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ORIGINAL ARTICLE

Cytotoxicity and release ions of endodontic sealers incorporated with a silver and vanadium base nanomaterial

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Abstract

The modification of endodontic sealers with nanoparticles to confer antimicrobial activity allow greater effect, with interaction at a molecular level. The nanostructured silver vanadate decorated with silver nanoparticles ($AgVO_3$) is a nanomaterial unprecedented in dentistry for this application. This study incorporated the $AgVO_3$ into three endodontic sealers of different compositions and evaluate the cytotoxicity and release of compounds. The groups of commercially available AH Plus, Sealer 26, and Endomethasone N and groups of the same sealers with incorporated $AgVO_3$ (at concentrations 2.5, 5, 10%) were prepared, and extracts of the specimens were obtained for 24 h. The cell viability (cytotoxicity) of human gingival fibroblasts (HGF) was assessed after 24 h, 7 and 14 days. Silver (Ag^+) and vanadium (V^{4+}/V^{5+}) ion release was quantified after 24 h by ICP-MS. Data were analyzed by Kruskal–Wallis and Dunn's post-hoc ($\alpha = 0.05$). The cell viability was inversely proportional to treatment time. The Sealer 26 and Endomethasone N groups were cytotoxic for HGF cells, regardless of the incorporation of the $AgVO_3$ ($p > 0.05$), and the incorporation reduced cell viability of AH Plus ($p < 0.05$). The release of ions was proportional to the concentration of $AgVO_3$. AH Plus released more Ag^+ ions, and Sealer 26 and Endomethasone N releases more V^{4+}/V^{5+} ions. In conclusion, it was not possible to confirm the influence of $AgVO_3$ on HGF cell viability to Sealer 26 and Endomethasone N, however, nanomaterial influenced cell-viability to AH Plus, so the commercial sealers can be cytotoxic in synergy with the nanomaterial. The release of Ag^+ and V^{4+}/V^{5+} was proportional to the $AgVO_3$ incorporated.

Keywords Cytotoxicity · Cell viability · Ions · Nanoparticles · Root canal sealer

Introduction

The complete elimination of bacteria in endodontic treatments only with chemo-mechanical preparation is practically impossible. The complexity of the root canal system and virulence factors of microorganisms may cause treatment failure and subsequent need for retreatment [1, 2]. The use of obturation materials with antimicrobial activity can be advantageous in the reduction of persistent bacteria [1].

Modified endodontic sealers with various antimicrobial agents still present limited information regarding their biocompatibility, long-term maintenance of their properties, and antibacterial effect [2–4]. Advances in this area include the incorporation of nanoparticles, since nanomaterials present higher chemical reactivity due to the small size, with more atoms on their surface relative to the nucleus [5–8].

The nanostructured silver vanadate decorated with silver nanoparticles ($AgVO_3$) is an innovative proposal in dentistry for the modification of endodontic sealers, presenting advantages over the use of silver nanoparticles [9]. According to Holtz et al. [10], this hybrid nanomaterial combines the action of silver nanoparticles and vanadate nanowires, which dissociate into silver (Ag^+) and vanadium (V^{4+} and V^{5+}) ions that interact at the cellular level with bacterial cells. Silver nanoparticles have been applied in nanobiotechnology and are known for their antimicrobial potential, however, problems such as the cluster formation and color change limit its use [10–13].

Previous studies indicated an antimicrobial activity of endodontic sealers incorporated with $AgVO_3$ [14, 15], being necessary to evaluate the cytotoxic effect on human cells and

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the amount of Ag^+ and V^{4+}/V^{5+} ions that are released for future application in clinical practice. The released ions and other sealer components interact with the bacterial membrane and are thus essential for the antimicrobial efficacy of sealers. In contrast, these substances can lead to inflammatory reactions in the periapical cells, affecting biocompatibility [16–18].

Given this perspective, the $AgVO_3$ can contribute to the seeks an endodontic sealer with ideal physicochemical properties, biocompatibility, and antimicrobial effectiveness. However, its cytotoxic effect on human cells is still unknown. Therefore, the objective of this study was to incorporate the $AgVO_3$ to three endodontic sealers of different compositions, at concentrations: 2.5%, 5% and 10%; and evaluate the cell viability of human gingival fibroblasts (HGF) in contact with extracts of the specimens for 24 h, 7 and 14 days. The concentration of Ag^+ and V^{4+}/V^{5+} ions released in distilled water in 24 h was also assessed. The null hypothesis tested was that the cell viability of HGF and the amount of ions released are not affected by the concentration of the nanomaterial incorporated into the endodontic sealers.

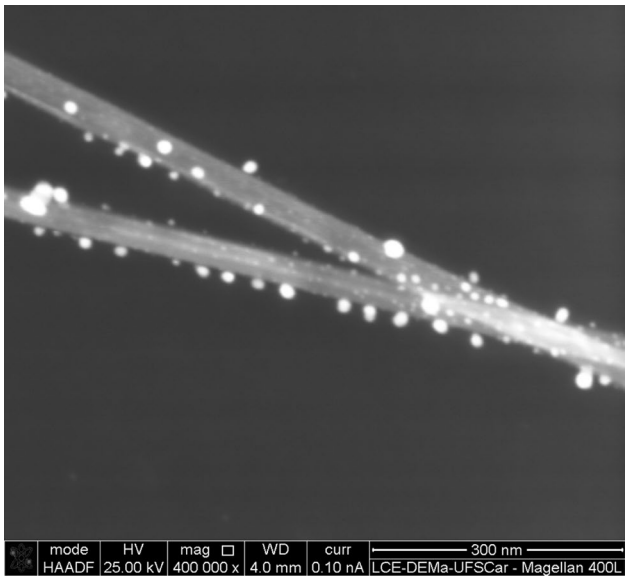

Fig. 1 Photomicrograph of the nanostructured silver vanadate decorated with silver nanoparticles ($AgVO_3$) obtained by scanning transmission electron microscopy (400,000× magnification)

Materials and methods

Specimens preparation

The $AgVO_3$ was synthetized by dissolving 1.3569 g of silver nitrate ($AgNO_3$, Merck, 99.8%; Merck KGaA, Darmstadt, Germany) and 0.9736 g of ammonium vanadate (NH_4VO_3; 99%; Merck KGaA, Darmstadt, Germany) each in 200 mL of distilled water, and then these solutions were mixed. The silver vanadate solution was vacuum filtered, washed with distilled water and absolute ethanol, and vacuum dried for 10 h [11, 19]. The morphology of the powder obtained was analyzed by scanning transmission electron microscopy (Magellan 400L, FEI Company, Hillsboro, OR, EUA), and demonstrated semi-spherical silver nanoparticles with an average size of 25 nm coated the nanowires of silver vanadate with dimensions nano and micrometric, and an average diameter of 150 nm (Fig. 1).

The endodontic sealers commercially available AH Plus, Sealer 26, and Endomethasone N were incorporated with $AgVO_3$ in concentrations of 2.5, 5 and 10% (by mass), based on previous pilot studies. A commercial group of each sealer without the incorporation of $AgVO_3$ were prepared. The powder or base paste of the sealers and the $AgVO_3$ were weighed on a precision scale, and mixed with their respective liquid or catalyst paste in an unpolished glass plate, to facilitate the incorporation of the nanomaterial (Table 1). The mixed sealers were inserted into silicon matrices (Zetalabor© Zhermack SpA, Badia Polenise, RO, Italy) of Ø7.75 × 1.5 mm and incubated in a stove (DeLeo, B2DG) at 37 °C for 7 days for setting [9].

Cell viability

The specimens ($n = 3$) were sterilized with ethylene oxide, and to obtain the extracts, the specimens were placed in polypropylene tubes (BD Falcon, Juiz de Fora, Brazil) with 436 µL of DMEM (Dulbecco's Modified Eagle Medium–Gibco, Thermo Fisher Scientific, Waltham, MA, USA) (3 cm^2/mL area-volume ratio, ISO 10993-5 Part 5: Cytotoxicity tests), and incubated at 37 °C in 5% CO_2 and 95% oxygen (Series, Shell Lab, Cornelius, OR, USA) for 24 h.

The culture of human gingival fibroblasts (HGF) was performed in DMEM supplemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil) and 1% penicillin and streptomycin (Sigma, St. Louis, MO, USA) at 37 °C in 5% CO_2 and 95% O_2 (Series). 3×10^3 cells were seeded per well of a 96-well plate, incubated for 24 h. The cells were treated for 24 h, 7 and 14 days with 200 µL of the specimens extracts, DMEM (without FBS) for the negative control, and distilled water for the positive control group ($n = 3$). After treatment, the wells were washed with PBS solution, and 500 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg of MTT/1 mL of DMEM without phenol red) was added for 4 h at 37 °C. The MTT solution was removed and dimethyl sulfoxide solvent (DMSO, Synth, Diadema, SP, Brazil) was added for 20 min

Table 1 Composition and quantities of silver vanadate and endodontic sealers used

Endodontic Sealer	Manufacturer	Composition	[$AgVO_3$][a]	Powder/paste A (g)	Powder $AgVO_3$ (g)	Liquid (drops)/paste B (g)
AH Plus	DENTSPLY, Konstanz, Germany	Epoxide paste: diepoxide, calcium tungstate, zirconium oxide, aerosil, pigment Amine paste: 1-adamantane amine, *N*,*N*'-dibenzyl-5-oxa-nonandiamine-1,9, TCD-Diamine, calcium tungstate, zirconium oxide, aerosil, silicone oil	Commercial	0.1300	–	0.1300
			2.5%	0.1267	0.0033	
			5%	0.1235	0.0065	
			10%	0.1170	0.0130	
Sealer 26	DENTSPLY, Petrópolis, Brazil	Powder: bismuth trioxide, calcium hydroxide, hexamethylene tetramine, titanium dioxide. Liquid: epoxy bisphenol	Commercial	0.0600	–	1 drop
			2.5%	0.0585	0.0015	
			5%	0.0570	0.0030	
			10%	0.0540	0.0060	
Endomethasone *N*	SEPTODONT Saint-Maur-des-Fossés, France	Powder: hydrocortisone acetate, thymol iodide, barium sulfate, zinc oxide and magnesium stearate Liquid: eugenol	Commercial	0.1453	–	2 drops
			2.5%	0.1416	0.0037	
			5%	0.1380	0.0073	
			10%	0.1307	0.0146	

[a][$AgVO_3$] concentration of nanostructured silver vanadate decorated with silver nanoparticles

at room temperature. Absorbance reading was performed on a spectrophotometer (SYNERGY MX Monochromator-based reader, Biotek, Winooski, VT, USA) at 550 nm. The experiment was performed in triplicate, and cell viability was reported as percentage, considering the negative control as 100% cell viability [20].

Release of metal ions

The release of Ag^+ and V^{4+}/V^{5+} ions was evaluated by inductively coupled plasma mass spectrometry (ICP-MS) on NexIon 300X equipment (PerkinElmer, Waltham, MA, USA) [21]. The samples were suspended by a nylon strand in polypropylene tubes (BD Falcon) with 9 mL of deionized water, and incubated at 37 °C for 24 h. Samples were then removed from the tubes and the liquid was quantitatively analyzed by calibration curves of the equipment. All experiments were performed independently in triplicate ($n=3$).

Statistical analysis

Data analysis was performed using the Kruskal–Wallis and Dunn's post-hoc ($p=0.05$), using SPSS software v 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Cell viability

All groups presented a reduction in cell viability of HGF in comparison to the negative control ($p<0.05$), regardless of treatment time and $AgVO_3$ concentration. The Sealer 26 and Endomethasone N groups presented a reduction of more than 95% in HGF cell viability regardless of treatment time. After 24 h of treatment, the AH Plus commercial group presented 55.17% of cell viability, which is greater than the cell viability of the AH Plus groups incorporated with $AgVO_3$ ($p<0.05$). However, after 7 and 14 days of treatment, all AH Plus groups presented a reduction of more than 95% in HGF cell viability. The Sealer 26 and Endomethasone N groups presented statistical similarity in comparison to the positive control after 24 h of treatment ($p>0.05$) indicating cytotoxicity. The AH Plus groups presented higher cell viability than positive control in 24 h treatment ($p<0.05$) (Table 2).

Release of metal ions

The release of Ag^+ and V^{4+}/V^{5+} was proportional to the concentration of $AgVO_3$ incorporated in the sealers. The commercial groups and the AH Plus groups had the lowest release and the Endomethasone N groups had the highest release, especially the 10% group.

Table 2 Cell viability (%) of human gingival fibroblasts treated with extracts of endodontic sealers incorporated with different concentrations of $AgVO_3$, for three time periods (24 h, 7 and 14 days), determined by MTT assay ($n = 3$)

Group	[$AgVO_3$]†	24 h (%)	7 days (%)	14 days (%)
Negative control		100.00^{A}	100.00^{A}	100.00^{A}
Positive control		4.42 [− 0.54; 8.95]^{A}	4.38 [3.52; 4.93]^{A}	3.01 [1.62; 4.26]^{A}
AH Plus	Commercial	55.17 [47.54; 64.94]$^{A, B}$	4.42 [2.18; 6.13]*$^{A, B}$	2.17 [− 1.34; 6.95]^{A}
	2.5%	48.74 [37.36; 57.35]$^{A, B}$	4.24 [3.56; 5.22]$^{A, C}$	3.33 [1.02; 5.17]^{A}
	5%	25.00 [14.90; 37.39]$^{A, B}$	2.86 [1.53; 4.69]*^{A}	3.21 [1.61; 4.75]^{A}
	10%	34.62 [7.33; 58.27]$^{A, B}$	2.34 [0.58; 4.51]$^{A, B, C}$	3.38 [2.54; 4.30]^{A}
Sealer 26	Commercial	1.16 [-6.78; 12.33]*$^{A, B}$	2.34 [1.90; 2.72]^{A}	1.44 [− 0.16; 4.00]^{A}
	2.5%	4.25 [− 4.11; 10.81]*$^{A, B}$	2.99 [2.58; 3.26]*^{A}	2.02 [− 0.68; 4.32]^{A}
	5%	2.55 [− 1.97; 5.40]*$^{A, B}$	2.89 [1.33; 4.80]*^{A}	2.65 [0.92; 4.28]^{A}
	10%	12.57 [− 4.17; 25.76]*$^{A, B}$	2.76 [2.41; 3.06]^{A}	2.17 [0.00; 4.56]^{A}
Endomethasone N	Commercial	0 [− 2.43; 3.90]*$^{A, B}$	2.89 [2.17; 3.83]$^{A, D}$	2.50 [− 0,09; 6.22]^{A}
	2.5%	0 [− 1.68; − 2.70]*$^{A, B}$	3.90 [3.36; 4.58]*^{A}	2.65 [0.19; 5.90]^{A}
	5%	0.68 [− 2.03; 4.08]*^{AB}	4.16 [0.15; 10.42]^{AD}	2.97 [1.12; 4.71]^{A}
	10%	1.53 [− 1.58; 6.29]*$^{A, B}$	3.90 [2.92; 5.29]*^{A}	3.26 [1.60; 5.17]^{A}

Negative control: DMEM; Positive control: distilled water

^{A,B}Equal capital letters represents statistical difference between groups in the same column ($p < 0.05$)

*Represents statistical similarity in comparison to the positive control in the same column ($p > 0.05$)

†[$AgVO_3$] concentration of nanostructured silver vanadate decorated with silver nanoparticles. Kruskal–Wallis and Dunn's post-hoc. Median [interquartile range]

The AH Plus and Endomethasone N incorporated with 10% of $AgVO_3$ presented the highest release of Ag^+ and significant difference of 2.5% group ($p < 0.05$). The Sealer 26–10% group released significantly more Ag^+ than the other groups of this sealer ($p < 0.05$), and the Sealer 26–2.5% group showed no statistical difference in comparison to the commercial group ($p > 0.05$) (Fig. 2).

The AH Plus groups incorporated with $AgVO_3$ presented no release of V^{4+}/V^{5+} ($p > 0.05$) and showed a statistical difference of Sealer 26 and Endomethasone N groups with and without nanomaterial incorporation ($p < 0.05$). The highest V^{4+}/V^{5+} release was significantly observed in the Sealer 26–10% and Endomethasone N—5% and 10% groups ($p < 0.05$) (Fig. 2).

Discussion

The modification of endodontic sealers to confer long-term effective antimicrobial activity requires the continued release of components and maintenance of properties and biocompatibility. In this study, the biocompatibility and ion release of endodontic sealers added with an $AgVO_3$-based nanomaterial was investigated. The antimicrobial activity and physicochemical properties of these sealers have been reported elsewhere [14, 15, 22]. These conventional root canal sealers, with different chemical compositions, were selected for the initial study with the incorporation of $AgVO_3$, because the objective was to verify the best type of composition to incorporate the nanomaterial. Besides, the AH Plus is considered the gold standard in endodontics; sealers based on zinc oxide and eugenol, such as Endomethasone, were the first types of sealer to appear in dentistry; and the Sealer 26 with calcium hydroxide and epoxy resin in the composition, it is a low cost sealer widely commercialized [23, 24].

The null hypothesis tested of this study was partially accepted, since the incorporation of $AgVO_3$ to the AH Plus sealer influenced HGF viability. However, the different concentrations of $AgVO_3$ incorporated in Sealer 26 and Endomethasone N sealers, did not affect cell viability compared to the commercial group, indicating that these endodontic sealers are cytotoxic for HGF cells, regardless of the modification. The cell viability was inversely proportional to the exposure time with the extracts, and the concentration of released silver and vanadium ions was proportional to the concentration of incorporated $AgVO_3$.

The cytotoxicity evaluation with extracts was performed according to ISO 10993-5 [25], which considers that a material is potentially cytotoxic if cell viability is reduced to less than 70% and has a cytotoxic effect if cell viability is reduced to less than 30%. All experimental groups presented HGF viability reduction compared to the negative control, including the commercial groups.

The biocompatibility of endodontic materials directly influences treatment success. The contact of materials with periapical tissues should not interfere with the repair process [5], but some induce irritation or degeneration of the surrounding tissues [18, 26]. Biocompatibility is determined

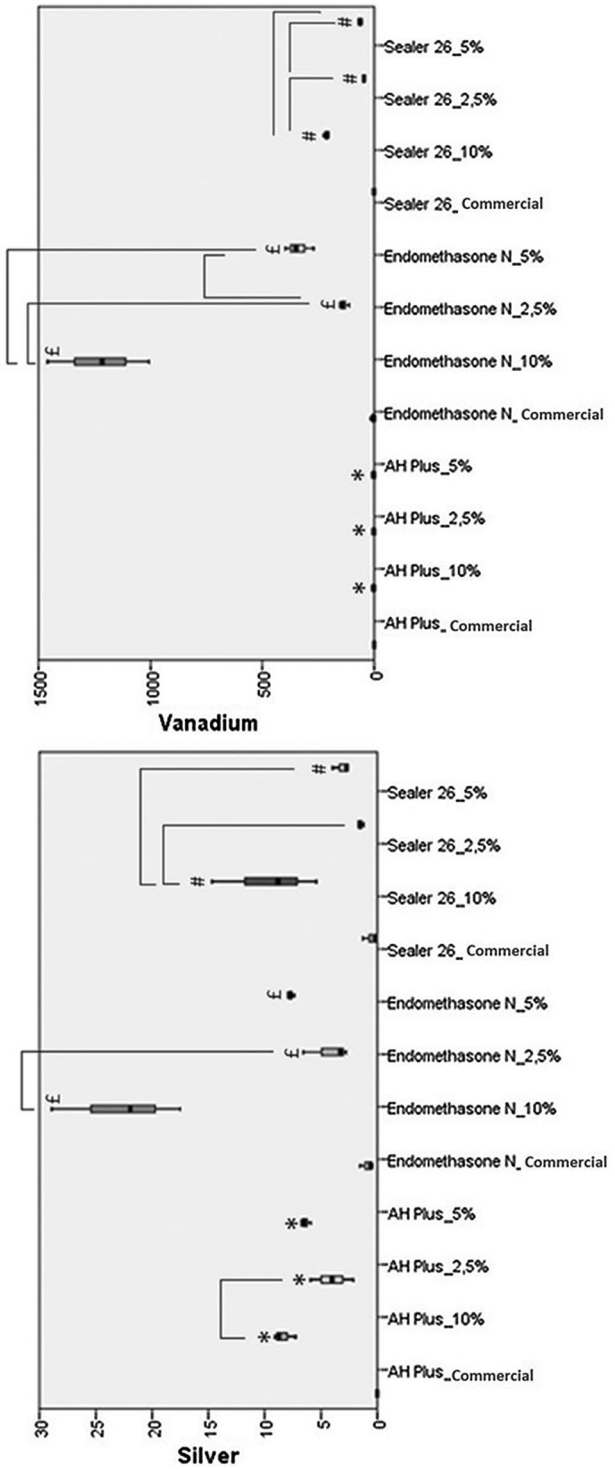

Fig. 2 Release Ag^{+} and V^{4+}/V^{5+} ions (µg/L) in distilled water for 24 h, of endodontic sealers incorporated with different $AgVO_3$ concentrations determined by ICP-MS (n = 3). Median [interquartile range]. *Represents statistical difference in comparison to AH Plus commercial group ($p < 0.05$). #Represents statistical difference compared to Sealer 26 commercial group ($p < 0.05$). £Represents a statistical difference compared to Endomethasone N commercial group ($p < 0.05$). ¯Represents statistical difference between the groups incorporated with $AgVO_3$ ($p < 0.05$)

by the physicochemical characteristics of materials (composition, element release, solubility, setting time) [27], and certain sealer components, such as zinc oxide, eugenol, formaldehyde, resinous components, and calcium hydroxide, have cytotoxic effects on human and animal cells [28–30].

The AH Plus groups had the lowest reduction of HGF viability, compared to the negative control, in 24 h of contact of the extracts with the cells, presenting only cytotoxic potential. The toxicity of AH Plus is attributed to the release of formaldehyde during the setting and to resinous components, such as amines that accelerate the polymerization reaction, besides the presence of bisphenol-A, epoxy resin component [16–18, 29]. With a longer exposure of cells to the extracts, all experimental groups showed a cytotoxic effect, with no significant difference among the commercial group, 2.5, 5 and 10% concentrations.

In addition to the cytotoxicity of sealers components, nanoparticles can also have this effect depending on the chemistry of their surface, since they interact with cellular DNA, in addition to proteins and lipids, which may damage the integrity of the membrane [31]. The incorporation of $AgVO_3$ increased the cytotoxic potential of AH Plus, compared to the commercial group in 24 h of treatment, an effect that can be attributed to silver. Once Artal et al. [32] evaluated the cytotoxicity of the $AgVO_3$ to *Daphinia similis*, an aquatic organism, and it found that the silver IC_{50} (concentration capable of inhibiting 50% of cell viability of *D. similis*) was 1.1 µg/L, while the vanadium IC_{50} was 1400 µg/L, proving the high cytotoxicity of silver. This study was to estimate that the silver IC_{50} for HGF was approximately 6.67 µg/L (based on the concentration of the ions released and the cell viability values for the AH Plus 10% in 24 h of treatment—Table 2 and Fig. 2). The silver IC_{50} estimated in this study is higher than the silver nanoparticle IC_{50} for HGF reported in the literature, whose concentration is approximately 0.04 µg/L [33, 34], giving preference to the use of $AgVO_3$.

Besides, the AH Plus commercial group caused a reduction in HGF cell viability in 44.83% in 24 h of treatment, while AH Plus 10% caused a reduction of 65.38% of HGF viability. Thus, the incorporation of 10% of $AgVO_3$ was responsible for reducing just 20.55% of HGF viability, acting in synergy with the components of the AH Plus commercial sealer.

For Sealer 26 and Endomethasone N, it cannot be affirmed that $AgVO_3$ influenced the cytotoxicity of these sealers since all the experimental groups had a cytotoxic effect. The observed cytotoxicity may be inherent to the components of these materials. The Sealer 26, is an epoxy resin-based sealer containing calcium hydroxide. This sealer came from a modification in the AH 26 (Dentsply), whose silver present in the original formulation was replaced by 20% of calcium hydroxide. Although it is an epoxy resin sealer as AH Plus, it is polymerization reaction is

characterized by polycondensation between the bisphenol epoxy resin and hexamethylenetetramine [24, 35]. Its initial irritating effect is due to the substances present in the formulation, mainly calcium hydroxide which, in contact with the culture medium, dissociates into calcium and hydroxyl ions, resulting in a higher pH [36, 37].

Endomethasone sealer (Septodont, Saint-Maur-des-Fossés, France) is based on zinc oxide and eugenol (ZOE) and has hydroquinone acetate in its composition to inhibit tissue inflammatory responses. Initially, this sealer contained paraformaldehyde, which despite the potent antimicrobial action, it was highly cytotoxic [26], inducing COX-2 expression and causing inflammation [30]. Endomethasone N was then developed with a formaldehyde-free composition. However, this sealer showed a cytotoxic effect for HGF in the present study, which may be due to the sealer base formula. ZOE extracts have been related to chronic inflammation of the periodontal ligament and shown to promote the release of signaling molecules that lead to bone resorption [18]. In addition, they have been reported to be cytotoxic for dental pulp stem cells, gingival fibroblasts, and keratinocytes [28], and released eugenol has a cytotoxic effect and might be related to the development of periapical lesions [18, 29].

Despite their cytotoxic effects, the release of chemical components of endodontic sealers is essential for the antimicrobial effect. Positively charged metal ions interact electrostatically with the negatively charged bacterial cell membrane, causing cell death [38]. The $AgVO_3$ presents this activity, since the silver ions and vanadium ions in the 5^+ state of oxidation (V^{5+}) interact with the thiol groups present in the enzymes of bacterial metabolism, forming stable complexes [11]. In addition, the contact of Ag^+ ions with bacterial DNA prevents its replication. Moreover, the oxidation–reduction reaction between V^{4+} and V^{5+}can cause oxidative stress to the bacterial cell [11].

In the present study, the concentration of released silver and vanadium ions was evaluated in the 24 h period, corresponding to the release time of the extracts for cell viability. It was found that the release was proportional to the concentration of $AgVO_3$ incorporated. The AH Plus modified groups showed the lowest concentration of released ions. This may have been due to the way this sealer is mixed; the manufacturer recommendations ask for equal proportions of the two pastes, and the higher proportion of resinous components could prevent the release of ions from the nanomaterial [39].

Sealer 26 and Endomethasone N released higher concentrations of vanadium ions, especially for the groups incorporated with 10% $AgVO_3$. The greater solubility of Endomethasone N, verified in a previous study [14], corroborates the findings of this study, and can be related to the higher cytotoxic effect. In this study, only the release of silver and vanadium was evaluated, however, the chemical elements of the original sealer might also influence the cytotoxicity, as mentioned previously.

Despite the low concentrations of Ag^+ released by the AH Plus groups, these were higher than the concentrations of V^{4+} and V^{5+}. Thus, the influence of $AgVO_3$ on the cytotoxicity of modified AH Plus groups could be attributed to silver, although present in low concentrations. For Sealer 26 and Endomethasone N sealers, high concentrations of vanadium ions were released. However, the cytotoxicity cannot be attributed to vanadium, as there was no statistical difference between groups with and without $AgVO_3$.

The results observed in this study support that, despite the effects observed on HGF viability, the original compositions of the commercial sealers are cytotoxic for HGF cells. In addition, the release of silver and vanadium ions assists in the antimicrobial activity of these sealers. Future investigations about genotoxic potential should be undertaken.

Conclusion

HGF cells viability was inversely proportional to the exposure time of the cells to the extracts. It was not possible to confirm the influence of $AgVO_3$ on HGF cell viability to Sealer 26 and Endomethasone N, however, nanomaterial influenced cell viability to AH Plus, so the commercial sealers can be cytotoxic in synergy with the nanomaterial. Nevertheless, the release of Ag^+ and V^{4+}/V^{5+} was proportional to the $AgVO_3$ incorporated, being essential for the maintenance of the antimicrobial effect.

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Author contributions The authors ABVT and ACDR contributed to the study conception and design. Material preparation, data collection and analysis were performed by ABVT, DTDC and MAS. The first draft of the manuscript was written by ABVT and ACDR. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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