Effect of Silver Nanoparticles on Microleakage and cytotoxicity of New Universal Adhesive

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Abstract

Introduction: Despite the incredible popularity of dental composites, their application is associated with several difficulties, one of the most important of which is the microleakage phenomenon. Incorporation of silver nanoparticles in composites exerts antibacterial effects; nonetheless, the impact of silver nanoparticles on microleakage has not yet been studied. This research conducted to assess the effect of silver nanoparticles application as a precursor on the microleakage of composite-filled clV cavities using two different approaches of universal bond application. **Methods:** Sixty non-carious human molars were collected, and clV cavities were provided on the buccal surfaces. The samples were categorized to 4 groups (n=15 in each group) and were restored using four different methods. After placement in 2% basic fuchsin solution for 24 h, permeability was determined between the cavity wall and restorative material in the gingival and occlusal margins. Thereafter, one sample of each group was selected. After preparation, the specimens were analyzed using backscattered electron scanning electron microscopy. Finally, the cytotoxicity was assessed by the Methyl-thiazolyl-tetrazolium (MTT) assay. **Results:** In both gingival and occlusal margins, if the silver nanoparticles were used, microleakage significantly decreased ($P \leq 0.001$). The addition of silver nanoparticles had no adverse effect on human gingival fibroblasts (HGF) cell viability. **Conclusion:** It seems that the use of silver nanoparticles in the total-etch method can improve phosphoric acid penetration into dentinal tubules, increases the depth of etching, and promotes the bond quality.

Keywords: ClV Restoration, Microleakage, MTT assay, Silver Nanoparticles, Universal Bond.

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Introduction

One of the most significant contributions to dentistry has been the development of the new generation of dental adhesive technology. Manufacturers have endeavored to make adhesives more efficient by introducing modifications in their composition to improve their characteristics and make them easier to use (1, 2). According to recent studies, universal adhesives have been explained as: "the no-mix, single-bottle adhesives which can be used in self-etch, total-etch or selective-etch mode" (3). Polymerization shrinkage is one of the most important considerations of composites, resulting in the formation of a gap between the margins of teeth and the restorations, that leads to recurrent caries and failure of restorations (4, 5).

The transmission of bacteria, molecules, ions, and oral fluids between the restoration and cavity walls is referred to as microleakage (6, 7). Many postoperative failures, such as recurrent caries, sensitivity and breakdown of the restoration, are associated with microleakage (8, 9). The current trend in using adhesive systems is to invent the simple self-etching systems which are obtained by reducing the number of steps to a one-step (2). Serious attempts have done to add some antibacterial materials, such as nanoparticles, to composite resins and dental adhesives in order to gear dental materials with therapeutic effects and antibacterial activity in (10-14).

Metallic silver is one of the elements that have proven antibacterial characteristics compared to other metals (15-18). Previous studies have demonstrated that when silver is converted into nanoscales, its surface-to-volume ratio increases, and this enhances its antibacterial activity (14, 19, 20). The evaluation of the silver nanoparticles cytotoxicity on fungi, viruses, protozoa, and different oral bacteria, such as *lactobacillus* and *streptococcus mutans* confirms the bactericidal properties of silver nanoparticles; nonetheless, its effects on human gingival fibroblasts (HGF) have not yet been investigated enough (21, 22). In addition, some researches have demonstrated the antibacterial effects of silver nanoparticles in composite restorations against oral cariogenic bacteria (15, 23).

In some previous studies, silver nanoparticles showed different effects on various aspects of resin-dentin bond characteristics (24-26). Nevertheless, the effect of silver nanoparticles on microleakage has not yet been studied, and no attention has been paid to the response of new universal adhesives to the application of silver nanoparticles. The present research conducted to assess the influences of silver nanoparticles on the microleakage of clV cavities using two different approaches of the universal bond. The null hypothesis of this research was that perhaps a positive effect would be seen on microleakage in silver nanoparticle-added groups compared to that in conventional bonding method groups.

Materials and Methods

Preparation of samples and silver nanoparticles

The materials composition used in the present research is illustrated in Table I. Silver nanoparticles were prepared and characterized according to the instruction in our recent study. Thereafter, 1g/L silver nitrate was mixed with sodium dodecyl sulfate (SDS) (Merck, Darmstadt, Germany) in an aqueous solution under a nitrogen atmosphere. SDS molecules were used to stabilize the formation of shaped silver nanoparticles and their dispersion. Oxygen was removed from water using nitrogen bubbling, and the electrolyte was combined under a N2 atmosphere. SDS was added at the 40 g/L level to avoid aggregation. The products were washed with distilled water and collected by centrifugation at 15000 rpm for 10 min (Hettich Universal 320) (27). The sample size was counted according to the estimated effect size among with study groups based on the previous researches (28).

Table I. Test materials, manufacturers, and composition

Name	Manufacture	Composition
Adhesive System (Singlebond Universal Adhesive)	3M, ESP, St Paul, MN, USA	MDP phosphate monomer, dimethacrylate resins, HEMA, Vitrebond copolymer, filler, ethanol, water, initiators, silane
Composite resin (Filtek Z-250)	3M, ESP, St Paul, MN, USA	UDMA (urethane dimethacrylate), Bis-EMA (bisphenol A polyethylene glycol diether dimethacrylate), TEGDMA (tri-ethylene glycol dimethacrylate) and inorganic filler
Etchant 37%	Ivoclar Vivadent, Germany	H_3PO4 37%
Silver nanoparticle	-	Mean size 20 nm

Sixty human molars were collected. The teeth were non-carious and extracted in the last six months. They were placed in chloramine T at 4°C for seven days and were then kept in distilled water. ClV cavities (3 mm length, 1.5 mm depth and 2 mm height) were done on the buccal surfaces, the height of the cavity concluded 1 mm high, and 1 mm below the cementoenamel junction (CEJ) (29). Cavities were done by a 0.9 mm fissure diamond bur in the presence of air-water coolant and high-speed handpiece. The cavities were randomly categorized to four groups (n =15).

Group 1: In each tooth, after etching for 15 sec, the cavity was rinsed and dried; thereafter, the universal adhesive (3M ESPE; St. Paul, MN, USA) was rubbed by micro brush according to the manufacturer's instruction. The air was used slowly for 5 sec to decrease the thickness of the bonding layer. Following that, the bonding was cured for 10 sec, and the Z250 (3M) composite was applied in the cavity and cured for 40 sec (VIP Junior, Bisco, Schaumburg, IL, USA) at 600 mW/cm2.

Group 2: After the preparation of cavities, the 3M universal adhesive was used in a self-etch approach and the cavities were then restored using Filtek Z250 composite.

Group 3: All the steps are the same for group 1; nonetheless, before etching, silver nanoparticles (1% W/W) were applied on dentin surfaces for 60 sec and then rinsed for 2 min.

Group 4: All the steps are the same for group 2, using silver nanoparticles on the teeth before the application of the universal bond in the aforementioned order.

After the cavities were restored, the teeth were stored in distilled water for 24 h at 25˚C; subsequently, the samples were polished and finished. Then, the sticky wax was used for sealing the apical foramen; All surfaces of the teeth were covered using two layers of nail varnish, except the one-millimeter margins of the restorations, then the teeth were stored in 2% basic fuchsine (Merck) for 24 h at 25˚C. Following that, to remove the additional dye, the samples were thoroughly washed with water. A Mecatome machine (PRESI, Mecatome T330, France) was used for cutting samples in a buccolingual direction in the presence of water and air coolant. Following that, a stereomicroscope (Motic K-500L, Motic Incorporation Ltd, Hong Kong) was used for measuring the permeability between the restoration and the teeth with 40x magnification by two observers. Microleakage was quantitatively determined by a specific gauge in occlusal and gingival margins.

Scanning electron microscopy analysis

For scanning electron microscopy (SEM) analysis, one sample of each group was randomly selected and acid-etched with 10% phosphoric acid (3M) for 10 sec to remove the smear layer produced by the cavity preparation. Subsequently, sodium hypochlorite 5% was applied on specimens for 2 min. The desiccation of samples was performed by immersion in a series of various concentrations of alcohol (Merck). Thereafter, the specimens were fixed onto a metal stub; their surfaces were coated with a thin layer of gold, observed under 1500 magnification of scanning electron microscope (TESCAN Brno, Brno - Czech Republic), and examined by backscattered electron images.

Cell culture and MTT assay

A total of 12 cylindrical specimens were prepared by placing the Filtek Z250 composite into stainless steel mold 2mm thick and 5mm in diameter. In half of the samples, 5% silver nanoparticles were added to the composite using ultrasonic and hand manipulation. The HGF, human gingival fibroblast cell line, cells were cultured in RPMI-1640 medium supplemented with 10% FBS, antibiotics, and glutamine (Gibco, Paisley, Scotland) at 37ºC in an incubator containing 5% CO_2. The cells (10000 cells/well) were incubated by samples at 24 h (30). The cytotoxicity was determined using methyl thiazolyl tetrazolium bromide (MTT, Merck) assay (31, 32).

Statistical analysis

The data were analyzed in SPSS software (version 18.0) using one-way ANOVA and Post Hoc Tukey to compare the effect of the etching method and the use of silver nanoparticles on the microleakage average. The mean scores of cytotoxicity of two different composite samples were evaluated by the Kruskal-Wallis test. A P-value less than 0.05 was considered statistically significant.

Results

Microleakage measurement

In gingival and occlusal margins in all groups, using silver nanoparticles was better (Figure 1). The microleakage reduction was significant in occlusal and gingival margins. The use of silver nanoparticles led to a microleakage reduction in both occlusal and gingival margins ($P \leq 0.001$). The microleakage of occlusal margins was less than gingival margins while using silver nanoparticles ($P \leq 0.001$). Moreover, in the absence of silver nanoparticles, it was demonstrated that the microleakage of occlusal margins was less compared to gingival margins ($P \leq 0.001$).

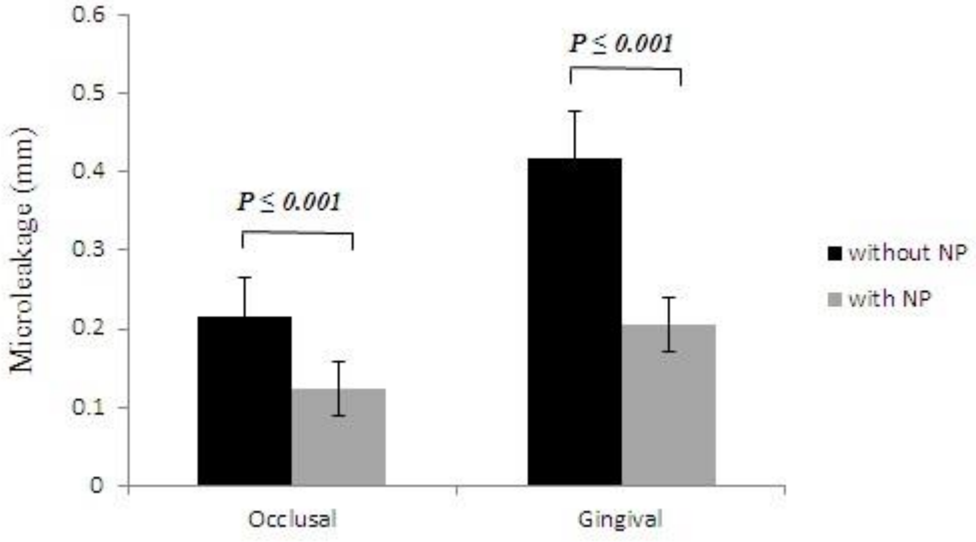

Figure 1. In both occlusal and gingival margins in all groups, the application of silver nanoparticles was superior to no application. This reduction in microleakage is significant in gingival and occlusal margins ($P \leq 0.001$). Mean± SD of microleakage (mm) is expressed for 15 independent experiments

In the enamel margins, when using the total-etch method and the silver nanoparticles prior to acid etching, a significant decrease was detected in the average amount microleakage ($P \leq 0.001$) (Figure 2). In the total-etch method in gingival margins, although the use of silver nanoparticles led to a decrease in the mean microleakage ($P \leq 0.001$), in the self-etch method, it led to a more significant microleakage reduction ($P \leq 0.001$). Silver nanoparticles in both self-etch and total etch methods, led to a decrease in microleakage values ($P \leq 0.001$).

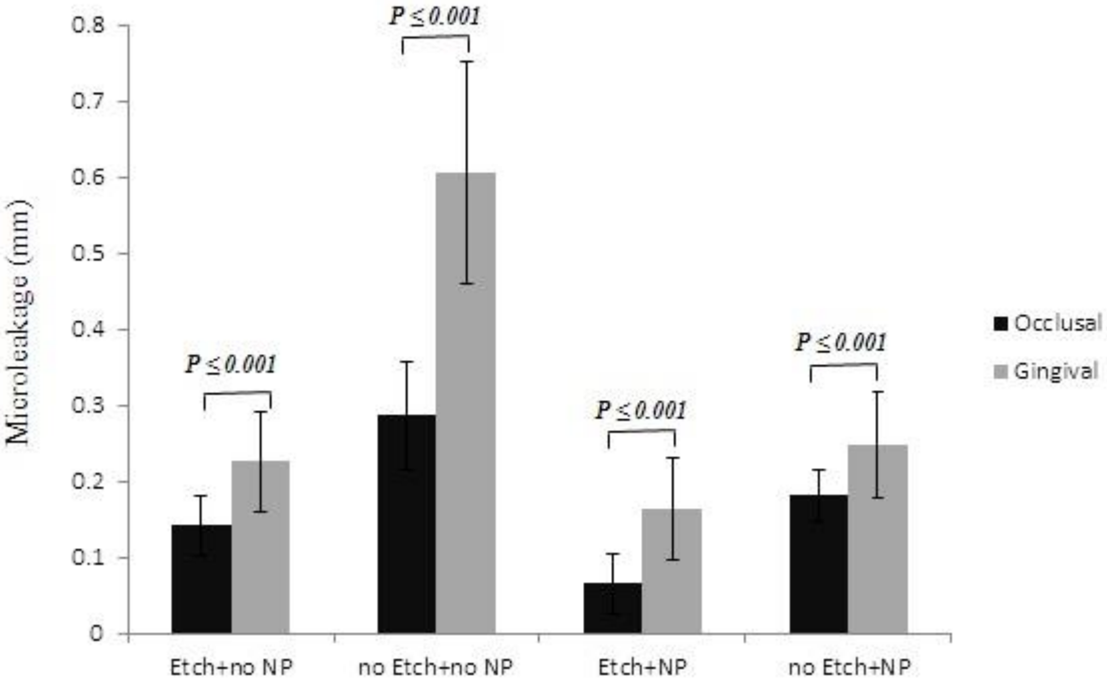

Figure 2. Comparison of microleakage scores between occlusal and gingival margins. Mean± SD of microleakage (mm) in the occlusal and gingival margins are displayed for 15 samples.

Cell cytotoxicity

In the present study, we evaluated the cytotoxicity of Filtek Z250 dental restorative materials incorporating silver nanoparticles by treating the HGF cells for 24 h, followed by an MTT assay. However, compared to the controls, the total cell number of Filtek Z250 and Filtek Z250 incorporated silver nanoparticles decreased by19.58% (P≤0.001) and 22.86% (P≤0.001), respectively. This decrease is not significant between the two test groups (*P=0.06*). The outcome is expressed as mean± SD from 12 independent experiments. In addition, the cytotoxicity of dental materials was assessed by Kruskal-Wallis test (Figure 3).

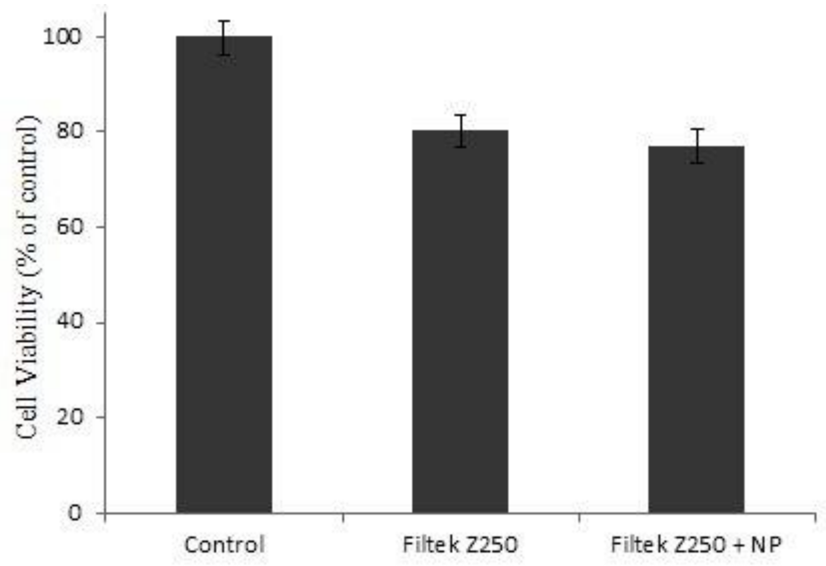

Figure 3. Compared to the controls, the Filtek Z250 and Filtek Z250 incorporating silver nanoparticles, 19.58% (*P-value* ≤0.01) and 22.86% (*P* ≤0.01), decreased in total cell number, respectively. Results are expressed as a percentage of viability compared to control. Statistical evaluation of cytotoxicity of dental materials was assessed by the Kruskal-Wallis test.

Scanning electron microscope

The margins (occlusal and gingival) of both total-etch and self-etch approaches of universal adhesive with and without silver nanoparticles were assessed, and the gaps was noted. The gaps between the restoration and tooth structure were not similar in both groups (Figure 4). The bond interface of self-etch approach specimens displayed an indistinct interface between the margin of the restoration and the tooth structure. Acid etching resulted in more mineral removal, more irregular surfaces and open dentinal tubules (Figure 4A). In groups using silver nanoparticles, areas impregnated with moderate amounts of resin were detected beyond the hybrid layer (Figures 4C and 4D). However, more resin tag impregnation was detected in most areas of the margins after etching and silver nanoparticle application. The resin tags were uniform in shape and oriented in different directions (Figure 4C). Due to the slight roughness of the dentin, fewer open tubules were observed in self-etch mode compared to pretreatment with phosphoric acid. In addition, less resin impregnation was detected compared to the etch-and-rinse mode (Figure 4B and 4D).

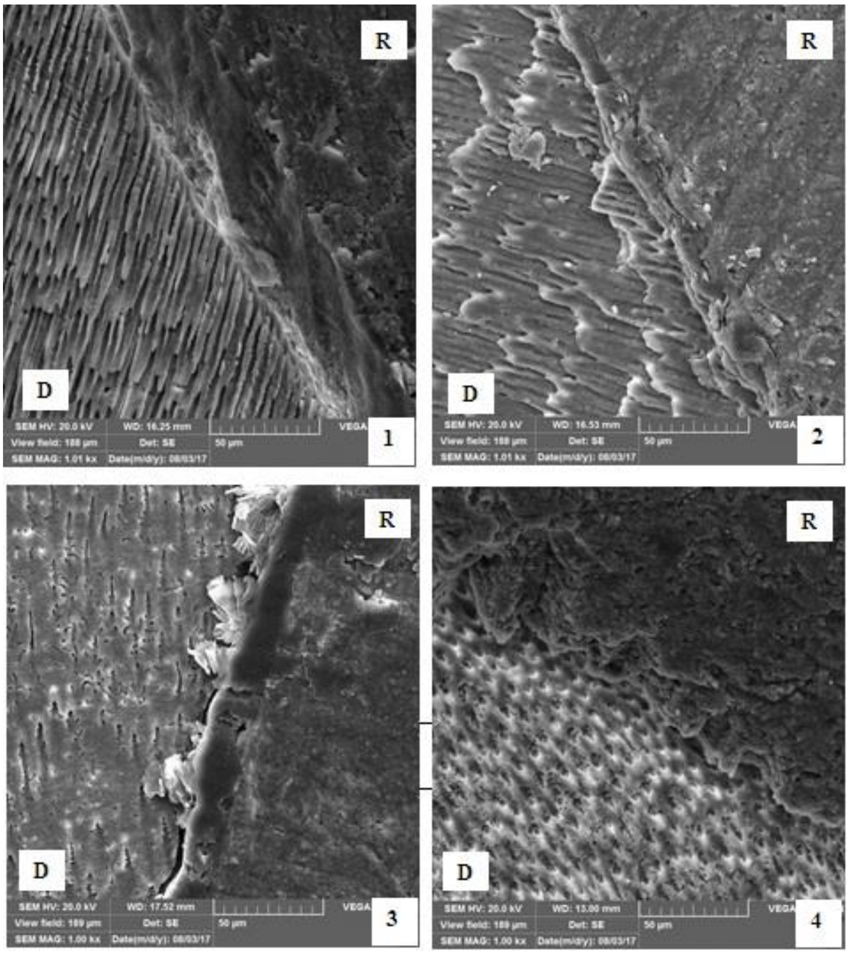

Figure 4. Group 1, 2, 3, and 4 specimens' × 1000. Scanning electron microscopy illustrating the resin-dentin interface (R=restorative material, A=adhesive layer, D=dentin)

Discussion

Silver is a safe ingredient with antimicrobial effects; it is innocuous and harmless for host cells; nonetheless, it is very toxic to microorganisms. The antibacterial properties of silver nanoparticles have been recently assessed in various fields of medical sciences and dentistry (33). Jowkar et al. state that pretreatment with silver nanoparticles can be recommended as an approach to achieve potent antibacterial activities without compromising the bond strength (25). Another advantage of the small size is that silver nanoparticles can penetrate to the cell membranes, resulting in greater antibacterial function. It seems that silver ions can cause structural changes in the cell wall, increase membrane permeability, and induce cell death. Furthermore, silver nanoparticles can preclude DNA replication by interaction with the bacterial proteins. As a result, they have acceptable biocompatibility and low toxicity with human cells. These reports are consistent with the findings of the current study regarding the minor cytotoxic effect of silver nanoparticles on human gingival fibroblasts (34).

This study is the first report on using silver nanoparticles as a pretreatment on the cavity walls for microleakage measurement of new universal bonding agents. Khairy et al. detected no difference in microleakage between ClinproTM pit fissure sealant with adjunct silver nanoparticles and the conventional sealant when applied to permanent teeth (28). Along the same lines, in a study by Quiroga et al., it was established that no significant differences in the microleakage were noted between the conventional sealant and silver nanoparticle-containing sealant. The addition of silver nanoparticles did not change the adhesion of the sealant (35). Daneshkazemi et al. suggested that in groups containing 0.1% concentration of Cu_2O_3 nanoparticles, the microleakage elevated by increasing the number of thermocycling up to 15,000 cycles (36).

Very specific and cross-linking monomers are needed to develop a universal adhesive. The monomers must have the capacity to react with different substrates; moreover, they have hydrophilic properties and copolymerize with resin-based restorative materials in order to "wet" dentin properly. However, to prevent bond hydrolysis over time, they must be as hydrophobic as possible. Ideally, universal adhesives must be enough acidic to be efficient in a self-etching approach but not so acidic that they separate initiators required for the polymerization of dual-cure and self-etch resin types of cement. Universal adhesives should also include water since it is necessary for the functional monomers dissociation in order to create a self-etching approach (3).

In total-etch adhesive systems, the demineralization of superficial dentin by mineral acids leads to hybrid layer formation and exposure of collagen fibrils that infiltrated by hydrophilic monomers (37). Jowkar et al. found that silver nanoparticles improved the micro shear bond strength for etch-and-rinse and self-etch an adhesive system (26). In another study, they indicated that application of silver nanoparticles after the conditioner was associated with significantly greater micro shear bond strength values (38).

One of the problems of universal adhesives is when little water is needed; too much may change the adhesive chemistry, leading to reduced shelf-life and incomplete evaporation during the air-drying. Residual water may lead to defective polymerization of the adhesive, compromised adhesive interface and more hydrolysis after polymerization. These reasons may lead to phase separation, as well as the hydrolysis and degradation of bonding layer. Although the incorporation of acetone or ethanol into universal adhesive can promote infiltration of tooth tissues, other important factors, such as initiator and solvent, pH, as well as monomer types, also have a major role to play in these systems efficacy.

It seems that using silver nanoparticles in the self-etch approach of universal adhesive leads to improved dentin wetting by adhesive and increased bond quality, resulting in the reduction of microleakage. Presumably, due to these reasons, the response of the total-etch approach of universal adhesive when applying silver nanoparticles is lower compared to that of the self-etch approach (3). The extent and depth of demineralization by self-etch adhesives are limited in comparison with the total-etch. Based on the studies, the functional monomers in self-etch adhesive systems may have some chemical interactions with hydroxyapatite in an acceptable clinical time. These chemical interactions have been improved by the infiltration of silver nanoparticles into dentinal tubules and maybe provide better resistance to degradation by microleakage prevention (23).

As demostrated by the results, it seems that the use of silver nanoparticles in the total-etch approach can enhance phosphoric acid penetration into dentinal tubules, increases the etching depth, and promotes the quality of the bond. Compared to total-etch approach, the extent and depth of demineralization are lower in the self-etch approach (39). Investigation has pointed out that the monomers of the universal adhesive have good reaction with hydroxyapatite in the presence of silver nanoparticles (40). This reaction helps the penetration of silver nanoparticles into dentinal tubules and may prevent microleakage. In conclusion, dentin pretreatment with silver nanoparticles decreased the microleakage in self-etch and total-etch methods of universal adhesive compared to the control groups. Moreover, silver nanoparticles had no adverse effects on HGF cell viability.

Conflicts of Interest

The authors declare no conflict of interest

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