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Antimicrobial Efficacy of Silver Nanoparticles Incorporated in an Orthodontic Adhesive: An Animal Study

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A B S T R A C T

Objectives: This study assessed the antimicrobial efficacy of silver nanoparticles (AgNPs) incorporated in Transbond XT orthodontic adhesive used in rats.

Materials and Methods: Transbond XT orthodontic adhesive containing 0%, 1%, 5% and 10% AgNPs was experimentally produced. Twenty-eight male Wistar rats were randomly divided into four groups (n=7) of control (0% AgNPs), 1% AgNPs, 5% AgNPs and 10% AgNPs. After anesthetizing the rats, one drop (10 µm) of the adhesive was applied on the central incisor, and light-cured for 20 s. Transbond XT composite (1×1×1 mm) was also applied. Another 10-µm drop was applied over it, and light-cured for 40 s. Biofilm test was carried out, and the number of colony forming units (CFUs) of *Streptococcus sanguinis* (*S. sanguinis*), *Streptococcus mutans* (*S. mutans*) and *Lactobacillus acidophilus* (*L. acidophilus*) in the saliva of rats was counted at baseline and 24 h after the application of adhesive. The data were analyzed using one-way ANOVA and Tukey's test.

Results: In presence of 5% and 10% AgNPs, *S. sanguinis* and *L. acidophilus* counts were significantly lower than those in the control and 1% AgNP groups (P<0.05). The *S. mutans* colony count was significantly lower in presence of all concentrations of AgNPs compared with the control group (P<0.05). The *S. mutans* colony count in 10% AgNP group was significantly lower than that in 1% and 5% AgNP groups (P<0.05).

Conclusion: Silver nanoparticles have dose-dependent antimicrobial effects; 5% concentration is the minimum concentration of AGNPs with optimal antimicrobial efficacy against all strains evaluated in this study.

Keywords: Nanoparticles; Silver; Anti-Bacterial Agents; Dental Bonding; Orthodontic Brackets; Rats

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INTRODUCTION

Despite recent advances in orthodontics, application of fixed orthodontic appliances still carries a high risk of development of white spot lesions, which are often irreversible and are a major concern for patients and orthodontists [1].

Composite resins are routinely used for orthodontic bracket bonding; however, their major drawback is that they enhance the accumulation of bacterial biofilm, especially at the margins. Acid production by the

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bacteria present in the biofilm can cause white spot lesions and secondary caries [2].

Several methods have been proposed to prevent plaque accumulation around composite resin restorations. Incorporation of antibacterial agents in the formulation of adhesives or composite resins is one suggested strategy for this purpose. Silver nanoparticles have been recommended for incorporation in the formulation of restorative materials due to their excellent antimicrobial properties [3]. They are extensively used in clothing, cosmetic, and food industries, as well as in medicine and dentistry due to their optimal antimicrobial properties. Due to their small size, they easily pass through the cell membrane and affect cell function. Moreover, silver nanoparticles (AgNPs) have antimicrobial activity stronger than that of silver particles because of providing a larger surface area [4].

They release silver ions, which exert their antimicrobial effects by binding to sulfur-containing proteins on the bacterial cell membrane and changing the absorption properties, morphology and respiratory cycle of the cells, causing eventual death of microorganisms. Bacteria cannot develop resistance to AgNPs, which further adds to the antimicrobial efficacy of AgNPs [4]. Strong antibacterial activity of AgNPs against *Streptococcus mutans* (*S. mutans*), *Streptococcus sanguinis* (*S. sanguinis*), and *Lactobacillus acidophilus* (*L. acidophilus*) cariogenic microorganisms has been well documented [5]. Optimal antimicrobial efficacy of AgNPs incorporated in methacrylate acrylic resin has been previously demonstrated [5]. Also, Ahn et al. [6] showed that addition of nano-silver oxide particles to a bonding agent decreased plaque accumulation.

S. mutans has been frequently isolated from carious teeth [7]. Also, a direct correlation has been noted between *Lactobacillus* count and rate of caries [8]. Streptococci and lactobacilli are the main acid-producing cariogenic bacteria in the oral cavity [9].

As mentioned earlier, antibacterial efficacy of AgNPs has been confirmed in many in vitro studies [10-12]. However, prior to their use in the clinical setting, their efficacy and safety must be tested in animal models. Thus, this study sought to assess the antimicrobial efficacy of AgNPs incorporated in an orthodontic adhesive against *S. mutans*, *S. sanguinis*, and *L. acidophilus* in rats.

MATERIALS AND METHODS

The AgNPs were obtained from Pishgaman Nano Company (Tehran, Iran), and were evaluated by scanning electron microscopy (in terms of thickness), transmission electron microscopy (in terms of microscopic and crystalline structure) and X-ray powder diffraction (in terms of crystallinity) by Pishgaman Nano Company. Next, 0%, 1%, 5%, and 10% concentrations of AgNPs were experimentally incorporated in the formulation of Transbond XT bonding agent (3M ESPE, St. Paul, MN, USA) in the Gamma Radiation Center of the Iranian Atomic Energy Organization. The prepared bonding agents were supplied in micro-tubes and wrapped in aluminum wraps to prevent contact with air and exposure to light.

All animal experiments were carried out in accordance to the protocols approved by the animal ethics committee of Tehran University of Medical Sciences. Twenty-eight male Wistar rats (150-200g; Pasteur Institute; Tehran; Iran) were housed one rat per cage under sanitary conditions at 22-25°C with 12 h light/dark cycles, and ad libitum access to sanitized food and water. The rats were acclimated to room condition for 1 week prior to each experiment to increase the accuracy of microbiological assessments. The cages were disinfected with 10% povidone iodine solution.

Since the oral microbial flora of the rats and humans is not the same, the rats had to become germ-free. For this purpose, a 7-day antibiotic therapy protocol was carried out according to a previous study [13]. During the first 4 days, each rat received 20 mg gentamicin along with 20 mg ampicillin daily, which were added to their daily drinking water (70cc), followed by 3 days of no antibiotics [13].

A microbial suspension of *S. mutans* (ATCC 25175), *S. sanguinis* (ATCC 10556) and *L. acidophilus* (ATCC 4356), obtained from the Pasteur Institute of Iran, was prepared, which contained 3×10^8 colony forming units (CFUs)/mL of *S. mutans* and *S. sanguinis*, and 3×10^9 CFUs/mL of *L. acidophilus*. A swab was dipped in the suspensions and used to inoculate the oral cavity of the rats [11]. Twenty-four hours after colonization of the oral cavity of the rats, saliva samples were collected by a swab, and transferred to brain heart infusion broth (Merck, Darmstadt, Germany). The suspension was cultured in mitis salivarius mutans valinomycin agar (Merck, Darmstadt, Germany) for *S. mutans*, modified medium 10-sucrose agar for *S. sanguinis*, and de Man, Rogosa and Sharpe clindamycin ciprofloxacin agar (Merck, Darmstadt, Germany) for *L. acidophilus* [14,15]. After ensuring bacterial colonization, the rats were randomly divided into four groups (n=7) of control (adhesive containing 0% AgNPs), adhesive containing 1% AgNPs, adhesive containing 5% AgNPs, and adhesive containing 10% AgNPs.

The rats were anesthetized by intraperitoneal injection of 50 mg/kg ketamine and 6 mg/kg xylazine [16]. The maxillary central incisors of the rats were dried with a cotton pellet and etched with 37% phosphoric acid (3M ESPE, St. Paul, MN, USA) for 40 s [17]. The etchant gel was then removed by a moist cotton pellet, and the teeth were dried. Care was taken to avoid saliva contamination [18]. Using a sampler, a 10-µm drop of Transbond XT with the respective concentration of AgNPs was applied on the tooth surface, and light-cured for 20 s (Bluphase 16i; Ivoclar Vivadent, AG, Australia).

A small piece of Transbond XT composite (3M ESPE, St. Paul, MN, USA) measuring 1×1×1 mm was placed on the teeth and spread to obtain a very thin uniform increment on the surface of both central incisors. Another 10-µm drop of Transbond XT with the respective concentration of AgNPs was applied on the tooth surface and light-cured for 40s. To prevent contact with the opposing teeth, the mandibular central incisors were reduced by 2mm.

After 24h, the rats were examined to ensure that the composite remained on their tooth surfaces. Saliva samples were collected again by a swab, dissolved in brain heart infusion broth, and cultured as explained earlier; the number of CFUs was counted [19].

The colony counts were reported at baseline and 24h after the application of bonding agent and composite in the four groups using descriptive statistics. The data were analyzed using SPSS version 21 (SPSS Inc., IL, USA).

The four groups were compared in terms of colony counts by one-way ANOVA. Since one-way ANOVA revealed significant differences among the four groups, pairwise comparisons were carried out using the Tukey's HSD test. $P<0.05$ was considered statistically significant.

RESULTS

Table 1 shows the mean and standard deviation of colony counts in the four groups. In the control group with 0% AgNPs, the mean count of *L. acidophilus* at 24h after the application of bonding agent and composite was 1.02 times the rate at baseline; this value was 1.11 times for *S. sanguinis* count and 1.44 times for *S. mutans* count.

In the adhesive group with 1% AgNPs, the mean count of *L. acidophilus* was 1.09 times the baseline value at 24 h after the intervention; this value was 0.84 times for *S. sanguinis* and 0.64 times for *S. mutans* count. In the adhesive group with 5% AgNPs, the mean count of *L. acidophilus* was 0.08 times the baseline value after the intervention. This value was 0.04 times the baseline value for *S. sanguinis* and 0.47 times the baseline value for *S. mutans*. In the adhesive group with 10% AgNPs, the mean count of *L. acidophilus* was 0.02 times the baseline value after the intervention. All *S. sanguinis* colonies were eliminated and the mean count of *S. mutans* was 0.04 times the baseline value after the intervention.

Pairwise comparisons of the groups (Table 2) in terms of *L. acidophilus* count were carried out using the Tukey's HSD test.

Table 1. Mean and Standard deviation (SD) of *Lactobacillus acidophilus, Streptococcus sanguinis,* and *Streptococcus mutans,* and colony counts in the four groups with 0%, 1%, 5%, and 10% concentrations of AgNPs before and after composite bonding

	Mean (SD) change in the colony count of bacteria							
	Before (CFUs/mL)				After (CFUs/mL)			
AgNP (%)	0	1	5	10	0	1	5	10
Lactobacillus acidophilus	114.29 (85.02)	108.57 (97.54)	91.43 (46.34)	101.43 (105.10)	110.00 (110.75)	98.57 (62.29)	7.14 (9.51)	2.86 (4.80)
Streptococcus sanguinis	91.43 (108.54)	92.86 (73.19)	128.57 (120.47)	114.29 (114.43)	77.14 (62.64)	71.43 (48.10)	12.86 (23.60)	0
Streptococcus mutans	242.86 (320.45)	410.00 (243.65)	442.86 (434.61)	114.29 (82.43)	165.71 (143.85)	264.29 (176.62)	172.86 (148.06)	11.43 (26.09)

No significant difference was noted in *L. acidophilus* count between the control and 1% AgNP groups; however, the *L. acidophilus* colony count in 5% and 10% concentrations of AgNPs was significantly lower than that in the control and 1% AgNP groups (P<0.05). No significant differences were noted in this regard between 5% and 10% concentrations of AgNPs (P>0.05).

The results of pairwise comparisons of the groups in terms of *S. sanguinis* count using the Tukey's HSD test are shown in Table 2.

Table 2. Pairwise comparisons of the four groups in terms of *Lactobacillus acidophilus (L. a.), Streptococcus sanguinis (S. s.),* and *Streptococcus mutans (S. m.)* colony counts

		P-value		
AgNP (%)	Groups	*L. a.*	*S. s.*	*S. m.*
0	1%	0.989	0.497	0.146
	5%	0.001	<0.001	0.059
	10%	0.001	<0.001	0.004
1	0%	0.989	0.497	0.146
	5%	0.001	0.001	0.967
	10%	<0.001	0.001	0.365
5	0%	0.001	<0.001	0.059
	1%	0.001	<0.001	0.967
	10%	0.994	0.995	0.632
10	0%	0.001	<0.001	0.004
	1%	<0.001	0.001	0.365
	5%	0.994	0.995	0.632

No significant difference was noted in this regard between the control and 1% AGNP groups; but the *S. sanguinis* count in presence of 5% and 10% AgNPs significantly decreased compared with its count in the control and 1% AgNP groups (P<0.05); 5% and 10% AgNP concentrations were not significantly different in this respect (P>0.05). Pairwise comparisons of the groups (Table 2) in terms of *S. mutans* count using the Tukey's HSD test showed a significant reduction in *S. mutans* count in presence of 10% AgNPs compared with the control, 1% and 5% AgNP groups (P<0.05); 1% and 5% AgNP groups were not significantly different in this respect (P>0.05). Reduction in *S. mutans* count was also noted in 5% and 1% concentrations compared with the control group (P<0.05). The exact P-values are presented in Table 2.

DISCUSSION

Researchers have long been in search of methods to decrease the microbial plaque accumulation around orthodontic brackets and prevent the formation of white spot lesions. Composite resins, commonly used for adhesive bonding of brackets, have a polymer matrix, which can serve as a suitable environment for growth and proliferation of aerobic and anaerobic microorganisms, causing subsequent decalcification of enamel and periodontal disease [20].

Antimicrobial agents such as benzalkonium chloride, chlorhexidine, and triclosan were

first added to composites with variable degrees of success [21]. Addition of AgNPs was later proposed for this purpose due to optimal antimicrobial activity of AgNPs [2-5,22-25]. However, most previous studies in this respect had an in vitro design [10-12]; thus, the current animal study is unique in this field. The AgNPs release silver ions, which inactivate the critical bacterial enzymes and inhibit bacterial proliferation as such. Low toxicity, high biocompatibility, substantial antimicrobial activity, and substantivity are among the major advantages of AgNPs; moreover, compared with antibiotics, bacteria less commonly develop resistance against AgNPs [2].

Streptococci (particularly *S. mutans*) and lactobacilli (particularly *L. acidophilus*) are the main acid-producing cariogenic bacteria present in dental plaque [7-9]. The mutans streptococci are mainly responsible for initiation of caries while lactobacilli play a role in progression of carious lesions. Presence of *S. sanguinis* in the oral environment decreases the population of mutans streptococci, and these two are in equilibrium [26]. Thus, the current study focused on the antimicrobial effects of different concentrations of AgNPs on the aforementioned three main cariogenic bacteria. The AgNPs in different concentrations were added to Transbond XT, since it is the gold standard for orthodontic bracket bonding [27].

Kreth et al. [26] discussed that *S. mutans* and *S. sanguinis* are in competition in dental plaque, and increase in the population of one strain would result in a reduction in the population of the other; however, the current study showed reduction in the count of both microorganisms due to the non-selective antimicrobial activity of AgNPs. One might claim that antibacterial effect of AgNPs on *S. sanguinis* may not be favorable since *S. sanguinis* has an inhibitory effect on the activity of *S. mutans* (by controlling its population); however, it should be noted that *S. sanguinis* is a pioneer in the formation of dental plaque and thus, leads to caries development due to plaque accumulation.

Selection of rats in the current study was based on their extensive use in dental research, low maintenance cost, proper size of their mouth, and easy inoculation of their oral cavity with the respective bacteria [28]. However, since the oral microbial flora of rats is different from that of humans, and *S. mutans* and *S. sanguinis* are only found in gingival crevices of rats in small amounts, we made them germ-free first, and then inoculation with the respective bacteria was performed. A previous study successfully used an antibiotic therapy protocol to make the rats germ-free; thus, the same protocol was applied in the current study for this purpose [10].

Despite taking measures to maintain composites in the mouth of rats for longer periods of time (by reduction of height of mandibular incisors, application of a thin increment of composite, and providing rats with very soft diet), all composites were detached at 48 h. Thus, we could only assess the antibacterial efficacy of AgNPs at 24 h, and could not evaluate the effect of time on their antibacterial activity.

The selected concentrations of AgNPs in the current study were based on a previous study by Sodagar et al [29]. Also, in the current study, the biofilm inhibition test was carried out to assess the antimicrobial activity of AgNPs because it has been shown that bacteria in the form of biofilm are four times more resistant to antibacterial agents compared with the planktonic form. In the biofilm state, bacteria result in initiation of carious lesions and their progression by forming resistant bacterial accumulations [30].

The current results revealed that AgNPs decreased the count of microorganisms in a dose-dependent manner. The colony counts of *L. acidophilus* and *S. sanguinis* significantly decreased in presence of 5% and 10% concentrations of AgNPs compared with 0% and 1% concentrations. Since no significant difference was noted in this respect between 5% and 10% concentrations, 5% concentration of AgNPs seems to be more suitable because it exerts antimicrobial effects in a lower dose. The *S. mutans* count significantly decreased at all concentrations of AgNPs compared with the control group. The

difference in this respect between 1% and 5% concentrations was not significant; but, the magnitude of reduction in presence of 10% AgNPs was the highest. This indicates that despite higher reduction in presence of 10% AgNPs, concentrations as low as 1% can also exert antimicrobial effects on mutans streptococci.

Cheng et al. [25] added 0.028%, 0.042%, 0.088%, and 0.175% concentrations of AgNPs to composite resin and concluded that the mechanical properties of the composite with 0.028% and 0.042% concentrations of AgNPs were similar to those of the control group; while, the colony count of *S. mutans* in presence of 0.042% AgNPs was 75% lower than that in the control group. Their findings revealed that AgNPs have significant antimicrobial activity even in low concentrations without adversely affecting the mechanical properties of composites [25].

Ahn et al. [6] compared an adhesive composite containing AgNPs with two conventional composites, and reported significantly higher antimicrobial efficacy of the composite containing AgNPs. Hernández-Sierra et al. [31] concluded that AgNPs exert higher antimicrobial efficacy in lower concentrations compared with gold nanoparticles and zinc oxide; due to lower concentration, the toxic effects would be lower while maintaining optimal clinical efficacy. Miresmaeili et al. [32] evaluated the effect of incorporation of 0%, 0.5%, 1%, and 2.5% AgNPs in Opallis Flow light-cure composite on antimicrobial properties and bracket bond strength.

They reported that 1% concentration of AgNPs had the highest antimicrobial effects on *S. mutans*. They explained that lower antimicrobial activity of 2.5% AgNPs was attributed to the fact that AgNPs were not well mixed and were not homogenously distributed in the composite. Our study showed dose-dependent antimicrobial effects of 1%, 5%, and 10% AgNPs on *S. mutans*. The difference in this respect may be due to the difference in the design of the two studies since their study had an in vitro design. Zhang et al. [24] assessed the antibacterial efficacy of 5% methacryloyloxydodecylpyridinium bromide plus 0.05% AgNPs incorporated in primer against microorganisms in human oral biofilm in an in vitro study and concluded that methacryloyloxydodecylpyridinium bromide and AgNPs decreased the biofilm both alone and in combination with one another; they showed significant synergistic effects and caused a reduction in bacterial biofilm by 10 folds when used together. Li et al. [2] compared the antibacterial effects of 10% quaternary ammonium and 0.05% AgNPs incorporated in adhesive and primer on *S. mutans* on the surface of resin (contact inhibition) and in areas far from the resin surface (long-distance inhibition), and also evaluated their cytotoxicity and effects on microtensile bond strength. They found that the bonding agent containing quaternary ammonium only exerted antimicrobial activity on resin surface (contact inhibition), and did not cause long-distance inhibition; while, AgNPs decreased the bacterial count both on the surface of resin and in farther areas (both contact and long-distance inhibition). They added that quaternary ammonium and AgNPs had no adverse effect on microtensile bond strength, and were not toxic for human gingival fibroblasts [2]. These findings further add to the optimal antimicrobial efficacy of AgNPs in low concentrations, even in areas far from the resin surface. Zhang et al. [23] evaluated the antimicrobial effects of TiO2 alone and in combination with AgNPs on *S. mutans* and *S. sanguinis*. The bacteria were exposed to stainless steel brackets coated with a thin layer of nanoparticles. After 20 min, TiO2 plus AgNPs eliminated 79% of the bacteria and after 30-60 min, all bacteria were eliminated. However, TiO2 alone could not eliminate all the bacteria even after 240 min. Their findings further confirm the antimicrobial efficacy of AgNPs and support our results. Burgers et al. [17] fabricated round composite samples containing 0%, 0.3%, and 0.6% AgNPs and showed the highest accumulation of *S. mutans* around the composite sample with 0% AgNPs. The highest count of dead mutans streptococci was seen around the composite sample containing 0.6% AgNPs. Similar to the current study, their

results pointed to the dose-dependent antimicrobial efficacy of AgNPs; although the concentrations of AgNPs were not the same in the two studies.

The cytotoxicity of AgNPs has been the subject of extensive investigations. Due to small size, AgNPs may enter into the cells and cause cell damage. The level of free silver ions is mainly responsible for the possible cytotoxicity of AgNPs [33]. However, the level of free silver ions rarely reaches a hazardous state. It has been shown that AgNPs affect DNA replication [34]. However, eukaryotic cells are less sensitive to AgNPs than prokaryotic cells, and it is believed that AgNPs can invade the bacteria with no adverse effect on eukaryotic cells. However, future studies are still warranted in this respect. Further studies are recommended to evaluate the effect of time on the antibacterial efficacy of AgNPs using eluted component test, and also assess the important parameters and properties of the modified adhesive such as its biocompatibility (by the methyl thiazolyl tetrazolium assay and alkaline phosphatase test), and physical (degree of conversion) and mechanical (bond strength to orthodontic brackets) properties. Also, assessment of the antibacterial efficacy of AgNPs in combination with other nanoparticles may be an interesting topic for future studies.

CONCLUSION

The current results showed dose-dependent antibacterial effects of AgNPs incorporated in an orthodontic adhesive. Based on the current results, 5% concentration is the minimum concentration of AGNPs with optimal antimicrobial efficacy against all strains evaluated in this study.

CONFLICT OF INTEREST STATEMENT

None declared.

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