Evaluation of the cell viability and antimicrobial effects of orthodontic bands coated with silver and zinc oxide nanoparticles: an *in vitro* study

Behrad Tanbakuchi[1], Maryam Pourhajibagher[2], Alireza Badiei[3], Reza Masaeli[4], Rashin Bahrami[1]*

1- Department of Orthodontics, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

2-Dental Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran

3- School of Chemistry, College of Science, University of Tehran, Tehran, Iran

4- Department of Dental Biomaterials, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran.

*** Correspondence to:**
Rashin Bahrami. Orthodontic resident, Department of Orthodontics, School of Dentistry, Tehran University of Medical Sciences, North Kargar St, Tehran, Iran. Tel: +989370053618; Email: bahramirashin@yahoo.com

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Abstract

Objective: The present study evaluated the cell viability and antimicrobial effects of orthodontic bands coated with silver and zinc oxide nanoparticles. **Methods:** In this experimental study, thirty orthodontic bands were divided into three groups (n=10) of control (uncoated band), silver (Ag) coated band and zinc oxide (ZnO) coated band. The electrostatic spray-assisted vapor deposition method was used to coat silver (Ag) and zinc oxide (ZnO) nanoparticles on orthodontic bands. The biofilm inhibition test assessed the antimicrobial effectiveness of nano-Ag and nano-ZnO against *Streptococcus mutans, Lactobacillus acidophilus*, and *Candida albicans*. Biocompatibility tests were carried out using an MTT assay. A One-way ANOVA with a Post-hoc test was used for the comparison between groups.

Results: The reduction in the number of *L. acidophilus, C. albicans, and S. mutans* colonies around nano-Ag coated bands was significantly higher than around nano-ZnO coated bands (P= 0.015, 0.003, and 0.005, respectively). Compared to the control group, Ag showed a reduction of all microorganisms' ability to replicate at least 2 $\log_{10}$ steps; but ZnO, except for *S. mutants*, reduced the other two microorganisms to less than 2 $\log_{10}$ steps. The lowest cell viability mean was observed in nano-Ag coated bands, but the difference between the groups was insignificant (P>0.05).

Conclusion: Coating orthodontic bands with ZnO and Ag nanoparticles induced antimicrobial properties against oral pathogens. Among these nanoparticles, the Ag group showed the best antimicrobial activity and the ZnO group showed the highest biocompatibility.

Keywords: Biocompatibility, SEM, Microbiology, Biomaterial science

Introduction:

Oral hygiene is greatly complicated following the placement of fixed orthodontic appliances[1]. Molar bands in place are considered for nearly all patients experiencing fixed orthodontic therapy over the whole treatment period, which normally lasts 1.5 to 2 years[2].

Microbiologically, the oral ecosystem may be altered by the insertion of bands through incrementing the number of cariogenic microorganisms (*Lactobacillus acidophilus* and *Streptococcus mutans*), accumulation of plaque, and improvement of *Candida albicans* colonization, and reducing plaque pH[3]. Clinically, such ecological alterations cause a higher occurrence of oral soft tissue and white spot lesions disease[4]. White spots can be formed around orthodontic attachments into treatment as early as less than a month[5].

Dental biofilm is removed through various regular prevention methods, including mechanical biofilm removal, tooth brushing, and antimicrobial or antiplaque agents[6]. Nonetheless, these approaches may not entirely remove microorganisms, and biofilm formation may not be prevented. Thus, drug-resistant microorganisms will exist, possibly disrupting the natural bacterial flora[7].

White spot lesions during orthodontic treatment have a documented etiology. Briefly, the accumulation of plaque and food around brackets, bands, wires, and other attachments caused decreased pH and increased *S. mutans* colonization, which led to the possibility of clinical demineralization[8-10].

Several traditional prevention approaches for dental biofilm removal include tooth brushing, mechanical biofilm removal, and antiplaque or antimicrobial agents[11]. Still, these methods might not get rid of all microorganisms or stop biofilms from forming. They might also cause drug-resistant organisms to grow, which would mess up the natural bacterial flora[12].

Thus, methods with minimum side effects and maximum benefit are preferred clinically; several ways, such as surface treatments of the metal appliance, including coating with nanoparticles, have been used to decrease or prevent bacterial aggregation around the teeth[13]. Because of their small size, surface-to-volume ratio, and increased contact with external environments, metal nanoparticles have many antimicrobial properties[14].

Previous studies have shown that Ag nanoparticles have more antimicrobial activity than other metal nanoparticles[15]. Studies have evaluated the cytotoxicity of Ag nanoparticles against fungi, protozoa, Gram-negative and Gram-positive bacteria such as *S. mutans* and *L. acidophilus*. As Ag nanoparticles were confirmed to possess antimicrobial properties, especially against *S. mutans*, they were used as an antimicrobial additive in dental materials[16, 17].

ZnO has significant antimicrobial properties against Gram-positive and Gram-negative bacteria and is an essential mineral for humans. ZnO nanoparticles have been for have antimicrobial properties and to be safe for humans and non-polluting to the environment due to their use as an antimicrobial agent[18].

Furthermore, coating orthodontic bands with ZnO and Ag nanoparticles does not reduce the number of bacteria in the oral cavity but may lead to less colonization and plaque formation on these bands. Since these bands come into contact with the oral mucosa and fluids for a long time, they must be biocompatible.

The antimicrobial properties and biocompatibility of orthodontic brackets and wires coated with Ag or ZnO nanoparticles have been studied[19]. However, studies on bands and comparisons between the two nanoparticles are lacking.

This study aimed to evaluate and compare the cell viability and antimicrobial effects of orthodontic bands coated with Ag and ZnO nanoparticles since no study has ever assessed both the antimicrobial and biocompatibility properties of Ag and ZnO nanoparticles simultaneously.

Method and material:

This was an experimental, *in vitro* study.

- **Ag and ZnO nanoparticles:**

The nanoparticle was supplied from the Pishgaman Iranian Nanomaterials Company. X-Ray Diffraction (XRD; TESCAN MIRA3, Australia) was used to confirm the nature of the nanoparticles. Nanoparticle size and shape were confirmed using field emission scanning electron microscopy (FESEM; TESCAN MIRA3, Australia, 15 kV accelerating voltage) and transmission electron microscopy (TEM; TESCAN MIRA3, Australia).

- **Coating procedure:**

One group was not coated and served as the control group, but other groups included the bands coated with Ag or ZnO nanoparticles. The electrostatic spray-assisted vapor deposition method was used to coat Ag and ZnO nanoparticles on orthodontic bands (American Orthodontics, Sheboygan, WI, USA). The ZnO suspension was prepared by mixing 0.1 g of ZnO powder with 3 mL of acetone; Ag suspension was prepared similarly. The suspension was pumped at a flow rate of 10 mL/hr using a syringe pump at a distance of 3 cm from the bands. An input voltage of 8 kV was applied at the nozzle tip and counter electrode. FESEM and energy-dispersive X-ray spectroscopy (EDX) tests were used to confirm the presence of a nanoparticle coating on the surface of the bands.

- **The coating binding:**

To check the adhesion of the coating to the surface of the band, a simulator of the regular oral hygiene procedures such as brushing was performed. In this way, after confirming the presence of nanoparticles on the band's surface (by FESEM), the coated band was immersed in 2 mL of artificial saliva for 30 days; specimens were brushed with a soft tooth brush using distilled water for 2 minutes, twice daily, over one month. Then, FESEM was used to re-examine the nanoparticles' presence on the bands' surface.

Microorganisms and growth conditions:

The strains of *S. mutans* ATCC 35668, *L. acidophilus* ATCC 314 and *C. albicans* ATCC 14053 were obtained from the Pasteur Institute of Iran. *S. mutans and L. acidophilus* were incubated in Tryptic Soy Broth (TSB; Merck, Germany) in the presence of 5% CO_2 and *C. albicans* in brain heart infusion (BHI) broth medium (Merck, Germany) under aerobic conditions for 48 hours at 37°C.

- **Antimicrobial assay:**

The microbial suspensions were added to the bands in the tubes with a concentration of 0.5 McFarland (1.5×10^8 colony forming unit (CFU)/mL for *S. mutans* and *L. acidophilus*; 1.5×10^5 CFU/mL for *C. albicans*), and then incubated at 37 °C according to the growth conditions of each microorganism to form the microbial biofilms.

After 48 hours of incubation, the bands were washed under aseptic conditions in 1 mL of sterile normal saline to remove microorganisms in the planktonic phase and microorganisms with loose bonding. The bands were then placed in tubes containing 1 mL of sterile BHI and vortexed at high speed for 1 minute to separate the microbial biofilm from the surface of the bands. The obtained microbial suspensions were serially diluted and 10-µL aliquots were inoculated into BHI agar (Merck, Germany). The plates containing *S. mutants* and *L. acidophilus* were incubated at 37°C in the presence of 5% CO_2, and plates containing *C. albicans* were aerobically incubated. After 24 hours, the CFU/mL of each sample was calculated[20]. The experiment was repeated three times.

- **MTT [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay:**

MTT assay was performed as outlined in ISO 10993-5[21]. Human gingival fibroblasts (10459 HGF, CELL NO.IBRCC) were seeded in 96 well plates using Dulbecco's modification of Eagles medium culture medium (Idehzist, Iran) containing 10% Fetal Bovine Serum (Capricorn, Germany) and 1 % penicillin/streptomycin (Biosera,

France). The cell density was 10×10^6 cell/well. The cells were incubated for 24 hours at 37°C and %5 CO_2 humidified atmosphere.

To evaluate the indirect cytotoxicity of coated bands, the extracts were prepared by incubating the samples with a medium containing serum at an extraction ratio of 0.75 cm^2/mL for 24 hours (Figure 1). The experiment was repeated three times.

After 24 hours of incubation with eluents, the eluents were removed from each well, and 40 µL of MTT solution (5 mg/mL MTT [Sigma, Germany] in phosphate saline) was added followed by reincubation for 3-4 hours at 37°C and %5 CO_2. Finally, the MTT solution was removed, 60 µL of DMSO solution was added to each well, and the absorbance was determined at 570 nm using Microplate Reader (BioTek, USA).

For evaluate the morphological changes of HGF, the morphology of the cells that were not in contact with the band was considered to be normal, and that of the cells in contact with it was compared to that.

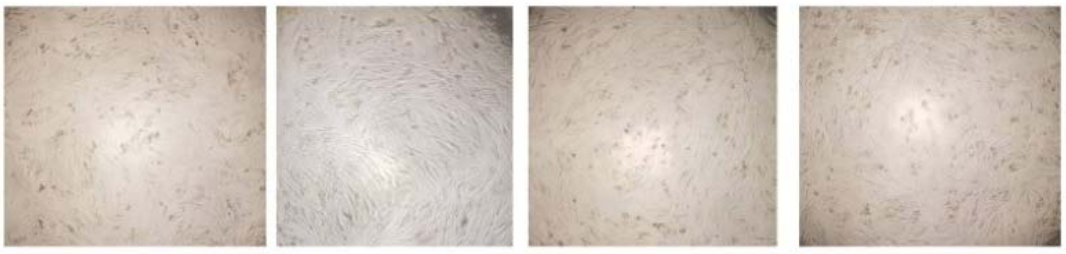

Figure 1: Cells after addition of eluents - before MTT

- **Statistically analysis:**

Statistical Package for the Social Sciences version 29.0 software (SPSS Inc., Chicago, Illinois, USA) was used to analyze the data. Descriptive statistics were used to describe the data and one-way ANOVA, repeated measures analysis of variance, and post hoc Games-Howell and Tukey tests were used to analyze the data. The level of significance was set at $P < 0.05$.

Results:

Confirmation of synthesized Ag and ZnO nanoparticles:

The size and morphology of Ag and ZnO nanoparticles were shown in Figures 2 and 3. As demonstrated in Figures 2a and 3a, the spherical morphology and uniform shape were dominant and the distribution of particles was visually acceptable. According to the results of FESEM, the particles are smaller than 60 nm (Figures 2b and 3b). The XRD diagram of each nanoparticle confirms its nature (Figures 2c and 3c).

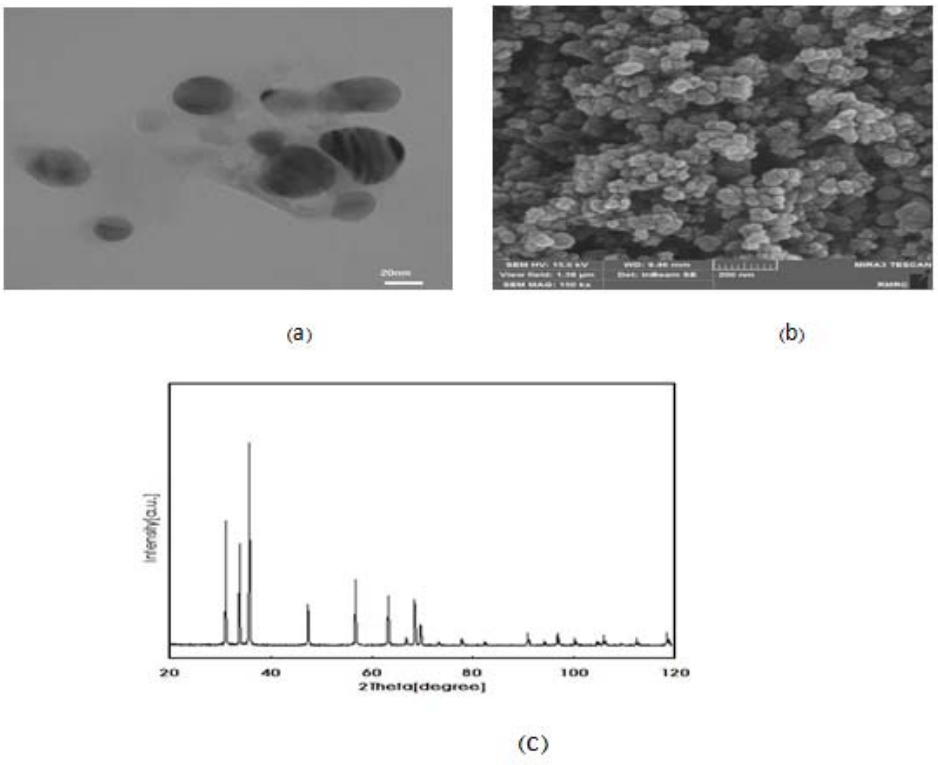

Figure 2: Characterization of synthesized Ag nanoparticles; a. TEM image of Ag nanoparticles in optimal conditions (scale bar 20 nm), b. FESEM image of Ag nanoparticles in optimal conditions (scale bar represents 200 nm), c. XRD graph of Ag nanoparticles.

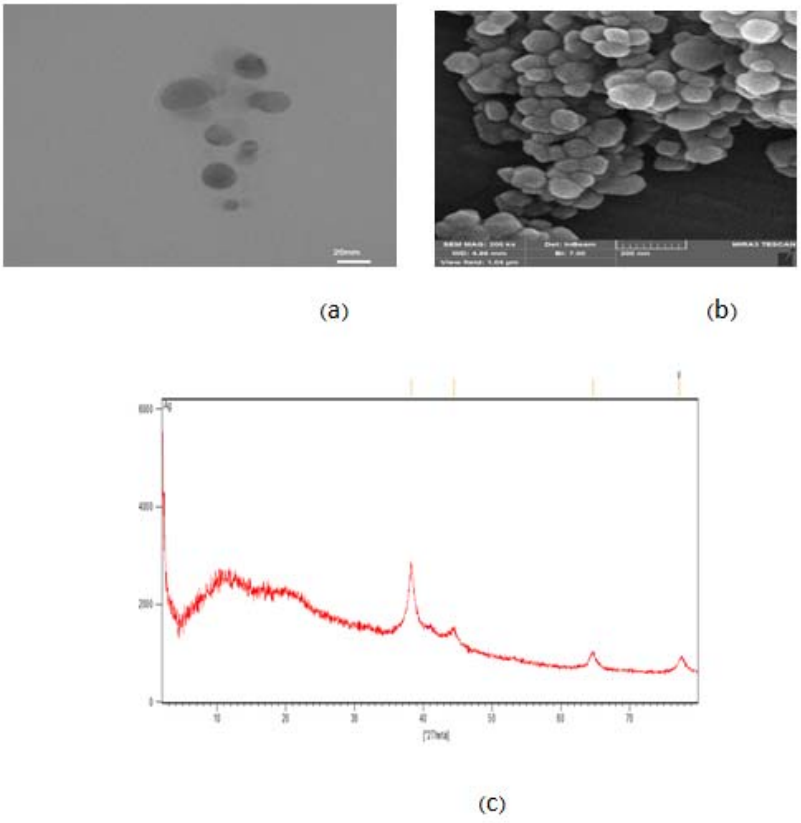

Figure 3: Characterization of synthesized ZnO nanoparticles; a. TEM image of ZnO nanoparticles in optimal conditions (scale bar 20 nm), b. FESEM image of ZnO nanoparticles in optimal conditions (scale bar represents 200 nm), c. XRD graph of ZnO nanoparticles.

Band surface evaluation:

FESEM and EDX:

FESEM images of nano-Ag and nano-ZnO coated bands show that these particles are present on the band surface (Figures 4a and 5a). The EDAX spectra for the stainless-steel bands coated with Ag and ZnO nanoparticles, respectively, showed the presence of Ag and Zn ions in addition to the normal composition of the band (Figures 4b and 5b). The value of Ag is estimated to be 62.53% by weight and the value of Zn is estimated to be 64.02% by weight. After brushing, the FESEM views confirmed the nanoparticles' presence on the band surface.

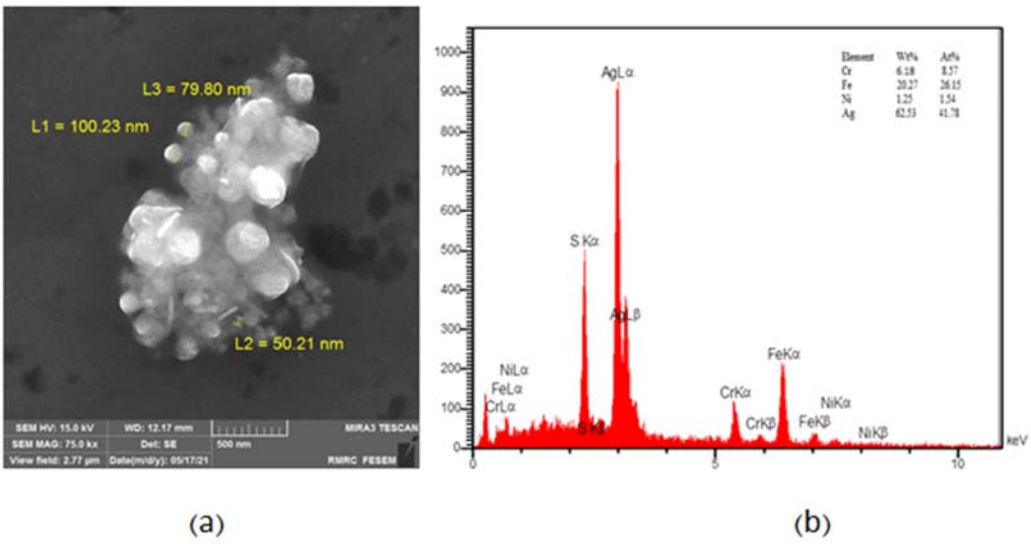

Figure 4: Nano-Ag coated band surface; (a) FESEM images; (b) EDX analysis.

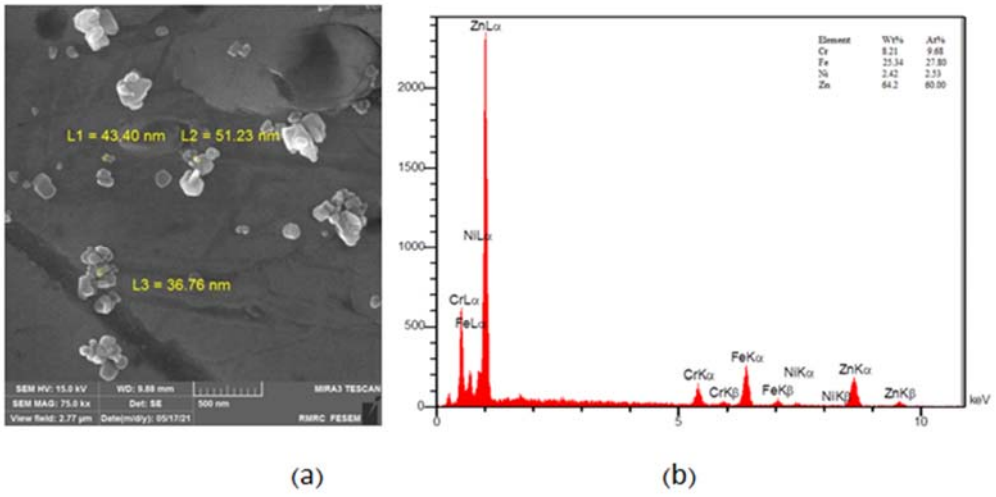

Figure 5: Nano-ZnO coated band surface; (a) FESEM images; (b) EDX analysis.

Antimicrobial effect:

According to the results (Figure 6 and Table 1), the mean number of grown *L. acidophilus* colonies in culture medium containing nano-Ag coated bands was 4.3 ± 0.55 × 10^7 CFUs/mL, in culture medium containing nano-ZnO coated bands 31.0 ± 4.35 × 10^7 CFUs/mL and in culture medium containing uncoated bands 656.6 ± 61.1 × 10^7 CFUs/mL. Mean number of grown *C. albicans* colonies in culture medium containing nano-Ag coated bands 2.96 ± 0.71 × 10^4 CFUs/mL, in culture medium containing nano-ZnO coated bands 111.0 ± 8.18 × 10^4 CFUs/mL and in culture medium containing uncoated bands 460.0 ± 55.7 × 10^4 CFUs/mL. The mean number of

grown *S. mutans* colonies in culture medium containing nano-Ag coated bands, nano-ZnO coated bands and uncoated bands were 2.6 ± 0.7 × 10^6, 46.3 ± 4.51 × 10^6 and 6166.7 ± 1106.04 × 10^6 CFUs/mL, respectively (Table 1). The difference between all groups was significant (P<0.05).

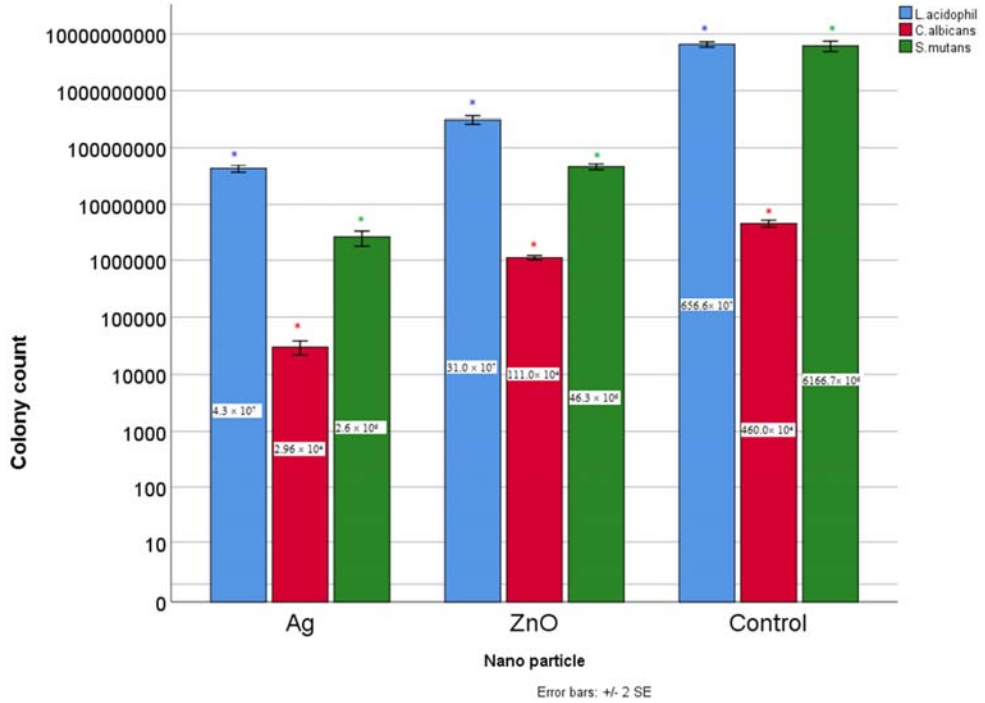

Figure 6: Colony count of microorganism (CFUs/mL). *Significantly different with P<0.05.

Table 1:
Descriptive values of colony count for each microbial strain in three groups (CFUs/mL)

Groups	Dependent variable	Maximum	Minimum	Median	Mean	Standard Deviation	95% Confidence Interval of the Difference	
							Lower bound	Upper bound
Ag	***L. acidophilus***	4.9×10^7	3.8×10^7	4.3×10^7	4.3×10^7	0.55×10^7	2.9×10^7	5.7×10^7
	C. albicans	3.6×10^4	2.2×10^4	3.1×10^4	2.96×10^4	0.71×10^4	1.2×10^4	4.7×10^4
	S. mutans	3.3×10^6	1.9×10^6	2.5×10^6	2.6×10^6	0.7×10^6	0.82×10^6	4.3×10^6
ZnO	***L. acidophilus***	36.0×10^7	28.0×10^7	29.0×10^7	31.0×10^7	4.35×10^7	20.1×10^7	41.8×10^7
	C. albicans	118.0×10^4	102.0×10^4	113.0×10^4	111.0×10^4	8.18×10^4	90.7×10^4	131.3×10^4
	S. mutans	51.0×10^6	42.0×10^6	46.0×10^6	46.3×10^6	4.51×10^6	35.1×10^6	57.5×10^6
Control	***L. acidophilus***	710.0×10^7	590.0×10^7	670.0×10^7	656.6×10^7	61.1×10^7	504.9×10^7	808.4×10^7
	C. albicans	520.0×10^4	410.0×10^4	450.0×10^4	460.0×10^4	55.7×10^4	321.7×10^4	598.3×10^4
	S. mutans	7200.0×10^6	5000.0×10^6	6300.0×10^6	6166.7×10^6	1106.04×10^6	3419.1×10^6	8914.2×10^6

- **Antimicrobial effect in comparison to the control group:**

Compared to the control group, 3.4 and 2.14 $\log_{10}$ step reduction of *S. mutans* were observed for nano-Ag and nano-ZnO coated bands, respectively; also, 2.18 and 1.36 $\log_{10}$ reduction in the number of *L. acidophilus* were shown for nano-Ag and nano-ZnO coated bands comparison to the control group, respectively. 2.2 and 0.6 $\log_{10}$ steps of reduction in the number of *C. albicans* were calculated for nano-Ag and nano-ZnO coated bands in comparison to the control group, respectively.

Cell viability:

Figure 7 shows the mean cell viability in each group; the lowest mean cell viability was found in Ag nanoparticles group (0.42 ± 0.02), followed by ZnO nanoparticles group (0.45 ± 0.02), and the control group (0.48 ± 0.04). As relative percentages of untreated control (100%), cell viability in uncoated, nano-ZnO, and nano-Ag coated bands groups were 98%, 91.8%, and 85.7%, respectively. The mean cell viability of nano-Ag coated bands was lower than the other two groups; but there were no significant differences among groups ($P>0.05$).

The control sample of a monolayer of 10459 HGF fibroblast cells (normal morphology) was observed in the indirect contact assay using monolayer cultures of

the 10459 HGF cell line (Figure 8a). In contact with the nano-Ag and nano-ZnO coated bands, the morphology of the cells did not change (Figures 8b and 8c).

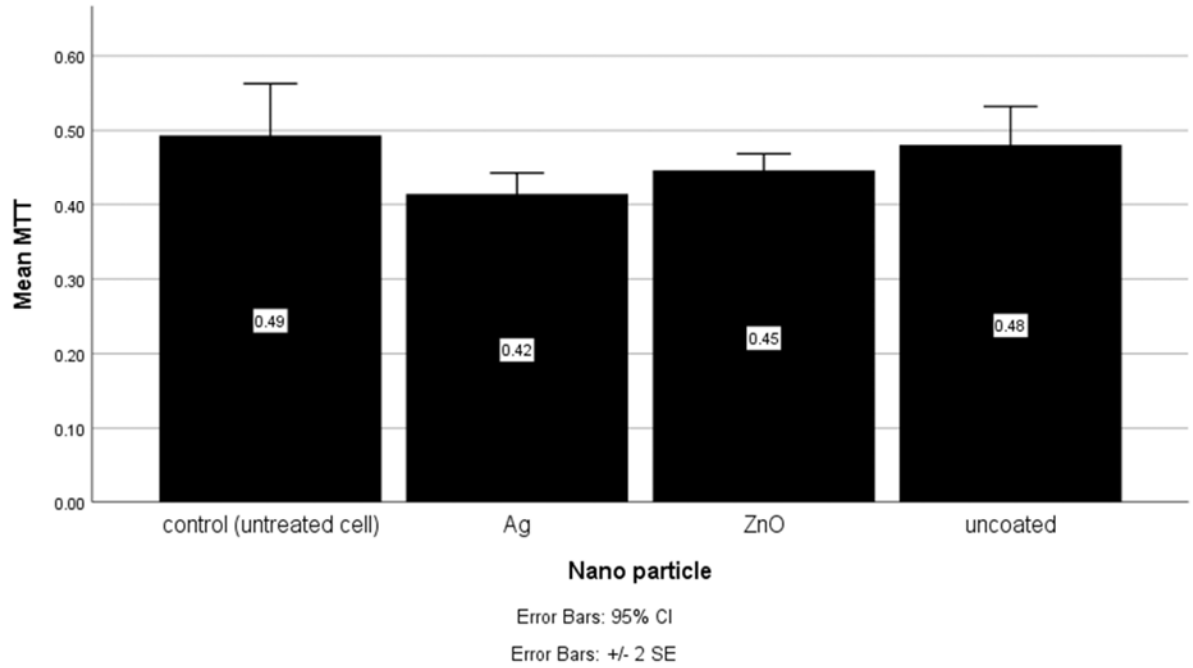

Figure 7: Mean cell viability in each group.

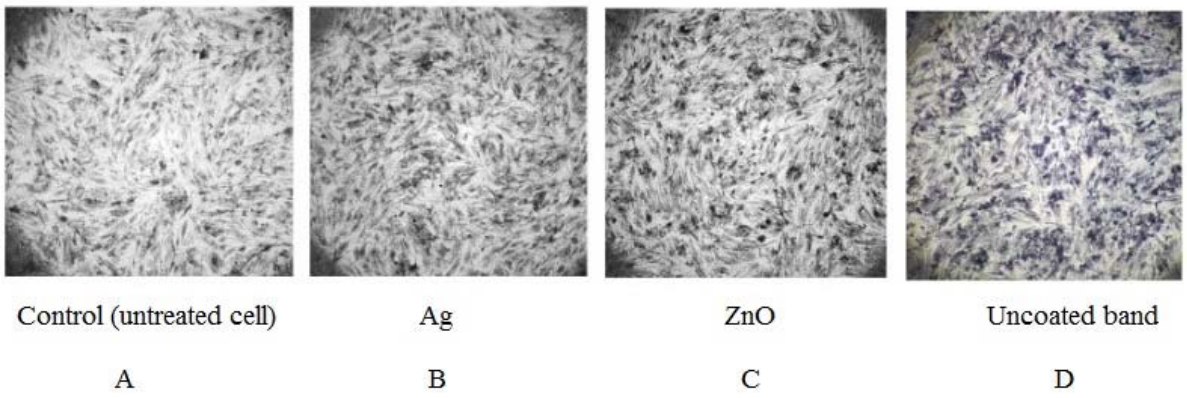

Figure 8: Monolayer culture of the 10459 HGF cell line was used for indirect contact assay. A) The control sample consists of a confluent layer of fibroblast cells. The majority of cells are spindle-shaped, which is considered normal. B) The nano-Ag coated band sample did not change the morphology of the cells when in contact with the 10459 HGF confluent layer. C) The 10459 HGF cells contacting the nano-ZnO coated band also showed the normal morphology of the cells.

Discussion:

Oral hygiene maintenance has long been an important challenge in fixed orthodontic treatment. It has led clinicians to search for methods that are less dependent on patient cooperation. The use of fluoride-releasing materials is appropriate for caries-prone patients, but they are primarily used in dental offices, and their number of uses is also limited[22].

Some studies have assessed the antibacterial properties of nanoparticles[23- 26]. Adding nanoparticles to orthodontic adhesives can be problematic and negatively effect on mechanical properties[27, 28]; When ZnO nanoparticles containing Ag ions are used in orthodontic composites, the shear bond strength is decreased[29]. The discoloration of composite resins caused by Ag nanoparticles (1 mM) is problematic for dental applications[30]. For these reasons, nanoparticles have recently been added to orthodontic metal components. But it should be noted that different properties of nanoparticles (such as size, shape, consistency of particles, and surfactant types) can affect the antimicrobial efficacy of nanoparticles[31, 32]; therefore, if added to different materials, their properties should be re-evaluated.

Among the fixed metal orthodontic appliances, orthodontic bands should be mentioned, which remain in the mouth from the very beginning through the very end of treatment; Due to the large area of the bands compared to the bonded attachments and also due to the food being trapped around the bands, having an orthodontic band with antimicrobial properties makes it possible to prevent tooth decay and oral lesions.

In this study, stainless steel orthodontic bands were coated with ZnO and Ag nanoparticles to produce a potent antimicrobial effect against *S. mutans, L. acidophilus*, and *C. albicans*; also, the present study evaluated the cell viability of these bands.

According to the results of the present study, the reduction in the number of all three microorganisms' colonies (*S. mutans, L. acidophilus, and C. albicans*) around nano-Ag coated bands (0.1 g nano-Ag in 3 mL acetone) was significantly higher than those of nano-ZnO coated bands (nano-ZnO in 3 mL acetone) and control group.

Statistically, the decrease in the number of colonies of all three microorganisms by both nanoparticles was significant compared to the control group; but clinically, to use the term 'antimicrobial', dental materials need to show a reduction in bacterial ability to replicate at least 2 $\log_{10}$ steps) compared with the control group[33]. According to this, the term antimicrobial can be used for nano-Ag coated band because at least 2 $\log_{10}$ step reduction in the number of all three microorganisms' colonies was observed compared to the control group. The term 'antimicrobial' for nano-ZnO coated band only applies to *S. mutans* because the reduction of *L. acidophilus* and *C. albicans* has been less than 2 $\log_{10}$. For the bands, smaller reductions may have clinical relevance as well.

On the other hand, nano-Ag biocompatibility was lower than nano-ZnO coated bands, but this difference was not significant (0.42 ± 0.02 vs. 0.45 ± 0.02, $P>0.05$). This result agreed with the study of Hernández-Sierra et al. (2008), in which they evaluated the effects of Ag (25 nm), and ZnO (125 nm) nanoparticles on *S. mutans* and reported that the antibacterial activity of Ag nanoparticles is much higher than those of ZnO and gold nanoparticles[34].

The results of this study were contrary to the other studies[35- 37]; Kasraei et al. (2014) concluded that ZnO had a more significant antimicrobial effect than Ag against *S. mutans*, but there was no difference between the two nanoparticles against *L. acidophilus*[35]. But, in the present study, a significant difference was found in the mean colonies of *S. mutans* and *L. acidophilus* in a culture medium containing nano-Ag coated bands compared to a culture medium containing ZnO coated bands. This difference in results may be due to differences in size and concentration of the nanoparticles; they add 1% ZnO and 1% Ag nanoparticles with an average particle size of 50 nm and 20 nm in composite, respectively. Reducing the particle size increases the specific surface area of a dose of nanoparticles. Hence, more interaction of significant material is allowed with the surrounding environment. Besides, cell wall penetration is facilitated by the smaller particle sizes. So, the antimicrobial effect of substances like Ag and ZnO, which are naturally antimicrobial, is enhanced by increasing the surface/volume ratio.

Hailan et al. (2019) showed a reduction in the number of *S. mutans* colonies around primer discs containing ZnO and Ag nanoparticles, which agreed with our results. However, in their study, the antimicrobial properties of ZnO nanoparticles against *S. mutans* were significantly higher than Ag nanoparticles, which was inconsistent with the results of the study[36]. These differences might be attributed to the size of the applied nanoparticles; in their study, the particle size of ZnO nanoparticles (50 nm) was relatively smaller than the size of Ag nanoparticles (80 nm); while in the present study, the average size of both nanoparticles was 20 nm. The concentration of both nanoparticles in the study of Hailan et al. was 1% added to the primer. In the present study, the bands were coated with 0.1 g of Ag and 0.1 g of ZnO in 3 mL of acetone.

A study by Prabha et al. (2017) demonstrated the antibacterial activity of Ag nanoparticles against Gram-positive pathogens. Their study used the thermal evaporation method to coat orthodontic bands (vacuum of 5 × 10−5millibar at 961°C for 10 min); their results also showed the biocompatibility of bands coated with nanoparticles[38]. In the present study, the antimicrobial and biocompatibility properties of both Ag and ZnO nanoparticles were compared; also, the difference between the FESEM views of the two studies was due to the method of coating the bands. In the current study, the bands were coated with the electrostatic spray-assisted vapor deposition method (distance: 3 cm, rate: 200 rpm, and voltage: 8 kV), which is cost-effective and is a suitable method for coating alloys and metal objects[39].

Several in vitro studies have been performed on the toxicity mechanisms of ZnO and Ag nanoparticles in mammalian cells[40-42]. These studies revealed the metal's intrinsic toxicity and the solubility of nanoparticles dictated by the metal's chemical features, uptake, and the potential to induce oxidative stress as the main properties causing the toxicity of the two nanoparticles.

A clinical trial is the most reliable way to determine biocompatibility. Nonetheless, ethical considerations limit its use. It is essential to assess the toxicity and biocompatibility of dental materials before they are used in the clinic. There is no or rarely any danger associated with using biocompatible dental materials[43]. MTT tests indicated that the cytotoxicity of Ag and ZnO nanoparticles coated on orthodontic

bands had no major and significant effect on the cells. Considering the results of this study, it is evident that all coated bands prevent the primary bacteria responsible for dental caries (*S. mutans*, *Lactobacillus spp*, and *C. albicans*) from growing to a significant extent; due to the long duration of orthodontic treatments, plaque accumulation and dental caries are less likely to occur. Nano-ZnO coated bands are considered to be more useful than nano-Ag coated bands because they are less toxic and have antimicrobial properties that are semi-similar to those of nano-Ag coated bands.

Based on a comprehensive literature search in the dental database, this is the first study to compare the antimicrobial properties of nano-Ag and nano-ZnO coated bands. The strong point of this study is the Investigation and comparison of the antimicrobial effect of these nanoparticles against the main microorganisms in the formation of dental caries and the biocompatibility of these nanoparticles. However, certain limitations exist; first, there was a lack of a brushing machine to equalize the speed and force of brushing when checking the coating binds on the surface. Second, only the antimicrobial effect on single species was investigated and multispecies biofilms were not evaluated. Third, in this study, the impact of nanoparticle coating on cariogenic microorganisms was assessed, and the study of peri pathogenic bacteria will be investigated in a future study.

Further studies are needed on the durability of ions released from nanoparticle-coated bands, and the changes in the physical properties of the material such as its long-term stability in the oral environment and the retention of nanoparticles during clinical application.

Conclusion:

According to the results of this study, the antimicrobial properties of coated orthodontic bands were significantly higher than uncoated bands. The antimicrobial properties of nano-Ag coated bands were significantly higher than those of nano-ZnO coated bands. In the case of cell viability, the lowest rate was observed in the bands

coated with Ag nanoparticles and the highest rate in the orthodontic bands without coating, but this difference was not statistically significant.

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