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Influence of Adding Nanoparticles of Silver Vanadate on Antibacterial Effect and Physicochemical Properties of Endodontic Sealers

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ABSTRACT

Introduction: The aim of this study was adding the nanostructured silver vanadate decorated with silver nanoparticles ($AgVO_3$) (0, 2.5, 5, and 10%) to the endodontic sealers AH-Plus, Sealer 26 and Endomethasone N, and evaluate the antimicrobial activity, solubility and pH. **Methods and Materials**: The antimicrobial activity of freshly mixed sealers (n=10) and set sealers (n=9) against *Enterococcus faecalis* (*E. faecalis*) was evaluated by colony forming units per milliliter and epifluorescence microscopy. Solubility (n=9) and pH (n=10), 6 and 24 h and 7, 14, and 30 days were also evaluated. The Kruskal-Wallis and Dunn's post-test were applied for the antimicrobial activity of fresh sealers. ANOVA and Tukey's post-test was used for set sealers and solubility, and Friedman's two-way analysis of variance for pH (α=0.05). **Results**: The fresh sealers inhibited *E. faecalis*. Set Sealer 26 (5 and 10% $AgVO_3$) and Endomethasone N (2.5, 5, and 10% $AgVO_3$) presented higher activity then the corresponding controls. Modification with $AgVO_3$ did not influence the solubility of AH Plus and Sealer 26, but Endomethasone N (5%) presented reduced solubility. The AH-Plus groups showed acidic pH, and Sealer 26, basic pH after 30 days. Endomethasone N (5 and 10% $AgVO_3$) presented statistical difference compared to 0% (P<0.05). **Conclusion:** In this *in vitro* study all fresh sealers and set Sealer 26 (5 and 10%) and Endomethasone N (2.5, 5, and 10%) presented higher antimicrobial activity than controls. The modification with 5% and 10% $AgVO_3$ decreased solubility and pH of Endomethasone N, but did not affect the other groups.

Keywords: Antimicrobial Activity; Endodontic Sealer; Nanoparticles; Silver Vanadate; Solubility

Introduction

The complexity of the root canal system and virulence factors of gram-positive anaerobic and facultative microorganisms prevent the complete elimination of bacteria by means of chemo-mechanical preparation in endodontic treatments [1-4], resulting in retreatment rates between 18-26 % [5].

Among the persistent microorganisms, *Enterococcus faecalis* (*E. faecalis*) is found in 24 to 77% of treated canals [2], presenting resistance to irrigants, intracanal drugs, and antimicrobial compounds, as well as being capable of penetrating the dentinal tubules [1, 2, 6, 7]. Thus, the use of obturation materials with antimicrobial activity can be advantageous in the reduction of persistent bacteria [1-3]. However, the effect of sealers with antimicrobial activity or modified with antimicrobials is reduced over time [6], increasing the risk of recontamination [8].

Incorporation of micro and nanoparticles to endodontic sealers facilitates the penetration of these particles into the dentin tubules [9] and allows the prolonged release of antimicrobial components [1]. Nanostructured silver vanadate decorated with silver nanoparticles ($AgVO_3$) [10] is a nanomaterial with a recent application in dentistry [11], and unprecedented association with

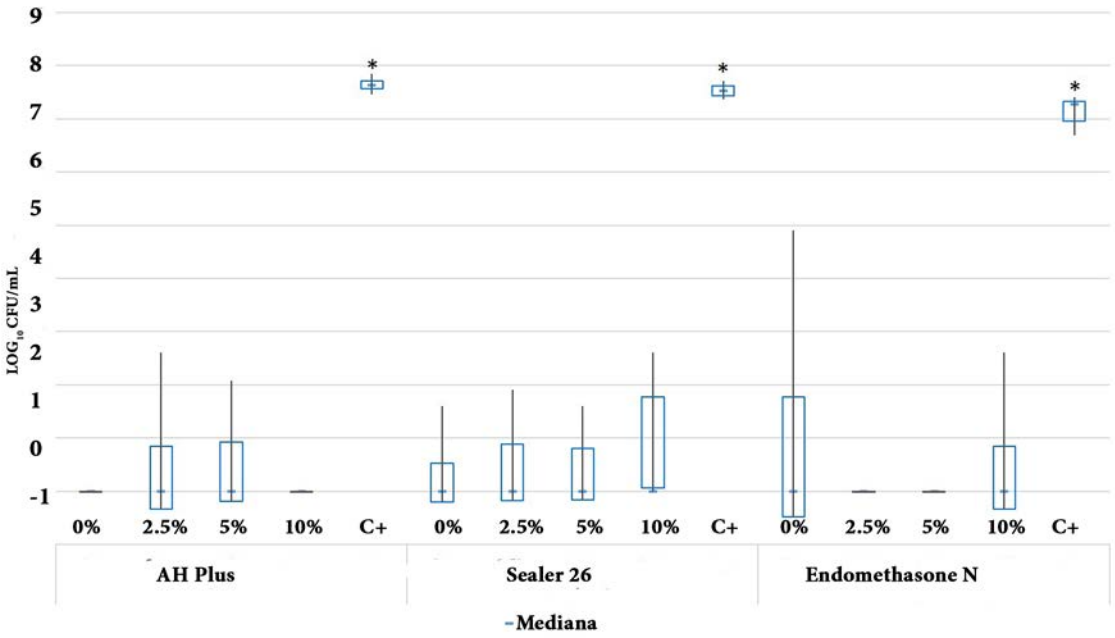

Figure 1. Colony forming units per milliliter of *Enterococcus faecalis* in direct contact with endodontic sealers freshly mixed modified with different concentrations of $AgVO_3$. C+: positive control group. * represent statistical difference of C+ group compared to the other experimental groups

endodontic sealers. Produced with nanowires of vanadate and silver nanoparticles, this material presents antimicrobial activity against gram-positive and gram-negative microorganisms [12, 13]. This nanomaterial presents the antimicrobial effectiveness of silver nanoparticles and also solves the limitation of the aggregation of these nanoparticles in vanadate nanowires [12, 14].

The addition of antimicrobial agents to sealers may alter their composition and influence their physicochemical properties [15, 16]. High solubility is often associated with disintegration of the material and formation of gaps that compromise the sealing of the canal. Moreover, the release of chemicals that affect pH can cause irritation to the periapical tissues [17-19]. On the other hand, an alkaline pH also assists in the elimination of persistent bacteria [18].

In this study, we added 2.5, 5, and 10% $AgVO_3$ to three endodontic sealers in order to evaluate the antibacterial activity of freshly mixed sealers and set sealers, their solubility and pH, aiming at finding an endodontic sealer with an effective bacterial reduction capacity. The null hypothesis was that $AgVO_3$ does not increase the antibacterial activity or causes changes in the physicochemical properties of the sealers.

Materials and Methods

The nanostructured silver vanadate decorated with silver nanoparticles ($AgVO_3$) was synthesized [11] and added at the concentrations of 0 (control group) 2.5, 5, and 10% to the endodontic sealers AH-Plus (Dentsply DeTrey GmbH, Konstanz, Germany), Sealer 26 (Dentsply, Petrópolis, RJ, Brazil), and Endomethasone N (Septodont, Barueri, SP, Brazil). The powder or base paste of the sealers and the $AgVO_3$ were weighed on a precision scale (Micronal S/A, model AB 204, São Paulo, SP, Brazil) and added to the liquid or catalyst paste. Mixing was performed on an unpolished glass slab following the manufacturer's instructions.

Antibacterial activity

The antibacterial activity of fresh sealers and set sealers was evaluated by direct contact test (DCT) with *Enterococcus faecalis* (ATCC 29212), obtained from a recent culture and standardized in a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA), with an absorbance of 0.150 at 625 nm wavelength (10^8 CFU/mL bacteria). For evaluation of fresh sealers the applied methodology was similar to Arias-Moliz *et al.* [15]. Endodontic sealers were prepared aseptically and 0.2 mL of each material was added with sterile syringe to a well of a 96-well plate (n=10). Then, 50 µL of *E. faecalis* bacterial suspension was added to each well and incubation was performed at 37°C for 1 h in a microbiological oven. As a positive control, 50 µL of the bacterial suspension was used. After the incubation period, 100 µL of sterile TSB (Tryptic Soy Broth, Difco, Sparks, MD, USA) was added to the wells and gently pipetted for 1 min. A 100-µL aliquot was taken from each well for evaluation of colony forming units per milliliter (CFU/mL) and epifluorescence microscopy.

For biofilm formation on the samples of the set sealers, the methodology of de Castro *et al.* [11] was applied. The specimens (7.75 mm×1.5 mm) were prepared, incubated at 37°C for 7 days

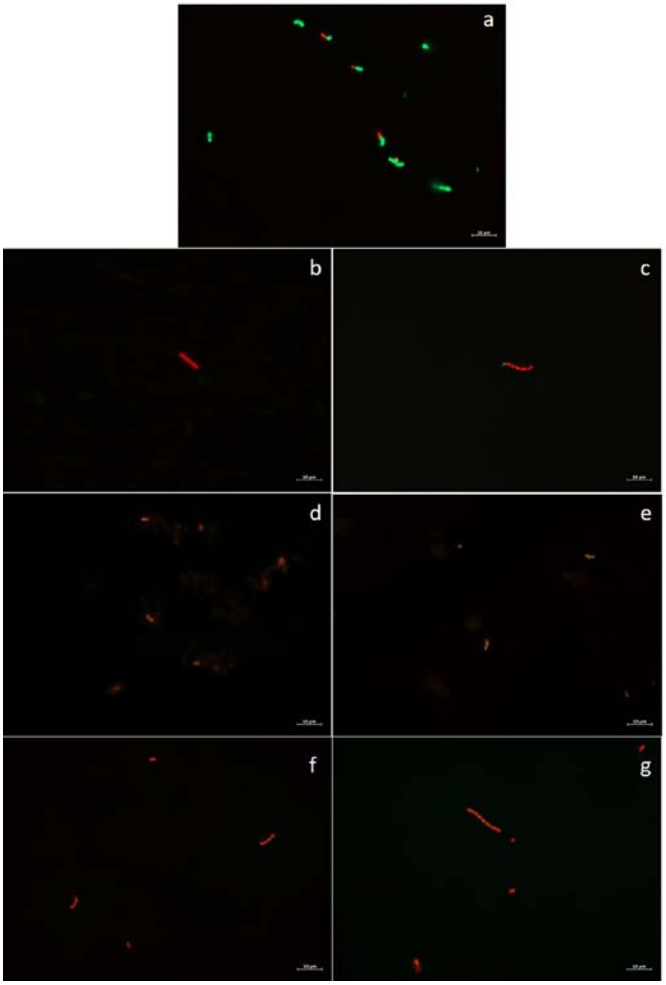

Figure 2. Epifluorescence microscopy of freshly mixed sealers in contact with *Enterococcus faecalis* (magnification 63×); *A)* positive control; *B)* 0% AH-Plus; *C)* 10% AH-Plus; *D)* 0% Sealer 26; *E)* 5% Sealer 26; *F)* 0% Endomethasone N; *G)* 2.5% Endomethasone N

for complete setting, and sterilized with ethylene oxide (ACECIL, Central de Esterilização Comércio e Indústria Ltda., Campinas, Brazil). Specimens (n=9) were inserted into 24-well plates, and 1500 µL of the *E. faecalis* suspension was added to each well. The plates were maintained in a microaerophilic environment and incubated (Shaker, Cienlab, Campinas, SP, Brazil) for 1 h and 30 min at 37°C under agitation at 75 rpm for adhesion of the microorganism to the specimen. Then, the contaminated culture medium was removed and 1500 µL of sterile TSB was added. The plates were incubated for 48 h under the same conditions for growth and maturation of the *E. faecalis* biofilm; the culture medium was renewed every 24 h. After biofilm formation, each specimen was washed, added to a microtube containing 1000 µL of PBS and sonicated in ultrasonic bath (Altsonic, Clean 9CA, Ribeirão Preto, SP, Brazil) (200 watts/40 Hz) for 20 min for biofilm detachment. Afterwards, 25 µL was collected from each microtube for determination of CFU/mL and epifluorescence microscopy.

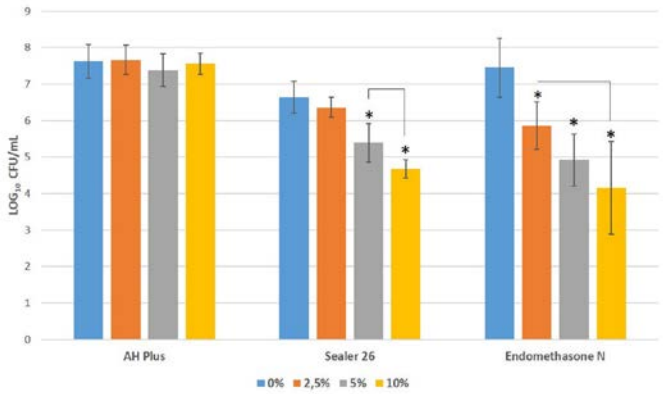

Figure 3. Colony forming units per milliliter of *Enterococcus faecalis* in direct contact with endodontic sealers modified with different concentrations of $AgVO_3$;* Statistical difference between control group (0%) and the other experimental groups within the same type of sealer; represent statistical difference between groups modified with $AgVO_3$

The number of viable cells was quantified in CFU/mL after seeding aliquots of serial dilutions (10^{-1} to 10^{-4}) in TSA culture medium (Trypticase Soy Agar, Difco, Sparks, MD, USA). The plates were incubated at 37°C for 24 h in a microaerophilic environment. After incubation, the number of colonies at each dilution was counted, and the CFU value was obtained based on a dilution yielding 1-300 colonies as follows: CFU/mL=number of colonies×10n/q, where: n=absolute dilution value (0, 1, 2, 3, or 4) and q=amount of coated suspension (0.025 mL). The CFU/mL values were converted to log10 [11].

Fluorescence microscopy was done to obtain a qualitative complement of the antimicrobial activity analysis. The biofilms formed on the surface of the specimens were stained with the BacLight ™ Live/Dead® Cell Viability Kit (L 7007, Molecular Probes, Inc., Eugene, OR, USA). The dye was prepared with 2 µL of the A component (Syto 9) and 2 µL of the B component (propidium iodide) for the fresh sealers, and 3 µL of the A and B components diluted in 15 mL of distilled water for the set sealers. The plates were incubated at room temperature in the dark for 15 min. After incubation, the specimens were rinsed with PBS, mounted on 0.14-mm thick glass coverslips (24×60 mm) and observed on an inverted microscope with filters at excitation of 490 nm and 546 nm wavelengths (Axio Observer A1; Carl Zeiss, Gottingen, Germany) with a 63× magnification. The images were captured and analyzed using ZEN 2.3 lite software (Carl Zeiss® Microscopy Ltd., Oberkochen, Germany).

Solubility

The initial mass of the specimens was measured in a precision scale (Micronal S/A, model AB 204 - São Paulo, SP, Brazil). Specimens were then immersed in 20 mL of deionized and

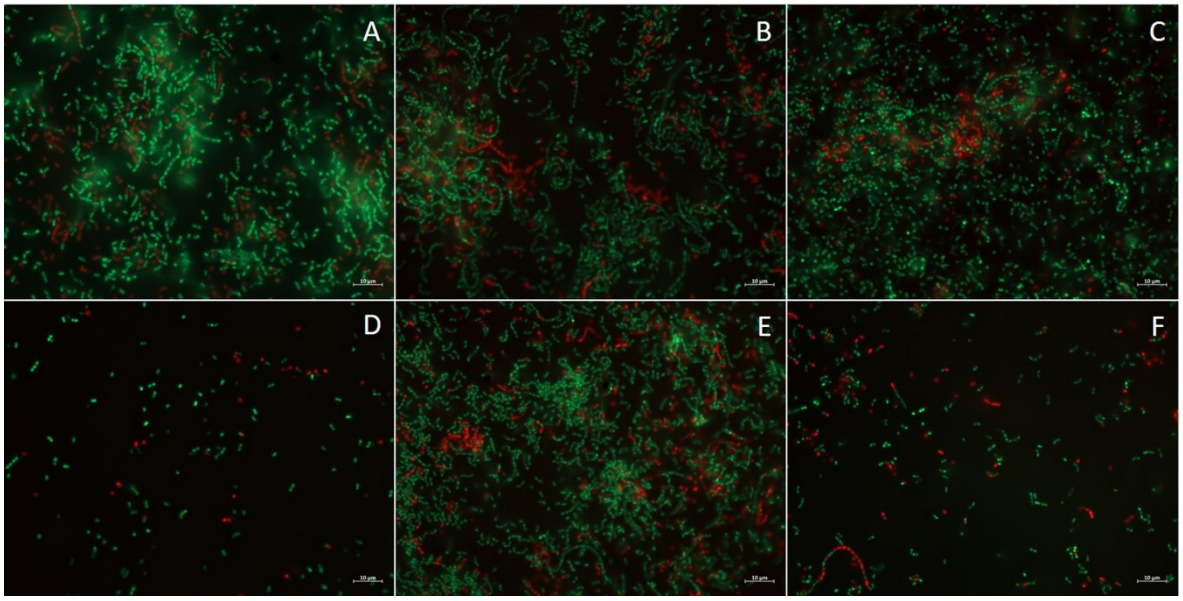

Figure 4. Epifluorescence microscopy of set sealers in contact with *Enterococcus faecalis* (magnification 63×); *A)* 0% AH-Plus; *B)* 2.5% AH-Plus; *C)* 0% Endomethasone N; *D)* 10% Endomethasone N; *E)* 0% Sealer 26; *F)* 10% Sealer 26

distilled water in polypropylene tubes (BD Falcon, Juiz de Fora, Brazil) suspended by a nylon thread so that samples did not touch the walls of the tube. The tubes were kept in an oven at 37°C for 24 h, and then the specimens were washed with distilled water and dried with absorbent paper. Afterwards, specimens were kept in a silica desiccator (Pyrex, Corning, NY, USA) for 48 h, and the final mass was measured to calculate the solubilization percentage [17].

pH variation

Based on the methodology of Vitti *et al.* [18], specimens were placed in sterile polypropylene tubes (BD Falcon, Juiz de Fora, Brazil) containing 10 mL of distilled water and stored at 37°C. The pH of the solutions was measured initially and after 6 and 24 h, and 7, 14 and 30 days using a previously calibrated digital pH meter (Ultrabasic, Denver Instrument Company, Arvada, Colorado, USA). The pH variation was calculated as the difference between the initial pH and the pH measured at each time-point.

Statistical analysis

Data distribution (Shapiro-Wilk) was first verified. For the analysis of the antimicrobial activity of the fresh sealers, the Kruskal-Wallis test was used followed by Dunn's post-test. For pH versus time analysis, Friedman's two-way analysis of variance for related samples was used (α=0.05). ANOVA followed by the Tukey's post-test was applied to the antimicrobial activity of the set sealers and solubility (α=0.05), using PASW Statistics software v.22.0 (SPSS, SPSS Inc., Chicago, IL, USA).

Results

Antibacterial activity

The control (0%) fresh sealers presented the inherent antibacterial activity and the nanomaterial-modified groups maintained this activity, totally inhibiting the growth of *E. faecalis* (P>0.05). Figure 1 shows that the median bacterial growth of all the experimental groups was zero and only the positive control (C +) presented statistical difference. This result was also observed qualitatively in epifluorescence microscopy images (Figure 2), where green fluorescence represents viable bacteria and red non-viable bacteria. In Figure 2A, a greater number of viable bacteria in the positive control group can be observed.

For set sealers, a greater inhibition of *E. faecalis* was found in 5 and 10% $AgVO_3$-modified Sealer 26 groups, and 2.5, 5, and 10% $AgVO_3$-modified Endomethasone N groups in relation to the control groups (P<0.05); the increased inhibition was proportional to the $AgVO_3$ concentration. On the other hand, the modified AH-Plus groups presented similar results to the control (P>0.05) (Figure 3). Epifluorescence microscopy images (Figure 4) showed more viable bacteria in the groups without the nanomaterial (Figures 4A, 4C, 4E), and a reduction of bacteria for the Endomethasone N and Sealer 26 groups modified with 10% $AgVO_3$ (Figures 4 D, 4F).

Solubility

In Table 1, AH-Plus and Sealer 26 did not present a significant difference (P>0.05) in solubility, and the Endomethasone N sealer modified with 5% $AgVO_3$ presented lower solubility in relation to the other groups (P<0.05).

pH variation

AH-Plus showed a significant difference in pH with time (Table 2) between the modified and control groups ($P<0.05$) except at 7 and 30 days ($P>0.05$). Within the same concentration of $AgVO_3$ (Table 2), pH varied in the initial time and in the final time of 30 days, all groups showing an acidic pH in relation to the initial pH.

For Sealer 26, a small pH variation occurred between groups, except for the 10% group, which presented a difference at 6 h ($P=0.001$), and 5% group, which presented a difference at 7 days ($P=0.024$). Comparing the pH variation within the same concentration of $AgVO_3$ (row), an increase in pH over time was observed, with significant difference at 6 h in relation to 7, 14, and 30 days ($P<0.05$) (Table 2).

For Endomethasone N, a greater variation in pH was found in relation to the time factor of groups modified with 5 and 10% $AgVO_3$ in relation to the control group. The 5% group had a significant difference between 24 h, 14 days and 30 days in relation to the control, and the 10% group at 7, 14 and 30 days ($P<0.05$). Within the same $AgVO_3$ concentration (row), there was a small increase in pH in all groups over time (Table 2).

Discussion

Endodontic sealers with ideal characteristics should have an antimicrobial effect and physicochemical properties that allow adequate root canal filling and success of treatment [20-22]. Although no sealer meets all the desired conditions, modified materials are an option in the search for the best properties [21, 22]. The association with antimicrobial agents can improve bacterial control, but studies have found varied responses. Thus, the addition of micro and nanoparticles are a viable alternative for the prolonged release of antimicrobial agents [1].

The results obtained with the addition of $AgVO_3$ to three endodontic sealers of distinct compositions led to the rejection of the null hypothesis that the nanomaterial does not increase the antibacterial activity or causes changes in the physicochemical properties of the sealers.

The direct contact test (DCT) is based on the direct contact between bacteria and the endodontic sealers without the influence of the soluble sealer components [23]. The test is a quantitative and reproducible method that can be used in

Table 1. Mean (SD) of Solubility of endodontic sealers modified with different concentrations of silver vanadate

Silver Vanadate	AH-Plus	Sealer 26	Endomethasone N
0%	0.061 (0.648)	-0.101 (2.271)	2.639 (1.424) a
2.5%	-0.003 (0.460)	0.138 (0.845)	2.354 (2.265) b
5%	0.470 (0.868)	0.843 (3.046)	0.017 (1.834) abc
10%	0.364 (0.967)	-0.378 (1.740)	2.847 (1.718) c

Similar letters represent statistical difference in column

Table 2. pH variation of endodontic sealers modified with different concentrations of $AgVO_3$

Sealer	Silver Vanadate	6 h	24 h	7 days	14 days	30 days	Mean of pH after 30 days
AH-Plus	**0%**	0.29 [0.19; 0.43] ^{Aab}	1.06 [0.94; 1.25] ^{Ac}	0.59 [0.25; 1.29] ^{Abc}	0.96 [0.36; 1.63] ^{Abc}	-0.13 [-0.45; 0.46] ^{Aa}	5.99
	2.5%	0.25 [0.18; 0.34] ^{Ab}	0.90 [0.82; 0.96] ^{Ac}	0.50 [0.33; 0.94] ^{Ac}	-0.41 [-0.59;-0.29] ^{ABba}	-0.58 [-0.74; -0.27] ^{Aa}	5.48
	5%	0.61 [0.50; 1.20] ^{Bb}	1.12 [0.91; 1.68] ^{Ac}	0.49 [0.16; 1.08] ^{Ab}	-0.74[-1.03;-0.26] ^{BCa}	-0.78 [-0.99; -0.13] ^{Aa}	5.43
	10%	0.38 [0.34; 0.42] ^{ABbc}	0.62 [0.51; 0.67] ^{Bc}	0.29 [-0.14; 0.78] ^{Ac}	-1.05 [-1.20; -0.94] ^{Ca}	-0.81 [-0.99; -0.18] ^{Ab}	540
Sealer 26	**0%**	2.90 [2.69; 3.04] ^{Aab}	2.73 [1.92; 2.94] ^{Aa}	4.03 [3.63; 4.46] ^{ABcd}	3.33 [3.04; 4.29] ^{Abc}	3.82 [3.45; 4.98] ^{Ad}	10.21
	2.5%	2.74 [2.43; 2.90] ^{ABa}	2.56 [2.01; 2.77] ^{Aa}	4.16 [4.01; 4.23] ^{ABc}	3.31 [3.08; 3.58] ^{Ab}	3.83 [3.24; 4.06] ^{Ab}	9.65
	5%	2.69 [2.64; 2.75] ^{ABa}	2.72 [2.69; 2.82] ^{Aa}	4.15 [4.07; 4.29] ^{Bc}	3.93 [3.53; 4.15] ^{Ab}	4.57 [3.84; 4.84] ^{Ac}	10.34
	10%	2.63 [2.57; 2.65] ^{Ba}	2.79 [2.66; 2.86] ^{Ab}	3.81 [3.48; 4.01] ^{Ad}	3.38 [2.96; 3.73] ^{Ac}	4.05 [3.42; 4.42] ^{Ad}	9.91
Endomethasone N	**0%**	0.33 [0.14; 0.80] ^{Aab}	0.32 [0.25; 0.46] ^{Aa}	0.37 [0.31; 0.42] ^{ABab}	0.45 [0.36; 0.48] ^{ABb}	0.43 [0.35; 0.46] ^{Aab}	7.19
	2.5%	-0.06 [-0.18; 0.01] ^{Aa}	0.26 [0.18; 0.32] ^{ABb}	0.45 [0.43; 0.48] ^{Bc}	0.46 [0.45; 0.48] ^{Ac}	0.34 [0.31; 0.37] ^{ABb}	7.13
	5%	-0.40 [-0.44; -0.27] ^{Ba}	0.10 [0.02; 0.24] ^{Bb}	0.16 [0.05; 0.35] ^{ACbc}	0.22 [0.17; 0.33] ^{BCc}	0.14 [0.09; 0.22] ^{BCb}	6,94
	10%	-0.33 [-0.36; -0.30] ^{Ba}	0.19 [0.15; 0.27] ^{ABd}	0.13 [0.09; 0.18] ^{Cc}	0.19 [0.16; 0.20] ^{Cd}	-0.06 [-0.12; 0.02] ^{Cb}	6,73

Median (confidence interval). ^{AB} Same capital letters represent statistical similarity in the column within the same type of sealer. ^{ab} Same lowercase letters represent statistical similarity in the line

different stages of setting [2]. The results showed that the antimicrobial activity of the endodontic sealers was higher soon after its mixing, which is expected since fresh sealers exhibit increased antimicrobial activity within 24 h against *E. faecalis* [2], and planktonic bacteria are less resistant than biofilms bacteria [6].

Endodontic sealers with antimicrobial activity have a maximum effect of 1 week, with a decrease of this property after complete setting; the addition of nanoparticles is an attempt to prolong this activity [5]. In this study, the $AgVO_3$-modified set sealers presented a higher inhibition of *E. faecalis* at 5 and 10% concentrations for Sealer 26, and at 2.5, 5, and 10% for Endomethasone N. An intrinsic antimicrobial effect was expected for these sealers due to their components, such as eugenol and zinc oxide in Endomethasone N [9], and calcium oxide in Sealer 26, since the release of hydroxyl ions promotes pH increase [2]. However, the negative control of the sealers did not present antibacterial effect, making the addition of $AgVO_3$ a promising alternative for the elimination of resistant bacteria.

The silver nanoparticles and vanadium nanowires of $AgVO_3$ act synergistically in reactions of oxidative stress and bond to the bacteria cell membrane causing their death [12]. It is also reported that nanoscale silver exhibits antibacterial potency similar or higher to broad-spectrum antibiotics, thus reducing bacterial adhesion and making biofilm formation difficult [4].

Epoxy resin sealers, such as AH Plus, exhibit antimicrobial activity due to bisphenol diglycidyl ether and the release of formaldehyde during polymerization reaction [2]. In addition, this sealer presents strong crosslinks between resin polymers [15]. This suggests that with setting of the material, the resin matrix of this sealer may hinder the release of the nanomaterial, since no bacterial inhibition was observed. The bonds among resin polymers are reported to contribute to the solubility resistance of this type of sealer [15].

The solubilization of endodontic sealers is desirable to a certain extent, because it may contribute to the release of the antimicrobial components and pH increase. However, an excess solubility of the material is detrimental [15, 21]. Endomethasone N presented higher solubility compared to AH-Plus and Sealer 26, and the addition of 5% $AgVO_3$ influenced this property by decreasing its solubilization. However, all groups presented a satisfactory solubility according to the recommendation of the American National Standards Institute/American Dental Association (ANSI/ADA, specification 57, 2000) of not exceeding ±3% of the total mass of the specimen [17]. Despite the observed solubility rates, the antimicrobial properties of all groups were preserved.

The antimicrobial action is also influenced by pH, since an alkaline pH assists in the elimination of persistent bacteria [18]. Sealers based on calcium hydroxide, such as Sealer 26, present basic pH due to the release of calcium ions, confirmed in this study at 30 days evaluation; no influence of $AgVO_3$ was observed. For Endomethasone N, the addition of 5 and 10% $AgVO_3$ caused a slight decrease of pH compared to the neutral pH of the control group at 30 days. The AH-Plus groups also presented an alkaline pH, which is an advantage, as a high pH that prevents mineralized tissue dissolution, neutralizes the lactic acid from osteoclasts and activates alkaline phosphatase, contributing to the material biocompatibility [21].

The biocompatibility of the modified endodontic sealers proposed in this study should be investigated, once nanoscale particles, such as silver nanoparticles, may have greater ease of penetration and cellular internalization, providing greater toxicity against bacteria. However, this reactivity can be harmful to biological tissues [22, 24].

As demonstrated in this study, the antimicrobial properties of both fresh and set endodontic sealers with varied compositions can be improved with the addition of $AgVO_3$ maintaining the physicochemical properties of solubility and pH within recommended standards. Thus, the use of this nanomaterial in clinical practice.

Conclusion

Freshly mixed sealers, 5 and 10% $AgVO_3$-modified set Sealer 26, and 2.5, 5, and 10% $AgVO_3$-modified set Endomethasone N, showed higher antimicrobial activity than control groups. Endomethasone N had a decreased solubility with the addition of 5% $AgVO_3$ and decreased pH with the addition of 5 and 10% $AgVO_3$. The other endodontic sealers evaluated in this study were not affected by the nanomaterial.

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Conflict of Interest: 'None declared'.

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