dental Materials* **29**, 580-93.

Chiang TY, Ding SJ (2010) Comparative physicochemical and biocompatible properties of radiopaque dicalcium silicate cement and mineral trioxide aggregate. *Journal of Endodontics* **36**, 1683–7.

Cintra LTA, Benetti F, de Azevedo Queiroz ÍO *et al*. (2017) Cytotoxicity, Biocompatibility, and Biomineralization of the New High-plasticity MTA Material. *Journal of Endodontics* **43**, 774-8.

Coomaraswamy K, Lumley PJ, Hofmann MP (2007) Effect of Bismuth Oxide Radioopacifier Content on the Material Properties of an Endodontic Portland Cement–based (MTA-like) System. *Journal of Endodontics* **33**, 295-8.

Cutajar A, Mallia B, Abela S, Camilleri J (2011) Replacement of radiopacifier in mineral trioxide aggregate; characterization and determination of physical properties. *Dental Materials* **27**, 879–91.

Eid AA, Hussein KA, Niu LN et al. (2014) Effects of tricalcium silicate cements on osteogenic differentiation of human bone marrow-derived mesenchymal stem cells in vitro. *Acta Biomaterialia* **10**, 3327–34.

Elashiry M, Meghil MM, Kalathingal S, *et al.* (2017) Development of radiopaque, biocompatible, antibacterial, micro-particle fillers for micro-CT imaging of simulated periodontal pockets. *Dental Materials* doi: 10.1016/j.dental.2017.12.002. [Epub ahead of print]

Espir CG, Guerreiro-Tanomaru JM, Spin-Neto R, Chávez-Andrade GM, Berbert FL, Tanomaru-Filho M (2016) Solubility and bacterial sealing ability of MTA and root-end filling materials. *Journal Applied Oral Science* **24**, 121-5.

Gomes-Cornélio AL, Rodrigues EM, Salles LP *et al.* (2017) Bioactivity of MTA Plus, Biodentine and an experimental calcium silicate-based cement on human osteoblast-like cells. *International Endodontic Journal* **50**, 39-47.

Gandolfi MG, Shah SN, Feng R, Prati C, Akintoye SO (2011) Biomimetic calcium-silicate cements support differentiation of human orofacial mesenchymal stem cells. *Journal of Endodontics* **37**, 1102–8.

Gandolfi MG, Taddei P, Modena E, Siboni F, Prati C (2013) Biointeractivity-related versus chemi/physisorption-related apatite precursor-forming ability of current root end filling materials. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **101**, 1107–23.

Grech L, Mallia B, Camilleri J (2013) Characterization of set Intermediate Restorative Material, Biodentine, Bioaggregate and a prototype calcium silicate cement for use as root-end filling materials. *International Endodontic Journal* **46**, 632–41.

Grech L, Mallia B, Camilleri J (2013b) Investigation of the physical properties of tricalcium silicate cement-based root-end filling materials. *Dental Materials* **29**, 20-8.

Gregory CA, Gunn WG, Peister A, Prockop DJ (2004) An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem* **329**, 77-84.

Guerreiro Tanomaru JM, Storto I, Da Silva GF *et al.* (2014) Radiopacity, pH and antibacterial activity of Portland cement associated with micro- and nanoparticles of zirconium oxide and niobium oxide. *Dental Materials Journal* **33**, 466–70.

Guerreiro-Tanomaru JM, Cornélio AL, Andolfatto C, Salles LP, Tanomaru-Filho M (2012) pH and Antibacterial Activity of Portland Cement Associated with Different Radiopacifying Agents. *ISRN Dentistry* 2012, 469019.

Guerreiro-Tanomaru JM, Storto I, Da Silva GF *et al.* (2014) Radiopacity, pH and antibacterial activity of Portland cement associated with micro- and nanoparticles of zirconium oxide and niobium oxide. *Dental Materials Journal* **33**, 466-70.

Guerreiro-Tanomaru JM, Trindade-Junior A, Costa BC *et al.* (2014) Effect of zirconium oxide and zinc oxide nanoparticles on physicochemical properties and antibiofilm activity of a calcium silicate-based material. *Scientific World Journal* 2014, 975213.

Guerreiro-Tanomaru JM, Vázquez-García FA, Bosso-Martelo R, Bernardi MI, Faria G, Tanomaru-Filho M (2016) Effect of addition of nano-hydroxyapatite on Physicochemical and antibiofilm properties of calcium silicate cements. *Journal Applied Oral Science* **24**, 204-10.

Huffman BP, Mai S, Pinna L *et al.* (2009) Dislocation resistance of ProRoot Endo Sealer, a calcium silicate-based root canals sealer, from radicular dentine. *International Endodontic Journal* **42**, 34–46.

Húngaro Duarte MA, de Oliveira El, Kadre GD, Vivan RR, Guerreiro Tanomaru JM, Tanomaru Filho M (2009) Radiopacity of portland cement associated with different radiopacifying agents. *Journal of Endodontics* **35**, 737-40.

Húngaro Duarte MA, Minotti PG, Rodrigues CT, Zapata RO, Bramante CM, Tanomaru Filho (2012) Effect of diferente radiopacifying agentes on the physicochemical properties of white Portland cement and white mineral trioxide aggregate. *Journal of Endodontics* **38**, 394-

International Organization for Standardization Dentistry (ISO). ISO 6876 (2002). Root Canal Sealing Materials. London, UK: British Standards Institution.

International Organization for Standardization (ISO). ISO 10993-5 (2005). Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity. Switzerland.

Jung JY, Woo SM, Lee BN, Koh JT, Nör JE, Hwang YC (2015) Effect of Biodentine and Bioaggregate on odontoblastic differentiation via mitogen-activated protein kinase pathway in human dental pulp cells. *International Endodontic Journal* **48**, 177-84.

Katara G, Hemvani N, Chitnis S, Chitnis V, Chitnis DS (2008) Surface disinfection by exposure to germicidal UV light. *Indian Journal of Medical Microbiology* **26**, 241–2.

Lee SJ, Monsef M, Torabinejad M (1993) Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. *Journal of Endodontics* **19**, 541-4.

Lee BN, Lee KN, Koh JT *et al.* (2014) Effects of 3 endodontic bioactive cements on osteogenic differentiation in mesenchymal stem cells. *Journal of Endodontics* **40**, 1217–22.

Marciano MA, Costa RM, Camilleri J, Mondelli RF, Guimarães BM, Duarte MA (2014) Assessment of color stability of white mineral trioxide aggregate angelus and bismuth oxide in contact with tooth structure. *Jounal of Endodontics* **40**, 1235-40.

Marciano MA, Duarte MA, Camilleri J (2016) Calcium silicate-based sealers: Assessment of physicochemical properties, porosity and hydration. *Dental Materials* **32**, 30-40.

Mestieri LB, Gomes-Cornélio AL, Rodrigues EM, Faria G, Guerreiro-Tanomaru JM, Tanomaru-Filho M (2017) Cytotoxicity and Bioactivity of Calcium Silicate Cements Combined with Niobium Oxide in Different Cell Lines. *Brazilian Dental Journal* **28**, 65-71.

Mestieri LB, Tanomaru-Filho M, Gomes-Cornelio AL, Salles LP, Bernardi MI, Guerreiro-Tanomaru JM (2014) Radiopacity and cytotoxicity of Portland cement associated with niobium oxide micro and nanoparticles. *Journal of Applied Oral Science* **22**, 554-9.

Min KS, Chang HS, Bae JM *et al.* (2007) The induction of heme oxygenase-1 modulates bis- muth oxide-induced cytotoxicity in human dental pulp cells. *Journal of Endodontics* **33**, 1342–6.

Scelza MZ, Linhares AB, da Silva LE, Granjeiro JM, Alves GG (2012) A multiparametric assay to compare the cytotoxicity of endodontic sealers with primary human osteoblasts. *International Endodontics Journal* **45**, 12-8.

Silva EJ, Perez R, Valentim RM *et al.* (2017) Dissolution, dislocation and dimensional changes of endodontic sealers after a solubility challenge: A micro-CT approach. *International Endodontic Journal* **50**, 407-14.

Silva EJNS, Carvalho NK, Zanon M *et al.* (2006) Push-out bond strength of MTA HP, a new high-plasticity calcium silicate-based cement. *Brazilian Oral Reserch* **30**, 1-5.

Silva GF, Tanomaru-Filho M, Bernardi MI, Guerreiro-Tanomaru JM, Cerri OS (2015) Niobium pentoxide as radiopacifying agent of calcium silicate-based material: evaluation of physicochemical and biological properties. *Clinical Oral Investigation* **19**, 2015-25.

Taddei P, Modena E, Tinti A, Siboni F, Prati C, Gandolfi MG (2011) Vibrational investigation of calcium-silicate cements for endodontics in simulated body fluids. *Journal of Molecular Structure* **993**, 367–75.

Tanomaru-Filho M, Jorge EG, Guerreiro-Tanomaru JM, Gonçalves M (2007) Radiopacity evaluation of new root canal filling materials by digitalization of images. *Journal of Endodontics* **33**, 249-51.

Vazquez-Garcia F, Tanomaru-Filho M, Chávez-Andrade GM, Bosso-Martelo R, Basso-Bernardi MI, Guerreiro-Tanomaru JM (2016) Effect of Silver Nanoparticles on Physicochemical and Antibacterial Properties of Calcium Silicate Cements. *Brazilian Dental Journal* **27**,508-14.

Viapiana R, Flumignan DL, Guerreiro-Tanomaru JM, Camilleri J, Tanomaru-Filho M (2014) Physicochemical and mechanical properties of zirconium oxide and niobium oxide modified Portland cement-based experimental endodontic sealers. *International Endodontic Journal* **47**, 437-48.

Wang Z, Lee Y H, Wu B *et al.* (2010) "Anti-microbial activities of aerosolized transition metal oxide nanoparticles," *Chemosphere* **80**, 525-9.

TABLES

Table 1 Experimental Materials, Manufacturers, and Proportions used

Material	Manufacturer/Proportion
MTA HP	Angelus, Londrina, Brazil / 1g powder: 300 µL liquid
TCS	Mineral Research Processing, Meyzieu, France / 1g powder: 330 µL Distilled water
TCS + 30% ZrO_2	ZrO_2 (Sigma Aldrich, St Louis, MO, USA) / 1g powder: 330 µL Distilled water
TCS + 30% $CaWO_4$	$CaWO_4$ (Sigma Aldrich, St Louis, MO, USA) / 1g powder: 340 µL Distilled water
TS + 30% Nb_2O_5	Nb_2O_5 (Sigma Aldrich, St Louis, MO, USA) / 1g powder: 420 µL Distilled water

TCS- tricalcium silicate; ZrO_2- zirconium oxide; $CaWO_4$- calcium tungstate; Nb_2O_5- niobium oxide.

Table 2 Mean and Standard Deviation (±) of radiopacity, setting time and solubility results of the materials evaluated.

	Radiopacity (mmAl)	**Setting time (minutes)**	**Solubility (%)**
MTA HP	3.20^{d} (±0.18)	32.50^{d} (±1.80)	-1.10^{c} (±0.90)
TCS	1.70^{e} (±0.06)	44.00^{b} (±1.63)	2.20^{a} (±1.02)
TCS + ZrO_2	4.20^{b} (±0.13)	40.06^{bc} (±2.45)	1.47^{ab} (±0.55)
TCS + $CaWO_4$	4.61^{a} (±0.01)	61.78^{a} (±4.07)	0.81^{b} (±0.28)
TCS + Nb_2O_5	3.55^{c} (±0.17)	38.00^{c} (±4.96)	-1.06^{c} (±0.39)

TCS- tricalcium silicate; ZrO_2- zirconium oxide; $CaWO_4$- calcium tungstate; Nb_2O_5- niobium oxide. Different letters in the same column indicate statistically significant difference among the experimental groups ($P<0.05$).

Table 3 Mean and Standard Deviations of pH values results found in the different time intervals evaluated.

pH	3 hours	12 hours	24 hours	7 days	14 days	21 days
MTA HP	10.5^{c}	9.9^{ab}	9.3^{ab}	10.3^{a}	10.3^{a}	9.1^{a}
	(±0.34)	(±0.36)	(±0.44)	(±0.27)	(±0.44)	(±0.47)
TCS	11.2^{a}	9.6^{ab}	9.5^{a}	10.2^{ab}	10.2^{a}	8.7^{a}
	(±0.21)	(±0.13)	(±0.62)	(±0.66)	(±0.65)	(±0.79)
TCS + ZrO_2	10.9^{ab}	10.0^{a}	9.0^{ab}	9.2^{c}	9.1^{b}	9.3^{a}
	(±0.43)	(±0.40)	(±0.52)	(±0.93)	(±0.71)	(±0.42)
TCS + $CaWO_4$	10.9^{ab}	9.0^{c}	8.8^{b}	$9.8^{(abc)}$	10.0^{a}	9.0^{a}
	(±0.24)	(±0.56)	(±0.53)	(±0.66)	(±0.60)	(±0.60)
TCS + Nb_2O_5	10.7^{bc}	9.4^{bc}	8.7^{b}	9.4^{bc}	9.8^{ab}	9.0^{a}
	(±0.28)	(±0.42)	(±0.54)	(±0.90)	(±0.57)	(±0.69)
Control	6.4^{d}	6.5^{d}	6.3^{c}	6.4^{d}	6.1^{c}	6.6^{b}
	(±0.14)	(±0.41)	(±0.27)	(±0.22)	(±0.17)	(±0.46)

TCS- tricalcium silicate; ZrO_2- zirconium oxide; $CaWO_4$- calcium tungstate; Nb_2O_5- niobium oxide. Different letters in the same column indicate statistically significant difference among the experimental groups ($P<0.05$).

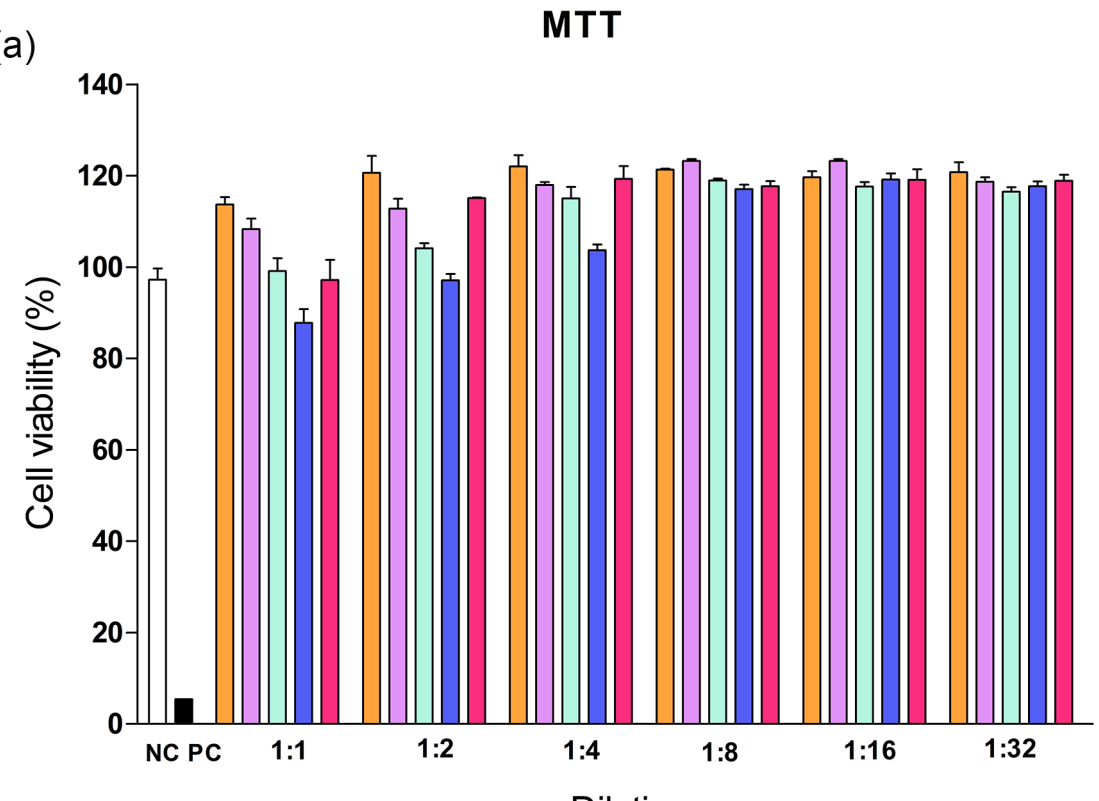
Table 4 Mean and standard deviation Colony Forming Units (CFU).

Groups	**MTA HP**	**TCS**	**TCS + ZrO_2**	**TCS + $CaWO_4$**	**TCS + Nb_2O_5**	**Control**
CFU mL^{-1} log_{10}	1.48 (±1.59)[b]	0.57 (±1.07)[b***]	0.66 (±1.24)[b**]	0.00 (±0.00)[b****]	0.00 (±0.00)[b****]	7.74 (±0.32)[a]

TCS- tricalcium silicate; ZrO_2- zirconium oxide; $CaWO_4$- calcium tungstate; Nb_2O_5- niobium oxide. Different letters in the same column indicate statistically significant difference among the experimental groups (P<0.05). *Kruskall-Wallis and Dunn (P<0.05). **Kruskall-Wallis and Dunn (P<0.01). ***Kruskall-Wallis and Dunn (P<0.001) ****Kruskall-Wallis and Dunn (P<0.0001).

FIGURE LEGENDS

(a)

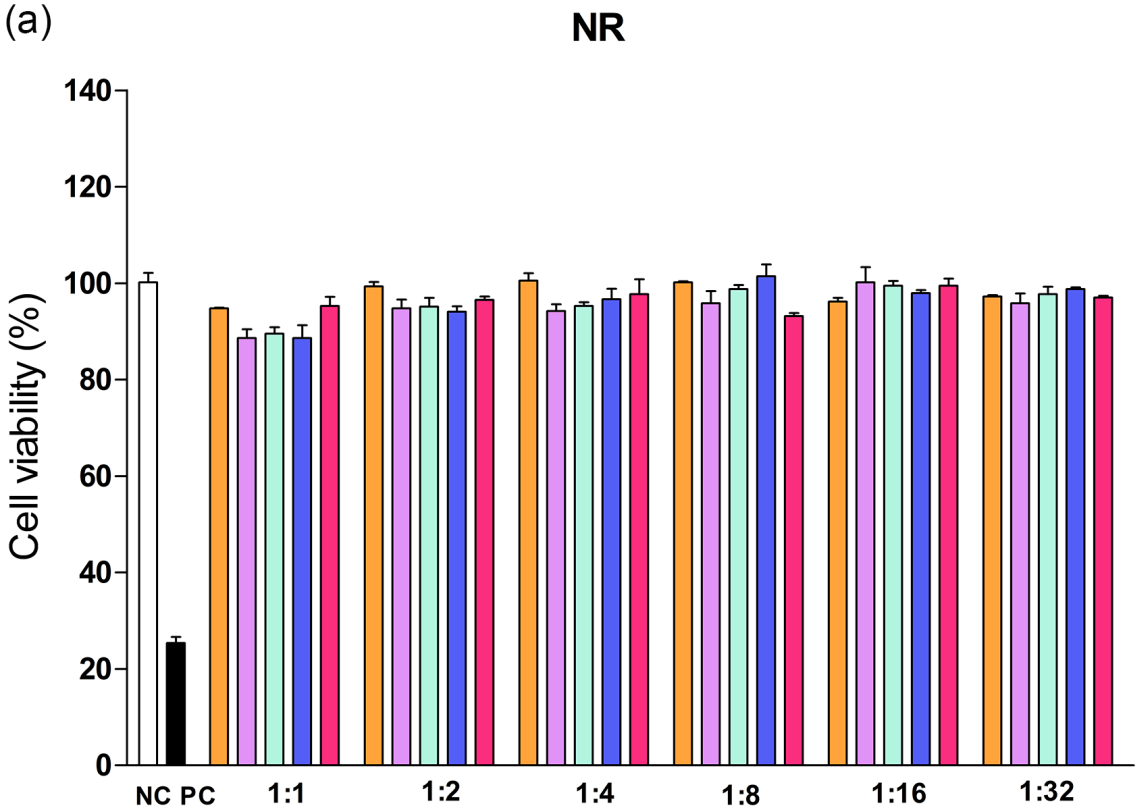


(b)

Dilutions		1:1	1:2	1:4	1:8	1:16	1:32
TCS		c	c	c	a,c	a,c	c
TCS + ZrO_2		c	d	c	c	c	c
TCS + NbO_2		d	a	c	a,c	a,c	c
TCS + $CaWO_4$		e	e	a	a,c	a,c	c
MTA HP		a,d	c,d	c	a,c	a,c	c
Negative Control (NC)		a	a	a	a	a	a
Positive Control (PC)		b	b	b	b	b	b

Figure 1 (a) Cell Viability (%) evaluated by the MTT assay after 24 hours exposure of Saos-2 cells to the cements extracts in different dilutions, and to the negative and positive controls. (b) Statistical comparison of cell viability. Different letters in the columns indicate statistically significant difference among the groups in each dilution. TCS- tricalcium silicate; ZrO_2- zirconium oxide; $CaWO_4$- calcium tungstate; Nb_2O_5- niobium oxide and C- control.

(a)



(b)

Dilutions		1:1	1:2	1:4	1:8	1:16	1:32
TCS		a	a	a	a	a	a
TCS + ZrO_2		a	a	a	a	a	a
TCS + Nb_2O_5		a	a	a	a	a	a
TCS + $CaWO_4$		a	a	a	a	a	a
MTA HP		a	a	a	a	a	a
Negative Control (NC)		a	a	a	a	a	a
Positive Control (PC)		b	b	b	b	b	b

Figure 2 (a) Cell Viability (%) evaluated by the NR assay after 24 hours exposure of Saos-2 cells to the cements extracts in different dilutions, and to the negative and positive controls. (b) Statistical comparison of cell viability. Different letters in the columns indicate statistically significant difference among the groups in each dilution. TCS- tricalcium silicate; ZrO_2- zirconium oxide; $CaWO_4$- calcium tungstate; Nb_2O_5- niobium oxide and C- control.

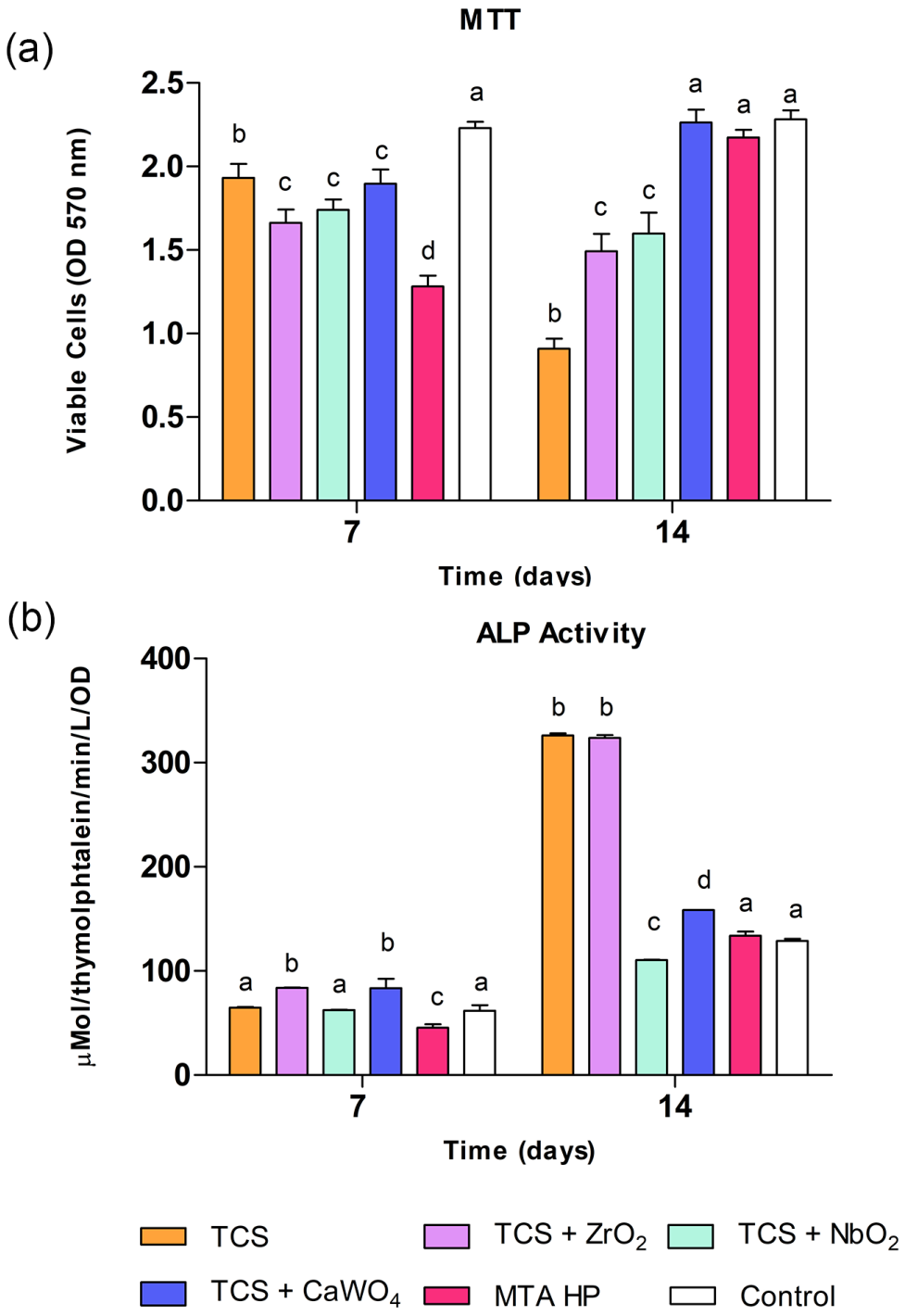


Figure 3 (a) MTT assay and (b) alkaline phosphatase activity (ALP) in Saos-2 osteoblast cells after exposure to the cements extracts and culture medium: negative control in different periods. Bars with different letters in each time interval indicate statistically significant difference among the groups. TCS- tricalcium silicate; ZrO_2- zirconium oxide; $CaWO_4$- calcium tungstate; Nb_2O_5- niobium oxide and C- control.

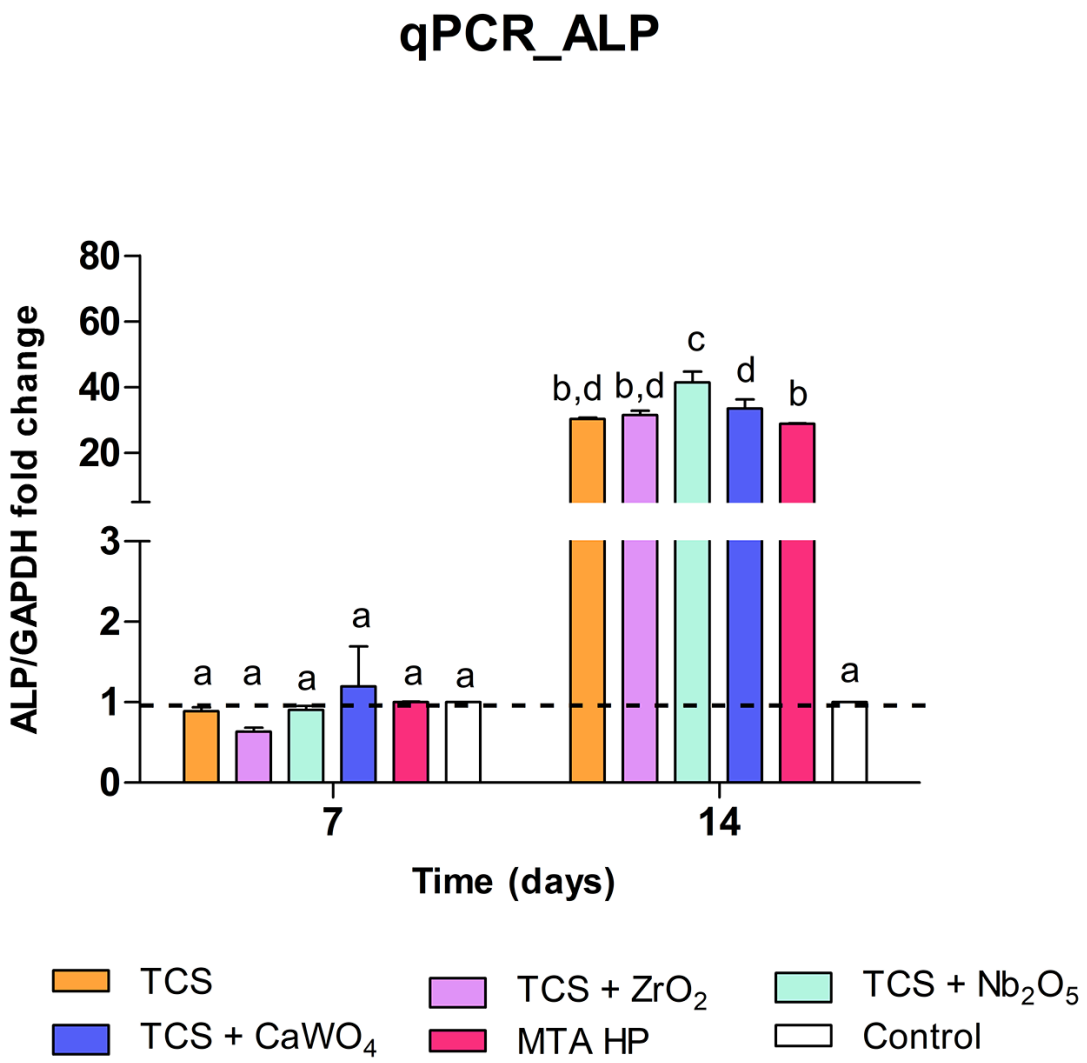


Figure 4 mRNA expression levels of target genes in Saos-2 cells exposed to TCS - tricalcium silicate; ZrO_2- zirconium oxide; $CaWO_4$- calcium tungstate; Nb_2O_5- niobium oxide and MTA HP at 1:8 dilution and to culture medium (control) for 7 and 14 days. Bars with different letters indicate statistically significant difference among the groups. ALP = alkcaline phosphatase genes.

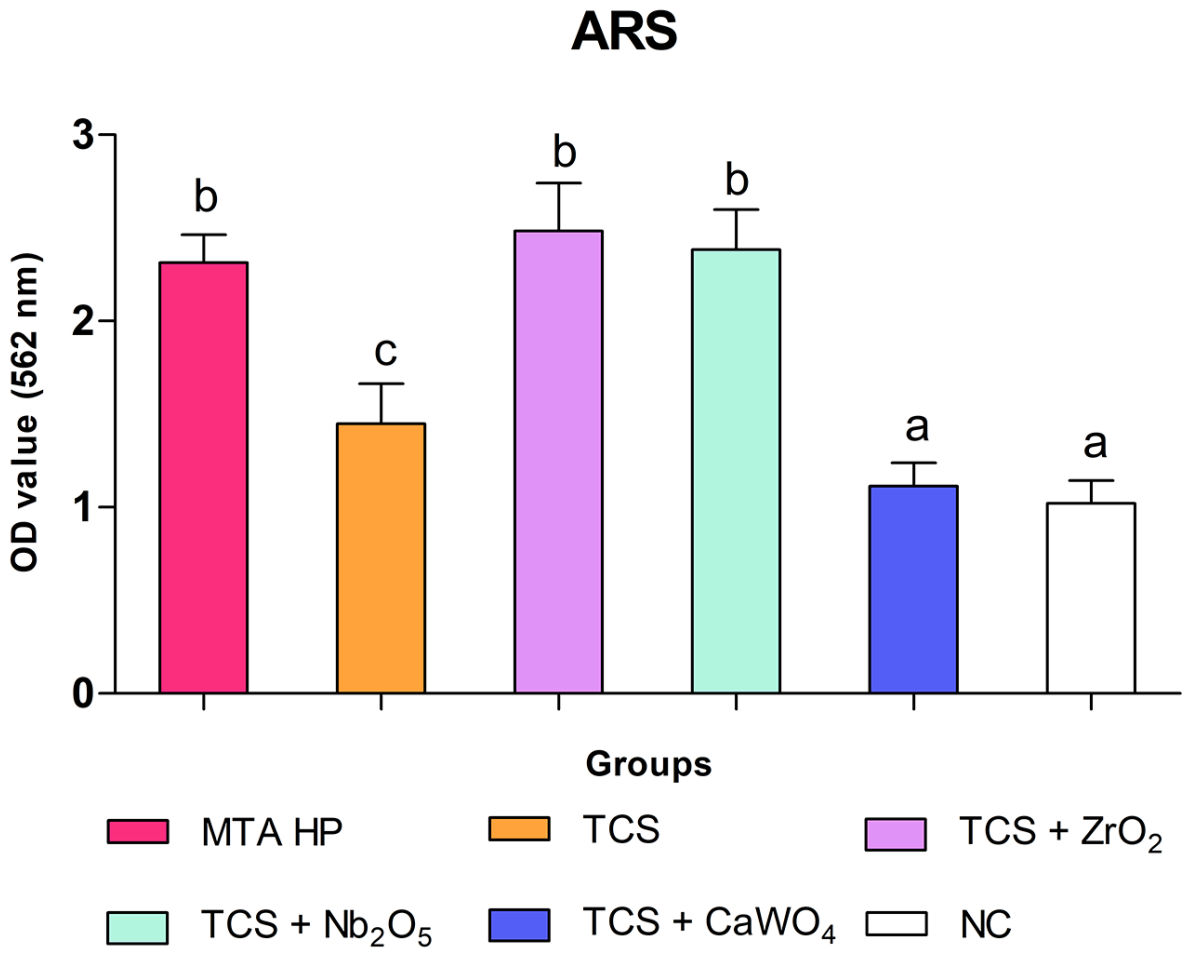


Figure 5 Mineralization nodule formation in human Saos-2 osteoblast cells after 21 days of exposure to the cement extracts and osteogenic DMEM culture medium: control (C) by Alzarin Red staining. Bars with different letters indicate statistically significant difference among the groups. TCS- tricalcium silicate; ZrO_2- zirconium oxide; $CaWO_4$- calcium tungstate; Nb_2O_5- niobium oxide and C- control.

4 Publication 2*

Physicochemical, biological, and antibacterial evaluation of tricalcium silicate-based reparative materials associated with Biosilicate

Abstract

Introduction: Biosilicate is a bioactive glass-ceramic used in the fields of medicine and dentistry. This study evaluated reparative materials composed of pure tricalcium silicate (TCS), 30% zirconium oxide (ZrO_2) and 10% or 20% Biosilicate, in comparison with Biodentine. **Methods:** Setting Time (ST) and radiopacity were evaluated based on ISO 6876/2002 standard. Solubility was evaluated according to the method proposed by Carvalho-Júnior *et al.* (2007) modified. The pH was analyzed with a digital pHmeter. Cell viability was evaluated by the MTT and Neutral Red tests; bioactivity, by alkaline phosphatase activity and alizarin red; real time PCR was used to evaluate gene expression (osteocalcin and alkaline phosphatase); and antimicrobial activity, was evaluated on *Enterococcus faecalis* by means of the direct contact test. The data were analyzed by ANOVA and Tukey; Two-Way ANOVA; Bonferroni and Kruskal-Wallis, and Dunn statistical tests ($\alpha = 05$). **Results:** Pure TCS presented a longer setting time than the other materials ($P < .05$). The association of 10% or 20% Biosilicate with TCS + ZrO_2 demonstrated radiopacity and solubility in accordance with the ISO 6876/2002 standard; alkaline pH; antimicrobial action and presented no cytotoxic effects. Pure TCS and Biodentine showed higher ALP activity in 14 days than the control ($P < .05$) All the cements produced mineralized nodules. **Conclusions:** The addition of Biosilicate to TCS ZrO_2 demonstrated proper radiopacity, solubility, setting time and pH, in addition to presenting antibacterial action, biocompatibility and bioactive potential. Thus, this material presents perspectives for clinical application.

Key Words

Physical properties, chemical properties, cytotoxicity, calcium silicate

*article in the norms of the Journal of Endodontics

Tricalcium silicate-based cements have been developed due its bioactivity (1). Association of tricalcium silicate with zirconium oxide radiopacifier (ZrO_2) presented proper physicochemical and mechanical properties, such as calcium release, hydration, radiopacity, setting time, solubility and pH (1, 2), as well as biocompatibility (2). Biodentine (Septodont, Saint Maur des Fossés, France) is a tricalcium silicate and a dicalcium silicate- based biomaterial that presents ZrO_2 as radiopacifier, with indications similar to those of Mineral Trioxide Aggregate (3, 4). Different components have been incorporated to hydraulic calcium silicate and ZrO_2-based materials in order to improve their antimicrobial and biological properties (5, 6).

A completely crystallized bioactive glass-ceramic denominated Biosilicate (Federal University of São Carlos, São Carlos) has been developed by controlled heat treatment, for use in the medical and dental areas (7). Biosilicate has a stimulatory effect on bone cell metabolism (8), and is bioactive, osteoconductive, and osteoinductive; is not cytotoxic or genotoxic; has the capacity to increase pH (9), and stimulate osteogenic activity, thereby accelerating bone repair (10, 11). Biosilicate has shown antimicrobial activity against a broad spectrum of microorganisms, including anaerobic bacteria (12).

Although bioglasses have bioactivity, the handling properties of these materials for clinical use are limited (13). Therefore, the association of Biosilicate powder with a hydrosoluble polymer (14) and carboxymethylcellulose (13) has been proposed with the purpose of improving the consistency of this material and its applicability.

Considering that Biosilicate has excellent biological properties, in addition to antimicrobial action and limited consistency for application, the purpose of the present study was to develop and evaluate the physicochemical, biological, and antibacterial properties of the association between tricalcium silicate and Biosilicate for use as a reparative material, in comparison with Biodentine cement.

Materials and Methods

Biosilicate is a completely crystallized (99.5%) quaternary bioactive glass ceramic P_2O_5-Na_2O-CaO-SiO_2 (1 to 20μm). It was produced by the Glass Ceramic Innovation Institute - “Instituto de Inovação em Vitro-cerâmicas” (Vitrovita, São Carlos, SP, Brazil) in accordance with the process of preparation described in the Patent deposit (7). Biodentine was

manipulated according to the manufacturer's instructions. The reparative materials, their respective manufacturers and proportions used are described in Table 1.

Physicochemical tests

Setting Time

This test was based on ISO Standard 6876/2002 (15). After manipulation, the cements were inserted in metal rings measuring 10 mm in diameter and 1 mm high (n=6). A Gilmore needle with mass of 100 ± 0.5g and a tip diameter of 2 ± 0.1 mm was used, supported on the cement surface. The materials were kept in an oven (37°C and 95% humidity) during analysis and the needle was cleaned between the analyses. The setting times were determined as the time when the marks of needle could not be observed on the sealer surface.

Radiopacity

The radiopacity was determined based on based on ISO Standard 6876/2002 (15). After manipulation, the cements were inserted in rings with an internal diameter of 10 mm and height of 1 mm and kept in an oven at 37°C and 95% humidity for 24 hours. A sample of each material (n=6) and an aluminum scale were placed on an occlusal film (Insight – Kodak Comp, Rochester, NY, USA) to take the radiograph (X-ray appliance -X GE 1000 - General Electric, Milwaukee, WI, USA). The parameters used were 60 kV, 7 mA, 0.32 pulses per second and focus-film distance of 33 cm. The films were processed, digitized, and evaluated by using Image J for Windows software, to determine the radiopacity equivalence of the cements in millimeters of aluminum (mm Al). Thus, the radiopacity of the evaluated cements was estimated by using a conversion equation as performed by Tanomaru-Filho *et al.* (16).

Solubility

Solubility was evaluated in accordance with Carvalho-Junior *et al.* (17) modified. Circular molds measuring 1.5 mm high and 7.75 mm in internal diameter were fabricated. Each mold was placed on a glass plate covered with cellophane paper film and filled with the material (n=6). A Nylon thread was included in the cement mass and another glass plate also enveloped in cellophane was placed on the mold. This unit was kept at 37°C and 95% humidity for three times the duration of their setting time. The test specimens were removed from the molds and weighed on a precision balance (HM-200, A & D Engineering, Inc., Bradford, MA, USA). Then, they were placed in closed plastic flasks containing 7.5 mL of distilled and deionized water and kept in an oven at 37°C for 7 days. The mass was measured

before and after the samples were immersed in distilled water, and every 24 h thereafter, until the mass was stabilized. The loss of mass was expressed as a percentage of the original mass.

pH

For the pH test, polyethylene tubes 10 mm long and 1 mm in diameter were filled with each material (n=10). Each tube was immersed in 10 mL of distilled water and kept in an oven. At each period, the tubes were removed from the flasks and put into new flasks each containing 10 mL of distilled water. Flasks containing only distilled water were used as control. The experimental time intervals were 3, 12, and 24 hours, 7, 14 and 21 days after manipulation of the materials. Immediately after the end of each experimental time interval, the pH of the solutions was measured with a previously calibrated digital pH meter (Digimed Analítica Ltda. Grupo Digicrom Analítica. São Paulo, Brazil).

Biological Tests

For the tests, 0.5 g of each material was manipulated in the proportions described in Table 1. These portions were then placed on the bottoms of 12-well culture plates (Corning, New York, NY, USA) and hydrated with humidified gauze. The culture plates were kept at 37 °C at 95% humidity, 5% CO_2, for 24 hours to allow complete setting of the materials. After this period, the cements were exposed to U.V. Light for 30 minutes to prevent contamination. Then 5mL DMEM culture medium (Sigma/Aldrich) serum-free (without the presence of fetal bovine serum - FBS) were placed in each well of the plates, in which the material was accommodated, and thus remained at 37˚C, 95% humidity and 5% CO_2 for 24 hours to allow formation of the extract of each material. The extracts were collected and diluted in DMEM without FBS, thereby obtaining the 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions for performing the cytotoxicity tests. As negative control, the DMEM culture medium serum-free; and as positive control 20% DMSO were used.

Viability and Biomineralization Potential Assays

MTT

Human osteoblast cells (Saos-2) were plated ($1x10^5$ cells/mL) in 96-well plates (Corning Inc, NY, USA) and kept at 37 °C at 95% humidity, 5% CO_2, for 24 hours. After this period, the cells were exposed to the cement extracts in cell culture for 24 hours. For the MTT assay, the medium was replaced by DMEM serum-free, containing 5 mg/mL of MTT solution (Sigma-Aldrich) and cells were incubated at 37 °C at 95% humidity, 5% CO_2, for 3 hours. After this period, each well was washed with 1 mL phosphate buffer solution (PBS) and 100 µl isopropyl alcohol (acidified 0.04N HCl) was added to solubilize the formazan. The optical density was measured at 570 nm in an automatic microplate reader (ELx800; Instruments Bio-Tek, Winooski, VT, USA). Three independent experiments were performed in triplicate for each experimental group and outcome. The same methodology was used for the MTT assay in the time intervals of 7 and 14 days, in which the cells were plated at $7x10^4$ cells/mL.

Neutral Red

The cells ($1x10^5$ cells/mL) were plated in 96-well plates (Corning) in DMEM medium supplemented with 10% FBS and kept at 37 °C at 95% humidity, 5% CO_2, for 24 hours. After the cells had remained in contact with the cement extracts in their different concentrations for 24 hours, the extracts were replaced by 100 µL DMEM serum-free, containing 50 µg NR/mL (Sigma-Aldrich), followed by incubation at 37º C, 95% humidity and 5% CO_2 for 3 hours. The coloring agent was removed and the colorimetric product was solubilized in 100 µL of 50% ethanol and 1% acetic acid solution (Sigma-Aldrich). The optical density was measured in the plate reader at 570 nm (Asys-UVM 340, Biochrom – MikroWin 2000, USA). Three independent experiments were performed in triplicate for each experimental group and outcome.

Alkaline Phosphatase

Alkaline phosphatase activity was determined by using the commercial Labtest kit (Labtest, Lagoa Santa, MG, Brazil). After plating for 24 hours, ($7x10^4$ cells/mL in 96-well plates), the Saos-2 cells were exposed to the cement extracts for 7 and 14 days. The extracts were renewed every two days throughout the exposure period. After each experimental period, the cells were washed with 200 µL of PBS 1X, followed by the addition of 200 µL of

a sodium lauryl sulphate solution (1% in distilled water, Sigma-Aldrich) to each well. The samples remained at rest at room temperature for 30 minutes, and then 12.5 µL of each sample was transferred to Eppendorf tubes with reagents of the commercial Alkaline Phosphatase kit, according to the manufacturer's instructions (Labtest).

The optical density was evaluated in an automatic miniplate reader (ELx800, Bio-Tek Instruments, Winooski, VT, USA) at 590 nm. The data were expressed as ALP activity normalized by the number of viable cells determined in the MTT assay, in the respective culture period. Three independent experiments were performed in sextuplicate for each experimental group and outcome.

Alizarin Red

With the purpose of evaluating the calcium deposition induced by the materials, Saos-2 cells were plated ($1x10^4$ cells/mL) in 24-well culture plates (Corning, New York, NY, USA) in D-MEM medium supplemented with 10% FBS. For 21 days, the cement extracts prepared with osteogenic DMEM culture medium (DMEM 10% FBS; 100 IU/mL penicillin; 100 mg/mL streptomycin; 0.023 g/mL β-Glycerophosphate; 0.055 mg/mL ascorbic acid – Sigma Chemicals St Louis MO, USA) were renewed every 2 days. The medium was then aspirated, the wells washed with PBS 1X, and the cells fixed with 70% ethanol (Sigma/Aldrich) at 4^0C, for 1 hour. The cells were washed twice with distilled water before the addition of 300 µL Alizarin Red S (at 2% and pH 4.1). The cells were incubated at room temperature for 2 minutes. After this they were washed 4 times with 1 mL distilled water/well for 5 minutes. The plates were left at an angle for 2 minutes, to facilitate the removal of excess water, and were then photographed (Canon EOS-Rebel T6i, Canon Inc, Tokyo, Japan). For quantitative analysis of mineralization nodule formation, the nodules were solubilized in 0.5 mL Cetylpyridinium chloride (Sigma-Aldrich) under agitation for 15 minutes. After homogenization, three aliquots of 100 µL from each well were transferred to a 96-well plate. Mineral nodule formation was analyzed in accordance with the absorbance determined at 562 nm in a plate reader (ELx800, Bio-Tek Instruments). Three independent experiments were performed in triplicate for each experimental group and outcome.

Antibacterial Test

Test of direct contact on planktonic *E. faecalis* cells

Microplates with 96 wells were used, in which the experimental materials were manipulated and inserted on the lateral wall of the well in a standardized manner. The materials were submitted to decontamination under ultraviolet light for 30 minutes. After this, 10 μL of each inoculum was deposited on the materials and on the positive control, to which only the inoculum was added. The plates were kept in an oven at 37°C for 1 hour and 30 minutes. Afterwards, 250 μL of *brain heart infusion* (BHI) were added to each contaminated well, serial decimal dilution was performed, and the plates containing *Tryptic soy agar* (TSa) were seeded. After incubating the plates in an oven at 37°C for 48 hours, cells were counted in CFU mL^{-1}.

Statistical Analysis

For the physicochemical tests the data obtained were submitted to a normality test, and afterwards to the ANOVA and Tukey tests, with 5% level of significance. For the antimicrobial test, the Kruskall-Wallis and Dunn multiple comparison tests were performed, with 5% significance, after logarithmic transformation (log 10). For the cell viability and bioactivity, the data were analyzed by using Two-way ANOVA and Bonferroni tests (α=0.05). Alizarin red staining (ARS) was compared by ANOVA one-way and Tukey tests.

Results

Physicochemical Tests

The radiopacity, setting time and solubility results are described in Table 2. No statistically significant difference between TCS ZrO_2 + 10% Biosilicate and TCS ZrO_2 + 20% were observed for radiopacity, with values higher than 3 mm aluminum. The experimental cements containing the different proportions of Biosilicate presented a setting time similar to that of Biodentine ($P > .05$). The materials presented low solubility, except for Biodentine ($P < .05$).

All the materials presented an alkaline pH in the different time intervals evaluated, as demonstrated in Table 3.

Cell Viability

In the MTT assay, in the 1:1 and 1:2 dilutions, there was lower cell viability in all the cements in comparison to the negative control ($P < .05$). At the 1:4 dilution, there was no statistically significant difference among the cements ($P > .05$), with the exception of pure TCS that presented lower cell viability than the negative control ($P < .05$). At the 1:8, 1:16 and 1:32 dilutions, there was no difference in cell viability among the groups ($P > .05$), however, at the 1:16 dilution, the Biodentine group presented higher cell viability values in comparison with those of the negative control ($P < .05$), as shown in Figure 1.

NR Assay, in the 1:1 dilution, presented lower cell viability in all the cements, except to TCS ZrO_2 + 20% Biosilicate, that was no statistically different in comparison to the negative control ($P < .05$). Biodentine presented lower cell viability in comparison to the negative control ($P < .05$) at the 1:1, 1:2 and 1:4 dilutions. At the 1:8 dilution, the cell viability of Biodentine was similar to the negative control ($P > .05$). In the same dilution, TCS ZrO_2 + 20% Biosilicate was significantly higher when compared to the negative control ($P < .05$). At the other dilutions, 1:16 and 1:32, there was no statistically significant difference ($P > 05$) in cell viability of all materials when compared to the negative control (Figure 2).

ALP Activity

In the MTT assay of 7 days, all the groups presented lower cell viability in comparison with the control ($P < .05$). In 14 days, the cell viability was similar (pure TCS) or lower (TCS ZrO_2 + 10% Biosilicate and TCS ZrO_2 + 20% Biosilicate), in comparison with the control ($P > .05$). Only Biodentine presented higher cell viability in comparison to the negative control ($P < .05$). In the period of 7 days of exposure to the cements, only Biodentine presented higher ALP activity in comparison with the negative control ($P < .05$). At 14 days, the Groups TCS and Biodentine presented higher ALP activity than the control *($p < 05$)*, as shown in Figure 3.

Alizarin Red

As shown in Figure 4, after 21 days of exposure, all the cements induced a large production of mineralized nodules when compared to the control group ($P < .05$). Biodentine were highlighted by its ability to induce more mineralized nodules than the other ($P < .05$)

Antibacterial Activity

For the test of direct contact on planktonic *E. faecalis* cells, all the materials presented antibacterial activity, differing from the control group ($P < .05$). Biodentine and

TCS ZrO_2 + 20% Biosilicate presented lower bacterial count values ($P < .0001$) than pure TCS and TCS ZrO_2 + 10% Biosilicate ($P < .01$), when compared with the control. The data are presented in Table 4.

Discussion

The present study demonstrated that in the two concentrations of Biosilicate associated with tricalcium silicate and the ZrO_2 radiopacifier, the experimental materials presented proper physicochemical, biological and antibacterial properties, with potential for use as reparative materials. We chose to add zirconium oxide to the TCS, because it has been shown to be inert, release calcium ions (1), has a setting time similar to MTA Angelus, low solubility, and suitable radiopacity (19). In accordance with the ISO 6876/2002 standard, an endodontic material must present radiopacity of over 3 mm Aluminum (mmAl). In this study, the pure TCS and Biodentine cements presented radiopacity values lower than those recommended by ISO 6876/2002, according to previous studies (19,20). Although Biodentine contains ZrO_2 as radiopacifier, the proportion is 5% (1), which may explain its low radiopacity. In the present study, 30% of this radiopacifier was added to obtain an experimental cement with good radiopacity. The incorporation of 10% or 20% of Biosilicate promoted no significant change in the radiopacity of the experimental cements.

Pure TCS was the material with the longest setting time in comparison with the other materials. The addition of ZrO_2 and Biosilicate in the two proportions accelerated the setting process of TCS. The shorter setting time of Biodentine when compared with TCS is related to the calcium chloride present in its liquid, which decreases the setting time (21). Moreover, the calcium carbonate present in the Biodentine powder acts on the mechanism of hydration of this cement, also reducing its setting time (1). The Biosilicate might have acted on accelerating the hydration reaction of TCS. Biodentine is known to have the potential to form hydroxycarbonate apatite (11), and when associated with a vehicle, it promotes a faster calcium ion release when compared with Biosilicate (22).

Biodentine was the material that showed the highest solubility values, corroborating the findings of previous studies (23,24). The high solubility of this material may be related to the incorporation of a hydrosoluble plasticizer into a polymer, which has a tensoactive effect, dispersing the particles of the cement and thus promoting a higher level of solubility (23). Regarding the cements associated with Biosilicate, the solubility values found were in accordance with ISO 6876/2002, i.e., below 3%.

Biosilicate added to TCS + ZrO_2 in the concentration of 10%, presented a gain in mass after the solubility test, differing from pure TCS, whereas, in the concentration of 20%, its solubility was similar to that of pure TCS. The results obtained with the experimental cements containing Biosilicate were in disagreement with the study of Vivan *et al*. (25) who observed a correlation between the properties of solubility and setting time. Although the addition of Biosilicate and ZrO_2 radiopacifier accelerated the hydration process, and consequently, decreased the setting time of TCS, it solubility was not changed at the concentration of 20%, whereas, there was water absorption by the cement with the lower concentration (10% Biosilicate) and corroborated with the study of Cutajar *et al*. (26) who showed that the water/cement proportion effect on the solubility of the material. Gandolfi *et al.* (26) evaluated a new endodontic sealer with bioactive glass incorporated into its composition (GuttaFlow Bioseal) and observed a shorter setting time for this material, when compared with similar sealers without bioactive glass.

Biodentine and the hydraulic calcium silicate-based cements have a known capacity for alkalinization (19, 20). In this study, all the cements had an alkaline pH (approximately 10). Except for the period of 3 hours, Biosilicate incorporated into tricalcium silicate and the ZrO_2 radiopacifier in both concentrations promoted no significant change in increasing the pH in comparison with pure TCS.

The cements evaluated presented no cytotoxic effects in both the cytotoxicity assays, with the exception of Biodentine that showed higher cytotoxicity at the lower dilutions (1:1, 1:2) when compared to the negative control. Previous studies have shown lower cell viability for Biodentine at the lower dilutions of the cement extracts (1:1, 1:2 and 1:4) when compared to the negative control (27, 28).

The highest ALP activity was detected for the pure TCS and Biodentine cements in the period of 14 days. When the tricalcium silicate cements come into contact with the tissue fluids, they produce hydrated hydraulic calcium silicate and calcium hydroxide (29), which releases calcium ions essential for cell migration, adhesion, proliferation and differentiation, and contribute to the formation of hydroxyapatite (30). Tricalcium silicate cement associated with tantalum oxide (Ta_2O_5), was biocompatible and induced ALP activity in Saos-2 cells (31). Biodentine presented biocompatibility and osteogenic bioactivity when compared with MTA and tricalcium silicate-based materials (27,28).

In the literature, there are no studies that showed the deposition of mineralization nodules in pure tricalcium silicate cement. However, our results showed that Pure TCS, Biodentine, TCS ZrO_2 + 10% Biosilicate and TCS ZrO_2 + 20% Biosilicate stimulated the

production of mineralization nodules. The largest deposition of calcium nodules was observed for Biodentine. Biodentine was capable of depositing calcium nodules when in contact with Saos-2 cells (27, 28), and human dental pulp cells (hDPCs) (32).

All the materials were effective against *E. faecalis.* Biodentine and the association of 20% Biosilicate showed a lower bacterial count. These findings are in agreement with previous studies that observed antimicrobial effectiveness for Portland Cement associated with ZrO_2 (5, 6, 33), and for Biodentine (34). Our results for Biosilicate corroborate those of the study of Martins *et al.* (12), which showed that this material had a broad spectrum of antimicrobial properties; and its association with TCS in our study allowed a significant increase in the antimicrobial effect of this material against *E. faecalis.* The antimicrobial action of the materials evaluated may be explained by the release of hydroxyl ions, which create an environment unfavorable to bacterial proliferation and survival (35).

Based on the results obtained in the present study, the association of Biosilicate (10% or 20%) with TCS+ ZrO_2 showed adequate physicochemical properties, demonstrating the capacity to reduce setting time and adequate solubility. In addition, the above-mentioned materials demonstrated antimicrobial activity, bioactivity, and were not cytotoxic, suggesting their potential for clinical use.

Acknowledgments

This study was supported by FAPESP (2015/03437-6, 2016/11036-4 and 2016/00321-0) and CAPES (2016/1604159). The authors deny any conflicts of interest related to this study.

References

1. Camilleri J, Sorrentino F, Damidot D. Investigation of the hydration and bioactivity of radiopacified tricalcium silicate cement, Biodentine and MTA Angelus. Dent Mater 2013;29:580-93.

2. Li X, Yoshihara K, De Munck J, *et al.* Modified tricalcium silicate cement formulations with added zirconium oxide. Clin Oral Investig 2017;21:895-905.

3. Laurent P, Camps J, About I. Biodentine(TM) induces TGF-beta1 release from human pulp cells and early dental pulp mineralization. Int Endod J 2012;45:439-448.

4. Tran XV, Gorin C, Willig C, *et al.* Effect of a calcium-silicate-based restorative cement on pulp repair. J Dent Res 2012;91:1166-71

5. Vazquez-Garcia F, Tanomaru-Filho M, Chávez-Andrade GM, *et al.* Effect of Silver Nanoparticles on Physicochemical and Antibacterial Properties of Calcium Silicate Cements. Braz Dent J 2016;27:508-14.

6. Guerreiro-Tanomaru JM, Vázquez-García FA, Bosso-Martelo R, *et al.* Effect of addition of nano-hydroxyapatite on physico-chemical and antibiofilm properties of calcium silicate cements. J Appl Oral Scie 2016;24:204-10.

7. Zanotto ED *et al.* Fundação universidade federal de são carlos; universidade de são paulo. Process and compositions for preparing particulate, bioactive or resorbable biosilicates for use in the treatment of oral ailments. Int. C. C03C10/00, 20 Feb. 2004, WO2004/074199.

8. Moura J, Teixeira LN, Ravagnani C, *et al.* In vitro osteogenesis on a highly bioactive glass-ceramic (Biosilicate). J Biomed Mater Res A 2007;82:545-57.

9. Stoor P, Söderling E, Salonen JI. Antibacterial effects of a bioactive glasspaste on oral microorganisms. Acta Odontol Scand 1998;56:161-5.

10. Pinto KN, Tim CR, Crovace MC, *et al.* Effects of biosilicate® scaffolds and low-level laser therapy on the process of bone healing. Photomed Laser Surg 2013;31:252-60.

11. Azenha MR, de Lacerda SA, Marão HF, *et al.* Evaluation of Crystallized Biosilicate in the Reconstruction of Calvarial Defects. J Maxillofac Oral Surg 2015;14:659-65.

12. Martins CH, Carvalho TC, Souza MG, *et al.* Assessment of antimicrobial effect of Biosilicate® against anaerobic, microaerophilic and facultative anaerobic microorganisms. J Mater Sci Mater Med 2011;22:1439-46.

13. Gabbai-Armelin PR, Renno AC, Crovace MC, *et al.* Putty-like bone fillers based on CaP ceramics or Biosilicate® combined with carboxymethylcellulose: Characterization, optimization, and evaluation. J Biomater Appl 2017;32:276-88.

14. Kido HW, Oliveira P, Parizotto NA, *et al.* Histopathological, cytotoxicity and genotoxicity evaluation of Biosilicate® glass-ceramic scaffolds. J Biomed Mater Res A 2013;101:667-73.

15. International Organization for Standardization Dentistry (ISO). ISO 6876. Root Canal Sealing Materials. London, UK: British Standards Institution; 2002.

16. Tanomaru-Filho M, Jorge EG, Guerreiro Tanomaru JM, *et al.* Radiopacity evaluation of new root canal filling materials by digitalization of images. J Endod 2007;33:249-51.

17. Carvalho-Junior JR, Correr-Sobrinho L, Correr AB, *et al.* Solubility and dimensional change after setting of root canal sealers: a proposal for smaller dimensions of test samples. J Endod 2007;33:1110-6.

18. International Organization for Standardization (ISO). ISO 10993-5. Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity. Switzerland; 2009.

19. Bosso-Martelo R, Guerreiro-Tanomaru JM, Viapiana R, *et al.* Physicochemical properties of calcium silicate cements associated with microparticulate and nanoparticulate radiopacifiers. Clin Oral Invest 2016;20:83-90.

20. Lucas CP, Viapiana R, Bosso-Martelo R, *et al.* Physicochemical Properties and Dentin Bond Strength of a Tricalcium Silicate-Based Retrograde Material. Braz Dent J 2017;28:51-6.

21. Grech L, Mallia B, Camilleri J. Characterization of set Intermediate Restorative Material, Biodentine, Bioaggregate and a prototype calcium silicate cement for use as root-end filling materials. Int Endod J 2013;46:632-41.

22. Kido HW, Brassolatti P, Tim CR, *et al.* Porous poly (D,L-lactide-co-glycolide) acid/biosilicate(®) composite scaffolds for bone tissue engineering. J Biomed Mater Res B Appl Biomater 2017;105:63-71.

23. Dawood AE, Manton DJ, Parashos P, *et al.* The physical properties and ion release of CPP-ACP-modified calcium silicate-based cements Aust Dent J 2015;60:434-44.

24. Singh S, Podar R, Dadu S, *et al.* Solubility of a new calcium silicate-based root-end filling material. J Conserv Dent 2015;18:149-53.

25. Vivan RR, Zapata RO, Zeferino MA, et al. Evaluation of the physical and chemical properties of two commercial and three experimental root-end filling materials. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;110:250-6.

26. Cutajar Cutajar A, Mallia B, Abela S, Camilleri J. Replacement of radiopacifier in mineral trioxide aggregate; characterization and determination of physical properties. Dent Mater. 2011;27:879-91.

27. Gandolfi MG, Siboni F, Prati C. Properties of a novel polysiloxane-guttapercha calcium silicate-bioglass-containing root canal sealer. Dent Mater 2016;32:e113-26.

28. Gomes-Cornelio AL, Rodrigues EM, Salles LP *et al.* Bioactivity of MTA Plus, Biodentine and an experimental calcium silicate-based cement on human osteoblast-like cells. Int Endod J 2017;50:39–47.

29. Rodrigues EM, Gomes-Cornélio AL, Soares-Costa A, *et al.* An assessment of the overexpression of BMP-2 in transfected human osteoblast cells stimulated by mineral trioxide aggregate and Biodentine. Int Endod J 2017;50:e9-e18.

30. Camilleri J. Staining potential of Neo MTA Plus, MTA Plus and Biodentine used for pulpotomy procedures. J Endod 2015;41:1139-45.

31. Gandolfi MG, Taddei P, Modena E, et al. Biointeractivity-related versus chemi/physisorption-related apatite precursor-forming ability of current root end filling materials. Appl Biomater 2013;101:1107-23.

32. Tanomaru-Filho M, Andrade AS, Rodrigues EM, *et al.* Biocompatibility and mineralized nodule formation of NeoMTA Plus and an experimental tricalcium silicate cement containing tantalum oxide. Int Endod J 2017;50:e30-e39.

33. Luo Z, Kohli MR, Yu Q, *et al.* Biodentine induces human dental pulp stem cell differentiation through mitogen-activated protein kinase and calcium-/calmodulin-dependent protein kinase II pathways. J Endod 2014;40:937-42.

34. Guerreiro-Tanomaru JM, Storto I, Da Silva GF, *et al.* Radiopacity, pH and antimicrobial activity of Portland cement associated with micro- and nanoparticles of zirconium oxide and niobium oxide. Dent Mater J 2014;33:466-70.

35. Koruyucu M, Topcuoglu N, Tuna EB, *et al.* An assessment of antibacterial activity of three pulp capping materials on Enterococcus faecalis by a direct contact test: An in vitro study. Eur J Dent 2015;9:240-5.

36. Gandolfi MG, Siboni F, Prati C. Chemical-physical properties of TheraCal, a novel light-curable MTA-like material for pulp capping. Int Endod J 2012;45:571-9.

TABLES

TABLE 1. Experimental Materials, Manufacturers, and Proportions used

Group	Manufacturer/Proportion
Biodentine	Septodont, Saint Maur des Fossés, France **1 capsule of powder: 5 drops liquid**
TCS	100% tricalcium silicate- based cement (Mineral Research Processing, Meyzieu, France) **1g powder: 330 µL Distilled water**
TCS ZrO_2 + 10% Biosilicate	60% tricalcium silicate-based cement (TCS, Mineral Research Processing, Meyzieu, France) 10% Biosilicate (Vitrovita, São Carlos, SP, Brazil). 30% Zirconium Oxide (ZrO_2, Sigma-Aldrich Brasil Ltda., São Paulo, SP, Brazil) **1g powder: 300 µL Distilled water**
TCS ZrO_2 + 20% Biosilicate	50% tricalcium silicate-based cement (TCS, Mineral Research Processing, Meyzieu, France) 20% Biosilicate (Vitrovita, São Carlos, SP, Brazil). 30% Zirconium Oxide (ZrO_2, Sigma-Aldrich Brasil Ltda., São Paulo, SP, Brazil) **1g powder: 300 µL Distilled water**

TCS- tricalcium silicate; ZrO_2- zirconium oxide.

TABLE 2. Mean and Standard Deviation (±) of radiopacity (mmAl), setting time (minutes), and solubility results for the different materials evaluated.

	Biodentine	**TCS**	**TCS ZrO_2 + 10% Biosilicate**	**TCS ZrO_2 + 20% Biosilicate**
Radiopacity (mmAl)	2.26^{b} (± 0.12)	1.69^{c} (±0.06)	3.93^{a} (±0.20)	4.02^{a} (±0.13)
Setting time (minutes)	34.17^{b} (±2.40)	44.00^{a} (±1.63)	37.17^{b} (±2.60)	35.00^{b} (±2.51)
Solubility (%)	3.69^{a} (±0.77)	1.81^{b} (±0.64)	-0.14^{c} (±0.14)	1.16^{b} (± 0.13)

TCS- tricalcium silicate; ZrO_2- zirconium oxide. Different letters in the same column indicate statistically significant difference among the experimental groups ($P < .05$).

TABLE 3. Mean and Standard Deviations (±) of pH value results found in the different time intervals of analysis.

pH	Biodentine	TCS	TCS ZrO_2 + 10% Biosilicate	TCS ZrO_2 + 20% Biosilicate	Control
3 hours	11.7^{a} (±0.25)	11.2^{b} (± 0.21)	10.3^{c} (±0.26)	10.8^{b} (± 0.40)	6.8^{d} (±0.40)
12 hours	10.1^{a} (±0.39)	9.5^{a} (±0.27)	9.8^{a} (±0.67)	9.9^{a} (±0.49)	6.6^{b} (± 0.30)
24 hours	9.6^{a} (±0.24)	9.5^{a} (±0.62)	9.7^{a} (±0.47)	9.8^{a} (±0.48)	6.4^{b} (±0.10)
7 days	10.8^{ab} (±0.53)	10.2^{b} (±0.66)	11.1^{a} (±0.26)	11.2^{a} (±0.45)	6.6^{c} (±0.22)
14 days	10.6^{ab} (±0.31)	10.4^{b} (± 0.49)	11.0^{a} (±0.30)	10.7^{ab} (±0.25)	6.7^{c} (±0.23)
21 days	10.0^{a} (±0.43)	9.2^{b} (±0.71)	10.1^{a} (±0.25)	10.1^{a} (0.19)	6.7^{c} (±0.26)

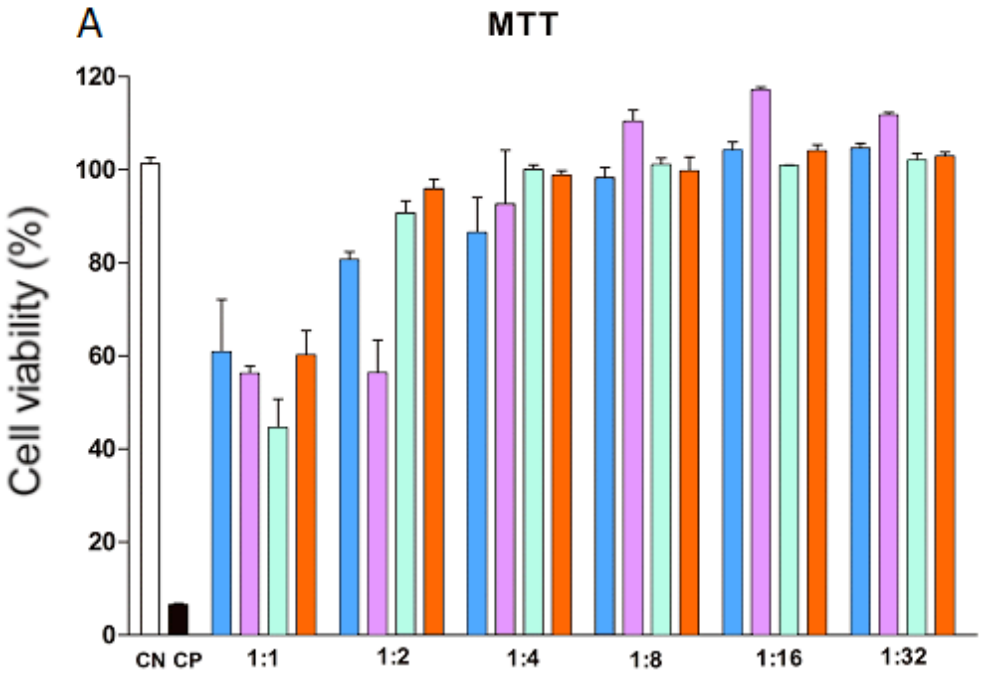
TCS- tricalcium silicate; ZrO_2- zirconium oxide. Different letters in the same line indicate statistically significant difference among the groups ($P < .05$).

TABLE 4. Mean and Standard Deviation (±) CFU (mL^{-1} log_{10}) of the materials evaluated.

Groups	Biodentine	TCS	TCS ZrO2 + 10% Biosilicate	TCS ZrO2 + 20% Biosilicate	Control
CFU mL^{-1} log_{10}	0.00^{b**}	0.67^{b*}	0.64^{b*}	0.00^{b**}	8.04^{a}
	(±0.00)	(±1.26)	(±1.19)	(±0.00)	(±0.18)

TCS- tricalcium silicate; ZrO_2- zirconium oxide. Different letters in the same line indicate statistically significant difference among the groups ($P < .05$). *Kruskall-Wallis and Dunn ($P < .01$). **Kruskall-Wallis and Dunn ($P < .0001$).

Figures

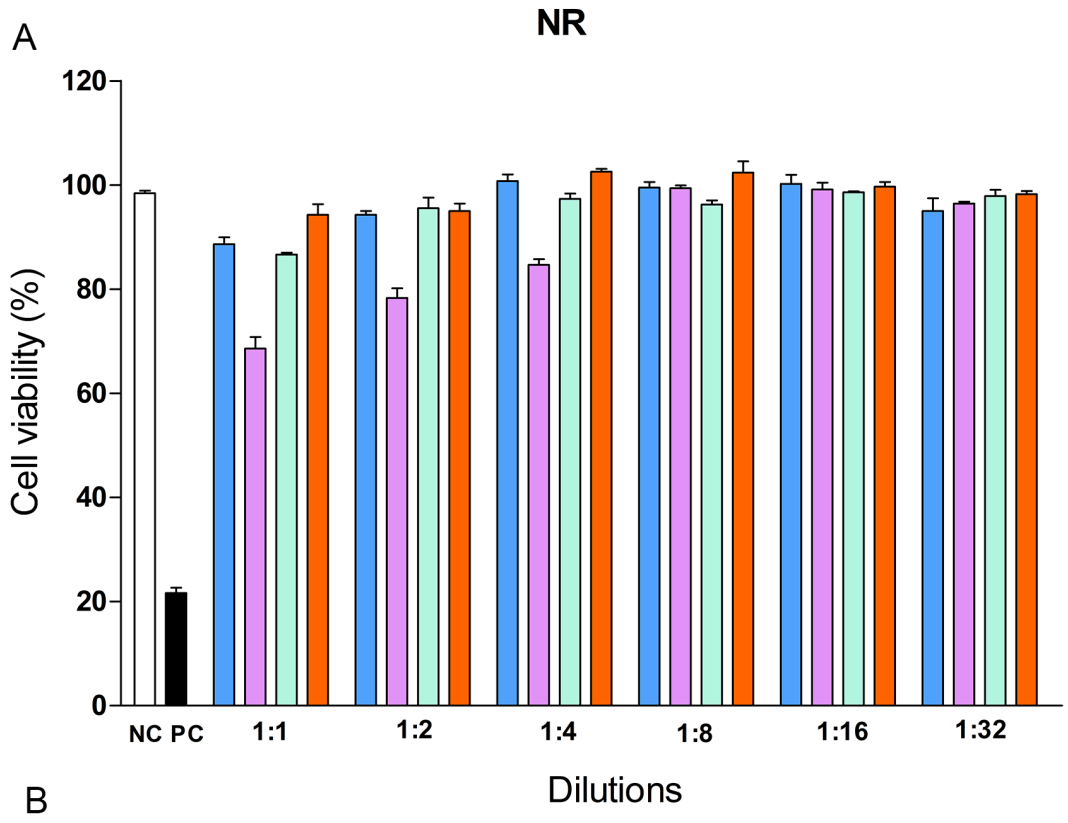


B

Dilutions		1:1	1:2	1:4	1:8	1:16	1:32
TCS		c	c	c	a	a,c	a
Biodentine		c,d	d	a,c	a	c	a
TCS ZrO_2 + 10% Biosilicate		d	a,c	a,c	a	a	a
TCS ZrO_2 + 20% Biosilicate		c	a	a,c	a	a,c	a
Negative Control (NC)		a	a	a	a	a	a
Positive Control (PC)		b	b	b	b	b	b

Figure 1. (A) Cell Viability (%) evaluated by MTT assay after 24 hours exposure of Saos-2 cells to the eluates of cements in different dilutions, and to the negative (NC) and positive controls (PC). (B) Statistical comparison of cell viability (%). Different letters in the columns indicate statistically significant difference among the groups in each dilution. TCS- tricalcium silicate; ZrO_2- zirconium oxide.

A



B

Dilutions		1:1	1:2	1:4	1:8	1:16	1:32
TCS		c	a	a,d	a,c	a	a
Biodentine		d	c	c	a	a	a
TSC ZrO_2 + 10% Biosilicate		c	a	a	a	a	a
TSC ZrO_2 + 20% Biosilicate		a	a	d	c	a	a
Negative Control (NC)		a	a	a,d	a	a	a
Positive Control (PC)		b	b	b	b	b	b

Figure 2. (A) Cell Viability (%) evaluated by NR assay after 24 hours exposure of Saos-2 cells to the eluates of cements in different dilutions, and to the negative (NC) and positive controls (PC). (B) Statistical comparison of cell viability (%). Different letters in the columns indicate statistically significant difference among the groups in each dilution. TCS- tricalcium silicate; ZrO_2- zirconium oxide.

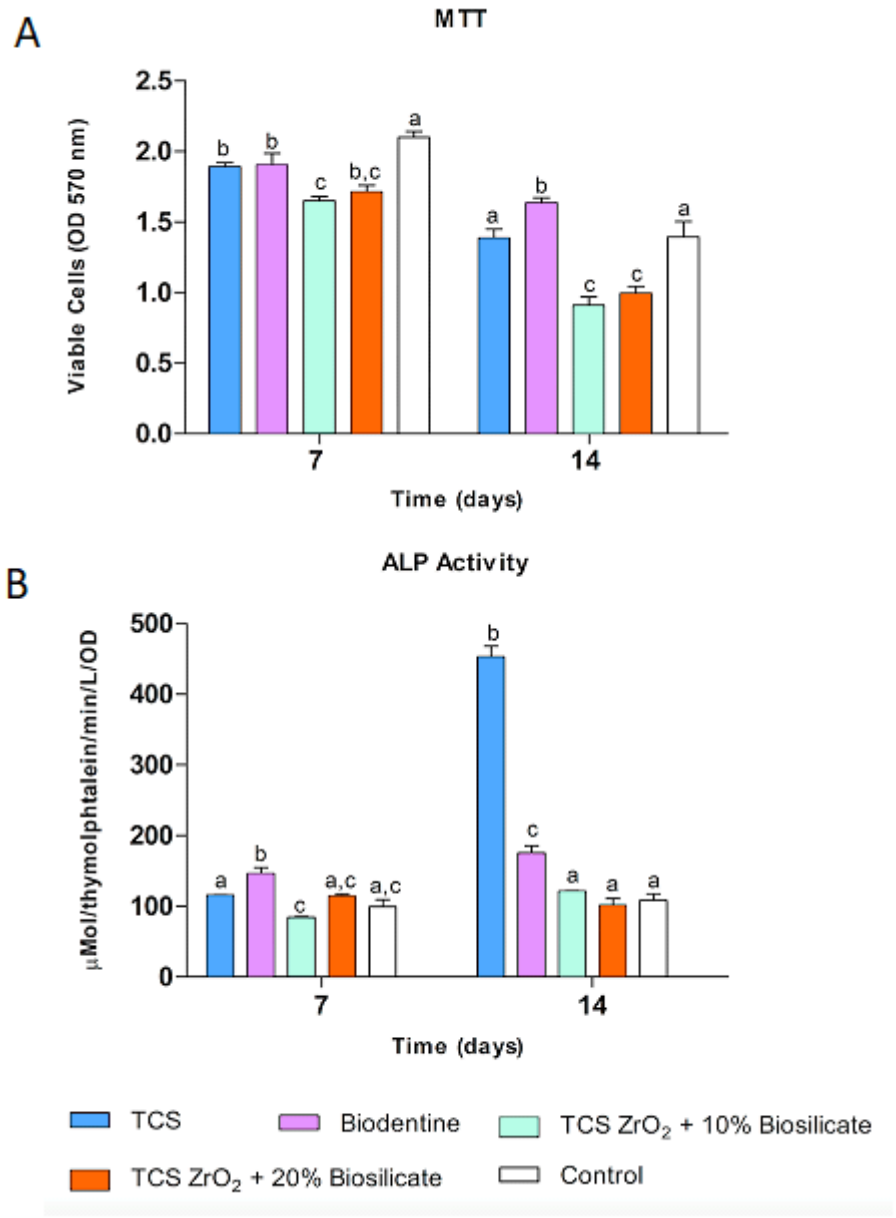


Figure 3. (A) MTT assay and (B) Alkaline Phosphatase Activity (ALP) in osteoblast cells of the human Saos-2 lineage after exposure to the eluates of cements and to the control (culture medium D-MEM + 1% FBS) in different time intervals. Bars with different letters in each time interval indicate statistically significant difference among the groups. TCS- tricalcium silicate; ZrO_2- zirconium oxide.

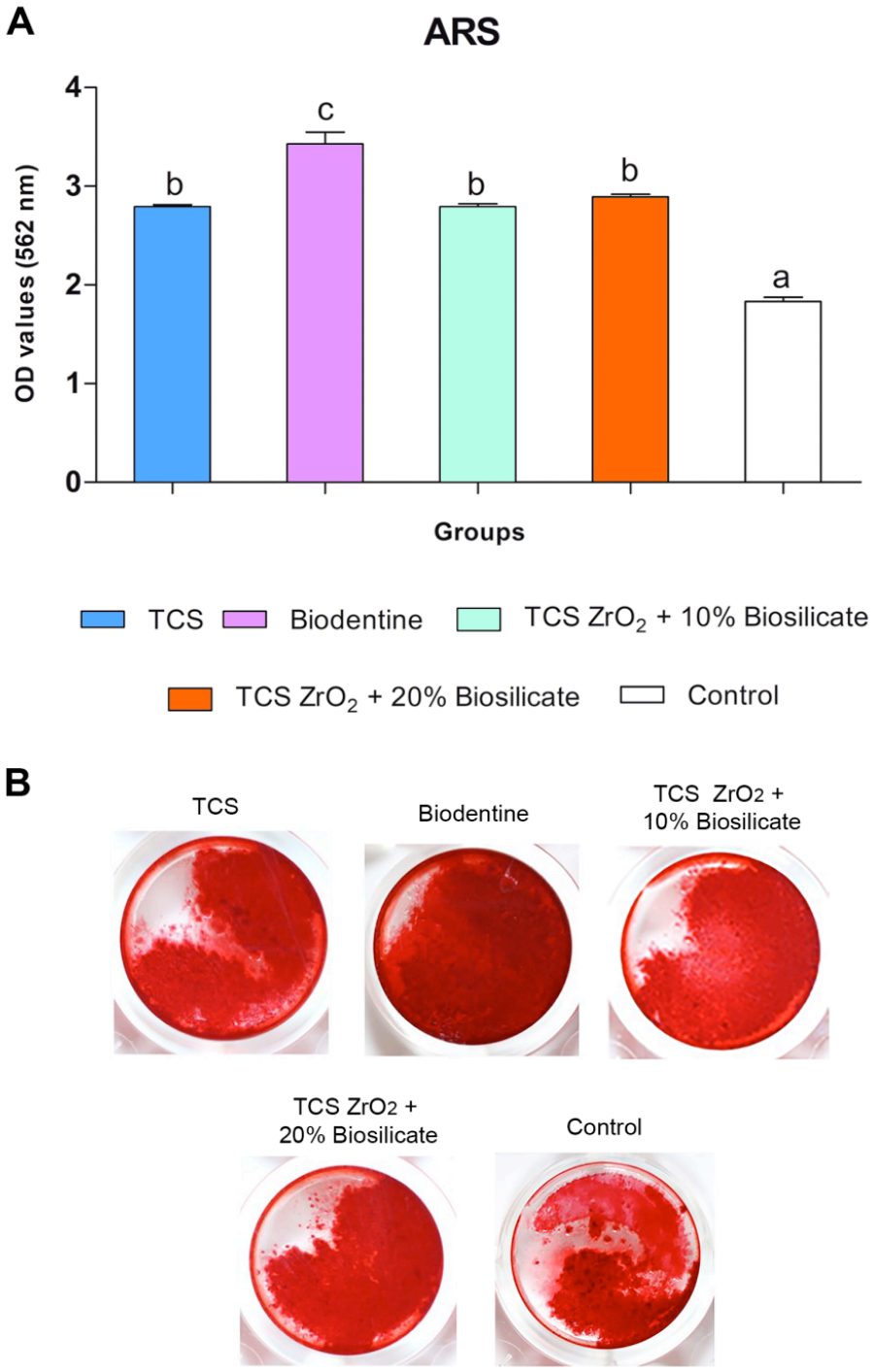


Figure 4. (A) Alizarin red staining (ARS) statistical analysis after 14 days exposure to the cement eluates. (B) Osteogenic culture of Saos-2 showing calcium deposition as demonstrated by positive ARS. Cells exposed to osteogenic culture medium were the control group. Bars with different letters represent significant differences among the groups. TCS- tricalcium silicate; ZrO_2- zirconium oxide.

5 DISCUSSION

Reparative materials must have adequate physico-chemical and biological properties Mineral Trioxide Aggregate (MTA) is a hydraulic calcium silicate-based reparative material[58] that is biocompatible and capable of inducing mineralized tissue formation[59.60]. At present, pure tricalcium silicate[7,8] has been indicated as a reparative material because of the possibility of incorporating heavy metals into it during hydration of this cement[58].

Tricalcium silicate (TCS) is used as the main constituent in the formulation of new biomaterials[38,61]. The bioactive potential of this material induces the formation of apatite precipitates, leaving to mineralized tissue formation[62,63]. The induction of cell growth and differentiation has also been demonstrated, with the formation of hydroxyapatite[38].

TCS has biological characteristics and bioactive potential similar to those of MTA[38,61,62,63], and in association with a radiopacifier, it is indicated as a substitute for MTA.

The consistency of MTA makes it difficult to manipulate[64], and the bismuth oxide used as radiopacifier in its composition, harms its physico-chemical properties and may cause tooth darkening[5,11,12]. Therefore, bioactive endodontic cements have been developed[65] using pure tricalcium silicate associated with different radiopacifiers such as zirconium oxide (ZrO_2), calcium tungstate ($CaWO_4$) and niobium oxide (Nb_2O_5).

Biodentine cement is an example of a hydraulic calcium silicate-based biomaterial developed as a substitute for MTA cement[7.] Biosilicate is a glass-ceramic that is highly bioactive, osteoconductive, non-cytotoxic, non-genotoxic and also presents an antibacterial property[48]; these are desirable characteristics for a biomaterial. Its use in association with hydraulic calcium silicate cements for reparative use in endodontics has not yet been studied.

In the present study, the different cements evaluated presented radiopacity in accordance with the specifications of the ISO Standard 6876/2002, with the exception of pure TCS and Biodentine. Our results corroborated those of studies that observed adequate radiopacity for the ZrO_2[8,17,39], $CaWO_4$[39] and Nb_2O_5[17,30,33] radiopacifiers when added to PC or to TCS; and values below those recommended for TCS[17,39] and Biodentine[25,66,67]. MTA HP contains $CaWO_4$ as radiopacifier,

however, this material presented lower radiopacity, which could be justified by the different proportion of radiopacifier added to the material.

The results of the present study demonstrated that the addition of Nb_2O_5 diminished the setting time of TCS, in agreement with the study of Bosso-Martelo et al.[35], while the addition of $CaWO_4$ increased its setting time, corroborating the findings of Hungaro-Duarte et al. [35]

The solubility of the cements was evaluated after 7 days of immersion in distilled water. With the exception of Biodentine, all the materials showed values in accordance with the standard ISO 6876/2002, with solubility lower than 3%. Previous studies evaluating the solubility of Biodentine also demonstrated high solubility values for this material[67-69]. As regards the cements associated with Biosilicate, the values found were promising, because this material obtained a low percentage of solubility, particularly in the proportion of 10%. All the cements presented an alkaline pH, in agreement with the study of Camilleri[70], who associated different radiopacifiers with Portland cement.

Furthermore, in vitro biological tests were performed in Saos-2 osteoblast cell cultures. An increase in the cell viability of MTA HP in comparison with the negative control was observed, in agreement with the study of Cintra et al.[42]. After 7 days exposure to the cements, the materials that presented the highest ALP activity values were TCS + ZrO_2 and TCS + $CaWO_4$ ($p<0.05$), corroborating the findings of Huffman et al.[71], who described that $CaWO_4$ was able to contribute to a higher level of calcium ion release, thus helping with the process of biomineralization. The highest ALP activity value was detected in the period of 14 days, in comparison with the control, with emphasis on the TCS, TCS + ZrO_2, TCS + $CaWO_4$ and Biodentine ($p<0.05$).

The cements evaluated presented no cytotoxic effects in both the cytotoxicity assays, with the exception of Biodentine that showed lower cell viability at the lower dilutions (MTT) and (NR) in comparison with the negative control. In a similar manner, Gomes-Cornélio et al.[38] observed that Biodentine in the dilutions of 1:1 and 1:2 presented lower cell viability than the negative control. Rodrigues *et al.*[26] in a study with Saos-2 cells, also observed a reduction in cell viability for Biodentine in the dilutions 1:2 and 1:4, when compared with the negative control. Chang et al.[72] demonstrated that Biodentine presented high ALP activity values in comparison with the control.

According to the results obtained in the present study, the association of TCS with the different radiopacifiers presented adequate physico-chemical properties; presented no cytotoxicity, and showed both bioactive and antimicrobial potential. Furthermore, the incorporation of Biosilicate in the concentration of 10% or 20% into TCS + ZrO_2 demonstrated adequate physico-chemical properties, capacity to reduce setting time and solubility of TCS, and presented antimicrobial action without cytotoxic effects. Considering that the materials showed excellent prospects for clinical use; and that the addition of alternative radiopacifiers and Biosilicate improved the manipulation properties of TCS, a possible complementation would be to perform later in vivo tests, with the purpose of proving the biocompatibility of these materials and confirming their indication for use in endodontic practice.

6 CONCLUSION

Based on the results obtained,

- The tricalcium silicate cements associated with the radiopacifiers ZrO_2, $CaWO_4$, Nb_2O_5 and with Biosilicate (10% or 20%), as well as MTA HP presented proper setting time, radiopacity, antimicrobial action and solubility.
- Biodentine was not in accordance to ISO 6876/2002 standards regarding solubility and radiopacity.
- All the materials evaluated presented alkaline pH.
- The materials TCS + ZrO_2; TCS + Nb_2O_5; TCS + $CaWO_4$; MTA HP, and Biodentine presented no cytotoxic effects and increased the biomineralization potential in Saos-2 cells.
- The association of Biosilicate (10% or 20%) with TCS + ZrO_2 demonstrated adequate physico-chemical properties and capacity to reduce setting time and solubility of TCS, in addition to presenting no cytotoxic effects.

REFERENCES*

1. Darvell BW, Wu RC. "MTA"- an hydraulic silicate cement: review update and setting reaction. Dent Mater. 2011; 27(5): 407-22.
2. Lee SJ, Monsef M, Torabinejad M. Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. J Endod. 1993; 19(11): 541-4.
3. Parirokh M, Torabinejad M. Mineral trioxide aggregate: a comprehensive literature review--Part I: chemical, physical, and antibacterial properties. J Endod. 2010; 36(1): 16-27
4. Camileri J. Hydration mechanisms of mineral trioxide aggregate. Int Endod J. 2007; 40(6): 462-70.
5. Camilleri J. Characterization of hydration products of mineral trioxide aggregate. Int Endod J. 2008; 41(5): 408–17.
6. Gandolfi MG, Taddei P, Tinti A, Prati C. Apatite- forming ability (bioactivity) of ProRoot MTA. Int Endod J. 2010b; 43(10): 917–29.
7. Camilleri J, Sorrentino F, Damidot D. Investigation of the hydration and bioactivity of radiopacified tricalcium silicate cement, Biodentine and MTA Angelus. Dent Mater. 2013; 29(5): 580-93.
8. Grech L, Mallia B, Camilleri J. Investigation of the physical properties of tricalcium silicate cement-based root-end filling materials. Dent Mater. 2013; 29(2): 20-8.
9. Camilleri J, Cutajar A, Mallia B. Hydration characteristics of zirconium oxide replaced Portland cement for use as a root-end filling material. Dent Mater. 2011; 27(8): 845–54.
10. Camilleri J, Mallia B. Evaluation of the dimensional changes of mineral trioxide aggregate sealer. Int Endod J. 2011; 44(5): 416-24.
11. Coomaraswamy K, Lumley PJ, Hofmann MP. Effect of bismuth oxide radioopacifier content on the material properties of an endodontic Portland cement–based (MTA-like) System. J Endod. 2007; 33(3): 295-8.

* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf

12. Marciano MA, Costa RM, Camilleri J, Mondelli RF, Guimarães BM, Duarte MA. Assessment of color stability of white mineral trioxide aggregate angelus and bismuth oxide in contact with tooth structure. J Endod. 2014; 40(8): 1235-40.
13. Cutajar A, Mallia B, Abela S, Camilleri J. Replacement of radiopacifier in mineral trioxide aggregate; characterization and determination of physical properties. Dent Mater. 2011; 27(9): 879–91.
14. Antonijevic D, Medigovic I, Zrilic M, Jokic B, Vukovic Z, Todorovic L. The influence of different radiopacifying agents on the radiopacity, compressive strength, setting time, and porosity of Portland cement. Clin Oral Investig. 2014;18 (6):1597-604.
15. Camilleri J, Grech L, Galea K, Keir D, Fenech M, Formosa L, *et al.* Porosity and root dentine to material interface assessment of calcium silicate-based root-end filling materials. Clin Oral Investig. 2014; 18(5): 1437-46.
16. Camilleri J. Evaluation of the effect of intrinsic material properties and ambient conditions on the dimensional stability of white mineral trioxide aggregate and Portland cement. J Endod. 2011; 37(2): 239-45
17. Bosso-Martelo R, Guerreiro-Tanomaru JM, Viapiana R, Berbert FL, Duarte MA, Tanomaru-Filho M. Physicochemical properties of calcium silicate cements associated with microparticulate and nanoparticulateradiopacifiers. Clin Oral Investig. 2016; 20(1): 83-90
18. Espir CG, Guerreiro-Tanomaru JM, Spin-Neto R, Chávez-Andrade GM, Berbert FL, Tanomaru-Filho M. Solubility and bacterial sealing ability of MTA and root-end filling materials. J Appl Oral Sci. 2016; 24(2): 121-5.
19. Laurent P, Camps J, De Meo M, Dejou J, About I. Induction of specific cell responses to a Ca(3) SiO(5)-based posterior restorative material. Dent Mater. 2008; 24(11): 1486–94.
20. Laurent P, Camps J, About I. Biodentine (TM) induces TGF-beta1 release from human pulp cells and early dental pulp mineralization. Int Endod J. 2012; 45(5): 439-48.
21. Koubi S, Elmerini H, Koubi G, Tassery H, Camps J. Quantitative evaluation by glucose diffusion of microleakage in aged calcium silicate-based open-sandwich restorations. Int J Dent. 2012; 2012: 105863.

22. Tran XV, Gorin C, Willig C, Baroukh B, Pellat B, Decup F, et al. Effect of a calcium-silicate-based restorative cement on pulp repair. J Dent Res. 2012; 91(12): 1166-71.
23. Koubi G, Colon P, Franquin JC, Hartmann A, Richard G, Faure MO, *et al.* Clinical evaluation of the performance and safety of a new dentine substitute, Biodentine, in the restoration of posterior teeth - a prospective study. Clin Oral Investig. 2013; 17(1): 243-9.
24. Butt N, Talwar S, Chaudhry S, Nawal RR, Yadav S, Bali A. Comparison of physical and mechanical properties of mineral trioxide aggregate and Biodentine. Indian J Dent Res. 2014; 25(6): 692-7.
25. Lucas CP, Viapiana R, Bosso-Martelo R, Guerreiro-Tanomaru JM, Camilleri J, Tanomaru-Filho M. Physicochemical properties and dentin bond strength of a tricalcium silicate-based retrograde material. Braz Dent J. 2017; 28(1): 51-6.
26. Rodrigues EM, Gomes-Cornélio AL, Soares-Costa A, Salles LP, Velayutham M, Rossa-Junior C, et al. An assessment of the overexpression of BMP-2 in transfected human osteoblast cells stimulated by mineral trioxide aggregate and Biodentine. Int Endod J. 2017; 50(2): e9-e18.
27. McMahon RE, Ma J, Verkhoturov SV, Munoz-Pinto D, Karaman I, Rubitschek F, et al. A comparative study of the cytotoxicity and corrosion resistance of nickel-titanium and titanium-niobium shape memory alloys. Acta Biomater. 2012; 8(7): 2863-70.
28. Guerreiro Tanomaru JM, Storto I, Da Silva GF, Bosso R, Costa BC, Bernardi MI, et al. Radiopacity, pH and antimicrobial activity of Portland cement associated with micro- and nanoparticles of zirconium oxide and niobium oxide. Dent Mater J. 2014; 33(4): 466–70.
29. Guerreiro-Tanomaru JM, Vázquez-García FA, Bosso-Martelo R, Bernardi MI, Faria G, Tanomaru-Filho M. Effect of addition of nano-hydroxyapatite on physico-chemical and antibiofilm properties of calcium silicate cements. J Appl Oral Sci. 2016; 24(3): 204-10.
30. Silva GF, Tanomaru-Filho M, Bernardi MI, Guerreiro-Tanomaru JM, Cerri OS. Niobium pentoxide as radiopacifying agent of calcium silicate-based material: evaluation of physicochemical and biological properties. Clin Oral Investig. 2015; 19(8): 2015-25.

31. Wang Z, Lee Y H, Wu B, Horst A, Kang Y, Tang YJ, et al. Anti-microbial activities of aerosolized transition metal oxide nanoparticles. Chemosphere. 2010; 80(5): 525-9.
32. Viapiana R, Flumignan DL, Guerreiro-Tanomaru JM, Camilleri J, Tanomaru-Filho M. Physicochemical and mechanical properties of zirconium oxide and niobium oxide modified Portland cement-based experimental endodontic sealers. Int Endod J. 2014; 47(5): 437-48.
33. Mestieri LB, Tanomaru-Filho M, Gomes-Cornelio AL, Salles LP, Bernardi MI, Guerreiro-Tanomaru JM. Radiopacity and cytotoxicity of Portland cement associated with niobium oxide micro and nanoparticles. J Appl Oral Sci. 2014; 22(6): 554-9.
34. Mestieri LB, Gomes-Cornélio AL, Rodrigues EM, Faria G, Guerreiro-Tanomaru JM, Tanomaru-Filho M. Cytotoxicity and bioactivity of calcium silicate cements combined with Niobium Oxide in different cell lines. Braz Dent J. 2017; 28(1): 65-71.
35. Húngaro Duarte MA, Minotti PG, Rodrigues CT, Zapata RO, Bramante CM, Tanomaru Filho. Effect of diferente radiopacifying agentes on the physicochemical properties of white Portland cement and white mineral trioxide aggregate. J Endod. 2012; 38(3): 394-7.
36. Tanomaru-Filho M, Andrade AS, Rodrigues EM, Viola KS, Faria G, Camilleri J, et al. Biocompatibility and mineralized nodule formation of NeoMTA Plus and an experimental tricalcium silicate cement containing tantalum oxide. Int Endod J. 2017; 50(2): e31-e39.
37. Guerreiro-Tanomaru JM, Vázquez-García FA, Bosso-Martelo R, Bernardi MI, Faria G, Tanomaru M Filho. Effect of addition of nano-hydroxyapatite on physico-chemical and antibiofilm properties of calcium silicate cements. J Appl Oral Sci. 2016; 24(3): 204-10.
38. Gomes-Cornélio AL, Rodrigues EM, Salles LP, Mestieri LB, Faria G, Guerreiro-Tanomaru JM, et al. Bioactivity of MTA Plus, Biodentine and an experimental calcium silicate-based cement on human osteoblast-like cells. Int Endod J. 2017; 50(1): 39-47.
39. Húngaro Duarte MA, de Oliveira El, Kadre GD, Vivan RR, Guerreiro Tanomaru JM, Tanomaru Filho M. Radiopacity of portland cement associated with different radiopacifying agents. J Endod. 2009; 35(5): 737-40.

40. Bosso-Martelo R, Guerreiro-Tanomaru JM, Viapiana R, Berbert FL, Tanomaru-Filho M. Calcium silicate-based cements associated with micro- and nanoparticle radiopacifiers: physicochemical properties and bioactivity. Int Sch Res Notices. 2015; 23: 874283.
41. Marciano MA, Duarte MA, Camilleri J. Calcium silicate-based sealers: assessment of physicochemical properties, porosity and hydration. Dent Mater. 2016; 32(2): 30-40.
42. Cintra LTA, Benetti F, de Azevedo Queiroz ÍO, de Araújo Lopes JM, Penha de Oliveira SH, et al. Cytotoxicity, biocompatibility, and biomineralization of the new high-plasticity MTA material. J Endod. 2017; 43(5): 774-8.
43. Hench LL, Wilson J. Surface-active biomaterials. Science. 1984; 226(4675): 630-6.
44. Hench LL. The story of Bioglass. J Mater Sci Mater Med. 2006; 17(11): 967-78.
45. Zanotto ED, Ravagnani C, Peith Filho O, Panzeri H, Guimarães LEH; Universidade Federal de São Carlos, Universidade de São Paulo. Process and compositions for preparing particulate, bioactive or resorbable biosilicates for use in the treatment of oral ailments. BR WO2004/074199 A1. 2004 Feb 20.
46. Moura J, Teixeira LN, Ravagnani C, Peitl O, Zanotto ED, Beloti MM, et al. In vitro osteogenesis on a highly bioactive glass-ceramic (Biosilicate). J Biomed Mater Res A. 2007; 82(3): 545-57.
47. Roriz VM, Rosa AL, Peitl O, Zanotto ED, Panzeri H, de Oliveira PT. Efficacy of a bioactive glass-ceramic (Biosilicate) in the maintenance of alveolar ridges and in osseointegration of titanium implants. Clin Oral Implants Res. 2010; 21(2): 148-55.
48. Kido HW, Tim CR, Bossini PS, Parizotto NA, de Castro CA, Crovace MC et al. Porous bioactive scaffolds: characterization and biological performance in a model of tibial bone defect in rats. J Mater Sci Mater Med. 2015; 26(2): 74.
49. Martins CH, Carvalho TC, Souza MG, Ravagnani C, Peitl O, Zanotto ED, et al. Assessment of antimicrobial effect of Biosilicate® against anaerobic, microaerophilic and facultative anaerobic microorganisms. J Mater Sci Mater Med. 2011; 22(6): 1439-46.
50. Stoor P, Söderling E, Salonen JI. Antibacterial effects of a bioactive

glasspaste on oral microorganisms. Acta Odontol Scand. 1998; 56(3): 161-5.

51. Allan I, Newman H, Wilson M. Antibacterial activity of particulate bioglass against supra- and subgingival bacteria. Biomaterials. 2001; 22(12): 1683-7.
52. Jabur RO, Barros VMR, Trivelatto AE, Peitl O. Osseointegration of Ti implants in buccal alveolar bone defect filled with bioactive glasses in dog mandible. Oral Surg. 2010; 2(4): 161-6.
53. Granito RN, Rennó AC, Ravagnani C, Bossini PS, Mochiuti D, Jorgetti V, et al. In vivo biological performance of a novel highly bioactive glass-ceramic (Biosilicate®): a biomechanical and histomorphometric study in rat tibial defects. J Biomed Mater Res B Appl Biomater. 2011; 97(1): 139-47.
54. Pinto KN, Tim CR, Crovace MC, Matsumoto MA, Parizotto NA, Zanotto ED, et al. Effects of biosilicate® scaffolds and low-level laser therapy on the process of bone healing. Photomed Laser Surg. 2013; 31(6):252-60.
55. Kido HW, Oliveira P, Parizotto NA, Crovace MC, Zanotto ED, et al. Histopathological, cytotoxicity and genotoxicity evaluation of Biosilicate® glass-ceramic scaffolds. J Biomed Mater Res A. 2013; 101(3): 667-73.
56. Hench LL, Jones JR. Bioactive glasses: frontiers and challenges. Front Bioeng Biotechnol. 2015; 3: 194.
57. Kido HW, Brassolatti P, Tim CR, Gabbai-Armelin PR, Magri AM, Fernandes KR *et al.* Porous poly (D, L-lactide-co-glycolide) acid/biosilicate(®) composite scaffolds for bone tissue engineering. J Biomed Mater Res B Appl Biomater. 2017; 105(1): 63-71.
58. Schembri M, Peplow G, Camilleri J. Analyses of heavy metals in mineral trioxide aggregate and Portland cement. J Endod. 2010; 36(7): 1210-5.
59. Tanomaru JM, Tanomaru-Filho M, Hotta J, Watanabe E, Ito IY. Antimicrobial activity of endodontic sealers based on calcium hydroxide and MTA. Acta Odontol Latinoam. 2008; 21(2): 147-51.
60. Tanomaru-Filho M, Luis MR, Leonardo MR, Tanomaru JM, Silva LA. Evaluation of periapical repair following retrograde filling with different root-end filling materials in dogteeth with periapical lesions. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006; 102(1): 127-32.
61. Saghiri AM, Orangi J, Asatourian, James LG, Garcia-Godoy F,Lofti M, et al. Calcium silicate-based cements and functional impacts of various constituents. Dent Mater J. 2017; 36(1): 8-18.

62. Camilleri J. Characterization and hydration kineties of tricalcium silicate cement for use a dental biomaterial. Dent Mater. 2011; 27(8): 836-44.
63. Camilleri J. Sealers and warm gutta-percha obturation techniques. J Endod. 2015; 41(1): 72-8.
64. Prati C, Gandolfi MG. Calcium silicate bioactive cements biological perspectives and clinical applications. Dent Mater. 2015; 31(4): 351-70.
65. Parirokh M, Torabinejad M, Dummer PMH. Mineral trioxide aggregate and other bioactive endodontic cements: an updated overview - part I: vital pulp therapy. Int Endod J. 2018; 51(2): 177-205.
66. Tanalp J, Karapınar-Kazandağ M, Dölekoğlu S, Kayahan MB. Comparison of the radiopacities of different root-end filling and repair materials. ScientificWorldJournal. 2013; 23: 594950.
67. Kaup M, Schäfer E, Dammaschke T. An in vitro study of different material properties of Biodentine compared to ProRoot MTA. Head Face Med. 2015; 11: 16.
68. Dawood AE, Manton DJ, Parashos P, Wong R, Palamara J, Stanton DP, et al. The physical properties and ion release of CPP-ACP-modified calcium silicate-based cements. Aust Dent J. 2015; 60(4): 434-44.
69. Singh S, Podar R, Dadu S, Kulkarni G, Purba R. Solubility of a new calcium silicate-based root-end filling material. J Conserv Dent. 2015; 18(2): 149-53.
70. Camilleri J. Evaluation of the physical properties of an endodontic Portland cement incorporating alternative radiopacifiers used as root-end filling material. Int Endod J. 2010; 43(3): 231–40.
71. Huffman BP, Mai S, Pinna L, Weller RN, Primus CM, Gutmann JL, et al. Dislocation resistance of ProRoot Endo Sealer, a calcium silicate-based root canals sealer, from radicular dentine. Int Endod J. 2009; 42(1): 34–46.
72. Chang SW, Lee SY, Ann HJ, Kum KY, Kim EC. Effects of calcium silicate endodontic cements on biocompatibility and mineralization-inducing potentials in human dental pulp cells. J Endod. 2014; 40(8): 1194-200.

Araraquara, March 16, 2018.

Marcela Borsatto Queiroz