UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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NANOTUBOS DE HALOISITA CARREADOS COM BROMETO DE TRIMETIL AMÔNIO COMO AGENTE ANTIBACTERIANO PARA CIMENTOS ENDODÔNTICOS

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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgião-Dentista.

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O sábio ditado 'A caneta é mais poderosa que a espada' é verdadeiro. Os extremistas têm medo dos livros e das canetas. É por isso que eles atacam escolas todos os dias: porque têm medo da mudança, da igualdade que vamos trazer para a nossa sociedade. Uma criança, um professor, uma caneta e um livro podem mudar o mundo.

Malala, 2013.

RESUMO

O objetivo deste estudo foi avaliar o efeito de nanotubos de haloisita (HNT) carreados com brometo de trimetil amônio (ATAB) nas propriedades físicas, químicas e biológicas de um cimento endodôntico experimental. Uma resina base de polimerização dual foi formulada com a incorporação de ATAB e HNT em diferentes proporções (1:1; 1:2; 2:1) e um grupo sem adição de ATAB:HNT foi usado como controle. A carga foi analisada através de microscopia eletrônica de transmissão. Os cimentos formulados foram avaliados através do grau de conversão, amolecimento em solvente, radiopacidade, escoamento, espessura de película, atividade antibacteriana contra biofilme e bactérias planctônicas e citotoxicidade. O grupo ATAB:HNT (1:1) aumentou significativamente o grau de conversão imediato ($p>0.05$) e o grau de conversão após 24 horas não diferiu entre os grupos. ($p>0.05$). Os grupos com ATAB:HNT apresentaram menor dureza inicial ($p>0.05$), porém sem diferença estatística entre os grupos no resultado de amolecimento em solvente ($p>0.05$). A radiopacidade de todos os grupos foi compatível a pelo menos 3mm de alumínio. Todos os grupos apresentaram pelo menos 17mm de escoamento e espessura de película menor que 50 µm, como requerido pela ISSO 6876:2012. Todos os grupos teste apresentaram efeito antimicrobiano contra o *E. faecalis* e quanto maior a proporção de ATAB adicionada, maior a capacidade antimicrobiana do material ($p>0.05$). A viabilidade celular foi maior que 70% para todos os grupos, sem diferença estatística entre os grupos ($p>0.05$). A incorporação de ATAB:HNT induziu atividade antibacteriana contra biofilme e bactérias planctônicas de *E. faecalis* sem efeitos na viabilidade de células humanas e nas propriedades físico-químicas dos cimentos endodônticos experimentais.

Palavras-chave: Antibacterianos. Compostos de Amônio Quaternário. Sistemas de Liberação de Medicamentos. Nanotecnologia.

ABSTRACT

The aim of this study was to evaluate the effect of alkyl trimethyl ammonium bromide (ATAB)-loaded halloysite nanotubes (HNT) in physical, chemical and biological properties of experimental resin sealers. An experimental dual-cure resin sealer was formulated with ATAB and HNT at different ratios (1:1; 1:2; 2:1) and one group remained without ATAB:HNT as control. Filler was analyzed by transmission electron microscopy. The sealers were evaluated for degree of conversion, softening in solvent, radiopacity, flow, film thickness, antibacterial activity for biofilm and planktonic bacteria and cytotoxicity. ATAB:HNT (1:1) significantly increased the immediate DC ($p<0.05$) and the DC after 24h did not differ among groups ($p>0.05$). ATAB:HNT groups showed lower initial Knoop hardness ($p<0.05$) without statistically significant difference among groups for softening in solvent ($p>0.05$). The radiopacity of all groups achieved at least 3mm of aluminum. All groups showed more than 17mm of flow and film thickness lower than 50µm, as required by ISO 6876:2012. All test groups presented antibacterial activity against *E. faecalis* and the higher the ATAB ratio, the greater the antibacterial activity of the resin sealer ($p<0.05$). Cell viability was higher than 70% for all groups, with no statistically significant difference among groups ($p>0.05$). The incorporation of ATAB:HNT induced antibacterial activity against biofilm and planktonic *E. faecalis* with no effects on pulp cell viability nor in the chemico-physical properties of the experimental resin sealers.

Keywords: Anti-bacterial agents. Quaternary Ammonium Compounds. Drug delivery systems. Nanotechnology.

SUMÁRIO

1 ANTECEDENTES E JUSTIFICATIVA

O tratamento endodôntico tem como objetivo eliminar ou evitar a colonização de micro-organismos no sistema de canais radiculares, através do preparo químico-mecânico e de uma obturação com bom selamento. Entretanto, devido a anatomia complexa dos canais radiculares, existem áreas inacessíveis para os instrumentos e irrigantes endodônticos durante o preparo químico-mecânico, como ramificações e deltas apicais[1]. Essas áreas que não são atingidas pelo processo de sanificação do canal radicular podem permanecer contaminadas e ser responsáveis pelo insucesso da terapia[2]. A persistência da infecção microbiana no sistema de canais radiculares é o principal fator associado a falha do tratamento endodôntico[1,2]. Além disso, pode ocorrer a infiltração de micro-organismos no canal radicular quando a restauração permanente ou provisória é defeituosa, independente da técnica de obturação e dos irrigantes utilizados[3].

Os tratamentos endodônticos primários falham entre 3%[4] a 8%[5] dos casos, enquanto que os retratamentos endodônticos falham em até 23%[6] dos casos. Portanto, o uso de materiais para preencher o espaço intra-radicular ou selar a interface material/dentina dentro do canal é proposto para diminuir a recontaminação bacteriana no sistema intrarradicular.

Enterococcus faecalis (*E. faecalis*) é uma bactéria gram-positiva facultativamente anaeróbia e é a principal bactéria relacionada com a infecção intrarradicular[7,8]. O *E. faecalis* possui diversos fatores de sobrevivência e virulência, incluindo sua habilidade para competir com outros micro-organismos, invasão de túbulos dentinários, resistência a privação nutricional e a capacidade de formar biofilmes[8]. Este micro-organismo é resistente a muitos agentes antimicrobianos usados na endodontia[7], como a clorexidina e o hidróxido de cálcio[9]. Nesse contexto, materiais com propriedades antimicrobianas capazes de eliminar o *E. faecalis* são necessários.

Os cimentos endodônticos comerciais (AH plus, EndoRez, Pulp Canal, Endomethasone, iRoot SP, MTA Fillapex, Sealapex) possuem atividade antibacteriana capaz de eliminar o *E. faecalis* quando recém misturados, porém no período entre 2-7 dias estes materiais não mostram mais inibição do crescimento bacteriano[10,11].

Compostos quaternários de amônio (CQA) são amplamente usados em hospitais, cosméticos e indústria devido à sua atividade antibacteriana[12,13]. Diversos tipos de CQA foram estudados em materiais dentários como resinas compostas, sistemas adesivos e ionômero de vidro. Além de excelente propriedade antimicrobiana e antifúngica, os CQA apresentam baixa toxicidade e alta permeabilidade. CQA têm a estrutura NR_4^+, sendo R um radical alquil[12]. O tamanho da cadeia alquil é importante na ação antibacteirana dos CQA. Compostos com a cadeia formada por 12-14 radicais de alquil são muito eficazes contra bactérias gram-positivas[13], como é o caso do *E. faecalis*. O brometo de trimetil amônio é um CQA, comercializado com cadeias de 14 radicais de alquil, que possui amplo espectro antimicrobiano e é eficaz na eliminação do *E. faecalis*[14]. A adição deste em soluções endodônticas desinfetantes aumentou sua capacidade antibacteriana contra o *E. faecalis* nos túbulos dentinários[15].

O efeito antibacteriano dos CQA, amplamente aceito como um agente de "morte por contato", é devido à interação eletrostática com bactérias. O CQA tem uma carga catiônica devido a apenas quatro grupos ligados ao nitrogênio e essa carga atrai a camada fosfolipídica de carga negativa da membrana celular da bactéria. Quando próximos, o CQA penetra na membrana bacteriana devido à sua longa cadeia de alquíl - um segmento hidrofóbico compatível com fosfolipídios da membrana celular[16]. Então, a difusão do CQA aumenta a pressão osmótica e causa o rompimento da membrana celular, levando ao vazamento citoplasmático e morte celular[17]. Uma forma de transportar agentes antimicrobianos sem a capacidade de copolimerização, como o brometo de trimetil amônio, é através de sistemas de liberação de fármacos, como nanocápsulas[18], microesferas[19] e nanotubos[20].

Os nanotubos de haloisita são um nanomaterial abundante na natureza que possui uma estrutura tubular perfeita e são originados de uma nanoargila. Eles vêm sendo estudados devido a suas propriedades físico-químicas como, por exemplo, estrutura tubular, troca de íons e hidrofobicidade, e mecânicas, que são melhoradas pela inclusão deste material inorgânico na matriz polimérica. [21]. Dentre suas aplicações, inclui-se o carreamento de antimicrobianos para que ocorra uma liberação continuada deste agente[22], tendo uma ação antimicrobiana mais duradoura. A liberação da droga quando carreada no nanotubo de haloisita pode durar de trinta a cem vezes mais do que a droga sozinha ou em outros carreadores[21]. Diversas substâncias química e biologicamente ativas já foram carreadas neste nanotubo, dentre elas o triclosan[20,23]. Além disso, devido à composição de sua superfície

externa, que é composta principalmente de grupos silanol, os nanotubos de haloisita são capazes de induzir deposição mineral em sua superfície, como mostrado anteriormente em um adesivo ortodôntico com nanotubos de haloisita carreados com triclosan. A bioatividade ocorre devido aos grupos silanol na superfície externa do nanotubo, que induzem uma diferença na eletronegatividade que favorece a reação com os grupos hidroxila quando em contato com o fluido corporal simulado (SBF)[20].

No entanto, não há relatos sobre o uso de nanotubos de haloisita carreados com brometo de trimetil amônio em cimentos endodônticos. Considerando a necessidade de diminuir a prevalência de infecções intrarradiculares e sabendo da característica antibacteriana do brometo de trimetil amônio contra a principal bactéria causadora destas infecções, o objetivo deste estudo foi avaliar a influência da adição de nanotubos de haloisita com brometo de trimetil amônio em um cimento endodôntico experimental.

2 OBJETIVO

O objetivo do presente estudo foi formular um cimento endodôntico experimental com adição de nanotubos de haloisita com brometo de trimetil amônio e avaliar suas características.

3 ARTIGO

O presente trabalho de conclusão de curso apresenta-se na forma de um artigo publicado no periódico Dental Materials em maio de 2019. https://doi.org/10.1016/j.dental.2019.02.018

Halloysite nanotubes loaded with alkyl trimethyl ammonium bromide as antibacterial agent for root canal sealers

ABSTRACT

Objective: Evaluate the effects of halloysite nanotubes (HNT) doped with alkyl trimethyl ammonium bromide (ATAB) on an experimental endodontic sealer.

Methods: An experimental dual-cure resin sealer was formulated and used as control, or loaded with ATAB and HNT at different ratios (1:1; 1:2; 2:1) and one group remained without as control. The ATAB:HNT filler was characterized through transmission electron microscopy (TEM). While, the sealers were evaluated for degree of conversion, softening in solvent, radiopacity, flow, film thickness, antibacterial activity for biofilm and planktonic bacteria and cytotoxicity.

Results: $G_{ATAB:HNT\ (1:1)}$ significantly increased the immediate DC ($p<0.05$), but no differ between the tested groups was encountered after 24h ($p>0.05$). All sealers containing ATAB:HNT showed lower initial Knoop hardness ($p<0.05$), but with no significant reduction after softening in solvent ($p>0.05$). The radiopacity of all groups achieved at least 3 mm of aluminum. All groups showed more than 17 mm of flow and film thickness lower than 50 µm, as required by ISO 6876:2012. All test ATAB:HNT sealers showed antibacterial activity against *E. faecalis;* the higher the ATAB ratio, the greater the antibacterial activity of the resin sealer ($p<0.05$). Cell viability was higher than 70% for all groups, with no statistically significant difference among groups ($p>0.05$).

Significance: The incorporation of ATAB:HNT induced antibacterial activity against biofilm and planktonic *E. faecalis* with no effects on pulp cell viability nor in the chemico-physical properties of the experimental resin sealers.

Keywords: Anti-bacterial agents. Quaternary Ammonium Compounds. Drug delivery systems. Nanotechnology.

INTRODUCTION

Endodontic treatment aims to disinfect the root canal system through chemomechanical debridement/instrumentation and intracanal medication. Moreover, it is crucial to obtain an adequate seal of the root canal in order to prevent bacterial recolonization [1]. Specific parts of the root canal system, such as ramifications, deltas and itshmus, that are not well disinfected may lead to persistent and/or secondary intraradicular infection, one of the main causes for endodontic treatment failure and retreatment [1,2]. Furthermore, regardless the obturation technique and the irrigators used, the infiltration of microorganisms in root canal may also occur when permanent or temporary restoration is defective [1,3]. Survival rate for primary endodontic treated teeth ranged from 31 to 96% [4] and the outcome of secondary root canal treatment was shown to reach 77% [5]. In attempt to improve endodontic treatments, filling and sealing materials for root canals with antibacterial properties have been studied [6-8].

The incorporation of antibacterial agents in materials to seal the material/dentin interface inside the root canal, such as in dual-cure resin sealers, contributes to eliminate remaining microorganisms and to prevent recontamination processes [9]. *Enterococcus faecalis* (*E. faecalis*) is the main microorganism related to endodontic infections [9] and it has shown resistance to previous used antimicrobial agents [10]. Thus, alternative antibacterial agents are required to assist in decontamination. Alkyl trimethyl ammonium bromide (ATAB) is a quaternary ammonium compound (QAC) with broad-antimicrobial activity [11]. ATAB is a non-antibacterial-agent-release that has already been tested for topical medications (wounds, burns, cleansing skin) [12], antiseptic for hand washing, disinfection of non-critical surfaces [11] and composition of root canal irrigants [13]. In addition, ATAB

showed to decrease biofilm stability and it indicated antibacterial activity against *E. faecalis* [13].

Drug release systems, such as microspheres [8] and nanotubes [14], have been used for carrying non-antibacterial-agent-release. Halloysite nanotubes (HNT) are nanoclays with tubular structure, abundant in nature, with biocompatibility, besides low cost [15]. HNT present 10-40nm inner diameter and 40-70nm outer diameter, allowing their use as drug carrier [15]. Also, HNT show high elastic modulus (140 GPa), improving resins' mechanical properties [16]. When encapsulated by HNT, the drug release can last from 30 to 100 times more than the drug alone or in other carriers [15]. However, there is no information on the use of HNT with ATAB in resin sealers.

The aim of this study was to evaluate the influence of addition of alkyl trimethyl ammonium bromide-loaded halloysite nanotubes (ATAB:HNT) in an experimental endodontic sealer. The null-hypothesis is that the use of ATAB:HNT would not have any beneficial effect on the chemico-physical and antibacterial properties of experimental resin-based sealers.

2 METHODS

2.1 Preparation of ATAB:HNT

Halloysite nanotubes (HNT, $Al_2Si_2O_5(OH)_4 \cdot 2H_2O$) and alkyl trimethyl ammonium bromide (ATAB, $C_{17}H_{38}BrN$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HNT was mixed with ATAB at the following proportions: 1:1 ($G_{ATAB:HNT\ (1:1)}$); 1:2 ($G_{ATAB:HNT\ (1:2)}$) and 2:1 ($G_{ATAB:HNT\ (2:1)}$). For the preparation of ATAB:HNT, ATAB and HNT were mixed under continuous magnetic stirring with ethanol PA until solvent evaporation. The product was stored in a desiccator at 25°C for three days.

2.2 Formulation of the experimental endodontic resin sealers

The experimental endodontic sealers were prepared by mixing 70 wt.% of urethane dimethacrylate (UDMA), 15 wt.% of glycerol 1,3-dimethacrylate (GDMA) and 15 wt.% of ethoxylated bisphenol Aglycol dimethacrylate (BisEMA). Camphorquinone (CQ), dihydroxyethyl-para-toluidine (DHEPT) and benzoyl peroxide (BP) were added as initiator system at 1 mol% according to monomer moles to formulate a dual-cure resin-sealer. Calcium tungstate ($CaWO_4$) at 100 wt.% was added as radiopacifier. All reagents, broth for antibacterial analyses and cytotoxicity were purchased from Sigma-Aldrich (St. Louis, MO, USA). The description of each test group, composed by different proportions of ATAB and HNT, and the control group ($G_{CONTROL}$) without any ATAB:HNT concentration is presented in Table 1. All specimens were photo-activated (Radii Cal, SDI Ltd., Bayswater, Victoria, Australia) for 40s on each side, using an irradiation value of 1200 mW/cm^2.

2.3 Transmission electron microscopy (TEM)

The powder of $G_{ATAB:HNT\ (1:1)}$ was dispersed in propanol PA at 5 wt.% and subsequently stirred for 15 min at ultrasonic bath. The propanol with ATAB:HNT was dispersed on square 400-mesh copper grid (TEM: Electron Microscopy Sciences, Hatfield, PA, USA). $G_{ATAB:HNT\ (1:1)}$ was analyzed by TEM (JEM 1200 EXll, JEOL, Tokyo, Japan) at 80 kV at 100000X and 500000X of magnification.

2.4 Degree of conversion

The degree of conversion (DC) of each experimental resin sealer was evaluated through Fourier-transform infrared spectroscopy with a spectrometer (Vertex 70, Bruker Optics, Ettlingen, Baden-Württemberg, Germany) equipped with Attenuated

Total Reflectance (ATR), a horizontal diamond crystal, and a 45° mirror angle. Three specimens per group (n=3) were dispensed onto the ATR crystal in a matrix with 4 mm of diameter and 1 mm of thickness. The light-curing device were fixed at 1 mm from the top of the specimen. Absorbance spectra were obtained using Opus software (Opus 6.5, Bruker Optics, Ettlingen, Baden-Württemberg, Germany), with Blackman-Harris 3-Term apodization over the range of 4000 to 400 cm^{-1} before and immediately after photoactivation (40s) of the specimens. Spectra were transferred to ImageJ software for the measurements of the peak height of the aliphatic C=C (1640 cm^{-1}) and carbonyl C=O bonds (1715 cm^{-1}) [17].

2.5 Softening in solvent

To evaluate the softening in solvent, five specimens per group (n=5) with 4 mm of diameter and 1 mm of thickness were prepared and embedded in acrylic resin. The specimens were polished using sandpapers (granulation of 600, 1200, 2000) under distilled water and a felt disc embedded with aluminum oxide suspension with 0.05 µm. The Knoop hardness was evaluated using a digital microhardness tester (HMV-2, Shimadzu, Tokyo, Tokyo, Japan). Three indentations (10g for 5s) were performed on the surface of each specimen to determine the initial Knoop hardness number (KHN1). The specimens were stored for 2h in an ethanol:water solution (50:50) and the final Knoop hardness (KHN2) was assessed. The percentage difference between KHN1 and KHN2 was calculated (ΔKHN%) for each group [18].

2.6 Radiopacity

To evaluate the radiopacity of the experimental resin sealers, five specimens per group (n=5) with 10 mm of diameter and 1 mm of thickness were tested according

to ISO 6876:2012 [19]. Radiographic images were obtained by a digital system with phosphor plates (VistaScan, Dürr Dental GmbH & Co. KG, Bietigheim-Bissingen, Baden-Württemberg, Germany) and the specimens were exposed along with an aluminum step-wedge in all images. An X-ray unit operating at 66 kV, 7,5 mA, exposure time of 0.4s, and focus-film distance of 400 mm was used. The images were digitized and analyzed using ImageJ software. The average and standard deviations of the grey levels (pixel density) of the aluminium (Al) step-wedge and the specimens were obtained in a standardized area and the mean value for each group was calculated [20].

2.7 Flow

To evaluate the flow of the experimental resin sealers, three specimens per group (n=3) were tested according to ISO 6876:2012 [19]. Using a graduated syringe, a total of 0.05 (± 0.005) mL of the freshly mixed sealer was placed on a glass plate (40x40x5 mm). At 180 ±5s after mixing started, another glass plate (40x40x5 mm) and a load of 100g were placed on the top of the material. After 10 min, the load was removed, the major and the minor diameters of the specimens were measured using a digital caliper. The mean value was recorded when it was observed a difference lower than 1 mm between the diameters [8].

2.8 Film thickness

To evaluate the film thickness of the experimental resin sealers, three specimens per group (n=3) were tested according to ISO 6876:2012 [19]. Two glass plates with 5 mm of thickness and 40 mm of length were placed together and the combined thickness was measured (F1). Using a graduated syring, 0.05 mL of the

experimental resin sealers was placed at the center of one plate and the second plate was placed on the top of the material. At 180 ± 5s after mixing started, 150N was applied on the top of the glass plate. After 10 min of mixing, the thickness of the two glass plates and the interposed resin sealer film was measured (F2). The difference between F1 and F2 was used to calculate the mean film thickness of the experimental resin sealers in three measurements [8].

2.9 Antimicrobial activity assays

Evaluation of antibacterial activity against biofilm formation

For the antibacterial activity assay against biofilm formation of *E. faecalis,* three specimens per group (n=3) were prepared with 4 mm of diameter and 1 mm of thickness. The specimens were attached on the lid of a test plate and the assembly was submitted to sterilization by hydrogen peroxide plasm. Each well of one test plate was inoculated with 900 µL of brain-heart infusion (BHI) broth and 100 µL of a suspension of an overnight broth culture of *E. faecalis* (INCQS 00234, ATCC 29212) corresponding to 9.2 x 10^8 CFU/mL. The lid with the specimens was placed on the sterile well-plate and the specimens' surfaces were exposed to the BHI broth with the bacteria at 37°C for 24h. The specimens were removed from the lid and vortexed in 1 mL of saline solution (0.9%) during 1 min to be subsequently diluted until 10^{-6} dilution. Two 25-µL drops of each dilution were platted in BHI-agar Petri dishes and incubated at 37°C for 48h. The number of colony forming units (CFUs) was visually counted and transformed to logCFU/mL [21].

Evaluation of antibacterial activity against planktonic bacteria

For the evaluation of the experimental resin sealers against planktonic bacteria, three specimens per group (n=3) with 4 mm of diameter and 1 mm of thickness were prepared, attached on the lid of a test plate and submitted to sterilization by hydrogen peroxide plasm. Each well of one test plate was inoculated with 900 µL of brain-heart infusion (BHI) broth and 100 µL of a suspension of an overnight broth culture of *E. faecalis* (INCQS 00234, ATCC 29212) corresponding to 9.2×10^8 CFU/mL. The lid with the specimens was placed on the sterile well-plate and the specimens' surfaces were exposed to BHI broth with the bacteria at 37°C for 24h. For the first dilution, 100 µL of each well was diluted in 900 µL of saline solution and the solutions were diluted until 10^{-6}. Three wells containing BHI and 100 µL of the suspension of overnight broth culture of *E. faecalis* were used as negative control. Two drops of 25 µL each one from each dilution were platted in BHI-agar on Petri dishes and incubated for 48h at 37°C to visually count and to calculate the logCFU/mL.

2.10 Cytotoxicity evaluation

After approval of the local ethics committee (n° 1.739.340), human pulp fibroblasts were collected from a third molar from a health person extracted for therapeutic reason. The person that provided the tooth assigned an inform consent and agreed to donate the tooth for research purpose. To evaluate the cytotoxicity of the experimental resin sealers, three specimens per group (n=3) with 1 mm of thickness and 4 mm of diameter were immersed in 1mL of Dulbecco's Modified Eagle Medium (DMEM) for 24h to eluates preparation. Pulp fibroblasts were placed at $5x10^3$ per well in a 96-well plate with 100µL of DMEM with the eluate previously prepared. After 72h, the cells were fixed with trichloroacetic acid at 10% and were incubated at 4 °C for 1h, washed six times with running water and dried at room temperature.

Sulforhodamide B at 4% was added to stain the cells and the plate was incubated for 30 min at room temperature. The plates were washed four times with acetic acid at 1% and dried at room temperature. Trizma solution was added and the plate was incubated for 1h to allow complete solubilization of the dye. The microplates were analyzed in a spectrotometer at 560nm. Wells containing pulp fibroblasts and DMEM, without eluate from specimens, were used as negative control to calculate the cell viability in wells with eluate from the experimental resin sealers [21].

2.11 Statistical analysis

Data normality was evaluated by Shapiro-Wilk test. One-way ANOVA and Tukey post hoc test were used to compare groups for KHN1, ΔKHN, radiopacity, flow, film thickness, antibacterial activity against biofilm formation, antibacterial activity against planktonic bacteria and cytotoxicity. Paired Student t test was used to compare KHN1 and KHN2 in each group. Two-way ANOVA and Tukey post hoc test were used to compare groups and different times for DC. All tests were performed at a level of 0.05 of significance.

3 RESULTS

Figure 1 shows the nanotubes morphology by TEM. In the lumen of HNT, brighter circles were observed suggestive of ATAB presence. The DC, Knoop hardness and softening in solvent values are presented in Table 2. The values of immediate DC ranged from 53.95 (±3.50)% for $G_{ATAB:HNT\ (2:1)}$ to 76.50 (±6.07)% for $G_{ATAB:HNT\ (1:1)}$, with higher value for $G_{ATAB:HNT\ (1:1)}$ compared to other groups ($p<0.05$). After 24h, $G_{ATAB:HNT\ (2:1)}$ increased the DC from 53.95 (±3.50) to 67.07 (±9.19), with statistically significant difference ($p<0.05$). The other groups showed no statistically

significant differences between immediate and 24h analyses ($p>0.05$). The values of KHN1 ranged from 11.88 (±0.62) for $G_{ATAB:HNT\ (1:2)}$ to 21.69 (±1.43) for $G_{CONTROL}$, with higher value for $G_{CONTROL}$ among groups ($p<0.05$). All groups decreased the Knoop hardness values after immersion in solvent ($p<0.05$) and there was no statistically significant difference among groups for ΔKHN ($p>0.05$).

The results of radiopacity, flow and film thickness analyses are shown in Table 3. For radiopacity, there was no statistically significant difference among groups ($p>0.05$) and all resin sealers reached values at least equal to 3 mm of aluminum, which is recommended by ISO 6876:2012. Flow values ranged from 18.26 (± 0.76) mm to 22.02 (± 0.60) mm and all groups reached values in accordance to ISO 6876:2012. All groups presented film thickness up to 50 μm, with no significant difference among groups ($p>0.05$).

Table 4 shows the results for antibacterial activity and cytotoxicity tests. There were statistically significant differences among groups in direct contact inhibition assay and in the planktonic viability analysis, with greater antibacterial effect as higher ATAB incorporation for both tests ($p<0.05$). The cell viability of all groups were normalized against the cell viability of cells in wells without the eluate and the values ranged from 80.07 (±10.82)% for $G_{CONTROL}$ to 100.47 (±9.68)% for $G_{ATAB:HNT\ (1:2)}$ with no statistically significant difference among groups ($p>0.05$).

4 DISCUSSION

The incorporation of antibacterial agents in resin sealers may benefit endodontic treatments by maintaining the root canal system aseptic [8,22]. The physico-chemical properties of the experimental root canal sealers were not compromised by the incorporation of ATAB:HNT. Moreover, the human cells viability remained higher with

no differences among groups and the resin sealer presented antibacterial activity against *E. faecalis* regardless of ATAB:HNT proportion. Therefore, the null hypothesis of this study must be rejected.

The morphological analysis of the particles carried out in the present study using TEM showed the presence of brighter circles inside the HNT, suggesting the presence of ATAB inside the lumen of the nanotubes [23]. However, we suppose that ATAB was also adsorbed on the outer surface of HNT [23]. In previous studies, when triclosan was added in HNT, the nanotubes presented the same characteristics representative of the presence of the drug content in nanotubes [23]. Usually, fillers with silica in the composition are silanized to be incorporated in resins. In this study, HNT was not silanized in order to maintain most of the surface available to interact with ATAB. In addition, HNT may better interact with the resins by chemical bonding between carbonyl groups from monomers and the electric deficient aluminum in the surface of HNT [14]. In this way, HNT could be available to interact with the monomers, may assisting the polymerization or the tensile strength distribution within the material [14].

It is well known that the polymerization of dual-cure endodontic resin sealers continues after the photoactivation due to their chemical mechanism [24]. In this study, the DC of the experimental endodontic sealers was evaluated by FTIR-ATR immediately and after 24h and it was observed that the incorporation of $G_{ATAB:HNT\ (1:1)}$ proportion presented a highest immediate DC ($p<0.05$). When the proportion of ATAB or HNT increased, the DC had no significant change compared to $G_{CONTROL}$ (ATAB:HNT/free resin). It is possible that a higher incorporation of ATAB or HNT may have altered the chain mobility compared to $G_{ATAB:HNT\ (1:1)}$, impairing the immediate polymerization for those groups [25]. Nevertheless, free radicals present in the polymer, especially due to amine and benzoyl peroxide used for chemical

polymerization, were able to react and continue the process over time, leading to non-difference among groups in the 24h evaluation. One could expect that with the increase of filler amount of ATAB:HNT groups compared to $G_{CONTROL}$, the DC would decrease due to the lower light transmission through the material [26]. Nevertheless, all groups reached reliable DC, with values according to commercial sealers [27]. These results are promising since higher DC increases material's stability over time, and provide reliable physical properties. Such a situation can also decrease unreacted monomers leaching, improving the materials' biocompatibility [28].

While the DC assess the conversion of aliphatic C=C double bonds in C-C single bonds, the Knoop hardness and softening in solvent evaluation assist at better understanding the stability of the polymer's network. Indeed, when a resin presents higher porosity, lower cross-link density and, consequently, more free volume in the network, more solvent is absorbed causing faster and more drastic hydrolytic degradation [29]. In this study, $G_{CONTROL}$ showed the highest initial Knoop hardness among groups. QAC with no methacrylate groups, as ATAB, may decrease Knoop hardness values [30] because of its non-copolymerization ability and lower degree of functionality, decreasing the cross-linking density [29]. Although the differences observed for KHN1, all experimental materials softened after solvent storage. The softening occurs due to higher forces of attraction between polymer and solvent than the attraction inside the polymer, leading to its swell and rupture of crosslinks [18]. However, there was no difference among groups for Knoop hardness variation after solvent immersion regardless the ATAB:HNT concentration.

An adequate radiopacity is one of the most important properties for a sealer, as this allows a proper diagnosis and enable the clinician to observe if empty spaces where left within the root canal system [31]. Inorganic radiopacifier agents such as

$CaWO_4$ are incorporated into resins to improve radiopacity of materials [7]. $CaWO_4$ is non-cytotoxic radiopacifier [32] and it presents a high atomic number (z=74), leading to greater absorption of X-rays so offering clinically relevant radiopacity to resin/based materials [20]. Current results showed that all groups tested in this study presented radiopacity equal or higher than 3 mm of aluminum, as required by ISO 6876:2012 [19]; this will allow adequate visualization of all experimental resin sealers in radiographic exams. In addition to the adequate radiopacity, the experimental resin sealers showed flow above 17 mm and film thickness under 50 μm, as required for resin sealers by ISO 6876:2012 [19]. In this way, the material is more prone to reach apical foramen and penetrates in difficult spaces such as accessory canals. Therefore, all experimental sealers were in accordance to ISO 6876:2012 standard, supporting their use for endodontic treatment.

In terms of antibacterial activity against biofilm formation and against planktonic bacteria the results of this study showed that the higher the concentration of ATAB at ATAB:HNT proportion, the greater antibacterial effect against *E. faecalis*. $G_{ATAB:HNT\ (2:1)}$ completely inhibited bacteria in the direct contact inhibition and in the planktonic bacteria assays. The antibacterial effect of QAC, widely accepted as a “contact killing” agent [33] is due to the electrostatic attraction between the cationic charge of QAC and the negatively charged phospholipids of the bacterial membrane [34]. Furthermore, QAC penetrate through the bacterial membrane by their long alkyl chain – a hydrophobic segment compatible with phospholipids [34]. The diffusion of QAC into the bacteria increases osmotic pressure and cause the disruption of bacteria, leading to cytoplasmic leakage and cell death [34]. Therefore, the charge density and the chain length are determinant factors in the antimicrobial activity of QAC [34]. The long alkyl chain with 14 carbons of ATAB induces a high hydrophobic property for this QAC,

assisting in its high antibacterial effect. In addition, ATAB presents an alkaline character, which is recommended for resin sealers to provide antibacterial activity [6,7].

The antibacterial effect of ATAB could be accompanied by cytotoxic effects, since human and bacteria cells present a lipid bilayer [35]. In addition, the longer the alkyl chain of QAC, the higher the possibility for cytotoxic effects [36]. The interaction between ATAB and bacteria membrane is facilitated due to its negative charges [35,33]. On the other hand, human cells are net neutral, assisting to non-cytotoxic effects of charged species [35]. In this study, the cytotoxicity for human pulp fibroblasts was evaluated by SRB method, which presents higher sensitivity and is less affected by exogenous factors than MTT tetrazolium test [37] preconized by ISO 10993-5 [38]. There was no significant difference in the cell's viability among groups and all resin sealers presented values above 70%, suggesting that the experimental materials presented no cytotoxicity according to ISO 10993-5 [38]. The use of a drug carrier system as HNT leads to slower release of the agent, increasing antibacterial activity over time [15] and reducing adverse effects against human cells. In addition to the antibacterial activity provided by ATAB, HNT may induce bioactivity for the experimental resin sealers due to the silanol groups on the outer surface of HNT [14]. Therefore, the incorporation of ATAB:HNT in root canal sealers is a promising alternative to improve therapeutic effects in endodontic treatments.

Persistent intraradicular or secondary infections are the major causes of failure in root canal treatment, regardless the quality of the primary treatment [1]. The present study showed that is possible to formulate an endodontic resin sealer with high antibacterial activity, no cytotoxic effects to human pulp cells, with reliable chemical and physical properties which could postpone the need for retreatment.

5 CONCLUSIONS

The incorporation of ATAB:HNT into new generation root canal sealers may be a good strategy to obtain materials with antibacterial activity against biofilm and planktonic *E. faecalis* without affecting their biocompatibility and their physico-chemical properties.

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Figure 1. Images of halloysite nanotubes with alkyl trimethyl ammonium bromide by MET at 100000X (a) and 500000X (b).

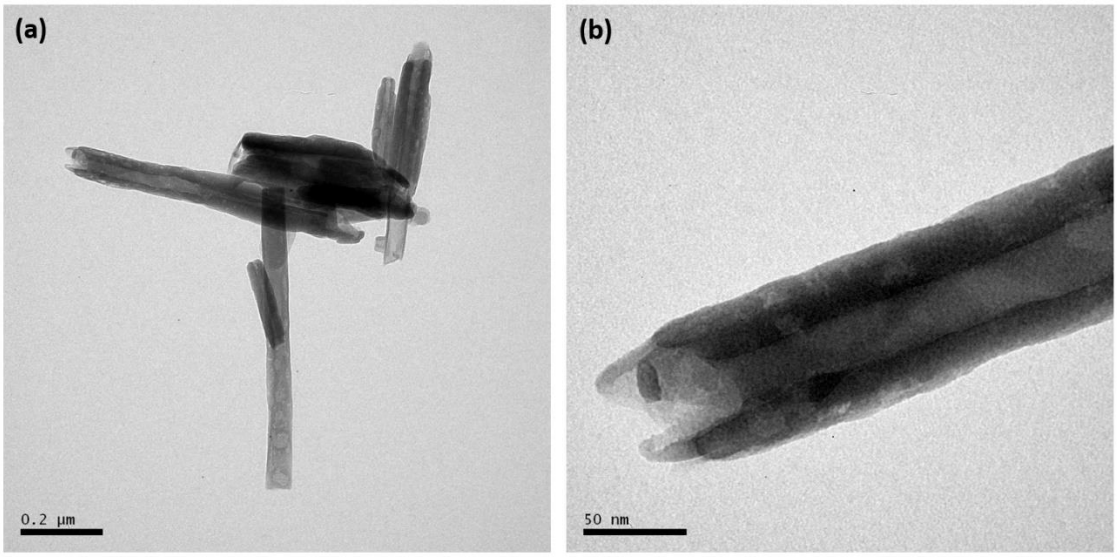

Table 1. Name of each experimental resin sealer and description according to each formulation.

Group	Description
$G_{CONTROL}$	Base resin (GDMA, UDMA, BisEMA, CQ, BP, DHEPT, $CaWO_4$)
$G_{ATAB:HNT\ (1:2)}$	Base resin (GDMA, UDMA, BisEMA, CQ, BP, DHEPT, $CaWO_4$) 3.3 wt%. ATAB and 6.6 wt.% HNT
$G_{ATAB:HNT\ (1:1)}$	Base resin (GDMA, UDMA, BisEMA, CQ, BP, DHEPT, $CaWO_4$) 5 wt%. ATAB and 5 wt%. HNT
$G_{ATAB:HNT\ (2:1)}$	Base resin (GDMA, UDMA, BisEMA, CQ, BP, DHEPT, $CaWO_4$) 6.6 wt%. ATAB and 3.3 wt.% HNT

Table 2. Mean and standard deviation values of initial Knoop hardness number (KHN1), final Knoop hardness number (KHN2), percentage of Knoop hardness variation (ΔKHN%) and degree of conversion (DC%) for experimental resin sealers.

Group	KHN1	KHN2	ΔKHN%	DC%	
				Immediate	24h
$G_{CONTROL}$	21.69 (±1.43) ^{Aa}	5.14 (±0.47) ^{b}	76.28 (±1.78) ^{A}	58.98 (±5.94) ^{Ba}	64.02 (±9.23) ^{Aa}
$G_{ATAB:HNT\ (1:2)}$	11.88 (±0.62) ^{Ca}	2.30 (±0.64) ^{b}	80.51 (±5.94) ^{A}	60.03 (±3.46) ^{Ba}	60.27 (±0.74) ^{Aa}
$G_{ATAB:HNT\ (1:1)}$	17.12 (±1.48) ^{Ba}	3.49 (±0.45) ^{b}	79.43 (±3.39) ^{A}	76.50 (±6.07) ^{Aa}	69.57 (±7.78) ^{Aa}
$G_{ATAB:HNT\ (2:1)}$	15.82 (±0.58) ^{Ba}	2.67 (±0.49) ^{b}	83.05 (±3.32) ^{A}	53.95 (±3.50) ^{Bb}	67.07 (± 9.19) ^{Aa}

Different capital letters indicate statistically significant difference in the same column ($p<0.05$).

Different small letters indicate statistically significant difference in the same row ($p<0.05$) for the same test.

Table 3. Mean and standard deviation values of Flow (mm) and Film thickness (μm) for experimental resin sealers.

Group	Radiopacity	Flow	Film thickness
$G_{CONTROL}$	125.75 (±5.85) ^{A}	21.71 (±1.66) ^{A}	30.00 (±0.00) ^{A}
$G_{ATAB:HNT\ (1:2)}$	118.48 (±6.31) ^{AB}	19.5 (±0.2) ^{AB}	36.70 (±11.54) ^{A}
$G_{ATAB:HNT\ (1:1)}$	116.33 (±2.97) ^{A}	18.26 (±0.76) ^{B}	36.70 (±5.77) ^{A}
$G_{ATAB:HNT\ (2:1)}$	119.30 (±5.53) ^{AB}	22.02 (±0.60) ^{A}	36.70 (±11.54) ^{A}
3 mmAl	108.40 (±3.37) ^{A}	-	-

Different capital letters indicate statistically significant difference in the same column ($p<0.05$).

Table 4. Mean and standard deviation values of direct contact inhibition assay and planktonic bacteria inhibition assay in colony forming units per milliliter with logarithmic transformation (log CFU/mL) and cytotoxicity test (%).

Groups	Direct contact inhibition assay	Planktonic bacteria inhibition assay	Cytotoxicity
$G_{CONTROL}$	5.94 (± 0.04) ^{A}	8.89 (±0.08) ^{A}	80.07 (±10.82) ^{A}
$G_{ATAB:HNT(1:2)}$	3.10 (±0.10) ^{B}	7.82 (±0.04) ^{B}	100.47 (±9.68) ^{A}
$G_{ATAB:HNT\ (1:1)}$	2.39 (±0.09) ^{C}	5.91 (±0.05) ^{C}	97.49 (±6.49) ^{A}
$G_{ATAB:HNT\ (2:1)}$	0.00 (±0.00) ^{D}	0.00 (±0.00) ^{D}	97.07 (±9.45) ^{A}
Negative control	-	9.00 (±0.05) ^{A}	100.00

Different capital letters indicate statistically significant difference in the same column ($p<0.05$).

4 CONSIDERAÇÕES FINAIS

Grande parte das bactérias presentes nos canais radiculares são eliminadas através do preparo químico-mecânico e do uso de agentes irrigantes. Contudo, bactérias residuais permanecem em canais laterais/colaterais, anastomoses, delta apical e outros locais na cavidade pulpar que não são atingidos pelo processo de sanificação e podem levar a uma nova infecção pulpar. Nesse contexto, o uso de cimentos endodônticos com atividade antimicrobiana pode assistir na eliminação dessas bactérias[12].

Cimentos com atividade antibacteriana podem aumentar a taxa de sucesso dos tratamentos endodônticos, especialmente frente a infecções secundárias ou persistentes. In vitro, a maioria dos cimentos endodônticos apresentam atividade antibacteriana para o *E. faecalis* quando recém misturados. Porém, essa atividade é perdida com a polimerização do material e não existe mais em 2 a 7 dias para todos os tipos de cimento comumente utilizados[11]. Sabe-se que a liberação de partículas carreadas nos HNT ocorre de forma 30-100x mais lenta[21]. Portanto, a adição de ATAB:HNT em um cimento endodôntico resinoso poderia levar a uma atividade antibacteriana prolongada. A ação antibacteriana a longo prazo do cimento experimental formulado neste estudo não foi avaliada, entretanto, corpos de prova para este teste foram preparados e estão armazenados em água destilada em uma estufa a 37ºC para avaliação em um ano. Além disso, estudo clínicos são necessários para avaliar se a ação antibacteriana prolongada e constante pode eliminar bactérias que tenham permanecido em lugares de difícil acesso, como deltas apicais ou ramificações, e aumentar a taxa de sucesso dos tratamentos endodônticos.

Além da capacidade antimicrobiana promovida pelo ATAB, o cimento endodôntico com ATAB:HNT pode ser capaz de promover deposição mineral. Os HNT ($Al_2Si_2O_5(OH)_4$), assim como outros compostos a base de sílica, são capazes de induzir precipitação mineral[20,24]. HNT têm a parede externa do tubo composta principalmente por grupamentos silanol (SiOH) que podem ser responsáveis por uma diferença em eletronegatividade que pode induzir deposição mineral. Outra hipótese é que a precipitação ocorra por reatividade dos grupamentos hidroxila (OH^-) quando imersos em SBF[20]. Para um cimento endodôntico, é vantajoso ter precipitação mineral, uma vez que necroses pulpares com presença de lesão periapical são

comuns na prática clínica e pode haver reparo acelerado da porção periapical com auxílio de materiais bioativos.

A excelente atividade antibacteriana encontrada para todos os cimentos com ATAB:HNT formulados faz com que estes sejam materiais promissores para o uso em endodontia. Do mesmo modo, outros materiais usados na odontologia poderiam ser beneficiados com a incorporação desta carga. A interface de união de resinas compostas e adesivos aos tecidos dentários é o elo fraco das restaurações, sendo muitas vezes acometida por cárie secundária. Das restaurações realizadas em consultórios, entre 50-70% são substituições de restaurações que falharam[25]. Usar materiais restauradores com propriedades antibacterianas pode auxiliar no controle da atividade de cárie nas margens da restauração. Com o uso de ATAB – que é capaz de eliminar o *Streptococcus mutans*[26] - esses materiais poderiam reduzir as bactérias relacionadas com a cárie nas margens da restauração, prevenindo a desmineralização e a ocorrência de cáries secundárias. Com a bioatividade relatada para os HNT[20], precipitação mineral poderia ocorrer nas margens da restauração, auxiliando na prevenção ao repor minerais perdidos no processo de desmineralização dentária.

Foi possível formular e avaliar cimentos endodônticos experimentais com a adição de ATAB:HNT, tendo o objetivo do estudo sido alcançado, apesar das limitações relatadas. Poderia ser interessante, após a conclusão dos testes *in vitro*, realizar um estudo clínico *in vivo* para avaliar o potencial reparador do cimento endodôntico formulado.

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