



Original Article

Evaluation of Microleakage and Biocompatibility of Universal Adhesive Containing Titanium Dioxide Nanoparticles: A Comparative In Vitro Study

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Abstract

Background & Objective: Although dental composites have undergone a high level of development in recent years, there are still difficulties including microleakage. The incorporation of nanoparticles (NPs) in dental materials can produce antibacterial effects, but the effect of TiO₂ NPs on microleakage has not yet been investigated. The present study aimed to determine the effect of TiO₂ NPs incorporation in the universal single bond and pretreatment with them on microleakage of cV cavities using both total-etch and self-etch approaches.

Materials & Methods: Standard cV cavities were prepared on the buccal surfaces of the sixty non-carious human molar teeth. The restoration of samples was carried out with 6 different methods and randomly divided into six groups of ten subjects. Microleakage in both gingival and occlusal margins was determined after placement in 2% basic fuschin solution for 24h. Biocompatibility of a universal single bond was evaluated by MTT assay.

Results: In both occlusal and gingival margins in all groups, the application of TiO₂ NPs was better than no application. We observed the relatively low cytotoxic effect of TiO₂ NPs incorporated in the universal adhesive on NIH-3T3 cell viability.

Conclusion: Regarding the limitations of the present investigation, using TiO₂ NPs with both total-etch and self-etch approaches to universal single bond may lead to a decrease in the microleakage of resin composite restorations.

Keywords: TiO₂ NPs, Microleakage, Universal Bond, NIH-3T3

Introduction

Microleakage is the most frequently encountered problem with posterior composites mainly due to polymerization shrinkage (1). According to Kidd, microleakage is defined as the clinically undetectable passage of bacteria and bacterial products, fluids, molecules, or

ions from the oral cavity along with the various spaces present in the cavity restoration interface (2). Producing polymer chains from monomers leads to composite shrinkage. These conditions can lead to the fracture of composite restoration and recurrent caries (3).

Despite the significant improvements in composite and adhesive structures over the recent years, some drawbacks related to polymerization shrinkage and microleakage still remain that

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have to be improved (4, 5). Currently used composites demonstrated the volumetric shrinkage from 2 to 5%. This results in marginal gap formation leading to microleakage (6). If the bonding strength at the interface is lower than contraction forces, interfacial spaces can lead to recurrent caries, staining, postoperative sensitivity and marginal leakage. Therefore, the bonding systems have an important role in reducing microleakage (7).

There are different ways to prevent plaque accumulation at the interface and on resin composite restorations. One acceptable method is the incorporation of NPs to adhesive and composite resins, due to their antimicrobial properties (8, 9).

A protective insoluble and stable oxide layer (TiO₂) is forming on the titanium surface. TiO₂ NPs are mostly used in medical and in the field of dentistry (10). TiO₂ has some specific characteristics such as exhibition of a highly efficient photocatalytic effect, chemical stability and low toxicity (11). TiO₂ NPs are considered as inorganic additives in resin composites that have many promising properties (12). To achieve opalescence, it was incorporated into cold-curing resin composites. A recent investigation showed that dental resin composites with TiO₂ NPs, demonstrated better mechanical properties (13). It was shown that the number of adherent bacteria reduces significantly on stable Titania (titanium/zirconia NPs) (14).

This investigation, thus, aimed to evaluate the effect of incorporating TiO₂ NPs and pretreatment with TiO₂ NPs on microleakage of

dental universal adhesive and their cytotoxicity using culture media of fibroblast cell line NIH-3T3.

Materials & Methods

Preparation of samples and TiO₂ NPs

The composition of the materials used in this study is shown in table 2. TiO₂ NPs were purchased from the Iranian Nanomaterials Pioneers Company, Mashhad, Iran, with an average diameter of <30 nm. The characterization tests were validated by X-ray diffraction (XRD) test (DX-1000X, Dandong Fangyuan, China) and transmission electron microscope (TEM) (JEM-1011, JEOL, Japan). Dilution of Titanium isopropoxide 97% (Aldrich) in isopropyl alcohol (Merck) was done as starting solution. After the addition of distilled water (pH 8), TiO₂ NPs were precipitated. These precipitates were centrifuged and heated.

A total of 60 non-carious human molars that were extracted in the last 6 months were collected. Chloramine T solution was used to keep teeth (1 week at 4 °C). The teeth were placed in distilled water before cavity preparation. standard cIV cavities (2 mm height, 1.5 mm depth, 4 mm length) were prepared on the buccal surfaces of the teeth, such that 2 mm of a height of the cavity, 1 mm high and 1 mm below CEJ. Cavities were prepared by a high-speed handpiece and a diamond bur (836G. FG. 010; Jota AG; Switzerland) with a diameter of 1 mm in the presence of air-water coolant. The teeth were divided into six groups according to table 1.

Table 1. Six different tested groups (n=10)

Groups	Protocols of using TiO ₂ NP and Universal adhesive
A	Acid etching+ Universal single Bond + Composite Z250
B	No acid etching+ Universal single Bond + Composite Z250
C	Acid etching+ TiO ₂ NP Incorporation+ Universal single Bond + Composite Z250
D	No acid etching+ TiO ₂ NP Incorporation + Universal single Bond + Composite Z250
E	Acid etching+ TiO ₂ NP Pretreatment+ Universal single Bond + Composite Z250
F	No acid etching+ TiO ₂ NP Pretreatment + Universal single Bond + Composite Z250

Table 2. Test materials name, manufacturers, composition and mode of application

Name	Manufacture	Composition	Application Mode
(Singlebond Adhesive System Universal Adhesive)	3M, ESP, St Paul, MN, USA	MDP phosphate monomer, dimethacrylate resins, HEMA, Vitrebond copolymer, filler, ethanol, water, initiators, silane	Acid etch(20 s- optional); rinse (15 s); air-dry (30 s); dentin rewetted with water (1.5 µl) (60 s); one coat of adhesive; air-dry (10s); light cure (10s)
Composite resin (Filtek Z-250)	3M, ESP, St Paul, MN, USA	UDMA (urethane dimethacrylate), Bis-EMA (bisphenol A polyethylene glycol diether dimethacrylate), TEGDMA	air-dry the dentin surface; two coats of primer with slight agitation (20s);
		(tri-ethylene glycol dimethacrylate) and inorganic filler	air-dry (20s); one coat of the adhesive with slight agitation (20s); light cure (10s)
Etchant 37%	3M, ESP, St Paul, MN, USA	H ₃ PO ₄ 37%	

In every tested group, using TiO₂ NPs was followed by water rinsing (60s). The application of adhesive systems was followed by the manufacturer's instructions (Table 2). After the application of the bonding system on the dentin surface, one block of tested composite (Z250; A1, 3M ESPE, St. Paul, MN, USA) was used over the bonded dentin cavity walls and light-cured for 40s at 600 mW/cm² (VIP Junior, Bisco, Schaumburg, IL).

After the restoration of cavities, teeth were placed in distilled water for 24h at room temperature, and then restorations were finished and polished. In the next step, the sticky wax was used for sealing apical foramens. All surfaces of the teeth were covered by two layers of nail varnish, except one-millimeter margin of the restorations, and then the teeth were immersed in a 2% solution of basic fuchsine (Merck, Germany) for 24h at room temperature. After that, teeth were completely washed with water to remove the additions of dye. The samples were sectioned in a buccolingual direction by a diamond disc of Mecatome cutting machine (Mecatome, France) with the presence of water and air coolant. Then, permeability was measured between the teeth and the restoration by a stereomicroscope (Motic K series) with 40x

magnification by two observers. Microleakage was determined quantitatively in occlusal and gingival margins, measured by a specific gauge.

Cell Treatment and MTT Assay

In the present study, we used the NIH-3T3 cell line. The culture medium was RPMI-1640 supplemented with 10% FBS, glutamine and antibiotics (Gibco, Scotland). The cultures were incubated at 37 °C, with 100% humidity and 5% CO₂. In half of samples, 0.5% TiO₂ NPs was added to dentin bonding using both ultrasonic and hand manipulation. A micropipette was used to measure 3 and 5µL of dentin bonding agents. Subsequently, they were placed in the center of petri dishes (5 cm inner diameter) and irradiated with a visible light curing unit for 20s at 600 mW/cm² (VIP Junior, Bisco, Schaumburg, IL). Thereafter, 5 mL per vial of serum-free RPMI-1640 was added (37 °C for 24 h). A 0.22 mm syringe filter was used to sterile the extract medium. 100 µL of extract medium was added to the 104 cells (final volume 200 µl supplemented with 10% FBS) and incubated for a further 48 h. The methyl thiazolyl tetrazolium bromide (MTT, Sigma, USA) assay was used to determine the cell viability (15-17).

Statistical Analysis

Kolmogorov-Smirnov test was used to check the normal distribution of continuous variables. Continuous variables with or without normal distribution were presented as mean (standard deviation) and median (interquartile range), respectively. SPSS 18.0 software (SPSS Inc. USA) was used to analyze the data. The results of the study were analyzed using the Kruskal-Wallis test to compare the effect of the etching method and the use of TiO₂ NPs on the microleakage median by the statistics expert. Mean \pm SD of cytotoxicity of two different bonding groups were assessed by Student's t-test ($P < 0.05$).

Results

TEM and XRD analysis

The size and morphology of particles were assessed using a TEM (Figure 1a). TEM image shows TiO₂ NPs with an average diameter of about 30 nm and with a spherical shape. A typical XRD pattern of the TiO₂ NPs is shown in Figure 1b. The 2θ at peak of 27.2° confirms the TiO₂ rutile structure (18). The intensity of XRD peaks of the sample reflects that the formed nanoparticles are crystalline and broad diffraction peaks indicate very small size crystallite.

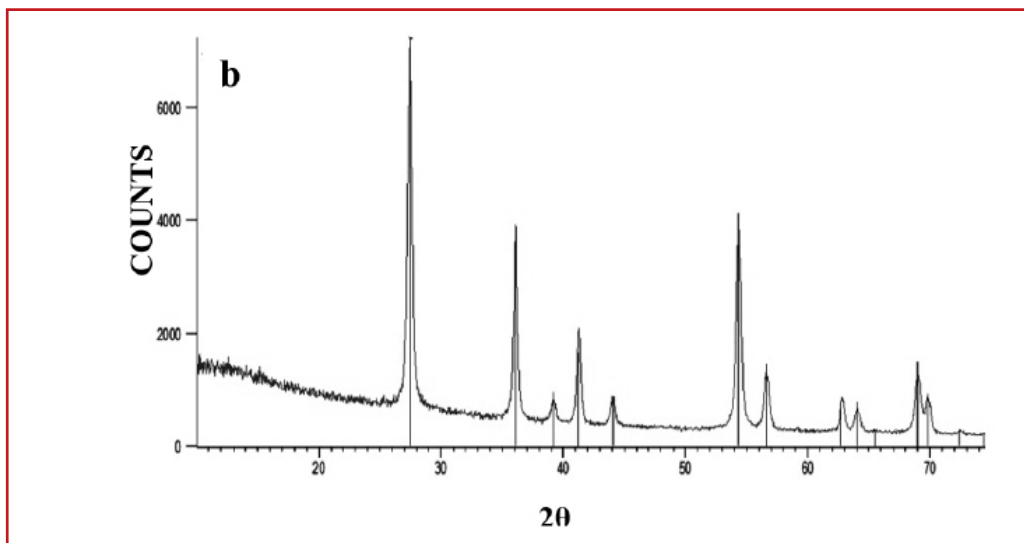
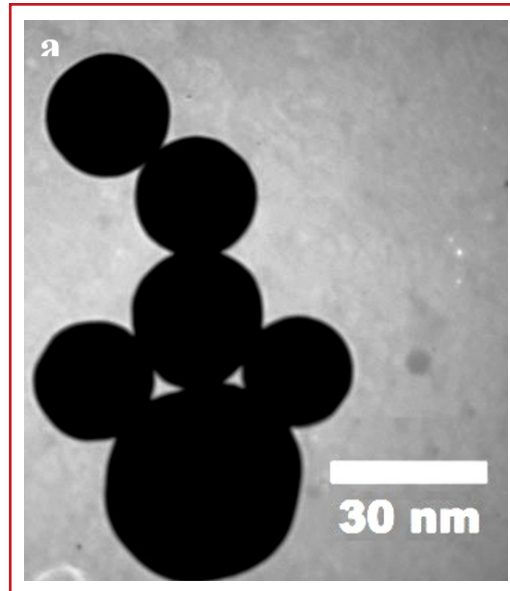


Figure 1. (a) TEM image; (b) XRD pattern of TiO₂ nanoparticles

Microleakage Measurement

In both occlusal and gingival margins in all groups, the application of TiO₂ NPs was better than no application (Chart 1). This reduction in microleakage is significant in occlusal margins (P<0.001). In the case of margins located in the enamel, we have found a

significant decrease in

the median of microleakage in the groups that underwent pretreatment of TiO₂ NPs when using the total-etch and self-etch method (P<0.001) (Chart 2). A pairwise comparison test was showed significant differences in microleakage of occlusal margins in all tested groups.

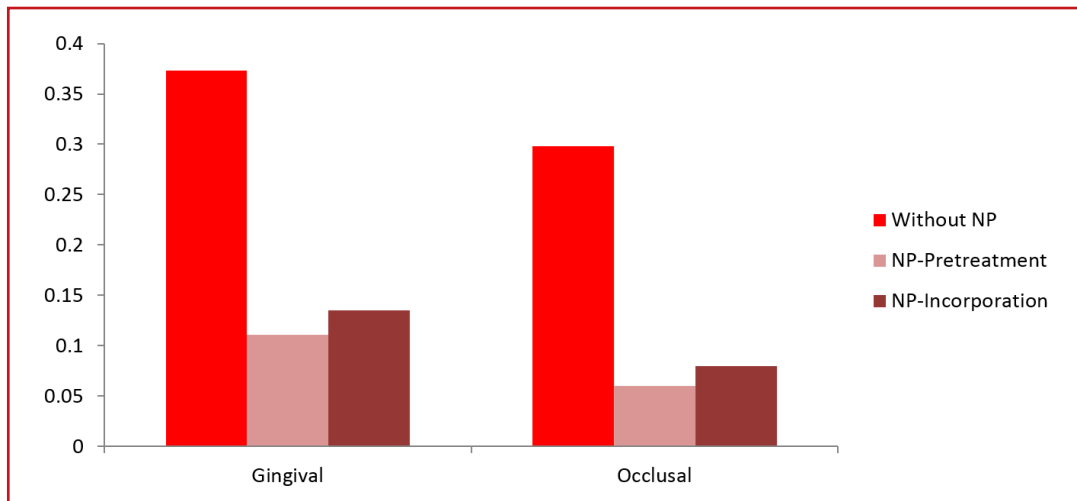


Chart 1. Microleakage in both gingival and occlusal margins decreases when using TiO₂ NPs

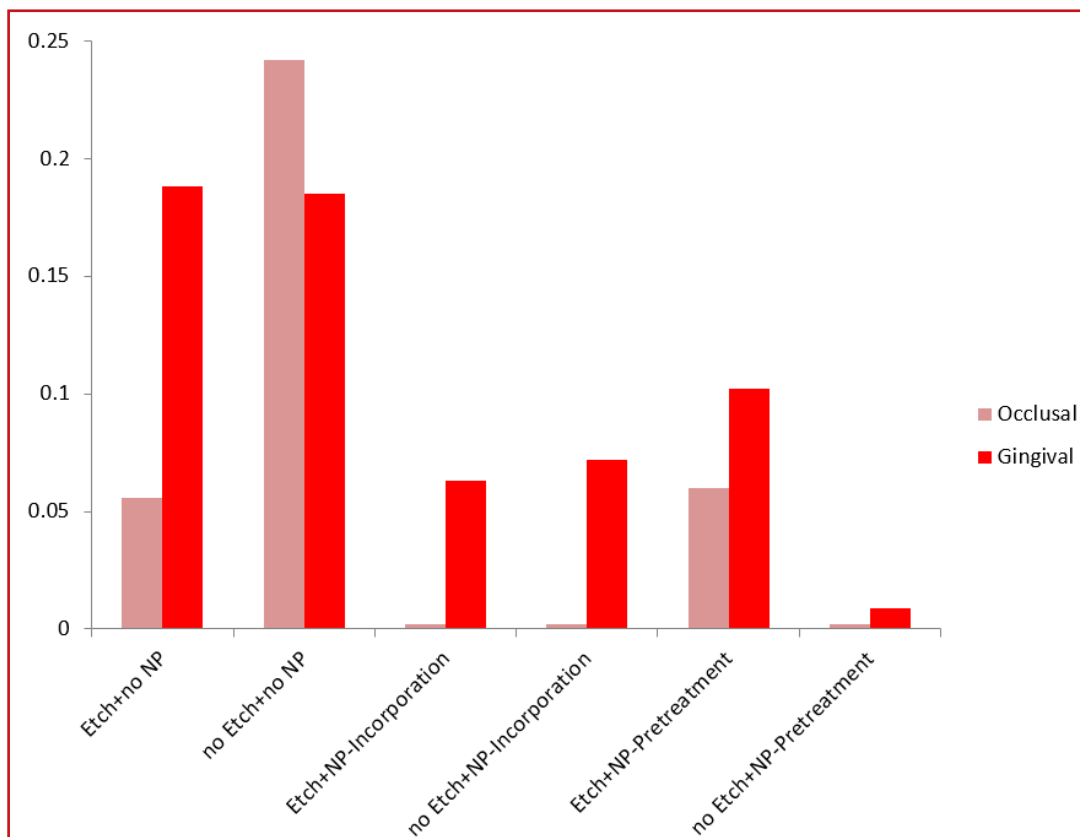


Chart 2. The mean of microleakage in the occlusal and in the gingival. TiO₂ NPs in both total-etch and self-etch approaches led to a decrease in the microleakage. Kruskal-Wallis test showed significant differences in the microleakage of occlusal margins (P<0.001) but in the gingival margins, we did not any significant differences (P=0.128)

In gingival margins, although the incorporation of TiO₂ NPs in the total-etch and self-etch method led to a decrease in

the median of microleakage, it was not a significant microleakage reduction (P=0.128) (Table 3).

Table 3. Amount of microleakage in test groups

Group	Code	Microleakage	
		Occlusal margin	Gingival margin
Etch	A	0.056	0.188
No etch	B	0.242	0.185
Etch + NP	C	0.000	0.063
No etch + NP	D	0.000	0.072
Etch + Pretreatment	E	0.102	0.060
No etch + Pretreatment	F	0.009	0.000

Cell cytotoxicity

In this study, we determined the cytotoxicity of universal single bond dental adhesive with and without incorporation of TiO₂ NPs by treating the NIH-3T3 cells for 24 h followed by MTT

assay. However compared to the controls, the total cell numbers of universal single bond and TiO₂ NPs incorporated universal single bond, were 103.10% (P>0.05) and 76.35% (P<0.05) respectively (Chart 3).

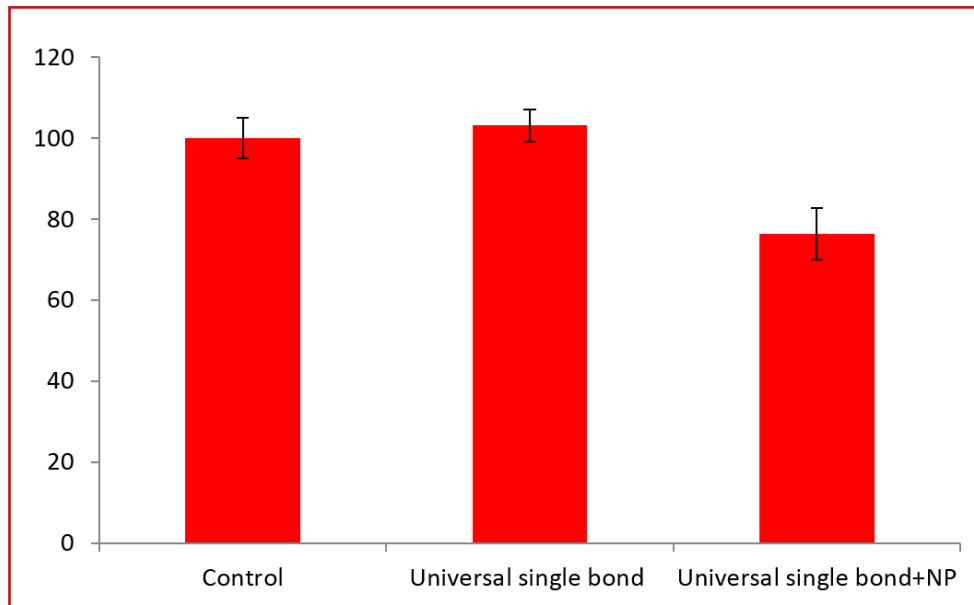


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Discussion

Metal oxide NPs are stable under harsh processing conditions, they are considered as unique antibacterial and safe agents (19, 20). In particular, TiO₂ is a non-expensive and chemically stable compound. It was showed that because of opposite charge of the metal oxides

and microorganisms, the electromagnetic attraction can lead to oxidization and death of microorganisms (21). Deactivation of the DNA and cellular enzymes also can denature bacterial cell walls that lead to increase permeability and cell death (22).

Using nanoscale fillers in resin composites

can influence their mechanical and biological properties. Due to the high biocompatibility and pleasing color of TiO₂ NPs, they are optimal for this application in dentistry. Previous studies that used TiO₂ NPs in epoxy resins, revealed that they can overcome the drawbacks of traditional tougheners and lead to improvement in its toughness, stiffness and strength (23).

It was shown that different factors such as polymerization contraction stresses and chewing forces during mastication can influence the marginal leakage of dental composites (24). These conditions can deform the tooth structure and create marginal gaps. Dentin -bonding systems are mainly used to improve the bonding strength between tooth structure and composite resin (25). The incorporation of TiO₂ NPs in bonding systems may act as fillers. It was shown that increasing in filler level may contribute to improve the retention of dental restorations, distribute the occlusal stresses and reduce the microleakage across resin and dentin interface (26). After incorporating the NPs, adhesive layer may acts more efficiently as an intermediate layer between dental composites and the cavity walls. This intermediate layer can probably compensate the polymerization shrinkage stresses and shocks (27). It seems that using filled adhesives may improve internal and marginal seals and increase the mechanical properties of composite restoration. Perhaps because of these reasons the incorporation of TiO₂ NPs in universal bonding agents can lead to acceptable results in reducing microleakage.

This investigation was the first report on incorporation and pretreatment with TiO₂ NPs for microleakage evaluation of new universal single bond. Some multifunctional cross-linking monomers are needed in order to produce a universal adhesive. These monomers can react and copolymerize with dental cements and resin-based materials in order to have good dentin wetting and hydrophilic properties (28). Also, the hydrophobicity is important to prevent hydrolysis of bond over time. For better responses of universal adhesive systems in a self-etching approach, we need enough acidity

but it should not be so acidic that separation of initiators in self-cure and dual-cure resin cements was occurred(29). Universal adhesive systems must also contain water, for the dissociation of acidic functional monomers in self-etching approach (30).

In total-etch adhesives, the hybrid layer is formed by demineralization of superficial dentin with inorganic acids, then collagen fibrils are exposed and infiltrated by hydrophilic monomers (31, 32). One of the difficulties of universal adhesive systems is that more than needed water may change the chemistry of adhesive while little water is required. This problem can lead to incomplete evaporation during the air-drying and phase separation of monomers and compromise the interface of adhesive (33). It also seems that the use of TiO₂ NPs in the self-etch method of universal adhesive systems results in better dentin wetting by adhesives and increases the quality of bonding systems that leads to reduction of microleakage. These reasons are responsible for lower responses of total-etch approach to universal adhesive systems when applying TiO₂ NPs compared to self-etch approach.

According to the results, it seems that the use of TiO₂ NPs as pretreatment agent in the total-etch method of universal adhesive helps to better penetration of phosphoric acid into dentinal tubules and increase the depth of etching, which results in the formation of longer resin tags and promotion of bond quality. The depth and extent of demineralization in the self-etch method is lower than that of total-etch. Research has shown that the monomer used in the universal adhesive can better react with hydroxyapatite in the presence of TiO₂ NPs, and this chemical reaction allows the penetration of TiO₂ NPs into dentinal tubules and may help to prevent microleakage.

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Previous studies have shown lower cytotoxicity and better responses of oral soft tissues in self-etch adhesives compared to total-etch adhesive systems (34). The present study shows that adhesive systems can have metabolic effects in NIH-3T3 fibroblasts. Due to the speed and ease of cell growth, NIH-3T3 cell line is chosen to evaluate the biological responses of monomer materials; on the other hand, it is in line with ISO recommendations for assessment of toxicity of dental materials (35). It was shown that whereas the TiO₂ NPs incorporated universal single bond had moderate cytotoxic effects, the universal adhesive system alone was fully tolerated by NIH-3T3 fibroblasts. This universal adhesive system contains specific functional monomers such as Vitrebond Copolymer, silane and MDP. The presence of silane in the universal adhesive allows direct application of it. MDP is a monomer with a long carbon chain that is responsible for hydrolytic stability and hydrophobicity. Thus, cell viability in this type of adhesive systems may be related to the presence of multiple components. One previous study also demonstrated that differences in the type of solvents, adhesive concentration, molecular weight and monomer type can have a considerable effect on the adhesive cytotoxicity (36). Other in vitro investigations have demonstrated that monomers such as UDMA and Bis-GMA were strongly cytotoxic, whereas TEGDMA and HEMA are less cytotoxic to fibroblasts (37). It has been reported that camphoroquinone (the most common photoactivator) may also leach from dental adhesive systems and induce mutagenic and cytotoxic effects (37, 38). In the previous studies, the addition of 0.5% Ag NPs or 10% quaternary ammonium dimethacrylate into the bonding agents could not induce considerable cytotoxic effects compared to the conventional bonding agents (39). Further investigations are needed to elucidate the probable risks of using NPs in dental materials.

Conclusions

In conclusion, considering the limitations of



the present study, using TiO₂ NPs with both self-etch and total-etch approaches to universal adhesive seems to be reasonable for decreasing the microleakage of resin composite restorations. Moreover, TiO₂ NPs have negative effect on NIH-3T3 cell viability.

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Conflict of interests

The authors declare no conflict of interest.

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