Original Research
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Evaluation of in vitro experimental model for analysis of bioceramic sealers

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Abstract: This study aimed to evaluate physicochemical properties of three ready-to-use calcium silicate-based endodontic sealers: Endosequence BC Sealer, Bio C Sealer, and Sealer Plus BC. Radiopacity was evaluated using specimens of 10 mm in diameter and 1 mm in height, along with an aluminum step wedge. For the flow test, 0.5 mL of each sealer was deposited between glass plates and the mean of the measurement of two diameters was considered the cement flow value. For pH and calcium release, root canals of 30 prototypes of upper incisor acrylic resin teeth were filled with sealer and gutta percha point and then immersed in containers with 13 mL of ultrapure water. Both pH and release of calcium ions (atomic absorption spectrophotometer) were measured at 3, 24, 72, and 168 h. Acrylic resin teeth were scanned by Micro-CT 1174 at the time of pH and calcium ion readings for volumetric change analysis. The data were analyzed by ANOVA, Tukey's, Kruskal-Wallis, and Dunn's tests. Endosequence BC Sealer presented the lowest, and Bio C Sealer the highest volumetric change after 72 h and 168 h ($p < 0.05$). Endosequence BC Sealer presented higher radiopacity ($p < 0.05$). All materials showed alkalinization capacity. All of them presented calcium ion release, with a higher value for Sealer Plus BC. All materials presented alkalinization, calcium release capacity, radiopacity, and flow above the minimum values required by the ISO standard. The highest volumetric loss was experienced by Bio C Sealer and the lowest one by Endosequence BC Sealer.

Keywords: Root Canal Filling Materials; X-Ray Microtomography; Solubility.

Introduction

Calcium silicate-based cements are known to have biocompatibility and bioactivity[1] and their bioactive potential is associated with alkalinization and release of calcium ions. These ions are released during the hydration reaction, producing calcium hydroxide, which is slightly soluble in water.[2] The solubility of cement may, however, compromise the sealing of a root canal against recontamination by microorganisms or penetration into tissue fluids.[3]

New, premixed, and ready-to-use injectable endodontic calcium silicate-based sealers include Endosequence BC Sealer (Brasseler, Savannah, USA), Bio C Sealer (Angelus, Londrina, Brazil), and Sealer Plus BC (MK Life, Porto Alegre, Brazil). Endosequence BC Sealer is composed of tricalcium silicate, dicalcium silicate, colloidal silica, monobasic calcium phosphate, calcium hydroxide, and zirconium oxide. As demonstrated in studies, Endosequence BC Sealer was biocompatible, especially[3] within some days after its application;[4] presented lower induction capacity of mineralized tissue formation than that of MTA, but higher than that of AH Plus;[5] was effective against *E. faecalis*; and showed antimicrobial activity that was probably due to the combination of a high level of pH, hydrophilia, and active release of calcium hydroxide.[6] Bio C Sealer contains tricalcium silicate, dicalcium silicate, tricalcium aluminate, silicon oxide, polyethylene glycol, iron oxide, and zirconium oxide. This sealer showed greater biocompatibility and ability to induce mineralized tissue formation when compared to AH Plus,[7] and adequate flow, setting time, and radiopacity, but high solubility.[8] Sealer Plus BC is composed of tricalcium silicate, dicalcium silicate, calcium hydroxide, and zirconium oxide and presents biocompatibility when used in contact with periapical tissues, inducing a mild inflammatory reaction and favoring repair[9] and solubility above that recommended by ISO 6876.[10]

However, studies on solubility and volumetric change have employed a vast number of specimens and tested materials only after their setting. Testing volume change, calcium release, and pH immediately after root canal filling, thus simulating clinical practice, would provide a closer to real-world scenario. Standard solubility tests are based on the difference between weights before and after cement placement in ultrapure water.[11,12] These tests have methodological limitations because material particles can detach from cements during the storage period or cement can absorb water, which could influence weight and, consequently, the outcomes.[13] Micro-CT has been used to evaluate volumetric change in endodontic materials, correlating solubility and dimensional alteration between different time intervals, allowing a more reliable observation of the dimensional behavior of the materials.[14,10,15]

In order to simulate the behavior of these sealers in clinical practice, it is important that they have adequate physicochemical properties. Therefore, the aim of this study was to evaluate volumetric change, release of calcium and hydroxyl ions, and flow and radiopacity of these sealers. The null hypothesis is that the materials would have similar physicochemical properties.

Methodology

The following premixed calcium silicate-based endodontic sealers were used in this study: Endosequence BC Sealer, Bio C Sealer, and Sealer Plus BC. Because they are premixed and ready-to-use materials, no prior manipulation was required.

Radiopacity

Cylindrical specimens were produced in accordance with ISO 6876:2012 standard using three rings measuring 10 ± 0.1 mm in diameter and 1 ± 0.1 mm in thickness. The molds were supported on a glass plate, filled with the injected sealer, and covered with another glass plate. The specimens and the aluminum step wedge were positioned on occlusal films (Speed F; Kodak Comp, Rochester, USA), and X-rays were then taken using a radiographic unit (Gnatus XR 6010; Gnatus Ribeirão Preto, Brazil) operated at 60 Kv and 10 mA for 0.3 s. The film focus distance was 30 cm. After processing, the radiographs were scanned using a Canon t5i still camera and imported into software (Adobe Photoshop CS5 Extended version 12.0 x32), where a tool was used to identify density areas on the radiographic images. This procedure allowed comparing radiographic data, sealer density, and radiopacity of the different thicknesses of the aluminum step wedge. Radiopacity was determined according to radiographic density and converted to Al millimeters, following the conversion equation proposed by Duarte.[16]

For solubility, pH, and calcium release tests, 30 prototypes of maxillary central incisors printed in transparent resin (IM from Brazil, São Paulo, Brazil) were used, which were divided into three groups (n = 10). The root canals of all specimens were prepared by automated asymmetric oscillatory motion using an Endodontic Reciproc VDW Silver motor (VDW, Munich, Germany) using an R#25 instrument (VDW, Munich, Germany) up to the limit of the foramen, and the preparation was complemented with an R#50 instrument working 1 mm below the apical foramen limit. During mechanical preparation, the canals were irrigated with distilled water and final cleaning was done by shaking the volume of water present with an Easy Clean plastic instrument (Bassi Easy, Belo Horizonte, Brazil) activated in rotational motion by a pneumatic handpiece for 20 s, in order to remove any remaining resin debris from mechanical preparation. The canals were then irrigated once again and aspirated with capillary tips (Ultradent do Brasil, Indaiatuba, Brazil). The sealers were injected into the simulated canals using a syringe and its injection tip, which penetrated up to 4 mm below the actual working length of the canal and was slowly removed as the sealer was deposited until adequate refilling could be observed. Dia Pro R R50 gutta percha points (Diadent, Burnaby, BC, Canada) then had their tips wrapped in sealer and inserted into the canal to the working length (1 mm below the foramen); the cervical excess was cut with a heated instrument; and the opening of the pulp chamber was sealed temporarily with Coltosol (Coltene Brasil, Rio de Janeiro, Brazil). Once filled, all specimens were placed in vials containing 13 mL of ultrapure water. The resin crowns were transfixed and glued to the lid of the containers so that the root apices would be immersed in ultrapure water throughout the experimental period, maintaining constant humidity. Solubility, pH level, and calcium release were tested following the methodology used by Guimarães et al.[17]

Flow

The flow test was performed according to ISO 6876:2001. A total volume of 0.5 mL of sealer was deposited at the center of a glass plate. After a 3-minute application with the syringe, another glass plate weighing 20 ± 2 g was placed on the plate containing the sealer and a weight corresponding to 100 g. After 10 min of application, the weight was removed and the largest and smallest diameters of the cement was measured using a digital caliper (Mitutoyo MTI Corporation, Tokyo, Japan). The mean measurement of the two diameters was considered the cement flow value. Three measurements were made for each cement variable. Throughout the experimental period, the samples were kept in an oven at 37 ºC.

Volumetric change analysis

The methodology used by Cavenago et al.[18] was applied for volumetric change analysis. Solubility was evaluated by volumetric measurements of sealers using micro-CT images. Thirty prototypes of upper incisor acrylic resin teeth (n=10) were used and each specimen was scanned four times after root canal filling. They were filled with injected sealer and gutta percha point, with proper care to avoid bubble formation during this step. After filling the canals, the specimens were scanned using a microfocus computerized tomographic desktop X-ray device (SkyScan 1174v2; SkyScan, Kontich, Belgium). The scanning procedure was completed using a 50 Kv X-ray tube and an 800 µA anode current. The image capture parameters were voxel size of 14.1 µm with rotation step of 0.7º, rotation of 180º, and 16.82 µm pixel size. Each scan consisted of 266 Tiff images with 1304 x 1024 pixels. The digital data were further elaborated by the reconstruction software (NReconv1.6.4.8, SkyScan) used for volume measurements. In the CTAn software, the specimens were analyzed separately. The analysis was limited to the region of interest (ROI) of each specimen, and the new ROI data were saved in separate folders. The data were then accessed and the binary value was adjusted according to the raw images. This value was recorded to be used later in the second scan, thus allowing for a quantitative analysis of the material volume through 3D plug-in analysis. This tool performs an automated calculation of the total volume (mm^3) of the three-dimensional (3D) image of the selected binary objects (white color). These data were reported on a list of results after the '3D analysis' function was performed. Before the scanning procedure and the final setting time, the specimens were individually immersed in acrylic vials containing 13 mL of ultrapure water and scanned immediately and at intervals of 24, 72, and 168 h. Between the intervals, the specimens were taken from the vials, dried with filter paper, and scanned again using exactly the same parameters adopted for the first scan. The volume of the sealer after immersion in water was calculated in the same way as in the first scan. Thus, solubility was determined by calculating the volume of sealers lost during immersion, and the results found were converted to percentage values to show the proportion of dissolved material.

pH and calcium release

Thirty prototypes of upper incisor teeth printed in acrylic resin (10 per group) were used. The materials were injected into the root canals and the filling was completed with a gutta percha point wrapped in the same sealer and condensed vertically. After filling the canals, the specimens were placed individually in an acrylic vial containing 13 mL of ultrapure water and stored at 37ºC throughout the experimental period. The acrylic teeth had their crowns fixed on the lid of the vial so that the apex of the roots would be always immersed in water. To avoid any interference in the results, all acrylic vials were previously washed with ultrapure water. To obtain pH and calcium release levels, readings were performed after 3, 24, 72, and 168 h. After each experimental period, the teeth were transferred to a new vial with the same volume of ultrapure water. The pH level was measured with a pH meter (Thermo model Orion 3 Star, City, USA), previously calibrated using controls with pH at values of 4.7 and 14. After removal of the specimens, the vial was placed in a shaker (model 251; Farmem, São Paulo, Brazil) for 5 s before measuring the temperature of the room during reading at 25ºC. Deionized water was used as a control for the pH level measurements for all periods analyzed. Calcium ion release was performed using an atomic absorption spectrophotometer (AA6800; Schimadzu, Tokyo, Japan) equipped with a hollow cathode lamp. For the wavelength and crack parameters, several tests were analyzed to determine the most appropriate one. Standard calcium solutions were prepared at the following concentrations: 20 mg, 10 mg, 5 mg, 2.5 mg, 1.25 mg, and 1 mg. Two milliliters of lanthanum nitrate solution were added to 6 mL of standard calcium or test solution. For the preparation of the blank solution, the same amount of lanthanum nitrate solution was added to 6 mL of ultrapure water. Calcium and blank and test solutions were quantified by atomic absorption spectrophotometry. A nitric acid solution was used to adjust the device to zero absorbance. The calcium ion release readings were compared with a standard curve obtained from the standard solution readings (Figure 1).

Statistical analysis

The test results were then subjected to the Kolmogorov-Smirnov test for normality assessment. Radiopacity and flow data showed normal distribution, and ANOVA and Tukey's test were used for statistical comparison. The data on pH, calcium release, and volumetric change did not present a normal distribution, and the Kruskal-Wallis and Dunn's tests were used for comparison between the groups. The significance level was set at 5%. GraphPad Prism 6.0 Software was used for the analyses.

Results

Flow and radiopacity test results are displayed in Table 1. All tested sealers presented flow and radiopacity above the minimum values recommended by ISO 6876:2001. Flow was similar for the three materials studied ($p < 0.05$). Endosequence BC Sealer presented the highest radiopacity (9.0 mmAl) followed by Bio C Sealer (6.9 mmAl) and, finally, by Sealer Plus BC (4.8 mmAl), with the lowest radiopacity. There were significant differences in radiopacity among the three materials ($p < 0.05$).

Figure 1. Filled root canal of a prototype with root apex in constant contact with moisture.

Table 1. Median and standard deviation of flow (mm) and radiopacity (mmAl) tests.

Variable	Flow	Radiopacity
Endosequence BC	25.47 +/-0.3[a]	9.0 +/-1.6[a]
Bio C Sealer	25.46 +/-2.2[a]	6.9 +/-0.6[b]
Sealer Plus BC	27.84 +/-4.7[a]	4.8 +/-0.4[c]

Different lowercase letters indicate significant differences between materials ($P<0.05$).

The median and the minimum and maximum values for solubilization are shown in Table 2. Regarding volumetric change, all sealers exhibited volume loss above 3%, with statistically significant difference among them. The fillings made by Bio C Sealer had the highest volumetric loss, while the fillings performed with Endosequence BC Sealer showed the lowest volumetric loss (Figure 2).

The results for pH and release of calcium ions are shown in Tables 3 and 4. The pH of the water in which the materials were immersed was 5.5. All materials in the present study demonstrated alkalinization capacity, mainly in the first 3 h of analysis, but alkalinity decreased to a level close to neutral after 24 h and remained so until the end of the experimental period (168 h). The canal fillings performed with Sealer Plus BC showed the highest pH values, followed by Bio C Sealer, while the fillings performed with Endosequence BC Sealer had lower alkalinization power. Regarding calcium release, canal fillings performed with Sealer Plus BC released a greater amount of calcium ions throughout the experimental period, mainly in the initial periods of analysis (up to 24 h), followed by fillings performed with Endosequence BC Sealer, which showed higher values of calcium ion release at the end of the experimental period. The canal fillings using Bio C Sealer presented the lowest value in the analyzed periods.

Table 2. Median, minimum and maximum values of volumetric change percentage.

Variable	Volumetric change (%)		
	24h	72h	168h
Endosequence BC	-5,000 (-8,460 - -2,780)[a]	-7,295 (-10,51 - -4,860)[a]	-7,050 (-10,49 - -4,440)[a]
Bio C Sealer	-11,43 (-15,17 - -9,780)[b]	-15,96 (-21,08 - -14,91)[b]	-16,92 (-21,08 - -15,42)[b]
Sealer Plus BC	-8,760 (-19,58 - -4,090)[b]	-13,36 (-21,96 - -8,170)[c]	-14,42 (-23,28 - -9,620)[c]

(-) identifies percentage of volumetric loss. Different lowercase letters demonstrate significant differences between the analyzed materials (p < 0,05).

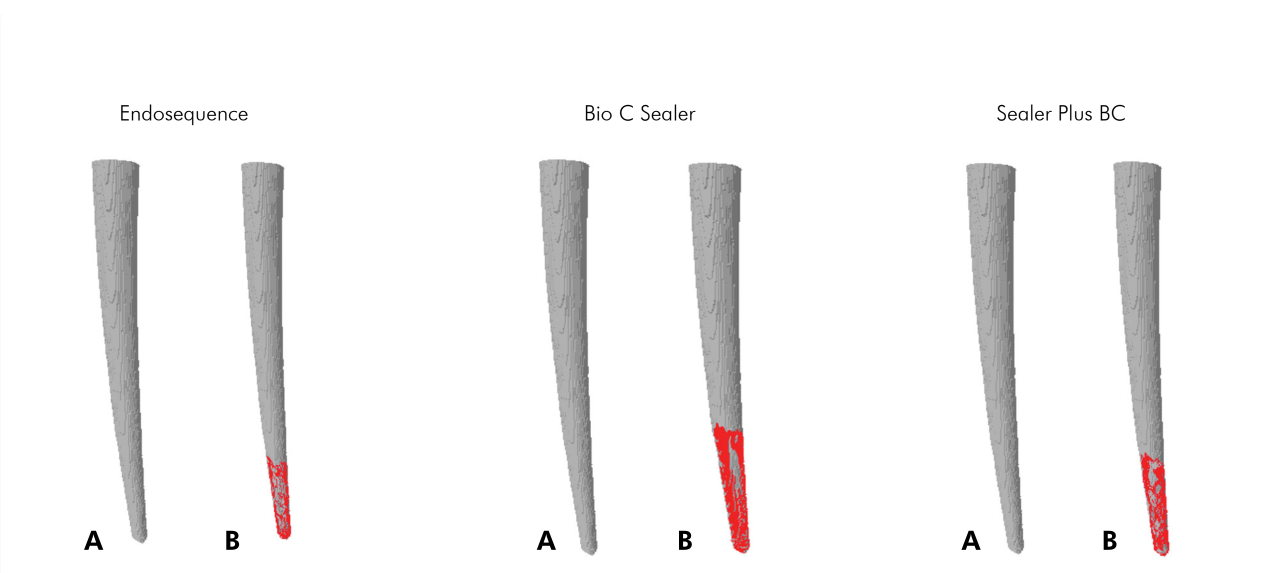

Figure 2. Representative images of volumetric change of materials. A) material in the initial scan and B) change in volume after 168 h.

Table 3. Median, minimum and maximum pH values of studied materials in the different experimental periods.

Variable	pH level			
	3h	24h	72h	168h
Endosequence BC	6,775 (5,900-7,450)[aA]	5,590 (5,340-5,650)[bA]	5,660 (5,600-5,810)[cA]	5,945(5,790-6,550)[dA]
Bio C Sealer	8,475 (6,360-9,500)[aB]	7,145 (6,670-8,600)[bB]	7,015 (6,650-7,410)[bB]	6,950 (6,210-7800)[bB]
Sealer Plus BC	9,310 (6,840-9,980)[aB]	7,545 (6,580-9,490)[bC]	7,015 (6,470-8,590)[cB]	7,180 (6,430-8.770)[bcB]

Different lowercase letters demonstrate significant differences between the periods analyzed within the same material (p <0,05).

Table 4. Median, minimum and maximum calcium values (mg/L) released form the studied materials in the different experiment period.

Variable	Calcium ion release			
	3 h	24 h	72 h	168 h
Endosequence BC	1.850 (0.9900-2.990)[aAB]	1.025 (0.9600-1.500)[bAB]	1.250 (1.010-1.520)[abA]	1.770 (1.310-13.13)[aA]
Bio C Sealer	0.9200 (0.3200-2.910)[aA]	0.5850 (0.1700-1.360)[aA]	0.5450 (0.2600-18.38)[aB]	0.6200 (0.2200-3.770)[aB]
Sealer Plus BC	2.745 (0.3900-14.28)[aB]	1.600 (0.4100-2.920)[abB]	0.8550 (0.6900-1.240)[bAB]	1.095 (0.750-3.770)[abAB]

Different lowercase letters demonstrate significant differences between the periods analyzed within the same material (p < 0,05).

Discussion

Calcium silicate-based sealers have been introduced into endodontics very recently, with the prospect of expanding the biological properties of endodontic fillings. These properties are dependent on the sealers' ability to alkalize the environment and to promote the release of calcium ions, factors that may be related to the solubility of the material. Flow is also important for sealers to penetrate and fill more critical anatomical areas of the root canal system such as isthmus, lateral canals, and dentinal tubules, leading to better sealing. However, flow should not be highly elevated to prevent extravasation into periapical tissues, which could interfere in the repair process, depending on the level of cytotoxicity of the material.[19] The three studied sealers presented flow rates above the minimum required by ISO 6876:2012 and similar behavior among them. The results for Sealer Plus BC and Endosequence BC Sealer corroborate those of some studies.[20,21]

Radiopacity values above those of the dentin make it easier to visualize the filling quality of the root canal system by the sealers. All studied materials presented radiopacity above the ideal values established by the ISO 6876/2001 standard. These results corroborate those of previous studies.[8,20,22] All the studied sealers have the same radiopacifier (zirconium oxide) in their compositions. The choice of zirconium oxide as a radiopacifier in the new calcium silicate-based sealers takes aesthetics into account. Moreover, zirconium oxide does not affect the other physicochemical and biological properties of calcium silicates;[12] it can still be effective in inhibiting fungal infection by *Candida albicans*.[23] The different radiopacity values of the materials can be explained by the variable amount of radiopacifier used in each sealer, which makes the material more or less radiopaque.[24]

Manufacturers claim that the pH value of these sealers is near 12 in the first few hours. The present study, however, analyzed the precise value of hydroxyl ion that would be released by the sealer through the foramen, simulating clinical practice. It was verified that the pH released to the apical region as a result of hydration was close to 10 in the first 3 h of application, decreasing alkalinity to a level close to neutral after 24 h, remaining like that until the end of the experimental period. Sealer Plus BC and Bio C Sealer showed higher capacity for hydroxyl ion release, unlike Endosequence BC Sealer. On the other hand, Endosequence BC Sealer was the one with the lowest volumetric solubility. Regarding calcium release, it was the highest for Sealer Plus BC and the lowest for Bio C Sealer. Possibly, the absence of calcium hydroxide in the composition of Bio C Sealer might have contributed to this finding. It is important to highlight that the alkalinization capacity and release of calcium ions could have been limited by the methodology used in this study, in which the contact of the material with the external environment (ultrapure water) was restricted to the foramen diameter of acrylic tooth canals (about

0.25 mm), which would be closer to that observed in clinical practice.

The solubility of filling materials is related to the dissociation of the components of these materials or the degradation of particles when in contact with tissue fluids, which can undesirably form gaps that can facilitate microbial colonization and lead to reinfection.[11] In the present study, all sealers presented volumetric losses above 3% within the first 24 h. Moisture can alter the properties of premixed hydrophilic calcium silicate-based sealers.[25] Until sealers complete their total setting time, the influence of tissue fluids and blood on them can contribute to their solubilization.[11] Simulation of tissue fluids may reveal greater similarity to clinical application.[26] Thus, the methodology of the present study was targeted at simulating clinical practice by injecting the premixed sealer into the root canal, allowing for immediate contact of the sealer with moisture through the apical foramen. Endosequence BC Sealer presented the lowest solubility throughout the experimental period. In this study, the total volumetric solubility of this material was similar to that observed by Zhou et al.[27] Some factors may explain the lower solubility of this sealer in relation to the other two sealers. Endosequence BC Sealer has calcium phosphate in its composition, which improves its setting time and whose chemical composition and crystalline structure are similar to those of apatites.[28] Another reason could be particle size; both Endosequence and Sealer Plus BC have nanoparticles, which tend to produce more efficient hydration reactions, given that their surface area is more susceptible to an inrush of water molecules, further optimizing their setting process and, consequently, minimizing their solubility. On the other hand, Bio C Sealer presented the highest solubility among the three investigated sealers. In addition to not having calcium phosphate in its composition, Bio C Sealer can be composed of particles larger than those of Endosequence BC and Sealer Plus BC. The larger the particle, the greater the volume loss of the material, as it is solubilized. The solubility observed in the present study was even higher than that presented by Zordan-Bronzel et al.,[8] who used a different methodology, waiting for the complete setting of sealers before putting them in contact with moisture, unlike the present work, in which the specimens were immersed in water immediately after the insertion of the filling materials, thereby explaining the higher solubility obtained.

The use of prototypes in this study aimed to reduce some biases during the physicochemical tests, such as diameter of dentinal tubules and variations in internal anatomy (root canal shape, root canal length, and apical foramen diameter). The model allowed the amount of filling material and foramen size to be standardized, providing all groups with a similar clinical setting. Although we kept the canals moist before filling them and maintained the foramen in contact with moisture throughout the experimental period, the model may present limitations for tests with bioceramic sealers. The natural root dentin moisture and the presence of dentinal tubules could contribute to a greater diffusion of calcium or hydroxyl ions and also influence the process of hydration and setting of the sealers. Thus, future comparative tests with natural teeth are suggested so that this type of model can be consolidated as a methodological tool.

Conclusions

The null hypothesis was rejected, except for the flow test, because there were significant differences between the materials. All materials presented alkalinization, calcium release capacity, radiopacity, and flow rates above the minimum required by the ISO standard. Bio C Sealer showed the largest volumetric loss, while Endosequence BC Sealer had the smallest volumetric loss. Both pH and calcium release were higher for the Bio C sealer and Sealer Plus BC.

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