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Original Article

Comparison of subcutaneous inflammatory response induced by elastomeric orthodontic ligatures coated with silver and zinc oxide nanoparticles with control group on rats

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ABSTRACT

Background: Silver and zinc oxide (ZnO) nanoparticles have recently become common to coat ligatures in order to take advantage of positive properties of nanoparticles, although there are concerns about their cytotoxicity. This study tended to compare subcutaneous inflammatory response induced by elastomeric orthodontic ligatures coated with silver and ZnO nanoparticles with a control group in rats.

Materials and Methods: In this *in vitro* and animal cross-sectional descriptive-analytical study, silver nanoparticles were synthesized by chemical reduction of silver nitrate solution in the presence of sodium borohydride and ZnO nanoparticles by the same method and by chemical reduction of zinc sulfate solution with sodium hydroxide and were coated on elastomeric ligatures. Subcutaneous inflammation degrees were assessed after 15 and 30 days and were compared in the groups by Kruskal–Wallis test and ordinal generalized estimation equation with exchangeable correlation matrix. All tests were performed with a significance level ($P = 0.05$).

Results: There was a significant difference in terms of degrees of inflammation in the groups coated with ZnO nanoparticles ($P = 0.003$) and silver nanoparticles ($P = 0.04$) compared to the control group in 15- and 30-day samples. Zinc nanoparticles caused 3.22 times more inflammation than silver nanoparticles ($P = 0.053$). The decrease in inflammation was significant over time in all groups ($P = 0.001$).

Conclusion: There was a significant more inflammation in the groups receiving ZnO and silver nanoparticles compared to the control group in 15- and 30-day samples. Silver nanoparticles are probably safer than zinc nanoparticles for tissue and a better material to choose for antibacterial effects.

Key Words: Elastomeric ligatures, silver nanoparticles, subcutaneous inflammation, zinc oxide nanoparticles

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INTRODUCTION

Nanomaterials are materials that are <100 nm long in at least one dimension and include nanometer clusters, films, and plates <100 nm thick.[1] Nanoparticles have superior physical, chemical, mechanical, and optical properties compared to microparticles and can be used to make dental materials with

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high mechanical properties and antimicrobial and anti-decay effects.[1] Larger surfaces in nanoparticles provide a higher contact surface with other organic and inorganic molecules.[2] Addition of nanoparticles to dental materials has been considered in orthodontic knowledge in order to benefit from properties of these materials. These include the application of nanoparticles in materials to increase antimicrobial effects, improve the bond strength of composites and orthodontic adhesives, reduce friction between wires and brackets, improve physical properties of acrylics, make visible orthodontic adhesives, and improve physical properties of orthodontic elastomers as well as mouthwashes.[3,4] The antibacterial property is due to increase in surface-to-volume ratio of these particles and is inversely related to size of the nanoparticles.[5] All nanoparticles including silver, zinc oxide (ZnO), curcumin, titanium oxide, chitosan, and quaternary ammonium derivatives have been reported to have good antibacterial properties.[4] Elastomeric orthodontic ligatures, despite practical advantages such as speed and ease, have problems compared to steel ligatures due to their rough surface and adsorbent properties, higher accumulation of bacterial plaque, and more microorganisms in the plaque around the brackets.[5] Recently, elastomers with the property of releasing silver ions (OrthoShield Safe-T-Tie-Ortho Organizers) have been introduced. These technological improvements in elastomers have reduced the binding of *Streptococcus mutans* and lactobacilli to elastomers, inhibited their growth, and reduced periodontal pathogens and gingivitis in orthodontic patients.[6-8] Improvement has been reported in physical properties of these elastomers compared to the control group.[8] The inhibitory effects of zinc ions have been confirmed on oral bacteria including cariogenic bacteria, and ZnO nanoparticles have been introduced as an antibacterial agent.[9-12] Zinc is added to mouthwashes and toothpaste as an antimicrobial and plaque control agent, preventing plaque formation and reducing halitosis (bad breath). This substance accumulates in microbial plaque of teeth and saliva and its high concentrations can remain in the mouth for hours after application of health products.[13] In orthodontic knowledge, antibacterial effects of ZnO nanoparticles added to orthodontic composites and coated on orthodontic brackets against *S. mutans* and *Lactobacillus* have been investigated in some cases,[14-16] and in one case, antibacterial effects of ZnO against *S. mutans* were more than silver particles.[15] Zinc in nanoparticle form is often toxic to bacteria relative to its micron equivalents.[17] For human cells, ZnO nanoparticles are a biologically safe and nontoxic substance,[10] and if ZnO nanoparticles are used in biological applications in the range of normal concentration, biocompatibility and biosafety will be maintained.[18] Due to the increasing use of ZnO nanoparticles in industries, more studies are needed to confirm the safety issues associated with these applications.[19] When the particle size decreases to the nanoparticle level, some neutral substances may exhibit cytotoxic effects.[20] The present study tends to compare reaction and subcutaneous inflammation induced by elastomeric orthodontic ligatures coated with silver and ZnO nanoparticles with the control group on rats.

MATERIALS AND METHODS

Population and data collection

To perform this test, 120 elastomeric ligatures (Clear Transparent Orthodontic Ligature Ties O'Ring, ORTHO Organizers, USA) were prepared and were divided into 3 groups of 40 (control, coated with silver nanoparticles, and coated with ZnO nanoparticles). Elastomeric ligatures were immersed in isopropyl alcohol and then were kept in the ultrasonics detergent for 30 min and then were washed with Distilled water. Moreover, 30 healthy male rats with an average weight of 250 g and an average age of 120 days were selected and randomly assigned to 3 groups of 10; in each group, a certain type of nanoparticles was randomly used. For the control group, nanoparticles were not used.

Procedure

In this cross-sectional descriptive-analytical *in vitro* and animal intervention study, silver and ZnO nanoparticles were synthesized using chemical reduction and coated on ligature elastomers. Then, these elastomers were placed under the skin of rats and tissue samples were removed at two intervals of 15 and 30 days and examined for degree of infiltration and the number of subcutaneous inflammatory cells. Silver nanoparticles were synthesized using chemical reduction of silver nitrate solution (Merck KGaA, Frankfurt, Germany) in the presence of sodium borohydride (reducing agent),[21] and ZnO nanoparticles were synthesized by this method and by chemical reduction of zinc sulfate solution (Merk, Frankfurt, Germany) with sodium hydroxide.[15] For this test, 120 clear elastomeric

ligatures (Clear Transparent Orthodontic Ligature Ties O'Ring, ORTHO Organizers, USA) were prepared and divided into 3 groups of 40 (control, coated with silver nanoparticles, and coated with ZnO nanoparticles). Forty elastomeric ligatures in silver nitrate solution and 40 in zinc sulfate solution were immersed. In order to reduction of silver and zinc ions, respectively we added sodium borohydride solution to silver nitrate solution and sodium hydroxide solution to zinc sulfate solution. Synthesis of silver and ZnO nanoparticles was performed for 12 h. The color of the solution after the production of silver nanoparticles had changed to dark brown. This change was a sign of the production of silver nanoparticles. milky solution in zinc sulfate solution is formed and formation of the white precipitate indicates the synthesis of ZnO nanoparticles. In the next step, the elastomeric ligatures were removed out of the solution and dried at room temperature.

The presence of nanoparticles on elastomeric ligatures and coated surface of the elastomers with them was confirmed by field emission scanning electron microscope [Figures 1 and 2] and X-ray diffraction spectroscopy (Philips, Eindhoven, The Netherlands) [Figures 3 and 4].[14,15] The average size of silver nanoparticles was measured in scanning electron microscope (SEM) image; according to the estimates, the average size of silver particles was 82.85 nm and size of ZnO particles was 66.19 nm. The spectrum prepared by energy-dispersive spectroscopy device clearly showed that there were deposits of silver and zinc on the elastomer.

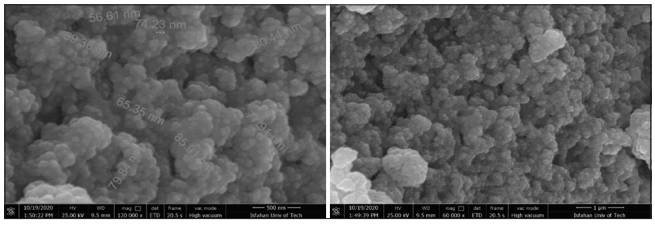

Figure 1: Field emission scanning electron microscope view of silver nanoparticles on elastomer with magnification of ×60,000 and ×12,000.

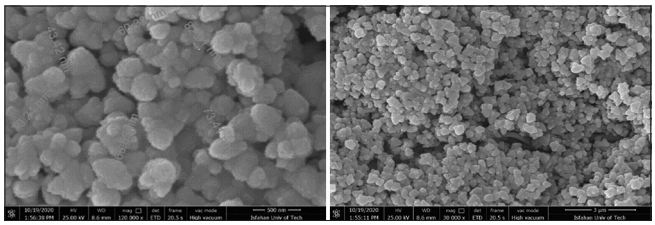

Figure 2: Field emission scanning electron microscope view of zinc oxide nanoparticles on elastomer with magnification of ×60,000 and ×120,000.

All groups of mice were under controlled conditions (temperature: 21°C–23°C, humidity: 50%–70% in a 12-h artificial cycle light and darkness) and were kept in cages. Animals were fed with water and ordinary food. After anesthesia, the back area of mice was disinfected with 70% isopropyl alcohol and 4 sites with a suitable distance (at least 30 mm) behind the selected mice and sectioned with a length of 10 mm and a depth of 5 mm. Elastomeric ligatures located in sites using aseptic method and finally, the skin stitched by suture.

After 15 and 30 days, mice were anesthetized and elastomers located at the sites along with adjacent skin and connective tissue were removed and the wounds were sutured again. On the 15th day, 2 samples were prepared from the back of each mouse, and on the 30th day, 2 samples were prepared from the same mouse [Figures 5 and 6]. Samples were fixed in 10% formalin for 48 h and then were evaluated for inflammatory responses and foreign body reactions.

In these evaluations, the observer was informed about the nature of the groups.

Tissues cut longitudinally using a microtome in dimensions of 5–6 microns and were stained with H and E for histological evaluation of inflammatory cells, including mast cells, macrophage, neutrophil, eosinophil, and giant cell.

Histological evaluation using light microscope

Infiltration of inflammatory cells at ×40 magnification was evaluated as follows:

- 0 = does not have any inflammatory cells with ×40 magnification
- 1 = mild, with an average of <25 inflammatory cells with ×40 magnification
- 2 = moderate, with an average of 25–124 inflammatory cells with ×40 magnification
- 3 = severe, with an average of 125 or more inflammatory cells with ×40 magnification.

The study was conducted using descriptive-analytical method. This method has been part of laboratory and animal intervention studies (Ethical Code: IR.IAU.KHUISF.REC.1399.084). Considering the following equation and considering $\beta = 0.01$ and $\alpha = 0.05$ as well as the difference between the groups with a maximum error of $d = 0.3$, the number of samples in each group

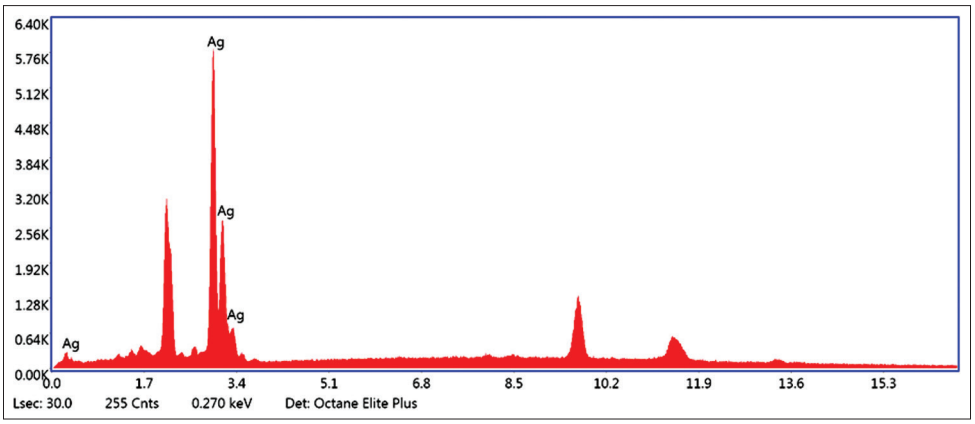

Figure 3: Graph of silver particles drawn by X-ray diffraction device.

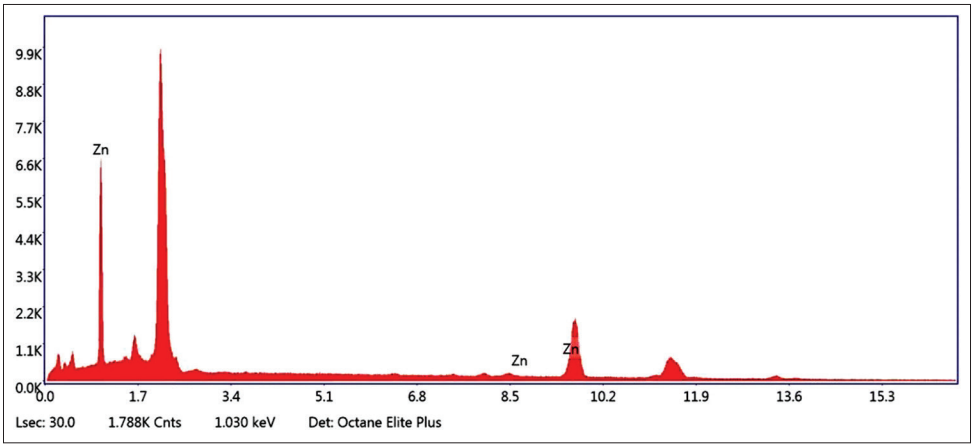

Figure 4: Graph of zinc oxide particles drawn by X-ray diffraction device.

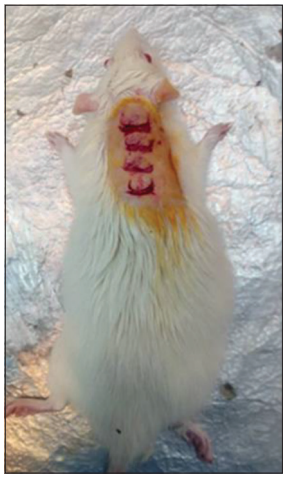

Figure 5: Elastomer cultured under the skin and preparing tissue sample.

was estimated to be 10 (30 for the whole groups). In this study, animals and elastomeric ligatures coated with ZnO nanoparticles and silver nanoparticles were selected nonrandomly based on inclusion criteria. The animals were randomly assigned to three groups.

$$n = \frac{\left[Z_{1-\alpha} + Z_{1-\beta}\right]\left[P_1\left(1-P_1\right) + P_2\left(1-P_2\right)\right]}{d^2}$$

Where is coefficient of confidence 95% which was set at 1.96; is minimum difference of means which shows a significant difference; is an estimate of prevalence.

Statistical analysis

SPSS software version 24.0(IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armond, NY: IBM Corp.) was used for statistical analysis of data. For this purpose, frequency and percentage of degrees of inflammation in different groups of silver nanoparticles, ZnO nanoparticles, and control were calculated and reported in 15- and 30-day samples. Frequency and percentage of degrees of inflammation were assessed by Kruskal–Wallis test. In order to investigate the relationship between treatment groups and rate of inflammation in 15 and 30 days later, ordinal generalized estimation equation test with exchangeable correlation matrix was used, because the rate of inflammation is ordinal, and odds ratio

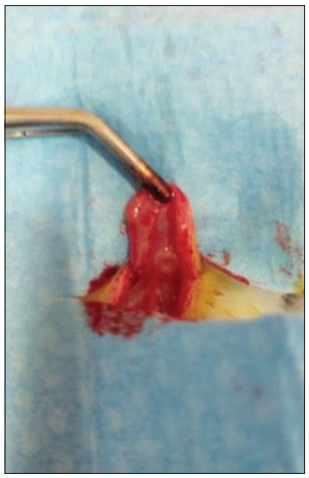

Figure 6: Elastomer cultured under the skin and preparing tissue sample. From a close view.

was reported as mild inflammation or moderate inflammation to noninflammation.

RESULTS

Table 1 and 2 shows Frequency of different degrees of inflammation in 15-day tissue samples and 30-day tissue samples based on Kruskal–Wallis Ordinal generalized estimation equation and exchangeable correlation matrix were used to evaluate the odds of mild inflammation and moderate inflammation compared to noninflammation. Relationship between the groups and rate of inflammation on days 15 and 30 was examined in two stages: once by placing the control group and noninflammation on day 15 as reference [Table 3] and once by placing the silver group and moderate inflammation on day 30 as reference [Table 4]. The results showed significant differences in terms of degrees of inflammation in the groups coated with ZnO nanoparticles and silver nanoparticles and the control group in 15- and 30-day tissue samples. The odds of inflammation in the silver group is 4.2 times higher than the control group (P = 0.04), and the odds of inflammation in the zinc group is 13.8 times higher than the control group (P = 0.003). Inflammation of the ZnO nanoparticle group was 3.22 times higher than that of silver nanoparticles, but the difference was not significant (P = 0.053). Most of the inflammations were mild (3.5 times greater than noninflammation), and the odds of developing moderate inflammation was 71% lower than noninflammation. On day 30 compared to day 15, the rate of inflammation is 72% lower and the odds of inflammation decreases over time (P = 0.001). The reduction of inflammation in 30-day samples was significant compared to 15-day samples in each of the groups (P = 0.001).

Table 1: Frequency of different degrees of inflammation in 15-day tissue samples based on Kruskal-Wallis

Inflammation group	No inflammation (%)	Mild (%)	Moderate (%)	Sum (%)
Control	2 (10.0)	10 (50.0)	8 (40.0)	20 (100.0)
Coated with zinc oxide nanoparticles	2 (10.0)	2 (10.0)	16 (80.0)	20 (100.0)
Coated with silver nanoparticles	2 (10.0)	8 (40.0)	10 (50.0)	20 (100.0)
Sum	6 (10.0)	20 (33.3)	34 (56.7)	60 (100.0)

Unit: Number of tissue samples. significance level (*P*) was 0.05

Table 2: Frequency of different degrees of inflammation in 30-day tissue samples based on Kruskal-Wallis

Inflammation group	No inflammation (%)	Mild (%)	Moderate (%)	Sum (%)
Control	8 (40.0)	6 (30.0)	6 (30.0)	20 (100.0)
Coated with zinc oxide nanoparticles	4 (20.0)	8 (40.0)	8 (40.0)	20 (100.0)
Coated with silver nanoparticles	2 (10.0)	16 (80.0)	2 (10.0)	20 (100.0)
Sum	14 (23.3)	30 (50.0)	16 (26.7)	60 (100.0)

Unit: Number of tissue samples. significance level (*P*) was 0.05

Table 3: Relationship between groups and inflammation rate on days 15 and 30

variables	OR	*P*	CI	
			Lower bound	Upper bound
Inflammation				
Non	Reference			
Mild	3.5	0.03	1.09	11.2
Moderate	0.29	0.01	0.11	0.75
Intervention				
Control	Reference			
Zinc	13.87	0.003	2.51	76.46
Silver	4.29	0.04	1.03	17.82
Time				
Day 15	Reference			
Day 30	0.28	0.001	0.14	0.58

CI: Confidence interval, OR: Odds ratio

DISCUSSION

Nanoparticles containing molecules with anti-decay and antibacterial properties in structure of elastomeric ligatures have been considered in recent years. Silver and ZnO nanoparticles are suitable for these purposes

Table 4: Relationship between groups and inflammation rate on days 15 and 30

Variables	OR	*P*	CI	
			Lower bound	Upper bound
Inflammation				
Non	0.23	0.002	0.096	0.58
Mild	2.85	0.016	6.69	1.21
Moderate	Reference			
Intervention				
Control	0.23	0.04	0.96	0.056
Zinc	3.22	0.053	10.56	0.98
Silver	Reference			
Time				
Day 15	3.49	0.001	7.14	1.71
Day 30	Reference			

CI: Confidence interval, OR: Odds ratio

because of their ability to reduce the demineralization of tooth enamel due to accumulation of bacterial plaque without adversely affecting the properties of the substances.[8] Silver nanoparticles are not highly toxic and are well compatible with human cells, although more studies are needed.[8] On the other hand, ZnO has inhibitory effects on a wide range of bacteria,[22] and this substance has a strong antibacterial activity, especially at the nanoscale.[10,23] Although metal oxide nanomaterials hold potential for improving human health, there are still multiple challenges to bring these materials to the clinic. One of the obstacles is that there is current misunderstanding regarding the biological effects and cytotoxicity profiles of ZnO nanoparticles. The discrepancies in the literature are likely attributable to the lack of common understanding between life scientists and material scientists regarding the other's limitations and capabilities. Nanoparticles are not necessarily identical from batch to batch and may display alterations in surface chemistry or size distribution. Life scientists might not appreciate the difficulty in controlling the synthesis process, while nanotechnologists might not appreciate the sensitivity of mammalian cells to these variations. There is also concern that researchers may treat ZnO nanoparticles made by different synthesis methods as a single entity with insufficient regard to their potential to exert different biological responses. Other confounding factors include differences in handling, pH variations of the dispersion media, long-term stability versus freshly prepared nanoparticles, impurities, humidity variations during the synthesis, and variations in aspect ratio or agglomeration potential. In sum, a lack of careful surface and physiochemical characterizations of ZnO nanoparticles has led to much of the current confusion regarding the biological responses elicited from these materials. What is needed to avoid these types of problems is a better understanding of the intersecting areas of science between nanomaterial scientists and biologists, such that collaborations allow for the effective exchange of information and methodology to advance the field.[23]

Inflammatory responses are triggered by a severe reaction due to the surgical procedure and foreign object. Because the reaction is not specific in the 1st h after surgery, the cells are not counted, and after 7 days of waiting, inflammatory response is mediated by foreign object through inflammatory cell count.[24] The present study was performed by subcutaneous placement test in rats by counting the number of inflammatory cells on days 15 and 30. Biocompatibility is defined as the ability to produce biological reactions in materials, and whether or not a foreign object causes harmful effects on body tissues. This property is determined by biocompatibility tests. In this regard, biocompatibility characteristics of most materials in contact with the body, such as implants or restorative materials, are evaluated through mucosal implantation in the body of animals such as rats.[25,26] Material biocompatibility analysis is also based on inflammation assessments.[27] In this study, this method was used because elastomeric ligatures are located near the gums or oral epithelium during orthodontic treatment. Meanwhile, it was tried to use a smaller number of animals in these experiments due to accuracy of the research protocol and use of optimal research methods. On the other hand, because ligatures are changed during routine orthodontic treatments every 4 weeks, we also chose a 30-day period for maximum time the ligatures stay under the skin.

In order to evaluate the biocompatibility of brackets coated with silver nanoparticles, Metin-Gürsoy *et al*. (2016) examined inflammatory reactions by counting inflammatory cells on days 7, 14, 30, and 60. Overall, orthodontic brackets coated with silver nanoparticles had better biocompatibility than conventional orthodontic brackets, and in general, no signs of granuloma or necrosis were seen during the study.[28] In the present study, no degree of severe inflammation was seen in any of the groups. In the present study, the number of inflammatory cells around ligatures containing ZnO ($P = 0.003$) and silver ($P = 0.04$) nanoparticles was significantly higher

than the control group, which is inconsistent with Metin-Gürsoy *et al*. Different results of studies can be due to the type of material used, different techniques for adding nanoparticles, the size of nanoparticles used, and different concentration. In the present study, mild inflammation was observed in all groups in all periods; the number of inflammatory cells significantly decreased over time (P = 0.001), which is consistent with Metin-Gürsoy *et al*.[28] Chen *et al*. observed good biological effects on days 7 and 14 in rats treated with nanoparticles and microparticles. However, adverse biological effects were seen on day 30 and the tissues around the silver nanoparticles showed severe inflammation. Chen *et al*. reported higher severity of subcutaneous inflammation than that of reported by Metin-Gürsoy and the present study; over time, adverse effects were observed in the tissue, which is in contrast to Metin-Gürsoy and our study.[29]

Sadeghian *et al*. performed a study to determine the biocompatibility of orthodontic retainers containing silver nanoparticles at concentrations of 2.5% and 5% and observed that the number of inflammatory cells around nanocomposites containing silver nanoparticles was significantly higher than the control group, which is consistent with the present study.[30] Sadeghian *et al*. also reported that inflammatory reactions were more severe and the number of inflammatory cells was higher on the 7th day after placement, and the rate of inflammatory reactions in all groups decreased after 2 months.[30] The reduction in inflammation in the groups over time is consistent with the present study. This gradual decrease in inflammatory events on day 30 is consistent with other studies.[31] Mozayeni *et al*. (2017) studied the biocompatibility of gutta-percha coated with silver nanoparticles and showed that inflammation had decreased over time in both control and case groups, which was also observed in the present study.[32] In short-term acute inflammation, macrophages will also disappear rapidly if the stimuli are removed from the environment. In chronic inflammation, accumulation of macrophages occurs continuously. In early stages of tissue reaction, neutrophil cells predominate and are adsorbed to the site by chemokines released from damaged cells. In later stages, monocytes, macrophages, and fibroblasts predominate, leading to formation of dense fibrous tissue, which primarily consists of fibroblasts and collagen matrix. In this regard, observations of tissue reactions in the present study are consistent.[33] In the present study, no signs of neutrophils were seen. This finding shows that acute inflammation did not persist until day 15. In the research of Matin-Gursoy *et al*.,[28] the sign of neutrophils was not seen until day 7. This finding is similar to our study.

In the present study, there was a significant difference in terms of degrees of inflammation in different groups of coating with ZnO nanoparticles (P = 0.003) and silver nanoparticles (P = 0.04) or the control group on days 15 and 30. These results are inconsistent with Moreira *et al*. (2014)[34] and Prabha *et al*. (2016).[35] Moreira *et al*. reported biocompatibility of cement containing silver nanoparticles equal to control groups.[34] Prabha *et al*. also showed that orthodontic braces covered with silver nanoparticles have good biocompatibility and are not toxic for mice fibroblasts.[35] The results of the present study on inflammatory properties of ZnO nanoparticles are also consistent with Reddy *et al*.,[36] Hackenberg *et al*.,[37] and Ng *et al*.[19] Reddy *et al.* examined Cytotoxicity of ZnO nanoparticles on human alveolar epithelial cells (A549) and human embryonic renal cells in laboratory condition and demonstrated that ZnO nanoparticles indicate inflammatory response and prevent cell growth dose-dependently.[36] Hackenberg *et al*. examined the effects of ZnO nanoparticles on cells of the human nasal mucosa. In this study, Induction of injury to DNA, cytotoxicity and genotoxicity and inflammatory potential of ZnO nanoparticles on nasal mucosal cells, was Approved.[37] Ng *et al*. also reported reduction of Cell survival, cytotoxicity and genotoxicity in human lung fibroblasts due to exposure to ZnO nanoparticles in laboratory conditions.[19] In the present study, the average particle size of ZnO was 66.19 nm and the average size of silver nanoparticles was 82.85 nm (based on SEM images). This relatively small particle size can intensify inflammatory reaction in tissues. This argument is consistent with Park *et al*.,[38] Asare *et al*.,[39] and Raju *et al*.[40]

Park *et al*.[38] investigated cytotoxicity and inflammatory reactions after oral administration of silver nanoparticles. silver particles with smaller sizes (42, 22, and 71 nm) in brain, lungs, liver, and kidneys were distributed but silver nanoparticles with larger size (323 nm) were not detected in these tissues.

We suggest future studies to investigate physical and mechanical properties of elastomeric ligatures coated

with ZnO and silver nanoparticles and their color change.

CONCLUSION

There was a significant more inflammation in different groups of coating with ZnO nanoparticles and silver nanoparticles in comparison with the control group in 15- and 30-day samples. Zinc nanoparticles caused more inflammation than silver nanoparticles (although the difference between them was not significant, it was obvious, 3.22 times more inflammation). The reported inflammation was moderate in coated groups at day 15, which decreased significantly after 15 days (day 15 to day 30) (72%). Degree of inflammation was mild at day 30. As a result, silver nanoparticles may be safer for the tissue than zinc nanoparticles and may be a better material to use for antibacterial effects. However, more research is needed.

Furthermore, to minimize the application of animals in the present study, in order to comply with ethical values, the minimum number of animal samples was used for the present study, which is the reason for the wide range of results.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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