The cytotoxicity of self-etching primer bonding agents in vitro

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Objective. This study evaluated the cytotoxicity of 3 self-etching bonding systems: Optibond Solo Plus SE primer, Xeno III, and *i* Bond.
Study design. The test materials were applied on the dentine discs of dentine barrier models in the same way as in the clinical procedures recommended by each manufacturer. 3-D cell culture of Bovine pulp–derived cells transfected with Simian virus 40 Large T antigen and perfusion condition were conducted in this experiment. Cell viability after exposure to the bonding agents was determined by dimethylthiazolediphenyltetrazolium bromide (MTT) assay.
Results. The results revealed that cell survival with the above-mentioned bonding agents was 99.66%, 72.59%, and 10.65%, respectively. *i* Bond is the most toxic material ($P < .05$). Xeno III is less toxic than *i* Bond but more toxic than Optibond Solo Plus SE primer ($P < .05$).
Conclusions. Among the 3 test self-etching bonding systems, Optibond Solo Plus SE primer should be selected in cases where the remaining dentin above pulp tissue is 0.5 mm or less. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;107:e86-e90)**

Adhesive dentistry has led to changes in cavity preparation design under the concept of minimal intervention. This concept of bonding resin to enamel has been credited to Buonocore.[1] Etched enamel with phosphoric acid permits the penetration and mechanical lock of low-viscosity resins. Bonding to dentine can be conducted in 2 ways: bonding to inorganic or organic components of dentine. Bowen et al.[2] achieved the first dentine bonds of shear bond strength to dentine of approximately 14 Mpa.

Nakabayashi[3] proposed adhesion of a bonding agent through microretention. The hydroxyapatite layer is removed by acid etching and acquires a highly porous structure. Application of the dentin-bonding agent results in diffusion of the bonding agent into the spaces depleted by the acid etching agent. The applied dentin-bonding agent does not thoroughly diffuse and penetrate into those regions where the hydroxyapatite has been removed by the acid-etching procedure. Consequently, the patients experience postoperative sensitivity and debonding of restorations.

Self-etching bonding systems were introduced to resolve these problems. The demineralization and resin deposition procedures are performed almost simultaneously, rather than being carried out sequentially. The reduction of sensitivity is an additional advantage of this system.

The concentration of each monomer in the combination of each bonding system influences toxicity. Both exposure times and the interactions (synergism or antagonism) between the bonding components may be important parameters in determining the cytotoxicity of dentine bonding agents.[4] Ratanasathien et al.[4] showed that TC_{50}value for these 4 resins could be ranked from most toxic to least toxic as BisGMA, UDMA, TEGDMA, and HEMA after exposures of 24 and 72 hours. The concentration of BisGMA antagonized the cytotoxicity of HEMA after 24-hour exposure. On the other hand, synergistic reaction occurred in combinations of 25 μmol/L of BisGMA regardless of the concentration of HEMA. In addition, low molecular weight resins such as HEMA, 4-META, and TEGDMA may also act as solvents for more viscous resins such as BisGMA and UDMA that make them more diffusible to cells and tissues.[5]

The pH of self-etching primer solution is low to demineralize the smear layer and underlying dentinal surface so that the infiltration of resin occurs simultaneously with the self-etching process. In addition, water-rinsing after etching, as used in the total-etching system, is eliminated. Based on levels of etching ag-

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gressiveness, self-etching bonding systems were subdivided into 2 groups of strong and mild self-etching adhesives. The Optibond Solo Plus self-etching adhesive system, Xeno III, and *i* Bond were classified as intermediary strong self-etching adhesives.[6] The pH of these adhesives are 1.5, 1.4, and 1.6, respectively. The typical pattern of bonding is the 2-fold build-up of the dentinal hybrid layer with a completed demineralized top layer and a partially demineralized base. Better bonding of micromechanical interlocking resin at enamel and dentin and the chemical intermolecular interaction at the hybrid layer base is achieved. Some investigations of self-etching bonding systems of marginal permeability[7] and bonding effectiveness[8] have been performed but there have been few reports of cytotoxicity testing. Until now there has not been a report of whether strong self-etching primer bonding agents of pH lower than 2 have any adverse effect on cultured cells.

The aim of this study is to evaluate the cytotoxicity of 3 self-etching bonding systems: Optibond Solo Plus SE primer adhesive systems, Xeno III, and *i* Bond by using 3-dimensional (3-D) cell culture technique in simulated model with a perfusion condition.

MATERIALS AND METHODS

Cell preparation

The target cells used in this experiment were TCPC SV40 (Bovine fibroblast pulp derived cells transfected with Simian virus 40 Large T-antigen).[9] The polyamide mesh, diameter 8 mm, was cleaned with 0.1 M acetic acid and washed with sterile water 3 times. Fibronectin (0.03 mg/mL in water) was coated on the mesh, and the mesh was left to dry for 2 hours in a Biohazard Safety Cabinet. A 1.25-mL volume of MEM α (Minimum Essential Media, Gibco, New York Grand Island, NY, USA), including 20% of fetal calf serum was added to each well of a 6-well tissue culture plate. Then, Millicell membrane (Minucells and Minutissue, Bad Abbach, Germany) size 30 mm, pore size 0.45 μm was inserted in each well.

Four dried meshes were put in each insert. Twenty microliters of 4×10^6 cells/mL were dropped on each mesh in the insert. The plate was incubated at 37°C, 5% CO_2 and 100% humidity for 48 hours. Then, each mesh was separately put into each well of a 24-well tissue culture plate. Cells on the meshes were fed with 1 mL of 20% MEM α containing 50 μg/mL of ascorbic acid, and the medium was changed every other day. After growing for 14 days in the incubator, the 3-D cells on the mesh were ready for use in the experiment.

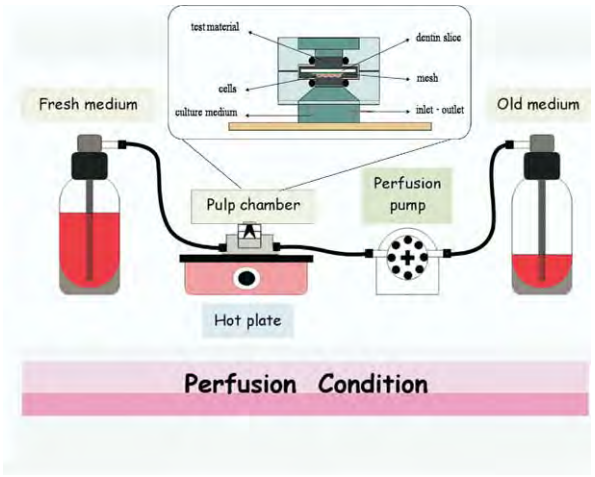

Fig. 1. Diagram of the perfusion condition.

Dentine disc preparation

A dentine disc 500 μm thick, close to the pulp cavity, was longitudinally sectioned from a bovine incisor. A dentine disc of 6 to 7 mm under the cemento-enamel junction was cut for the experiment. The pulpal side of each disc was etched with 50% citric acid for 30 seconds and soaked in normal saline and autoclaved before the experiment.

Test materials

Three self-etch bonding systems, Optibond Solo Plus SE primer (Kerr, Orange, CA), Xeno III (Dentsply/Caulk, Millford, DE), and *i* Bond (Heraeus Kulzer, Armonk, NY) are experimental materials. Negative and positive controls are President (Coltene AG, Alstatten, Switzerland) and Vitrebond (3M Medica GmbH, Broken, Germany), respectively.

In vitro perfusion chamber

A commercial cell culture chamber (Minucells and Minutissue) was used as the in vitro model.[10] The chamber was separated into the pulpal side and cavity side by the dentine disc mentioned above. The pulpal side of the disc was placed over the cultivated cell mesh. These 2 compartments were held together by a stainless steel holder. Cells in the mesh were fed with 20% MEM α contained in the lower part of the chamber. The model was set in this manner on the hot plate (37°C ± 2°C) with the perfusion of culture medium through the lower part of the chamber. The perfusion pump (Ismatec UK Co., Weston-super-Mare, England) was connected and adjusted to the perfusion rate of 0.2 mL/h for 24 hours before test material application. A cotton pellet soaked with culture medium was put on the dentine in the cavity side (Fig. 1).

The test procedure

After 24 hours of perfusion, the medium was adjusted to the rate of 2 mL/h of perfusion through the model for pulpal blood flow simulation. The cotton pellet in the cavity side was removed and the dentine in the cavity side was cleaned with sterile water and dried with gently blown air. Three self-etching bonding systems, as well as President and Vitrebond, were applied on the dentine as in clinical practice, according to each manufacturer's recommendation. Each material and control group was used in 5 models and repeated in triplicate. The enzyme activity of target cells was analyzed by using dimethylthiazolediphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assay after 24 hours of test material application.

MTT assay

The mesh was removed from the stainless-steel holder of the perfusion chamber and immediately inserted into freshly prepared 0.5-mL MTT solution (1 well/1 mesh) in a 48-well tissue culture plate. The plates were incubated for 2 hours. Mitochondrial dehydrogenase enzymes in living cells convert the yellow water-soluble tetrazolium salt 3-(4, 5-dimethylthiazol-2, 5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) into dark blue formazan crystals stored in the cytoplasm of cells. Then, MTT solution was removed and the mesh was washed twice with 0.5 mL phosphate buffer saline solution. Two hundred and fifty microliters of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan from the cells. The plate was agitated on a shaker for 30 minutes to enhance the dissolution of MTT formazan. A 200-μL aliquot was drawn from each well and transferred into a 96-well tissue culture plate and the spectrophotometric absorbance was measured at 540 nm using DMSO as the blank.

Statistical analysis

The mean optical density of the negative control group was set to represent 100% viability. Results of the experimental groups and positive control were expressed as percentages of the negative control. Statistical analysis was performed by applying the nonparametric Mann-Whitney test ($P < .05$).

RESULTS

Fig. 2 shows survival rates of 3-D cell cultures in perfusion chambers after diffusion of 3 bonding agents for 24 hours. The average percentage of cell viability after exposure to Optibond Solo Plus SE primer, Xeno III, *i* Bond, and Vitrebond were 99.66, 72.59, 10.65, and 59.90, respectively. Optibond Solo Plus SE primer was the least toxic agent ($P = .000$ to .002), whereas *i* Bond was the most toxic agent ($P = .000$). Xeno III was less toxic than *i* Bond ($P = .000$) but more toxic than Optibond Solo Plus SE primer ($P = .000$). Vitrebond was more toxic than Optibond Solo Plus and Xeno III ($P = .000$) but less toxic than *i* Bond ($P = .000$).

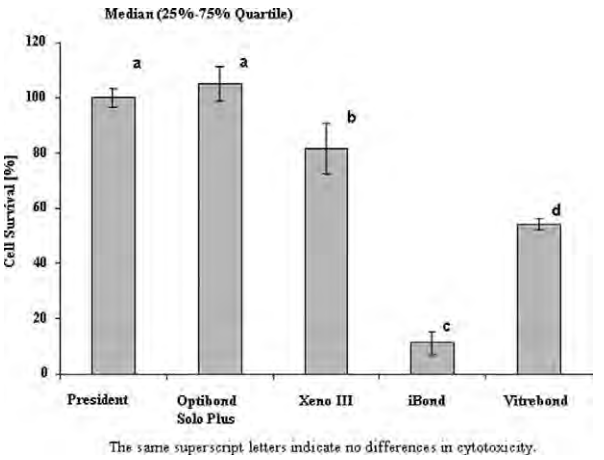

Fig. 2. Cell survival after exposure to test bonding agents, as well as positive and negative controls.

DISCUSSION

Cell culture assays provide a convenient, controllable, and repeatable method to assess the biocompatibility of materials. Increasing public concern regarding the use of animals in biocompatibility evaluation of dental materials has made in vitro testing more reasonable and more ethically acceptable. Spangberg and Pascon[11] showed that the preparation of material for the experiments significantly altered the apparent cytotoxicity effects of a material. They stressed the importance of material preparation in the process of testing the target cells. Every effort has been made to simulate in vitro conditions in the laboratory; however, it is impossible to create an environment that is a total replication.

The dentine barrier cytotoxicity test is recommended in ISO 7405[12] for testing the cytotoxicity of dental restorative materials by a cell culture technique. The cells and materials are separated by a dentine disc to simulate the clinical situation of a tooth cavity filled with a restorative material. The positive control should reduce cell viability by approximately 50% after 24 hours of exposure; the negative control should have no effect on cell viability. Our pilot study revealed no toxicity of President, whereas Vitrebond was approximately 50% cytotoxic.

Some articles have claimed poor correlations between in vivo and in vitro toxicity evaluation of dental materials. This initial test was performed to simulate

the in vivo situation of both cell layers in pulpal tissue and pulpal blood flow. A previous study by Schmalz et al.[13] revealed a concentration-dependent decrease of cell survival in 48-well tissue culture plates in the dose-response curves of the 3-D cell culture to phenol. The TC_{50} of L 929-monolayer was 0.09% phenol, whereas the dose response curves in 3-D cell culture were 0.53% phenol. The discrepancies might be because of a several-fold higher cell density in the 3-D cell culture. Cross-sections of the 3-D cell culture revealed about 15 to 20 cell layers. Wataha et al.[14] also showed higher TC_{50} values for copper ions with increasing cell density. As such, multiple cell layers, as in 3-D cell culture, should simulate the in vivo pulpal cell layer, giving a better response to toxicity than monolayers.

Pulpal blood flow is one of the most important factors in diluting leachable toxic substances from an exposed site. In the present study, the in vitro perfusion chamber was perfused with nutrient medium to simulate pulpal blood flow. A normal pulpal blood flow of 100 g of tissue is 20.0 to 82.4 mL/m, which corresponds to a perfusion of 0.6 to 5.0 mL/h.[15] A perfusion rate of 0.2 mL/h was chosen before material application because the experiment showed similar cytotoxicity to test materials as static experiments, whereas perfusion conditions of 5 mL/h led to increased cell death.[13] As such, the pefusion rate of 2 mL/h was chosen for use after the testing material application onto the dentine discs.

The remaining dentine thickness in vivo would decrease the pulpward diffusion rate and the total diffusion of leachable toxic substances.[16] Stanley and his coworkers[17] revealed that pulpal reactions to restorative materials were inconsistent if the thickness of the remaining dentine over the pulp was 1 to 2 mm. A dentine thickness of 0.5 mm can reduce material toxicity to 75%, and 1 mm dentine can reduce toxicity to 90% of the control value when dentine is not present.[18] As such, 500 μm of coronal human dentine is recommended for in vitro cytotoxicity evaluation. Bovine dentine near the cemento-enamel junction seems to be a suitable alternative for coronal human dentine with respect to transdentinal permeability characteristics.[19]

A number of investigations have revealed pulpal responses following adhesive bonding system restorations. Those results showed differences in the pulpal responses to the different adhesive bonding systems.[13,20,21] We could conclude that the pulpal response is affected by the type of material, not only by the presence of bacteria.

Both Optibond Solo Plus SE primer and Xeno III contain alcohol as the resin primer solvent, while acetone is the solvent in *i* Bond. Acetone has water-chasing properties that are better than alcohol, so it can carry the primer agent through dentine more than alcohol does.[22] *i* Bond can diffuse into the target cells under the dentine discs better than the other 2 systems. Including the mixture of UDMA and 4-META in the composition of this bonding agent may cause more toxicity to the target cells. *i* Bond exhibited significantly less leakage than other test self-etching adhesives because its composition includes gluteraldehyde.[7] Gluteraldehyde, a cell-fixing substance, is toxic to cells.

An in vitro experiment presents a higher degree of controlling test conditions than an in vivo experiment. The standardized and well-designed simulations of the clinical applications will bring meaningful insights to clinical practice. Tests of 3 self-etching bonding systems were conducted in dentine barrier models with 3-D cell culture under perfusion conditions. The results of this investigation certainly give some toxicity information about applying these bonding agents in patients. If remaining dentine over the pulp tissue is as thin as 0.5 mm, lining material should be used before applying high-toxicity bonding agents to protect pulp from the leachable toxic substances in some bonding systems. Hence, other than bacterial penetration, bond strength, and microleakage, pulpal irritation should be considered when choosing a self-etching bonding agent system in clinical practice.

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