



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



Victor Manuel Ochoa Rodríguez

**Physicochemical and biological properties of Biodentine associated with
radiopacifiers**

Araraquara

2018



UNESP - Universidade Estadual Paulista
Faculdade de Odontologia de Araraquara



Victor Manuel Ochoa Rodríguez

**Physicochemical and biological properties of Biodentine associated with
radiopacifiers**

Dissertação apresentada à Universidade Estadual Paulista (UNESP), Faculdade de Odontologia de Araraquara, para obtenção do grau de Mestre em Odontologia, na Área de Endodontia.

Orientador: Profa. Dra. Gisele Faria

Araraquara

2018

Ochoa Rodriguez, Victor Manuel

Physicochemical and biological properties of Biodentine associated
with radiopacifiers / Victor Manuel Ochoa Rodríguez. – Araraquara:
[s.n.], 2018

40 f.; 30 cm

Dissertação (Mestrado em Odontologia) – Universidade Estadual
Paulista, Faculdade de Odontologia

Orientadora: Profa. Dra. Gisele Faria

1. Dental cements 2. Endodontics 3. Materials testing I. Título

Victor Manuel Ochoa Rodríguez

Physicochemical and biological properties of Biodentine associated with radiopacifiers

Comissão julgadora

Dissertação para obtenção do grau de Mestre em Odontologia

Presidente e orientador: Gisele Faria

2º Examinador: Profa. Dra. Raquel Assed Bezerra Segato

3º Examinador: Prof. Dr. Mário Tanomaru-Filho

Araraquara, 29 de março de 2018.

CURRICULAR DATA

Victor Manuel Ochoa Rodríguez

BIRTH DATE: 23/11/1991 – Arequipa, Arequipa, Perú

AFFILIATE:

Victor Raúl Haya Ochoa Cuentas

Ana Maritza Trinidad Rodríguez Pérez

2009 - 2013

Graduated in Odontology.

Universidad Catolica de Santa Maria, U. SANTA MARIA, Arequipa-Peru

2016 - 2018

Specialist in Endodontics.

Araraquara School of Dentistry, São Paulo State University - UNESP, São Paulo, Brazil

2016-2018

Master in Dentistry.

Araraquara School of Dentistry, São Paulo State University - UNESP, São Paulo, Brazil

ACKNOWLEDGMENTS

To my advisor, Profa. Dra. Gisele Faria

Thanks for receiving me with open arms, showing me the way in difficult moments, for the effort you put in everything you do, for the time you give, regardless of how tired you are. I have been privileged to experience first-hand how critical and passionate you can be and make a big difference. You inspire me with your enthusiasm, support and confidence. You have set an example of excellence as a researcher, friend, mentor, instructor, and role model.

To Prof. Dr. Mario Tanomaru-Filho

I infinitely thank you for the opportunity given to me, for the guidance as professional, person and mentor. Thanks for the friendship and confidence. I am proud to have worked by your side.

To Prof. Hair Salas Beltrán

Thank you, my friend, for giving me the opportunity to learn from you. It was you and your encouragement to follow the endodontics path, that have brought me here.

To Dra. Elisandra Rodrigues

Thank you for the support, for the patience, the trust, confidence and friendship you have given me. Thanks for sharing all the smiles, laughs, and sometimes, the unhappy moments in the laboratory, you are an example and inspiration to me.

To the Teachers of the Discipline of Endodontics of this Faculty, **Prof. Dr. Fábio Luiz Camargo Vilella Berbert, Prof. Dr. Idomeo Bonetti-Filho, Profa. Dra. Juliane Maria Guerreiro-Tanomaru, Prof. Dr. Mário Tanomaru-Filho and Prof. Dr. Renato de Toledo Leonard.**

Thanks for the friendship, the happy moments, the laughs and stories shared. Thanks for the knowledge and wisdom you have left imprinted in me.

To my parents and little sister

Dr. Victor Ochoa Cuentas, Dra. Ana Rodríguez Pérez and Geraldine Ochoa Rodríguez who always supported me, encouraged and trusted me. Thank you for the good advices, the calls at night to ask about your patience, your life lessons, your trust and me. I still take your lessons with me, every day.

To the friends and colleagues of the Endodontic post grade

Thank you so much for the dedication, convivence, parties, laughs, advices, and good memories of each one. I thank you for the harmonious willingness to always help.

To the staff of the Technical Postgraduate Section, **José Alexandre Garcia and Cristiano Afonso Lamounier and the employees of the Department of Restorative Dentistry**

Thanks for the availability, efficiency, patience and joyfulness.

To the São Paulo State University "JÚLIO DE MESQUITA FILHO", UNESP, in UIC people of the Magnificent Rector Prof. Dr. **Sandro Roberto Valentini**

To the School of Dentistry at Araraquara (FOAr / UNESP), in the persons of the Director Profa. Dra. Elaine Maria Sgavioli Massucato.

**To the Postgraduate Program in Dentistry, FOAr / UNESP, in the people of the
Coordinator Prof. Dr. Joni Augusto Cirelli**

“Experience life in all possible ways; goodbad, bitter-sweet, dark-light, summer-winter. Experience all the dualities. Do not be afraid of experience, because the more experience you have, the more mature you become”

Rajneesh

Ochoa-Rodríguez VM. Propriedades físico-químicas e biológicas do Biodentine associado a radiopacificadores [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2018.

RESUMO

Biodentine™ (BD) apresenta bioatividade, biocompatibilidade e propriedades físico-químicas adequadas; no entanto, não possui radiopacidade adequada. Os objetivos foram avaliar (1) a radiopacidade de BD e BD associado com 15% de tungstato de cálcio (BDCaWO₄) ou óxido de zircônio (BDZrO₂), empregando sistemas de radiografia convencional e digital; e (2) as propriedades físico-químicas de tempo de presa, pH e solubilidade, e as propriedades biológicas de citocompatibilidade e potencial para induzir mineralização desses cimentos. Para a avaliação da radiopacidade, cada corpo de prova foi radiografado ao lado de uma escada de alumínio usando filme oclusal, placa de fósforo ou sensores digitais. As radiografias convencionais foram digitalizadas por câmera fotográfica ou scanner. Os valores médios de cinza dos materiais foram expressos em milímetros de alumínio (mm Al). A solubilidade foi avaliada após 7 dias de imersão dos espécimes em água destilada e expressa em porcentagem de perda de massa. O tempo de presa foi avaliado empregando a agulha de Gillmore (105 ± 0,5 g) e o pH foi mensurado com um medidor de pH. A citocompatibilidade e a bioatividade celular foram avaliadas em células de linhagem osteoblástica (Saos-2) utilizando os ensaios de metiltetrazólio (MTT), vermelho neutro (NR), atividade de fosfatase alcalina (ALP) e coloração de vermelho de alizarina. Os dados foram avaliados utilizando ANOVA de um fator e pós-teste Tukey ou ANOVA de dois fatores e pós-teste de Bonferroni ($\alpha=0,05$). A radiopacidade do BD foi inferior a 3 mm Al e do BDZrO₂ e BDCaWO₄ foi acima de 3 mm Al em todos os sistemas de radiografia utilizados. A solubilidade foi de 2,28% para BD, 2,27% para BDZrO₂ ($p>0,05$) e 3,63% para BDCaWO₄ ($p<0,05$). O tempo de presa foi de 27,5 min para BD, 33,5 minutos para BD ZrO₂ e 30 minutos para BDCaWO₄. Os ensaios MTT e NR revelaram que os extratos de cimentos, nas diluições 1: 2, 1: 4, 1: 8 e 1:12, apresentaram citocompatibilidade maior ($p<0,05$) ou similar ($p>0,05$) ao grupo controle (meio de cultura). A atividade de ALP nos grupos dos cimentos foi semelhante ($p>0,05$) ou maior ($p<0,05$) que o grupo controle aos 1, 3 e 7 dias. Aos 7 dias, a maior atividade de ALP foi detectada para o grupo BD seguido de BDZrO₂ ($p<0,05$) e do BDCaWO₄ ($p<0,05$). Não houve diferença significativa entre BDCaWO₄ e grupo controle ($p>0,05$). Todos os materiais induziram maior produção de nódulos mineralizados que grupo controle ($p<0,05$) sem diferença significativa entre eles. Em conclusão, a radiopacidade de BD foi inferior a 3 mm de Al em todos os sistemas radiográficos, e a adição de 15% de ZrO₂ ou CaWO₄ foi suficiente para aumentar a radiopacidade de BD para valores maiores que o mínimo recomendado pelo ISO 6876 (>3mm Al). BD associado a radiopacificadores mostrou propriedades adequadas do tempo de presa, pH e solubilidade, exceto BDCaWO₄, que apresentou maior solubilidade que BD e BDZrO₂. Todos os cimentos apresentaram citocompatibilidade e potencial de induzir mineralização em células Saos-2. Os resultados sugerem que a adição de 15% de ZrO₂ pode ser uma boa opção para aumentar a radiopacidade do BD sem alterar suas propriedades físico-químicas e biológicas.

Palavras-chave: Cimentos dentários. Endodontia. Teste de materiais.

Ochoa-Rodríguez VM. Physicochemical and biological properties of Biodentine associated with radiopacifiers [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2018.

ABSTRACT

Biodentine™ (BD) presents bioactivity, biocompatibility and suitable physicochemical properties; however, it does not have adequate radiopacity. The objectives were to evaluate (1) the radiopacity of BD and BD associated with 15% calcium tungstate (BDCaWO₄) or zirconium oxide (BDZrO₂), employing conventional and digital radiography systems; and (2) the physicochemical properties of setting time, pH and solubility, and biological properties of cytocompatibility and potential to induce mineralization of these cements. For radiopacity evaluation, each cement specimen was radiographed alongside an aluminum step-wedge using occlusal film, photostimulable phosphor plates or digital sensors. The conventional radiographies were digitized by digital photographic camera or scanner. Mean grey values of materials were expressed in millimeters of aluminum (mm Al). Solubility was evaluated after 7 days of specimens' immersion in distilled water and expressed as percentage of mass loss. Setting time was evaluated employing a Gillmore needle (105 ± 0.5 g) and pH was evaluated with pH meter. The cytocompatibility and cell bioactivity were evaluated in osteoblasts-like cells (Saos-2) using methyl-thiazol-tetrazolium (MTT), neutral red (NR), alkaline phosphatase (ALP) activity and alizarin red staining assays. The data were evaluated using one-way ANOVA and Tukey post-test or two-way ANOVA and Bonferroni post-test ($\alpha=0.05$). BD radiopacity was below 3 mm Al and BDZrO₂ and BDCaWO₄ was above 3 mm Al in all radiography systems used. Solubility was 2.28% for BD, 2.27% for BDZrO₂ ($p>0.05$) and 3.63% for BDCaWO₄ ($p<0.05$). All cements showed alkaline pH with no statistical difference between them ($p>0.05$). The setting time was 27.5 min. for BD, 33.5 min. for BDZrO₂ and 30 min. for BDCaWO₄. MTT and NR assays revealed that cements extract at dilutions of 1:2, 1:4, 1:8 and 1:12 had greater ($p<0.05$) or similar ($p>0.05$) cytocompatibility in comparison to control group (culture medium). The ALP activity of cements groups at 1, 3 and 7 days was similar ($p>0.05$) or greater ($p<0.05$) than the control group. At 7 days, the highest ALP activity was detected for BD group followed by BDZrO₂ ($p<0.05$) and BDCaWO₄ group ($p<0.05$). There was no significant difference between BDCaWO₄ and control group ($p>0.05$). All materials induced greater production of mineralized nodules than control group ($p<0.05$) without significant difference among them. In conclusion, BD radiopacity was below 3 mm Al in all radiography systems, and addition of 15% ZrO₂ or CaWO₄ was sufficient to increase the radiopacity of BD to values greater than the minimum recommended by ISO 6876 (> 3 mm Al). BD associated with radiopacifiers showed suitable properties of setting time, pH and solubility, except BDCaWO₄, which exhibit a higher solubility than BD and BDZrO₂. All cements had cytocompatibility and potential to induce mineralization in Saos-2 cells. The results suggest that the addition of 15% ZrO₂ may be a good option to increase the radiopacity of BD without altering its physicochemical and biological properties.

Key words: Dental cements. Endodontics. Materials testing.

SUMMARY

1 INTRODUCTION	11
2 OBJETIVES.....	15
3 MATERIAL AND METHODS	16
4 RESULTS	22
5 DISCUSSION.....	28
6 CONCLUSIONS.....	31
REFERENCES	32

1 INTRODUCTION

MTA is mainly composed of Portland cement (PC) and contains 53.1% of tricalcium silicate, 22.5% of dicalcium silicate, 21.6% of bismuth oxide (Bi_2O_3) as radiopacifier and traces of calcium sulfate (Torabinejad, White⁸⁷, 1998; Camilleri et al.¹⁶, 2005; Camilleri²⁰, 2007; Camilleri¹⁹, 2008). It is considered the gold standard material for diverse treatments in endodontics, such as root perforation, root-end filling, among others (Hwang et al.⁴⁶, 2011; Torabinejad et al.⁸⁸, 2018), due to its sealing capability, biocompatibility and ability to induce mineralization (Parirokh, Torabinejad⁶⁶, 2010; Tanomaru-Filho et al.⁸³, 2017; Rodrigues et al.⁷³, 2017). However, MTA is difficult to manipulate and insert into cavities (Parirokh, Torabinejad⁶⁶, 2010), low compressive strength (Parirokh, Torabinejad⁶⁵, 2010), has a long setting time (Parirokh, Torabinejad⁶⁵, 2010; Tanomaru-Filho et al.⁸⁶, 2012) and causes tooth discoloration (Belobrov, Parashos⁸, 2011; Akbari et al.¹, 2012; Felman, Parashos³², 2013; Kang et al.⁵¹, 2015) derived from the chemical reaction between the collagen in the dentin matrix and Bi_2O_3 (Marciano et al.⁵⁹, 2014). There is evidence that Bi_2O_3 causes structural damages capable of compromising the longevity of the material, increasing the porosity degree, and consequently reducing the compressive strength (Coomaraswamy et al.²⁷, 2007).

Tricalcium silicate, the principal active component in MTA (Camilleri¹⁶, 2005), has been used with or without additives as bone cement (Huan, Chang⁴², 2008; Zhao et al.⁹⁷, 2008), posterior restorative material (Laurent et al.⁵⁶, 2008) and reparative dental material (Wang et al.⁹³, 2008; Camilleri et al.¹⁷, 2013). It has shown suitable physicochemical properties (Wang et al.⁹³, 2008; Huan, Chang⁴², 2008), bioactivity and biocompatibility (Peng et al.⁶⁹, 2011; Camilleri et al.¹⁷, 2013; Tanomaru-Filho et al.⁸³, 2017), besides promoting odontoblastic differentiation of human dental pulp cells (Peng et al.⁶⁹, 2011). The hydration of the tricalcium silicate after chemical reaction with tissue fluids forms hydrated calcium silicate gel and calcium hydroxide, thus, being the tricalcium silicate phase responsible for the bioactivity of this material (Camilleri¹⁸, 2011; Khalil et al.⁵⁴, 2016). Dental materials based on tricalcium silicate have been developed. These materials are synthesized in the laboratory from high purity raw materials unlike the Portland cement in MTA. One such formulation is BiodentineTM – BD (Septodont, Saint-Maurdes-Fossés, France) which was developed for use as a bioactive dentin substitute and has been indicated for coronal and radicular restorations, pulp capping, pulpotomy, root and furcation perforations, apexification, root resorption and as root-end filling (Rajasekharan et al.⁷⁰, 2014). BD is composed of a powder and liquid system. The powder contains 80% tricalcium silicate (main component), 15% calcium carbonate

(filler material), 5% zirconium oxide (radiopacifier), dicalcium silicate (traces), calcium oxide (traces), iron oxide (traces). The mixing liquid is an aqueous solution of a hydrosoluble polymer (water reducing agent) with calcium chloride, which decreases the setting time of the cement (Septodont)⁷⁶. Studies show that this cement has biocompatibility (Fonseca et al.³³, 2016), bioactivity (Grech et al.³⁹, 2013), with better handling conditions (Butt et al.¹³, 2014) and lower setting time in relation to MTA (Kaup et al.⁵³, 2015).

The biological properties of BD have been studied, showing positive responses. BD presents cytocompatibility (Chang et al.²⁴, 2014; Daltoe et al.³⁰, 2016; Rodrigues et al.⁷⁴, 2017) and in vitro potential to induce mineralization (Gomes- Cornélio et al.³⁷, 2017) higher than MTA (Collado-González et al.²⁵, 2017; Rodrigues et al.⁷⁴, 2017). In vivo, BD promotes formation of collagenous capsules when implanted in the subcutaneous tissue of rats (Fonseca et al.³³, 2016) and induces the formation of mineralized tissue when used as pulp-capping material in human and dog teeth (Nowicka et al.⁶³, 2013; De Rossi et al.³¹, 2014; Cuadros-Fernández et al.²⁸, 2016) or when used for the sealing of furcation perforations (Silva et al.⁸⁰, 2017).

The physicochemical properties of BD have benefit in relation to MTA. The initial setting time ranges from 9 minutes (Septodont)⁷⁶ to 16 minutes (Lucas et al.⁵⁸, 2017) and the final setting time from 35 minutes (Lucas et al.⁵⁸, 2017) to 85.6 minutes (Kaup et al.⁵³, 2015), which is lower than MTA (Parirokh et al.⁶⁷, 2018). The polycarboxylate-based hydrosoluble polymers in the liquid of BD acts as water reducing agent and allows low water/powder ratio. As a result, BD has lower porosity and, consequently, higher compressive strength than MTA (Camilleri et al.¹⁷, 2013; Lucas et al.⁵⁸, 2017). BD presents alkaline pH similar to MTA (Lucas et al.⁵⁸, 2017). This pH is derived from the hydration reaction of tricalcium silicate which forms calcium hydroxide and calcium silicate hydrate gel (Camilleri et al.¹⁷, 2013; Khalil et al.⁵⁴, 2016).

Despite of the good properties, some in vitro studies, using conventional film (Lucas et al.⁵⁸, 2017) or photostimulable phosphor plates (Tanalp et al.⁸², 2013), have shown that BD presents lower radiopacity than that recommended by the International Standards Organization (ISO 6876)⁴⁸. According to ISO standard, the endodontic sealers must have a radiopacity equivalent to not less than 3 mm Al (ISO 6876)⁴⁸. Moreover, researchers, who have used BD as a retrograde obturation material in human teeth, have reported that low radiopacity is the primary clinical limitation of BD, which makes radiographic assessment of treatment and follow-up difficult (Bachoo et al.⁵, 2013; Caron et al.²², 2014). Considering the appropriate properties of tricalcium silicate-based cements associated with zirconium oxide

(ZrO₂) and calcium tungstate - CaWO₄ (Cutajar et al.²⁹, 2011; Gomes-Cornélio et al.³⁸, 2011; Húngaro Duarte et al.⁴⁴, 2012; Camilleri et al.¹⁷, 2013; Bosso-Martelo et al.¹¹, 2015; Silva et al.⁷⁹, 2017), an option to improve BD's radiopacity is to associate it with these radiopacifiers.

CaWO₄ has been used as an alternative radiopacifier to Bi₂O₃ for calcium silicate-based cements (Marciano et al.⁶⁰, 2016). Studies have reported that CaWO₄ associated with Portland cement, promotes alkaline pH (Húngaro-Duarte et al.⁴⁴, (2012), decreases the solubility, increases the compressive strength, does not affect the final setting time (Tanomaru-Filho et al.⁸⁶, 2012) and is not cytotoxic for periodontal and osteoblast-like cells (Gomes-Cornélio et al.³⁸, 2011). CaWO₄, associated with calcium silicate-based cement, presents bioactivity (Bosso-Martelo et al.¹¹, 2015) and maintains physicochemical properties similar to MTA (Bosso-Martelo et al.¹², 2016).

ZrO₂ was initially introduced as a biomaterial for use in joint implants in orthopedic surgery. In restorative dentistry, ZrO₂ is used to replace the metal framework in crown and bridges and as radiopacifier in glass ionomer cements (McCabe et al.⁶¹, 2003). It is commonly used in combination with tricalcium silicate cements for endodontic use (Viapiana et al.⁹¹, 2014; Tanomaru et al.⁸⁵, 2017) including BD. ZrO₂ does not participate in the hydration process of Portland cement thus being inert when compared to Bi₂O₃ (Camilleri et al.¹⁴, 2011; Camilleri et al.¹⁷, 2013). The association of Portland cement with 30% ZrO₂ resulted in a material with physicochemical properties comparable to those of MTA (Cutajar et al.²⁹, 2011). ZrO₂ in association with white Portland cement induced lower inflammatory reaction than Bi₂O₃ (Silva et al.⁷⁸, 2014), fibroblast proliferation and accelerated the regression of the inflammatory reaction when compared to MTA (Silva et al.⁷⁹, 2017) in subcutaneous rat tissue.

Radiopacity of endodontic materials should be sufficient to allow distinction from dentin or cortical bone (American National Standard/American Dental Association - ANSI/ADA)³. For quantifying the radiopacity of endodontic materials, specimens should be prepared in standard discs and radiographed along with an aluminum (Al) step-wedge reference with at least 98% pure, using type D or E occlusal films (ISO 6876)⁴⁸. Values in terms of Al equivalent thickness minimize the influence of exposure time and film development time (Rasimick et al.⁷¹, 2007; Akcay et al.², 2012). ISO standard recommends that radiopacity must be evaluated in conventional radiographic films using an optical densitometer (ISO 6876)⁴⁸. However, nowadays, the radiopacity of dental materials has been performed using digital images obtained by indirect (Akcay et al.², 2012; Siboni et al.⁷⁷, 2017) or direct technique (Baksi et al.⁶, 2007; Akcay et al.², 2012; Khalil et al.⁵⁴, 2016, Versiani et

al.⁹⁰, 2016). In the indirect technique, the conventional radiographic image is converted into digital sign using radiographic scanner (Tanomaru-Filho et al.⁸⁴, 2007; Akcay et al.², 2012; Siboni et al.⁷⁷, 2017) or digital photographic camera (Húngaro Duarte et al.⁴³, 2009; Candeiro et al.²¹, 2012; Wang et al.⁹⁴, 2014). In the direct technique, digital radiography is obtained using digital sensors or photostimulable phosphor plates (Baksi et al.⁷, 2008; Akcay et al.², 2012; Grech et al.⁴⁰, 2013; Khalil et al.⁵⁴, 2016, Versiani et al.⁹⁰, 2016).

Although several studies have assessed the radiopacity of endodontic materials by using digital systems (Rasimick et al.⁷¹, 2007; Baksi et al.⁷, 2008; Akcay et al.², 2012; Grech et al.⁴⁰, 2013, Camilleri et al.¹⁷, 2013; Khalil et al.⁵⁴, 2016, Versiani et al.⁹⁰, 2016), there is no consensus on how digital radiography influences the radiopacity of materials. Rasimick et al.⁷¹ (2007) reported that barium-containing materials tended to be 13% more radiopaque in radiographs obtained by digital sensor than on the conventional film type. On the other hand, other endodontic materials appeared less radiopaque on digital radiography, ranging from 7% to 20% difference between conventional and digital radiography obtained by photostimulable phosphor plates (Baksi et al.⁷, 2008). Therefore, it is important to evaluate the radiopacity of BD and BD associated with radiopacifiers using conventional and digital radiography systems. In addition, it is important to evaluate the effect of the addition of the radiopacifiers on the physicochemical and biological properties of BD.

2 OBJETIVES

The aim of this study was to evaluate (1) the radiopacity of BD and BD associated with CaWO_4 or ZrO_2 using conventional and digital radiography systems, and (2) the physicochemical properties of setting time, pH and solubility, and biological properties of cytocompatibility and potential to induce mineralization of these cements. The null hypothesis was that there is no difference in the radiopacity values of BD using conventional or digital radiography systems, and that CaWO_4 or ZrO_2 associated with BD would not change its radiopacity, biological and physicochemical properties.

3 MATERIAL AND METHODS

The materials evaluated were BD and BD associated with radiopacifiers ZrO_2 or $CaWO_4$, in proportion of 85% BD and 15% ZrO_2 (BD ZrO_2) or 15% $CaWO_4$ (BD $CaWO_4$) by weight. The composition, manufacturer, and powder/liquid proportion used for materials are shown in Table 1. To mix the cements, six drops of liquid supplied by the were placed in the capsule with powder and the set was ground for 30 seconds using a mixing device (SDI Ultramat 2, Bayswater, Victoria, Australia) as instructed by the manufacturer.

Table 1 - Materials, composition, manufacturer and proportion used

Material	Manufacturer	Powder-liquid proportion
Biodentine™ (BD)	Powder: tricalcium silicate (main component), calcium carbonate (filler material), zirconium oxide (radiopacifier), dicalcium silicate (traces), calcium oxide(traces), iron oxide(traces) (Sepodont, Saint-Maur-des-Fossés, France) Liquid: aqueous solution of a hydrosoluble polymer (water reducing agent) with calcium chloride (decreases the setting time) (Sepodont, Saint-Maur-des-Fossés, France)	0,7 g / 6 drops
BD (85%) + zirconium oxide (15%) (BD ZrO_2)	Powder: BD (Sepodont); zirconium oxide (Sigma-Aldrich, Co., St. Louis, Missouri, United States) Liquid: solution BD (Sepodont)	0.7 g BD + 0.12g ZrO_2 / 6 drops
BD (85%) + calcium tungstate (15%) (BD $CaWO_4$)	Powder: BD (Sepodont); calcium tungstate (Sigma-Aldrich) Liquid: solution of BD (Sepodont)	0.7 g BD + 0.12g $CaWO_4$ / 6 drops

Source: Author

Physicochemical properties

Radiopacity

Five specimens measuring 10 mm in diameter by 1 mm thickness were made for each tested material, according to ISO 6876⁴⁸ specification. The specimens were stored at 37 °C and 95% humidity for 24 hours and, subsequently, they were radiographed using conventional or digital radiography systems.

- Conventional radiography

The specimens were placed on occlusal radiographic E-speed films (Insight – Kodak Co., Rochester, NY, USA) along with an aluminum step-wedge, with an 8-step wedge with 2 mm incremental steps, for radiographic exposure. The standard geometric configuration was fixed at 320 mm source-to-object distance and zero degrees vertical and horizontal angulations of the X-ray beam. A GE-1000 X-ray unit (General Electric, Milwaukee, WI, USA), operating at 65 kVp and 7 mA using an exposure time of 0.25 seconds was used (Ackay et al.², 2012). The radiographic films were digitalized with a scanner (Ackay et al.², 2012) or with a digital photographic camera (Húngaro Duarte et al.⁴³, 2009). Scanner (Microtek ScanMaker i800, Hsinchu City, Taiwan) with 300 DPI resolution and Microtek Scan Wizard 5 (Microtek) software were used. The digital photographic camera (Canon EOS T1, Tokyo, Japan) with macro lens of 100 mm was used with the following parameters: lens-to-object distance of 58 cm, ISO 200, aperture of 6.3 shutter and speed of 1/40 s.

-Digital radiography

Each specimen along with an aluminum step-wedge with 8 steps of 2 mm increment each, were placed on digital sensors CMOS Fona (CDR Elite, Fona, Germany), CMOS Kodak (rvg 6100, Kodak Co., Rochester, NY, USA) or on photostimulable phosphor plates (Digora, Soredex, Nahkelantie, Tuusula, Finland) for radiographic exposure. The standard geometric configuration was fixed at 320 mm source-to-object distance and zero degrees vertical and horizontal angulations of the X-ray beam. A GE-1000 X-ray unit (General Electric), operating at 65 kVp and 7 mA using an exposure time of 0.16 seconds was used (Ackay et al.², 2012).

The images obtained by means of all radiography systems were evaluated using the software Photoshop CC 2015 for Windows (Adobe Systems Incorporated, Mountain View, California, USA), by measuring the grayscale to determine the equivalence of radiopacity of the cements in millimeters of aluminum (mm Al), using the mathematical formula of Hungaro-Duarte et al.⁴³ (2009).

pH analysis

For pH analysis, polyethylene tubes measuring 10 mm long and 1 mm in diameter were filled with each material (n=10). Each tube was immersed in 10 mL of deionized water and maintained at 37 °C, throughout the experimental time intervals of 1, 3, 10, 20 and 30 days. At each time interval, the tubes were removed from the flasks and conditioned in a new flask with 10 mL of deionized water. At each time interval, the pH of the solution was measured with a previously calibrated digital pH meter (Ultrabasic; Denver Instrument Company, Arvada, Colorado, USA) in a room temperature of 25 °C. As control group, the pH of deionized water without immersed material was measured.

Setting time

The cements were inserted into ring-shaped metal molds measuring 10 mm in diameter and 1 mm thickness (n=6) and were kept at 37 °C and 95% humidity. To determine the setting time, the Gillmore needle technique with 100g weight and 2mm diameter was used according to ISO 6876⁴⁸ specification. The setting time of each cement was established by calculating the averaged time elapsed from time manipulation until the Gillmore needle no longer caused indentations marks on the surface of the specimens.

Solubility

The solubility assay was performed according to the methodology of Carvalho-Junior et al.²³ (2007) methodology modified. Cements were prepared and then, and placed in a silicone mold, measuring 7.75 mm in diameter and 1.5 mm thickness (n=6). A 5-cm nylon thread was placed in the center of the specimens when the material was placed into the mold. The specimens were maintained at 37 °C and 95% humidity for 3 times the length of their setting time. Right after, the specimens were removed from the mold, weighed on a precision balance HM-200 (A & D Engineering, Inc., Bradford, MA, EUA and suspended from the lid by means of nylon wires, inside plastic flasks, containing 7.5 mL of deionized water. The flasks were maintained at 37 °C for seven days. Then, the specimens were removed, rinsed and placed in a silica dehumidifier. The mass was measured every 24 hours after the experiment, until the mass stabilized, in a silica desiccator. The material solubility was expressed as mass loss of the original mass and expressed as the percentage for each specimen.

Cytocompatibility and potential to induce mineralization

Cell culture and preparation of cements extracts

Saos-2 cells (ATCC HTB-85) were cultured in flasks containing Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% foetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY, USA), penicillin (100 IU/mL), streptomycin (100 µg/mL) in an atmosphere consisting of 5% CO₂, 95% humidity at 37 °C.

The cements were proportioned according to table 1. After manipulation, 0.7 g of each material was placed in empty wells of a 12-well culture plates (314.0 mm² area and 3.0 mm height) and hydrated with humidified gauze. The plates were kept at 37 °C and 95% humidity for 48 hours. After this time, the cements were exposed to ultraviolet light (UV) under laminar flow for 30 minutes to prevent contamination (Katara et al.⁵², 2008). Five mL of serum-free DMEM were added in each well of the plates in which the material was accommodated and maintained. For 24 h, the plate was maintained at 37 °C, 95% humidity and 5% CO₂ to create the extract of each cement (ISO 10993-5)⁴⁷. DMEM was used as negative control and 20% dimethyl sulfoxide (DMSO) as positive control. (Margunato et al.⁶², 2015).

Cell viability assays

Cell viability were assessed by methyl-thiazol-tetrazolium (MTT) and neutral red (NR) assays. Saos-2 cells were seeded at a density of 1x10⁵ cells/mL in a 96-well plate containing DMEM with FBS 10% for 24 hours to adhere to the plates. After that, the cells were exposed to the cement extracts at 1:1, 1:2, 1:4, 1:8 and 1:12 dilutions (v:v) in serum-free DMEM for 24 h (Tanomaru-Filho et al.⁸³, 2017; Andolfatto et al.⁴, 2017).

MTT assay was performed by replacing the cement extracts with 100 µL of a 5 mg/mL MTT solution (Sigma-Aldrich) followed by incubation at 37°C, 95% humidity and 5% CO₂ for 3h. The well content was removed, and the colorimetric product was solubilized in 100 µL of acidified isopropanol 0.04 N (Sigma-Aldrich). The optical densities of the solutions were measured in a spectrophotometer (Elx800; Bio-Tek Instruments, Winooski, VT, USA) at 570 nm.

NR assay (Repetto et al.⁷², 2008) was performed by replacing the cement extracts with 100 µL DMEM containing 50µg NR/mL (Sigma-Aldrich). The cells were incubated at 37 °C, 95% humidity and 5% CO₂ for 3h, the well content was removed to proceed with

solubilization of the colorimetric product in 100 μL of an ethanol solution mixture (50% ethanol and 1% acetic acid, Sigma-Aldrich). The optical densities of the solutions were measured in a spectrophotometer (Elx800) at 570 nm. Three independent experiments were performed for both assays.

Alkaline phosphatase (ALP) activity

Alkaline phosphatase (ALP) activity was evaluated by using a commercial kit (Labtest; Lagoa Santa, MG, Brazil). Saos-2 (1×10^5 cells/mL) were cultivated in a 96-well plate for 24 hours to adhere to the plates and were exposed to the cement extracts at 1:8 dilution for one, three and seven days. The cement extracts were renewed every two days. After each experimental period, the cells were washed with 200 μL of phosphate buffered saline solution (PBS) and 200 μL of a sodium lauryl sulfate solution (1% in distilled water, Sigma-Aldrich) were added to each well. Then the samples were rested for 30 minutes at room temperature. Each sample (5 μL) in lauryl sulfate solution was transferred to a microtube (Eppendorf, Hamburg, Germany) containing substrate and the enzyme buffer. Absorbance was measured in a spectrophotometer at 590 nm. Data were expressed as ALP activity normalized with the number of viable cells detected in the MTT assay in the respective culture period (Westgard et al.⁹⁵, 1981).

Alizarin red staining (ARS)

Saos-2 cells were cultivated (1×10^4 cells/mL) in 12-well culture plates using DMEM supplemented with 50 $\mu\text{g/mL}$ L-ascorbic acid (Sigma-Aldrich) and 10 mM β glycerophosphate (Sigma-Aldrich). The cells were exposed to the cement extracts at 1:8 dilution for 21 days. The cement extracts were renewed every two days. Afterwards cells were washed with PBS, fixed with 10% paraformaldehyde (Sigma) and stained with 2% ARS (pH 4.1). The plate was incubated in room temperature for 20 minutes, the dye was aspirated, the wells were washed 4 times with 1 mL of distilled water/ well for 5 minutes. The plates were left angled for 2 minutes to facilitate the removal of excess of water. Then, the mineralization was quantified by dissolution of the nodules with 1 mL of 10% solution of cetylpyridinium chloride (Sigma/Aldrich) was added to each well and the plate was incubated for 15 minutes, under shaking at room temperature. Three aliquots of 100 μL of the resuspension of each well were transferred to a 96-well plate and the reading was performed in a spectrometer with 562 nm wavelength filter (Elx800; Bio-Tek Instruments, Winooski, VT, USA). Three independent experiments were performed.

Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) and Tukey post-test or two-way ANOVA and Bonferroni post-test. ($\alpha = 0.05$), by using of the statistical program GraphPad Prism (GraphPad Software Inc. San Diego, CA, USA).

4 RESULTS

Physicochemical properties

Radiopacity

In all digital and convectional radiography systems used, the BD radiopacity did not amount to 3 mm Al, as specified by ISO 6876⁴⁸. BD associated with radiopacifiers ZrO₂ or CaWO₄ had radiopacity higher than 3 mm Al shown in all radiographic systems. (Table 2). The radiopacity of the materials obtained with the use of a Kodak digital sensor was higher than the values obtained by means of the other systems ($p < 0.05$).

Table 2 - Mean and standard deviation of the materials radiopacity (mm Al) evaluated by digital or convectional radiography systems and compliance to ISO 6876⁴⁸

	BD	BD ZrO₂	BD CaWO₄
Kodak digital sensor	2.52 (0.09)	4.20 (0.32)	4.26 (0.42)
Fona digital sensor	2.17 (0.02)	3.81 (0.15)	3.52 (0.39)
Photostimulable phosphor plates	2.39 (0.38)	3.70 (0.08)	3.55 (0.40)
Oclusal film scanned	2.21 (0.04)	3.59 (0.23)	3.88 (0.25)
Oclusal film photographed	2.08 (0.06)	3.70 (0.22)	3.58 (0.28)
ISO 6876		>3	

Source: Author

pH

According to Table 3, the deionized water containing the materials had alkaline pH in all periods. Differences between groups were not found ($p > 0.05$), except control group (deionized water) that had significantly lower pH values than other groups in all time intervals ($p < 0.05$).

Table 3 - Mean and standard deviation of pH values of the materials and control in the evaluation periods

	BD	BD ZrO₂	BD CaWO₄	Control
1 day	11.21 (0.47) ^a	11.28 (0.34) ^a	11.21 (0.35) ^a	6.53 (0.14) ^b
3 days	9.91 (0.72) ^a	9.63 (1.08) ^a	9.48 (0.97) ^a	6.69 (0.19) ^b
10 days	9.53 (1.44) ^a	9.42 (1.32) ^a	9.48 (1.24) ^a	6.69 (0.27) ^b
20 days	9.83 (0.98) ^a	9.06 (1.05) ^a	9.71 (0.95) ^a	6.92 (0.43) ^b
30 days	8.92 (1.37) ^a	8.95 (1.02) ^a	9.75 (1.11) ^a	6.72 (0.18) ^b

Different letters in the lines indicate statistically significant differences between cements ($p < 0.05$).
Source: Author

Solubility and setting time

According to Table 4, there was no significant difference between BD and BD ZrO₂ ($p > 0.05$) and both displayed lower than 3% mass lost, and showed no sign of disintegration. BD CaWO₄ showed higher mass lost (3.63%) in relation the other materials ($p < 0.05$), and no sign of disintegration. BD had the lower setting time than other materials ($p < 0.05$). The addition of CaWO₄ and ZrO₂ to the BD increased the setting time by 2.5 and 6minutes, respectively ($p < 0.05$).

Table 4 -Mean and standard deviation of solubility (% of mass lost) and initial setting time (in minutes) of the materials

	Solubility	Initial setting time
BD	2.28 (0.26) ^a	27,50 (0.57) ^a
BD ZrO₂	2.27 (0.22) ^a	33.50 (1.73) ^b
BD CaWO₄	3.63 (0.67) ^b	30.00 (0.81) ^c

Different letters in the columns indicate statistically significant differences between cements ($p < 0.05$).

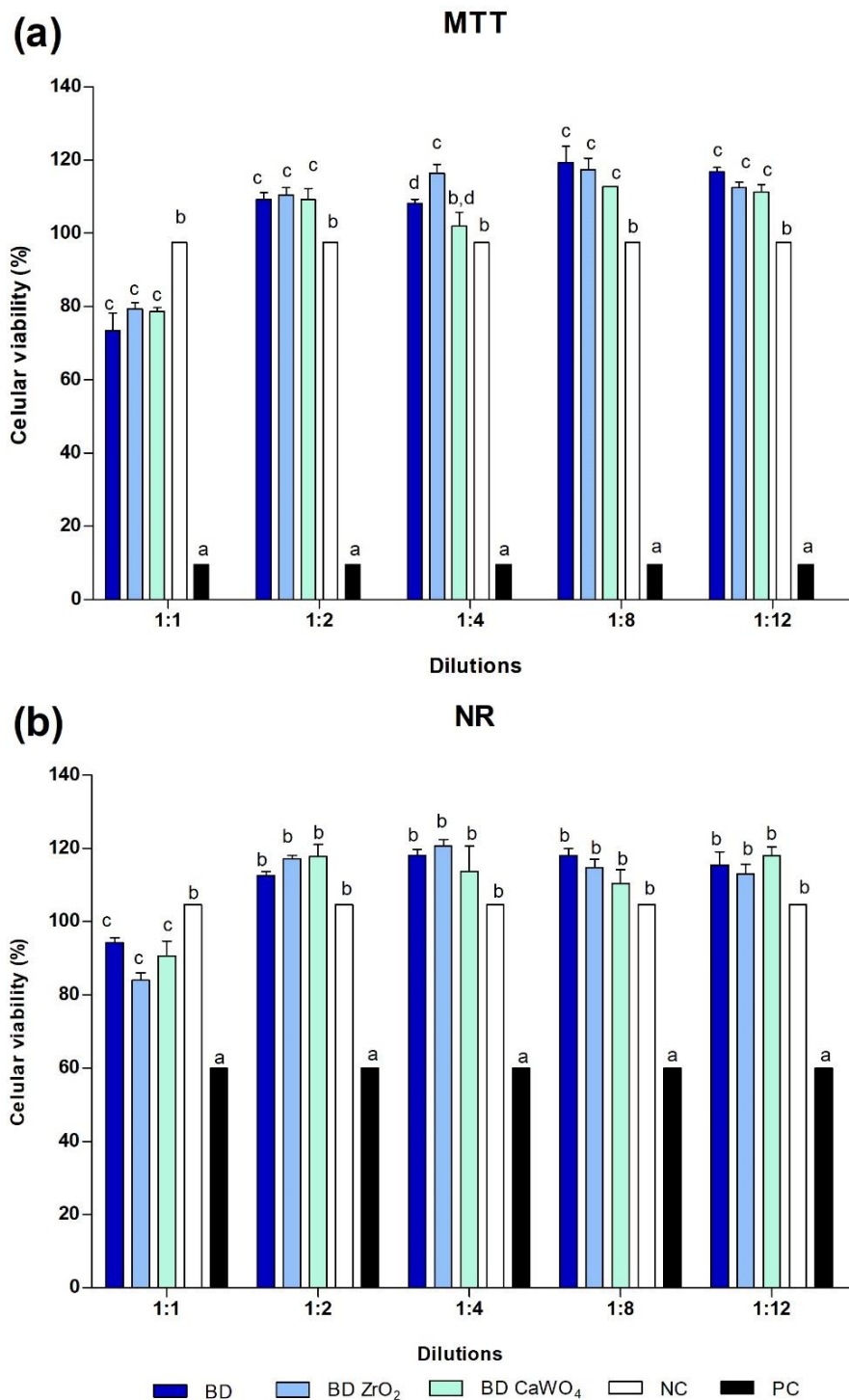
Source: Author

Cytocompatibility and potential to induce mineralization

Cell viability assays

MTT and NR assays revealed that at 1:1 dilution all material groups had lower cell viability values when compared with culture medium - negative control ($p > 0.05$). At 1:2, 1:4, 1:8 and 1:12 dilutions, the cytocompatibility of the materials was greater than ($p < 0.05$) or similar ($p > 0.05$) to negative control. In all dilutions, there was no significant difference among BD, BD CaWO₄ and BD ZrO₂ ($p > 0.05$), except for the 1: 4 dilution in which BD ZrO₂ presented the highest cytocompatibility, in the MTT test. In the positive control group (DMSO) there was low cell viability (Figure 1a and 1b). Considering the results of MTT, the 1:8 dilution was chosen for the ALP activity and ARS assays.

Figure 1 - Saos-2 cell viability evaluated by (a) methyl-thiazol-tetrazolium (MTT) and (b) neutral red (NR) assays. after 24 hours of exposure to BD, BD ZrO₂ and BD CaWO₄ and controls



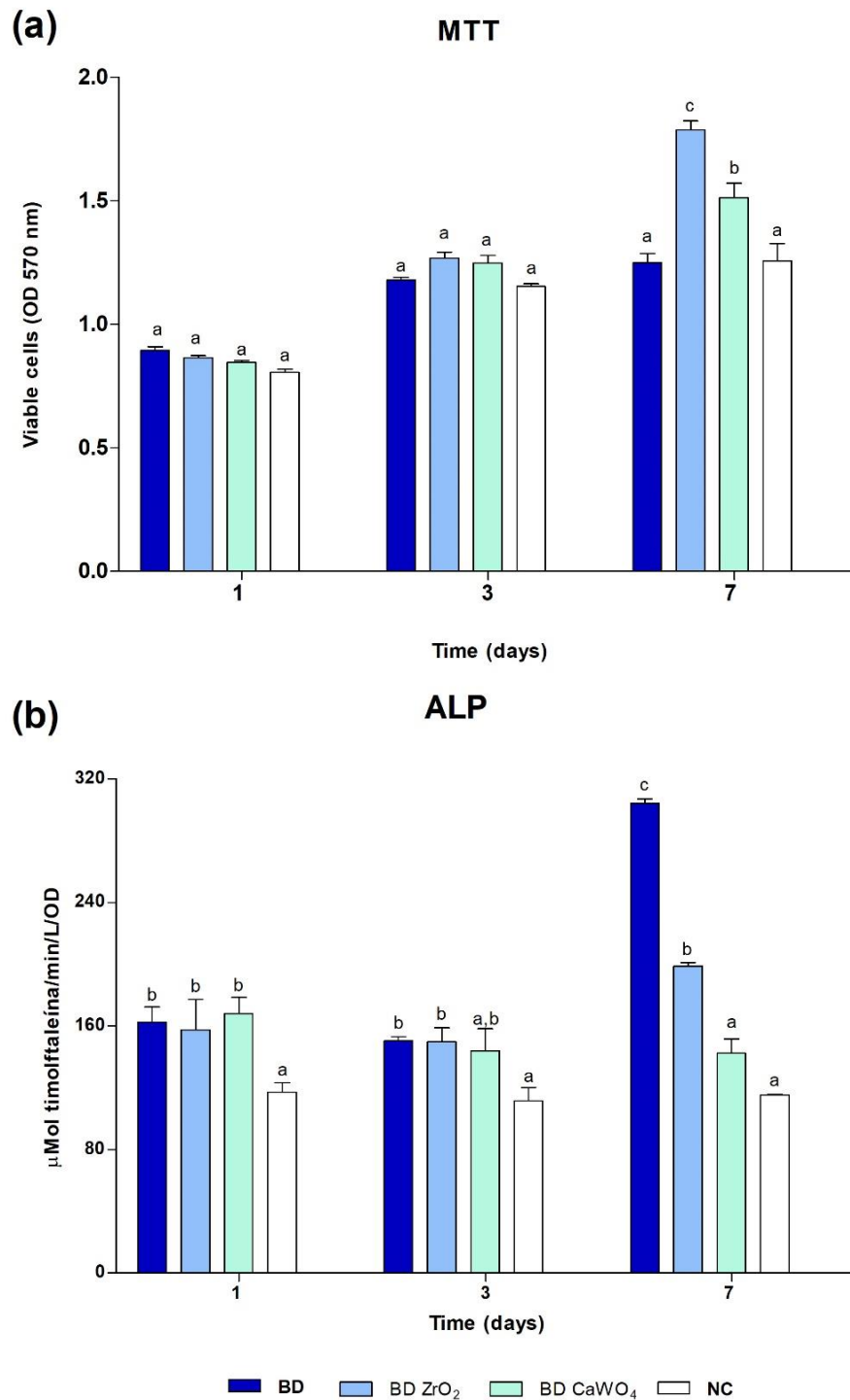
At 1:2, 1:4, 1:8 and 1:12 dilutions, the cytocompatibility of cements was greater than or similar to that of the negative control. Bars with different letters represent significant difference between groups in each dilution. BD, Biodentine; BD ZrO₂, BD with addition of 15% zirconium oxide; BD CaWO₄, BD with addition of 15% calcium tungstate; NC, negative control; PC, positive control

Source: Author

ALP activity

According to Figure 2a, Saos-2 cells exposed to cements extracts had viability similar ($p > 0.05$) or greater ($p < 0.05$) than the control group at 1, 3 and 7 days. The lowest cell viability was detected on the first day of cell exposure to the cement extracts, increasing over the periods of 3 and 7 days. At 7 days, At 7 days, Groups BD ZrO₂ and BD CaWO₄ showed higher cell viability values than BD and control group ($p < 0.05$), whereas there was no significant difference between BD and control group ($p > 0.05$). The ALP activity (Figure 2b) of cements groups at 1, 3 and 7 days was similar ($p > 0.05$) or greater ($p < 0.05$) than that of the control group. At 7 days, the highest ALP activity was detected for BD followed by BD ZrO₂ ($p < 0.05$) and BD CaWO₄ group ($p < 0.05$). There was no significant difference between BD CaWO₄ and control group ($p > 0.05$).

Figure 2 - Saos-2 cell viability evaluated by methyl-thiazol-tetrazolium (MTT) assay (a) and alkaline phosphatase (ALP) activity (b) evaluated after exposure to BD, BD ZrO₂ and BD CaWO₄ at 1:8 dilution and DMEM (negative control) for 1, 3 and 7 days



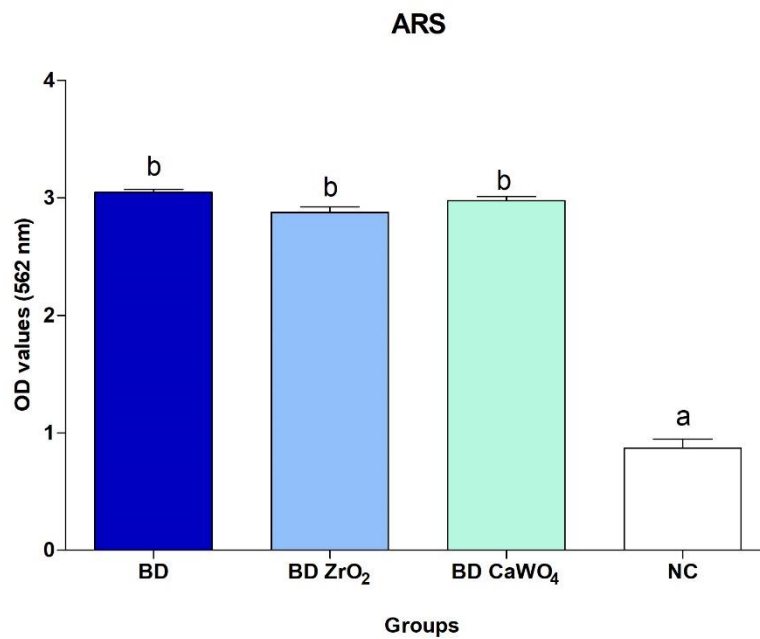
The ALP activity of cement groups at 1, 3 and 7 days was similar to or greater than that of the control group. At 7 days, the highest ALP activity was detected for Group BD followed by Groups BD ZrO₂ and BD CaWO₄. There was no significant difference between Group BD CaWO₄ and control group. Bars with different letters represent significant differences between groups in each period. BD, Biodentine; BD ZrO₂, BD with addition of 15% zirconium oxide; BD CaWO₄, BD with addition of 15% calcium tungstate; NC, negative control.

Source: Author

Alizarin red staining

As observed in the Figure 3, all materials induced a greater production of mineralized nodules when compared to the negative control group ($p < 0.05$) after 21 days of cell exposure to cement extracts. There was no significant difference among the cements groups ($p > 0.05$)

Figure 3 - Alizarin red staining (ARS) assay. Comparison of mineralized nodules production after 21 days of cell exposure to BD, BD ZrO₂ and BD CaWO₄ extracts and negative control group



All cements induced a greater mineralized nodules production when compared to the negative control group. Bars with different letters represent significant differences between groups. BD, Biodentine; BD ZrO₂, BD with addition of 15% zirconium oxide; BD CaWO₄, BD with addition of 15% calcium tungstate; NC, negative control.

Source: Author

5 DISCUSSION

The first aim of this study was to evaluate the radiopacity of BD and BD associated with 15% CaWO₄ or ZrO₂. The radiopacity was evaluated using conventional radiography and different digital radiography systems, because according to literature, the radiopacity of materials may vary between 7% a 20% depending on the radiography systems used (Baksi et al.⁷, 2008). In the present study, depending on the X-ray system employed, BD showed radiopacity ranging between 2,08 to 2,52 mm Al, which is smaller than 3 mm Al recommended by ISO 6876⁴⁸. This meant that 5% of ZrO₂ present in BD is not sufficient to provide an adequate radiopacity. The radiopacity of BD measured in the present study was in agreement with the values showed in previous studies, that showed radiopacity of 2.79 mm Al using digitized conventional radiography (Lucas et al.⁵⁸, 2017) and 2.80 mm Al employing photostimulable phosphor plates (Tanalp et al.⁸², 2013). Conversely, studies using photostimulable phosphor plates showed radiopacity of BD around 4 mm Al (Camilleri et al.¹⁷, 2013; Grech et al.⁴⁰, 2013). The differences could be due diverse factors as X-ray machine, exposure time, tube voltage and source to object distance (Lucas et al.⁵⁸, 2017).

BD ZrO₂ and BD CaWO₄ had radiopacity between 3.52 e 4.26 mm Al, showing that addition of the radiopacifiers in proportion of 15%, resulting in approximately 20% in weight, was sufficient to increase the radiopacity of BD to values higher than the minimum recommended by ISO (ISO 6876)⁴⁸. The amount of radiopacifier added to the BD was based on studies which showed that tricalcium silicate associated with 20% ZrO₂ or Portland cement with 20% ZrO₂ or CaWO₄ exhibited radiopacity greater than 3 mm Al, in addition to adequate physicochemical properties and cytocompatibility (Bortoluzzi et al.⁹, 2009; Hungaro-Duarte et al.⁴⁴, 2012; Gomes-Cornelio et al.³⁸, 2011; Camilleri et al.¹⁷, 2013).

The second objective of this study was to evaluate the physicochemical properties of setting time, pH and solubility, and biological properties of cytocompatibility and potential for induction of mineralization of BD and BD CaWO₄ or BD ZrO₂.

An alkaline medium enhances the mineralization activity of human dental pulp cells (Okabe et al.⁶⁴, 2006) and also contribute to osteogenic potential, biocompatibility and antibacterial activity of material (Zhou et al.⁹⁸, 2013). The addition of radiopacifiers did not change the pH of BD CaWO₄ or BD ZrO₂ when compared with BD; all cements had alkaline pH in all time intervals. Alkaline pH of BD has been shown in the literature (Khan et al.⁵⁵, 2012; Grech et al.³⁹, 2013; Lucas et al.⁵⁸, 2017) and it results from the hydration reaction of tricalcium silicate, which forms calcium hydroxide that dissociates liberating Ca⁺² and (OH)⁻,

thereby, alkalinizing the medium (Camilleri et al.¹⁷, 2013; Khalil et al.⁵⁴, 2016).

Materials that exhibit high solubility may provide inadequate sealing and the presence of voids in the filling. The confection of specimens to evaluate solubility followed the methodology of Carvalho-Junior et al.²³ who showed that smaller specimens than the size recommended by ISO 6876⁴⁸ do not affect the accuracy of the methodology. ISO 6876⁴⁸ establishes that solubility cannot be greater than 3% of total mass after 24 hours of immersion of specimens in water. In the present study, BD showed mass loss of 2.28 % after being immersed in water for 7 days. This result is in line with findings of a previous study, that showed BD mass loss of 2.74%, 2.74% and 2.90% at 24 hours, 3 and 10 days of immersion in water, respectively (Singh et al.⁸¹, 2015). Opposite to our results, some researchers reported solubility of BD higher than 3% evaluated in periods between 24 hours to 7 days of immersion in water (Kaup et al.⁵³, 2015; Torres et al.⁸⁹, 2017). The addition of ZrO₂ did not change the solubility of BD, and the addition of CaWO₄ increased the solubility to 3.63%. Studies have shown that solubility of tricalcium silicate-based cement was not altered by the addition of ZrO₂ or CaWO₄ (Hungaro Duarte et al.⁴⁴, 2012; Marciano et al.⁶⁰, 2016).

The setting time of root canal sealers should be long enough to allow the manipulation and placement in the root canal system (Collares et al.²⁶, 2013). On the other hand, cements with long setting time were more susceptible to dissolution (Bosso-Martelo et al.¹², 2016). The initial setting time of BD in the present study was 27.5 minutes. Previous studies have shown initial setting time of 9 minutes (Septodont)⁷⁶ 16 minutes (Lucas et al.⁵⁸, 2017) or 85.66 minutes (Kaup et al.⁵³, 2015) for BD. The initial setting time of BD CaWO₄ and BD ZrO₂ were 30 minutes and 33.5 minutes respectively, which represent an increase of setting time in relation to the BD of 2.5 minutes for BD CaWO₄ and 6 minutes for BD ZrO₂. It is important to inform that BD associated with radiopacifiers presented a better consistency and greater ease of handling in relation to BD.

Considering the relevance of osteoblast response for mineralized tissue repair, human osteoblast-like cells (Saos-2) were used in the present study (Gomes-Cornélio et al.³⁷, 2017; Tanomaru-Filho et al.⁸³, 2017). Simultaneous evaluation of different cell parameters is necessary to provide reliable information about the cytotoxicity of materials (Scelza et al.⁷⁵, 2012). MTT is a colorimetric test for assessing cell metabolic activity; it is based on succinate dehydrogenase mitochondrial enzyme activity, which converts the yellow tetrazolium salt into insoluble formazan crystals that are violet colored. The absorbance of solubilized formazan crystals is proportional to the amount of living cells (ISO 10993-5)⁴⁷. NR is a cell viability

assay that is based on the incorporation of NR dye into the lysosomes membranes. Thus, the loss of NR uptake corresponds to loss of cell viability (Repetto et al.⁷², 2008).

According to MTT and NR results, all cements evaluated were cytocompatible. The cytocompatibility of BD has been showed in human dental pulp cells (Chang et al.²⁴, 2014), osteoblast-like cells (Jung et al.⁵⁰, 2015; Gomes-Cornélio et al.³⁷, 2017; Rodrigues et al.⁷⁴, 2017) and immortalized murine pulp cells (Zanini et al.⁹⁶, 2012). The addition of radiopacifiers ZrO_2 and $CaWO_4$ did not prejudice the cytocompatibility of BD. A direct comparison of the present results was not possible due to lack of studies evaluating the cytocompatibility of BD associated radiopacifiers. However, calcium silicate-based cements associated with these radiopacifiers have shown good biological properties. Gomes-Cornélio et al.³⁸ (2011) reported that 20% $CaWO_4$ or ZrO_2 in association with white Portland cement was not cytotoxic for periodontal and osteoblast-like cells. Silva et al.⁷⁹ (2017) showed that 30% ZrO_2 in association with white Portland cement induced fibroblast proliferation and accelerated the regression of the inflammatory reaction when compared to MTA in subcutaneous rat tissue.

ALP activity and ARS assays were performed to evaluate cell bioactivity. ALP has a critical role in the mineralization (Golub et al.³⁶, 2007). After 7 days of Saos-2 cell exposure to the cement extracts, ALP activity increased, especially for BD and BD ZrO_2 , when compared to negative control group. These results are in accord with previous studies that showed that BD presented potential to induce mineralization even higher than MTA (Chang et al.²⁴, 2014; Gomes-Cornélio et al.³⁷, 2017; Collado-González et al.²⁵, 2017 Rodrigues et al.⁷⁴, 2017). The association of 30% ZrO_2 with calcium silicate-based cement induced suitable cell bioactivity (Gomes-Cornélio et al.³⁷, 2017)

ARS is a test used to evaluate calcium deposits in cell culture. This test detects microcrystalline or nanocrystalline calcium phosphate salts, apatite crystal clumps and small calcium pyrophosphate dihydrate crystals (Paul et al.⁶⁸, 1983). In the present study all materials induced greater production of mineralized nodules when compared to control group after 21 days of cell exposure to cements extracts. These results are in line with studies, which revealed that BD induced similar, or greater production of mineralized nodules than unexposed cells (Jung et al.⁵⁰, 2015; Gomes-Cornélio et al.³⁷, 2017). In summary, BD, BD associated to ZrO_2 or $CaWO_4$ had cytocompatibility, induced ALP activity and production of mineralized nodules, which are necessary to promote endodontic repair.

6 CONCLUSIONS

BD radiopacity was lower than 3 mm Al in the conventional and digital radiography systems, and addition of 15% ZrO_2 or CaWO_4 was sufficient to increase the radiopacity of BD to values greater than the minimum recommended by ISO 6876 (> 3 mm Al). BD associated with radiopacifiers showed suitable properties of setting time, pH and solubility, except BD CaWO_4 , which exhibit higher solubility than BD and BDZrO_2 . All cements evaluated had citocompatibility and potential to induce mineralization in Saos-2 cells. The results suggest that the addition of 15% ZrO_2 may be a good option to increase the radiopacity of BD, allowing its radiograph detection in clinical practice, without altering its physicochemical and biological properties.

REFERENCES*

1. Akbari M, Rouhani A, Samiee S, Jafarzadeh H. Effect of dentin bonding agent on the prevention of tooth discoloration produced by mineral trioxide aggregate. *Int J Dent.* 2012; 2012:563203
2. Akcay I, Ilhan B, Dundar N. Comparison of conventional and digital radiography systems with regard to radiopacity of root canal filling materials. *Int Endod J.* 2012; 45(8):730-6.
3. American National Standard/American Dental Association (ANSI/ADA). Specification n.57: endodontic sealing material. Chicago: ANSI/ADA; 2000
4. Andolfatto C, Bonetti-Filho I, Carlos IZ, Guerreiro-Tanomaru JM, Kuga MC, Tormin FBC, et al. Cytocompatibility, physical properties, and antibiofilm activity of endodontic sealers with amoxicillin. *Microsc Res Tech.* 2017; 80(9):1036-48.
5. Bachoo IK, Seymour D, Brunton P. Clinical case reports using a novel calcium-based cement. *Br Dent J.* 2013; 214(2):61-4.
6. Baksi BG, Ermis RB. Comparison of conventional and digital radiography for radiometric differentiation of dental cements. *Quintessence Int.* 2007; 38(9): 532-6.
7. Baksi BG, Sen BH, Eyuboglu TF. Differences in aluminum equivalent values of endodontic sealers: conventional versus digital radiography. *J Endod.* 2008; 34(9):1101-4.
8. Belobrov I, Parashos P. Treatment of tooth discoloration after the use of white mineral trioxide aggregate. *J Endod.* 2011; 37(7):1017-20
9. Bortoluzzi EA, Broon NJ, Bramante CM, Felipe WT, Tanomaru Filho M, Esberard RM. The influence of calcium chloride on the setting time, solubility, disintegration, and pH of mineral trioxide aggregate and white Portland cement with a radiopacifier. *J Endod.* 2009; 35(4):550-4.
10. Bortoluzzi EA, Guerreiro-Tanomaru JM, Tanomaru-Filho M, Duarte MA. Radiographic effect of different radiopacifiers on a potential retrograde filling material. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2009; 108(4):628–32.

* 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>

11. Bosso-Martelo R, Guerreiro-Tanomaru JM, Viapiana R, Berbert FL, Basso Bernardi MI, Tanomaru-Filho M. Calcium silicate-based cements associated with micro- and nanoparticle radiopacifiers: physicochemical properties and bioactivity. *Int Sch Res Notices*. 2015; 2015:874283.
12. Bosso-Martelo R, Guerreiro-Tanomaru JM, Viapiana R, Berbert FLC, Húngaro-Duarte MA, Tanomaru-Filho M. Physicochemical properties of calcium silicate cements associated with microparticulate and nanoparticulate radiopacifiers. *Clin Oral Investig*. 2016; 20(1):83-90.
13. 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.
14. 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
15. Camilleri J, Gandolfi MG. Evaluation of the radiopacity of calcium silicate cements containing different radiopacifiers. *Int Endod J*. 2010; 43(1):21–30.
16. Camilleri J, Montesin FE, Brady K, Sweeney R, Curtis RV, Ford TR. The constitution of mineral trioxide aggregate. *Dent Mater*. 2005; 21(4):297-303.
17. 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.
18. Camilleri J. Characterization and hydration kinetics of tricalcium silicate cement for use as a dental biomaterial. *Dent Mater*. 2011; 27(8):836–44.
19. Camilleri J. Characterization of hydration products of mineral trioxide aggregate. *Int Endod J*. 2008; 41(5):408-17.
20. Camilleri J. Hydration mechanisms of mineral trioxide aggregate. *Int Endod J*. 2007; 40(6):462-70.
21. Candeiro GT, Correia FC, Duarte MA, Ribeiro-Siqueira DC, Gavini G. Evaluation of radiopacity, pH, release of calcium ions, and flow of a bioceramic root canal sealer. *J Endod*. 2012; 38(6):842-5.
22. Caron G, Azérad J, Faure MO, Machtou P, Boucher Y. Use of a new retrograde filling material (Biodentine) for endodontic surgery: two case reports. *Int J Oral Sci*. 2014; 6(4):250-3.
23. Carvalho-Junior JR, Correr-Sobrinho L, Correr AB, Sinhoreti MA, Consani S, Sousa-Neto MD. Solubility and dimensional change after setting of root canal sealers: a proposal for smaller dimensions of test samples. *J Endod*. 2007; 33(9):1110-6.

24. 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.
25. Collado-González M, García-Bernal D, Oñate-Sánchez RE, Ortolani-Seltenerich PS, Álvarez-Muro T, Lozano A, et al. Cytotoxicity and bioactivity of various pulpotomy materials on stem cells from human exfoliated primary teeth. *Int Endod J.* 2017; 50 Suppl 2:19-30.
26. Collares FM, Klein M, Santos PD, Portella FF, Ogliari F, Leitune VC, et al. Influence of radiopaque fillers on physicochemical properties of a model epoxy resin-based root canal sealer. *J Appl Oral Sci.* 2013; 21(6):533-9.
27. Coomaraswamy KS, LumLey PJ, Hofmann MP. Effect of bismuth oxide radiopacifier content on the material properties of an endodontic Portland cement-based (MTAlike) system. *J Endod.* 2007;33(3): 295–8.
28. Cuadros-Fernández C, Lorente Rodríguez AI, Sáez-Martínez S, García-Binimelis J, About I, Mercadé M. Short-term treatment outcome of pulpotomies in primary molars using mineral trioxide aggregate and Biodentine: a randomized clinical trial. *Clin Oral Investig.* 2016; 20(7):1639-45.
29. 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.
30. Daltoé MO, Paula-Silva FW, Faccioli LH, Gatón-Hernández PM, De Rossi A, Bezerra Silva LA. Expression of mineralization markers during pulp response to Biodentine and Mineral Trioxide Aggregate. *J Endod.* 2016; 42(4):596-603.
31. De Rossi A, Silva LA, Gatón-Hernández P, Sousa-Neto MD, Nelson-Filho P, Silva RA, et al. Comparison of pulpal responses to pulpotomy and pulp capping with biodentine and mineral trioxide aggregate in dogs. *J Endod.* 2014; 40(9):1362-9.
32. Felman D, Parashos P. Coronal tooth discoloration and white mineral trioxide aggregate. *J Endod.* 2013; 39(4):484–7.
33. Fonseca TS, da Silva GF, Tanomaru-Filho M, Sasso-Cerri E, Guerreiro-Tanomaru JM, Cerri PS. In vivo evaluation of the inflammatory response and IL-6 immunoexpression promoted by Biodentine and MTA Angelus. *Int Endod J.* 2016; 49(2):145-53.
34. Gandolfi MG, Siboni F, Prati C. Properties of a novel polysiloxane-guttapercha calcium silicate-bioglass-containing root canal sealer. *Dent Mat.* 2016; 32(5):113-26.
35. Gandolfi MG, Taddei P, Modena E, Siboni F, Prati C. Biointeractivity-related versus chemi/physisorption-related apatite precursor-forming ability of current root end filling materials. *J Biomed Mater Res B Appl Biomater.* 2013; 101(7):1107-23

36. Golub E, Boesze-Battaglia K. The role of alkaline phosphatase in mineralization. *Curr Opin Orthop*. 2007; 18(5):444-8.
37. 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.
38. Gomes Cornélio AL, Salles LP, Campos da Paz M, Cirelli JA, Guerreiro-Tanomaru JM, Tanomaru Filho M. Cytotoxicity of Portland cement with diferente radiopacifying agents: a cell death study. *J Endod*. 2011; 37(2):203-10.
39. 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(7):632-41.
40. 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.
41. Guerreiro-Tanomaru JM, Cornélio AL, Andolfatto C, Salles LP, Tanomaru-Filho M. pH and antimicrobial activity of Portland cement associated with different radiopacifying agents. *ISRN Dent*. 2012; 2012:469019
42. Huan Z, Chang J. Study on physicochemical properties and in vitro bioactivity of tricalcium silicate- calcium carbonate composite bone cement. *J Mater Sci Mater Med*. 2008; 19(8):2913-8.
43. Húngaro Duarte MA, de Oliveira El Kadre GD, Vivian RR, Guerreiro Tanomaru JM, Tanomaru Filho M, de Moraes IG. Radiopacity of Portland cement associated with different radiopacifying agents. *J Endod*. 2009; 35(5):737-40.
44. Hungaro Duarte MA, Minotti PG, Rodrigues CT, Zapata RO, Bramante CM, Tanomaru Filho M, et al. 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.
45. Hungaro Duarte MA, Tanomaru Filho M, Gomes de Moraes I. 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(2):250-6.
46. Hwang YC, Kim DH, Hwang IN, Song SJ, Park YJ, Koh JT, Son HH, Oh WM. Chemical constitution, physical properties, and biocompatibility of experimentally manufactured Portland cement. *J Endod*. 2011, 37(1):58-62.

47. International Organization for Standardization. ISO 10993-5: biological evaluation of medical devices - Part 5. Tests for in vitro cytotoxicity. London, UK: British Standards Institution; 2009.
48. International Organization for Standardization. ISO 6876: dentistry: root canal sealing materials. London, UK: British Standards Institution; 2012.
49. Ioannidis K, Mistakidis I, Beltes P, Karagiannis V. Spectrophotometric analysis of coronal discoloration induced by grey and white MTA. *Int Endod J.* 2013; 46(2):137-44.
50. Jung JY, Woo SM, Lee BN, Koh JT, Nör JE, Hwang YC. Effect of Biodentine and Bioaggregate on odontoblastic differentiation via mitogen-activated protein kinase pathway in human dental pulp cells. *Int Endod J.* 2015; 48(2):177-84.
51. Kang SH, Shin YS, Lee HS, Kim SO, Shin Y, Jung IY, Song JS. Color changes of teeth after treatment with various mineral trioxide aggregate-based materials: an ex vivo study. *J Endod.* 2015; 41(5):737-41.
52. Katara G, Hemvani N, Chitnis S, Chitnis V, Chitnis DS. Surface disinfection by exposure to germicidal UV light. *Indian J Med Microbiol.* 2008; 26(3):241-2.
53. Kaup M, Schäfer E, Dammaschke T. An in vitro study of different material properties of Biodentine compared to ProRoot MTA. *Head Face Med.* 2015; 2:11-6.
54. Khalil I, Alfred Naaman, DDS, Josette Camilleri. Properties of tricalcium silicate sealers. *J Endod.* 2016; 42(10):1529-35.
55. Khan SIR, Ramachandran A, Deepalakshmi M, Kumar KS. Evaluation of pH and calcium ion release of mineral trioxide aggregate and a new root-end filling material. *E-Journal of Dentistry.* 2012; 2(2):166-9.
56. Laurent P, Camps J, De Méo M, Déjou J, About I. Induction of specific cell responses to a Ca_3SiO_5 -based posterior restorative material. *Dent Mater.* 2008; 24(11):1486-94.
57. Li Q, Deacon AD, Coleman NJ. The impact of zirconium oxide nanoparticles on the hydration chemistry and biocompatibility of white Portland cement. *Dent Mater J.* 2013; 32(5):808-15
58. 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.
59. 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.

60. Marciano MA, Duarte MA, Camilleri J. Calcium silicate-based sealers: assessment of physicochemical properties, porosity and hydration. *Dent Mater.* 2016; 32(2): e30-40.
61. McCabe JF, Walls AWF. *Applied dental materials.* 9th ed. Oxford: Blackwell; 2008.
62. Margunato S, Taşlı PN, Aydın S, Karapınar Kazandağ M, Şahin F. In vitro evaluation of ProRoot MTA, Biodentine, and MM-MTA on human alveolar bone marrow stem cells in terms of biocompatibility and mineralization. *J Endod.* 2015; 41(10):1646-52.
63. Nowicka A, Lipski M, Parafiniuk M, Sporniak-Tutak K, Lichota D, Kosierkiewicz A, et al. Response of human dental pulp capped with biodentine and mineral trioxide aggregate. *J Endod* 2013; 39(6):743-7.
64. Okabe T, Sakamoto M, Takeuchi H, Matsushima K. Effects of pH on mineralization ability of human dental pulp cells. *J Endod.* 2006; 32(3):198-201.
65. 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.
66. Parirokh M, Torabinejad M. Mineral trioxide aggregate: a comprehensive literature review--Part III: Clinical applications, drawbacks, and mechanism of action. *J Endod.* 2010; 36(3):400-13.
67. 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.
68. Paul H, Reginato AJ, Schumacher HR. Alizarin red S staining as a screening test to detect calcium compounds in synovial fluid. *Arthritis Rheum.* 1983; 26(2):191-200.
69. Peng W, Liu W, Zhai W, Jiang L, Li L, Chang J, et al. Effect of tricalcium silicate on the proliferation and odontogenic differentiation of human dental pulp cells. *J Endod.* 2011; 37(9):1240-6.
70. Rajasekharan S, Martens LC, Cauwels RG, Verbeeck RM. Biodentine™ material characteristics and clinical applications: a review of the literature. *Eur Arch Paediatr Dent.* 2014; 15(3):147-58.
71. Rasimick BJ, Shah RP, Musikant BL, Deutsch AS. Radiopacity of endodontic materials on film and a digital sensor. *J Endod.* 2007; 33(9):1098-101.
72. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc.* 2008; 3(7):1125-31.

73. Rodrigues EM, Gomes -Cornélio AL, Mestieri LB, Fuentes ASC, Salles LP, Rossa-Junior C, et al. Human dental pulp cells response to mineral trioxide aggregate (MTA) and MTA Plus: cytotoxicity and gene expression analysis. *Int Endod J.* 2017; 50(8):780-9.
74. 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 Suppl 2:9-18.
75. Scelza MZ, Linhares AB, da Silva LE, Granjeiro JM, Alves GG. A multiparametric assay to compare the cytotoxicity of endodontic sealers with primary human osteoblasts. *Int Endod J.* 2012; 45(1):12-8.
76. Septodont's Research Group. Biodentine™: Active Biosilicate Technology™: scientific file. Saint-Maur-des-Fossés Cedex: R&D; 2010.
77. Siboni F, Taddei P, Prati C, Gandolfi MG. Properties of Neo MTA Plus and MTA Plus cements for endodontics. *Int Endod J.* 2017; 50 Suppl 2:83-94.
78. Silva GF, Bosso R, Ferino RV, Tanomaru-Filho M, Bernardi MI, Guerreiro-Tanomaru JM, et al. Microparticulated and nanoparticulated zirconium oxide added to calcium silicate cement: Evaluation of physicochemical and biological properties. *J Biomed Mater Res A.* 2014; 102(12):4336-45.
79. Silva GF, Guerreiro-Tanomaru JM, da Fonseca TS, Bernardi MIB, Sasso-Cerri E, Tanomaru-Filho M, et al. Zirconium oxide and niobium oxide used as radiopacifiers in a calcium silicate-based material stimulate fibroblast proliferation and collagen formation. *Int Endod J.* 2017; 50 Suppl 2: e95-e108
80. Silva LAB, Pieroni KAMG, Nelson-Filho P, Silva RAB, Hernández-Gatón P, Lucisano MP, et al. Furcation perforation: periradicular tissue response to Biodentine as a repair material by histopathologic and indirect immunofluorescence analyses. *J Endod.* 2017; 43(7):1137-42.
81. 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.
82. Tanalp J, Karapınar-Kazandağ M, Dölekoğlu S, Kayahan MB. Comparison of the radiopacities of different root-end filling and repair materials. *ScientificWorldJournal.* 2013; 2013:594950.
83. Tanomaru-Filho M, Andrade AS, Rodrigues EM, Viola KS, Faria G, Camilleri J, et al. Biocompatibility and mineralized nodule formation of Neo MTA Plus and an

- experimental tricalcium silicate cement containing tantalum oxide. *Int Endod J.* 2017; 50 Suppl 2:31-9.
84. Tanomaru-Filho M, Jorge EG, Guerreiro Tanomaru JM, Gonçalves M. Radiopacity evaluation of new root canal filling materials by digitalization of images. *J Endod.* 2007;33(3):249-51.
85. Tanomaru-Filho M, Torres FFE, Chávez-Andrade GM, de Almeida M, Navarro LG, Steier L, et al. Physicochemical properties and volumetric change of silicone/bioactive glass and calcium silicate-based endodontic sealers. *J Endod.* 2017; 43(12):2097-101
86. Tanomaru-Filho M, Morales V, da Silva GF, Bosso R, Reis JM, Húngaro-Duarte MA, et al. Compressive strength and setting time of MTA and Portland cement associated with different radiopacifying agents. *ISRN Dent.* 2012; 2012:898051.
87. Torabinejad M, White DJ inventors; Loma Linda University, assignee. Tooth filling material and method of use. United States patent 5769638.1998 Jun 23.
88. Torabinejad M, Parirokh M, Dummer PMH. Mineral trioxide aggregate and other bioactive endodontic cements: an updated overview - part II: other clinical applications and complications. *Int Endod J.* 2018; 51(3):284-317.
89. Torres FFE, Bosso-Martelo R, Espir CG, Cirelli JA, Guerreiro-Tanomaru JM, Tanomaru-Filho M. Evaluation of physicochemical properties of root-end filling materials using conventional and Micro-CT tests. *J Appl Oral Sci.* 2017; 25(4):374-80.
90. Versiani MA, Abi Rached-Junior FJ, Kishen A, Pécora JD, Silva-Sousa YT, de Sousa-Neto MD. Zinc oxide nanoparticles enhance physicochemical characteristics of Grossman Sealer. *J Endod.* 2016; 42(12):1804-10.
91. 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.
92. Vivian RR, Zapata RO, Zeferino MA, Bramante CM, Bernardineli N, Garcia RB, 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(2):250-6.
93. Wang X, Sun H, Chang J. Characterization of $\text{Ca}_3\text{SiO}_5/\text{CaCl}_2$ composite cement for dental application. *Dent Mater.* 2008; 24(1):74-82.
94. Wang CW, Chiang TY, Chang HC, Ding SJ. Physicochemical properties and osteogenic activity of radiopaque calcium silicate-gelatin cements. *J Mater Sci Mater Med.* 2014; 25(9):2193-203.

95. Westgard JO, Barry PL, Hunt MR, Groth T. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem*. 1981; 27(3):493-501.
96. Zanini M, Sautier JM, Berdal A, Simon S. Biodentine induces immortalized murine pulp cell differentiation into odontoblast-like cells and stimulates biomineralization. *J Endod*. 2012; 38(9):1220-6.
97. Zhao W, Chang J, Zhai W. Self-setting properties and in vitro bioactivity of $\text{Ca}_3\text{SiO}_5/\text{CaSO}_4 \cdot 1/2\text{H}_2\text{O}$ composite cement. *J Biomed Mater Res*. 2008; 85(2):336-44.
98. Zhou HM, Shen Y, Zheng W, Li L, Zheng YF, Haapasalo M. Physical properties of 5 root canal sealers. *J Endod*. 2013; 39(10):1281-6.

Não autorizo a reprodução deste trabalho pelo prazo de 2 anos após a data de defesa
(Direitos de publicação reservado ao autor)

Araraquara, 29 de março de 2018.

Victor Manuel Ochoa Rodríguez