Cytotoxicity evaluation of dentin bonding agents by dentin barrier test on 3-dimensional pulp cells

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Objective. The aim of this study was to evaluate the effects of 4 dentin-bonding agents on the cell viability of bovine derived cells.

Study design. Cytotoxicity of dentin-bonding agents (G-Bond [GB], Adper Prompt Self-Etch [APSE], Clearfil DC Bond System [CDCB], and Quadrant University-1-Bond [UB]) was analyzed with a dentin barrier test device using 3-dimensional (3D) pulp cell cultures. A commercially available cell culture perfusion chamber was separated into 2 compartments using a 500 μm dentin disk. The 3D cultures were placed on a dentin disk and held in place with a special biocompatible stainless steel holder. Test materials were introduced into the upper compartment in direct contact with the cavity side of the dentin disks according to the manufacturer's instructions. Subsequently, the pulpal part of the perfusion chamber containing the cell cultures was perfused with a medium (2 mL/h). After an exposure period of 24 hours, cell survival was determined by using the MTT assay. Statistical analyses were performed using the Mann-Whitney *U* test.

Results. In the dentin barrier test, cell survival rates of UB and CDCB were similar to the control group ($P > .05$). However, all other tested materials were cytotoxic for the 3D pulp-derived cell cultures ($P < .05$).

Conclusions. Dentin-bonding agents include biologically active ingredients and may modify pulp cell metabolism when the materials are used in deep cavities in spite of a dentin barrier. If these adhesive agents are used in deep cavities, a biocompatible cavity liner should be used. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112: e83-e88)**

The development of dental technology has resulted in many materials on the market. Besides their esthetics and durability, these materials must be biocompatible with dental tissues. Materials used should not have detrimental effects on tooth structures. Until the introduction of new materials to the market based on the biocompatibility evaluation made after the production stage, the use of more tissue-friendly materials continues to be sought.[1]

Several in vivo studies have identified factors, such as cavity preparation or bacterial contamination, that

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can substantially modify the biologic responses to these agents,[2] but these in vivo studies have not successfully isolated the effects of the agents themselves. In vivo investigations have generally been carried out on nonhuman primates where pulpal physiology and dentin anatomy differ from humans.[3] Factors such as remaining dentin thickness, effect of short-term biologic response, and quantification of the results have proved to be problems when interpreting the results.[4]

The risk of toxicity from a dental material may be acceptable, but the occurrence of other types of adverse reactions, such as hypersensitivity, complement activation, or alteration of gene expression in odontoblasts, cannot be ruled out. On the other hand, most eluted substances are found to be cytotoxic in vitro; therefore, the materials may not necessarily be cytotoxic in vivo. From a clinical point of view, there are limitations regarding the correlation between in vitro testing and clinical usage tests. However, the in vitro cytotoxicity test is important in understanding the biologic risk of these materials at the initial setting stage.[5]

The permeability of dentin plays an important role in toxicity of adhesive materials by allowing increased diffusion of the released components through dentin to the pulp. The main factors that influence permeability

are the thickness and age of the remaining dentin. High permeability should increase the toxicity of adhesives by allowing increased diffusion of the released components through dentin.[4]

Dentin-bonding agents are recommended in the placement of resin-based restorative materials. The agents are used to improve the contact between the restorative material and the walls of the prepared cavity of the tooth. Because dentin-bonding agents come into close and prolonged contact with vital dentin, their influence on pulp tissue is of great interest. Therefore, dentin-bonding agents should have good biocompatibility.[6]

MATERIAL AND METHODS
Cell culture
The target cells used in this experiment were TCPC SV40 (bovine fibroblast pulp–derived cells transfected with simian virus 40 large T-antigen).[7] Bovine dental-papilla–derived cells were maintained in a growth medium (MEM, PAN; Biotech, Aidenbach, Germany) supplemented with 20% fetal bovine serum [FBS], 10,000 IU/mL penicillin/streptomycin, and 0.1 mg/mL geneticin) in a humidified atmosphere at 37°C with 5% CO_2.

Preparation of 3D cultures
Polyamide meshes (0.5 cm^2; Reichelt Chemietechnik, Heidelberg, Germany) were immersed in 0.1 mol/L acetic acid for 30 minutes, washed 3 times with phosphate-buffered saline solution, and air-dried. Cell culture inserts (Millipore, Eschborn, Germany) were placed in 6-well plates with 1.25 mL medium (MEMα with 20% FBS) per well. The fibronectin-coated meshes were placed on the inserts and 8×10^4 cells in a volume of 25 μL/mesh were seeded on them. After 48 hours of incubation (37°C, 5% CO_2, 100% humidity), meshes were transferred to 24-well plates and incubated for up to 21 days. The culture medium (supplemented with 0.05 mg/mL ascorbic acid) was changed 3 times a week.

Cytotoxicity testing
After 14 ± 2 days, 3D cultures were introduced into a dentin barrier test system as previously described[8]; a commercially available cell culture perfusion chamber (Minucells and Minutissue, Bad Abbach, Germany) made of polycarbonate with a base of 40 × 40 mm and a height of 36 mm was modified. The 3D cultures placed on a dentin disk were held in place by a special biocompatible stainless steel holder, resulting in a dentin barrier test situation (Fig. 1). The dentin disk (500 ± 20 μm thick) was cut from a bovine incisor, etched on pulpal side with 50% citric acid for 30 seconds, and autoclaved as previously described.[9] Thus, the cell culture chamber was separated into 2 compartments by the dentin disk. The cell culture tissues were placed in direct contact with the etched side of the dentin disk and held in place with a stainless steel holder. All chambers were perfused with a 0.3 mL assay medium (a growth medium with 5.96 g/L HEPES buffer) per hour for 24 hours at 37°C. Perfusion was switched off; test materials were introduced into the upper compartment in direct contact with the "cavity" side of the dentin disk (Fig. 1).

Test materials
The materials used for cytotoxicity testing are listed in Table I. The dentin in the cavity side was cleaned with sterile water and dried with gently blown air. The bonding systems were applied on the dentin as in clinical practice and light cured (380-515 nm LED light curing unit; Bluephase; Ivoclar Vivadent, Liechtenstein), according to each manufacturer's recommendation. A silicon impression material (President Coltene, Altstatten, Sweden) was used as a negative control (100% cell viability). The cytotoxicity of test materials was recorded after the pulpal part of the in vitro pulp chamber was perfused with a cell culture medium (2 mL/h) for 24 hours of incubation at 37°C. Each material and control group was used in 5 models, and each experiment was carried out ≥2 times. The enzyme activity of target cells was analyzed by using dimethylthiazolediphenyltetrazolium bromide (MTT) assay after 24 hours of test material application.

MTT (Methyltetrazolium test) assay
Cell viability of 3D cultures was determined by enzyme activity (MTT assay). The tissues were removed from the pulp chambers, placed into 48-well plates containing 500 μL prewarmed MTT solution (0.5 mg/mL in MEMα), and incubated for 2 hours at 37°C. Then, the tissues were washed 2 times with phosphate-buffered saline solution. The blue formazan precipitate was extracted from the mitochondria by using 250 μL dimethyl sulfoxide on a shaker at room temperature for 30 minutes, and 200 μL of this solution was transfered to a 96-well plate and the absorption at 540 nm determined spectrophotometrically. The mean values of control tissues (cell cultures exposed to silicone impression material) were set to represent 100% viability. Results of the cytotoxicity experiments were expressed as a percentage of control tissues. A statistical analysis between control and test materials was performed using the nonparametric Mann-Whitney U test ($P < .05$).
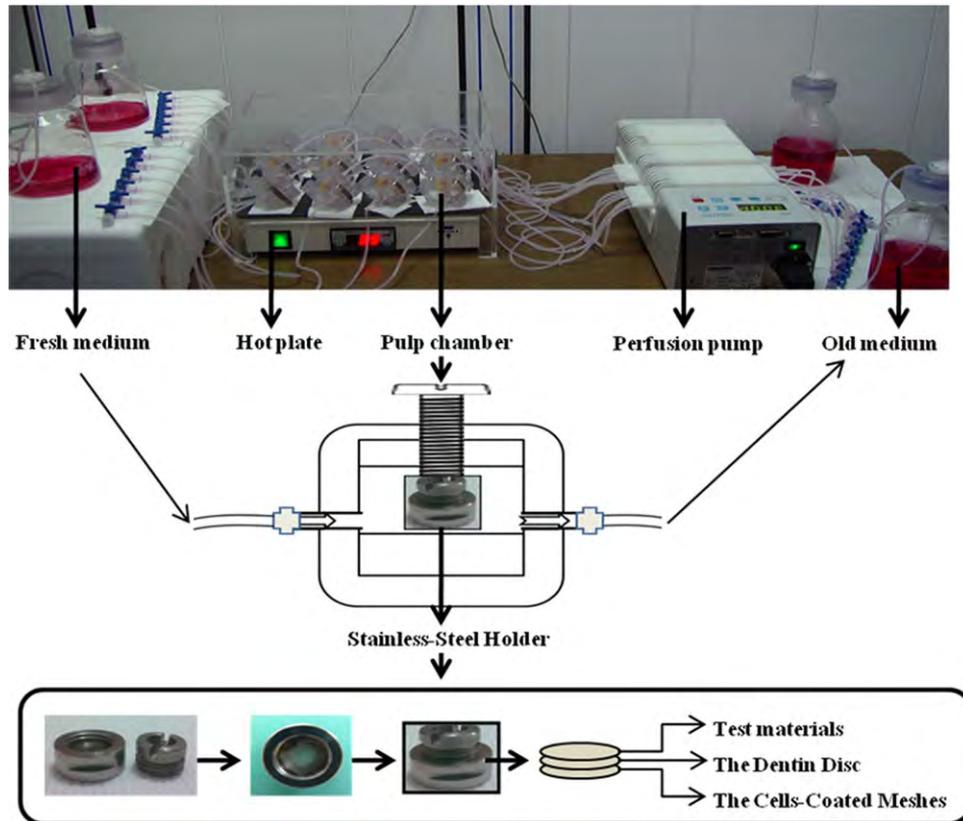
Fig. 1. Dentin barrier test system.

Table I. Test materials, components, and manufacturers

Bonding	Components	Manufacturer
G-Bond (GB)	4-MET, UDMA, silica, phosphoric acid ester monomer, acetone, water, photoinitiator	GC Corp., Tokyo, Japan
Adper Prompt Self-Etch (APSE)	Methacrylated phosphoric esters, bis-GMA, camphorquinone, water, HEMA, Polyalkenoic acid,	3M Espe, St. Paul, MN
Clearfil DC Bond System (CDCB)	Bis-GMA, TEGDMA, silanated colloidal silica, barium glass, D,L-camphorquinone, benzoyl peroxide	Kuraray Medicine, Tokyo, Japan
Quadrant University-1-Bond (UB)	4-META monomer, based on ethanol-water	Cavex Holland, Harmenjansweg, Haarlem, The Netherlands

RESULTS

The results of the dentin barrier test with dentin-bonding agents are summarized in Fig. 2. A vinyl polysiloxane material (President) was used as a negative control material. The Clearfil DC Bond System (CDCB) reduced cell survival rate to 86.2%. It was not found to be toxic compared with cell cultures exposed to President ($P > .05$). Statistically, Adper Prompt Self-Etch (APSE) and G-Bond (GB) were more toxic than the other tested materials ($P < .05$). APSE and GB were toxic materials compared with cell cultures exposed to President ($P < .05$). Quadrant University-1-bond (UB) was not found to be toxic. Exposure of the cell cultures to UB resulted in 87.56% cell survival, which was statistically the same as the control material ($P > .05$).

DISCUSSION

Several in vitro methods have been developed during the past 30 years to overcome the limitations of in vivo tests that measure the biologic response to dentin adhesives. In early tests, materials were placed in direct contact with cells in a monolayer culture, and cell number was used to monitor cytotoxic effects. How-
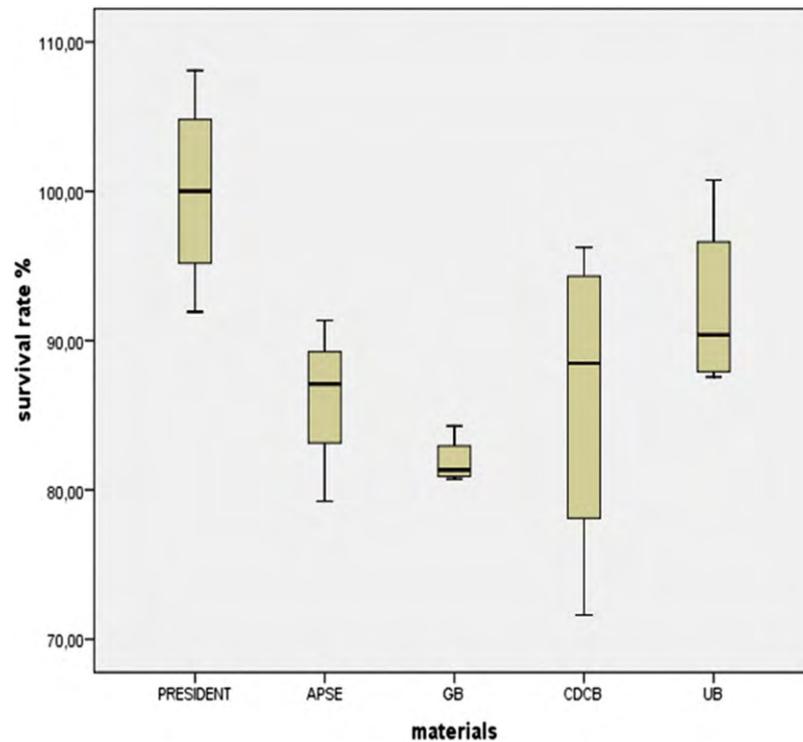
Fig. 2. Cell survival of 3-dimensional cultures in the dentin barrier test device after exposure to bonding agents. Data are expressed as percentage of the negative-control cultures.

ever, direct-contact tests have been suspected as being inappropriate for dental materials that are placed on dentin,[10] because the dentin markedly changes the biologic response. If barriers between the material and the cells are incorporated into the in vitro model, then these models appear to be more appropriate for estimating the in vivo response. Several types of barrier have been used, including agar, Millipore filters, powdered dentin, tooth crowns, and dentin disks. If barriers are used, then direct contact between the eluate from the dentin and cells has been shown to be of value in assessing the cytotoxic potential of adhesive agents.[4,11]

To date, the sensitivity of cultured human pulp cells to dentin-bonding agents has not been adequately studied. It is important to clarify the effects of dentin-bonding agents on cells derived from oral tissues, such as pulp cells, because bonding agents come into close contact with pulp tissues.

Studies have shown that dentin can reduce the toxicity of resins and bonding adhesives by limiting diffusion of those substances from the cavity preparation to the pulp.[12] Dentin probably adsorbs substances in the tubules and further limits the traverse of substances.[13] Despite the demonstration of differences in permeability of dentin of different thickness and locus,[14] there is no evidence that relates permeability to the ability of resins to cause a cytotoxic effect.

Smear layers on dentin have been shown to reduce diffusion through dentin by 25%-30%,[15] but most current bonding adhesives use primers that remove the smear layer. Other evidence shows that some adhesive components diffuse rapidly through dentin,[16,17] Therefore, there are compelling reasons to question whether these adhesives can cause cytotoxicity by diffusion through the dentin.[4]

The clinical relevance of identifying the potential of dental materials and their components to induce damage in cells and tissues in vitro has been recently emphasized.[18] Advances in the analysis of the cellular toxicology of resin monomers, as discussed, have also provided new insights into the interpretation of the risk factors for oral cavity tissue. For example, the degree of the monomer diffusion across dentin is modified by parameters such as the remaining dentin thickness, dentin permeability, or dentin location.[19,20] It has been estimated that sufficient amounts of the monomers triethylene-glycoldimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) are probably eluted from clinically used bonding agents to cause cellular toxicity.[17]

It has been shown that 4 dentin adhesive systems release sufficient components to cause suppression of cellular metabolism through dentin in an in vitro model. High-permeability dentin was generally more permis-

sive in allowing the diffusion of these components, but the effect of permeability on cytotoxicity depended on the material. On the other hand, the time interval between application of the bonding agent and collection of the eluant was consistently important for all materials. Materials were most cytotoxic at early intervals and were generally less cytotoxic at later intervals, although there were exceptions and there was persistent (>15%) suppression of cellular metabolism even at late (168 h) intervals. The results suggest that application of these materials to dentin, and particularly dentin with high permeability, poses a potential risk to the health of pulpal tissues.[21]

The cytotoxicities of the polymerized bonding agents Scotchbond 1 (3M Espe), Prime and Bond NT (Dentsply DeTrey), Xeno III (Dentsply DeTrey), and Clearfil Protect Bond (primer and bond parts; Kuraray) on mouse fibroblast cells have been compared.[22] Xeno III was also tested using thin dentin disks. In an in vitro culture, all 4 dentin-bonding agents were cytotoxic. Xeno III was the most toxic, even using dentin disks. It seems that the cytotoxicities depend on the compositions of the materials tested. The most toxic part of Clearfil Protect Bond was the primer, which contains the antibacterial pyridinium molecule.

The cytotoxic effects of different dentin-bonding systems on L 929 cells with dentin barrier test have been evaluated.[1] According to that study, all dentin-bonding systems applied to the 0.5-mm-thick dentin disks have cytotoxic effects ($P < .05$). Clearfil SE Bond and Protect Bond had lower cytotoxic effects than the others ($P < .05$) and showed no statistically significant difference between each other ($P > .05$). Clearfil SE Bond was the least cytotoxic bonding system among all materials applied to a 1.5-mm-thick dentin disk ($P < .05$). Adper Prompt was the most cytotoxic system to the cells ($P > .05$).

The possible cytotoxicity of 9 different adhesives by agar-overlay technique have been compared.[23] In that study, Adhese 2 Bonding (AB), Clearfil SE Bond, Clearfil Protect Bond (CPB), Optibond Solo Plus (OBSP), GB, APSE, Clearfil S3 Bond (CTSB), CDCB, and Hybrid Bond (HB) were tested. CTSB, OBSP, and CDCB caused higher lysis scores than other materials ($P < .01$). However, GB, HB, and AB showed lower lysis scores than the others ($P < .01$). Whereas CTSB had the largest decolorization zone of the fibroblast cells, CPB was found to have low decolorization compared with the other tested materials. According to the lysis and zone index, CTSB was found to be a highly cytotoxic material.

It has been reported[24] that 2,2-bis[4'-(x-hydroxy-3'—methacryloyoxy) phenyl] propane (bis-GMA) showed the highest toxicity against mouse fibroblasts,

followed by urethane dimethacrylate (UDMA), TEGDMA, and HEMA in order of decreasing toxicity. Based on the study by Ratanasathien et al.,[24] the cytotoxicity of monomers was ranked as follows: bis-GMA > UDMA > TEGDMA (3G) > HEMA.

In the present study, 4-methacryloxyethyl trimellitate anhydride (4-META [4-methacryloyloxyethy trimellitate anhydride])–containing dentin-bonding agent UB caused the lowest cytotoxic effects to the 3D cell cultures. Also, 4-META–containing dentin-bonding agent GB caused less cytotoxic effects than the other dentin-bonding agents compared with the control group.

This study concluded that the most intense cytotoxic effects were caused by bis-GMA–containing dentin-bonding agent APSE. In a comparison of the cytotoxicity of 35 resin components, Geurtsen et al.[25] found that UDMA was more cytotoxic than TEGDMA on human gingival fibroblasts and periodontal ligament cells, but less toxic on human dental pulp cells.

The cytotoxicity of 5 1-step dentin-bonding agents on human dental pulp and odontoblast-like cells (MDPC-23) has been compared.[26] Photopolymerized and unpolymerized samples of these dentin-bonding agents were prepared and incubated with dental pulp or MDPC-23 cells. All polymerized dentin-bonding agents exhibited lower cytotoxicity by 2%-65% than their unpolymerized counterparts. The appearance of the cytotoxicity of dentin-bonding agents was time dependent, and cell viability was lower at 72 hours by 2%-46% than at 24 hours. The cytotoxicity to MDPC-23 cells was ~5%-24% higher than that to pulp cells. These results indicate that 1-step dentin-bonding agents differ markedly in their cytotoxicity. Differential cytotoxic effects of 1-step dentin-bonding agents should be considered during clinical application of operative restoration.

The biocompatibility of 3 kinds of dentin-bonding agents, Xeno III (XO), Adper Prompt (AP), and Single Bond 2 (SB), were compared and evaluated through cell culture in vitro.[27] The results showed that all 3 kinds of dentin-bonding systems had cytotoxicity to human pulp fibroblast to different degrees in vitro. The cytotoxicities of XO and AP were less than that of SB ($P < .05$). The results of the cell culture in vitro indicated that total-etching adhesives systems create more irritation to pulp than self-etching adhesives system. The monomer type and the interactions between these monomers are found to be important in the cytotoxic effects. Even in the presence of a barrier between the material and cells, monomers can diffuse through the dentinal tubules and affect the cells. But when the dentin thickness increases, the harmful effect is decreased.[1]

The different cytotoxic effects of dental adhesives should be considered when selecting an appropriate

adhesive for operative restorations.[28] Dentin-bonding agents include biologically active ingredients and may modify pulp cell metabolism when the materials are used in deep cavities, despite a dentin barrier. If these adhesive agents are used in deep cavities, a biocompatible cavity liner should be used.

CONCLUSIONS

Within the limitations of this in vitro study, it appears that components of the dentin-bonding systems tested may be capable of causing cellular damage, even when an interposing layer of dentin separates the material from the pulp. Clinicians should therefore consider the application of a lining agent to the depths of their cavity preparations before applying a resin bonding agent.

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