UNIVERSIDADE DE SÃO PAULO
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Physical-chemical properties and cytotoxicity analysis of 5 different endodontic sealers

Propriedades físico-químicas e análise da citotoxicidade de 5 diferentes cimentos endodônticos

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Orientador: Prof. Dr. Norberti Bernardineli

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FOLHA DE APROVAÇÃO

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RESUMO

Propriedades físico-químicas e análise da citotoxicidade de 5 diferentes cimentos endodônticos

O objetivo do presente estudo foi analisar a radiopacidade, tempo de presa, escoamento, pH, liberação de íons cálcio, solubilidade e citotoxicidade dos cimentos biocerâmicos Totalfill BC Sealer e Totalfill BC RRM e compará-los ao AH Plus, MTA Fillapex e MTA Angelus. Os grupos foram divididos e comparados entre si de acordo com as funções dos cimentos de obturação e retro-obturação. Comparamos o cimento obturador Totalfill BC Sealer com os cimentos AH Plus e MTA Filapex, e o cimento retrobturador Totalfill BC RRM com o cimento retrobturador MTA Angelus. Para análise da radiopacidade, os espécimes foram colocados em anéis metálicos medindo 10x1 mm, dispostos sobre um filme oclusal com uma escala de alumínio. O software Digora 1.51 foi utilizado para avaliar as imagens digitalizadas e determinar a densidade radiográfica. O tempo de presa foi realizado de acordo com as especificações da American Society for Testing and Materials C266-08 standard specifications, mas os espécimes foram feitos de acordo com a International Organization for Standardization 6876: 2001. O escoamento foi realizado de acordo com as especificações ANSI/ADA N^0 57/2000. Trinta dentes acrílicos foram preenchidos com cimentos obturadores e vinte dentes de acrílico (com cavidade retrógrada) foram preenchidos com cimentos retro-obturadores e imersos em água ultrapura para mensuração do pH e liberação de íons cálcio (espectrofotômetro de absorção atômica) no período de 1, 3, 24, 72, 168 e 360 horas. Para o teste de solubilidade, foram escaneados 50 dentes acrílicos e digitalizados duas vezes pelo Micro-CT, antes e após a imersão em água ultrapura nos períodos de 168, 360 e 720 horas. As imagens foram reconstruídas e o volume (mm^3) das amostras foi obtido usando o software CTan (CTan v1.11.10.0, SkyScan). Os efeitos celulares *in vitro* foram analisados nas concentrações de 100, 50, 10, 5, 1 mg/mL e 0 mg / mL-grupo controle negativo e registados nos períodos de 24, 48 e 72 horas através do ensaio de redução de MTT. Os resultados foram analisados estatisticamente pelos testes ANOVA, Tukey, Kruskal-Wallis e Dunn ($p < 0.05$). Todos os valores de radiopacidade estavam de acordo com a norma ISO 6876/2001, sendo o AH Plus (7.86 mm Al) o mais radiopaco seguido dos demais cimentos; Totalfill BC Sealer

(4.84 mm Al), MTA Filapex (3.41 mm Al), Totalfill BC RRM (6,8 mm Al), MTA Angelus (6,7 mm Al). Os valores obtidos para o tempo de presa inicial e final foram respectivamente, AH Plus (8 e 15 horas), Totalfill BC Sealer (11 e 24 horas), MTA Filapex (13 e 26 horas), Totalfill BC RRM (3 horas e 22 horas) e MTA Angelus (10 e 120 minutos). Na análise de escoamento os cimentos se comportaram da seguinte forma: AH Plus (33 mm), MTA Filapex (47 mm), Totalfill BC Sealer (41,5 mm), Totalfill BC RRM (33,5 mm), e MTA Angelus (17,5 mm) ($p < 0.05$). A análise do pH mostrou que o cimento AH Plus de um modo geral, foi o que apresentou os menores valores, seguido do Totalfill BC RRM, MTA Angelus, MTA Filapex e Totalfill BC Sealer. A maior liberação de Ca^{2+} do AH Plus foi no período de 1 hora (1.38 mg/L), MTA Filapex foi em 360 horas (3.81 mg/L), Totalfill BC Sealer 360 horas (6.77 mg/L), Totalfill BC RRM 360 horas (3.81 mg/L) e MTA Angelus em 1 hora (1.38 mg/L). Todos os cimentos apresentaram solubilidade menor que 3% em todos os períodos, como recomendado pela ISO 6876/2001. Entretanto, os valores de solubilidade do MTA Fillapex excedeu mais que 5% em todos os períodos. Com relação à citotoxicidade, todos os cimentos mostraram-se tóxicos na concentração de 100 mg/mL, porém o Totalfill BC Sealer e Totalfill BC RRM apresentaram melhor resultado de viabilidade celular comparado aos demais cimentos testados. Concluiu-se que os cimentos de obturação e retro-obturação cumpriram os requisitos de radiopacidade, tempo de presa, escomento, pH, liberação de íons cálcio, solubilidade e citotoxicidade. Com exceção do MTA Fillapex que não cumpriu somente o requisito de solubilidade. Dos cimentos obturadores, o que melhor se portou foi o Totalfill BC Sealer, apresentando maior pH e liberação de íons cálcio e menor citotoxicidade. Dentre os cimentos retro-obturadores, Totalfill BC RRM foi o que melhor se destacou, mantendo seu pH elevado, possuindo maior liberação de Ca^{2+} e menor citotoxicidade.

Palavras-chave: Endodontia; Microtomografia por Raio-X, cultura de célula e citotoxicidade

ABSTRACT

Physical-chemical properties and cytotoxicity analysis of 5 different endodontic sealers

The aim of this study was to analyze the radiopacity, setting time, flowability, pH, calcium ion release, solubility and cytotoxicity of bioceramic cements Totalfill BC Sealer and Totalfill BC RRM, and compare them with AH Plus, MTA Fillapex and MTA Angelus. The groups were divided and compared among them according to the filling and retro-filling cement functions. Totalfill BC Sealer was compared with AH Plus and MTA Fillapex; and Totalfill BC RRM retrofilling cement with MTA Angelus. For radiopacity analysis, specimens were placed in metal rings measuring 10x1 mm placed on occlusal film together with the aluminum scale. Digora 1.51 software was used to evaluate the digitized images and determine radiographic density. Setting time was tested in accordance with the American Society for Testing and Materials C266-08 standard specifications, but specimens were fabricated in accordance with the International Organization for Standardization 6876: 2001. Flow was tested in accordance with ANSI/ADA Nº.57/200 specifications. In total 30 acrylic teeth were filled with filling-cements and 20, with (retrograde cavity) retro-filling cements. All teeth were immersed in ultrapure water for pH and calcium ion release measurement (atomic absorption spectrophotometer) for time intervals of 1, 3, 24, 72, 168 and 360 hours. Solubility was tested by scanning and digitizing 50 acrylic teeth twice by Micro-CT, before and after immersion in ultrapure water for time intervals of 168, 360 and 720 hours. The images were reconstructed and volume (mm^3) values of samples obtained by means of CTan software (CTan v1.11.10.0, SkyScan). The *in vitro* effects on cells were analyzed at concentrations of 100, 50, 10, 5, 1 mg/mL, and 0 mg / mL-negative control group and recorded in time intervals of 24, 48 and 72 hours by MTT reduction assay. The results were statistically analyzed by the ANOVA, Tukey, Kruskal-Wallis and Dunn tests ($P<0.05$). All radiopacity values according to ISO 6876/2001, AH Plus (7.86 mm Al) being the most radiopaque followed by Totalfill BC Sealer (4.84 mm Al), MTA Fillapex (3.41 mm Al), Totalfill BC RRM (6.8 mm Al), and MTA Angelus (6.7 mm Al). The following values were the initial and final setting time (in hours), respectively: AH Plus (8 and 15); Totalfill BC Sealer (11 and 24);

MTA Fillapex (13 and 26); MTA Angelus (10 and 120 minutes) and Totalfill BC RRM (3 hours and 22 hours). In flow analysis, the cements behaved as follows: MTA Fillapex (47 mm), Totalfill BC Sealer (41.5 mm), Totalfill BC RRM (33.5 mm), AH Plus (33 mm) e MTA Angelus (17.5 mm) ($p < 0.05$). pH analysis showed in general the lowest values for AH Plus cement, followed by Totalfill BC RRM, MTA Angelus, MTA Fillapex and Totalfill BC Sealer. AH Plus showed the highest Ca^{2+} release in time interval 1 hour (1.38 mg/L); MTA Fillapex, in 360 hours (3.81 mg/L); MTA Angelus, 1 hour (1.38 mg/L); Totalfill BC Sealer, 360 hours (6.77 mg/L) and Totalfill BC RRM, 360 hours (3.81 mg/L). Almost all the sealers presented solubility lower than 3% in all periods, as recommended by ISO 6876/2001. Whereas, the MTA Fillapex solubility value was higher than 5% in all periods. Relative to cytotoxicity, all the cements were shown to be toxic at the concentration of 100 mg/mL, however, Totalfill BC Sealer and Totalfill BC RRM showed the best cell viability result compared with the other cements tested. We concluded that all root canal filling and root retro-filling complied with the requisites of radiopacity, setting time, flow, pH, calcium ion release, solubility and cytotoxicity. With the exception of the MTA Fillapex that not only fulfilled the requirement of solubility. Of the sealers, Totalfill BC Sealer was outstanding: it showed the highest pH and Ca^{2+} release, and lowest cytotoxicity. Among the retro-filling cements, Totalfill BC RRM maintained its high pH, higher Ca^{2+} release, and lower cytotoxicity.

Keywords: Endodontics; X-Ray Microtomography, cell culture and cytotoxicity

LISTA DE ABREVIATURA E SIGLAS

%	percentagem
+	mais
-	menos
<	menor
>	maior
=	igual
º	grau
#	número
2D	duas dimensões
3D	três dimensões
Al	alumínio
BC	bioceramic (biocerâmico)
Ca	cálcio
cm	centímetro
CO_2	gás carbônico
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide-Synth
FBS	fetal bovine sérum (soro fetal bovino)
Fe	ferro
g	grama (s)
h	hora
kV	quilovolt
L	litro
mA	miliampére (s)
mg	miligrama (s)
mL	mililitro (s)
mm	milímetro (s)
mm^2	milímetro quadrado

mm^3	milímetro cúbico
min	minuto
Micro-CT	microtomografia computadorizada
MTA	mineral trioxide aggregate
	(agregado de trióxido material)
MTT	dimethylthiazol diphenyltetrazolium bromide
n	número
nm	micronanometro
ºC	grau Celsius
P	significância estatística
PBS	phosphate buffer solution
	(solução salina tamponada com fosfato)
pH	potencial hidrogeniônico
ROI	region of interest
	(região de interesse)
RPM	rotações por minuto
RRM	root repair material
	(material de reparação da raiz)
s	segundos
µm	micrometro
µA	microampere

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

One of the requirements for successful endodontic treatment is complete filling of root canal systems (BUCKLEY; SPANGBERG, 1995). This is possible when the sealing materials seal the spaces of the root canal and all the entrance ports between the canal and the periodontium, preventing the entry of microorganisms or tissue fluids into the root canal (da SILVA NETO et al., 2007; SIQUEIRA et al. 1999).

Endodontic treatment consists of several stages and its final purpose is to remove the pulp tissue, residues resulting from the biomechanical preparation, the inflammatory or infectious process, and finally, to fill of the root canal system; therefore, failure at any stage can compromise the entire treatment (FRIEDMAN; MOR 2004).

In general, the failure of endodontic treatment is associated with the persistence of microorganisms harbored in the void spaces of the root canal system and the periradicular region (SIQUEIRA et al., 2001; SUEDQVIST et al., 1998). Faced with this situation, in some cases, the best indication of treatment may be to perform parendodontic surgery. In addition to performing the surgical procedure correctly, another factor determining the success of retrograde filling, if indicated, is the choice of retro-filling material to be used.

Materials used for root canal sealer and repair of root canals must meet the basic requirements of physical, chemical, biological properties, and if possible, have an antimicrobial effect. The purpose of root canal sealers is to promote a seal along the root structures and avoid infiltration of microorganisms and their toxic products into the periapical tissues, favoring their repair, and preventing perpetuation of the periapical lesions (BRANSTETTER; VON FRAUNHOFER 1982; SCHILDER, 1967). In order to achieve these objectives, it is necessary to consider not only the filling technique itself, but also the filling material, always taking into account its physical, chemical and biological properties.

According to GROSSMAN (1976), a suitable endodontic material should have good physical properties, good flow, good insertion ability, excellent sealing capacity, setting time compatible with ensuring sufficient working time, excellent radiopacity not exceeding that of dentin, insolubility in liquids from the tissues and adequate adherence to the canal walls. The values of these properties, which the sealers should present, are recommended by ISO 6876/2001 standards and by ANSI/ADA specification nº 57/2000. Chemical (pH value and the ability to release calcium) and biological (biocompatibility) properties are also important in the selection of the material. The biological compatibility of these materials is important because they are in close contact with the periapical tissues. The tissue response to these materials may influence the end result of endodontic treatment (KHASHABA; CHUTKAN; BORKE, 2009)

There are some tests to measure cytotoxicity, among them, the MTT-based colorimetric assay. MTT is 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium and exists as a yellow tetrazolium salt. MTT is reduced in metabolically active cells by a mitochondrial enzyme to form insoluble purple formazan crystals that are solubilized by the addition of a detergent. The resulting purple color can be quantified by measuring the absorbance. There is a linear relationship between the number of cells and absorbance, so that the amount of MTT formed in cell cultures is correlated with absorbance; as lower as the absorbance, higher is the cytotoxic potential. The MTT assay is a standard method for determining the cytotoxicity of dental materials in cells culture (AL ANEZI et al., 2010; ZHANG; LI; PENG, 2010).

At present, there are several types of root canal sealers available on the market. For filling root canal systems, we have sealers based on glass ionomer, zinc oxide and eugenol, resin, calcium hydroxide, silicone; and bioceramic resin-based sealer; for retro-filling, apicification, apicigenesis, direct pulp capping and perforations (ROBERTS et al., 2008). We have Portland sealer associated with a radiopacifier, known as MTA (CAMILIERI et al., 2008, DUARTE et al., 2012, TORABINEJAD et al., 1995, TORABINEJAD et al., 1997) and bioceramic resin. In particular, these bioceramic materials result from the combination of calcium silicate and calcium phosphate, widely used in the medical and dental areas (KOCH et al., 2010). They have attracted considerable attention due to their physical and biological properties such as alkaline pH, chemical stability in the biological environment, as they are biocompatible. Another advantage of these materials is the ability to form hydroxyapatite during the fixation process, establishing a bond between the filling material and dentin (CANDEIRO et al., 2012; LOUSHINE et al., 2011; ZHANG et al., 2009).

Totalfill BC Sealer and Totalfill BC RRM (FKG Dentaire SA, La Chaux-de-Fonds, Switzerland) are bioceramic sealer composed of zirconium oxide, calcium silicate, calcium phosphate monobasic, calcium hydroxide, fillers and thickening agents. According to the manufacturer, these sealers are hydrophilic and use the moisture of the dentinal tubules and periapical tissues, respectively, to initiate and complete their setting reaction (ZHANG et al., 2009). Since dentin is composed of approximately 20% water, it facilitates fixation of the material (LOUSHINE et al., 2011; ZHANG et al., 2009). In addition, they have biological characteristics, such as antibacterial activity and biocompatibility.

However, to date, there are no studies on the physico-chemical and biological properties of Totalfill BC Sealer and Totalfill BC RRM. Therefore, the objective of the present study was to analyze the radiopacity, setting time, flow, pH, calcium ion release, solubility and cytotoxicity of these sealers and to compare them with AH Plus, MTA Fillapex and MTA Angelus. The groups were divided and compared with each other according to the functions of the filling and retro-filling sealers. We compared the Totalfill BC Sealer with the AH Plus and MTA Fillapex, and the Totalfill BC RRM with the MTA Angelus.

2 Articles

2 ARTICLES

2.1 Article 1- ***Analysis of the physical-chemical properties and cytotoxicity of 3 root canal Sealers***

The article presented in this Thesis was written according to the International Endodontic Journal instructions and guidelines for article submission

ABSTRACT

Aim To evaluate the radiopacity, setting time, flow, pH, calcium ion release, solubility and in vitro cytotoxicity of Totalfill BC Sealer, AH Plus and MTA Fillapex root canal filling materials.

Methodology For the analysis of radiopacity, the 10x1 mm specimens were arranged on occlusal films with a cylinder of dentine and an aluminium stepwedge. The digitized images captured were evaluated by Digora software 1.51 to determine the radiographic density. The setting time was established in accordance with the American Society of Testing and Materials C266-08 standard specifications, but the specimens were made in accordance with the International Organization for Standardization 6876: 2001. Flow was tested in accordance with the ANSI / ADA Specification Nº. 57/2000. The release of Ca^{2+} and pH were measured at periods of 1, 3, 24, 72, 168 and 360 hours with spectrophotometer and pH meter, respectively. For the solubility test, 30 acrylic teeth were scanned twice by Micro-CT, before and after immersion in ultrapure water for time intervals of 168, 360 and 720 hours. The images were reconstructed and volume (mm^3) the samples were obtained using CTan software (CTan v1.11.10.0, SkyScan). Effects on cells were analyzed in vitro at the concentrations of 100, 50, 10, 5, 1 mg / mL and 0 mg / mL- negative control group and recorded in time intervals of 24, 48 and 72 hour by MTT reduction assay. The results were analyzed statistically by the ANOVA, Tukey, Kruskal-Wallis and Dunn tests ($p < 0.05$). **Results** MTA Fillapex showed lower radiopacity (3.41 mm Al), followed by Totalfill BC Sealer (4.84 mm Al) and AH Plus (7.86 mm Al). The following were the initial and final setting time values (in hours), respectively: AH Plus (8 and 15); Totalfill BC Sealer (11 and 24); MTA Fillapex (13 and 26). In the flow analysis, AH Plus, Totalfill BC Sealer, and MTA Fillapex presented flow of 33 mm, 41.5 mm and 47 mm respectively ($p < 0.05$). pH analysis showed that Totalfill BC Sealer presented higher pH and Ca^{2+} release, followed by MTA Fillapex and AH Plus ($p < 0.05$). For the solubility, Totalfill BC Sealer and AH Plus were lower than 3% in all periods, as recommended by ISO 6876/2001. Whereas, the MTA Fillapex solubility value was higher than 5% in all periods. Totalfill BC Sealer presented better cell viability compared with the other sealers tested. **Conclusions** The radiopacity, setting time, flow and solubility were fulfilled by AH Plus and Totalfill BC Sealer. In addition, Totalfill BC Sealer presented higher pH and Ca^{2+} release values, and lower cytotoxicity compared with the other sealers.

INTRODUCTION

Root canal sealers are used to fill root canal systems with the purpose of promoting sealing along the root structures and filling the irregularities of the dentin walls, reducing the possibility of residual bacteria present inside the canals invading the periapical tissues, and consequently reducing the possibility of causing periapical lesions (Branstetter & von Fraunhofer 1982). Should there be leakage of filling material into the periapical tissues, it is important for the material to be biocompatible with the tissues (Hungaro Duarte *et al*. 2009; Zhang *et al*. 2009)

According to Grossman (1976) a suitable sealant material must have a good flow, good insertion and excellent sealing capacity, setting time compatible with ensuring sufficient working time, excellent radiopacity not exceeding that of dentin, insolubility of liquids to allow adequate bond to the root canal walls, and biocompatibility. At present, various types of root canal sealers are available, including glass ionomer-based sealer, zinc oxide and eugenol, resin, calcium hydroxide, silicone, and bioceramic resin-based sealer. In particular, these bioceramic materials resulting from the combination of calcium silicate and calcium phosphate are widely used in the medical and dental fields (Koch *et al*. 2010), and have attracted considerable attention due to their physical and biological properties such as alkaline pH, chemical stability in the biological environment and biocompatibility. Another advantage of these materials is their ability to form hydroxyapatite during the fixation process, thereby establishing a connection between the filling material and dentin (Zhang *et al*. 2009; Candeiro *et al*. 2012).

Totalfill BC Sealer (FKG Dentaire SA, La Chaux-de-Fonds, Switzerland) is a bioceramic sealer composed of zirconium oxide, calcium silicate, calcium phosphate monobasic, calcium hydroxide, fillers and thickening agents. According to the manufacturer, this sealer is hydrophilic and uses the moisture of the dentinal tubules to initiate and complete its setting reaction (Zhang *et al*. 2009). Since the dentin is composed of approximately 20% water, it promotes the fixation of the material (Zhang *et al*. 2009; Loushine *et al*. 2011). Furthermore, Totalfill BC Sealer has biological characteristics, such as antibacterial activity and biocompatibility.

However, to date, there are no studies on the physical-chemical and biological properties of Totalfill BC Sealer. Therefore, the objective of the present study was to analyze the radiopacity, setting time, flow, pH, calcium ion release, solubility and cytotoxicity of Totalfill BC Sealer and to compare it with AH Plus and MTA Fillapex**.**

MATERIALS AND METHODS

Preparation and handling of the sealers was carried out in accordance with the guidelines of each manufacturer

Radiopacity

Nine samples of each material were made by using a metal ring with an internal diameter of 10 mm, and 1 mm thick. The molds were placed on a glass plate, then filled with freshly spatulated sealers, covered and pressed with another glass plate, then held at 37° C. After the sealer had set completely, the specimen thicknesses were confirmed with a Digital caliper (Mitutoyo Corp., Tokyo, Japan). The root canal sealer specimens, a 1 mm thick dentin block (used as a control) and an aluminum scale (graded from 2 to 16 mm Al) were radiographed on occlusal films (F-velocity; Kodak Comp, Rochester, NY, USA), within the following parameters: 60 kV and 10 mA for 0.3 seconds (Gnatus XR 6010; Gnatus, Ribeirão Preto, SP, Brazil), with a focus-film distance of 30 cm. After processing, the radiographs were scanned with a digital scanner and imported into the Digora 1.51 software (Orion Corporation Soredex, Helsinki, Finland).

Setting time

The test was conducted under controlled temperature and humidity conditions of 37° C ± 1° C and 95% ± 5%, respectively. The settling time was calculated according to the American Society for Testing and Materials Specifications (ASTM-C266-08), but the samples were made in accordance with the ISO 6876: 2001 specification. Nine metal rings (n = 3) with 10 mm diameter and 2 mm thickness were filled with each previously spatulated sealer (ASTM 2008). After 180 seconds, a Gilmore 113.4g needle was used in each specimen at 60 second intervals. Once it was not possible to check any mark on the sample surface, the initial settling time was established. A Gilmore 453.6g needle was used in the same manner to determine the final set time. Three specimens were evaluated for each group.

Flowability

A volume of 0.5 mL of sealer was placed on a glass plate in accordance with ANSI / ADA Specification Nº. 57/2000. Three minutes after starting the spatulation, another plate with a mass of 20 g ± 2 g and a load of 100 g plus was applied centrally on top of the plate. Ten minutes after the start of mixing, the load was removed, and the average of the major and minor diame- ters of the compressed sealer was measured using a digital caliper (Mitutoyo MTI Corporation, Tokyo, Japan). Three mea- surements were performed for each sealer.

pH level and calcium release

Thirty acrylic teeth (10 per group) were used and filled with the sealers. The specimens were individually placed in glass vials containing 10 mL of ultrapure water (Milli-Q water; Purelab, Analítica, Brazil) and stored at 37° C where they remained throughout the experimental period. To avoid any interference in the results, all glass vials were pre-treated with nitric acid. The levels of pH and calcium release readouts were performed at time intervals of 1, 3, 24, 72, 168 and 360 hours. After each experimental period, the teeth were transferred to a new flask with the same volume of ultrapure water.

The pH was measured with a pH meter (model 371: Micronal, São Paulo, SP, Brazil), previously calibrated using controls with pH values 4, 7 and 14. After the specimens were removed, the containers were placed in an agitator (Model 251; Farmem, São Paulo, SP, Brazil) for 5 seconds before each measurement. The room temperature during readouts was 25° C. Deionized water was used as a control for the pH level measurements in all time intervals analyzed.

Calcium ion release was evaluated by means of an atomic absorption spectrophotometer (AA6800; Schimadzu, Tokyo, Japan) equipped with a calcium ion-specific hollow cathode lamp. All samples were analyzed at the same time as pH level analyses were performed. To avoid possible alkali metal interference, a lanthanum solution was prepared by diluting 9.8 g of lanthanum nitrate in 250 mL of acid solution. A stock solution of calcium was prepared by diluting 2.4972 g of calcium carbonate in 50 mL of ultrapure water. To this solution, 10 mL of concentrated hydrochloric acid was added, diluted with 1000 mL of ultrapure water, so that 1 mL of this solution corresponded to 1 mg of calcium. From this solution, calcium solutions were prepared in the following concentrations: 20 mg / L^{-1}; 10 mg / L^{-1}; 5 mg / L^{-1}; 2.5 mg / L^{-1}; 1.25 mg / L^{-1}; and 2 mL of the lanthanum nitrate solution was added to 6 mL of calcium or test solution. To prepare the blank, 6 mL of ultrapure water was added to the same amount of the lanthanum nitrate solution. The levels of calcium, blank and test solutions were read in an atomic absorption spectrophotometer. Calcium ion release readouts were compared with a standard curve obtained from standard solution readouts.

Determination of solubility

Solubility was determined by volumetric micro-CT images (Cavenago *et al*. 2014). Each specimen was scanned four times. Thirty acrylic central incisors (n = 10) were used, which were previously prepared with Flex-Gold rotary instruments up to instrument # 30.09 (Easy, Belo Horizonte, MG, Brazil). Samples were filled up to the single cone working length. Subsequently the samples were scanned with a desktop X-ray microfocus CT scanner (SkyScan 1174v2; SkyScan, Kontich, Belgium). The images were digitized by using a 50 kV X-ray tube voltage, 800 A anode currrent. Three samples were scanned at one time. The following image capture parameters were used: voxel size of 14.1 m with 1.1° rotation step using a 360° rotation. Each scan generated 327.tif images with 1024 X 1304 pixels. Digital data were compiled by reconstruction software (NRec-onv1.6.4.8. SkyScan), and the CTAN software (CTAN v1.11.10.0, SkyScan) was used for volume measurements. The images were analyzed individually using the CTAN software. The area of interest (ROI) was delimited for each sample and these images were binarized. Quantitative analysis of material volume (mm^3) was obtained. After this initial scanning process, each sample was individually immersed in a glass vial containing 15 mL of ultrapure water, and then stored at 37° C for time intervals of 168, 360 and 720 hours. After each experimental period the samples were scanned and analyzed again using exactly the same parameters as those set up for the first examination. Solubility of the samples from each group was determined by calculating the volume that was lost during immersion in ultrapure water, and the results found were converted to percentages to show the proportion of the material that was dissolved.

Specimen preparation for cytotoxicity evaluation

The root canal sealer samples were prepared in accordance with the manufacturer's recommendations. Three sealers were used to prepare the specimens, Totalfill BC Sealer (FKG Dentaire SA, La Chaux-de-Fonds, Switzerland), MTA Fillapex (Angelus Indústria de Odontológicos S / A, Londrina, Brazil) and AH Plus (Dentsply De Trey Gmbh, Konstanz, Germany). The initial concentration used for the experiment was 100 mg of cement in 1mL DMEM (Dulbecco's Modified Eagle's Medium - Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Fetal Bovine Serum - Gibco). The sealers conditioned medium was kept overnight at 37° C, under sterile conditions, with 5% CO_2 for 24 hours. After these steps, serial dilutions of the media conditioned with the sealers were performed, in accordance with the ISO 10993-5 (ISO 2009) recommendations (Slompo *et al*. 2015). The concentrations used were 100 mg / mL, 50 mg / mL, 10 mg / mL, 5 mg / mL , 1 mg / mL and 0 mg / mL- negative control group.

Cell culture

In vitro cytotoxicity of the sealers was evaluated by using NIH3T3 murine fibroblasts from the ATCC-American Type Culture Collection (Donated by the cell bank of the research laboratory, Department of Biological Sciences, Bauru School of Dentistry - University of São Paulo). These fibroblasts were cultured in DMEM culture medium 10% FBS, and incubated at 37 ° C containing 5% CO_2. After reaching subconfluence, the cells were subcultured using the trypsin enzyme (0.25% porcine trypsin (1:250) in HBSS, with 0.1% EDTA- Sigma-Aldrich), and the cell count was performed by using trypan blue dye. Subsequently, the cells were used for cytotoxicity assays (Volpato *et al*. 2011).

Cytotoxicity assay

For viability assays $2x10^3$ cells / well were plated in 96-well plates. After incubation for 24 hours, the wells were refreshed with the culture media conditioned with sealers, in addition to the negative control (DMEM 10% SFB). The MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) reduction assays were performed according to Mosmann T. (1983). In each experimental time (24, 48 and 72 hours) the wells were washed with 1X PBS (Phosphate buffered saline), then the cells were incubated in a 1 mg MTT solution to 1 mL DMEM without FBS. After these procedures, the plates were left at 37^o C for 4 hours; then the solution was removed, the pigment was extracted with DMSO (Dimethylsulfoxide-Synth, Labsynth, São Paulo) and left at room temperature for 30 minutes. The absorbance was measured in spectrophotometer at 562 nm (Synergy ™ Monochrome-Based Multi-Mode Microplate Reader, BioTek Instruments Inc, Winooski, Vermont, USA) (Silva *et al*. 2017). All assays were repeated twice.

Statiscal analysis

The values of radiopacity, setting time, flowabillity and cytotoxicity were compared by means of the ANOVA and Tukey tests. For the pH, calcium ion release and solubility the Kruskal-Wallis test was used; and the post hoc analysis was performed using the Dunn test, for the multiple comparisons ($P < 0.05$). Prism 5.0 Software (GraphPad Software Inc, La Jolla, CA, USA) was used as an analytical tool ($P < 0.05$).

RESULTS

Radiopacity, Setting time, Flowability, pH, calcium release and solubility tests

The radiopacity values of all the sealers were higher than those recommended by ISO 6876/2001. The AH Plus presented the highest radiopacity, followed by Totalfill BC Sealer and MTA Fillapex, with a statistically significant difference ($P < 0.05$) (Table 1).

The settling time of the sealers are shown in Table 2, which represents the initial and final setting time between the groups. AH Plus differed from other sealers and had the shortest working time.

The flowability results are shown in Table 1. The sealers tested had a flow rate higher than 30 mm, which was in accordance with ISO 6876/2001 recommendations. The MTA Fillapex was the one with the highest flow, followed by Totalfill BC Sealer and AH Plus. There was no statistically significant difference between the groups ($P > 0.05$).

pH analysis showed in general the lowest values for AH Plus, followed by MTA Fillapex and Totalfill BC Sealer (Table 3)

AH Plus showed the highest Ca^{2+} release in time interval 1 hour (1.38 mg/L); MTA Fillapex, in 360 hours (3.81 mg/L) and Totalfill BC Sealer, 360 hours (6.77 mg/L) (Table 3).

The solubility values of Totalfill BC Sealer and AH Plus were lower than 3% in all periods, as recommended by ISO 6876/2001. Whereas, the MTA Fillapex solubility value was higher than 5% in all periods (Table 4).

Cytotoxicity assay

All sealers used in this study were cytotoxic at the concentration of 100 mg / mL in all experimental periods. Totalfill BC Sealer at the concentrations of 1, 5, 10 and 50 mg / mL presented lower cytotoxicity in all periods compared with the other sealers tested and with the control group ($P < 0.05$) (Fig 1).

DISCUSSION

New root canal sealers are continually being introduced in the market, making it necessary to understand their biological physico-chemical properties and compatibility, the latter being one of the most important. The endodontic end result depends on the tissue response to these materials (Camilleri *et al*. 2011). Laboratory studies contribute to a better understanding of the clinical behavior and manipulation of endodontic root canal sealer. Bioceramic materials were introduced into daily dental practice, mainly because of their high level of biocompatibility (Damas *et al*. 2011) and strong antibacterial activity (Zhang *et al*. 2009). The present study used Totalfill BC Sealer bioceramic root canal seler and compared it with AH Plus and MTA Fillapex. AH Plus was selected because of its widespread use, and MTA Fillapex because it also contained bioceramic resin in its composition.

Physical properties were evaluated according to the ISO 6876/2001 specification. In addition radiopacity was evaluated; this fundamental physical property allows visualization of the endodontic filling material by radiographic examination to verify the quality of the filling (Candeiro *et al*. 2012). The results obtained in this study showed that the radiopacity of AH Plus was 7.86 mm Al – a higher values than that found in another study (Duarte *et al*. 2010); the value for Totalfill BC Sealer was 4.84 mm Al, and for MTA Fillapex, it was 3.41 mm Al. All the sealers complied with the ISO 6876/2001 specification that established the minimum radiopacity value of 3.00 mm Al. The radiopacifier in MTA Fillapex is bismuth oxide, whereas Totalfill BC Sealer and AH Plus have the same radiopacifier, zirconia, and in spite of this, Totalfill BC Sealer had a significantly lower radiopacity than AH Plus. This difference in radiopacity between the sealers can be explained by the proportion and the type of radiopacifier present in the sealer (Hungaro Duarte *et al*. 2009; Silva *et al*. 2013). Possibly, the reason why AH Plus showed a higher radiopacity value was because it contains two radiopacifiers, calcium tungstate and zirconia (Candeiro *et al*. 2012).

The setting time was determined by following the ASTM-C266-08 recommendations. The AH Plus presented initial and final setting times of 8 and 15 hours, respectively, being in agreement with the findings of previous studies (Zhang *et al*. 2009; Zhang *et al*. 2010, Camilleri *et al*. 2011; Loushine *et al.* 2011, Massi *et al*. 2011, Zoufan *et al*. 2011). For MTA Fillapex the setting time were 13 and 26 hours, and for Totalfill BC Sealer, 11 and 24 hours, in agreement with previous study (Zhou *et al.* 2013). AH Plus is a paste-to-paste material that has an amine polymerization reaction contained in the epoxy resin (Lin-Gibson *et al*. 2006). Totalfill BC Sealer is a premix of inorganic components and radiopacifiers that are premixed with a water-free liquid carrier; water is required for it to reach its final setting time (Loushine *et al*. 2011). On the other hand, MTA Fillapex is a paste-to-paste material, and when the two pastes come into contact, this promotes two chemical reactions that are responsible for the setting of the material (Abdullah *et al.* 2002; Gomes-Filho *et al*. 2012). The differences in setting time between s sealers may be associated with resin proportions, since a higher proportion of resin in the root canal sealer requires a long working time (Scarparo *et al*. 2010).

Regarding the flow, all sealers presented acceptable values in accordance with the ISO 6876/2001 recommendations, in which the minimum flow required for sealers is 20 mm, however, there was no statistical difference between the sealers. Flow is an extremely important physical property, as it promotes filling of the isthmuses and accessory canals (Candeiro *et al*. 2012). The present study showed higher flow values for MTA Fillapex (47 mm), followed by Totalfill BC Sealer (41.5 mm) and AH Plus (33 mm). Due to variations in the physico-chemical properties, and depending on the portion of the AH Plus tube used, this results in a different flow (Baldi *et al*. 2012). The flowability of the MTA Fillapex may be explained by the size of the sealer particles, as smaller as the particle, greater is the flow. (Silva *et al*. 2013). For Totalfill BC Sealer - a calcium phosphate-based silicate sealer - this could be attributed to the intrinsic properties of the materials.

An alkaline pH promotes the elimination of bacteria, such as *Enterococcus faecalis* capable of surviving even after chemical and mechanical disinfection (McHugh *et al*. 2004; Stuart *et al*. 2006). In the present study, the AH Plus presented lower pH values than the other sealers. Perhaps, this difference reflected the fact that Totalfill BC Sealer and MTA Fillapex contained pure bioceramic resin and a combination of bioceramic resin, respectively. The slightly cytotoxic effects of Totalfill BC Sealer may occur due to its high surface pH which causes denaturation of adjacent cells reported that when sealer pH decreased, cell lesions ceased (De Deus *et al*. 2005). It has been reported that the mechanism of stimulation of periapical repair by deposition of mineralized tissue depended on pH and Ca^{2+} release capacity (Okabe *et al*. 2006). Moisture facilitates calcium silicate hydration reactions (Yang *et* *al*. 2002; Zhang *et al*. 2009). The highest amount of Ca^{2+} release for Totalfill BC Sealer occurred in the time interval of 360 hours (6.77 mg / L) showing higher values than those of the other sealers. This factor may be related to the final setting time of this material (Loushine *et al*. 2011; Candeiro *et al*. 2012). Further studies should be conducted to confirm this observation. Another possible explanation for the large quantity Ca^{2+} released by bioceramic sealer may be the hydration of the calcium silicate. The calcium and hydroxyl ion release from sealers that contain calcium silicate, results in the formation of an apatite layer that chemically binds to calcium silicate sealer and the dentin wall (Sarkar *et al*. 2005). Therefore, calcium silicate in the composition of Totalfill BC Sealer has the potential to chemically adhere to the dentin, thereby reducing voids (Zhang *et al*. 2009).

The solubilization of sealer can cause the release of chemical compounds that may irritate the periapical tissues (McMichen *et al*. 2003) and produce spaces in the filling mass inside the root canals, resulting in bacterial a infiltration and proliferation (Vitti *et al*. 2013). The solubility test usually follows a technical standard. Currently, the ANSI / ADA Nº. 57/2000 specification is utilized (Hungaro Duarte *et al*. 2012). According to this method, the solubility values of Totalfill BC Sealer and AH Plus were not higher than 3%, according to the ISO 6876/2001 specification that recommends that dimensional change should not exceed 3%. Whereas, the MTA Fillapex solubility value was higher than 5%. For AH Plus and MTA Fillapex, the values were higher than those found in some studies (Duarte *et al*. 2010). This is one of the pioneering studies using Totalfill BC Sealer, so it is difficult to compare the results obtained with previous studies. Perhaps this difference between the level of solubility is due to the methodology used, since most of the studies used ANSI / ADA No. 57 or ISO 6876, which are based on the difference between the weights, before and after placing the sealer in ultrapure water (Hungaro Duarte *et al*. 2012, Torabinejad *et al*. 1995a, Torabinejad *et al*. 1995b; Vivan *et al.* 2010). We can make a correlation with the cell viability test, since the more soluble the sealer, the more of its components can come into contact with the cells.

Some uncontrolled inflammatory reactions caused by periapical extrusion of filling material have been a frequent complication during endodontic treatment (Yamaguchi *et al*. 2007). Thus, as physical-chemical properties, biological characteristics are essential for clinical success. The cytotoxicity of sealers can cause cell degeneration and delayed healing due to direct contact of the sealers with the periapical tissues (Sousa *et al.* 2006), making a satisfactory periapical repair difficult. Bioceramic materials are considered promising for this repair, due to their excellent physico-chemical and biocompatibility properties (Sagsen *et al.* 2011; Zoufan *et al*. 2011; Candeiro *et al*. 2012).

At present, there are few reports regarding the biocompatibility of root canal sealers compared cytotoxicity between Totalfill BC Sealer, AH Plus and MTA Fillapex, the cell viability analysis was performed on mouse fibroblasts (NIH3T3). In this method the sealers were in indirect contact with the cells, and this was the initial step in the evaluation of the response of cells versus endodontic cements. This methodology was closer to clinical, since sealer will not preferentially come into contact with the cells. Tissue fluids or blood will come into contact with the sealer, and carry on components to nearby cells. An important point was that the data obtained in this study revealed significant differences in cytotoxicity among the three different sealers used.

The results of this study revealed minor or nonexistent cytotoxic effects for Totalfill BC Sealer. This sealer exhibited good biocompatibility with NIH3T3 cells, as well as a lower cytotoxic potential compared with AH Plus. One possible explanation for this could be that Totalfill BC Sealer is sealer composed of fine hydrophilic particles, calcium-free aluminum silicate, calcium phosphate, zirconium oxide and calcium hydroxide, some of which are similar to dentin. The humidity should facilitate the hydration reactions of the calcium silicates to produce calcium silicate hydrogel and calcium hydroxide (Zhang *et al*. 2009), calcium hydroxide partially reacts with the phosphate to form hydroxyapatite and water (Yang *et al*. 2002). The water is used to restart the cycle, reacting with the calcium silicate to produce calcium silicate hydrogel and calcium hydroxide. This study showed that AH Plus and MTA Fillapex showed higher cytotoxicity at high concentrations (100 and 50 mg / mL) than Totalfill BC Sealer in all time intervals. This may have been caused mainly by small amounts of formaldehyde present in the resin or by release of the amine and epoxy resin components (Merdad *et al*. 2007).

CONCLUSION

In this study, the root canal sealers tested met the requirements of radiopacity, setting time and flow. For the solubility almost all sealers were in accordance ISO 6876/2001. In addition, the Totalfill BC Sealer presented higher pH; Higher Ca^{2+} release and lower cytotoxicity compared with AH Plus and MTA Fillapex.

CONFLICT OF INTEREST STATEMENT

The authors have declared no conflicts of interest.

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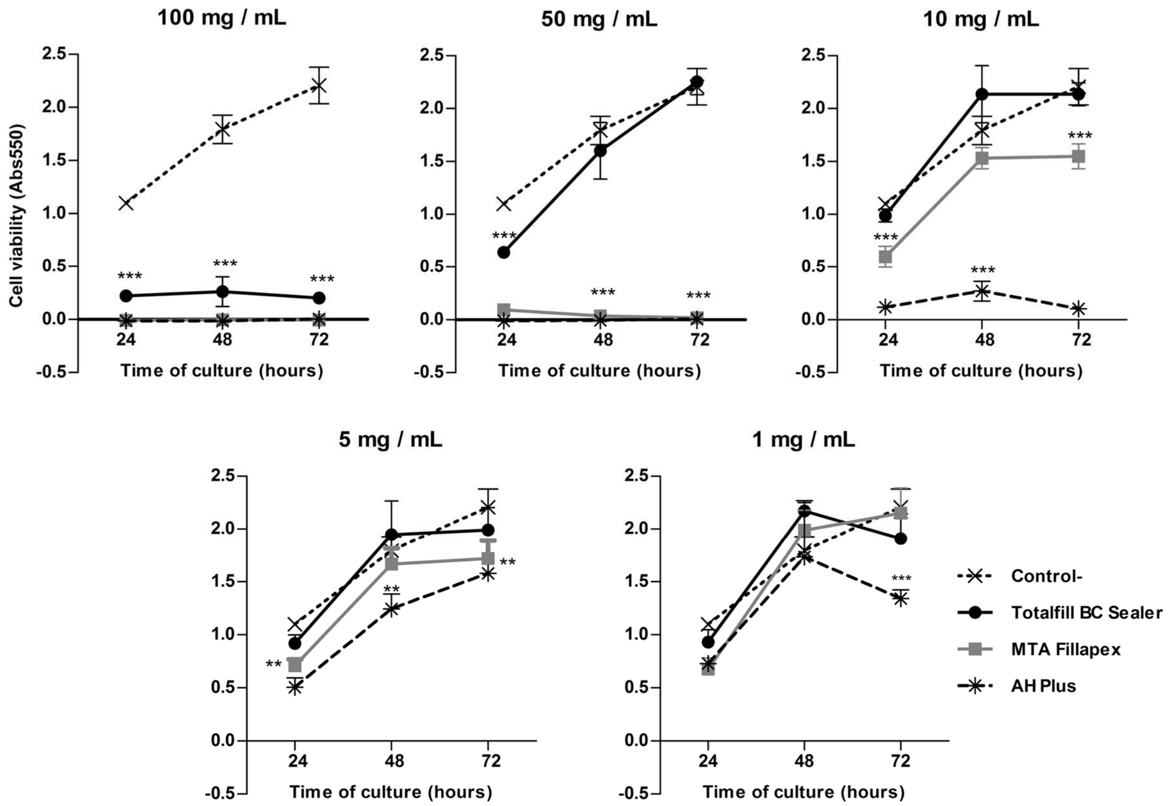

Figure 1. Cytotoxicity of root canal sealer in NIH3T3 fibroblast cells. The results demonstrated the mean and standard deviation at different cement concentrations in different time intervals. Data are compared with negative control group: **p < 0.01, ***p < 0.001

Table 1 Median, minimum and maximum values of the radiopacity (mm Al) and Flowability (mm) of the tested groups

Sealer	Radiopacity	Flowability
AH Plus	7.86 (7.08-7.96)[a]	33.0 (31.0-34.5)[a]
MTA Fillapex	3.41 (3.02-3.92)[b]	47.0 (37.0-55.5)[a]
Totalfill BC Sealer	4.84 (4.28-6.32)[b]	41.5 (41.0-43.5)[a]

Different letters in each column indicate statistical differences (P < 0.05).

Table 2 Median, minimum and maximum values of the setting time (hours) of the tested groups

Sealer	Initial	Final
AH Plus	8.0 (7.0-10.0)^{a}	15.0 (14.0-18.0)^{a}
MTA Fillapex	13.0 (12.0-14.0)^{b}	26.0 (24.0-28.0)^{b}
Totalfill BC Sealer	11.0 (10.0-13.0)^{b}	24.0 (22.0-26.0)^{c}

Different letters in each column indicate statistical differences ($P < 0.05$).

Table 3 Median, minimum and maximum values of the pH and calcium release values (mg L^{-1}) found in the different periods

Sealer	1h	3h	24h	72h	168h	360h
pH						
AH Plus	6.46 (6.00-6.73)^{a}	7.39 (6.91-7.63)^{a}	7.34 (7.06-7.58)^{a}	7.46 (7.09-7.70)^{a}	6.70 (6.50-6.90)^{a}	6.81 (6.54-7.00)^{a}
MTA Fillapex	6.93 (6.64-7.07)^{b}	7.47 (7.07-7.55)^{a}	7.77 (7.09-8.15)^{b}	7.36 (7.33-7.44)^{ab}	7.37 (6.80-8.16)^{a}	7.05 (6.84-7.15)^{a}
Totalfill BC Sealer	7.17 (7.04-7.43)^{c}	7.39 (7.32-7.51)^{a}	7.59 (7.53-7.70)^{b}	7.07 (6.24-7.42)^{b}	8.60 (8.31-9.01)^{b}	7.58 (7.14-7.88)^{b}
Calcium release						
AH Plus	1.38 (0.46-3.53)^{a}	0.00 (0.00-0.88)^{a}	0.54 (0.11-3.04)^{a}	0.12 (0.10-4.72)^{a}	0.00 (0,00-1.07)^{a}	0.05 (0.0-3.60)^{a}
MTA Fillapex	1.61 (1.22-3.31)^{a}	0.85 (0.56-3.24)^{b}	3.71 (2.96-10.33)^{b}	3.59 (2.10-5.03)^{ab}	3.77 (2.13-4.84)^{b}	3.81 (2.57-4.73)^{a}
Totalfill BC Sealer	3.29 (2.02-6.00)^{b}	0.83 (0.56-2.64)^{b}	3.74 (2.87-6.52)^{b}	5.23 (4.07-6.66)^{b}	6.10 (5.53-8.61)^{c}	6.77 (5.53-8.78)^{b}

Different letters in each column indicate statistical differences ($P < 0.05$).

Table 4 Median, minimum and maximum values of the solubility (%) of the tested groups

Sealer	168h	360h	720h
AH Plus	0.59 (-4.31-8.03)[a]	0.51 (-12.66-2.97)[a]	2.05 (-4.00-25.64)[a]
MTA Fillapex	5.81 (0.29-7.75)[b]	5.43 (-3.26 -10.30)[ab]	6.83 (-0.02-10.68)[a]
Totalfill BC Sealer	1.16 (-4.10-4.40)[a]	1.70 (-2.40-7.25)[a]	2.11 (0.00-13.11)[a]

Different letters in each column indicate statistical differences ($P < 0.05$).

2.2 Article 2- *Evaluation of radiopacity, setting time, flow, pH, calcium ion release, solubility and cytotoxicity of the Totalfill BC Root Repair Material*

The article presented in this Thesis was written according to the International Endodontic Journal instructions and guidelines for article submission

ABSTRACT

Aim The aim of the present study was to evaluate the radiopacity, setting time, flow, pH, calcium ion release, solubility and cytotoxicity of the Totalfill BC RRM in comparison with MTA Angelus.

Methodology For radiopacity analysis, specimens were placed in metal rings measuring 10x1 mm placed on occlusal film together with the aluminum scale. Digora 1.51 software was used to evaluate the digitized images and determine radiographic density. Setting time was tested in accordance with the American Society for Testing and Materials C-266-08 standard specifications, but specimens were fabricated in accordance with the International Organization for Standardization 6876: 2001. Flow was tested in accordance with ANSI/ADA Nº 57/200 specifications. In total 20 acrylic teeth were filled with retro filling sealers (retrograde cavity) (n=10 per group). All teeth were immersed in ultrapure water for pH and calcium ion release measurement (atomic absorption spectrophotometer) for time intervals of 1, 3, 24, 72, 168 and 360 hours. Solubility was tested by scanning and digitizing 20 acrylic teeth twice by Micro-CT, before and after immersion in ultrapure water for time intervals of 168, 360 and 720 hours. The images were reconstructed and volume (mm^3) values of samples obtained by means of CTan software (CTan v1.11.10.0, SkyScan). The *in vitro* effects on cells were analyzed at concentrations of 100, 50, 10, 5, 1 mg/mL and 0 mg/mL- negative control group and recorded in time intervals of 24, 48 and 72 hours by MTT reduction assay. The results were statistically analyzed by the ANOVA, Tukey, Kruskal-Wallis and Dunn tests ($P<0.05$).

Results All radiopacity values met the ISO 6876/2001 standard: Both materials present similar radiopacity values, Totalfill BC RRM (6.8 mm Al) and MTA Angelus (6.7 mm Al). The following were the initial and final setting time values (in minutes), respectively: MTA Angelus (10 and 120 minutes) and Totalfill BC RRM (3 hours and 22 hours). In flow analysis, the sealers behaved as follows: Totalfill BC RRM (33.5 mm) and MTA Angelus (17.5 mm) ($P > 0.05$). pH analysis showed the lowest values for Totalfill BC RRM in the first 3 hours (1h (6,56 mg L^{-1}) and 3h (6,41 mg L^{-1}) and in the other periods presented high pH (24h (7,57 mg L^{-1}), 72h (7,38 mg L^{-1}), 168h (7,17 mg L^{-1}) and 360h (7,75 mg L^{-1}), while the MTA Angelus showed the highest values in the first 3 hours (1h (7,63 mg L^{-1}) and 3h (7,34 mg L^{-1}) and in the other periods presented low pH (24h (7,26 mg L^{-1}) 72 and 168 h (7,14 mg L^{-1}) and 360h (7,11 mg L^{-1}). MTA Angelus showed the highest Ca^{2+} release in time interval 1 hour (1.38 mg/L) and Totalfill BC RRM in time interval 360 hours (3.81 mg/L). The solubility values of Totalfill BC RRM were lower than 3% in all periods, as recommended by ISO 6876/2001. Whereas, the MTA Fillapex solubility value was higher than 5% in the period 720 hours. Relative to cytotoxicity, all the sealers were shown to be toxic at the concentration of 100 mg/mL, however, Totalfill BC RRM showed the best cell viability result in different concentrations (50, 10, 5, 1 mg / mL) compared with the MTA Angelus. **Conclusion** Both retro-filling sealers complied with the requisites of radiopacity, setting time, flow, pH, calcium ion release and cytotoxicity. MTA was the only one that was not in accordance with ISO 6876/2001 in respect to solubility. Totalfill BC RRM maintained its high pH, higher Ca^{2+} release, and lower cytotoxicity.

INTRODUCTION

An ideal material for periapical repair must have dimensional stability, biocompatibility, radiopacity, antibacterial action, easy manipulation, adequate working time, fast setting time and ability to bond to dentin. The following are most important properties that this material requires: the capacity to induce or lead to bone deposition; promote efficient sealing; be insoluble in the periapical tissue fluids; and not be reabsorbed over time (Torabinejad & McDonald 2009). Mineral trioxide aggregate (MTA) was introduced in endodontics in 1993 by Torabinejad. It has been used in retrofilling procedures; direct pulp capping; root and furcation perforations, and in teeth with incomplete root formation (Roberts *et al*. 2008). MTA consists of either tricalcium silicate or dicalcium silicate; bismuth oxide; tetracalcium aluminoferrite; tricalcium aluminate, and calcium sulfate (Camilleri 2008, Torabinejad & Chivian 1999; Jafarnia *et al*. 2009). In presence of moisture, the calcium oxide promotes calcium hydroxide formation, leading to a high alkaline pH, which might explain the antimicrobial action of MTA. (Roberts *et al*. 2008, Holland *et al*. 1999; Hauman & Love 2003; Parirokh & Torabinejad 2010a). However, despite its good properties, MTA - introduced to the market under the commercial name MTA Angelus (MTA White, MTA, Angelus, Londrina, Brazil) - presented some undesirable characteristics such as, difficult manipulation and insertion (Bozeman *et al*. 2006; Santos *et al*. 2005). In addition, the setting time of MTA is short (Vivan *et al*. 2010) and the difficulty of maintaining the consistency of the mixture (Roberts *et al*. 2008). Another well-cited disadvantage of MTA (mixed with sterile water in a 3: 1 (w / v) ratio according to manufacturer's instructions) is the long setting time of 3-4 hours (Parirokh & Torabinejad 2010b, Roberts *et al*. 2008, Torabinejad *et al* 1995). Thus, considering the composition of MTA Angelus (80% Portland cement and 20% bismuth oxide), some studies have been conducted in an attempt to improve MTA handling characteristics (Jafarnia *et al*. 2009; Kogan *et al*. 2006).

A new root repair material has become available on the market; Totalfill BC Root Repair Material (Totalfill BC RRM; FKG Dentaire SA, La Chaux-de-Fonds, Switzerland). According to the manufacturer, it is composed of calcium silicates, calcium phosphate monobasic, zirconium oxide, tantalum oxide, proprietary fillers, and thickening agents. The manufacturer claims that the material is biocompatible and hydrophilic. According to the manufacturer, Totalfill BC RRM has a high pH, but without further details relative to pH; the material also has excellent radiopacity and the working time is 30 minutes. Totalfill BC RRM is available in a preloaded syringe in the form of a moldable putty. The preloaded syringe also has intracanal tips that can be bent to facilitate placement in clinical situations. The aim of the present study was to evaluate the radiopacity, setting time, flow, pH, calcium ion release, solubility and cytotoxicity of the Totalfill BC RRM in comparison with MTA Angelus (MTA Branco, MTA, Angelus, Londrina, Brazil).

MATERIALS AND METHODS

Preparation and handling of the sealers was carried out in accordance with the guidelines of each manufacturer.

Radiopacity

Six samples of each material were made by using a metal ring with an internal diameter of 10 mm, and 1 mm thick. The molds were placed on a glass plate, and then filled with freshly spatulated sealers, covered and pressed with another glass plate, then held at 37° C. After the sealers had set completely, the specimen thicknesses were confirmed with a Digital caliper (Mitutoyo Corp., Tokyo, Japan). The root repair material specimens, a 1 mm thick dentin block (used as a control), and an aluminum scale (graded from 2 to 16 mm Al) were radiographed on occlusal films (F-velocity; Kodak Comp, Rochester, NY, USA), within the following parameters: 60 kV and 10 mA for 0.3 seconds (Gnatus XR 6010; Gnatus, Ribeirão Preto, SP, Brazil), with a focus-film distance of 30 cm. After processing, the radiographs were scanned with a digital scanner and analyzed using the Digora 1.51 software (Orion Corporation Soredex, Helsinki, Finland).

Setting time

The test was conducted under controlled temperature and humidity conditions of 37° C ± 1° C and 95% ± 5%, respectively. The setting time was calculated according to the American Society for Testing and Materials Specifications (ASTM-C266-08), but the samples were made in accordance with the ISO 6876: 2001 specification. Six metal rings (n = 3) with 10 mm diameter and 2 mm thickness were filled with each previously spatulated sealer (ASTM 2008). After 180 seconds, a Gilmore 113.4g needle was used in each specimen at 60 second intervals. Once it was not possible to check any mark on the sample surface, the initial settling time was established. A Gilmore 453.6g needle was used in the same manner to determine the final setting time.

Flowability

A volume of 0.5 mL of sealers was placed on a glass plate in accordance with ANSI / ADA Specification Nº 57/2000. Three minutes after starting the spatulation, another plate with a mass of 20 g ± 2 g and a load of 100 g plus was applied centrally on top of the plate. Ten minutes after the start of mixing, the load was removed, and the average of the major and minor diame- ters of the compressed sealer was measured using a digital caliper (Mitutoyo MTI Corporation, Tokyo, Japan). Three measurements were performed for each sealer.

pH level and calcium release

Twenty acrylic teeth (10 per group) were used and retro-filling (retrograde cavity). The specimens were individually placed in glass vials containing 10 mL of ultrapure water (Milli-Q water; Purelab, Analytical, Brazil) and stored at 37 ° C where they remained throughout the experimental period. To avoid any interference in the results, all glass flasks were pre-treated with nitric acid. After 1, 3, 24, 72,168 and 360 hours, the teeth were placed in new flasks containing an equal volume of new deionized water. The pH of the water in which the teeth had been kept was measured with a pH meter (model 371; Micronal, São Paulo, SP, Brazil), previously calibrated using buffer solutions of pH 4, 7 and 14. After the removal of the specimens, the container was placed in a shaker (model 251; Farmem, São Paulo, SP, Brazil) for 5 s before measuring. The temperature of the room during the reading was 25◦C.

Calcium ion release was evaluated by means of an atomic absorption spectrophotometer (AA6800; Schimadzu, Tokyo, Japan) equipped with a calcium ion-specific hollow cathode lamp. All samples were analyzed at the same time as pH level analyzes were performed. To avoid possible alkali metal interference, a lanthanum solution was prepared by diluting 9.8 g of lanthanum nitrate in 250 mL of acid solution. A stock solution of calcium was prepared by diluting 2.4972 g of calcium carbonate in 50 mL of ultrapure water. To this solution, 10 mL of concentrated hydrochloric acid was added, diluted with 1000 mL of ultrapure water, So that 1 mL of this solution corresponds to 1 mg of calcium. From this solution, calcium solutions were prepared in the following concentrations: 20 mg L^{-1}; 10 mg L^{1}; 5 mg L^{-1}; 2.5 mg L^{-1}; 1.25 mg L^{-1}. Then, 2 mL of the lanthanum nitrate solution was added to 6 mL of calcium or test solution. To prepare the blank, 6 mL of ultrapure water was added to the same amount of the lanthanum nitrate solution. To bring the device to zero absorbance, a solution of nitric acid was used. The readings of the calcium ion release were compared with a standard curve obtained from readings of the standard solutions. This reading was performed in the same periods used for the pH level measurement.

Determination of solubility

The solubility test was performed using volumetric micro-CT measurements (Cavenago *et al*. 2014). Twenty acrylic teeth (n = 10) with a standardized root-end cavity 3-mm deep and 1.5-mm in diameter were used. The samples were filled with the freshly mixed sealer and the samples were scanned using a X-ray microfocus CT scanner (SkyScan 1174v2; SkyScan, Kontich, Belgium). The scanning was performed using 50 kV X-ray tube voltages, 800 μA anode current. Four samples were scanned at a time. The image capture parameters used were: a voxel size of 14.1 μm, with 1.1° rotation step in a 360° rotation. Each scan consisted of 327 .tif images with 1024 x 1304 pixels. Digital data were further transformed by reconstruction software (NReconv1.6.4.8, SkyScan), and the CTan software (CTan v1.11.10.0, SkyScan) was used for the volume measurements. The region of interest (ROI) was delimited for each sample and the binarization of these images was performed. Quantitative analysis of material volume (mm^3) was obtained. After this initial scanning process, each sample was individually immersed in a glass vial containing 15 mL of ultrapure water, and then stored at 37 ° C for the periods of 168, 360, 720 hours. After each experimental period the samples were scanned and analyzed again using exactly the same parameters adopted for the first examination. Solubility was determined by calculating the volume that was lost during immersion of samples from each group, and the results found were converted to percentages to show the proportion of the material that was dissolved.

Specimen preparation for cellular viability evaluation

The root canal sealer samples were prepared in accordance with the manufacturer's recommendations. Two endodontic sealers were used to prepare the specimens, MTA Angelus and Totalfill BC RRM. The initial concentration used for the experiment was 100 mg of sealer in 1mL DMEM (Dulbecco's Modified Eagle's Medium - Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Fetal Bovine Serum - Gibco). The sealer -conditioned medium was kept overnight at 37 ° C, under sterile conditions, with 5% CO_2 for 24 hours. After these steps, serial dilutions were performed of the media conditioned with the sealers, in accordance with the ISO 10993-5 (ISO 2009) recommendations (Slompo *et al*. 2015). The concentrations used were 100 mg / mL, 50 mg / mL, 10 mg / mL, 5 mg / mL, 1 mg / mL and 0 mg / mL- negative control group.

Cell culture

In vitro cytotoxicity of the sealers was evaluated by using NIH3T3 murine fibroblasts from the ATCC-American Type Culture Collection (Donated by the cell bank of the research laboratory, Department of Biological Sciences, Bauru School of Dentistry - University of São Paulo). These fibroblasts were cultured in DMEM culture medium 10% FBS, and incubated at 37 ° C in an environment containing 5% CO^2. After reaching subconfluence, the cells were subcultured using the trypsin enzyme (0.25% porcine trypsin (1: 250) in HBSS, with 0.1% EDTA-Sigma-Aldrich), and the cell count was performed by using trypan blue dye. Subsequently, the cells were used for cellular viability assays (Volpato *et al*. 2011).

Cellular viability assay

For viability assays $2x10^3$ cells / well were plated in 96-well plates. After incubation for 24 hours, the wells were refreshed with the culture medium conditioned with sealers in addition to the negative control (DMEM 10% SFB). The MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) reduction assays were performed according to Mossmann (1983). In each experimental time (24, 48 and 72 hours) the wells were washed with 1X PBS (Phosphate Buffered Saline), then the cells were incubated in 1 mg MTT solution to 1 mL DMEM without FBS. After these procedures, the plates were left at 37º C for 4 hours; DMSO (Dimethylsulfoxide-Synth, Labsynth, São Paulo) and left at room temperature for 30 minutes. The absorbance was measured in spectrophotometer at 562 nm (Synergy ™ Monochrome-Based Multi-Mode Microplate Reader, BioTek Instruments Inc, Winooski, Vermont, USA) (Silva *et al*. 2017). All assays were repeated twice.

Statistical analysis

For the radiopacity, setting time, flow, pH, calcium ion release, and solubility values, the U-Mann Whitney and Tukey test was used; and the post hoc analysis was performed using the Dunn test, for the multiple comparisons (P <0.05). The cell viability was represented by mean ± standard deviation and analyzed by one-way ANOVA, and post hoc Tukey tests. Prism 5.0 Software (GraphPad Software Inc., La Jolla, CA, USA) was used as the analytical tool (P <0.05).

RESULTS

Radiopacity, Setting time and Flowability Tests

The radiopacity values of all sealers were higher than those recommended by ISO 6876/2001. Both materials present similar radiopacity values, Totalfill BC RRM (6.8 mm Al) and MTA Angelus (6.7 mm Al) without statistically significant difference (P> 0.05) (Table 1).

The setting time of sealers (Table 2), showed that the MTA Angelus differed from Totalfill BC RRM and had the shortest working time.

Regarding the flow test (Table 1), Totalfill BC RRM presented a flow 33,5 mm, which is in accordance with the ISO 6876/2001 recommendations, while the MTA Angelus presented lower flow (17,5 mm). There was no significant difference between the sealers (P> 0.05).

pH level and calcium release

The results of the pH level showed that, except for the 168 hour period, MTA Angelus was differed significantly from Totalfill BC RRM in all periods tested (P <0.05). Relative to calcium ion release, except for the 1-hour period, MTA Angelus presented significant differences compared with Totalfill BC RRM in the other periods tested (P <0.05) (Table 3).

Solubility

The solubility values of Totalfill BC RRM were lower than 3% in all periods, as recommended by ISO 6876/2001. Whereas, the MTA Fillapex solubility value was higher than 5% in the period 720 hours (P <0.05) (Table 4).

Cellular viability assay

Both sealers used in this study promoted reduction in cell viability, or death, at the concentration of 100 mg / mL in comparison with the negative control. All MTA concentrations, with the exception of 1mg / mL, at 72 hours promoted reduction in cell viability compatible with death of all cells. While Totalfill BC RRM presented similar results to those of the control without treatment in the majority of concentrations and periods. ($P < 0.05$) (Fig 1).

DISCUSSION

MTA has been noted to be one of the best root-end filling materials on the market (Ma *et al*. 2011). It has many ideal properties including minimal leakage, good antimicrobial activity, and capacity for cementum formation. When used as a root-end filling material in apical surgery, MTA has been shown to form a bacterial-resistant barrier (Parirokh & Torbinejad, 2010b). MTA has also been shown to have bactericidal properties (Damas *et al*. 2011). These properties seem to be related to MTA's ability to release hydroxyl ions, creating an environment with a high pH, thereby diminishing bacterial survival (Parirokh & Torbinejad 2010b). MTA also has the ability to promote cementum deposition. This material has a short working time, extended setting time, and difficult handling properties (Parirokh & Torbinejad, 2010a).

Totalfill BC RRM has been described as a bioceramic material, in which ceramic products or components are used, suitable for medical and dental applications, mainly because of its high biocompatibility (Damas *et al*. 2011, Loushine *et al*. 2011, Zoufan *et al*. 2011) and antibacterial activity (Zhang *et al*. 2009b). According to the manufacturer, this material has many of the same ideal properties as MTA, but with marked improvements in setting time and handling characteristics, and with more of putty consistency that has been reported to be easier to physically manage and to place. In the present study, the physico-chemical properties and cytotoxicity of Totalfill BC RRM, recently introduced on the market, were compared with the MTA Angelus. The results obtained in this study showed that the radiopacity of MTA Angelus was 6.7 mm Al – a higher value than that found in another study (Duarte *et al*. 2012) and Totalfill BC RRM was 6.8 mm Al, in agreement study (Alanezi *et al*. 2010). All sealers complied with the ISO 6876/2001. The little difference in radiopacity between the sealers can be explained by the different radiopacifiers, in MTA Angelus the radiopacifier is bismuth oxide (Hungaro Duarte *et al*. 2009) and in Totalfill BC RRM it is zirconium oxide. Another factor to be observed is the amount of each radiopacifying agent proportional of the material, which can result in a more or less opaque material (Zhang *et al*. 2009a). According to the manufacturer, bioceramic materials are bright white in color, rendering a highly radiopaque material. This property makes it easy to place the material during treatment and to identify it in radiographs.

The setting time was determined in accordance with the ASTM-C266-08 recommendations. MTA Angelus presented initial and final setting times of 10 and 120 minutes, respectively, being in agreement with the findings of previous studies (Duarte *et al*. 2012; Gandolfi *et al*. 2009; Torabinejad *et al*. 1995). Totalfill BC RRM demonstrated initial and final setting times of 180 and 1320 minutes, respectively, in agreement with previous studies (Damas *et al*. 2011, Alanezi et al. 2010, Hansen *et al*. 2011)

In accordance with ISO 6876/2001 recommendations, the minimum flow required for sealers is 20 mm, and there was no statistical difference. However, the values recommended by ISO are for sealers, which are more fluid (Marciano *et al*. 2011). The present study showed higher flow values for Totalfill BC RRM (33.5 mm) than for MTA Angelus (17.5 mm). This difference could perhaps have been be caused by the particle size of zirconium oxide in each root repair material. Previous research has shown high flow values when using micro and nano-particulate zirconium oxide (Viapiana *et al*. 2014).

An alkaline pH promotes elimination of bacteria capable of surviving after chemical and mechanical disinfection (McHugh *et al*. 2004, Stuart *et al*. 2006). This study showed that the MTA Angelus pH level decreased over time, remained alkaline, and was close to the values found in some studies (Duarte *et al*. 2012, Cavenago *et al*. 2014). When the calcium oxide present in MTA Angelus comes into contact with moisture, it promotes calcium hydroxide formation, leading to a highly alkaline pH, which may explain the antimicrobial action of the MTA. (Roberts *et al*. 2008, Holland *et al*. 1999, Hauman & Love, 2003, Parirokh & Torabinejad 2010a, Parirokh & Torabinejad 2010b). Alkaline pH has a destructive effect on the protein structures of some microorganisms, promoting some inactivation of enzyme cell membranes and loss of biological activity, followed by membrane damage (Holland *et al*. 1999). High pH activates and stimulates alkaline phosphatase, favoring the formation and deposition of mineralized tissue and allowing the formation of a fibrous capsule, with a lower inflammatory reaction (Duarte *et al*. 2003; Vivan *et al*. 2010). For Totalfill BC RRM, there was a gradual increase in pH level in all periods. Perhaps, this difference reflected the fact that Totalfill BC RRM contained pure bioceramic resin.

The release of Ca^{2+} and pH stimulates the periapical repair process by mineralized tissue deposition (Okabe *et al*. 2006), in which moisture facilitates these reactions. (Zhang *et al*. 2009a). The release of calcium ions from MTA Angelus was lower in comparison with that of Totalfill BC RRM, decreasing over the time intervals. The values presented by MTA Angelus were lower compared with those of other studies (De Vasconcelos *et al.* 2009, Duarte et al.2003) Perhaps this difference may be related to the methodology used. In the present study, acrylic teeth with retrograde cavity were used, while the other studies used polyethylene tubes that had a larger opening and contact surface. The highest amount of Ca^{2+} release from MTA Angelus was in the 1 hour period (1.38 mg / L); for Totalfill BC RRM this occurred in the 360 hour period (3.81 mg / L). This factor could be related to the final setting time of this material for MTA Angelus (120 minutes) and Totalfill BC RRM (22 hours) in a humid environment. Further studies should be conducted to confirm this observation. Another possible explanation for the large amount of Ca^{2+} released by bioceramic sealers may be the hydration of the calcium silicate. The calcium and hydroxyl ions released from sealer s that contain calcium silicate, result in the formation of an apatite layer that chemically binds to calcium silicate sealer and dentin walls (Sarkar *et al*. 2005). Therefore, the presence of calcium silicate in the composition of Totalfill BC RRM, may have had the potential to chemically adhere to the dentin, thereby reducing voids.

In the process of solubility, the material stimulates the mineralization process through alkalinization and the release of calcium ions (Mizuno & Banzai 2008). Since high solubilization can lead to voids, compromising the sealing obtained (Vivan *et al*. 2009, Vitti *et al*. 2013), the solubility test was performed in accordance with the ANSI / ADA Nº 57/2000 specification. In the present study, the change in volume was studied by using a methodology proposed by Cavenago *et al*. (2014), in which the volume of material lost was measured. The design of the samples simulated clinical conditions with root-end cavities in acrylic teeth. According to this method, the solubility values of Totalfill BC RRM not exceed 3% in all periods, as recommended by ISO 6876/2001. Whereas, the MTA Fillapex solubility value was higher than 5% in the period 720 hours. This solubilization of MTA Angelus is of great importance for alkalinization of the medium, because the calcium oxide present in the composition of this sealer reacts with the fluids of the periapical tissue and produces calcium hydroxide, which in turn dissociates into hydroxide and calcium. The hydroxide ions are responsible for the alkalinization of the medium, and consequently improve the conditions of alkaline phosphatase catalysis (Holland *et al*. 2001). Conversely, calcium ions react with the carbonate ions present in the periapical tissue, leading to the precipitation of calcite granules, which triggers the process of mineralized tissue deposition (De Vasconcelos *et al* 2009). The possible explanation for this result of Totalfill BC RRM could be the presence of bioceramic resin in the composition of this Material, making it more fluid. Few studies have been conducted with Totalfill BC RRM, therefore it is difficult to correlate the results found. This difference between the studies may perhaps be based on the methodology used, since the majority of the studies have used ANSI / ADA Nº 57 or ISO 6876 that are based on the difference between the weights before and after placing the sealer in ultrapure water (Torabinejad & Chivian 1999).

A retrograde root-filling material designed to be placed in permanent and close contact with periradicular tissue must be as biocompatible as possible. In the present study, the MTT assay was used, which is a standard assay to evaluate the cytotoxicity of endodontic materials (Jafarnia *et al.* 2009; Eldeniz *et al*. 2007; Kogan *et al*. 2006; Saidon *et al*. 2003; Pascon *et al*. 1990) who checked whether the material would release cytotoxic substances that inhibit cell activity and growth. The advantage of this method is that the material is evaluated in dilutions of various concentrations. A multiple serial dilution can provide the material with a cytotoxic grade (Mosmann 1983, Damas *et al*. 2011). The cell viability analysis performed on mouse fibroblasts (NIH3T3) was compared between Totalfill BC RRM and MTA Angelus. Our results showed that MTA Angelus was slightly more cytotoxic to 1 mg / ml NIH3T3 fibroblast cells compared to some studies (Alanezi *et al*. 2010, Roberts *et al*. 2008, Saidon *et al*. 2003, Camargo *et al*. 2009, Chen *et al.* 2010), considered to be a biocompatible and non-toxic material for NIH3T3 cells. This difference between the studies may perhaps be based on the methodology used. The biocompatibility of the white MTA has been attributed to the low concentrations of Fe_2O_3 (0.15%) when compared with gray MTA (2.5%), determining different cytotoxic effects (Aranha *et al*. 2006). Totalfill BC RRM revealed minor or nonexistent cytotoxic effects at low and medium concentrations (1 - 50 mg / mL), demonstrating good biocompatibility with NIH3T3 cells. This effect could be related to the biocompatible components of the materials and release of calcium from the hydrated materials (Shokouhinejad *et al*. 2012, Zhu *et al*. 2014a, Zhu *et al*. 2014b).

In the present study, both MTA Angelus and Totalfill BC RRM showed the highest cytotoxicity effect at high concentrations (100 and 50 mg / mL). A possible explanation would be that this was due to the higher release of toxic components from the sealer in aqueous solutions in high concentrations and the high values pH and calcium ion release, which would have affected the cell morphology and their capacity for adhesion. Thus, the toxic effect of MTA repair prior to its complete setting time may determine cellular irritation and apoptosis (Bin *et al*. 2012) or perhaps, high pH concentrations and release of calcium ions at different periods, can compromise cell viability. The cytotoxic behavior of Totalfill BC RRM at high concentrations may result from resin components such as salicylate present in its composition.

CONCLUSION

In summary, the results of the present study showed that the retro-filling sealers met the requirements of radiopacity, setting time, flow, pH and calcium ion release. For the solubility, only MTA Angelus was in disagreement with ISO 6876/2001. As regards the viability test, Totalfill BC RRM was less cytotoxic when compared to MTA Angelus.

CONFLICT OF INTEREST STATEMENT

The authors have declared no conflicts of interest.

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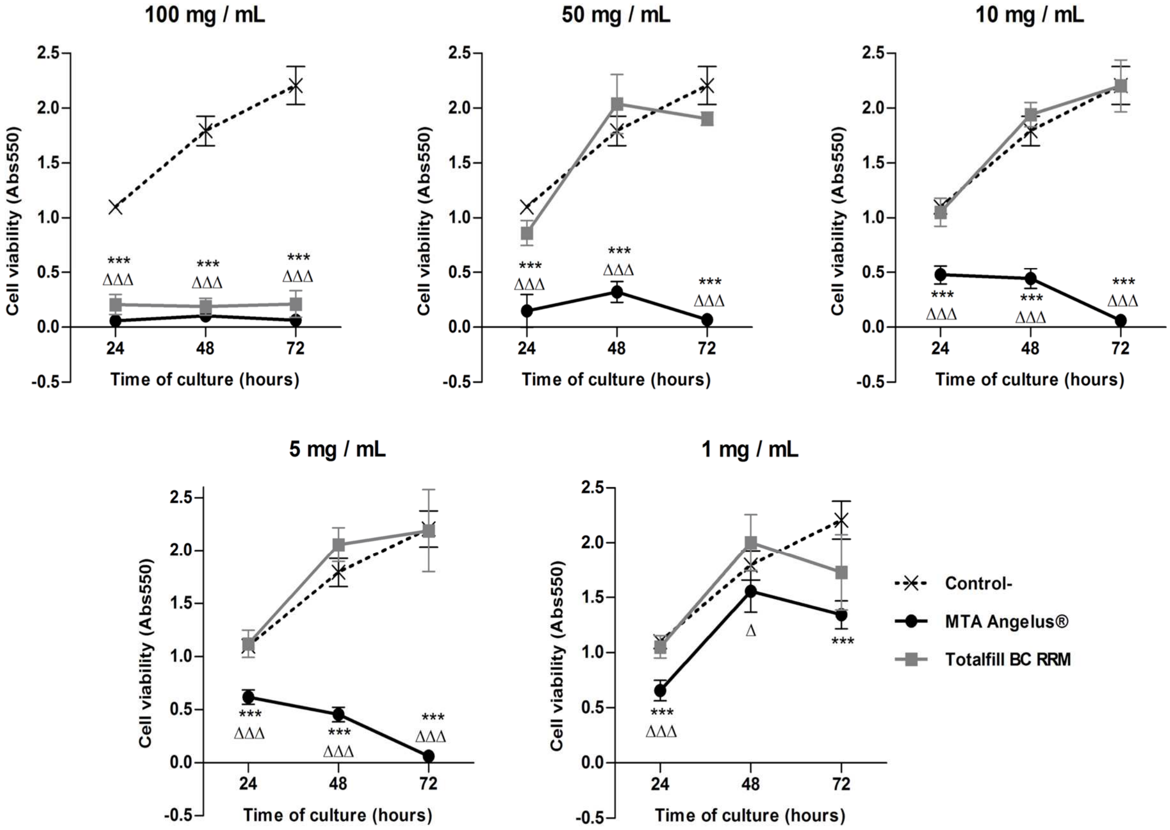

Figure 1. Cytotoxicity of root repair canal sealer in NIH3T3 fibroblast cells. The results demonstrated the mean and standard deviation at different cement concentrations in different time intervals. Data are compared with negative control group: $^{\Delta\Delta\Delta}$***$p < 0.001$

Table 1 Median, minimum and maximum values of the radiopacity (mm Al) and Flowability (mm) of the tested groups

Sealer	Radiopacity	Flowability
MTA Angelus	6.7 (6.0-6.9)[a]	17.5 (17.0-19.0)[b]
Totalfill BC RRM	6.8 (6.7-6.9)[a]	33.5 (33.5-33.5)[b]

Different letters in each column indicate statistical differences ($P < 0.05$).

Table 2 Median, minimum and maximum values of the setting time (min/ hour) of the tested groups

Sealer	**Initial**	**Final**
MTA Angelus (min)	10(8-12)[a]	120(120-180)[a]
Totalfill BC RRM (hour)	3(3-4)[b]	22(20-24)[b]

Different letters in each column indicate statistical differences ($P < 0.05$).

Table 3 Median, minimum and maximum values of the pH and calcium release values (mg L^{-1}) found in the different periods

pH						
Sealer	1h	3h	24h	72h	168h	360h
MTA Angelus	7,63(7,36-9,24)[a]	7,34(6,83-7,55)[a]	7,26(7,18-7,44)[a]	7,14(7,04-7,23)[a]	7,14(7,06-7,32)[a]	7,11(7,01-7,18)[a]
Totalfill BC RRM	6,56(4,26-9,68)[b]	6,41(5,2-6,65)[b]	7,57(6,76-9,75)[b]	7,38(7,23-7,54)[b]	7,17(7,10-7,39)[a]	7,75(7,06-9,09)[b]
Calcium release						
MTA Angelus	1,38(0,46-3,53)[a]	0,00(-0,30-0,88)[a]	0,54(0,11-3,04)[a]	0,10(0,0-4,72)[a]	0,00(0,00-1,07)[a]	0,05(0,0-3,60)[a]
Totalfill BC RRM	1,61(1,22-3,31)[a]	0,85(0,56-3,24)[b]	1,61(1,22-3,31)[b]	3,59(2,10-5,03)[b]	3,77(2,13-4,84)[b]	3,81(2,57-4,73)[b]

Different letters in each column indicate statistical differences ($P < 0.05$).

Table 4 Median, minimum and maximum values of the solubility (%) of the tested groups

Sealer	168h	360h	720h
MTA Angelus	-4,08(-39,55- 12,47)[a]	-3,58(-32,95- 16,03)[a]	10,49(-15,30- 31,37)[a]
Totalfill BC RRM	2,71(-27,67- 18,87)[a]	-8,88(-21,87- 22,14)[a]	2,9(-16,81- 33,11)[a]

Different letters in each column indicate statistical differences ($P < 0.05$).

3 Discussion

3 DISCUSSION

New root canal sealers are being introduced on the market, making it necessary to understand their physico-chemical properties and biological compatibility, the latter being one of the most important. The endodontic end result depends on the tissue response to these materials (CAMILLERI et al., 2011). Laboratory studies contribute to a better understanding of the clinical behavior and manipulation of root canal sealers. Bioceramic materials were introduced in the daily practice of Dentistry, mainly because of their high biocompatibility (DAMAS et al., 2011; LOUSHINE et al., 2011; ZOUFAN et al., 2011) and strong antibacterial activity (ZHANG et al., 2009). The present study used the bioceramic resin filling and retro-filling sealers Totalfill BC Sealer and Totalfill BC RRM, and compared them with other sealers already established on the market. The Totalfill BC Sealer was compared with the AH Plus and MTA Fillapex and the Totalfill BC RRM was compared to the MTA Angelus. AH Plus was selected because of its widespread use and the MTA Fillapex because it also has bioceramic resin in its composition. The MTA was selected as the main retro-filling sealer on the market.

Physical and chemical properties were evaluated in accordance with the ISO 6876/2001 specifications. Radiopacity is considered one of the fundamental physical properties, since it allows verifying the quality of the root canal filling by means of radiographic examination (CANDEIRO et al., 2012). All sealers evaluated were in accordance with the ISO 6876/2001 specification that established the minimum radiopacity value of 3.00 mm Al.

Totalfill BC Sealer, AH Plus and Totalfill BC RRM have the same radiopacifier, zirconium oxide, but the Totalfill BC Sealer radiopacity was significantly lower; MTA Fillapex and the MTA Angelus have bismuth oxide as radiopacifier. In the case of sealers with the same radiopacifier, the difference in radiopacificity between the sealers may probably be explained by the ratio and type of radiopacifier present in the sealers (HUNGARO DUARTE et al., 2009; SILVA et al., 2013), however, other materials present in the composition may be responsible for this variation. AH Plus possibly showed higher radiopacity, due to the presence of another radiopacifier, calcium tungstate in its composition (CANDEIRO et al., 2012).

The setting time was determined in accordance with the recommendations of ASTM C266-08 and this setting time should not exceed 10% of that specified by the manufacturer. The specimens were prepared in accordance with ISO 6876/2001, but standard hybridization was used due to the smaller amount of sealer used to make the sample (DUARTE et al., 2012; HUNGARO DUARTE et al., 2012; VIVAN et al., 2010). The initial and final setting times of AH Plus were 8 and 15 hours, respectively, in agreement with previous studies (CAMILLERI; CUTAJAR; MALLIA 2011; ZHANG et al., 2009; ZHANG; LI; PENG 2009) MTA Fillapex, 13 and 26 hours; and Totalfill BC Sealer, 11 and 24 hours, in agreement with previous study (ZHOU et al., 2013). AH Plus is a paste-to-paste material that has an amine polymerization reaction contained in the epoxy resin (LIN-GIBSON et al., 2006, RESENDE et al., 2009). Totalfill BC Sealer is a premix (with disposable tips) of inorganic components and radiopacifiers, which are premixed with a liquid water-free vehicle, and addition of water is required for it to reach its final setting time (LOUSHINE Et al., 2011). On the other hand, the MTA Fillapex is paste-to-paste and when the two pastes come into contact, this promotes two chemical reactions that are responsible for setting of the material. (GOMES-FILHO et al., 2002). The differences in the setting time between the products are associated with the physical characteristics of calcium silicate, which requires a long setting time (SCARPARO et al., 2010). Despite this requirement of the material, the result of its setting time does not escape the reality of the other two sealers, although they seem long, they provide us with a good working time. The setting time for the retro-filling sealers was: MTA Angelus, 10 and 120 minutes, in agreement with previous studies (DUARTE et al., 2012; GANDOLFI et al., 2009; ISLAM; CHNG; YAP 2006); Totalfill BC RRM (3 hours and 22 hours), in agreement with previous studies (AL ANEZI et al., 2010; DAMAS et al., 2011; MA et al., 2011). While the setting time of MTA Angelus seems to be appropriate, the values obtained for Totafill BC RRM are exaggerated for retrofilling material, due to the presence of blood or exudate in postoperative time.

In relation to flow, we followed the recommendations of ISO 6876/2001, according to which the minimum flow required for the sealers is 20 mm. The present study showed higher melt flow values for the MTA Fillapex (47 mm), followed by

Totalfill BC Sealer (41.5 mm), Totalfill BC RRM (33.5 mm), AH Plus (33 mm) and MTA Angelus (17.5 mm). The differences between the sealers tested and in relation to other researches were perfectly understandable since there are innumerable factors are involved in a research, and these will not be standardized by the researchers who conduct them, although they always try to simulate clinical reality. Not all sealers presented acceptable values according to the recommendations of ISO 6876/2001, but the values recommended by ISO are for sealers that have higher flow ability (MARCIANO et al., 2011). Due to variations in physico-chemical properties, and depending on the portion of the AH Plus tube used, there would be a different flow (BALDI et al., 2012). The flow ability of the MTA Fillapex may be explained by the size of the sealer particles: the smaller the particle, the greater the flow (SILVA et al., 2013). For Totalfill BC Sealer, this could be attributed to the intrinsic properties of the material that is a calcium phosphate-based silicate. Another possible explanation would be the difference in size of the zirconium oxide particle. Previous study has verified high flow values when using micro and nano-particulate zirconium oxide (VIAPIANA et al., 2014).

Microorganisms generally survive in an acid medium. An alkaline pH promotes elimination of bacteria resistant to chemical and mechanical disinfection (McHUGH et al., 2004; STUART et al., 2006). This alkalinity activates and stimulates alkaline phosphatase, favoring the formation and deposition of mineralized tissue, allowing the formation of a fibrous capsule, with a lower inflammatory reaction (DUARTE et al., 2003; VIVAN et al., 2010). Calcium silicate sealers are known to have high pH and calcium ion release values. (DUARTE et al., 2003). In this study, the AH Plus presented lower pH values than the other sealers. Perhaps, this difference reflected the fact that Totalfill BC Sealer and MTA Fillapex consist of a pure bioceramic resin and a bioceramic resin combination, respectively. For retro-filling sealers, the MTA Angelus pH decreased over time, remaining alkaline. According to previous studies (CAVENAGO et al., 2014; DUARTE et al., 2012), a possible explanation for the high alkalinity of MTA Angelus is the presence of calcium oxide, which promotes the formation of calcium hydroxide, when in contact with moisture, leading to a highly alkaline pH, increasing its antimicrobial action. (HAUMAN; LOVE 2003; HOLLAND et al., 1999; PARIROKH; TORABINEJAD 2010; ROBERTS et al., 2008). For Totalfill BC RRM, there was a gradual increase in the pH level in all time intervals. A possible explanation for this increase in alkalinity would be the fact that Totalfill BC RRM contains pure bioceramic resin.

The release of calcium hydroxide in the form of Ca^{2+} ions and hydroxyl ions results in an alkaline pH (CAMILLERI 2007; SANTOS et al., 2005;). High pH values are usually verified in the initial periods, before complete setting of the sealer.

The mechanism of stimulating periapical repair by deposition of mineralized tissue has been reported to depend on pH and Ca^{2+} release capacity (OKABE et al., 2006). Moisture facilitates Calcium hydroxide hydration reactions (ZHANG; LI; PENG 2009; ZHANG et al., 2009). For the bioceramic materials, this factor may be related to the final setting time of these materials (CANDEIRO et al., 2012; LOUSHINE et al., 2011). Probably the materials that had a higher calcium ion release value in the time interval of 360 hours, already started this release from the beginning; this seems perfectly valid for the repair process, which did not occur with the other materials tested. Further studies should be conducted to confirm this observation. Another possible explanation for the large amount of Ca^{2+} released by sealers may be the calcium silicate hydration. The release of calcium and hydroxyl ions from the sealers that contain calcium silicate results in the formation of an apatite layer that chemically binds to calcium silicate sealer and dentin walls (SARKAR et al., 2005). Therefore, the calcium silicate in the composition of Totalfill BC RRM has the potential to chemically adhere to the dentin, thereby reducing voids.

Solubility is generally evaluated by using the international standardization standards, such as ANSI / ADA N^{O}. 57/2000 or ISO 6876/2001 which determine that the material should not have solubility greater than 3%. However, these methods are based on the difference in the weight of the cement before and after the sealer immersion in ultrapure water (HUNGARO DUARTE et al.,2012; ; VIVAN et al., 2010; TORBINEJAD et al., 1995). The standard solubility test based on the weight of the samples presents limitations that can influence the results; some of the material particles can be released during storage or the sealer may absorb water (PARIROKH; TORABINEJAD 2010). Another disadvantage of the ISO and ADA methodology is the specimen used for the test, which has two faces with 20mm in contact with water, subject to a higher level of solubilization. In the present study, a methodology proposed by Cavenago et al. (2014) was used, in which the difference in volume between the sealers and the volume of lost material was measured. According to this method, Totalfill BC Sealer, Totalfill BC RRM and AH Plus presented solubility less than 3%, as recommended by the ISO 6876/2001 specification, with the exception of the MTA Fillapex and MTA Angelus (period 720 hours) that presented solubility higher than 5%. For the AH Plus and MTA Fillapex the solubility values were higher than some studies (DUARTE et al., 2003; DUARTE et al., 2012; VITTI et al., 2013). For MTA Angelus solubility values were lower in some studies (DUARTE et al., 2013; MARCIANO et al., 2014). This is one of the pioneering studies using Totalfill BC Sealer, so it is difficult to compare the results obtained with previous studies. This difference may perhaps be related to the methodology used. In the present study, acrylic teeth with retrograde cavity were used, while the other studies used polyethylene tubes that had a larger opening and contact surface. Furthermore, most of the studies used ANSI / ADA No.57 or ISO 6876/2001, which are based on the difference between the weights before and after placing the sealer in water.

When a material is extravasated into the periapical region during endodontic treatment, it causes some inflammatory reactions, making the repair process difficult (YAMAGUCHI; MATSUMAGA; HAYASHI, 2007). In addition to the physico-chemical properties, the biological characteristics are of extreme importance for the success of endodontic treatment. The cytotoxicity of sealers can cause cell degeneration and consequently delay healing due to the intimate contact of the sealers with the periradicular tissues, making repair difficult in this area (SOUSA et al., 2006). The same happens with the retro-filling sealers, since they are in close contact with the periradicular tissues, and therefore must be as biocompatible as possible. Bioceramic materials have been considered promising for this repair, due to their excellent physico-chemical and biocompatibility properties (CANDEIRO et al., 2012; SAGSEN et al., 2011; ZOUFAN et al., 2011).

At present, there are few reports regarding the biocompatibility of endodontic sealers. In the present study, the MTT assay was used, which is a standard assay to evaluate the cytotoxicity of endodontic materials (JAFARNIA et al., 2009; KOGAN et al., 2006) found that the material will release cytotoxic substances that inhibit cell activity and cell growth (PASCON et al., 1990; SAIDON et al., 2003). The advantage of this method is evaluation of the material diluted in various concentrations. A multiple serial dilution can provide the cytotoxic grade of the material (DAMAS et al., 2009; MOSMAN et al., 1983). Cell viability analysis was performed on mouse fibroblasts (NIH3T3) comparing the AH Plus, Totalfill BC Sealer, MTA Fillapex, Totalfill BC RRM and MTA Angelus. The results revealed minor or nonexistent cytotoxic effects for Totalfill BC Sealer and Totalfill BC RRM. These sealers exhibited good biocompatibility with NIH3T3 cells, as well as a lower cytotoxic potential compared with the other cements. A possible explanation for this is that these sealers are composed of fine hydrophilic particles, calcium aluminum silicate, calcium phosphate, zirconium oxide and calcium hydroxide, some of which are similar to dentin. Calcium hydroxide reacts with the phosphate to form hydroxyapatite and water. The water is used to restart the cycle, reacting with the calcium silicate to produce calcium silicate hydrogel and calcium hydroxide (ZHANG; LI; PENG, 2009). At high concentrations all sealers showed cytotoxicity. AH Plus, MTA Fillapex and MTA Angelus showed higher cytotoxicity at high concentrations (100 and 50 mg / mL) than all the other sealers in all time intervals. A possible explanation would be the greater release of toxic components from the sealers in aqueous solutions at high concentrations, such as the formaldehyde present in the resin-containing sealers or the release of the amine and epoxy resin components (MERDAD et al., 2007) thus affecting the cell morphology and its adhesion capacity.

Since some properties of the sealers did not behave in a completely satisfactory manner, further studies are necessary so that we can arrive at a material without restriction on its clinical indication.

4 CONCLUSIONS

4 CONCLUSIONS

1. In summary, the results of the present study showed that all root canal filling and root retro filling met the requirements of radiopacity, setting time, flow, pH, calcium ion release and cytotoxicity. With the exception of the MTA Fillapex that not only fulfilled the requirement of solubility

2. Totalfill BC Sealer and Totalfill BC RRM presented higher pH, higher calcium ion release and lower cytotoxicity.

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APPENDIXES

APENDIX A - DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article ***Analysis of the physical-chemical properties and cytotoxicity of 3 root canal Sealers*** will be included in the Thesis of the student (Marcela Milanezi de Almeida) and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, May 18th 2017.

Adriana Matos Arruda
Signature
Author

Kleber Kildare Teodoro Carvalho
Author — Signature

Marco Antonio Hungaro Duarte
Signature
Author

Rodrigo Cardoso de Oliveira
Author — Signature

Norberti Bernardineli

Author — Signature

APENDIX B - DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article ***Evaluation of radiopacity, setting time, flow, pH, calcium ion release, solubility and cytotoxicity of the Totalfill BC Root Repair Material*** will be included in the Thesis of the student (Marcela Milanezi de Almeida) and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, May 18th 2017.

Marcela Milanezi de Almeida
Author

Signature

Adriana Matos Arruda
Signature
Author

Kleber Kildare Teodoro Carvalho
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ANNEXES

ANNEX A – Article submitted letter from the International Endodontic Journal

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Responder

seg 27/03, 18:54

Você

Caixa de Entrada

27-Mar-2017

Dear Ms. MILANEZI DE ALMEIDA

Your manuscript entitled "Analysis of the physical properties and cytotoxicity of 3 root canal Sealers" has been successfully submitted online to the International Endodontic Journal.

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Please mention the above manuscript ID in all future correspondence or when calling the Editorial Office for questions. If there are any changes in your postal or e-mail address, please log in to ScholarOne Manuscripts at https://mc.manuscriptcentral.com/iej and edit your user information as appropriate.

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Thank you for submitting your manuscript to the International Endodontic Journal.

Kind regards

Paul Dummer
Editor, International Endodontic Journal
iejeditor@cardiff.ac.uk

ANNEX B – Article submitted letter from the International Endodontic Journal

International Endodontic Journal - Manuscript ID IEJ-17-00297

IJ International Endodontic Journal <onbehalfof+iejeditor+cardiff.ac.uk@manuscriptcentral.com>

Responder

Hoje, 21:06

Você

21-May-2017

Dear Ms. MILANEZI DE ALMEIDA

Your manuscript entitled "Evaluation of radiopacity, setting time, flow, pH, calcium ion release, solubility and cytotoxicity of the Totalfill BC Root Repair Material" has been successfully submitted online to the International Endodontic Journal.

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Thank you for submitting your manuscript to the International Endodontic Journal.

Kind regards

Paul Dummer
Editor, International Endodontic Journal
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