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Dentinal Tubule Penetration of a Calcium Silicate-Based Root Canal Sealer Using a Specific Calcium Fluorophore

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This study aimed to evaluate penetrability on dentinal tubule of a new bioceramic sealer through confocal laser scanning microscopy (CLSM). A specific fluorophore (Fluo-3) was mixed with the sealer. Forty distobuccal roots from maxillary molars were selected, and root canal preparation was carried out with Wave One Gold # 35.06 instruments. Roots were randomly assigned to 4 groups according to the filling procedures: Bioceramic/Lentulo (Sealer Plus BC); Bioceramic/EasyClean group, three activation of the sealer (3x20 s) with Easy Clean instrument; Bioceramic/Irrisonic: ultrasonic activation for 30 s; and AHplus/Lentulo: epoxy resin based sealer (AH Plus) was utilized with the same protocol as the BC/LE group. After 72 h, specimens were transversally sectioned at 2 and 7 mm from root apex and then analyzed through CLSM. Sealer penetration area on dentinal tubule was measured by Adobe Photoshop CC2018. Kruskal Wallis and Wilcoxon T tests were carried out. Penetrability results were similar for both sealers regardless of which technique was performed to activate them inside the root canal ($p>0.05$). It is reasonable to conclude that penetration of bioceramic and epoxy resin based sealers occurred unimpressively. The type of instrument used to activate bioceramic sealer did not affect penetrability. Fluo 3 should be recommended as the fluorophore to evaluate dentinal tubule penetration of bioceramic sealers.

Key Words: endodontics, bioceramic sealer, root canal filling, confocal laser scanning microscopy.

Introduction

Bioceramic materials have been gaining ground in Endodontics, and the reason may be a result of calcium silicate and calcium phosphate combination. In addition, bioceramic materials also contain alumina, zirconia, bioactive glass, glass ceramic, calcium hydroxyapatite, and resorbable phosphate (1,2). An earlier study demonstrated excellent physicochemical properties, such as pH, calcium ion release, flow radiopacity and setting time (3). In addition, bioceramic-based sealer demonstrated less cytotoxic and genotoxic effect in comparison with epoxy resin-based sealers (4).

The penetrability of endodontic sealers on dentinal tubules and complex anatomic areas is directly related to their flow property (5). A moderate flow is desirable in order to access areas that need to be filled and also not to leak into the periapical region. Periapical leakage might lead to tissue damage and interfere in apical healing (4,6).

Confocal laser scanning microscopy (CLSM) is an approach to evaluate sealers penetrability on dentinal tubule (7). To date, most studies have been demonstrating penetrability of bioceramic sealers inside dentinal tubule through this microscopy technology. They also use rhodamine B as a fluorophore to determine the magnitude of intratubular penetration (8-10).

The use of rhodamine B associated with calcium silicate based sealers might interfere the interpretation of the intratubular penetration data (11). The reason is that the tubular humidity and the water necessary for chemical reactions during setting of the sealer may bind to the rhodamine B instead of the bioceramic sealer. Consequently, false-positive results in terms of penetrability could be reported. In other words, fluorescent-tagged regions would not necessarily be filled with bioceramic sealer but with water inside dentinal tubule which is responsible for carrying the fluorophore.

These aspects cited above are crucial methodological concerns and, therefore, the employment of fluorophores that bind to the calcium present on sealer chemical composition becomes relevant. Furthermore, the fluorophore should also accurately demonstrate the penetration degree of bioceramic sealers on dentinal tubule and complex anatomic areas (11).

Some studies have been testing the effect of ultrasonic activation and other devices for intratubular penetration of endodontic sealers trying to address and to enhance the complete filling of the root canal system (12). The present study aimed to evaluate the penetrability on dentinal tubule of a new bioceramic sealer through CLSM working with a specific fluorophore (Fluo-3) for calcium ions after sealer insertion with Lentulo spiral, whether or not followed by activation of endodontic filling material.

Material and Methods

This study was approved by Research Ethics Committee from the Federal University of Rio Grande do Sul (2.421.115).

Teeth Selection

Forty human teeth were selected for this study, superior molars with three distinct roots, closed apex and a curvature less than 5 degrees which was visually evaluated. Teeth were stored in a 0.9% saline solution right after extraction and then submerged in a 2.5% sodium hypochlorite for 48 h. Following that, digital radiographs were performed to whether confirm or not the presence of root canal, the absence of internal reabsorption, calcifications and previous endodontic treatment. After all these procedures, teeth were kept in saline solution up to the following methodological steps from this study.

Root Canal Preparation

Standardized techniques were performed for the coronary opening of the selected teeth with spheric diamond bur number 1014 in high-speed. Only distobuccal roots were employed for this study. Working length was established by visualizing a size 15 K-File #15 at the apical foramen, and then 1 mm was subtracted to determine the real working length. Biomechanical preparation was carried out with #035.06 Wave One Gold rotary system (Dentsply-Sirona, Ballaigues, Switzerland) and the X-Smart Plus endodontic electric motor (Dentsply-Sirona, Ballaigues, Switzerland) at reciprocating mode with speed and torque as defined by the manufacturer.

Root canal was irrigated with 2 mL of 2,5% sodium hypochlorite using a syringe with 30 diameter needle (Navitip; Ultradent Products Inc. South Jordan, UT). At the end of preparation procedures, a final irrigation protocol was adopted with 5 mL of 2.5% NaOCl with passive ultrasonic irrigation (Irrisonic, Helse Technology, Ribeirão Preto, SP, Brazil) split into 3 stages: the first two periods were performed with 2 mL during 20 s of activation, and the last one was 1 mL and also an activation of 20 s.

Posteriorly, root canal was rinsed with physiologic saline solution and aspirated with aspirator Capillary Tips (Ultradent, Utah, USA). Afterward, 2 mL of 17% EDTA (Maquira, Maringá, PR, Brazil) was used to flush the root canal, then stirred for 3 min with a size 20 K-file (Dentsply-Sirona, Ballaigues, Switzerland). A rinse with saline solution was performed one more time and then aspirated and dried with sterile paper points in order to maintain a relative humidity as required by bioceramic sealer. In the control group, AH Plus sealer (Dentsply-Malleifer, Ballaigues, Switzerland) was used, and root canals were dried with as many paper points as necessary to properly dry them.

Root Canal Filling

Specimens were randomly assigned to 3 experimental groups and a control group with 10 teeth each. Single cone technique with Wave One Gold 35.06 gutta-percha cones (Dentsply-Sirona, Ballaigues, Switzerland) was performed for root canal filling.

Sealer Plus BC (MK LIFE, Porto Alegre, Brazil), a calcium silicate based sealer, was employed in this study. Fluorescent calcium indicator (Fluo-3; Thermo Fisher Scientific, USA) was added to the sealer in a ratio of 0.1% in order to increase fluorescence in the CLSM. Initially, 1 g of endodontic sealer was weighed on an analytic scale (Shimadzu, Tokyo, Japan) with a precision of 0.0001 g. Next, 0.002 g of the Fluo-3 indicator was weighed.

Root Canal Filling Protocol Was Proceeded According To The Following Experimental Groups:

Bioceramic/Lentulo - Bioceramic sealer was introduced into root canal by a Lentulo spiral number 30 (Dentsply-Sirona, Ballagues, Switzerland) and sequentially single cone technique was performed for root canal filling;

Bioceramic/EasyClean - Sealer was similarly inserted into the root canal by a Lentulo, then stirred 3 times for 20 s by Easy Clean instrument (Easy, Belo Horizonte, Brazil) with reciprocating movement with X-Smart Plus motor according to manufacturer specifications. Sequentially, root canal filling was carried out with single cone technique;

Bioceramic/Irrisonic - Sealer was added with Lentulo and passively stirred with Irrisonic ultrasonic tips (Irrisonic, Helse Technology, Ribeirão Preto, SP, Brazil) for 30 s;

AH Plus/Lentulo - Control group. AH Plus Sealer was inserted with a Lentulo spiral.

Following root canal filling procedures, vertical compaction was carried out with vertical compactors (Odous De Deus, Minas Gerais, Brazil). It is worth pointing out that fluorescent calcium indicator (Fluo-3; Thermo Fisher Scientific, USA) was also added to the sealer in the control group.

Evaluation by Confocal Laser Scanning Microscopy Evaluation (CLSM)

As root canal filling was concluded, teeth were sealed with Coltosol (Coltene, Switzerland) and stored with wet gauze pads for 72 h at room temperature to achieve sealer setting.

As finished this step, teeth coronary portion was sectioned under refrigeration with a double-sided diamond disk, producing 12 mm length roots. Specimens were horizontally sectioned at 2 and 7 mm from root apex using a diamond disk at a cutting machine (Extec Labcut 1010, Enfield, CT, USA) under refrigeration.

Posteriorly, surfaces were polished with Pop-on 4850SF 3/8 30D Azul Soflex disks (3M ESPE) to remove possible smear generated by cutting procedures. Samples were evaluated by Olympus Fluoview 100 confocal laser microscopy (Olympus Corporation, Tokyo, Japan) with excitation of 559 nm wavelength light.

Images were recorded at fluorescent mode, 10× magnification and a numeric aperture of 0.3 and 1.3 mm respectively.

Analysis of Sealer Penetrability

The area of sealer penetration inside dentinal tubule was generated by the images obtained at the FluoView 10-ASW 4.2 program (Olympus Corporation, Tokyo, Japan) with ×10 magnification.

Every image was imported to Adobe Photoshop CC2018 program in which the measurements were performed. Initially, pixels measurement was conducted for the total area of the images and then lasso tool delimit and measure the root canal circumference and area. The root canal measures were subtracted from the total area measures, and the result was the dentinal area which could be infiltrated by the sealer. Following that, the same procedures were carried out in order to calculate dentinal tubule area which was invaded by the sealer. Then, percentage of the sealer penetration area was obtained. Figure 1 shows each step used to determinate the sealer penetration.

Statistical Analysis

Data from penetration into dentinal tubules of bioceramic and AH Plus sealers in each root level were analyzed by Kruskal Wallis test. Wilcoxon T-test compared data from middle and apical thirds from the same group. Both significance levels were set at 5%.

Results

Table 1 summarizes the intratubular penetration of each experimental goup based on the root canal third evaluated.

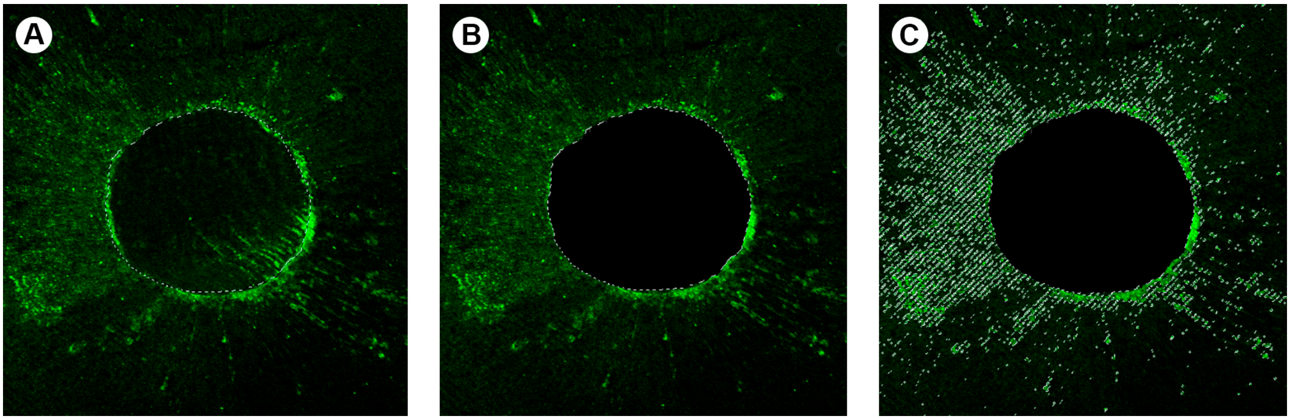

Figure 1. The CLSM images were evaluated using Adobe Photoshop CC2018. Initially, the total image area was measured. A: the root canal area was delimited and measured using the "lasso tool"; B: the root canal was cut out and subtracted from the total image area; and C: the sealer into the dentinal tubules was automatically marked by the program. Next, the percentage of dentin impregnated area was calculated based on the values of pixels marked in Figure 1C and the pixels present in Figure 1B.

Table 1. Median, 25 and 75 percentile, minimum and maximum values of the root canal sealer penetration from the 3 experimental groups and control groups at the middle and apical thirds

	Middle third (7 mm)			Apical third (2 mm)		
	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Bioceramic/Lentulo	8.46 Aa	4.77	10.25	7.92 Aa	6.59	9.55
Bioceramic/EasyClean	17.08 Aa	13.75	19.00	9.51 Aa	5.88	12.28
Bioceramic/Irrisonic	10.85 Aa	9.67	11.62	4.14 Aa	3.87	4.57
AH Plus/Lentulo	3.77 Aa	3.35	9.05	2.91 Aa	1.97	5.56

Same capital letters do not statistically differ when the comparison of the groups was performed into each root level (7 mm or 2 mm) using Kruskal Wallis test. Same lowercase letters indicate absence of significant differences after using Wilcoxon T test to compare data from 7 mm and 2 mm in each group. Wilcoxon T-test compared data from middle and apical thirds from the same group. Both significance levels were set at 5%.

No differences were observed when Bioceramic/Lentulo and Ah Plus/Lentulo groups were compared ($p>0.05$), regardless the root third evaluated. The method used to activate the bioceramic sealer did not improve its intratubular penetration ($p>0.05$). Finally, when each activation method was compared according to the root third no differences were observed ($p>0.05$).

Figure 2 and 3 show representative images from CLSM of the root canal sealers penetrability into dentinal tubule at the apical and middle thirds, respectively.

Discussion

For endodontic sealers, flow is an essential physicochemical property since it shows how root canal sealers penetrate complex anatomic areas such as canal isthmus, accessory, and lateral canals, apical delta and dentinal tubules (3,13-16).

The CLSM is an essential technology and a tool to evaluate the penetrability of root canal sealers on dentinal tubule (8,17). Fluo-3 introduction was a relevant consideration to this methodology because this fluorophore binds to calcium which is a decisive factor in assessing this research results. Using rhodamine B as dye with calcium silicate based sealer could show false-positive results since this sealer needs humidity to make chemical ligations during its setting and water would possibly react with rhodamine and not with the sealer of study instead.

Fluo-3 is a non-fluorescent compound, even though fluorescence significantly increases after binding to calcium. The calcium present in the calcium silicate based sealers binds to Fluo-3 and, consequently, fluorescence observed at microscopy belongs to the sealers (18). This

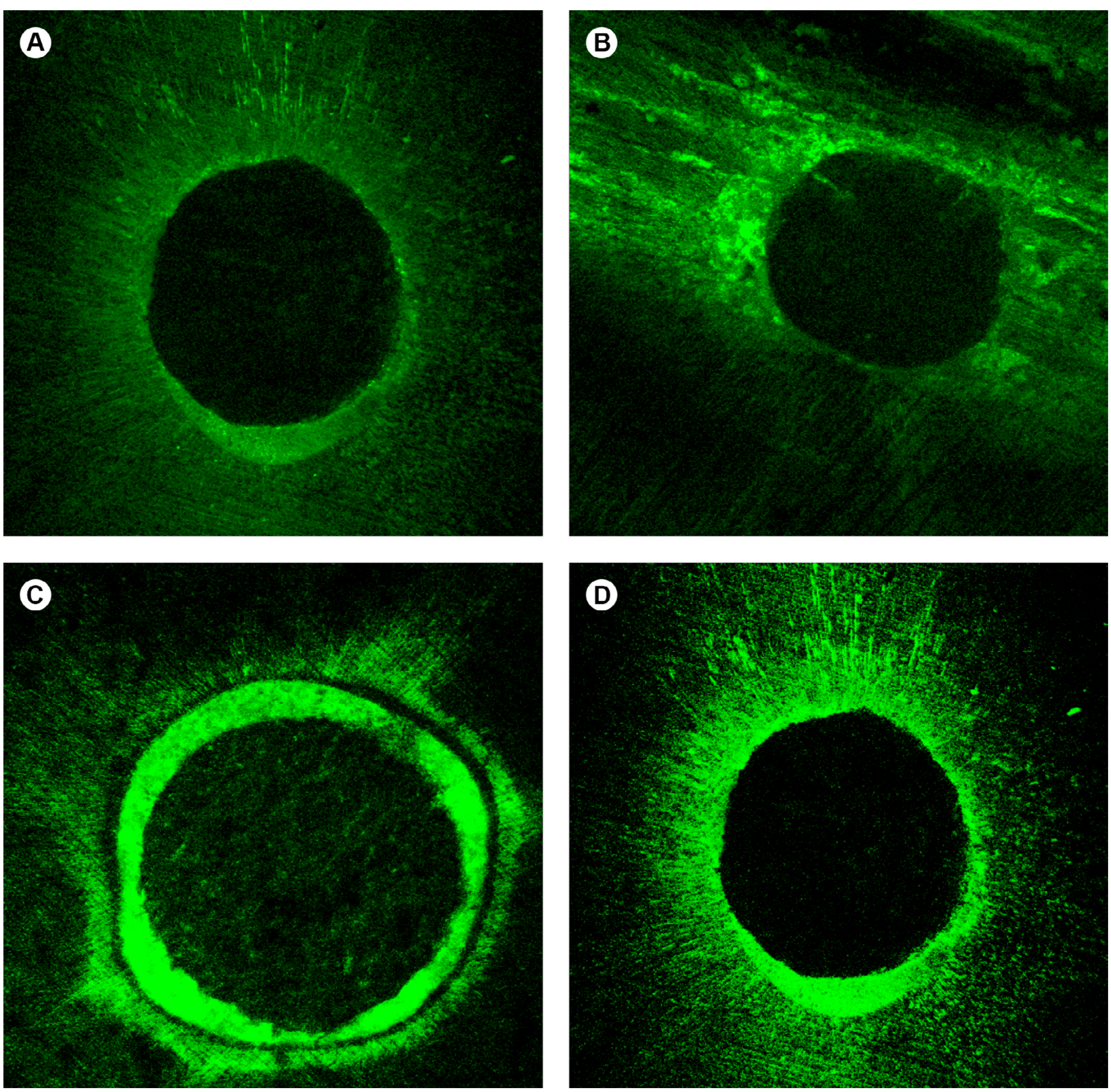

Figure 2. Representative CLSM images of dentinal tubule penetration in the apical third. A: Bioceramic/Lentulo; B: Bioceramic/Easy; C: Bioceramic/Irrisonic; D: AH Plus/Lentulo.

fluorophore may also be employed with AH Plus as it also presents calcium in the composition. In regard to AH Plus (epoxy resin-based sealer), it was chosen as the control group since it is considered as the gold standard sealer for various trials in endodontics research. A negative control group, only with deionized water and Fluo-3 without bioceramic sealer, was not performed because the indicator fluorescence only occurs when associated with calcium (11,19)

Previous researches evaluated penetration of other types of sealers (zinc oxide-eugenol-based and epoxy resin-based) in dentinal tubule through confocal laser microscopy (8,17,20). Rhodamine B was the chosen fluorophore since these materials require an absence of humidity during the insertion into the root canal, oppositely to bioceramic sealers (8,17,20). This is the first study that incorporated Fluo-3 dye to an epoxy resin-based sealer. Up to date, there was no reports regarding the intratubular penetration of these sealers associated with this fluorophore. The following investigations must compare the intratubular penetration of different endodontic sealers using both fluorophores (rhodamine B and Fluo-3).

The present study investigated the penetrability of Sealer Plus BC inside dentinal tubule of distobuccal root from superior molars. To our knowledge, only Jeong et al. (11) has used Fluo-3 and posterior visualization at CLSM when associated with calcium silicate based sealers. Therefore, it indicated that this Fluo-3 is the fluorophore of choice to fluoresce if bound to calcium and, consequently, only when this chemical element is present. CLSM produces light at a specific wavelength (488-600nm). The fluorophore is capable of increasing its fluorescence up to 6 times

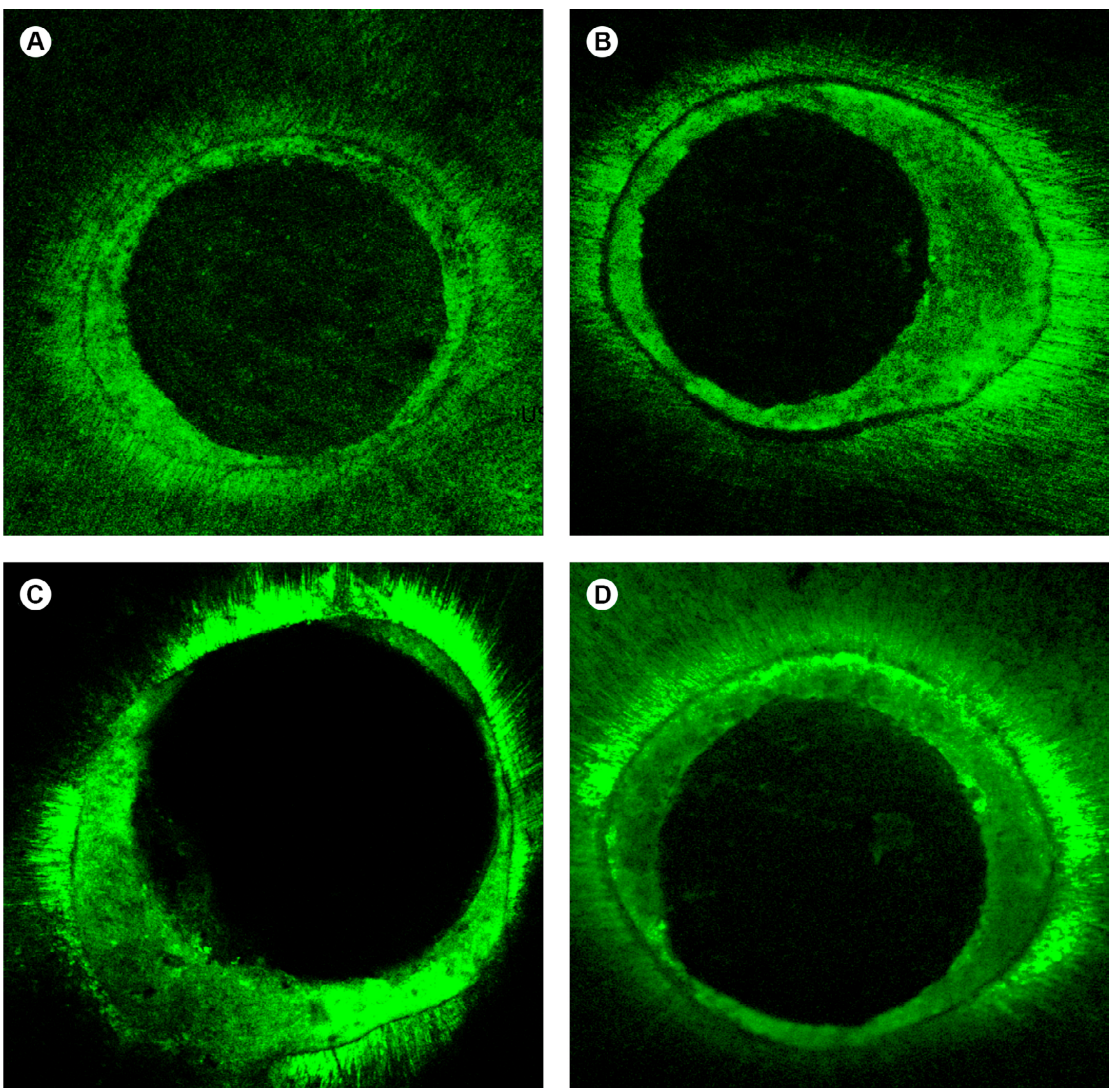

Figure 3. Representative CLSM images of dentinal tubule penetration in the middle third. A: Bioceramic/Lentulo; B: Bioceramic/Easy; C: Bioceramic/Irrisonic; and D: AH Plus/Lentulo.

depending on calcium quantity (19). Furthermore, it was initially created to detect gradients of intracellular calcium ions through CLSM and for flow cytometry in biochemistry (21,22). It is worth emphasizing that Fluo-3 is not able to detect calcium ions from dental structure; therefore, the obtained results consist only from calcium on bioceramic sealer composition (11).

Various authors investigated the interface adaption and penetrability of bioceramic sealers and used AH Plus sealer as control group (9,23). It should be mentioned that Rhodamine B was employed for visualization at CLSM in all of these studies. Therefore, results from those studies should not be confronted with the present findings because the marked areas may not adequately indicate the region infiltrated by the root canal sealers (9,23,24,25).

As previously mentioned, the flow rate of an endodontic sealer contributes to good penetration into dentinal tubules. Mendes et al. (3) demonstrated slightly lower flow rate of Sealer Plus BC than AH Plus ($p<0.05$). Probably for this reason, the penetrability observed in this study was similar for AH Plus and Sealer Plus BC, regardless the activation method employed over the bioceramic sealer. Contrary to our results, Alcalde et al. (26) found better quality of root canal filling and an increased intratubular penetration of the sealer, especially in the isthmus area, when AH Plus was ultrasonically activated. The differences between both studies probably occurred because Alcalde et al. (26) used mesial roots of mandibular molars with isthmus and the filling of this area is critical. In our study it was used distobuccal maxillary roots which present round cross sections more easily filled with the endodontic sealer, irrespective of the activation method. When intratubular penetration is evaluated, the studies found values that ranged between 1% up to 59% depending on the methodology used to measure this outcome (8,27). The values will depend on the irrigant used previously to root canal filling, activation method of the irrigant and the endodontic sealer, type of endodontic sealer, and magnification used for assessment.

Even though the it was observed higher percentage values for bioceramic sealers penetration than for epoxy resin-based sealer, statistical tests did not indicate significant differences regardless of the insertion method. Similarly, when middle and apical thirds were compared, there were also no significant differences (14-16). Furthermore, it should also be noted that sealer penetration close to surfaces of the canal walls in most of the samples with low percentage values of penetration for both sealers at middle or apical third. Another major concern from this research was the employment of different methods to activate root canal sealer (Lentulo, Easy Clean and ultrasonic insert). Guimarães et al. (12) researched the effect of ultrasonic activation on the intratubular activation of 4 different epoxy resin-based sealers. The adoption of ultrasonic activation promoted a more significant sealer penetration into dentinal tubules as well as fewer gaps. These effects improved the adaption on the interface between root canal sealer and dentinal walls.

In light of the present methodology and findings, it is licit to conclude that Fluo-3 should be recommended as the fluorophore to evaluate bioceramic sealers inside dentinal tubule. Penetration of bioceramic and epoxy resin-based sealers occurred barely unimpressively and the type of instrument to carry and activate bioceramic sealer did not influence on sealer penetrability.

Resumo

Este estudo objetivou avaliar a penetração nos túbulos dentinários de um novo cimento biocerâmico utilizando microscopia confocal de varredura a laser (MCVL). Um fluoróforo específico (Fluo-3) foi misturado com o cimento. Quarenta raízes distovestibulares de molares superiores foram selecionados e o preparo do canal radicular foi realizado com instrumentos Wave One Gold #35.06. As raízes foram divididas randomicamente em quatro grupos de acordo com os procedimentos obturadores: Bioceramic/Lentulo: cimento biocerâmico (Sealer Plus BC); Bioceramic/EasyClean: três ativações do cimento com instrumento Easy Clean (3 x 20 s); Bioceramic/Irrisonic: ativação ultrassônica do cimento por 30 s e AHplus/Lentulo: cimento à base de resina epóxica (AH Plus) foi utilizado com o mesmo protocolo que o grupo Bioceramic/Lentulo. Após 72h, os espécimes foram seccionados transversalmente em 2 e 7 mm do ápice radicular e analisados com MCVL. A área de penetração nos túbulos dentinários foi mensurada com Adobe Photoshop CC2018. O teste de Kruskal Wallis e T de Wilcoxon foram realizados. Os resultados de penetração foram similares para ambos os cimentos independentemente de qual técnica foi utilizada para ativá-los no interior do canal radicular ($p>0,05$). É razoável concluir que a penetração de cimentos biocerâmicos e à base de resina epóxica ocorreram de forma pouco expressiva. O tipo de ativação do cimento biocerâmcio não afeta sua penetrabilidade nos túbulos dentinários. Fluo 3 deve ser recomendado como o fluoróforo para avaliar a penetração intratubular de cimentos biocerâmicos.

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