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Evaluation of cytotoxicity, antimicrobial activity and physicochemical properties of a calcium aluminate-based endodontic material

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ABSTRACT

A calcium aluminate-based endodontic material, EndoBinder, has been developed in order to reduce MTA negative characteristics, preserving its biological properties and clinical applications. Objectives: The aim of this study was to evaluate the cytotoxicity, antimicrobial activity, pH, solubility and water sorption of EndoBinder and to compare them with those of white MTA (WMTA). Material and Methods: Cytotoxicity was assessed through a multiparametric analysis employing 3T3 cells. Antimicrobial activity against *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus*. (ATCC 25923) and *Candida albicans* (ATCC 10556) was determined by the agar diffusion method. pH was measured at periods of 3, 24, 72 and 168 hours. Solubility and water sorption evaluation were performed following ISO requirements. Data were statistically analyzed by ANOVA and Tukey`s test with a significance level of 5%. Results: EndoBinder and WMTA were non-cytotoxic in all tested periods and with the different cell viability parameters. There was no statistical differences between both materials (P>.05). All tested materials were inhibitory by direct contact against all microbial strains tested. EndoBinder and WMTA presented alkaline pH in all tested times with higher values of pH for WMTA (P<.05). Both materials showed values complying with the solubility minimum requirements. However, EndoBinder showed lower solubility than WMTA (P<.05). No statistical differences were observed regarding water sorption (P>.05). Conclusion: Under these experimental conditions, we concluded that the calcium aluminate-based endodontic material EndoBinder demonstrated suitable biological and physicochemical properties, so it can be suggested as a material of choice in root resorption, perforations and root-end filling.

Keywords: Endodontics. Biocompatible materials. Dental materials.

INTRODUCTION

Mineral trioxide aggregate (MTA) is a material that has been developed at Loma Linda University[14] initially as a root-end filling material and later has been used for pulp capping, pulpotomy, apexogenesis, apical barrier formation in teeth with open apexes, repair of root perforations, and as a root canal filling material[25]. MTA is a powder that consists of fine hydrophilic particles that set in the presence of moisture[34]. MTA has been recognized as a bioactive material[13] that is hard tissue conductive, hard tissue inductive, and biocompatible[17,23].

Mineral trioxide aggregate (MTA) has been shown to induce mineralization and to have favorable sealing properties[17,22,25,26]. Nevertheless, MTA remains subject to some concerns, such as its long setting time[11,25], poor handling characteristics, low resistance to compression, low flow capacity[11], high cost, and presence and release of arsenic[12,28]. These disadvantages lead to a need of ideal restorative materials, with adequate biological and mechanical properties[25,26].

A calcium aluminate-based endodontic material, EndoBinder (Binderware, São Carlos, SP, Brazil), has been developed with the intention of preserving the properties and clinical applications of MTA trying to reduce its negative characteristics[1,24]. EndoBinder is mostly composed of Al_2O_3 (≥68.5%), CaO (≤31.0%), SiO_2 (0.3-0.8%), MgO (0.4-0.5%), and Fe_2O_3 (<0.3%). The cement is produced by the process of calcining Al_2O_3 and $CaCO_3$ at temperatures between 1315°C and 1425°C to achieve a uniform composition. The product resulted of this process is cooled and then triturated until an adequate particle size is obtained. The final product is a result of the following chemical reaction: $CaCO_3+Al_2O_3=Ca(AlO_2)_2+CO_2$[21,24]. EndoBinder has good cell response, allowing greater development of cells at an advanced state of osteoblastic differentiation than the one obtained with MTA[7], it has less tissue reaction than MTA, it is biocompatible when tested in rat subcutaneous tissue and showed no gelatinolytic activity of MMP-2 and MMP-9[1,30]. However, up to now, there are limited publications about the physicochemical and biological properties of this calcium aluminate-based material and its possible use in clinical practice.

Thus, the aim of the present study was to evaluate the cytotoxicity, antimicrobial capability, pH, solubility and water sorption of EndoBinder and to compare them with those presented by WMTA (Angelus Indústria de Produtos Odontológicos, Londrina, PR, Brazil).

MATERIAL AND METHODS

Cytotoxicity evaluation was performed according to ISO 10993-5 specifications (2009)[20]. The agar diffusion method was used to measure the antimicrobial activity. pH was measured at periods of 3, 24, 72 and 168 hours. Solubility and water sorption evaluations were performed according to ISO 6876/2001 specifications (2001)[19]. Both materials were mixed according to the manufacturer's instruction, with a powder-to-liquid ratio of 3:1. The composition of the evaluated materials is shown in Figure 1.

Cytotoxicity

Under aseptic conditions, the materials were mixed on a glass slab for 1 min, and placed in Teflon rings (5 mm in diameter, 2 mm high). The specimens were allowed to set completely for 24 h at 37°C and 100% humidity under sterile conditions. After setting, the materials were placed in Dulbecco`s Modified Eagle`s Medium (DMEM) (Gibco, Life Technologies Corporation, Grand Island, NY, USA) with 10% Foetal Bovine Serum (Gibco, Life Technologies Corporation, Grand Island, NY, USA) using a 1.25 cm^2/mL ratio between the sample surfaces and the medium volume. Undiluted extracts were used for the test.

Cytotoxicity was evaluated with a commercial kit (Cytotox, Xenometrix AG, Allschwill, Switzerland) that evaluates three different cell viability parameters sequentially on the same cell culture: XTT, neutral red (NR), and crystal violet dye elution (CVDE). The XTT test is based on the ability of mitochondrial enzymes from metabolically active cells to reduce 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) molecules to a soluble salt of formazan, detectable by its absorbance at 480 nm, as measured by a spectrophotometer (Urit 660; URIT Medical Electronic CO, Guangxi, China). The same cells submitted to the XTT test were washed and assayed with the neutral red uptake test (NR), which determines the levels of viable cells through their membrane integrity. The vital dye NR is incorporated through endocytosis and accumulates preferentially on the lysosomes of membrane intact viable cells. After 3 h of exposure to the dye, cells were fixed and the NR was extracted and measured by the optical density (OD) of the supernatant at 540 nm, which directly relates to the proportion of viable cells. After the NR test, fixed cells were washed and evaluated for the total density of cells adhered, as estimated by the crystal violet dye exclusion test (CVDE). CVDE is a simple assay that evaluates cell density by staining DNA; after elimination of excess dye, the absorbance at 540 nm is proportional to the amount of cells in the well.

Fibroblast cells (lineage 3T3) were obtained from the American Type Culture Collection (ATCC) and cultivated in DMEM supplemented with 10% Foetal Bovine Serum (FBS) (Gibco, Life Technologies Corporation, Grand Island, NY, USA), 100 µg/ml streptomycin, and 100 mg/mL penicillin at 37°C in a humidified incubator under an ambient pressure air atmosphere containing 5% CO_2. Confluent cells

Material	Composition	Manufacturer
EndoBinder	Aluminium oxide, calcium oxide, silicon dioxide, magnesium oxide, iron oxide	Binderware (São Carlos, SP, Brazil)
WMTA	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrite, bismuth oxide, iron oxide, calcium oxide	Angelus (Londrina, PR, Brazil)

Figure 1- Materials tested and their composition

were detached with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid (Gibco, Life Technologies Corporation, Grand Island, NY, USA) for 5 min, and aliquots were subcultured. For the experimental set, $5x10^3$ cells were cultured in 96-well culture plates and allowed to achieve 80% confluence. After 24 h, the medium was removed from each well and replaced by 200 µl of one of the materials eluted in triplicate, as described above, for further 24 h.

Antimicrobial activity

The agar diffusion method was used to measure the antimicrobial activity of EndoBinder and WMTA against *E. faecalis* (ATCC 29212), *S. aureus*. (ATCC 25923) and *C. albicans* (ATCC 10556). Isolated for 24 h, colonies of pure culture of each microorganism were grown on Brain Heart Infusion (BHI; Oxoid, Basingstoke, U.K.) agar plates. Then, they were inoculated into tubes containing 5 mL of BHI broth (Oxoid Microbiology Products, Thermo Fisher Scientific, Basingstoke, U.K.). The suspension was adjusted spectrophotometrically at 800 nm to match the turbidity of 1.5×10^8 CFU mL^{-1} (equivalent to 0.5 McFarland turbidity standard). Five hundred µL of each test microorganism suspension was inoculated into glass bottles containing 50 mL of BHI agar at 46°C, vortexed, and poured onto 130-mm plates containing a previously set layer of Mueller Hinton Agar (MHA, Oxoid Microbiology Products, Thermo Fisher Scientific, Basingstoke, U.K.).

Sterilized stainless steel tubes of 8.0×1.0×10 mm (inner diameter 6 mm) were added to the surfaces of the media and filled with each tested substance. The plates were maintained for 2 h at room temperature in the appropriate gaseous conditions to allow the diffusion of the agents through the agar and then incubated at 37°C again under the appropriate gaseous conditions for an appropriate period of time: aerobe, 24 h; facultatives, 24–48 h in a CO_2 incubator (Jouan, Thermo Fisher Scientific, Saint Herblain, France), in an atmosphere of 10% CO_2. Zones of inhibition of microbial growth around the cylinder containing the tested substances were measured using a digital caliper and recorded after the incubation period. The inhibitory zone was considered to be the shortest distance (mm) from the outer margin of the cylinder to the initial point of the microbial growth. Three replicates were made for each microorganism.

pH analysis

Shortly after manipulation, the materials were carefully placed in plastic tubes (polyethylene) measuring 1.0 mm in internal diameter and 10.0 mm in length with only one open end with the aid of a lentulo spiral. Eight samples were used for each material. After being filled and weighed, each specimen was immediately immersed in test glass tubes containing 10 mL of deionized water, which were then sealed with parafilm (American National Can Company, Menasha, WI, USA) and placed in oven at 37°C, being kept throughout the study period. The pH was measured with pH meter (QM-400; Quimis Aparelhos Científicos, Diadema, SP, Brazil) previously calibrated with solutions of known pH (4, 7, 10). Previously to the immersion of specimens, pH of the deionized water was verified, showing pH 6.5. After removal of the specimens, the test tubes were shaken for 5 seconds before pH measurement. pH evaluations were performed always in fresh tubes containing deionized water at each evaluation period.

Solubility and water sorption

To determine the solubility (SL) and water sorption (WS), ISO 6876[19] (2001) specification was used. Five samples were prepared for each tested material, using teflon ring molds of 20 mm in diameter and 1.5 mm high. A nylon thread was inserted into the material before setting, allowing the sample to be hung and immersed in distilled water throughout the experimental period. The samples were kept on a cellophane-lined glass plate, and another cellophane-wrapped glass plate was placed on the top of the filled rings. The assembly was placed in a chamber with 95% relative humidity at 37°C for 24 hours. After setting, the specimens were removed from the rings and the residues and lose particles were removed. Samples were weighed in an analytical balance with 0.001 g precision (dry mass, m_1) and then placed in closed flasks with 50 mL of distilled water. Care was taken to avoid any contact between the samples and the inner surface of the container and the liquid. After 24 hours, the samples were removed from the flasks and weighted again to obtain the mass after saturation with water (m_2). The specimens were then placed in a desiccator at 37°C for 48 h and reweighed again (m_3). SL was calculated as:

$$\text{Solubility} = \frac{m_3 - m_1}{m_1} \times 100$$

WS was calculated as:

$$\text{Water Sorption} = \frac{m_2 - m_3}{m_3} \times 100$$

Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA) and Tukey's test by means of SPSS software 15.0 (SPSS Inc, Chicago, IL, USA). The significance level adopted was $P<.05$.

RESULTS

Cytotoxicity

Figure 2 shows the cell viability, evaluated by three different assays, in the periods of 24 and 48 hours. No significant difference was found among EndoBinder and WMTA in any experimental time (*P*>.05). No statistical difference was found between the different assays (*P*>.05).

Antimicrobial activity

The mean area of the zones of antimicrobial activity (mm) provided by EndoBinder and WMTA are presented in Table 1. All tested materials showed antimicrobial activity against all microbial strains tested. No statistical difference was observed between EndoBinder and WMTA against *E. faecalis* and *S. aureus* (*P*>.*05*). However, *C. albicans* was more susceptible to WMTA than to EndoBinder in 48 hours (*P*<.05).

pH

pH values at the different evaluation periods are shown in Table 2. EndoBinder and WMTA presented alkaline pH in all experimental times, with a maximum pH value at the 3 h evaluation. Both materials showed a decrease in pH values along the experimental times. WMTA showed higher pH values with statistical differences in all tested periods (*P*<.05).

Solubility and water sorption

EndoBinder showed an average weight loss of 1.47%, while WMTA showed a loss of 2.5%. Although both materials were in agreement with ISO 6876 statement[23], EndoBinder presented solubility lower than WMTA (*P*<.05). Regarding WS, EndoBinder and WMTA had an increase in mass of 9.47% and 9.17%, respectively, without statistical difference between them (*P*>.05).

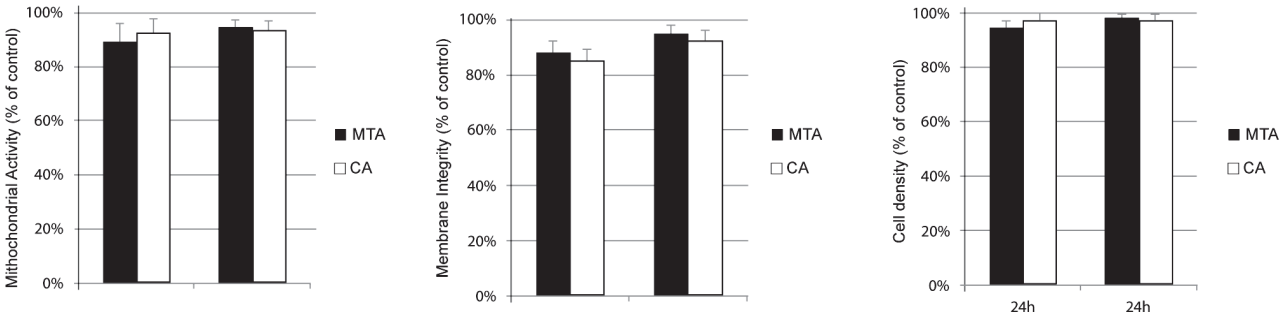

Figure 2- Cytotoxic effects of materials elutes on 3T3 cells by XTT, NR, and crystal violet tests, expressed as percentage of control (cells exposed to culture medium). Bars indicate mean±SD
SD=Standard Deviation; NR=neutral red

Table 1- Mean and standard deviation of the zones of microbial growth inhibition (mm) provided by the materials as well as statistical significance*

Materials	*S. aureus*		*E. faecalis*		*C. albicans*	
	24 h	48 h	24 h	48 h	24 h	48 h
EndoBinder	3.2±2.1^{Aa}	4.1±2.0^{Ab}	4.0±1.0^{Aa}	6.5±0.4^{Ab}	3.4±1.3^{Aa}	3.8±1.2^{Aa}
WMTA	3.0±0.8^{Aa}	4.5±1.5^{Ab}	4.4±0.7^{Aa}	7.2±1.4^{Ab}	4.1±0.4^{Aa}	6.5±0.9^{Bb}

Values are means of microbial growth inhibition (mm) from triplicate experiments. *Different capital letters represent significant differences between the materials in the same experimental time (P<.05). Different lowercase letters represent significant differences between the same material in different time points (P<0.05)

Table 2- Means and standard deviations of pH values at the different experimental times as well as statistical significance*

	3 hours	24 hours	48 hours	72 hours	168 hours
EndoBinder	8.96±0.42^{a}	8.94±0.43^{a}	8.80±0.50^{a}	8.78±0.33^{a}	8.46±0.45^{a}
WMTA	10.22±0.46^{b}	10.13±0.49^{b}	10.12±0.79^{b}	9.99±1.08^{b}	9.76±1.52^{b}
Control	6.50	6.50	6.50	6.50	6.50

*Values followed by different superscript letters indicate statistically significant differences (P<.05) in comparison between materials in the same experimental time

DISCUSSION

According to the manufacturer, EndoBinder has been developed to preserve the properties and clinical applications of MTA, without its negative characteristics[24]. The present study assessed the cytotoxicity, antimicrobial activity, pH, solubility and water sorption of EndoBinder and compared them with those presented by WMTA. Thereby, this study evaluated some of the main properties that should be considered for a suitable endodontic material. These tests must attend international standards. The International Organization for Standardization, also known as ISO, is the world's largest international standards developer. Cytotoxicity evaluation was performed according to ISO 10993-45 specifications and solubility and water sorption evaluation was carried out according to ISO 6876/2001[20].

Cytotoxicity was tested by employing a multiparametric assay, which evaluates in the same sample three different cell viability parameters, namely mitochondrial activity, membrane integrity and cell density. This method increases the chance of detection of cytotoxic effects, allowing correlation of different parameters, and provides a better understanding about toxicity mechanisms of biomaterials[10,27]. According to the present results, both materials were highly biocompatible in every parameter studied. These findings are in agreement with previous studies that demonstrated excellent biological properties of MTA, such as the ability to enhance proliferation of periodontal ligament fibroblasts, to induce differentiation of osteoblasts, to stimulate mineralization of dental pulp cells, to have a good biocompatibility and to be nontoxic to several cells linages[9,10,30]. In relation to EndoBinder, recent works have also showed good *in vitro* and *in vivo* biological properties, biocompatibility in tissues and absence of gelatinolytic activity for MMP-2[2,31]. One methodological aspect that needs to be discussed is the fact that sealers were exposed to cell culture media after 24 hours of manipulation. Endodontic cements are used in a freshly mixed condition in an incompletely polymerized stage. Thus, the results of the cytotoxicity test of the present study should not directly extrapolate to the clinical situation. However, previous studies demonstrated similar results using short time periods for comparative purposes of cytotoxicity[10].

In the present study, a modified agar diffusion test was used, which has been widely employed to assess the antimicrobial activity of several endodontic materials *in vitro* and allows direct comparisons between endodontic substances[3,4,15,32,33]. The microorganisms chosen were selected due to their known resistance to the endodontic procedures. *E. faecalis* and *C. albicans* are considered two of most resistant species in the oral cavity and are frequently associated with failure of root canal treatment[16]. Furthermore, *S. aureus* has also been isolated from primary and secondary or persistent endodontic infections[31]. Our results showed similar antimicrobial activity for EndoBinder and WMTA against *E. faecalis*, *S. aureus* and *C. albicans* at 24 h evaluation. A recent study[6], showed a higher susceptibility of *S. aureus* and *C. albicans* for MTA than EndoBinder, at 24 h evaluation, and no differences between materials for *E. faecalis*. The discrepant results could be explained by methodological differences. The antibacterial and antifungal properties of MTA have been extensively evaluated, with conflicting reports[4,24,32,33]. The differences in the results could be attributed not only to the bacterial source, difference between strains, amount of the bacteria inoculated, incubation time, metabolic activity of the microorganisms tested, but also to the molecular size, solubility, and diffusion of the materials through the aqueous agar medium, among others[3,4,15,32,33]. The antimicrobial activity of EndoBinder and MTA might be due to their high and constant pH. The influence of the composition of EndoBinder and MTA on antimicrobial activity requires further study.

Alkalinization of the medium occurs through the dissociation of calcium ions and hydroxyl ions when the material comes into contact with water. In this experiment, tubes of 1.0 mm in internal diameter were used to limit the contact surface of the materials to the surrounding water, simulating a clinical condition. Our results showed an alkaline pH for both materials, WMTA pH was significantly higher in all tested periods ($P<.05$). One possible explanation to the differences observed in the present study is related to EndoBinder synthesis. Phases with low Ca^+ ions content are privileged in EndoBinder synthesis and the material releases a smaller quantity of Ca^+ ions[7,24]. Although a higher pH promotes a better antimicrobial activity and a lower cytocompatibility, the present results showed that both materials are nonirritant to cells and have good antimicrobial capability.

High solubility of endodontic materials is undesirable because dissolution may cause release of the materials, allowing formation of gaps between them and the dental structure. Regarding the solubility test, both materials were within the recommended values of ISO 6786/2001[19], according to which the tested material should not have solubility greater than 3%. However, EndoBinder showed a significantly lower SL than WMTA ($P<.05$). Our results are in agreement with previous studies that showed MTA as a material with low solubility[5,18,25]. On the other hand, the higher solubility of MTA favored a higher pH level[8], confirming the results of the present study. As far as we know, this is the first time that solubility

has been evaluated for EndoBinder. It is important to point out that the solubility testing standards recommend immersion of the materials in water only after complete setting. However, this situation is impossible to be achieved clinically, since the materials are immediately placed in contact with fluids and blood. Therefore, solubility values in a clinical scenario are probably higher than the ones found *in vitro*[5]. Recently, a novel method was described to evaluate the solubility by the volumetric measurements of the cements using Micro-CT images[8]. This method could overcome the limitations of the ISO methodology and could be closed to simulate a clinical condition.

Water diffusion into cements may result in deterioration of their physical/mechanical properties, decreasing the life expectancy of the interfaces by hydrolysis and microcrack formation[29]. However, water sorption could be benefic as it promotes an expansion of the material, which may promote a proper sealing. Being both materials hydrophilic, a high water sorption was anticipated and confirmed by the results. No statistical differences were observed between the two tested materials in this aspect ($P<.05$). As the values of water sorption were similar between both materials, and as WMTA is a gold standard material, we might affirm that EndoBinder has good water sorption properties.

CONCLUSION

The present findings demonstrated the suitable cytotoxicity, physicochemical properties and the antimicrobial capability of this new calcium aluminate-based cement, which is known as EndoBinder.

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The authors deny any conflicts of interest related to this study.

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