UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

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Physicochemical and biological properties of a new bioceramic root canal filling material for primary teeth

Araraquara

2022

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Thesis presented to the Dentistry department of Araraquara, Universidade Estadual Paulista (Unesp) to obtain the title of Doctor in Dentistry, in the area of Endodontics

Advisor: Profa. Dra. Gisele Faria

Araraquara

2022

Ochoa Rodríguez, Victor Manuel
R696p Physicochemical and biological properties of a new bioceramic root canal filling material for primary teeth / Victor Manuel Ochoa Rodríguez. – Araraquara, 2022
70 f. : fotos

Thesis (Doctoral) – São Paulo State University (Unesp). School of Dentistry
Adivisor: Prof. Dr. Gisele Faria

1. Tooth, deciduous. 2. Endodontics. 3. X-Ray microtomography. 4. Chemical phenomena. I.Title.

Sistema de geração automática de ficha da Unesp. Biblioteca da Faculdade de Odontologia, Araraquara.
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Araraquara, June 27, 2022

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AKNOWLEDGEMENTS

To my advisor **Prof. Dr. Gisele Faria**, thank you so much for being the best advisor, person and friend. For always teaching me with patience and answering my questions. Learning from science by your side is an honor, your intelligence and reasoning ability is admirable. I will always be grateful for the time shared and its teachings in both science and life. Thank you for your patience and love!

To my parents, for their support, affection and unconditional love on my way in this great adventure in search of knowledge, wisdom and improvement.

To **Prof. Dr. Mário Tanomaru-Filho**, for his teachings and always being available to support me, answer my questions and for his professional and life advice.

To postgraduate professors **Prof. Dr. Juliane Maria Guerreiro-Tanomaru, Prof. Dr. Fabio Luiz Camargo Villela Berbert and Prof. Dr. Idomeio Bonetti-Filho**. It was a pleasure to learn from you. Thank you!

To friends/laboratory colleagues, **Jáder Camilo Pinto, Laura Andrea Gonzáles Maldonado, Eric Hernán Coaguila Llerena, Rafaela Honda Inada, Lívia Bueno Campi, Fernanda Ferrari Esteves Torres, Karina Inés Medina Carita Tavares, Ana Flávia Balestrero, Luana Raphael da Silva and Leandro Fernandes.** Thank you for the partnership and teachings.

To the employees of **FOAr-UNESP**, who with their kindness brightened my days at college. I am very grateful for the coexistence and for all the help for the development of my work and day to day.

To the **Faculty of Dentistry of Araraquara (FOAr-UNESP)**, all its professors, employees and students who allowed my professional and personal training in this postgraduate period.

To **CAPES**: This work was carried out with the support of the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) - Financing code 001.

To all those who directly or indirectly contributed to the development of this work. Thanks.

“Thy Life is a riddle, to bear rapture and sorrow
To listen, to suffer, to entrust unto tomorrow
In one fleeting moment, from the Land doth life flow
Yet in one fleeting moment, for anew it doth grow
In the same fleeting moment
Thou must live
Die
And know”

Answers, Final Fantasy XIV [*]

[*] Final Fantasy XIV, composed by Nobuo Uematsu, arranged by Tsutomu Narita, lyrics written by Yaeko Sato and Michael-Christopher Koji Fox, and performed by Susan Calloway.

Ochoa Rodríguez VM. Physicochemical and biological properties of a new bioceramic root canal filling material for primary teeth [Doctoral Thesis]. Araraquara: Dentistry departmet - São Paulo State University - UNESP; 2022.

ABSTRACT

An ideal root canal filling for primary teeth should not hinder the eruption of permanent successor teeth, but rather, should be resorbed as the primary teeth roots are physiologically resorbed. Bio-C Pulpecto - Bio CP (Angelus, Londrina, Brazil) was recently developed as the first bioceramic filling material for primary teeth. The objectives of **publication 1** were to evaluate radiopacity, setting time, pH, cytocompatibility and the potential for mineralization of Bio-CP compared to Calen thickened with zinc oxide (Calen-ZO) and zinc oxide eugenol (ZOE). The physicochemical properties were evaluated according to ISO 6876. Saos-2 (human osteoblast-like lineage) exposed to material extracts were subjected to methylthiazolyl tetrazolium, neutral red, alkaline phosphatase (ALP) activity and production of mineralized nodule. The results were analyzed using one-way or two-way ANOVA and Tukey or Bonferroni post-tests (α=0.05). All materials showed radiopacity greater than 3 mm Al. Bio-CP had a lower pH than Calen-ZO, but higher than ZOE. Calen-ZO and Bio-CP did not set. The ZOE setting time was 110 min. The order of cytocompatibility was Calen-ZO > Bio-CP > ZOE (1:2, 1:4 dilutions) and Calen-ZO > Bio-CP = ZOE (1:12, 1:24 dilutions) and Calen-ZO = Bio - CP>ZOE (dilution 1:32). Bio-CP induced greater ALP activity at 7 days, and greater production of Cal-mineralized proteins, compared to greater numbers of people ($p<0.05$). The objectives of **publication 2** were evaluated for solubility (mass loss), using modified methodology from ISO 6876; filling capacity, volumetric change and presence of voids, by means of micro computed tomography (micro-CT), of Bio-CP in comparison with Calen-ZO and ZOE. In addition, the distribution of elements on the surface of the materials and the crystalline phases of Calen-ZO and Bio-CP were evaluated by energy scattering X-ray scanning electron microscopy (SEM-EDX) and X-ray diffraction (XRD). The materials were placed in polyethylene tubes of 1 or 2 mm in diameter and immersed in water or PBS (Phosphate-Buffered Saline) for 30 days. Data were analyzed by two-way ANOVA and Tukey or Kruskal-Wallis and Dunn (α=0.05). Solubility was greater than 7% for all materials; the order was ZOE>Calen-ZO=Bio-CP. Calen-ZO and Bio-CP were more soluble in water than in PBS. Most materials showed higher solubility in 2 mm tubes, both in PBS and in water. Only Calen-ZO and ZOE were analyzed on micro-CT because Bio-CP separated into two phases during scanning. There was no difference in filling capacity between the materials. Calen-ZO had greater volumetric loss and presence of voids than ZOE in water, but there was no difference in PBS. Precipitates on the surface of BioCP and Calen-ZO, corresponding to hydroxyapatite, were drawn after printing in PBS. It was concluded that BioCP presented adequate physicochemical properties, citocompatibility and potential to induce mineralization. All materials showed adequate filling capacity. Bio-CP and Calen-ZO showed lower solubility than ZOE, regardless of immersion medium and tube diameter, except for the 2 mm tube immersed in PBS, and produced hydroxyapatite when immersed in PBS. Micro-CT was not suitable for Bio-CP evaluation. Although Bio-CP has the potential to become a suitable material for filling primary teeth, its composition needs to be revised to achieve chemical stability.

Keywords: Tooth, Deciduous. Endodontics. X-Ray microtomography. Chemical phenomena

Ochoa Rodríguez VM. Propriedades físico-químicas e biológicas de um novo material biocerâmico para obturação de canais radiculares de dentes decíduos [Doctoral Thesis]. Araraquara: Dentistry departmet - São Paulo State University - UNESP; 2022.

RESUMO

Bio-C Pulpecto - Bio CP (Angelus, Londrina, Brasil) foi recentemente desenvolvido como o primeiro material obturador biocerâmico para dentes decíduos. Os objetivos da **publicação 1** foram avaliar radiopacidade, tempo de presa, pH, citocompatibilidade e o potencial para induzir mineralização do Bio-CP em comparação com Calen espessado com óxido de zinco (Calen- ZO) e óxido de zinco e eugenol (ZOE). As propriedades físico-químicas foram avaliadas de acordo com ISO 6876. Saos-2 (linhagem de osteoblastos-*like* de humanos) expostos a extratos dos materiais foram submetidos a ensaios de metiltiazolil tetrazólio, vermelho neutro, atividade de fosfatase alcalina (ALP) e produção de nódulos mineralizados. Os resultados foram analisados empregando ANOVA *one-way* ou *two-way* e pós-testes de Tukey ou Bonferroni (α=0,05). Todos os materiais apresentaram radiopacidade superior a 3 mm Al. Bio-CP teve pH mais baixo que Calen-ZO, porém mais elevado que ZOE. Calen-ZO e Bio-CP não tomaram presa. O tempo de presa de ZOE foi de 110 min. A ordem de citocompatibilidade foi Calen-ZO> Bio-CP> ZOE (diluições 1: 2, 1: 4) e Calen-ZO> Bio-CP = ZOE (diluições 1:12, 1:24) e Calen-ZO = Bio- CP> ZOE (diluição 1:32). Bio-CP induziu maior atividade ALP em 7 dias, e maior produção de nódulos mineralizados, em comparação com Calen-ZO ($p < 0,05$). Os objetivos da **publicação 2** foram avaliar a solubilidade (perda de massa), empregando metodologia modificada da ISO 6876; e capacidade de preenchimento, alteração volumétrica e presença de vazios, por meio de microtomografia computadorizada (micro-CT), do Bio-CP em comparação Calen- ZO e ZOE. Além disso, foram avaliadas a distribuição de elementos na superfície dos materiais e as fases cristalinas de Calen-ZO e Bio-CP por microscopia eletrônica de varredura com raio-X por dispersão de energia (SEM-EDX) e difração de raios-X (XRD). Os materiais foram inseridos em tubos de polietileno de 1 ou 2 mm de diâmetro e imersos em água ou PBS (solução salina tamponada com fosfato) por 30 dias. Os dados foram analisados por ANOVA two-way e Tukey ou Kruskal-Wallis e Dunn (α=0,05). A solubilidade foi superior a 7% para todos os materiais; a ordem foi ZOE>Calen-ZO=Bio-CP. Calen-ZO e Bio-CP foram mais solúveis em água que em PBS. A maioria dos materiais apresentou maior solubilidade em tubos de 2 mm, tanto em PBS quanto em água. Apenas Calen-ZO e ZOE foram analisados em micro-CT porque Bio-CP se separou em duas fases durante o escaneamento. Não houve diferença na capacidade de preenchimento entre os materiais. Calen-ZO teve maior perda volumétrica e presença de vazios do que ZOE em água, mas não houve diferença em PBS. Precipitados na superfície de Bio-CP e Calen-ZO, correspondentes à hidroxiapatita, foram detectados após imersão em PBS. Concluiu-se que Bio-CP apresentou propriedades físico-químicas adequadas, citocompatibilidade e potencial para induzir mineralização. Todos os materiais apresentaram boa capacidade de preenchimento. Bio-CP e Calen-ZO apresentaram solubilidade menor que ZOE, independentemente do meio de imersão e diâmetro do tubo, com exceção do tubo de 2 mm imerso em PBS, e produziram hidroxiapatita quando imersos em PBS. Micro-CT não foi adequada para avaliação do Bio-CP. Embora Bio-CP tenha potencial para se tornar um material adequado para obturação de dentes decíduos, sua composição precisa ser revista para se obter estabilidade química.

Palavras-chave: Dente decíduo. Endodontia. Microtomografia por Raio-X. Fenômenos químicos.

SUMMARY

1 INTRODUCTION

To achieve success in endodontic therapy, a complete cleaning and shaping, a three-dimensional hermetic sealing throughout the canal and minor discrepancies between the dentinal wall of the root canal and the core filling material is required[1,2]. The ideal root canal sealer should have the following properties: ease of handling, be radiopaque, provide good adhesion between the canal walls and the core material, establish an hermetic seal, no shrinkage on setting, no staining of the tooth structure, be bacteriostatic or at least not encourage bacterial growth, insoluble in tissue fluids, be biocompatible and soluble in common solvent if a retreatment is required[3]. On the other hand, the ideal filling material for root canals of primary teeth should not impair the eruption of permanent successor teeth, should not be soluble in water, accompany the physiological resorption of the roots of primary teeth, be quickly reabsorbed when extruded beyond the apex, have antimicrobial properties, adhere to the root canal walls, should not suffer contraction, should be easily removed if necessary, have adequate radiopacity and do not cause teeth color alteration[4,5]. Many materials have been proposed for filling primary teeth, but none of them meet all the requirements for an ideal filling material[6,7].

Zinc oxide and eugenol (ZOE), for a long time, has been widely used as primary teeth filling material[6,8-12]. However, the deficiencies shown by this material have limited its use. ZOE is genotoxic[13] and cytotoxic[13,14], triggering inflammatory reactions in periapical tissues, particularly when extravasated beyond the radicular apex[14-16]. The irritating effect of ZOE has been attributed to eugenol[6,14]. In addition, ZOE has a low capacity to be phagocyted and eliminated, leaving particles in the periapical tissues when extravasated beyond the apex and also after the physiological root resorption of the root of primary teeth[11,12], taking months or even years to resorb[16-20].

Calcium hydroxide-based materials are also commonly used for filling root canals of primary teeth[6,8,21-24]. Calcium hydroxide [$Ca(OH)_2$] was introduced in Dentistry by Herman in 1920 for pulp capping[25] and, since then, it has been used in Endodontics for different purposes. The main reasons for using $Ca(OH)_2$ as a root canal filling material for primary teeth are its ability to stimulate mineralization and its antimicrobial effect[26,27]. These effects occur due to the dissociation of $Ca(OH)_2$ in calcium and hydroxyl ions[28]. The antimicrobial effect of $Ca(OH)_2$ is mainly attributed to its high pH, producing denaturation and damage to DNA and cytoplasmic membranes

in bacterial cells[29]. In addition, $Ca(OH)_2$ has the ability to neutralize the LPS produced by gram-negative bacteria[15,30-34] and neutralize lactic acid secreted by osteoclasts, preventing the loss of mineralized tissue[26,31]. The effect on mineralization happens because $Ca(OH)_2$ induces proliferation, migration, osteogenic differentiation of undifferentiated cells and mineralization[26,35], through mitogen-activated protein kinases – MAP[26].

Calen® paste (SS White, Rio de Janeiro, RJ, Brazil), an intracanal medication based on $Ca(OH)_2$, has antibacterial activity[27,36,37], high pH[38] and induces the formation of mineralized tissue or nodules[39-42]. Studies have shown adequate biocompatibility of the Calen® paste compared to other $Ca(OH)_2$-based pastes when implanted in subcutaneous tissue of rats[43-45]. However, the Calen® paste shows permeability to tissue fluids, is soluble in the root canal, has high flow and high viscosity and is, therefore, not suitable for clinical use as root canal filling material for primary teeth[23]. On the other hand, Calen® paste thickened with zinc oxide (Calen-ZO) has been used as root canal filling material in primary dentition[24,27,36,37]. showing adequate tissue response[24,44], antimicrobial activity[36] and radiopacity[23], in the proportions of 1:0.5 and 1:0.65 for Calen® paste and ZO respectively[23]. The addition of ZO reduces the phagocytosis of the Calen® paste, allowing its resorption to accompany the resorption of the roots of primary teeth[24]. A study has shown that Calen-ZO is more biocompatible than ZOE when used for root canal filling of dog teeth[24].

Iodoform-based pastes (KRI), or a combination paste of iodoform and calcium hydroxide are also used to fill the canals of primary teeth[8] because of its antibacterial effect[46], healing properties, and ability to be resorbed when in excess[47]. A recent systematic review and meta-alaysis showed that the clinical and radiographical success rates of $Ca(OH)_2$/iodoform paste are comparable with that of ZOE in primary teeth pulpectomy up to ≥18-month follow-up[10].

New endodontic cements, bioceramics among them, have been developed. Bioceramics, ceramic materials specifically manufactured for use in medicine and dentistry[48,49], can be classified into three major groups: almost inert, bioactive and resorbable[48]. They can be composed of alumina and zirconia, bioactive glass, glass ceramics, coatings and composites, hydroxyapatite, calcium phosphates or calcium silicates[49].

The main characteristics of bioceramic materials for endodontic use (calcium silicate-based bioceramics) are their alkaline pH, antibacterial activity, low contraction,

stability in a biological environment[49-51], biocompatibility[49,52-55] and bioactivity[52-55]. The hydration of calcium silicate-based cements with tissue fluids[56] or phosphate-containing solution[57] forms hydrated calcium silicate gel and $Ca(OH)_2$. $Ca(OH)_2$ suffers a chemical reaction with phosphate ions present in tissue fluids, which results in the formation of hydroxyapatite on the material surface[56,57]. This chemical apatite-forming reaction is thought to be useful for predicting in vivo bioactivity[57]. Hydroxyapatite finally provides a chemical bond with the root canal dentin[51,58]. In addition, bioceramic cements are hydrophilic, that is, they tolerate and need moisture for the setting reaction[2,51,59,60,61]. Although calcium silicate-based sealers have suitable physicochemical properties, studies have shown that they have high solubility[62,63] when evaluated by the ISO 6876[64] standards.

The biocompatibility and bioactivity of bioceramic sealers for permanent teeth has been studied in different types of cells. EndoSequence BC sealer cement (Brasseler USA, Savannah, GA, USA) showed low cytotoxicity in L929 fibroblasts[61], human gingival fibroblasts[2] and murine osteoblast precursor cells (IDG- SW3) and promoted osteoblastic differentiation[52]. In human stem cells from the apical papilla, iRoot FS (Innovate BioCreamix Inc.) promoted cell migration and enhanced their osteo/odontogenesis potential, via the Wnt/β-catenin pathway, without cytotoxicity when compared to ProRoot MTA (Dentsply Maillefer, Tulsa, OK)[65].

Biocompatibility and in vivo bioactivity of bioceramic materials have also been studied. Sealer Plus BC (Angelus), Bio-C Sealer (MK Life, Porto Alegre, Brazil), NeoMTA Plus (Avalon Biomed Inc., Bradenton, FL, USA), Bio-C Pulpo (Angelus), Biodentine (Sepdodont, Saint-Maur-des-Fossés, France) and White MTA (Angelus) showed biocompatibility and bioactive potential when implanted in dorsal subcutaneous sites of Holtzman rats[66-68].

Bio-C Pulpecto (Bio-CP; Angelus, Londrina, Paraná, Brazil), the first bioceramic root filling material for primary teeth, is composed of ester glycol salicylate, titanium oxide, calcium tungstate, silicon dioxide, toluene sulphonamide, and calcium silicate. According to the manufacturer, it has high alkalinity (pH 12.7), high radiopacity (9 mm of aluminium scale), and is resorbable, thus allowing simultaneous physiological resorption of the root and the material (Bio-C Pulpecto leaflet, Angelus, Basil, Londrina, Paraná, Brazil). A study showed that the Bio-CP is biocompatible and induced biomineralization in the subcutaneous tissue of rats, in a manner similar to MTA[69]. In addition, a root filling material for primary teeth developed by Angelus, with similar

composition of Bio-CP, but with the name not informed, showed adequate biocompatibility in the subcutaneous tissue of rats, low cytotoxicity in human gingival fibroblast, and satisfactory behaviour regarding the physicochemical properties studied[7]. However, the scientific literature about Bio-CP is still scarce[7,22,69].

Endodontic sealers should have low solubility, to prevent microbial and fluid leakage[70]. Furthermore, they should have minimal presence of voids, so it does not affect the obturation quality[71]. In case of a primary teeth root canal filling material, even though low solubility is a desirable property, the materials should also be resorbed as the primary teeth roots are physiologically resorbed and should also resorb readily if pressed beyond the apex[4,5]. Specifically, regarding solubility, they must not have high solubility, to avoid the formation of gaps, nor be insoluble so that they are not retained in the tissue, leading to inflammation, or impairing the eruption of the permanent successor[72,73].

Traditionally the solubility of endodontic materials for permanent teeth is evaluated complying the ISO guidelines[64] which recommends elaborating test specimens of the material with a diameter of 20 ± 1 mm and a height of 1,5 ± 0,1 mm. After setting, the test specimens are weighted and immersed in distilled water for 24 hours. Then, the specimens are dried in desiccators and the final weight is compared to the initial weight, determining the loss of mass. However, this model does not reproduce the clinical situation, mainly because tissue fluids are different from water, cement contact time with fluids is not restricted to 24 hours and sealers are immediately placed into contact with tissue fluids and/or blood during root canal treatment[70]. For this reason, to further understand the behavior of these materials, researchers have developed other methodologies to complement the solubility test of root canal sealers[70,74].

The methodologies to complement the solubility test of root canal sealers are based on micro computed tomography (micro-CT) to evaluate volumetric change and correlate it with the solubility of the material[74]. The root canals of permanent teeth are filled, and the teeth are immediately placed in the immersion medium[70]. The immersion time varies from 7 to 30 days[75], and the immersion medium can be distilled water[75] or phosphate-buffered saline - PBS -[70,74]. The use of PBS provides greater similarity to clinical situation than water[76]. In this model, the teeth are scanned using micro-CT immediately after filling and after the immersion period[70]. The use of micro-CT provided three-dimensional volumetric analysis (in mm^3), allowing correlation of volumetric

change with the solubility[72]. In addition, micro-CT may be used as a method for evaluating morphologic alterations and voids of the materials[40].

It is important to mention that to assess the solubility of primary teeth filling materials, it is not possible to use the ISO 6876[64] methodology, since many of them do not set. Thus, an evaluation of the solubility of these materials must be performed by a modified methodology. Researchers have evaluated the solubility of primary teeth filling materials inserted in polyethylene tubes with one end closed[23]. However, there is no defined protocol for performing the analysis of the solubility (expressed as loss of mass) of primary teeth filling materials in relation to the diameter of the polyethylene tubes and the immersion medium (water or PBS). In addition, there is no protocol for evaluating volumetric and morphological change in these materials using micro-CT. At first, one could consider using natural primary teeth filled with the studied materials, following methodology models used in permanent teeth[70]. However, as it is very difficult to obtain extracted primary teeth with non-resorbed roots, so alternative methodologies are used. One alternative is using polyethylene tubes[23] to simulate the root canals of primary teeth, because it's easy availability and access in the medical market and handling when prepared as study samples. Another is to evaluate those materials in 3D printed prototyped primary teeth, this is because, they have the potential to be used for educational purposes, endodontic training, and research due to the sample standardization[77,78].

Because the scientific literature on Bio-CP is still scarce, and its solubility is not known, studies are necessary to obtain information of its physicochemical and biological properties. In addition, as the absence of defined protocols for evaluating solubility and volumetric change of filling materials used for primary teeth can compromise the scientific impact of the studies, new methodologies must be developed.

2 PROPOSITIONS

To evaluate the physicochemical and biological properties of Bio-CP in comparison with Calen-ZO and ZOE.

2.1 Specific objectives

To evaluate the physicochemical properties of radiopacity, setting time and pH, and the biological properties of cytocompatibility and potential to induce mineralisation of Bio-CP, compared with Calen-ZO and ZOE (Publication 1);

To evaluate the solubility (% of mass loss), using a modified methodology derived from ISO 6876, and the volumetric change, presence of voids and filling capacity of Bio-CP, using micro-CT, in comparison to Calen-ZO and ZOE. Simultaneously, the effect of the size of the polyethylene tubes (1 or 2mm in diameter) in which the sealing materials were inserted and their immersion medium (distilled water or PBS) on the outcomes of solubility and micro-CT analysis were studied (Publication 2);

To compare chemical compounds and the crystalline phases of Bio-CP and Calen-ZO after 14 and 30 days of immersion in PBS through dispersive X-ray spectroscopy (SEM-EDX) and X-ray diffraction (XRD) (Publication 2).

3 PUBLICATIONS

3.1 Publication 1*

Physicochemical properties and effect of bioceramic root canal filling for primary teeth on osteoblast biology

Abstract

Bio-C Pulpecto (Bio-CP) was recently developed as the first bioceramic root filling material for primary teeth. **Objective:** To evaluate the physicochemical properties of radiopacity, setting time, pH, cytocompatibility and potential of Bio-CP to induce mineralisation, compared with (1) Calen thickened with zinc oxide (Calen-ZO), and (2) zinc oxide and eugenol (ZOE). **Methodology:** Physicochemical properties were evaluated according to ISO 6876. Saos-2 (human osteoblast-like cell line) exposed to extracts of the materials were subjected to assays of methyl thiazolyl tetrazolium, neutral red, alkaline phosphatase (ALP) activity and mineralised nodule production. The results were analysed using one-way or two-way ANOVA and Tukey's or Bonferroni's post-tests (α=0.05). **Results:** All the materials showed radiopacity higher than 3 mm Al. Bio-CP had lower pH than Calen-ZO, but higher pH than ZOE. Calen-ZO and Bio-CP did not set. The setting time for ZOE was 110 min. The cytocompatibility order was Calen-ZO > Bio-CP > ZOE (1:2, 1:4 dilutions) and Calen-ZO > Bio-CP = ZOE (1:12, 1:24 dilutions) and Calen-ZO = Bio-CP > ZOE (1:32 dilution). Bio-CP induced greater ALP activity at 7 days, and greater mineralised nodule production, compared to Calen-ZO ($p<0.05$). **Conclusions** Bio-CP showed adequate physicochemical properties, cytocompatibility and potential to induce mineralisation.

Keywords: Cytotoxicity. Endodontics. Primary teeth, Root Canal Filling Materials.

* The present publication follows the guidelines of the *Journal of Applied Oral Science*. The study has already been published. DOI: 10.1590/1678-7757-2020-0870

Introduction

An ideal root canal filling for primary teeth should not hinder the eruption of permanent successor teeth, but rather, should be resorbed as the primary teeth roots are physiologically resorbed, and should also resorb readily if pressed beyond the apex, be easily removed if necessary, be radiopaque and not discolour the tooth.[1]

Zinc oxide and eugenol (ZOE) are a combination that has been used as a root canal filling material in primary dentition for a long time.[2,3] However, ZOE is genotoxic and cytotoxic,[4] and cannot be completely phagocytised, leaving particles in periapical tissues when extravasated beyond the apex, and after physiological root resorption of the primary teeth.[2]

Calen (S.S. White, Rio de Janeiro, RJ, Brazil) is a commercial calcium hydroxide-based paste with a viscous vehicle (i.e., polyethylene glycol 400), and has suitable biological properties.[5] Calen thickened with zinc oxide (Calen-ZO) has been used as a root canal filling material in the primary dentition.[6-8] The addition of zinc oxide (ZO) reduces the phagocytosis of Calen, thus allowing Calen to accompany the physiological resorption of primary tooth roots, and improve its physicochemical properties.[7,9] Calen-ZO induces good tissue response,[7,10] has antimicrobial activity[6], and is more biocompatible than ZOE.[7,8]

New endodontic cements have been developed, especially highlighting the advance in improved bioceramics. The main characteristics of bioceramic materials for endodontic use are their alkaline pH, volumetric stability ~~shrink-free property~~, chemical stability within the biological environment,[11,12] biocompatibility and bioactivity.[13,14] Bio-C Pulpecto (Bio-CP) (Angelus, Basil, Londrina, Paraná, Brazil) is the first bioceramic root filling material for primary teeth. It is composed of ester glycol salicylate, titanium oxide, calcium tungstate, silicon dioxide, toluene sulphonamide, and calcium silicate (Material Safety Data Sheet information, product in development, file annexed). According to its manufacturer, it presents high alkalinity (pH 12.7), high radiopacity (9 mm of aluminium scale), complies with ISO 6876, [15 15 16] (16) (16)[15] and is resorbable, thus allowing simultaneous physiological resorption of the root and the material (Bio-C Pulpecto, Angelus, Basil, Londrina, Paraná, Brazil). A recent study showed that the Bio-CP is biocompatible and induced biomineralization in the subcutaneous tissue of rats, in a manner similar to MTA.[16] In addition, a root filling material for primary teeth developed by Angelus, with similar composition of Bio-CP, but with the name not informed, showed good biocompatibility in the subcutaneous tissue of rats, low

cytotoxicity in human gingival fibroblast, and satisfactory behaviour regarding the physicochemical properties studied.[8] However, the scientific literature about Bio-CP is still scarce.

Thus, the aim of this study was to evaluate (1) the physicochemical properties of radiopacity, setting time and pH, and (2) the biological properties of cytocompatibility and potential to induce mineralisation of Bio-CP, compared with Calen-ZO and ZOE, in vitro assays. The null hypothesis was that there would be no difference in the physicochemical and biological properties among the materials.

Methodology

Root filling materials, manufacturers and proportions for manipulation are shown in Figure 1.

Material	Manufacturer	Proportion for manipulation
Bio-C Pulpecto*	Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil	Ready for use
Calen + zinc oxide (Calen-ZO)	S.S. White Artigos Dentários Ltda., Rio de Janeiro, RJ, Brazil Biodinâmica Química e Farmacêutica LTDA, Ibiporã, PR, Brazil	0.24 g Calen + 0.12g ZO
Zinc oxide and eugenol (ZOE)	Biodinâmica Química e Farmacêutica	0.54 g ZO + 100 μL of eugenol

* Product under development, batch no. 190118

Figure 1 Root canal filling material, manufacturer and proportion of use

Radiopacity evaluation

Eight specimens of each material, measuring 10 mm diameter and 1 mm high were prepared according to ISO 6876:2012[1516] (Figure 1). The specimens were stored at 37°C and 95% humidity for 3 times the setting time for ZOE, until achieving maximum hardening for Bio-CP and Calen-ZO. Afterwards, they were placed on top of a KODAK CMOS digital sensor (6100, Kodak Co.), next to an aluminium step-wedge (98.5% of Al, 8 steps with 2 mm increments for each step) for radiographic exposure. Focus 50540 x-ray unit (Instrumentarium Dental; Tuusula, Finland) was used, and the parameters were set at 65 kVp, 7 mA, exposure time of 0.16 seconds and a 320 mm

source-to-object distance.[17]The digital images obtained were evaluated using methodology developed by Ochoa-Rodriguez, et al.[17]

pH analysis

Ten polyethylene tubes 10-mm long with 1 mm internal diameter were filled with each material. Each specimen was immersed in 10 mL of deionised water, inside of a plastic flask sealed individually with a lid, and then stored at 37°C. The experimental time intervals were 1, 3, 7, 14 and 28 days. The pH of the solution was assessed with a previously calibrated digital pH meter (Digimed; São Paulo, SP, Brazil) at each time interval. The control group consisted of deionised water with no immersed material.

Setting time

Calen-ZO and ZOE were inserted into ring-shaped metal moulds 1 mm wide and 10 mm diameter (n=6), kept at 37°C and 95% humidity. A plaster mould with the same dimensions was prepared for Bio-CP. The plaster mould was previously submerged in deionised water for 24 hours to provide the bioceramic material with moisture. The setting time was assessed using a Gillmore needle with a mass of 100 g ±0.5 and diameter of 2.0 mm ±0.1, according to ISO 6876:2012.[15] The setting time of the specimens was established as the time elapsing from initial mixing of Calen-ZO and syringe application of Bio-CP up to the maximum hardening of the specimens. At this point, the indentation marks made by the Gillmore needle were substantially reduced, but not eliminated. This is because the pastes had not set. Comparatively, the setting time for ZOE was considered as the time elapsing from when it started to be mixed until such time as the Gillmore needle failed to leave marks on the surface of the specimens.

Cell culture and preparation of root filling materials

Saos-2 (human osteoblast-like cell) immortalized cells line were grown in flasks containing Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Grand Island, NY, USA), penicillin (100 IU / mL), and streptomycin (100 µg / mL), in an atmosphere with 5% CO2 and 95% humidity at 37°C.

After handling of the specimens, 100 mg of each material (Figure 1) was placed in a 1.5 mL microtube (Eppendorf, Hamburg, Germany), to which 1.2 mL of DMEM was added, followed by placement of the microtubes in an oven at 37°C for 24 hours.[18] Afterwards, the supernatant was transferred to new microtubes centrifuged for 10 min at 20,800 g (5430, Eppendorf AG, Hamburg, Germany) to decant any particles of

material left in the supernatant. The supernatant was transferred to a new tube and was considered as the “stock solution/extract”. It was then diluted and placed in contact with Saos-2 cells.[19]

Cell viability analysis by methyl thiazolyl tetrazolium (MTT) and neutral red (NR) assays

Saos-2 cells were cultured at a density of $1x10^5$ cells/mL in a 96-well plate containing DMEM with 10% SFB for 24 hours to adhere to the plates. Thereafter, the cells were exposed to the cement extracts at the following dilutions in DMEM (v: v) 1:2; 1:4; 1:12; 1:24; 1:32. Cells exposed to DMEM were considered as the control group.

After 24 hours of cell contact with the material extracts and the control, 100 µL of 5 mg / mL MTT solution (Sigma-Aldrich) was added to each well, followed by incubation at 37°C, 95% humidity and 5% CO_2 for 3 hours. The colorimetric product was solubilised in 100 µl of 0.04 N acidified isopropanol (Sigma-Aldrich). A spectrophotometer (Elx800; Bio-Tek Instruments, Winooski, VT, USA) at 570 nm was used to measure the optical densities of the solutions. Absorbance readings were normalised to readings of cells exposed to DMEM, and represented the succinate dehydrogenase activity (cellular metabolism).

The NR assay was performed by applying 100 µL DMEM containing NR at 50µg / mL (Sigma-Aldrich) after 24 hours of cell contact with the materials and control extracts. The cells were incubated at 37°C, 95% humidity and 5% CO_2 for 3 hours, the contents of the wells were removed, and the colorimetric product was solubilised with 100 µL of a solution containing 50% ethanol and 1% acetic acid (Sigma-Aldrich). A spectrophotometer (Elx800; Bio-Tek Instruments, Winooski, VT, USA) at 570 nm was used to measure the optical densities of the solutions. Absorbance readings were normalised to readings of cells exposed to DMEM, and represented the ability to incorporate the dye into viable cell lysosomes.

Alkaline phosphatase (ALP) activity

Saos-2 ($5x10^4$ cells / mL) were cultivated in 96-well plates and exposed to the control (DMEM) and the extract of the materials at 1:24 dilution. This dilution was selected after observing the results of MTT assays performed after exposure of cells to cement extracts for 1, 3 and 7 days (data not shown). The 1:24 dilution was the highest extract concentration without cytotoxic effects. This is an essential consideration, since dead cells do not show ALP activity. ALP activity was evaluated at periods of 1, 3 and 7 days, using the commercial kit (Labtest, Lagoa Santa, MG, Brazil). Extracts of the materials were renewed every two days. Cells were washed

with 200 µL saline phosphate buffer (PBS) after each experimental period, and a 200 µL sodium sulphate solution (1% in distilled water, Sigma-Aldrich) was added to each well. Then the specimens were left to stand for 30 minutes at room temperature. Each sample (5 µL) in the sodium sulphate solution was transferred to a microtube (Eppendorf) containing a substrate and a buffer enzyme. Absorbance was measured using a spectrophotometer at 590 nm. Data were expressed as normalised ALP activity with total protein content, detected using the Total Protein Kit (Labtest) at the respective experimental periods. MTT assays were performed together with the ALP activity assay to follow up cell viability.

Alizarin Red Staining (ARS)

Saos-2 cells were cultivated ($1x10^4$ cells / mL) in 12-well culture plates with DMEM, supplemented with 50 µg / mL L-ascorbic acid (Sigma-Aldrich) and 10 mM β glycerophosphate (Sigma-Aldrich). The cells were exposed to material extracts for 21 days at the same dilution used for the ALP activity assay. Material extracts were renewed every two days. Then the cells were fixed with 4% paraformaldehyde (Sigma) and stained with 2% ARS (pH 4.1). The cells were incubated at room temperature for 20 minutes, and the dye was aspirated. The wells were washed 4 times with 1 mL of distilled water for 5 minutes. The water was removed and mineralisation was quantified by dissolving the nodules with 1 mL of a 10% cetylpyridinium chloride solution (Sigma Aldrich) added to each well, after which the plate was incubated for 15 minutes under stirring at room temperature. Three 100 µL aliquots of the suspension from each well were transferred to a 96-well plate and read with a 562 nm wavelength filter on a spectrometer (Elx800; Bio-Tek Instruments).

Statistical analyses

The results were shown as mean and standard deviation in at least two independent experiments performed from triplicate to sextuplicate. The results were analysed by one-way analysis of variance (ANOVA) and the Tukey's post-test, or two-way ANOVA and Bonferroni's post-test ($\alpha=0.05$), using GraphPad Prism statistical software (GraphPad Software; San Diego, CA, USA).

Results

Physicochemical properties

According to Table 1, all the materials showed radiopacity higher than 3 mm Al, in agreement with ISO 6876:2012.[15] There was no difference between Bio-CP and

Calen-ZO (p> 0.05), and ZOE showed higher radiopacity than the other materials (p<0.05). The ZOE setting time was 110 minutes. Calen-ZO and Bio-CP were considered to “set” (maximum hardening) at 192 hours and 240 hours, respectively. Although Calen-ZO and Bio-CP pastes had alkaline pH, Calen-ZO had a higher pH than Bio-CP (p<0.05). ZOE had a neutral pH (Table 2).

Table 1. Mean and standard deviation of the radiopacity in mm Al and initial setting time (in hours) of the materials evaluated

	Bio-CP	Calen-ZO	ZOE
Radiopacity	3.50 (0.15)[a]	3.51 (0.12)[a]	9.25 (0.31)[b]
Setting time	240.7 (0.1)[a*]	192.0 (0.2)[b**]	1.82 (0.01)[c]

Different letters in the lines indicate statistically significant differences among the materials (P<0.05) *The material did not set after the elapsed “setting” time. ** Partial areas of the material set at the evaluated time.

Table 2. Mean and standard deviation of pH values of materials and control group evaluated at different time intervals

	Bio-CP	Calen-ZO	ZOE	Control
1 day	10.07 (0.22)[b]	11.32 (0.16)[c]	7.30 (0.16)[d]	7.04 (0.08)[a]
3 days	9.92 (0.30)[b]	10.84 (0.22)[c]	7.18 (0.16)[a]	6.94 (0.07)[a]
7 days	8.57 (0.70)[b]	10.34 (0.38)[c]	7.03 (0.17)[a]	6.94 (0.08)[a]
14 days	9.65 (0.29)[b]	10.01 (0.68)[b]	6.87 (0.29)[a]	6.50 (0.25)[a]
21 days	8.70 (0.45)[b]	9.70 (0.63)[c]	6.99 (0.15)[a]	6.64 (0.16)[a]
28 days	8.08 (0.73)[b]	9.65 (0.73)[c]	6.52 (0.07)[d]	5.79 (0.14)[a]

Different letters in the lines indicate statistically significant differences among the materials (P<0.05)

Cell viability

As shown in Figure 2, the cell viability order at lower dilutions (1:2 and 1:4) was Calen-ZO > Bio-CP > ZOE for MTT and Calen-ZO > Bio-CP = ZOE for NR. As for the intermediate dilutions (1:12 and 1:24), the Calen group had the highest cell viability (p<0.05) in the MTT assay, whereas the Bio-CP and ZOE groups had similar cell viability (p>0.05). As for the NR assay, there was no difference between the materials and the control (p>0.05), except Calen-ZO at 1:24 dilution, that had higher cell viability than the other groups (p<0.05). At the highest dilution (1:32), there was no difference between Calen-ZO and Bio-CP (p>0.05) in the MTT assay, and both groups had higher cell viability than ZOE. In the NR assay, there was no difference among the materials evaluated at 1:32 dilution (p>0.05).

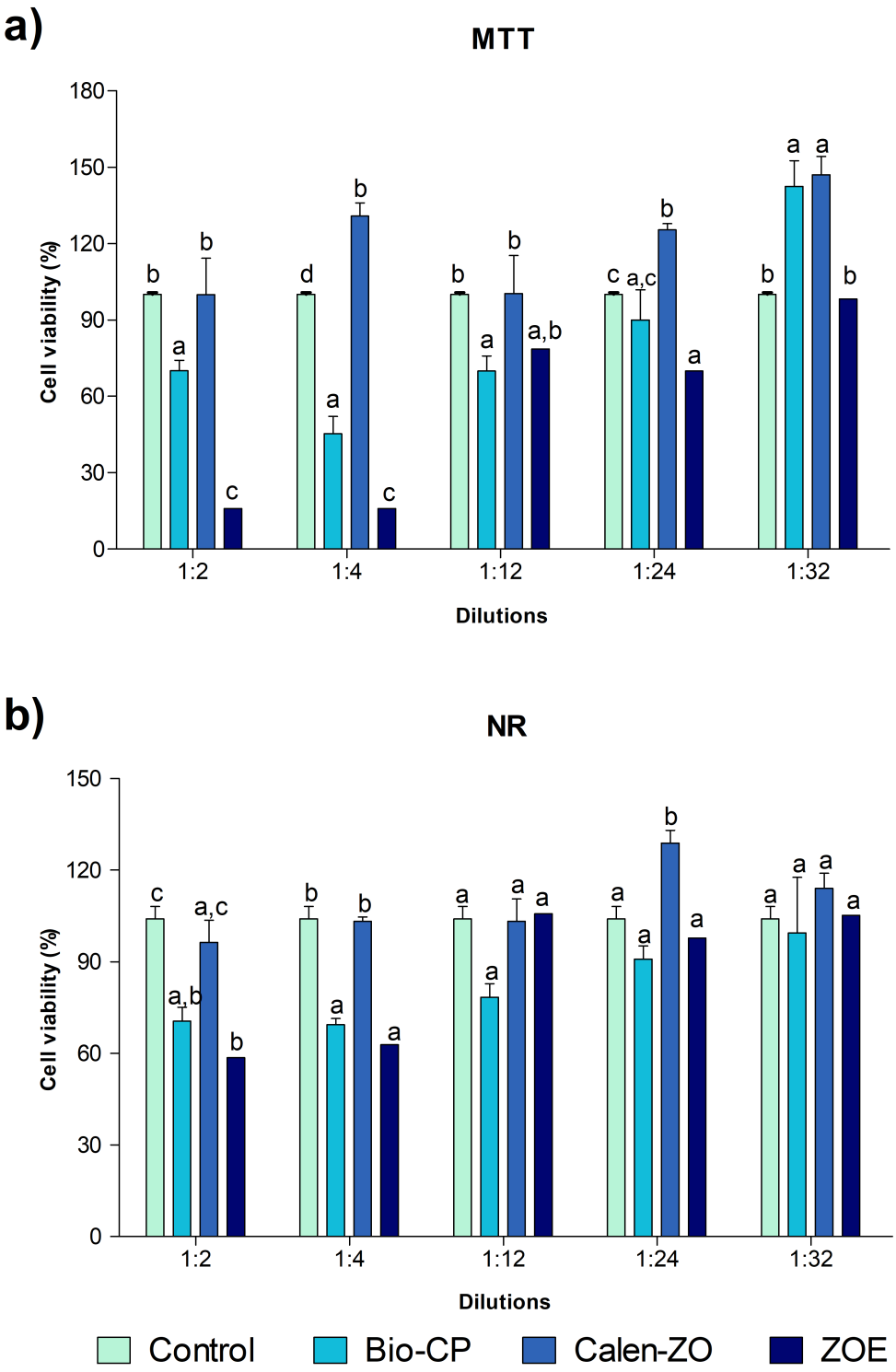

Figure 2. Saos-2 viability evaluated by (a) methyl-thiazol-tetrazolium (MTT) and (b) neutral red (NR) assays, after 24 hours of exposure to Bio-CP, Calen-ZO, ZOE and control (culture medium). Bio-CP = Bio C Pulpecto; Calen-ZO = Calen mixed with zinc oxide; ZOE = Zinc oxide and eugenol. Different letters in each dilution indicate statistically significant differences among the materials ($p<0.05$).

Potential to induce mineralisation

As shown in Figure 3, on the first and third day of Saos-2 exposure to the materials, all groups had similar ($p>0.05$) or greater cell viability than the control group ($p<0.05$), except for ZOE, which promoted lower cell viability than the control group ($p<0.05$). On the seventh day, there was no difference in the viability of the cells exposed to all the materials and the control ($p>0.05$). On the first day of cell culture, Calen-ZO induced higher ALP activity than Bio-CP and the control ($p<0.05$), whereas Bio-CP promoted ALP activity similar to that of the control ($p>0.05$). On the third day, there was no difference among the groups ($p>0.05$). On the seventh day, Bio-CP induced higher ALP activity than Calen-ZO ($p<0.05$), whereas Calen-ZO was no different from the control ($p>0.05$). In 21 days of cell exposure to the materials, Bio-CP and Calen-ZO induced higher mineralised nodule production than the control ($p<0.05$), although that of Bio-CP was higher than that of Calen-ZO ($p<0.05$).

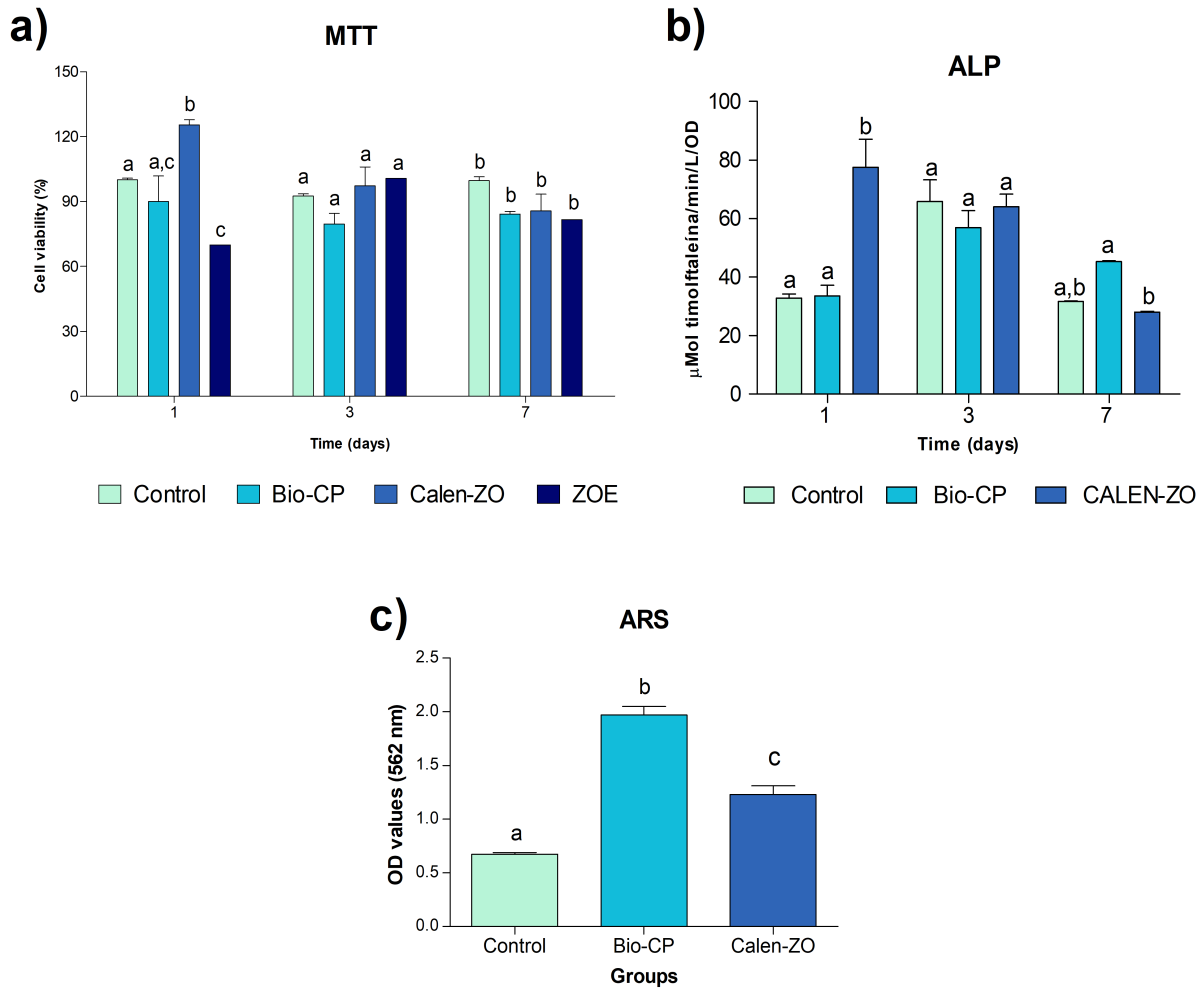

Figure 3. Effect of root canal filling materials (1:24 dilution) of primary teeth on the biology of Saos-2. MTT assay (a) and alkaline phosphatase (ALP) activity (b) were performed 1, 3 and 7 days after exposure of Soas-2 to extracts of materials and serum-free culture medium (control), whereas alizarin red stain (ARS) assay (c) was evaluated after 21 days of cell exposure to material extracts and osteogenic culture medium (control). Bio-CP = Bio C Pulpecto; Calen-ZO = Calen mixed with zinc oxide and eugenol; ZOE = Zinc oxide and eugenol. Different letters indicate statistically significant differences among the materials ($p<0.05$).

Discussion

In the present study, a comparison was made of the biological and physicochemical properties of Bio-CP, Calen-ZO and ZOE. Bio-CP exhibited adequate physicochemical properties, had cytocompatibility and revealed potential to induce mineralisation, when compared to the other evaluated root canal filling materials for primary teeth.

Radiopacity is an essential property for endodontic materials, because it allows endodontic filling material to be viewed by radiographic examination to evaluate the obturation quality,[12] and make a clear distinction among the materials and the

surrounding anatomical structures.[20] ISO 6876:2012[15] establishes that the root canal sealers must have at least 3 mm Al. In the present study, all the materials evaluated showed higher radiopacity than 3 mm Al. However, the radiopacity value of Bio-CP (3.50 mm Al) was less than that stated by the manufacturer (9 mm AL). On the other hand, it agrees with a previous study that showed that an experimental MTA-based material, with similar composition of Bio-CP, had radiopacity of 3.28.[8] The greater radiopacity of ZOE (9.25 mm Al) than that of the other materials (3.5 mm Al) is attributed to its zinc oxide used as a radiopacifier.[21]

An alkaline medium not only neutralises lactic acid from osteoclasts, but also prevents the mineral components of dentine from dissolving. In addition, it promotes antimicrobial activity, and may activate alkaline phosphatases, which play an important role in hard-tissue formation.[22] Both Bio-CP and Calen-ZO had alkaline pH; however, Calen-ZO had a higher alkaline pH compared with Bio-CP over the evaluated periods. Calcium hydroxide dissociates into calcium and hydroxyl ions, which raises the pH of the medium. Bioceramic materials form calcium hydroxide when they undergo a hydration reaction.[12] The lower pH of Bio-C compared to Calen-ZO is probably due to the lower amount of calcium hydroxide released into the medium by Bio-CP.

ZOE had a setting time of 110 minutes. The hardening time of Calen-ZO was 192 hours and that of Bio-CP was 240 hours; neither material set. The fact that Bio-CP does not set is not a disadvantage, since this phenomenon is observed in materials considered suitable for filling root canals of primary teeth.[8,9,23] Because Bio-CP was developed as a root filling material for primary teeth, is desirable that the material can be reabsorbed to accompany the physiological resorption of primary teeth roots. It can be hypothesized that the absence of set, along with the composition and solubility of the material, can allow simultaneous physiological resorption of the root and the material.

Saos-2 cells were used because they provide a suitable model to evaluate cytocompatibility and the potential to induce mineralisation of the materials.[17] MTT and NR assays were used to assess the cytocompatibility of the materials. MTT is based on the ability of viable cells to metabolise the MTT salt. NR assesses the ability of viable cells to incorporate dye into lysosomes.[17] Studies that evaluate different cell parameters make the cytocompatibility results more reliable[24] ALP activity and mineral nodule production assays were performed to evaluate the potential of materials to induce mineralisation. ALP activity assay is a biomarker of the enzyme alkaline

phosphatase produced by osteoblasts in their early maturation process,[25] and ARS identifies calcium deposits in cell culture.[17]

The cytocompatibility and the potential to induce mineralisation of bioceramic endodontic materials for permanent teeth has been studied in different cell types. EndoSequence BC sealer (Brasseler USA, Savannah, GA, USA) showed low cytotoxicity in L929 fibroblasts,[26] human gingival fibroblasts[27] and murine osteoblast precursor cells (IDG- SW3).[13] Moreover this material promoted osteoblastic differentiation,[13] showed anti-inflammatory effects and induced mineralisation in osteoblast precursor cells (MC3T3-E1).[14] Another study comparing mineral trioxide aggregate – MTA (ProRoot; Dentsply Tulsa Dental, Tulsa, OK, USA) and iRoot SP (Innovative BioCreamix, Vancouver, Canada) showed that neither material was cytotoxic in human tooth stem cells.[28]

Bio-CP cytocompatibility was lower compared to Calen-ZO, but higher compared to ZOE. Pilownic, et al.[8] (2017) showed no statistical difference in the cytotoxicity of Calen-ZO and the experimental MTA-based material with a similar composition of Bio-CP. This difference in result can be explained by the type of cells and also by the Calen-ZO proportion used. While in the present study Saos-2 cells (osteoblast-like cells), and Calen-ZO in a 2: 1 proportion were used, Pilownic, et al. [8] (2017) used human gingival fibroblasts and Calen-ZO in a 1: 1 proportion. It is important to emphasize that it is not clear if the manufacturer modified the concentrations of the components in Bio-CP, because at the time of the Pilownic, et al.[8] (2017) study, the material did not have a name yet and, unfortunately, the manufacturer did not disclose the concentrations.

Bio-CP induced lower ALP activity in 1 day, but greater ALP activity in 7 days, than Calen-ZO. In addition, Bio-CP induced greater mineralised nodule production than Calen-ZO. Calen-ZO induced higher calcium deposits than the control group. A recent study showed that Bio-CP was biocompatible and induced biomineralization similar to MTA in the subcutaneous tissue of rats.[16] Regarding Calen-ZO, the results of the present study are in line with previous studies performed on dogs' teeth and the subcutaneous tissue of mice, which showed that Calen-Zo was more biocompatible than ZOE.[7,10] ZOE was not evaluated in either mineralisation assay conducted in the present study, because it has no bioactivity.[29] The high mineralisation potential of calcium-silicate-based bioceramic materials is attributed to their hydration, that is, a chemical reaction with tissue fluids yields hydrated silicate gel (C-S-H) and calcium

hydroxide.[30] These compounds (C-S-H and calcium hydroxide) each have a mineralisation pathway. Calcium hydroxide induces the formation of calcite crystals that induce the formation of calcified areas. Calcite crystals are formed from the dissociation of calcium ions from calcium hydroxide, and carbon dioxide ions from tissues.[31] On the other hand, C-S-H induces hydroxyapatite deposition; this reaction is promoted by the hydration of tissue fluids. In bioceramic materials, calcium hydroxide reacts with the phosphate ions in tissue fluids to deposit calcium phosphate.[4,30]

The results obtained for cytocompatibility and mineralization induction should be confirmed by further *in vivo* studies. In addition, to complement the research on Bio-CP, studies should be carried out to evaluate its antibiofilm activity and the capacity to be reabsorbed to accompany the physiological resorption of primary teeth roots.

It was concluded that Bio-CP did not set, presented adequate radiopacity and alkaline pH, was cytocompatible and had the potential to induce mineralisation. Considering its good physicochemical and biological properties, Bio-CP has the potential to become an adequate material for the root canal filling of primary teeth.

Acknowledgements

This study was funded by fellowships from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) - Grant no. 2019/19766-0, and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Funding Code 001.

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3.2 Publicação 2*

Evaluation of solubility, volumetric change and morphological alterations of bioceramic filling material for primary teeth: a new methodological approach

Abstract

The objective was to evaluate the solubility (mass loss), using a modified methodology from ISO 6876, filling capacity, volumetric alteration, and presence of voids, by micro-computed tomography (micro-CT), of the bioceramic root canal filling material for primary teeth Bio-C Pulpecto (Bio-CP, Angelus, Brazil), compared to Calen thickened with zinc oxide (Calen-ZO) and zinc eugenol oxide (ZOE). Additionally, the surface element distribution and crystalline phases of Calen-ZO and Bio-CP were evaluated by energy scattering X-ray scanning electron microscopy (SEM-EDX) and X-ray diffraction (XRD). The materials were placed in polyethylene tubes of 1 or 2 mm in diameter and immersed in water or PBS for 30 days. Data were analyzed by two-way ANOVA and Tukey or Kruskal-Wallis and Dunn (α=0.05). Solubility was greater than 7% for all materials; the order was ZOE>Calen-ZO=Bio-CP. Calen-ZO and Bio-CP were more soluble in water than in PBS. Most materials showed higher solubility in 2 mm tubes, both in PBS and in water. Only Calen-ZO and ZOE were analyzed on micro-CT because Bio-CP separated into two phases during scanning. There was no difference between materials in filling capacity. Calen-ZO had greater volumetric loss and presence of voids than ZOE in water, but there was no difference in PBS. Precipitates on the surface of Bio-CP and Calen-ZO, corresponding to hydroxyapatite, were detected after immersion in PBS. It was concluded that, although Bio-CP has the potential to become a suitable material for filling deciduous teeth, its composition needs to be revised to obtain chemical stability.

Keywords: Endodontics. Micro-CT. Physicochemical properties. Primary teeth. Root canal filling materials.

* Manuscript still under evaluation for submission.

Introduction

Traditionally, calcium hydroxide-based, iodoform-based pastes, or a combination paste of iodoform and calcium hydroxide, and zinc oxide and eugenol (ZOE) materials have been used for root canal filling of primary teeth. However, they do not meet all the requirements for an ideal filling material (Barja-Fidalgo et al., 2011; Najjar et al., 2019).

Bioceramics (calcium silicate-based materials), used as sealer or repair materials on permanent teeth, have beneficial properties such as alkaline pH, antibacterial activity, low contraction, stability in a biological environment, biocompatibility and bioactivity (Al-Haddad et al., 2016). However, these materials have shown high solubility (Torres et al., 2019).

Bio-C Pulpecto (Bio-CP; Angelus, Londrina, Paraná, Brazil), the first bioceramic root filling material for primary teeth, is composed of ester glycol salicylate, titanium oxide, calcium tungstate, silicon dioxide, toluene sulphonamide and calcium silicate (Angelus: product in development). From a biological point of view, Bio-CP showed cytocompatibility and potential to induce mineralization comparable to Calen thickened with zinc oxide (Calen-ZO) in osteoblast like cells - Saos-2 - (Ochoa-Rodriguez et al., 2021); and biocompatibility and mineralization induction, in the subcutaneous tissue of rats, like MTA (Cosme-Silva et al., 2019). A recent study (Ochoa-Rodríguez et al., 2021) revealed that Bio-CP has alkaline pH, do not set, and has adequate radiopacity, that is, greater than 3 mm Al preconized by ISO 6876 (International Organization for Standardization. ISO 6876:2012). However, an important physicochemical property of Bio-CP, solubility, is not known yet.

In case of root filling materials for primary teeth, it is important they have a balance in solubility, as the roots of primary teeth are physiologically resorbed (Gupta & Das, 2011). This means that filling materials for primary teeth must not have high solubility, to avoid the formation of gaps (voids), or low solubility so that they are not retained in the tissue, leading to inflammation, or impairing the eruption of the permanent successor (Mortazavi et al., 2004; Bawazir et al., 2006).

To evaluate solubility of endodontic materials for permanent teeth, the ISO 6876 (International Organization for Standardization. ISO 6876:2012) guidelines have been employed; after setting, the specimens are weighed and immersed in distilled water for 24 hours and weighed again, after mass stabilization. However, this guidelines model does not reproduce a clinical situation, in which, the materials are placed in contact

with the tissue fluids and/or blood during the root canal treatment and is exposed to solubilization for more than 24 hours (Silva et al., 2017). For this reason, researchers have developed other methodologies, using micro computed tomography (micro-CT), to complement the evaluation of root canal sealers solubility (Silva et al., 2017; Torres et al., 2020). The root canals of permanent teeth are filled, and the teeth are immediately placed in the immersion medium (Silva et al., 2017). The immersion time varies from 7 to 30 days (Torres et al., 2019), and the immersion medium can be distilled water (Torres et al., 2019) or phosphate-buffered saline - PBS - (Silva et al., 2017; Torres et al., 2020). In this model, the teeth are scanned using micro-CT immediately after filling and after the immersion period, allowing correlation of volumetric and morphological changes with the solubility (Silva et al. 2017).

It has been reported that the solubility of calcium silicate-based sealers in contact with phosphate containing solutions was significantly lower than their solubility in distilled water (Urban et al., 2018; Torres et al., 2020; Silva et al., 2021b). This could be attributed to the mineral structure's precipitation on the surface of these materials that can be identified as apatite-like mineral structures (Han et al. 2015).

To assess the solubility of primary teeth filling materials, it is not possible to use the ISO 6876 (ISO 6876:2012) methodology, because for the use of this ISO the materials require to set so the samples can be produced and evaluated. However, there is no defined protocol for performing the analysis of the solubility (expressed as loss of mass) of primary teeth filling materials, so a new methodology, adapted for these materials should be created The absence of such protocols can compromise the scientific impact of the studies and, consequently, a methodology must be developed. As a method for evaluation for volumetric change employing to complement the solubility test of root canal sealers are based on micro computed tomography (micro-CT) to evaluate volumetric change and correlate it with the solubility of the material (Torres et al., 2020).

Thus, the aim of the study was to evaluate (1) the solubility (% mass loss), using a modified methodology derived from ISO 6876, and (2) the filling capacity, volumetric change, and presence of voids, using micro-CT, of Bio-CP in comparison to Calen-ZO and ZOE. Simultaneously, the effect of the size of the polyethylene tubes (1 or 2mm in diameter) in which the filling materials were inserted and their immersion medium (distilled water or PBS) on the outcomes of solubility and micro-CT analysis were studied. The third objective was to assess material surface element distribution and

the crystalline phases of Calen-ZO and Bio-CP through dispersive X‑ray spectroscopy (SEM‑EDX) and X-ray diffraction (XRD) when immersed in PBS.

Material and Methods

Root filling materials, manufacturers and proportions for manipulation are shown in Table 1 (Ochoa-Rodriguez et al., 2021).

Table 1. Root canal filling material, manufacturer, and proportion of use

Material	Manufacturer	Proportion for manipulation	Material composition
Bio-C Pulpecto*	Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil	Ready for use	Ester glycol salicylate, titanium oxide, calcium tungstate, silicon dioxide, toluene sulphonamide, and calcium silicate
Calen + zinc oxide (Calen-ZO)	S.S. White Artigos Dentários Ltda., Rio de Janeiro, RJ, Brazil Biodinâmica Química e Farmacêutica LTDA, Ibiporã, PR, Brazil	0.24 g Calen + 0.12g ZO	Calcium Hydroxide, zinc oxide, colophony, polyethylene glycol 400
Zinc oxide and eugenol (ZOE)	Biodinâmica Química e Farmacêutica	0.54 g ZO + 100 µL of eugenol	Zinc Oxide and Eugenol

* Produto em desenvolvimento batch nº 190118

Solubility Test

Transparent cylindrical polyethylene tubes (Embramed, Cremer S.A., São Paulo, SP, Brazil) of 1 mm or 2 mm in diameter and 13 mm in length, were closed at one end so that the final length was 10 mm (n = 12 for each material, polyethylene tube group and immersion medium). ZOE was inserted and lightly condensed inside the polyethylene tubes using Paiva condensers (Golgran, São Caetano do Sul, SP, Brasil). Calen-ZO was placed inside a 3 mm syringe (Becton Dickinson IND. CIRUR. LTDA., Curitiba, PR) and applied with a 21 G needle 0,80 x 30 mm (Becton Dickinson IND. CIRUR. LTDA., Curitiba, PR), so that it would be slowly inserted into the polyethylene tubes (Segato et al., 2016). Bio-CP, available in a ready-to-use syringe, was inserted slowly to the polyethylene tubes with the tips that come with it. Ultrasonic activation with the ultrasonic tip E1 - Irrisonic (Helse Ultrasonics, Santa Rosa de Viterbo, SP,

Brasil) using the ultrasonic device Ultrawave XS (Ultradent, Indaiatuba, SP, Brasil) at 20 % potency was employed to obtain a homogeneous filling of the tubes, without the presence of voids. Radiographs of all specimens were taken to previously assess the quality of the filling, so there were not radiolucent areas along the obturation. The specimens were weighed with the value registered as the initial mass. Afterwards, they were immersed in a flask containing 10 mL of phosphate buffered saline (PBS) or distilled water and stored in an oven at 37 ° C for 30 days (Torres et al., 2019). Then, they were removed from the flasks, placed in an oven at 37°C until the mass had stabilized. Afterwards, they were weighed, and this valor was recorded as the final mass value. The mass loss was determined by subtracting the initial and final weight values and expressed as percentage.

Volumetric change, voids, and filling capacity

Another set of polyethylene tubes as described before was used for this test (n = 12). After the tubes were filled with the materials and the quality of the filling verified by radiographs, they were immediately scanned by micro-CT (SkyScan 1176; Bruker-micro-CT, Kontich, Belgium). The micro-CT parameters were 18 μm voxel size, 80 kV, 125 μA, rotation step 0.5, frame 4, 1.0 mm aluminum filter, and 180° scanning. The time of scanning for every two specimens was 23 to 25 minutes. The reconstruction of the images was performed using NRecon software (V1.6.10.4; Bruker-micro-CT). The correction parameters for smoothing, beam hardening, and ring artefacts were defined for each material as follows; for Bio-CP, 0 for beam hardening, 4 for ring reduction, 0 for smoothing; for Calen-ZO, 20 beam hardening, 5 for ring reduction, 0 for smoothing and for ZOE, 50 for beam hardening, 5 for ring reduction and 0 for smoothing. After micro-CT scanning, the specimens were immersed in a flask containing 10 mL of PBS or distilled water and stored in an oven at 37 °C for 30 days (Torres et al., 2019). The specimens were removed from the flasks and immediately rescanned, using the same acquisition parameters used for the initial scan (Silva et al., 2017). The initial reconstructed images and the reconstructed images after the solubility challenge were superimposed using the Data Viewer software (V1.5.2.4; Bruker-micro-CT). The total volume (mm^3) of the root filling material, the total volume of the material in the determined ROI in percentage (% filling capacity) and voids (mm^3) was calculated by CTAn software (V1.15.4.0; Bruker-micro-CT). Volumetric change and voids were

expressed as the root filling material volume gained or lost in percentage (Silva et al., 2017; Torres et al., 2019; 2020).

Material surface element distribution: SEM-EDS analysis

Another set of polyethylene tubes (2 mm in diameter) (Embramed, Cremer S.A., São Paulo, SP, Brazil) as described before was used for this test (n = 3 for each material-Bio-CP and Calen-ZO, polyethylene tube group and immersion medium). The materials were placed in the tubes and immersed in PBS for 14 or 30 days inside plastic flasks containing 10 mL of the solution and stored in an oven at 37°C. Immediately after, the specimens were desiccated for 14 days in an oven at 37°C. Afterwards, the superficial element distribution of the specimens was assessed via scanning electron microscopy (SEM – JSM 6610LV, JEOL Ltd., Musashino, Akishima, Tokyo, Japan) and energy dispersive spectroscopy (EDS – Oxford instruments x-max, OIC Precision Labs, Winnipeg, Manitoba, Canada), without a carbon-coating process.

Crystalline phase identification: XRD analysis

The same specimens used for SEM-EDS analysis were used for XRD. The surface of each specimen was carefully scraped and crushed to fine powder, placed, and leveled with the holder surface to get enough materials to analyze. An automated x-ray diffractometer (D2 PHASER; Bruker-AXS, Karlsruhe, Germany), was employed with the scan parameters set at steps of 0.02 degrees every 0.2 seconds analyzed between 10 and 80 degrees 2 Theta; each analysis took about 12 minutes. Origin 2022 for windows OS (OriginLab Corporation, Northampton, MA, United States) was employed for qualitative analysis of the diffractogram. The peaks of each sample were matched with those of the International Center for Diffraction Data (ICDD) database (International Center for Diffraction Data, Newton square, PA, USA).

Statistical analysis

The data were evaluated using GraphPad Prism 9.0 statistical software for Windows OS (GraphPad Software Inc., San Diego, CA, USA). Whereas the Shapiro-Wilk test showed that the voids and solubility data did not fully comply with a normal distribution, Kruskal-Wallis followed by the Dunn’s test was used to detect differences

among groups. For volumetric change and filling capacity, ANOVA two-way and Tuckey post-test were used (α=0.05)

Results

The Bio-CP results were statistically compared with the other materials only in the solubility and filling capacity analysis. Bio-CP results were displayed in tables 3 and 4 but were not considered in the comparisons for volumetric change and presence of voids. This is because, during the scanning time on the micro-CT, the Bio-CP separated into two phases as shown in figure 1.

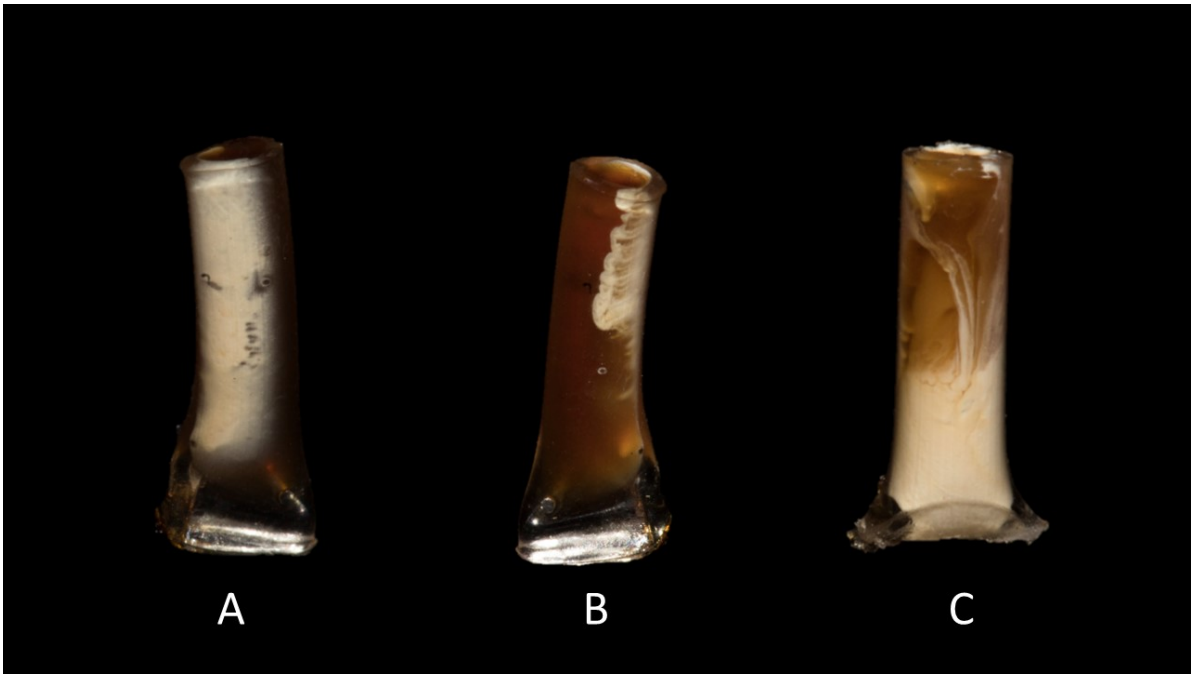

Figure 1. Polyethylene tube filled with Bio-CP left to rest for 45 minutes after filling to observe the phase separation events of the material. Front of the tube one (A), back of the tube one (B) and front of tube two (C).

Solubility

When the specimens were immersed in distilled water, ZOE was more soluble ($p<0.05$) than Bio-CP and Calen-ZOE in both polyethylene tubes diameter. Bio-CP and Calen-ZO had no difference in solubility ($p>0.05$) in 1mm diameter tubes. On the other hand, Calen-ZO was more soluble than Bio-CP ($p=0.0157$) in 2mm diameter tubes. In PBS, Bio-CP and Calen-ZO had no difference in solubility in both polyethylene tubes diameter. ZOE was more soluble ($p<0.05$) than Bio-CP in both polyethylene tubes diameter and Calen-ZO in polyethylene tubes of 1 mm diameter. Bio-CP and Calen-ZOE were more soluble in water ($p<0.05$) than PBS, and ZOE showed no difference

in solubility regardless of the immersion medium (p>0.05), but had higher solubility than other materials, in both polyethylene tubes diameter (p<0.0001). All materials showed more solubility in 2 mm diameter tubes in both PBS and distilled water, except Bio-CP immersed in water, which had no difference in solubility (p=0.2768) when comparing the diameter of tubes (1 mm vs 2 mm) (Table 2).

Table 2. Solubility comparison (% mass loss) of the materials inserted in polyethylene tubes (diameter 1mm or 2mm) and immersed in distilled water (DW) or PBS

	1 mm diameter		2 mm diameter	
	DW	PBS	DW	PBS
Materials	**M (Q1-Q3)**	**M (Q1-Q3)**	**M (Q1-Q3)**	**M (Q1-Q3)**
Bio-CP	9.206 (-8.459-10.14)^{A*}	7.524 (7.427-7.931)$^{A\#}$	9.70 (9.521-9.73)^{A*}	8.480 (8.126-8.965)^{A}
Calen-ZO	10.54 (-8.0 -11.46)$^{A*\#}$	7.554 (6.877-8.224)$^{A\#}$	13.03 (12.59-14.08)^{B*}	9.592 (9.129-9.664)^{A}
ZOE	16.57 (15.59-17.50)$^{B\#}$	17.82 (14.20-18.06)$^{B\#}$	22.35 (22.16-23.06)^{C}	20.45 (20.36-20.32)^{B}

The values are expressed as M(Q1-Q3), M, median; Q1, first quartile; Q3, third quartile. Different upper-case letters in the columns indicate statistically significant difference (p<0.05) between materials in each immersion mediums; * Indicates statistically significant difference (p<0.05) between immersion mediums for each material of the same polyethylene tube diameter (1mm or 2mm); # Indicates statistically significant difference (p<0.05) between polyethylene tube diameter (1mm or 2mm) in the same immersion mediums for each material (Statistical analysis made by Kruskal-Wallis and Dunn's test)

Filling capacity

There was no statistical difference in the filling capacity between the materials in each polyethylene tube diameter (p>0.05) and between 1 mm diameter and 2 mm diameter tubes (p>0.05) (Table 3).

Table 3. Filling capacity of the materials inserted in polyethylene tubes (diameter 1mm or 2mm)

	1 mm diameter	2 mm diamete
Materials	**M (Q1-Q3)**	**M (Q1-Q3)**
Bio-CP	99.30 (98.47-99.63)	98.42 (97.20-99.17)
Calen-ZO	98.87 (97.84-99.15)	98.30 (97.67-99.70)
ZOE	98.44 (97.87-98.98)	99.64 (97.15-99.17)

The values are expressed as M(Q1-Q3), M, median; Q1, first quartile; Q3, third quartile. The absence of letters indicates no statistical difference between materials in each polyethylene tube diameter (columns) and between 1 mm and 2 mm diameter tubes for each material (lines) (p>0.05). (Statistical analysis made by ANOVA two-way and Tuckey post-test, α=0.05).

Volumetric Change

All materials showed volumetric loss. When the specimens were immersed in water, Calen-ZO had more volumetric loss than ZOE (p<0.05) in polyethylene tubes of

1- and 2-mm diameter. When they were immersed in PBS, there was no difference in volumetric change between Calen-ZO and ZOE ($p>0.05$) for both tubes diameter. Calen-ZO showed greater volumetric loss in distilled water than PBS ($p<0.05$) in tubes of 2 mm diameter, and there was no difference ($p<0.05$) between distilled water and PBS for Calen-ZO in tubes of 1 mm diameter. ZOE had no difference in volumetric change between water and PBS in both tubes' diameter ($p>0.05$). When comparing internal diameter of tubes (1 mm vs 2 mm) in the same immersion medium, Calen-ZO had greater volumetric loss in tubes of 2 mm diameter than 1 mm when the immersion medium was water ($p<0.05$), and there was no statistical difference ($p>0.05$) when the immersion medium was PBS. ZOE had no statistical difference ($p>0.05$) when comparing internal diameter of tubes independent of the immersion medium (Table 4).

Table 4. Volumetric change (%) of the materials inserted in polyethylene tubes (diameter 1mm or 2mm) and immersed in distilled water (DW) or PBS

	1 mm diameter		**2 mm diamete**	
	DW	**PBS**	**DW**	**PBS**
Materials	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
Bio-CP	-35.89 (30.43)	-5.573 (2.529)	-25.36 (14.27)	-5.658 (2.406)
Calen-ZO	-4.711 (2.954)$^{A\#}$	-2.296 (1.032)^{A}	-15.14 (8.886)^{A*}	-1.930 (0.853)^{A}
ZOE	-0.921 (0.805)^{B}	-1.810 (1.813)^{A}	-5.048 (6.952)^{B}	-1.597 (1.274)^{A}

Bio-CP results were not considered in the statistical comparisons. Values expressed in mean and standard deviation (SD). Negative sign indicates volumetric loss. Different upper-case letters in the columns indicate statistically significant difference ($p<0.05$) between materials in each immersion mediums; * Indicates statistically significant difference ($p<0.05$) between immersion mediums for each material of the same polyethylene tube diameter (1mm or 2mm); # Indicates statistically significant difference ($p<0.05$) between polyethylene tube diameter (1mm or 2mm) in the same immersion mediums for each material. (Statistical analysis made by ANOVA two-way and Tuckey post-test, $\alpha=0.05$).

Voids

When immersed in water, Calen-ZO had more voids than ZOE ($p<0.05$) for polyethylene tubes of 1 mm diameter, and there was no difference ($p>0.05$) between the materials in tubes of 2 mm diameter. In PBS, both materials showed no difference ($p>0.05$) regardless of the diameter of the tubes. Calen-ZO showed greater presence of voids in water than PBS ($p<0.05$) in polyethylene tubes of 2 mm diameter, and there was no difference between water and PBS in tubes of 1 mm diameter. ZOE had no difference in volumetric change between water and PBS regardless of tubes diameter. Both materials did not show difference ($p>0.05$) in presence of voids when comparing internal diameter of tubes (1 mm vs 2 mm) in the same immersion medium (Table 5).

Table 5. Voids (%) comparison of the materials inserted in polyethylene tubes (diameter 1mm or 2mm) and immersed in distilled water (DW) or PBS

	1 mm diameter		2 mm diameter	
	DW	PBS	DW	PBS
Material	**M (Q1-Q3)**	**M (Q1-Q3)**	**M (Q1-Q3)**	**M (Q1-Q3)**
Bio-CP	2115 (562.2-12199)	629.2 (282.7-6294)	579.8 (325.4-6033)	179.7 (-54.68-583.4)
Calen-ZO	398.9 (302.3-475.8)^{A}	275.7 (194.7-363.4)^{A}	525.6 (229.5-829.6)^{A*}	116.8 (97.26-146.8)^{A}
ZOE	41.37 (29.72-135.8)^{B}	219.3 (127.4-311.2)^{A}	203.7 (67.85-507.9)^{A}	93.94 (77.94-195.4)^{A}

Bio-CP results were not considered in the statistical comparisons. The values are expressed as M(Q1-Q3), M, median; Q1, first quartile; Q3, third quartile. Different upper-case letters in the columns indicate statistically significant difference ($p<0.05$) between materials in each immersion mediums; * Indicates statistically significant difference ($p<0.05$) between immersion mediums for each material of the same polyethylene tube diameter (1mm or 2mm). (Statistical analysis made by Kruskal-Wallis and Dunn's test)

Material surface element distribution

On the Bio-CP specimens immersed in PBS, EDX detected oxygen (O), magnesium (Mg), phosphorous (P), chlorine (Cl), calcium (Ca), zinc (Zn) and Ytterbium (Yb) after 14 days of immersion in PBS. Apart from the elements detected at 14 days, also sodium (Na) was detected when the specimens were immersed for 30 days in PBS (Figure 2).

On the Calen-ZO specimens immersed in PBS, EDX detected oxygen (O), aluminum (Al), phosphorous (P), chlorine (Cl), calcium (Ca) and zinc (Zn) were identified. The surface of Calen-ZO specimens showed crystalline phase formations after immersion in PBS (Figure 2). The element identification on punctual analysis of crystalline formations did not differ from the total superficial analysis.

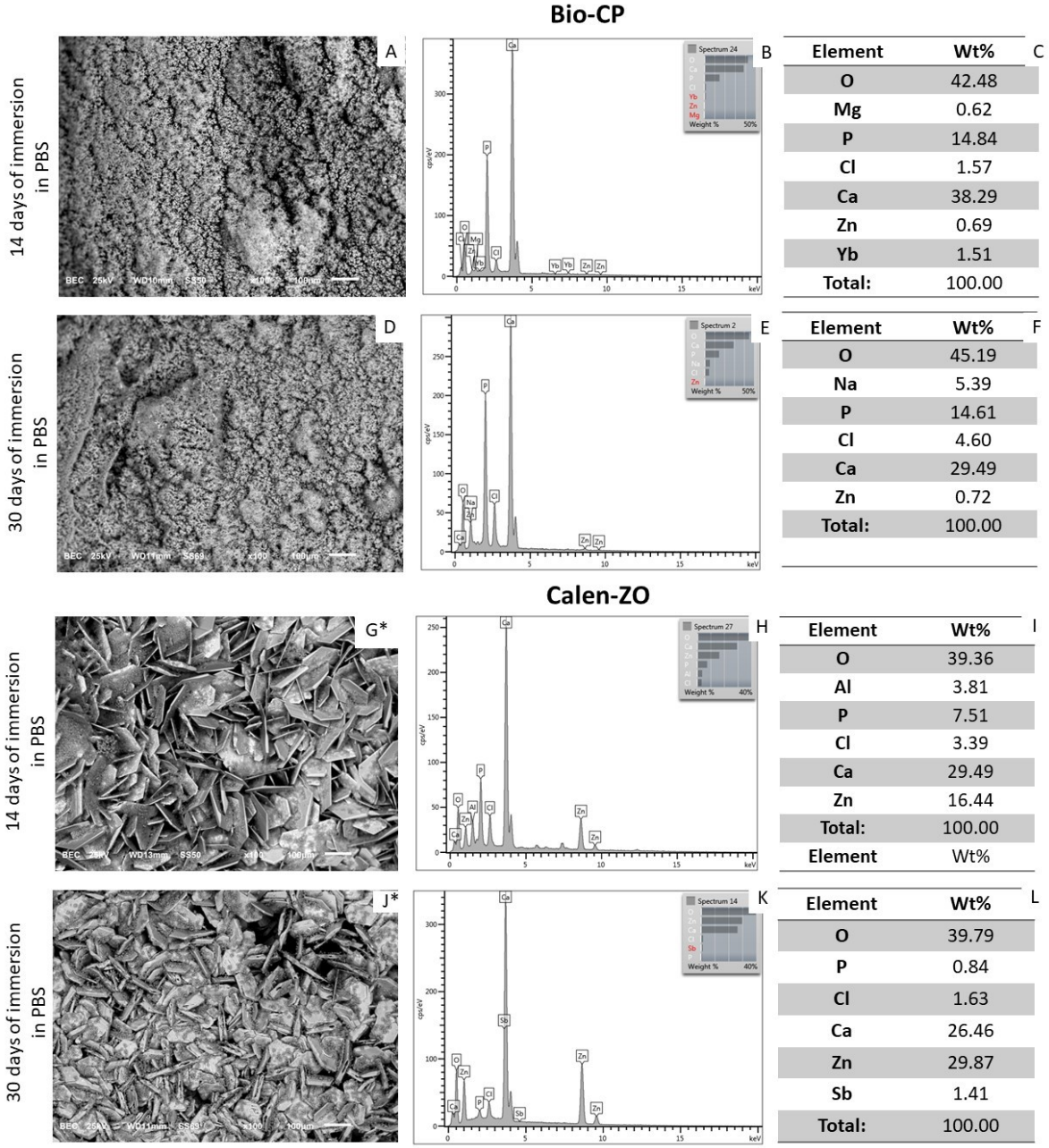

Figure 2. SEM examination of surface structure of Bio-CP (A-D) and Calen-ZO (G-J), EDX analysis of Bio-CP (B-E) and Calen-ZO (H-K); and element identification of Bio-CP (C-F) and Calen-ZO (I-L) after immersion in PBS for 14 and 30 days. Surface examination of crystalline formations in images D* and J* identified the same elements showed in the tables. Barr = 100 um

Crystalline phase identification: XRD analysis

After 14 of immersion of the Bio-CP specimens in PBS, XRD analysis detected carbonate-hydroxyapatite (C0.22 Ca5 O13.514 P2.823; 96-900-3552), Calcium Tungstate (CaOW4; 96-721-9230) and Doyleite ($Al(OH)_3$ 1980-041). After 30 days of immersion the same crystal phases were identified in Bio-CP (Figure 3). Calen-ZO had carbonate-hydroxyapatite (C0.22 Ca5 O13.514 P2.823; 96-900-3552), zinc oxide

(ZnO; 96-230-0113) and calcium silicate (Ca_2SiO_4; 1344-95-2) after 14 and 30 days after immersion in PBS (Figure 4).

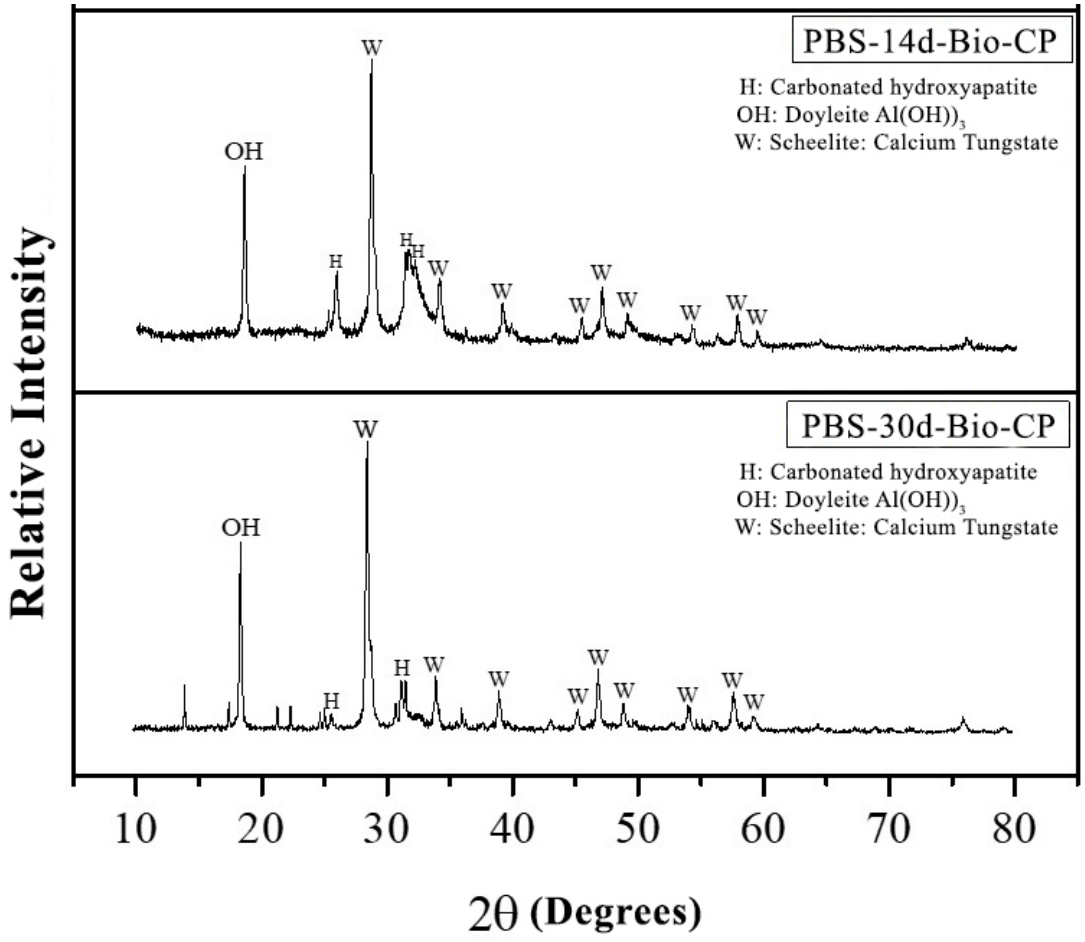

Figure 3. X-ray diffractogram (XRD). In both diffractograms, PBS-14d-Bio-CP and PBS-30d-Bio-CP, it is possible to identify the formation of peaks referring to the presence of carbonated hydroxyapatite (H), calcium tungstate (W) and Doyleite (OH) crystal phases.

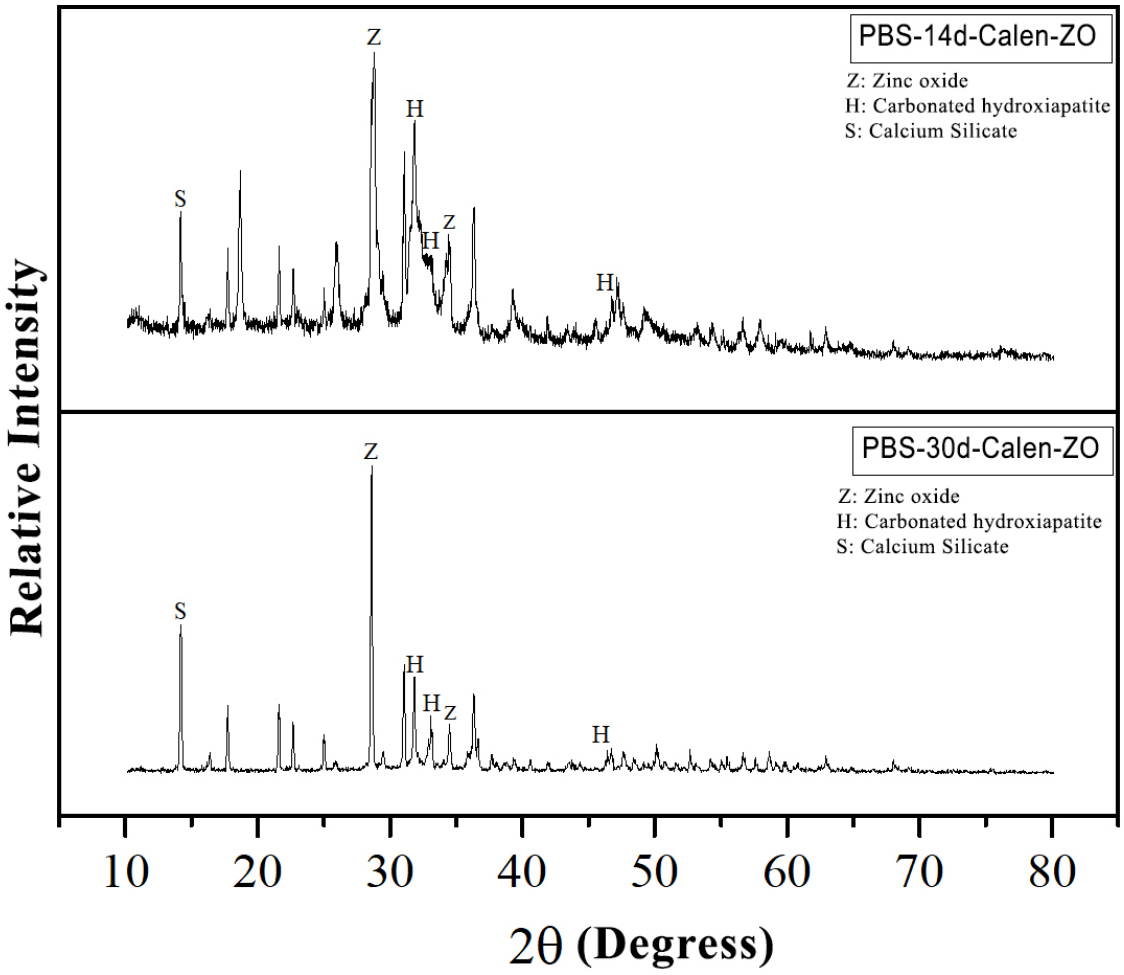

Figure 4.–X-ray diffractogram (XRD). In both diffractograms, PBS-14d-Calen-ZO and PBS-30d- Calen-ZO, it is possible to identify the formation of peaks referring to the presence of carbonated hydroxyapatite (H), zinc oxide (Z) and calcium silicate (S) crystal phases.

Discussion

The current study evaluated solubility, volumetric change, and presence of voids of Bio-CP, Calen-ZO and ZOE inserted into 1 or 2mm in diameter of the polyethylene tube and immersed in PBS or distilled water. The element distribution and the crystalline phases on the surface of Calen-ZO and Bio-CP after of immersion in PBS were also evaluated.

Traditionally, the solubility of root canal sealers is evaluated according to ISO 6876 (2012) guidelines and should be 3% or less after 24 hours of immersion of the set sealers in water. In the present study a period of 30 days and 2 immersion mediums, PBS and water, were employed to further understand the behavior of the materials and improve the clinical relevance of the results (Silva et al., 2017; Torres et al., 2019). Polyethylene tubes closed at one end were used to better simulate a clinical situation (Segato et al., 2016) and because Bio-CP and Calen-ZO do not set (Ochoa-Rodriguez et al., 2021). Some researchers have used prototyped 3D printed teeth to evaluate instrumentation protocols for primary (Moraes et al., 2019; 2021) and permanent teeth (Ordinola-Zapata et al., 2014). However, these prototyped teeth have a radiopacity similar to dentine. This could create a ROI delimitation problem when using micro-CT software because Calen-ZO and Bio-CP have a radiopacity close to 3 mm Al and, consequently, similar to the prototyped tooth. Also depending on the 3D printer, defects caused by printing resolution inaccuracies could be identified at the internal root canal surface (Moraes et al., 2021),–that could impair the micro-CT analysis. Another option would be to use natural teeth, but as the difficulty to get natural primary teeth with little root resorption, this option was discarded. Moreover, no interaction between the filling materials and dentine was evaluated in this study, so polyethylene tubes were chosen.

Filling material for root canals of primary teeth should accompany the physiological resorption of the primary teeth roots (Rifkin, 1980). However, there is not a definite valor or a study that correlates physiological root resorption speed to solubility of root canal filling materials. Bioceramic materials, used for endodontic permanent teeth treatment, show high solubility (Silva et al., 2021b), this is, more than 3% indicated by ISO (ISO 6876:2012). However, the value of 3% cannot be used as a parameter in the analysis of root canal filling materials for primary teeth.

All materials evaluated had more than 7% solubility, regardless of the immersion medium and the diameter of the tube. There was no difference between Bio-CP and

Calen-ZO, and both were less soluble than ZOE in both immersion mediums. A recent study showed that Calen-ZO, inserted in 1mm diameter polyethylene tubes, had a higher solubility than ZOE when immersed in water for 35 days; Calen-ZO and ZOE had solubility of 22.05% and 12.65% respectively (Pintor et al., 2021) compared to 10.54% (Calen-ZO) and 16.57% (ZOE) in tubes of 1 mm diameter in the present study. There are three possible explanations for the different results of Pintor et al. (2021). (1) Although not mentioned, they probably waited for the ZOE to set before immersing it in water, unlike the present study in which the specimens were immersed immediately after handling. Studies show that ZOE, after setting, has a solubility of approximately 4.5% after 30 days of immersion in distilled water (Torres et al., 208; Espir et al., 2016). (2) According to the table of materials studied, Pintor et al. (2021) used Calen in its commercial formulation, without the addition of zinc oxide, that is, more fluid. (3) They removed the specimens, placed them in a desiccator for 24 h, weighed them, and then immersed them again in water till the next experimental time, which were 7, 21, 28, and 35 days.

Bio-CP and Calen-ZO had significantly lower solubility in PBS compared to distilled water. The lower solubility of Bio-CP in PBS than in water can be explained by the formation of apatite-like structures on the surface of calcium-silicate based endodontic materials when in contact with phosphate buffered solutions (Camilleri 2011; Han et al. 2015; Torres et al., 2020; Silva et al.,2021a) that could potentially slow down their solubilization process. In relation to Calen-ZO, which is a material calcium hydroxide-based, $Ca(OH)_2$ suffers a chemical reaction with phosphate ions present in tissue fluids, which results in the formation of hydroxyapatite on the material surface (Kokubo & Takadama 2006; Gandolfi et al., 2010; Camilleri et al., 2011, Mohammadi & Dummer, 2011; Prüllage et al., 2016) .The formation of hydroxyapatite in Bio-CP and Calen-ZO immersed in PBS was confirmed in both materials by XRD analysis. On the other hand, it is important to mention the laboratory hydroxyapatite formation cannot be extrapolated directly to the clinical scenario, since the formation of crystals in vitro models may be an overestimation of the in vivo conditions (Moinzadeh et al. 2016; Silva et al., 2021a). Regarding the diameter of the tubes, the majority of the materials showed more solubility in 2 mm tubes both in PBS and in water. This is probably related to the difference in superficial contact with the immersion mediums (1 mm vs 2 mm diameter).

Micro-CT scanning is a high-resolution non-destructive method that allows quantification of volumetric change and voids, and a tridimensional evaluation of the quality of the filling (Başer Can et al., 2017). In the analyzes of volumetric change and voids, Bio-CP was not considered for the comparison between materials because after the time of scanning (23 to 25 minutes) it had separated into two phases and, in some specimens, a small portion of the material had leaked out of the tube. The phase separation of the material could be attributed to complete miscibility between the organic and inorganic components of the material that compromises its chemical stability (Baldi et al., 2012). This was not observed in the solubility test in which the tubes containing the material were immediately immersed in water or PBS.

ZOE had lower volumetric loss than Calen-ZO in distilled water; and there was no difference in volumetric change for both materials when immersed in PBS. Considering the solubility test results, it was expected that the ZOE had greater volumetric loss than Calen-ZO in water. However, in the tests involving micro-CT, the materials were not immersed in water or PBS immediately after manipulation, as the scanning time for every two specimens was 23 to 25 minutes. During this time, partial setting of ZOE may have occurred (ZOE has a setting time of 110min) (Ochoa-Rodriguez et al., 2021) leading to lower mass loss than Calen-ZO in water, since Calen-ZO does not set (Pilownic et al., 2017). The non-difference between Calen-ZO and ZOE in PBS and the lower volumetric change of Calen-ZO immersed in PBS than water is probably due to the formation of hydroxyapatite, which delays the solubility of Calen-ZO (Gandolfi et al., 2010; Mohammadi & Dummer, 2011; Prüllage et al., 2016).

Endodontic materials should not have a high percentage of voids (Gandolfi et al. 2016), since its presence may increase the probability of post-treatment reinfection (Başer Can et al. 2017). The occurrence of voids within the root filling may be influenced by several factors such as consistency and volume of material, the operator's expertise and the filling technique used (Keles et al.,2014). In the present study, polyethylene tubes closed at one end were filled by a single operator using the obturation technique preconized for each material. There was no difference in voids formation between Calen-ZO and ZOE, except for 1 mm diameter polyethylene tubes immersed in water, where Calen-ZO showed higher presence of voids compared to ZOE.

It is important to mention that even though Bio-CP is available in a ready-to-use syringe, it was necessary to use the ultrasonic tip to obtain a homogeneous filling of

the tubes, without the presence of voids (detected radiographically) that formed when the material was inserted in the polyethylene tubes through the syringe and the application tip. Ultrasonic activation can be employed for the agitation of intracanal sealers and intracanal medication pastes. This enhances the sealer's intratubular dentine penetration, antimicrobial activity against *E. faecalis* (de Andrade et al., 2021) and minimizes the presence of gaps in vitro (Guimarães et al., 2014).

The filling capacity of all materials was higher than 98% independent of the obturation technique applied. It should be noted that the phase separation of Bio-CP did not influence the % filling capacity of the material. Nonetheless, the composition of Bio-CP needs to be reviewed to achieve better chemical stability and filling capacity without the use of ultrasonics. A recent study compared filling effectiveness and voids of root filling materials and obturation techniques in resin-prototyped primary incisors by micro-CT. When employing lentulo to obturate the incisor with Calen-ZO, the authors found a filling capacity of 94.97% compared to a 99.77% when employing syringe (Aragão et al., 2021). These results are comparable to ours using a syringe which were 98.87 % for 1 mm diameter tubes and 98.42% for 2 mm diameter tubes. In the case of ZOE, they found a 96.35% using a lentulo and a 93.45% using a syringe (Aragão et al., 2021), and our study revealed a filling capacity higher than 98 % for 1 and 2-mm diameter tubes.

The present study search for adequate in vitro methodology for the evaluation of solubility (% of mass loss); and volumetric change of root canal filling for primary teeth. Insertion into tubes of 1 or 2 mm in diameter and immersion for 30 days in water or PBS made it possible to evaluate the solubility, volumetric change and voids of root canal filling materials for deciduous teeth. However, when one of the study materials is based on calcium silicate or calcium hydroxide, the immersion medium of choice should be PBS due to the formation of hydroxyapatite-like structures that can influence the solubility. (Gandolfi et al., 2010; Prüllage et al., 2016). Regarding the diameter of the tubes, in most cases, the materials inserted in 1 mm diameter tubes showed lower solubility than the ones inserted in 2 mm tubes so, further studies should be made to correlate the solubility of the materials, their resorption speed and the rhizolysis speed of primary teeth, to define the percentage of solubility that root canal filling materials should have. Theoretically, tubes of 1mm simulate better primary molar teeth, and 2 mm diameter tubes simulate better anterior primary teeth. The micro-CT analysis presents limitations when it is desired to compare materials that do not set with

materials that do set, as in the case of ZOE and Calen- ZO. This is because, even if the materials that set are placed inside the tubes and analyzed in micro-CT immediately after handling, they would set or at least have partial set, depending on their setting time. Thus, they can be placed in the immersion medium after setting which creates a bias for the comparison.

Conclusion

In general, there was no difference in the solubility of Bio-CP and Calen-ZO, and both had lower solubility than ZOE, regardless of the immersion medium (distilled water or PBS) and tube diameter (1 or 2mm in diameter). Bio-CP and Calen-ZO produced hydroxyapatite after 14 days of immersion in PBS. Micro-CT analysis of volumetric change and voids is not recommended for Bio-CP because it separates in two phases, creating a bias in the comparison of materials. There was no difference in volumetric loss and in the percentage of voids between Calen-ZO and ZOE when immersed in PBS. Bio-CP has the potential to become a suitable material for root canal filling in primary teeth. However, the formulation of Bio-CP should be adjusted to enhance both chemical stability and filling capacity without relying on ultrasonics.

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4 CONCLUSION

Publication 1

Bio-CP did not set, presented adequate radiopacity and alkaline pH, was cytocompatible and had the potential to induce mineralisation in osteoblast-like human cells (Saos-2).

Publication 2

In general, there was no difference in the solubility of Bio-CP and Calen-ZO, and both had lower solubility than ZOE, regardless of the immersion medium (distilled water or PBS) and tube diameter (1 or 2mm in diameter). Bio-CP and Calen-ZO produced hydroxyapatite after 14 days of immersion in PBS. Micro-CT analysis of volumetric change and voids is not recommended for Bio-CP because it separates in two phases, creating a bias in the comparison of materials. There was no difference in volumetric loss and in the percentage of voids between Calen-ZO and ZOE when immersed in PBS.

Considering the results of publications 1 and 2, Bio-CP has the potential to become a suitable material for root canal filling in primary teeth. Nonetheless, the composition of Bio-CP needs to be revised to achieve better ~~chemical stability and~~ filling capacity without the use of ultrasonics.

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APPENDIX

APPENDIX A- METHODOLOGY OF PUBLICATION 1

Root filling materials, manufacturers and proportions for manipulation are shown in Figure 1.

Figure 1 - Root canal filling material, manufacturer, and proportion of use

Material	Manufacturer	Proportion for manipulation
Bio-C Pulpecto*	Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil	Ready for use
Calen + zinc oxide (Calen-ZO)	S.S. White Artigos Dentários Ltda., Rio de Janeiro, RJ, Brazil Biodinâmica Química e Farmacêutica LTDA, Ibiporã, PR, Brazil	0.24 g Calen + 0.12g ZO
Zinc oxide and eugenol (ZOE)	Biodinâmica Química e Farmacêutica	0.54 g ZO + 100 μL of eugenol

* Product under development, batch no. 190118.
Source: Own elaboration.

Radiopacity evaluation

Eight specimens of each material, measuring 10 mm diameter and 1 mm high were prepared according to ISO 6876:2012[1,2] (Figure 1). The specimens were stored at 37°C and 95% humidity for 3 times the setting time for ZOE, until achieving maximum hardening for Bio-CP and Calen-ZO. Afterwards, they were placed on top of a KODAK CMOS digital sensor (6100, Kodak Co.), next to an aluminium step-wedge (98.5% of Al, 8 steps with 2 mm increments for each step) for radiographic exposure. Focus 50540 x-ray unit (Instrumentarium Dental; Tuusula, Finland) was used, and the parameters were set at 65 kVp, 7 mA, exposure time of 0.16 seconds and a 320 mm source-to-object distance.[3] The digital images obtained were evaluated using methodology developed by Ochoa-Rodriguez, et al.[3](2019).

pH analysis

Ten polyethylene tubes 10-mm long with 1 mm internal diameter were filled with each material. Each specimen was immersed in 10 mL of deionised water, inside of a plastic flask sealed individually with a lid, and then stored at 37°C. The experimental time intervals were 1, 3, 7, 14 and 28 days. The pH of the solution was assessed with

a previously calibrated digital pH meter (Digimed; São Paulo, SP, Brazil) at each time interval. The control group consisted of deionised water with no immersed material.

Setting time

Calen-ZO and ZOE were inserted into ring-shaped metal moulds 1 mm wide and 10 mm diameter (n=6), kept at 37°C and 95% humidity. A plaster mould with the same dimensions was prepared for Bio-CP. The plaster mould was previously submerged in deionised water for 24 hours to provide the bioceramic material with moisture. The setting time was assessed using a Gillmore needle with a mass of 100 g ±0.5 and diameter of 2.0 mm ±0.1, according to ISO 6876:2012.[1] The setting time of the specimens was established as the time elapsing from initial mixing of Calen-ZO and syringe application of Bio-CP up to the maximum hardening of the specimens. At this point, the indentation marks made by the Gillmore needle were substantially reduced, but not eliminated. This is because the pastes had not set. Comparatively, the setting time for ZOE was considered as the time elapsing from when it started to be mixed until such time as the Gillmore needle failed to leave marks on the surface of the specimens.

Cell culture and preparation of root filling materials

Saos-2 (human osteoblast-like cell) immortalized cells line were grown in flasks containing Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Grand Island, NY, USA), penicillin (100 IU / mL), and streptomycin (100 µg / mL), in an atmosphere with 5% CO2 and 95% humidity at 37°C.

After handling of the specimens, 100 mg of each material (Figure 1) was placed in a 1.5 mL microtube (Eppendorf, Hamburg, Germany), to which 1.2 mL of DMEM was added, followed by placement of the microtubes in an oven at 37°C for 24 hours.[4] Afterwards, the supernatant was transferred to new microtubes centrifuged for 10 min at 20,800 g (5430, Eppendorf AG, Hamburg, Germany) to decant any particles of material left in the supernatant. The supernatant was transferred to a new tube and was considered as the "stock solution/extract". It was then diluted and placed in contact with Saos-2 cells.[5]

Cell viability analysis by methyl thiazolyl tetrazolium (MTT) and neutral red (NR) assays

Saos-2 cells were cultured at a density of $1x10^5$ cells/mL in a 96-well plate containing DMEM with 10% SFB for 24 hours to adhere to the plates. Thereafter, the cells were exposed to the cement extracts at the following dilutions in DMEM (v: v) 1:2; 1:4; 1:12; 1:24; 1:32. Cells exposed to DMEM were considered as the control group.

After 24 hours of cell contact with the material extracts and the control, 100 µL of 5 mg / mL MTT solution (Sigma-Aldrich) was added to each well, followed by incubation at 37°C, 95% humidity and 5% CO_2 for 3 hours. The colorimetric product was solubilised in 100 µl of 0.04 N acidified isopropanol (Sigma-Aldrich). A spectrophotometer (Elx800; Bio-Tek Instruments, Winooski, VT, USA) at 570 nm was used to measure the optical densities of the solutions. Absorbance readings were normalised to readings of cells exposed to DMEM, and represented the succinate dehydrogenase activity (cellular metabolism).

The NR assay was performed by applying 100 µL DMEM containing NR at 50µg / mL (Sigma-Aldrich) after 24 hours of cell contact with the materials and control extracts. The cells were incubated at 37°C, 95% humidity and 5% CO_2 for 3 hours, the contents of the wells were removed, and the colorimetric product was solubilised with 100 µL of a solution containing 50% ethanol and 1% acetic acid (Sigma-Aldrich). A spectrophotometer (Elx800; Bio-Tek Instruments, Winooski, VT, USA) at 570 nm was used to measure the optical densities of the solutions. Absorbance readings were normalised to readings of cells exposed to DMEM, and represented the ability to incorporate the dye into viable cell lysosomes.

Alkaline phosphatase (ALP) activity

Saos-2 ($5x10^4$ cells / mL) were cultivated in 96-well plates and exposed to the control (DMEM) and the extract of the materials at 1:24 dilution. This dilution was selected after observing the results of MTT assays performed after exposure of cells to cement extracts for 1, 3 and 7 days (data not shown). The 1:24 dilution was the highest extract concentration without cytotoxic effects. This is an essential consideration, since dead cells do not show ALP activity. ALP activity was evaluated at periods of 1, 3 and 7 days, using the commercial kit (Labtest, Lagoa Santa, MG, Brazil). Extracts of the materials were renewed every two days. Cells were washed with 200 µL saline phosphate buffer (PBS) after each experimental period, and a 200 µL sodium sulphate solution (1% in distilled water, Sigma-Aldrich) was added to each

well. Then the specimens were left to stand for 30 minutes at room temperature. Each sample (5 µL) in the sodium sulphate solution was transferred to a microtube (Eppendorf) containing a substrate and a buffer enzyme. Absorbance was measured using a spectrophotometer at 590 nm. Data were expressed as normalised ALP activity with total protein content, detected using the Total Protein Kit (Labtest) at the respective experimental periods. MTT assays were performed together with the ALP activity assay to follow up cell viability.

Alizarin Red Staining (ARS)

Saos-2 cells were cultivated ($1x10^4$ cells / mL) in 12-well culture plates with DMEM, supplemented with 50 µg / mL L-ascorbic acid (Sigma-Aldrich) and 10 mM β glycerophosphate (Sigma-Aldrich). The cells were exposed to material extracts for 21 days at the same dilution used for the ALP activity assay. Material extracts were renewed every two days. Then the cells were fixed with 4% paraformaldehyde (Sigma) and stained with 2% ARS (pH 4.1). The cells were incubated at room temperature for 20 minutes, and the dye was aspirated. The wells were washed 4 times with 1 mL of distilled water for 5 minutes. The water was removed and mineralisation was quantified by dissolving the nodules with 1 mL of a 10% cetylpyridinium chloride solution (Sigma Aldrich) added to each well, after which the plate was incubated for 15 minutes under stirring at room temperature. Three 100 µL aliquots of the suspension from each well were transferred to a 96-well plate and read with a 562 nm wavelength filter on a spectrometer (Elx800; Bio-Tek Instruments).

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APPENDIX B – METHODOLOGY OF PUBLICATION 2

Root filling materials, manufacturers and proportions for manipulation are shown in Table 1.[1]

Table 1 - Root canal filling material, manufacturer and proportion of use

Material	Manufacturer	Proportion for manipulation	Material composition
Bio-C Pulpecto*	Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil	Ready for use	Ester glycol salicylate, titanium oxide, calcium tungstate, silicon dioxide, toluene sulphonamide, and calcium silicate
Calen + zinc oxide (Calen-ZO)	S.S. White Artigos Dentários Ltda., Rio de Janeiro, RJ, Brazil Biodinâmica Química e Farmacêutica LTDA, Ibiporã, PR, Brazil	0.24 g Calen + 0.12g ZO	Calcium Hydroxide, zinc oxide, colophony, polyethylene glycol 400
Zinc oxide and eugenol (ZOE)	Biodinâmica Química e Farmacêutica	0.54 g ZO + 100 µL of eugenol	Zinc Oxide and Eugenol

* Produto em desenvolvimento batch nº 190118.

Solubility Test

Transparent cylindrical polyethylene tubes (Embramed, Cremer S.A., São Paulo, SP, Brazil) of 1 mm or 2 mm in diameter and 13 mm in length, were closed at one end so that the final length was 10 mm (n = 12 for each material, polyethylene tube group and immersion medium).This was made by measuring polyethylene tubes with a calibrated digital paquimeter (Metrica – Comercial e Importadora Ltda., SP, Brazil), after marking the exact measure (13 mm), it was then cut with a 15 scalpel blade attached to an scalpel handle. ZOE was inserted and lightly condensed inside the polyethylene tubes using Paiva condensers (Golgran, São Caetano do Sul, SP, Brasil). Calen-ZO was placed inside a 3 mm syringe (Becton Dickinson IND. CIRUR. LTDA., Curitiba, PR) and applied with a 21 G needle 0,80 x 30 mm (Becton Dickinson IND. CIRUR. LTDA., Curitiba, PR), so that it would be slowly inserted into the polyethylene tubes (Segato et al., 2016). Bio-CP, available in a ready-to-use syringe, was inserted slowly to the polyethylene tubes with the tips that come with it. Ultrasonic activation

with the ultrasonic tip E1 - Irrisonic (Helse Ultrasonics, Santa Rosa de Viterbo, SP, Brasil) using the ultrasonic device Ultrawave XS (Ultradent, Indaiatuba, SP, Brasil) at 20 % potency for 30 seconds, was employed to obtain a homogeneous filling of the tubes, without the presence of voids. Radiographs of all specimens were taken to verify the quality of the filling. The specimens were weighed with the value registered as the initial mass. Afterwards, they were immersed in a flask containing 10 mL of phosphate buffered saline (PBS) or distilled water and stored in an oven at 37 ° C for 30 days (Torres et al., 2019). Then, they were removed from the flasks, placed in an oven at 37°C until the mass had stabilized. Afterwards, they were weighed, and this valor was recorded as the final mass value. The mass loss was determined by subtracting the initial and final weight values and expressed as percentage.

Volumetric change, voids, and filling capacity

Another set of polyethylene tubes as described before was used for this test (n = 12). After the tubes were filled with the materials and the quality of the filling verified by radiographs, they were immediately scanned by micro-CT (SkyScan 1176; Bruker-micro-CT, Kontich, Belgium). The micro-CT parameters were 18 μm voxel size, 80 kV, 125 μA, rotation step 0.5, frame 4, 1.0 mm aluminum filter, and 180° scanning. The time of scanning for every two specimens was 23 to 25 minutes. The reconstruction of the images was performed using NRecon software (V1.6.10.4; Bruker-micro-CT). The correction parameters for smoothing, beam hardening, and ring artefacts were defined for each material as follows; for Bio-CP, 0 for beam hardening, 4 for ring reduction, 0 for smoothing; for Calen-ZO, 20 beam hardening, 5 for ring reduction, 0 for smoothing and for ZOE, 50 for beam hardening, 5 for ring reduction and 0 for smoothing. After micro-CT scanning, the specimens were immersed in a flask containing 10 mL of PBS or distilled water and stored in an oven at 37 °C for 30 days.[2] The specimens were removed from the flasks and immediately rescanned, using the same acquisition parameters used for the initial scan (Silva et al., 2017). The initial reconstructed images and the reconstructed images after the solubility challenge were superimposed using the Data Viewer software (V1.5.2.4; Bruker-micro-CT). The total volume (mm^3) of the root filling material, the total volume of the material in the determined ROI in percentage (% filling capacity) and voids (mm^3) was calculated by CTAn software (V1.15.4.0; Bruker-micro-CT). Volumetric change and voids were expressed as the root filling material volume gained or lost in percentage.[2,3,4]

Material surface element distribution: SEM-EDS analysis

Another set of polyethylene tubes (Embramed, Cremer S.A., São Paulo, SP, Brazil) as described before was used for this test (n = 3 for each material-Bio-CP and Calen-ZO, polyethylene tube group and immersion medium). The materials were placed in the tubes and immersed in PBS or distilled water for 14 or 30 days inside plastic flasks containing 10 mL of each solution and stored in an oven at 37°C. Immediately after, the specimens were desiccated for 14 days in an oven at 37°C. Afterwards, the superficial element distribution of the specimens was assessed via scanning electron microscopy (SEM – JSM 6610LV, JEOL Ltd., Musashino, Akishima, Tokyo, Japan) and energy dispersive spectroscopy (EDS – Oxford instruments x-max, OIC Precision Labs, Winnipeg, Manitoba, Canada), without a carbon-coating process.

Crystalline phase identification: XRD analysis

The same specimens used for SEM-EDS analysis were used for XRD. The surface of each specimen was carefully scraped and crushed to fine powder, placed, and leveled with the holder surface to get enough materials to analyze. An automated x-ray diffractometer (D2 PHASER; Bruker-AXS, Karlsruhe, Germany), was employed with the scan parameters set at steps of 0.02 degrees every 0.2 seconds analyzed between 10 and 80 degrees 2 Theta; each analysis took about 12 minutes. Origin 2022 for windows OS (OriginLab Corporation, Northampton, MA, United States) was employed for qualitative analysis of the diffractogram. The peaks of each sample were matched with those of the International Center for Diffraction Data (ICDD) database (International Center for Diffraction Data, Newton square, PA, USA).

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Não autorizo a publicação deste trabalho pelo prazo de 27 de julho de 2024 (2 anos após data de defesa)

Araraquara, 27 de julho de 2022 .

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