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<Original Article>

In vitro Antibacterial Effect of Orthodontic Adhesives Mixed with Silver Nanoparticles

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• Abstract

Purpose: To examine the antibacterial effectiveness of silver nanoparticles (SNP) mixed with commercial orthodontic adhesives.
Materials and Methods: SNP was prepared by dissolving silver perchlorate in an organic solvent and reducing it with ultraviolet radiation. SNP was then mixed with four commercial orthodontic adhesives (Light Bond, Blugloo, Transbond XT, and Fuji Ortho LC) (0.05 wt %), which were then formed into disc-shape specimens (8.0 mm×3.0 mm). Commercial orthodontic adhesives containing no SNP were used as the control groups. Specimens of the four experimental and four control groups were incubated with *streptococcus mutans* and the medium turbidity was assessed at 3, 6, 9, 12, and 24 hours after incubation. The agar diffusion test was also performed to examine the growth inhibition zone of these groups. The data were statistically analyzed using a Wilcoxon rank sum test and t-test with a Bonferroni's correction ($P<0.05$).
Result: The SNP containing groups had a superior antibacterial effect compared to the control groups. In the agar diffusion test, the control groups without SNP did not produce an inhibition zone, whereas the SNP containing groups showed inhibition zone of 10~13 mm.
Conclusion: The incorporation of SNP into orthodontic adhesives can inhibit cariogenic bacterial growth.

• Key words : Anti-bacterial properties, Orthodontic adhesives, Silver nanoparticles

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Introduction

Enamel demineralization is one of the most frequent and easily recognized complications of orthodontic treatments with fixed appliances[1]. The occurrence of enamel demineralization is associated with cariogenic microorganisms, and the presence of an orthodontic bracket has been reported to provide an environment for bacterial adhesion, plaque retention and maturation[2]. The most susceptible site for bacteria and plaque retention is an excess of orthodontic adhesive left on the enamel surface because of their rough surface and the presence of a distant gap at the composite-enamel interface[2,3].

In this sense, one of the effective methods for preventing enamel demineralization is to use orthodontic adhesives resistant to bacterial accumulation. For this reason, many studies have examined the antibacterial properties of orthodontic adhesives or the effect of antibacterial agents incorporated into orthodontic adhesives[4-6]. In particular, fluoride and chlorhexidine are the most common ingredients incorporated into orthodontic adhesives for an antibacterial effect[7-10]. However, the limitation of fluoride and chlorhexidine as antibacterial agents is the short duration of the antibacterial effect[7,11].

Recently several reports have been published regarding the antibacterial effect of silver nanoparticles (SNP) in dentistry[5,12]. SNP have a large specific surface area, modified structure, controlled surface composition and reactivity, which endow them with remarkable physical, chemical and biological properties[13]. Several reports have demonstrated that silver ions are selectively toxic to prokaryotic microorganisms with little effect on eukaryotic cells[14,15]. These findings suggest that medical materials or devices with SNP coatings can prevent bacterial accumulation[16]. One study on the incorporation of SNP in experimental orthodontic adhesives reported promising results[5]. However, they did not clarify the method used to manufacture the SNP. The manufacturing method might affect the antibacterial characteristics of SNP. Therefore, precise information on the production method of SNP is important for readers interested in the use of SNP in orthodontic adhesives.

In this study, silver perchlorate, pluronic 123 and tetrahydrofuran (THF) were used to produce SNP by reduction with ultraviolet (UV) radiation. This study compared the antimicrobial activity of the four orthodontic adhesives mixed with SNP with those of as-received orthodontic adhesives without SNP.

Materials and Methods

1. Materials

Four commercial light-cured orthodontic adhesives were used in this study: Light Bond (LB) (Reliance Orthodontic Products, Itasca, IL, USA), Blugloo (BG) (Ormco Corp., Glendora, CA, USA), Transbond XT (TB) (3M/Unitek, Monrovia, CA, USA), and Fuji Ortho LC (FO) (GC Corp., Tokyo, Japan) (Table 1). The adhesive cements were divided into four experimental groups according to the orthodontic adhesives used. The experimental groups used SNP as the incorporated antibacterial agent. Orthodontic adhesives without SNP were employed as the control groups. The antibacterial effect of the experimental and control samples were measured at various incubation times (3, 6, 9, 12, and 24 hours).

Table 1. Four commercial light-cured orthodontic adhesives used in this study

Adhesive	Manufacturer	Composition
Light Bond (LB)	Reliance Orthodontic Products, Itasca, IL, USA	Fused silica Bisphenol A diglycidylmethacrylate Amorphous silica particles not otherwise classified (PNOC)
Blugloo (BG)	Ormco Corp., Glendora, CA, USA	Uncured methacrylate ester monomer Inert mineral fillers Fumed silica Activators and preservatives
Transbond XT (TB)	3M/Unitek, Monrovia, CA, USA	Silane treated quartz Bisphenol a diglycidyl ether dimethacrylate Triethylene glycol dimethacrylate Dichlorodimethylsilane reaction product with silica
Fuji Ortho LC (FO)	GC Corp., Tokyo, Japan	Alumino-silicate glass

2. Manufacturing SNP

A silver perchlorate solution was prepared by dissolving 5 mg of silver perchlorate and pluronic 123 in 20 ml of THF. The silver salt in the mixture was then reduced by radiation from a UV lamp (Black Ray longwave UV lamp model B100 AP, UVP Inc., Upland, CA, USA) for 2 h at 25°C. The appearance of a dark brown color indicated the formation of SNP. The reaction was quenched after 2 hours and the precipitate was centrifuged and dried under vacuum for 24 h at 60°C. Fig. 1 presents the method for manufacturing the SNP.

3. Incorporation of SNP into Orthodontic Adhesives

Each specimen was prepared using Teflon molds (inner dimension of 8 mm×3 mm). The Teflon molds were positioned on top of the lower glass slides. Each of the four orthodontic adhesives mixed with SNP (0.05% by weight) were placed into the mold until the material was level with the top of the template. A glass slide was placed on top of the Teflon mold, pushed down to make a flat surface, and then removed carefully. All materials were used according to the manufacturer's instructions and light cured for 40 s (20 s from the top and 20 s from the bottom). The control samples (orthodontic adhesives without SNP) were produced in the same manner described above. All the control and experimental samples were dried then sterilized with Ethylene Oxide gas.

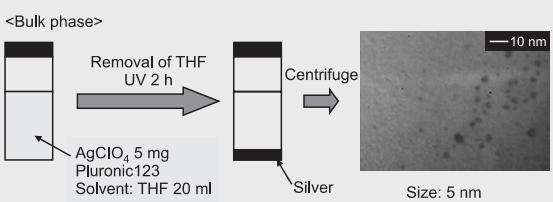

Figure 1. Method for manufacturing silver nanoparticles (SNP). Well dispersed SNP can be seen. UV: ultraviolet, THF: tetrahydrofuran.

4. Antibacterial Effect of Four Orthodontic Adhesives Mixed with or without SNP

The specimens for each of the four adhesives (n=10) were incubated in 5.0 ml of brain heart infusion (BHI) broth with each strain of *Streptococcus mutans* (*S. mutans* 25175) approximately 1×10^7 cells/ml for 3, 6, 9, 12, and 24 h at 37°C. In the control group, each of four orthodontic adhesives without SNP was incubated under same conditions. As a control, each bacterial suspension was incubated with sterile Teflon discs with the same size and shape as the experimental and control groups. After copious shaking of the test tubes, bacterial growth was then estimated spectrophotometrically at 570 nm. A blank medium without material was also used for comparison. Each experiment was repeated ten times (n=10).

5. Agar Diffusion Test

The agar diffusion test was designed to test the antibacterial effect of orthodontic adhesives with SNP that could readily diffuse through agar to produce a growth inhibition zone. Disc type (8.0 mm×3.0 mm) specimens were prepared and used. The test was performed on BHI agar plates. Each plate was spread on the top of the plate with 200 μm of

Table 2. Measurement of the optical density of the medium with *Streptococcus mutans* and four different orthodontic adhesives with or without silver nanoparticles after 3, 6, 9, 12, and 24 hours of incubation

		3 hrs	6 hrs	9 hrs	12 hrs	24 hrs
LB	Without SNP	0.055^{Aa}±0.003	0.066^{Ab}±0.002	0.122^{Ac}±0.008	0.213^{Ad}±0.011	0.250^{Ae}±0.019
	With SNP	0.046^{Ba}±0.002	0.056^{Bb}±0.002	0.060^{Bc}±0.003	0.062^{Bc}±0.003	0.069^{Bd}±0.004
BG	Without SNP	0.052^{Aa}±0.002	0.063^{Ab}±0.002	0.128^{Ac}±0.010	0.225^{Ad}±0.017	0.270^{Ae}±0.016
	With SNP	0.045^{Ba}±0.002	0.056^{Bb}±0.002	0.059^{Bc}±0.003	0.060^{Bc}±0.002	0.067^{Bd}±0.005
TB	Without SNP	0.051^{Aa}±0.003	0.061^{Ab}±0.002	0.122^{Ac}±0.009	0.209^{Ad}±0.015	0.272^{Ae}±0.012
	With SNP	0.046^{Aa}±0.006	0.057^{Ab}±0.006	0.062^{Bc}±0.005	0.065^{Bd}±0.006	0.069^{Be}±0.008
FO	Without SNP	0.060^{Aa}±0.003	0.071^{Ab}±0.003	0.141^{Ac}±0.010	0.196^{Ac}±0.013	0.235^{Ad}±0.019
	With SNP	0.044^{Ba}±0.004	0.050^{Bb}±0.001	0.053^{Bb}±0.002	0.059^{Bc}±0.003	0.067^{Bd}±0.004

LB: Light Bond, BG: Blugloo, TB: Transbond XT, FO: Fuji Ortho LC.
A Wilcoxon rank sum test was performed. The same superscript indicates no significant difference (P>0.05). The large alphabetic letter shows the results of a comparison between the groups with or without silver nanoparticles, the small alphabetic letter indicate the results of a comparison between the groups with different incubation periods within the same material.

freshly grown cariogenic *streptococci* 1×10^8 cells/ml. Five 5 mm diameter holes were punched in the agar surface of each plate. The respective adhesive was incubated at 37°C for 24 h and inspected visually for the presence of inhibition zones in the bacterial lawn. The bacterial inhibition zone halo was measured and expressed in millimeters. The tests were performed three times.

6. Statistical Analysis

A Wilcoxon rank sum test and t-test with a Bonferroni's correction were used to compare the antibacterial effect of the four orthodontic adhesives with or without SNP. Repeated measures data analysis using a mixed model was used to compare the change in antibacterial effect with time within each group. A P-value<0.05 was considered significant.

Result

1. Bacterial Growth Inhibition Test

Table 2 lists the results of the bacterial growth inhibition test. The incorporation of SNP had a significant effect on the inhibition of bacterial growth in the liquid media as shown by the optical density. Fig. 2 shows the results of the bacterial growth tests. At 3 and 6 hours of incubation, the LB, BG and FO groups with SNP showed a significantly lower optical density than the respective groups without SNP (P<0.05). In contrast, there was no significant difference in optical density between the SNP containing and non-containing TB groups. At 9, 12, and 24 hours of incubation, all four SNP containing groups showed significantly lower optical density than the respective groups without SNP (P<0.05).

The antibacterial effects of the orthodontic adhesives containing SNP at various incubation times were also compared. The LB, BG, and TB without SNP groups showed a difference in antibacterial effect at all incubation time. FO without SNP group showed a difference in the antibacterial effect at all incubation times except at 9 and 12 hours. In addition, TB with SNP group showed differences in antibacterial effect at all time periods of incubation. However, LB and BG with SNP group showed a difference in antibacterial effect at all incubation times except at 9 and 12 hours. FO with SNP group showed a difference at all incubation times except at 6 and 9 honors.

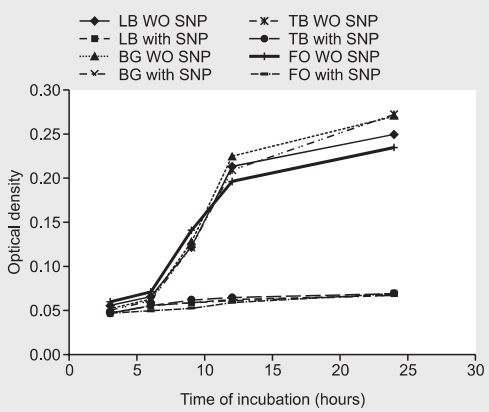

Figure 2. Results of the bacterial growth inhibition test, which shows that SNP containing orthodontic adhesives have a greater antibacterial effect than the as-received orthodontic adhesives. LB: Light Bond, BG: Blugloo, TB: Transbond XT, FO: Fuji Ortho LC, WO: without, SNP: silver nanoparticles.

2. Agar Diffusion Test

All four adhesives without SNP did not produce an inhibition zone. On the other hand, all the orthodontic adhesives that were mixed with SNP produced a 10~13 mm inhibition zone. Table 3 lists the results of the agar diffusion test.

Discussion

Metals, such as silver, gold and zinc, have been used as bactericidal and bacteriostatic agents for centuries[17-19]. Among these, silver is the most effective antibacterial agent[12] with broad antibacterial activity[20]. The antibacterial activity of metals depends on their contact surface. Recently developed SNP have a larger surface area that enables a wider range of

Table 3. Agar diffusion test (n=3)

Material		Inhibition zone (mm, mean±SD)
LB	With SNP	11±2[a]
	Without SNP	0±0[b]
BG	With SNP	10.67±1.53[a]
	Without SNP	0±0[b]
TB	With SNP	10.67±2.08[a]
	Without SNP	0±0[b]
FO	With SNP	11.33±1.53[a]
	Without SNP	0±0[b]

LB: Light Bond, BG: Blugloo, TB: Transbond XT, FO: Fuji Ortho LC.
A paired t-test was performed. The same superscript indicates no significant difference (P>0.05).

interactions with other organic and inorganic molecules. A size reduction from microparticles to nanoparticles results in a larger contact surface and reduced production cost, which may be advantageous to the routine use of SNP.

Previous studies[21,22] demonstrated the antimicrobial effect of SNP against microorganisms and human cells. Hernández-Sierra et al.[12] reported that SNP may be the most effective metallic agents for controlling *S. mutans*. Ahn et al.[5] showed that the incorporation of SNP in experimental orthodontic adhesives reduced the level of bacterial growth in a liquid medium significantly. Alt et al.[21] also reported that SNP were selectively toxic to bacteria but not cytotoxic to human cells. They attributed this phenomenon to eukaryotic cells being larger with higher structural and functional redundancy than prokaryotic cells, which means that more silver ions are needed to establish a comparable toxic effect than bacterial cells. They also suggested that this difference provides a "therapeutic window", in which bacterial cells are attacked successfully with minimal harmful effects on eukaryotic cells[12]. Another explanation for the difference in the susceptibility of eukaryotic and prokaryotic cells to the attack of silver ions is that the essential protein complex of the bacterial electron transport chains are located at the cellular surface and are easily accessible to silver ions, whereas those of eukaryotic cells are located in the intracellular mitochondrial organelles, which are protected from the attack of silver ions by the cellular membrane, which is a diffusion barrier[13].

This study demonstrated that four commercial orthodontic adhesives mixed with SNP had significantly higher antibacterial activity than the plain orthodontic adhesives without SNP. These results agree well with the study reported by Ahn et al.[5]. They also suggested that the incorporation of SNP in orthodontic adhesives may not have an adverse effect on the physical properties of orthodontic adhesives. The higher antimicrobial activity in orthodontic adhesives with SNP might be due to the effect of the catalytic action of silver, which change the oxygen into active oxygen that can denature the proteins and enzymes of bacteria[5]. In addition, the advantage of the incorporation of SNP is its lower tendency to induce the resistance of bacteria. As mentioned earlier[21], SNP binds to bacterial cellular structures, such as enzymes and other proteins, particularly to their SH-group. Accordingly, SNP might interfere with the bacterial cell integrity[23], production and conservation of energy[24]. Because of this multilevel antibacterial mode, resistance is not easily obtained from single point mutations, which is in contrast to antibiotics, such as aminoglycoside[16].

The antibacterial effect of SNP might be affected by the methods for manufacturing SNP. Unfortunately, the preparation of SNP was not clarified by previous report[5]. In the present study, as illustrated in Fig. 1, a silver perchlorate solution was prepared by dissolving 5 mg of silver perchlorate and pluronic 123 in 20 ml of THF. The silver salt was then reduced by radiation from a UV lamp. The formation of SNP particles, <5 nm in size, was confirmed by transmission electron microscopy.

The problem with SNP incorporation in orthodontic adhesives is mainly cosmetic changes, such as discoloration. However, the use of nanotechnology can eliminate this problem with new material properties[25]. The incorporation of an effective but not discoloring proportion of SNP to orthodontic adhesives might solve the problem[5], but further studies will be needed to determine the discoloration of the dentin and enamel caused by various concentrations of SNP along with methods for overcoming this limitation.

This study have several limitation, such as lack of mechanical strength test (bracket bonding test), the possible confounding antibacterial effect of remaining organic solvent (THF, Pluronic 123), relatively short observation time (24 h), no evaluation of the color change after incorporation of SNP. Furthermore, the experimental design which employs direct contact with SNP and bacteria would be better model for evaluation of antibacterial properties of SNP. A consecutive study with better experimental design is going to be carried out in this affiliation in near future.

Many of the current studies have been using *in vitro* methodologies, because biocompatibility of nanoparticles still did not prove enough[26]. Further *in vivo* studies will be necessary to investigate the long-term antibacterial effectiveness of SNP in orthodontic treatment.

Conclusion

The incorporation of SNP into orthodontic adhesives can be useful for inhibiting cariogenic bacterial growth around the brackets or adhesives.

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