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Comparison of Antibacterial Properties of an Orthodontic Composite Containing Silver and Amorphous Tricalcium Phosphate Nanoparticles against *Streptococcus mutans*: An In Vitro Study

Zahra Tavakolinejad [1], Mahmood Sheikh Fathollahi [2], Farzaneh Mirzaei [3,4] Elham Mirzaei [5]

[1] Department of Orthodontics, Faculty of Dentistry, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[2] Rajaie Cardiovascular Medical and Research Center, Iran University of Medical Sciences, Tehran, Iran
[3] Department of Parasitology and Mycology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[4] Department of Laboratory Sciences, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[5] Private Dentistry Practice, Yazd, Iran

Corresponding author:
Elham Mirzaei, Private Dentistry Practice, Yazd, Iran

mirzaei.elham2072@gmail.com

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Abstract

Background and Aim: Formation of white spot lesions, due to plaque accumulation and bacterial biofilm growth, is a common complication in orthodontic treatment. The present study aimed to compare the antibacterial properties of an orthodontic composite containing silver (Ag) and amorphous tricalcium phosphate (ATCP) nanoparticles against *Streptococcus mutans* (*S. mutans*).

Materials and Methods: In this in vitro study, 0.3% w/w Ag nanoparticles and 3% w/w ATCP nanoparticles were added to Transbond XT orthodontic composite. Totally, 48 composite discs were fabricated in three groups (n=16). The experimental groups included composite specimens containing nanoparticles and the control group included composite specimens without nanoparticles. The antibacterial effects of composite discs with and without nanoparticles against *S. mutans* (ATCC 35668) in the three groups were assessed by the direct contact test after 24 hours and 30 days. The number of bacterial colonies was visually counted in the three groups and compared. Data were analyzed by one-way ANOVA and Duncan's multiple comparisons test. P-values under 0.05 were considered significant.

Results: The antibacterial properties of nano-composites significantly increased in both experimental groups of composites containing Ag and ATCP nanoparticles, compared to the control group ($P<0.001$). The highest antibacterial activity was observed in the orthodontic composite containing ATCP nanoparticles.

Conclusion: Addition of Ag and ATCP nanoparticles to orthodontic light-cure composite increases its antibacterial activity against *S. mutans*.

Key Words: Anti-Bacterial Agents; Composite Resins; Nanoparticles; Silver; Streptococcus mutans

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Introduction

Formation of white spot lesions due to enamel demineralization caused by plaque accumulation and bacterial biofilm growth is a common complication in many patients undergoing orthodontic treatment [1-4].

Prevention of white spot lesions is a major concern for both patients and dental clinicians, because these lesions are sometimes irreversible [1, 5]. *Streptococcus mutans* (*S. mutans*) is the main microorganism responsible for the onset of tooth decay [6].

Patient cooperation in maintaining oral health, as a way of caries prevention, is always challenging during orthodontic treatment [1, 7]. Although good oral hygiene (toothbrushing, flossing, and using fluoride) is essential, antibacterial agents should be used around the brackets in patients with poor cooperation [5, 7, 8]. However, application of antibacterial agents, such as chlorhexidine and composites containing fluoride, is questionable due to their short-term antibacterial effects and suboptimal mechanical properties. Therefore, alternative modalities are required to overcome such shortcomings [9-11].

Alternative treatments to decrease dental caries include addition of metals such as silver (Ag), zinc, and copper in the form of nanoparticles, to composite resins [12, 13]. Silver has a higher antibacterial activity than other metals [14, 15]. Bacteria are less likely to resist metal nanoparticles than antibiotics [16]. Furthermore, nanoparticles reduce the surface roughness of orthodontic adhesives, which is an important factor in bacterial adhesion [5, 17, 18]. Another strategy to decrease caries is the application of composite resins containing calcium phosphate [19]. Beta-tricalcium phosphate nanoparticles with anti-caries and remineralization properties can be synthesized by using nanotechnology [20].

Beta-tricalcium phosphate, as a biocompatible material, can have different clinical applications [21]. A study showed that by increasing the concentration of beta-tricalcium phosphate from 1% to 5% added to fissure sealant, the remineralization potential of the fissure sealant increased with no reduction in its bond strength [20]. On the other hand, addition of beta-tricalcium phosphate to adhesives can improve their bond strength [21, 22].

Despite the advances in orthodontic materials and techniques, enamel demineralization around brackets during fixed orthodontic treatment is still a remarkable clinical problem that can jeopardize the esthetic results of an ideal orthodontic treatment. Therefore, finding a method that does not require patient cooperation has always been important. Since the color change of composite by silver nanoparticles can be esthetically undesirable, this study aimed to investigate the antibacterial properties of composites containing silver and amorphous tricalcium phosphate (ATCP) nanoparticles against *S. mutans*.

Materials and Methods

This in vitro study was carried out to evaluate the antibacterial properties of composite specimens containing silver and ATCP. The study was approved by the ethics committee of Rafsanjan University of Medical Sciences (IR.RUMS.REC.1396.141).

Synthesis of nanoparticles:

To synthesize ATCP, 1 g of hydroxyapatite was dissolved in 300 mL of deionized water and stirred vigorously to form a homogeneous suspension. Next, 3 molar solution of hydrochloric acid (Merck, Darmstadt, Germany) was added to the suspension to completely dissolve hydroxyapatite. In the next stage, one molar solution of sodium hydroxide (Merck, Darmstadt, Germany) was immediately added to the suspension to form tricalcium phosphate. The pH of the solution was adjusted with ammonium solution (Merck, Darmstadt, Germany). The resulting precipitate was stirred vigorously for 5 minutes and separated from the solution by centrifugation (Binder, Tuttlingen, Germany). The formed gel-like precipitate was washed three times, first with water, then with ethanol-water solution and eventually with water. Then 15 mL of ethanol

(Merck, Darmstadt, Germany) was added to the precipitate. The precipitate was dried after evaporation of ethanol and placed at 80°C for 1 hour [23, 24].

The co-precipitation method was used to synthesize Ag nanoparticles. For this purpose, 90 mg of silver nitrate (Merck, Darmstadt, Germany) was dissolved in water and heated to boiling temperature. At the same time, a reducing and stabilizing agent was added to the solution and stirred vigorously for 24 hours. The resulting precipitate was dried at 40°C for 4 hours [25].

Synthesis of nanocomposites:

To synthesize the nanocomposites, Ag nanoparticles with an average diameter of 40-60 nm and weight percentage of 0.3% as well as ATCP nanoparticles with an average diameter of 40-60 nm and weight percentage of 3% were weighed by a digital scale with an accuracy of 0.0001 g (Acculab digital scales, Edgewood, NY, USA). These nanoparticles were mixed uniformly with Transbond XT orthodontic light-cure composite (3M Unitek, CA, USA) by a high-speed mixer (3500 rpm) for 5 minutes in a semi-dark environment [8,13].

Figure 1 illustrates the field-emission scanning electron microscopic) FE-SEM) analysis, which is a microscopic analysis to take high-quality images from nanoparticulate materials at high magnification and resolution.

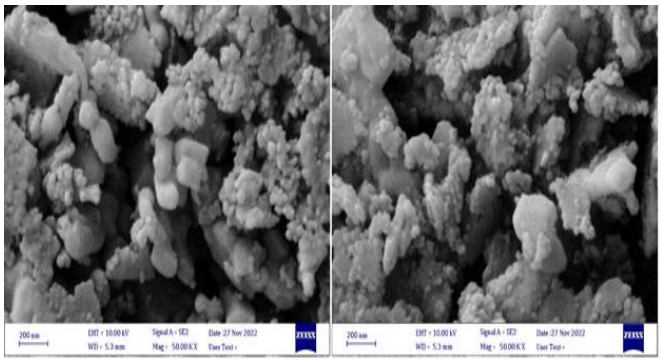

Figure 1. FE-SEM images of the synthesized silver nanoparticles

FE-SEM analyses showed that the synthesized material had very tiny aggregated particles. The mean particle size measured on FE-SEM images was 47 nm. The main reason for the aggregation of tiny particles is the high surface-to-volume ratio of the synthesized nanoparticles. In fact, the tiny particles had a high active surface energy, and they aggregated to decrease this surface energy.

Fabrication of composite discs:

Furthermore, 48 composite discs (4 mm in diameter and 3 mm in thickness) were fabricated in round Teflon molds. The discs were fabricated in a lathe workshop after ordering the desired diameter and thickness. To create a smooth surface in composite specimens, the molds were placed between glass slides. As a result, 32 discs were fabricated from nanocomposites (16 discs containing Ag nanoparticles and 16 discs containing ATCP nanoparticles) and 16 discs were fabricated from orthodontic light-cure composite without nanoparticles (as the control group). The required amounts of composites were placed in the desired molds, then the composites were cured using a light-curing unit (Woodpecker, LED.D China) with an output light intensity of 850 mW/cm^2 for 20 seconds. The surface of nano-composites was polished with 600, 800, and 1200-grit silicon carbide papers. Finally, the nanocomposites were removed from the molds and sterilized in an autoclave [1, 4, 7, 26].

Preparation of microbial suspension:

In order to evaluate the antibacterial properties of the synthesized composites, standard-strain *S. mutans* (ATCC 35668) was obtained from the Pasteur Institute of Iran, and was cultured in sterile brain heart infusion (BHI) broth (Merck, Darmstadt, Germany). In the next step, it was incubated at 37°C until the bacterial growth reached 0.5 McFarland concentration, containing approximately 1.5×10^8 bacteria [7, 26].

Anti-bacterial activity assessment:

The antibacterial properties of the fabricated composite discs were assessed 24 hours and 30 days after curing by the direct contact test and compared among the three groups. Composite discs were placed in sterile microtubes (n=16 per group) under sterile conditions. Next, 900 µL of sterile BHI broth and 100 µL of the bacterial suspension were added to each microtube and incubated at 37°C for 48 hours [7, 26].

Using a sampler, 10 µL of the BHI broth was collected from each microtube and diluted to 1.100 by sterile phosphate buffered saline, and sub-cultured on blood agar (Merck, Darmstadt, Germany) Petri dishes via lawn culture. The blood agar Petri dishes were incubated at 37°C for 48 hours [7, 26]. After 48 hours, the number of colonies on the surface of each plate was counted visually.

After performing the above-mentioned tests, all composite discs were rinsed with saline and dried. In the next step, the rinsed and dried composite discs were once again placed in sterile microtubes (n=16 per group) under sterile conditions, and the microbial test was performed after 30 days. The subsequent steps were performed as explained earlier.

Statistical analysis:

The results for *S. mutans* colony count were presented as mean ± standard deviation. The mean *S. mutans* colony count was compared using one-way ANOVA among the three study groups followed by the Duncan's multiple comparisons post hoc test. Paired t-test was applied to compare the mean *S. mutans* colony count at 24 hours and 30 days after curing in each group.

The non-parametric Kolmogorov-Smirnov test was employed to examine the normal distribution of *S. mutans* colony count data in each group, which indicated that the assumption of normality was met (P>0.05). The Levene's test was performed to assess the homogeneity of variances in the three groups, which confirmed the homogeneity assumption (P=0.416 and P=0.130 for 24 hours and 30 days after curing, respectively). For the statistical analysis, SPSS version 21.0 for windows (SPSS Inc., Chicago, IL) was used. All P values were 2-tailed, with statistical significance level defined at $P \leq 0.05$.

Results

Table 1 represents the mean number of *S. mutans* colony count in each group at 24 hours and 30 days after curing.

At each time point, different letters indicate significantly different mean *S. mutans* colony count between the study groups (P<0.001).

As shown in Table 1, the study groups differed significantly in terms of the mean number of *S. mutans* colonies at 24 hours and 30 days after curing (P<0.001). The Duncan's multiple comparisons test showed that the mean number of colonies in the composite group containing ATCP nanoparticles at 24 hours and 30 days after curing was significantly lower than the values in the composite group containing Ag nanoparticles and the control group (P<0.001). The same results were obtained when comparing the composite containing Ag nanoparticles with the composite without nanoparticles (P<0.001).

Moreover, paired t-test showed that the mean count of *S. mutans* colonies in the composite without nanoparticles was not significantly different at 24 hours and 30 days after curing (P=0.946). The same result was obtained in composites containing Ag and ATCP nanoparticles (P=0.861 and P=0.530, respectively).

Discussion

In the present study, the antibacterial effects of orthodontic composites containing Ag and ATCP nanoparticles against *S. mutans* were investigated and compared with those of orthodontic light-cure composite without nanoparticles. The findings showed that the antibacterial properties increased significantly in both groups of composites

Table 1. Comparison of the mean number of *S. mutans* colony count among the three groups and within each group at 24 hours and 30 days after curing

Group Duration	Without nanoparticles (n = 16)	Containing Ag nanoparticles (n = 16)	Containing ATCP nanoparticles (n = 16)	P value
24 hours after curing	2169.13 ± 170.98[a]	1592.75 ± 179.58[b]	1285.38 ± 122.62[c]	< 0.001
30 days after curing	2162.56 ± 290.03[a]	1580.94 ± 246.14[b]	1315.01 ± 175.43[c]	< 0.001
P-value	0.946	0.861	0.530	

containing Ag nanoparticles and ATCP nanoparticles. The highest antibacterial activity was noted in the orthodontic composite containing ATCP nanoparticles.

Microbial adhesion to the teeth and orthodontic appliances is among the important factors in initiation of enamel demineralization [27]. The retentive surface of orthodontic appliances can increase the risk of white spot development in case of poor oral hygiene [28-30]. Furthermore, *S. mutans* plays a major role in tooth decay due to its high adhesion to dental surfaces [31,32]. Application of orthodontic composites containing silver and calcium phosphate can reduce the adhesion of bacteria and formation of carious lesions around orthodontic brackets [33-35]. Meanwhile, nanotechnology has made great progress in prevention of enamel demineralization.

According to the present findings, 3% w/w ATCP nanoparticles significantly increased the short-term and long-term antibacterial properties of orthodontic composites. In other words, the composite containing ATCP nanoparticles had the highest short-term (24 hours) and long-term (30 days) antibacterial properties. Furthermore, the passage of time had no significant effect on the antibacterial properties of the tested nano-composites. Given that the orthodontic treatment process is long, long-lasting antibacterial properties are of particular importance.

Other studies [20, 36-38] revealed that composites containing different forms of calcium phosphate (amorphous calcium phosphate and tricalcium phosphate) were effective for tooth remineralization and caries inhibition, which was consistent with the results of the present study.

Alshammari and Sanea [39] found that amorphous calcium phosphate-containing adhesives can be effective in reducing enamel demineralization; these findings are also in line with the results of the present study.

A previous study [40] suggested that a cement containing amorphous calcium phosphate prevented enamel demineralization but this effect was not statistically significant. Differences in the results could be due to differences in material type as well as variations in the form of calcium phosphate used.

Some studies [41-43] evaluated the antibacterial properties of silver nanoparticles alone or in combination with other materials. The results of such investigations showed that silver nanoparticles were effective in inhibiting bacterial adhesion and reducing decay which confirms the results of the present study.

The present study showed that the orthodontic composite containing 0.3% silver nanoparticles had antibacterial properties after 24 hours and 30 days. In another study [8], growth inhibition zones were observed around 0.5% and 1% concentrations of silver

nanoparticles after 24 hours; while no such effect was seen after 30 days. Also, Sodagar et al. [1] indicated that adding 5% and 10% silver/hydroxyapatite nanoparticles created bacterial inhibition zones, but the same result was not reported following the use of 1% concentration of silver/hydroxyapatite nanoparticles. The difference in the results of the abovementioned studies with the present findings can be due to the difference in methodologies. On the other hand, lack of a significant reduction in short-term and long-term antibacterial properties of composites can be considered as a strength of this study.

Although addition of Ag and ATCP nanoparticles to orthodontic composite increased the antibacterial properties, in vitro studies on different concentrations of ATCP nanoparticles over longer periods of time are required to confirm the present results. Moreover, clinical trials are suggested to evaluate the antibacterial properties of orthodontic composites containing ATCP nanoparticles. Given that ATCP nanoparticles show positive effects in clinical studies, they may be recommended as a method for caries reduction in orthodontic patients.

Conclusion

Based on the present findings, addition of Ag and ATCP nanoparticles to orthodontic composite increased the antibacterial properties against *S. mutans*, but the highest antibacterial activity was related to the orthodontic composite containing ATCP nanoparticles.

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