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

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ABSTRACT

Objective. This study aimed at evaluating the effects of experimental endodontic sealers containing halloysite nanotubes (HNT) doped with alkyl trimethyl ammonium bromide (ATAB).

Methods. An experimental dual-cure resin sealer was formulated and used as control material. This resin was also filled with ATAB and HNT at different ratios (GATAB:HNT 1:1; 1:2; 2:1) generate three experimental resin sealers. The ATAB:HNT filler was characterized through transmission electron microscopy (TEM). While, the experimental and control sealers were evaluated for degree of conversion, softening ration, radiopacity, flow, film thickness, antibacterial activity for biofilm and planktonic bacteria and cytotoxicity in human pulpal cells.

Results. GATAB:HNT $_{(1:1)}$ significantly increased the immediate DC ($p < 0.05$), although no difference was encountered between the groups after 24 h ($p > 0.05$). All the experimental cements (ATAB/HNT) showed relatively low initial Knoop hardness ($p < 0.05$), but with no significant reduction ($p > 0.05$) after storage in ethanol (softening ratio). The radiopacity of all groups achieved at least 3 mm of aluminum. All groups showed more than 17 mm of flow, with a film thickness lower than 50 μm (ISO 6876:2012). All the experimental ATAB:HNT cements showed antibacterial activity against *E. faecalis;* the higher the ATAB ratio, the greater the antibacterial activity ($p < 0.05$). Cell viability was higher than 70% with no significant difference between the groups ($p > 0.05$).

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Significance. The incorporation of ATAB/HNT into the experimental resin sealers induced antibacterial activity against biofilm and planktonic *E. faecalis* without affecting the pulp cell viability or the chemo-mechanical properties.

1. Introduction

Endodontic treatments aim to disinfect the root canal system through chemo-mechanical procedues and intracanal medications. However, it is crucial to obtain an adequate seal of the root canal in order to prevent bacterial recolonization and achieve long lasting outcomes [1]. In case specific parts of the root canal system, such as ramifications, deltas and isthmus, have not been well disinfected, persistent and/or secondary intraradicular infection can cause treatment failures; one of the main causes for endodontic retreatment [1,2]. Furthermore, regardless the obturation technique and irrigation technique employed during the treatment, the infiltration of microorganisms in treated root canals may also occur when permanent or temporary restorations are characterized by leakage and marginal defects [1,3]. Survival rate for primary endodontic treated teeth ranged from 31 to 96% [4], while the outcome of secondary root canal treatment can reach up to 77% [5]. In attempt to improve endodontic treatments, innovative filling and sealing materials for root canal obturation with antibacterial properties have been developed [6–8].

The incorporation of antibacterial agents in materials generated to seal the dentin interface inside the root canal, such as in dual-cure resin sealers, may contribute to eliminate microorganisms and to prevent the recontamination endodontically-treated teeth [9]. *Enterococcus faecalis* (*E. faecalis*) is the main microorganism associated to endodontic infections [9], which shows resistance to several conventional antimicrobial agents generally used during endodontics treatments [10]. Thus, alternative antibacterial agents are required to assist in decontamination of the root canal system during endodontic treatments. Alkyl trimethyl ammonium bromide (ATAB) is a quaternary ammonium compound (QAC) with broad-antimicrobial activity [11]. ATAB is a non-antibacterial-agent-release that was tested for topical medications (wounds, burns, cleansing skin) [12], antiseptic for hand washing, disinfection of non-critical surfaces [11] and root canal irrigants [13]. In addition, ATAB has been advocated to decrease biofilm stability, as well as being an effective antibacterial agent against *E. faecalis* [13].

Microspheres [8] and nanotubes [14], have been used as drug delivery systems for antibacterial agents. Halloysite nanotubes (HNT) are biocompatible and "low-cost" nanoclays with tubular structure [15]. HNT present 10–40 nm inner diameter and 40–70 nm outer diameter, allowing their use as drug carrier [15]. Also, HNT nanotubes have high elastic modulus (140 GPa), and they are able to improve the mechanical properties of resin/based materials [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 for root canal obturations.

The aim of this study was to evaluate the influence of alkyl trimethyl ammonium bromide-loaded halloysite nanotubes (ATAB/HNT) incorporated in experimental endodontic sealers. The null-hypotheses is that the incorporation of ATAB/HNT in experimental resin-based root canal sealers: (1) would have no beneficial effect on their chemo-mechanical and antibacterial properties; (2) would increase their cytotoxicity to pulpal cells.

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 different ratios to generate three experimental fillers (GATAB:HNT (ratio: 1:1)); 1:2 (GATAB:HNT (ratio: 1:2)) and 2:1 (GATAB:HNT (Ratio: 2:1)). This was accomplished by maintaining the NHT and ATAB under continuous magnetic stirring with ethanol PA until solvent evaporation. The three GATAB:HNT fillers were maintained 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.% urethane dimethacrylate (UDMA) with 15 wt.% glycerol 1,3-dimethacrylate (GDMA) and 15 wt.% 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 dual-cure polymerization materials. Calcium tungstate ($CaWO_4$) was added (10 wt.%) as radio-pacifier. 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 concentrations of ATAB and HNT, as well as the control group ($G_{CONTROL}$) without any ATAB/HNT is presented in Table 1. A light-curing system with an irradiation output >1200 mW/cm^2 (Radii Cal, SDI Ltd., Bayswater, Victoria, Australia) was used for 40 s to polymerize the specimens used in this study.

2.3. *Transmission electron microscopy (TEM)*

The fillers ATAB:HNT were dispersed in propanol PA at 5 wt.% and subsequently stirred in an ultrasonic bath for 15 min.

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

These were then dispersed on a square 400-mesh copper grid (TEM: Electron Microscopy Sciences, Hatfield, PA, USA) and analyzed using TEM (JEM 1200 EXll, JEOL, Tokyo, Japan) at 80 kV at X100000 and X500000 magnifications.

2.4. Degree of conversion

The degree of conversion (DC) of each experimental resin sealer was evaluated through Fourier-Transform Infra-Red spectroscopy (FTIR: Vertex 70, Bruker Optics, Ettlingen, Baden-Württemberg, Germany) equipped with Attenuated Total Reflectance (ATR), a horizontal diamond crystal, and a 45° mirror. Five 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 previously mentioned was placed at a distance of 1 mm from the top of the resin specimens. 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 (40 s) 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 ratio in solvent

To evaluate the softening ratio of the tested materials, five specimens per group (n = 5; 4 mm of diameter and 1 mm of thickness) were photoactivated for 40 s and polished using SiC abrasive papers (granulation of 600, 1200, 2000) under continuous irrigation (distilled water), and finally finished using a felt disc embedded with aluminum oxide suspension (0.05 μm). The Knoop hardness was evaluated using a digital microhardness tester (HMV-2, Shimadzu, Tokyo, Tokyo, Japan). Three indentations (10 g for 5 s) were performed on the surface of each specimen to determine the initial Knoop hardness number (KHN1). Subsequently, the specimens were stored for 2 h in a solution containing ethanol and water (50 vol.%–50 vol.%) and the final Knoop hardness (KHN2) was assessed. The percentage difference (softening ratio) 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 ; 10 mm diameter and 1 mm 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). The specimens were exposed along with an aluminum step-wedge in all images using the X-ray unit operating at 66 kV, 7,5 mA, with exposure time of 0.4 s, and focus-film distance of 400 mm. The images were digitized and analyzed using ImageJ software. The average and standard deviations of the grey levels (pixel density) of the aluminum (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 flowing properties of the tested experimental resin sealers, five specimens per group (n = 5) were tested according to ISO 6876:2012 [19]. Using a standard syringe, a total of 0.05 (±0.005) mL of the freshly mixed sealer was placed on a glass plate (40 × 40 × 5 mm). At 180 ± 5 s after mixing, one more glass plate with 20 ± 2 g and a load of 100 g was placed on the top of the material. The load was removed after 10 min and 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, five specimens per group (n = 5) were tested according to ISO 6876:2012 [19]. Two glass plates (5 mm thickness; 40 mm length) were placed together and the combined thickness was measured (F1). Using a standard syringe, 0.05 mL of each experimental resin sealer was placed at the center of one plate and the second plate was placed on the top of the material. At 180 ± 5 s, 150 N was applied on the top of the glass plate. The thickness of the two glass plates and the interposed resin sealer film was measured (F2) after 10 min. 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

2.9.1. Evaluation of antibacterial activity against biofilm formation

For the antibacterial activity assay against biofilm formation of *E. faecalis*, five specimens per group (n = 5) were prepared (4 mm diameter; 1 mm thickness) as previously described. 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 the 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 9.2×10^8CFU/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 24 h. 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 48 h. The number of colony forming units (CFUs) was visually counted and transformed to log CFU/mL [21].

2.9.2. *Evaluation of antibacterial activity against planktonic bacteria*

For the evaluation of the experimental resin sealers against planktonic bacteria, five specimens per group (n = 5) 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 the 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 9.2×10^8CFU/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 24 h. 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 48 h at 37 °C to visually count and to calculate the log CFU/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 of a healthy person extracted for therapeutic reason. All patients who donated their teeth to perform such a test, were fully informed about the project agreed, and signed an informed consent approved by the research council board. To evaluate the cytotoxicity of the experimental resin sealers, five specimens per group (n = 5) with 1 mm of thickness and 4 mm of diameter were immersed in 1 mL of Dulbecco's Modified Eagle Medium (DMEM) for 24 h for eluates preparation. Pulp fibroblasts were placed at 5×10^3 per well in a 96-well plate with 100 μL of DMEM with the eluate previously prepared. After 72 h, the cells were fixed with trichloroacetic acid at 10% and incubated at 4 °C for 1 h, 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 1 h to allow complete solubilization of the dye. The microplates were analyzed in a spectrotometer at 560 nm. 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.

3. Results

The TEM ultramorphology of the fillers created in this study are depicted in Fig. 1. It was possible to note in the lumen of HNT, brighter circles of ATAB presence. The DC, Knoop hardness and softening ratio values are shown 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 the highest values obtained with $G_{ATAB:HNT\ (1:1)}$ ($p < 0.05$). After 24 h,

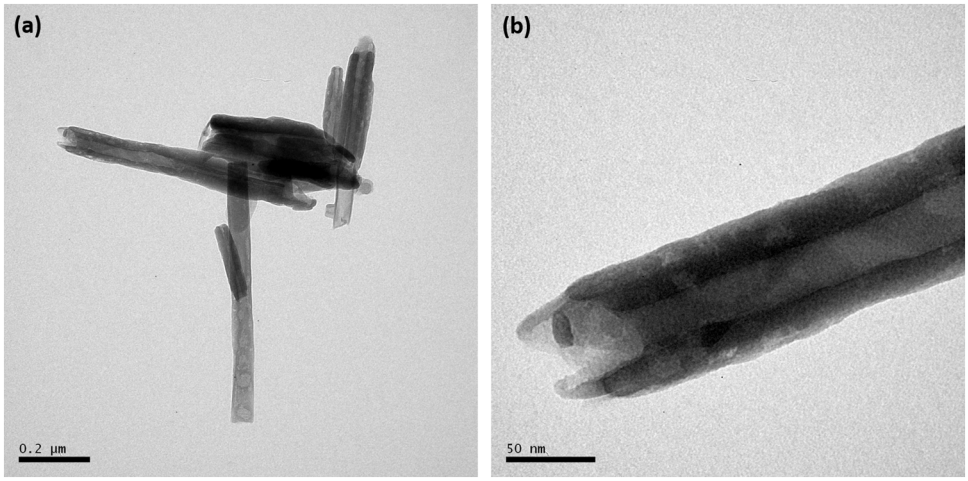

Fig. 1 – Images of halloysite nanotubes with alkyl trimethyl ammonium bromide by MET at 100,000× (a) and 500,000× (b).

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	24 h
$G_{CONTROL}$	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.

$G_{ATAB:HNT\ (2:1)}$ increased significantly the DC from 53.95 (±3.50) to 67.07 (±9.19) ($p < 0.05$). The other groups showed no significant differences between the values obtained at time 0 and those after 24 h ($p > 0.05$). The KHN1 results ranged from 11.88 (±0.62) for $G_{ATAB:HNT\ (1:2)}$ to 21.69 (±1.43) for $G_{CONTROL}$, which showed the greatest KHN1 values ($p < 0.05$). A decrease in Knoop hardness after immersion in solvent ($p < 0.05$) with no significant difference was observed between all the tested groups for ΔKHN ($p > 0.05$).

The results of radiopacity, flow and film thickness are shown in Table 3. For radiopacity, there was no significant difference between the groups ($p > 0.05$). However, all the experimental resin sealers reached values at least equal to 3 mm of aluminum, as required 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 ($p > 0.05$).

The results for antibacterial activity and cytotoxicity tests are presented in Table 4. Significant differences were encountered between the groups for both the direct contact inhibition assay and the planktonic viability analysis. The greatest antibacterial effect was obtained with the resin sealer containing high amount of GATAB:HNT for both tests ($p < 0.05$). The cell viability of all groups were normalized with the viability of cells in wells without the eluate. These values ranged from 80.07 (±10.82)% for $G_{CONTROL}$ to 100.47 (±9.68)% for GATAB:HNT $_{(1:2)}$ with no significant difference between the groups ($p > 0.05$).

4. Discussion

The incorporation of antibacterial agents in resin sealers may benefit endodontic treatments by preventing bacteria recontamination of the root canal system [8,22]. Indeed, the results of this study showed that the incorporation of GATAB:HNT in the experimental resin sealers tested in this study offered antibacterial activity against *E. faecalis* regardless the ATAB concentration in HNT. Moreover, the presence of GATAB:HNT within the composition of such experimental materials had no negative effects on their chemo-mechanical properties. Thus, the first null hypothesis must be rejected.

Furthermore, this study showed no adverse cytotoxic effect with the incorporation of ATAB:HNT in the resin sealers, since the human cells viability remained higher with no difference between the groups. Therefore, also the second null hypothesis of this study must be rejected.

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

It is well known that the polymerization reaction in dual-cure endodontic resin sealers continues after light-curing procedures [24]. In this study, the DC of the experimental endodontic sealers was evaluated by FTIR-ATR immediately and after 24 h and it was observed that the incorporation of ATAB in HNT with a concentration ratio of 1:1 (GATAB:HNT $_{(1:1)}$) presented greater immediate DC ($p < 0.05$). With the increase of ATAB in HNT, there was no significant change in the DC compared to the control group (GATAB:HNT-free resin). In this latter case, it was probably possible that the higher amount of GATAB in HNT incorporated in the resin-based material altered polymer chain mobility compared to GATAB:HNT $_{(1:1)}$, so impairing the immediate polymerization for those groups [25]. However, it is believed that free radicals present within the polymeric matrix of these tested materials, especially due to amine and benzoyl peroxide used for chemical polymerization, were able to react and continue the polymerization process over time, leading to no difference among groups for the evaluation at 24 h. One may expect that with the increase in ATAB in HNT concentration, the DC would decrease due to the lower light transmission through the material [26]. Nevertheless, this did not occur as all groups reached reliable DC, with values according to conventional resin sealers present currently in the market [27]. These are promising results since higher DC can also increase material's stability over time, and provide reliable physical properties. Such a situation can

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 ($p < 0.05$).

also decrease unreacted monomers leaching, so improving the materials' biocompatibility [28].

While DC assess the conversion of aliphatic C=C double bonds in C—C single bonds, the Knoop hardness and softening ratio in solvent evaluation offer a support to better understanding the stability of the polymer's network. Indeed, when a resin-based material presents higher porosity and lower cross-link density, it is likely that more solvent was absorbed and caused faster and more drastic hydrolytic degradation within the polymer matrix [29]. In this study, $G_{CONTROL}$ showed the highest initial Knoop hardness between all the tested groups. A possible explanation for the results attained with the experimental resins may be related to the fact that ATAB, a QAC with no methacrylate groups, may have decreased the cross-linking density and so, the Knoop hardness values [30] because of its lack of copolymerization ability and lower degree of functionality [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, leading to swelling and rupture of crosslinks between monomers [18]. However, there was no difference between the tested groups for Knoop hardness after solvent immersion, regardless the ATAB/HNT concentration.

Adequate radiopacity is a crucial and important characteristic for a root canal sealer, as this allows a proper diagnosis and enables the clinician to observe the presence of voids and porosity left within the root canal system [31]. Inorganic radio-pacifier agents such as $CaWO_4$ are usually incorporated within the composition of resin-based materials to improve their radiopacity [7]. $CaWO_4$ is a non-cytotoxic radiopacifiying agent [32] characterized by a high atomic number ($z = 74$), which leads to greater absorption of X-rays and clinically relevant radiopacity [20]. Current results showed that all groups tested in this study presented radiopacity equal or higher than 3 mm of aluminum; this is a suitable value as per ISO 6876:2012 [19], which allow an adequate visualization during radiographic exams. In addition to that, the experimental resin sealers showed a flow above 17 mm and film thickness under 50 μm, as required for resin sealers by ISO 6876:2012 [19]. Due to these specific characteristics, the experimental materials generated and tested in this study can be potentially able to reach the apical foramen and infiltrate those difficult spaces such as accessory canals. Therefore, all experimental sealers were in accordance to ISO 6076:2012 standard, supporting their use for endodontic treatments.

In terms of antibacterial activity against biofilm formation and planktonic bacteria, the results of this study showed that the higher the concentration of ATAB in HNT proportion, the greater antibacterial effect against *E. faecalis*. GATAB:HNT (2:1) inhibited bacteria growth completely when tested both in direct contact model and in the planktonic bacteria assay. The antibacterial effect of QACs, widely accepted as a "contact killing" agent [33], is due to the electrostatic attraction between the cationic charge of QACs and the negatively charged phospholipids of the bacterial membrane [34]. Furthermore, QACs 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 the osmotic pressure leading to cytoplasmic dispersion 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 16 carbons of ATAB results highly hydrophobic, and this improve its penetration into the cell, as well as its antibacterial effect. In addition, ATAB presents an alkaline character, which is recommended for resin sealers to provide antibacterial activity [6,7].

Cytotoxic might be correlated to the antibacterial effect of ATAB, since human and bacteria cells present a lipid bilayer membrane [35]. In addition, the longer the alkyl chain of QAC, the greater the cytotoxic effect [36]. The interaction between ATAB and bacteria membrane is facilitated due to its negative charges [35,33]. Conversely, human cells are not neutral, assisting to non-cytotoxic effects of charged species [35]. In this study, the cytotoxicity to human pulp fibroblasts was evaluated by SRB method, which presents higher sensitivity and it is less affected by exogenous factors rather than MTT tetrazolium test [37] adovcated by ISO 10993-5 [38]. There was no

significant difference in the cell viability between the tested groups, and all resin sealers presented values above 70%; this confirms that the experimental materials have no relevant 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 nanotubes [14]. Therefore, the incorporation of ATAB/HNT in root canal sealers may be a promising alternative to improve therapeutic effects in endodontic treatments.

5. Conclusions

The incorporation of ATAB/HNT into innovative resin-based root canal sealers may be a suitable strategy to generate materials with antibacterial activity against biofilm and planktonic *E. faecalis*, without affecting the biocompatibility and the main chemo-mechanical properties.

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