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Cytotoxicity of Low pH Dentin-Bonding Agents in a Dentin Barrier Test In Vitro

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The reaction of three-dimensional cultures of pulp-derived cells in a dentin barrier test was recorded after exposure to All-Bond 2, Prime & Bond NT, Syntac SC, Syntac Classic, and Prompt L-Pop. The materials were applied on bovine dentin disks in a perfusion chamber, and the experiments were performed with (0.3 ml/h, 2 ml/h) and without perfusion of the pulpal part of the chamber. The cell reaction was recorded (MTT assay) and related to noncytotoxic controls. Bonding agents with low pH did not show any cytotoxicity. Syntac Classic decreased the cell activities to 38% to 72%, depending on different experimental conditions, and was more cytotoxic than Syntac SC. Perfusion (2 ml/h) reduced the cytotoxicity for Syntac Classic and increased cell activities from 52% to 72%. Because low pH bonding agents did not show toxic reactions in this dentin barrier test, pulp damage caused by the tested substance is unlikely if a dentin layer protects the pulp.

As dentin-bonding agents come into close and prolonged contact with vital dentin, their influence on the pulp tissue is of great interest. It had been shown that hydrophilic components of dentin-bonding agents, such as triethylene glycol dimethacrylate (TEGDMA) (1) or 2-hydroxyethyl methacrylate (HEMA) (2) alone, as well as in combination with composite resins (3) in actual formulations, are able to diffuse through dentin to reach the dental pulp in concentrations that may cause pulp damage (1–3). Interestingly, diffusion does not only take place through sound dentin but also through dentin, from which caries have been removed (2). This indicates that the postulated sclerosis of underlying dentin after caries attack apparently does not reduce the diffusion of these substances through this underlying dentin and that it has no greater protective effect than sound dentin. The cumulative release of relevant dentin-bonding components through dentin, from which carious dentin was removed, was even higher (2).

The toxic potential of components of dentin-bonding agents has been shown in vitro. It was found that hydrophilic monomers such as HEMA or TEGDMA were cytotoxic but to a lesser degree than the more hydrophobic monomers bisphenoe A-diglycidye dimethacrylate or urethane dimethacrylate (4). Interaction of different monomers has been demonstrated, with the potential of increasing the toxicity of the single components (4). Less toxic hydrophilic monomers may act as carriers for more toxic hydrophobic monomers. Dentin-bonding components, such as HEMA or TEGDMA, may also have an influence on the immune system, leading to both immunosuppression and immunostimulation (5).

Dentin-bonding agents alone also proved to be cytotoxic (6), although there are indications that one-step dentin-bonding agents were less cytotoxic than their multistep counterparts (7). Cytotoxicity decreased in a series of dentin-bonding agents with time (6). After direct-pulp capping with dentin-bonding procedures, some animal studies reported no pulp pathology (8), but severe pulp damage was found by other authors (9). Corresponding studies on human pulps showed inflammation associated with foreign body reactions (10) up to severe pulp damage (11). However, it was consistently demonstrated that dentin is an effective diffusion barrier, preventing pulp damage not only from toxic substances, such as eugenol and phenol, but also from glutaraldehyde and HEMA (12–14).

Recently, new one-step dentin-bonding agents have been marketed with pH values as low as 1.0. Data after exposing the vital dentin/pulp complex to these substances were, however, not available. Ample evidence exists with the application of acids on dentin; the permeability increasing effect of dentin etching is dependent on several factors, e.g. dentin thickness of 0.5 mm and higher, no significant effect was measured (14). Correspondingly, no pulp reactions have been reported after the use of dentin bonding, including acid treatment, if the pulp was covered by an intact dentin layer (15). However, low pH acids (e.g. phosphoric acid) are applied only for a short time-period (up to 30 s) and are then rinsed away. Acidic monomers, however, remain on the dentin. The objective of this investigation was to study the effect of a series of dentin-bonding agents with a spectrum of different pH values in a recently developed in vitro pulp chamber by using transfected bovine pulp-derived cells.

MATERIALS AND METHODS

Test Materials

The materials that were used are listed in Table 1, and the compositions are presented in Table 2. They were applied accord-

TABLE 1. Test materials

Dentin Adhesive (Brand Name)	Lot Number	Dentin Conditioner	Lot Number	Manufacturer
Control: President regular (silicone impression material)	EK 454	None		Coltène AG, Altstätten, Switzerland
All-Bond 2	Primer A: 049217 Primer B: 049237 Enamel Bonding: 059125	UNI-ETCH	119266	BISCO, Köln, Germany
Prime & Bond NT	9802001062	DeTrey Conditioner 36	9602161	Dentsply/DeTrey, Konstanz, Germany
Syntac Single Component	901020	Email Preparator GS	924777	Vivadent, Schaan, Liechtenstein
Syntac Classic	Primer: 920395 Adhesive: 916561 Heliobond: 903376	None		Vivadent Schaan, Liechtenstein
Prompt L-Pop	FW 0048188	None		ESPE Dental-Medizin, Seefeld, Germany

TABLE 2. Composition of test materials

Dentin Adhesive (Brand Name)	Compound	Components	pH values
All-Bond 2	Primer A	*N*-tolylglycin-glycidylmethacrylate, ethanol, acetone, water	5.2*
	Primer B	3,4,3′4′-biphenyltetracarboxylic acid anhydride and 2-hydroxy ethyl methacrylate, or 3,3′ (or 4′)-dimethacryloxyethyl ester of 3,4,3′4′-biphenyltetracarboxylic acid, ethanol, acetone	
	Enamel Bonding	Bisphenol A diglycidyl dimethacrylate, urethane dimethacrylate, 2-hydroxy ethyl methacrylate	
Prime & Bond NT		PENTA, urethane dimethacrylate, silicone oxide, di- and trimethacrylic resins, initiator, stabilizer, cethyamine fluoride, acetone	
Syntac Single Component		2-hydroxyethylmethacrylate, methacrylic acid modified poly acrylic acid, maleic acid, water, fluoride	1.6
Syntac Classic	Primer	Tetraethylenglycoldimethacrylate, maleic acid, dimethyl keton, water	1.4
	Adhesive	Polyethyleneglycol dimethacrylate, glutaraldehyde, water	4.0
	Heliobond	Bisphenol A diglycidyl dimethacrylate, trietheyleneglycol dimethacrylate, initiator	
Prompt L-Pop		Methacrylic phosphates, initiator, stabilizer, fluoride, water	1.0

Initial pH values indicated for unpolymerized products were provided by the manufacturer. * Mixture of Primer A & B.

ing to the manufacturers' instructions. Before applying All-Bond 2 (BISCO, Köln, Germany), Prime & Bond NT (Dentsply/DeTrey, Konstanz, Germany), and Syntac Single Component (Vivadent, Schaan, Liechtenstein), the cavity side of the dentin discs (as described below) was etched with the appropriate acid gel (Table 1) for 15 s, rinsed with sterile water, and air-dried, thereby avoiding desiccating the dentin.

Cell Culture

Bovine pulp-derived cells transfected with SV40 large T-antigen were maintained in growth medium (MEMα, Gibco BRL, Karlsruhe, Germany) that was supplemented with 20% fetal bovine serum (FBS), 150 IU/ml of penicillin, 150 μg/ml of streptomycin, 0.125 μg/ml of amphotericin B, and 0.1 mg/ml of geneticin) in a humidified atmosphere at 37°C, 5% CO_2. For all experiments, cells within passages 18 to 26 were used.

Three-dimensional cultures of SV40 large T-antigen transfected pulp-derived cells were prepared as previously described (16). Polyamide meshes (0.5 cm^2; Reichelt Chemietechnik, Heidelberg, Germany) were placed in 48-well-plates, incubated in 0.1 M acetic acid for 30 min, washed three times with phosphate buffered saline, and air-dried. Next, meshes were coated with fibronectin (0.03 mg/ml; Sigma, Deisenhofen, Germany) and air-dried. Cell culture inserts (Millipore, Eschborn, Germany) were placed in 6-well-plates with 1.25 ml of growth medium per well. The meshes were placed on the inserts and 25 μl of cell suspension (4×10^6 cells/ml) were seeded on them. After 48 h incubation (37°C, 5% CO_2, 100% humidity), meshes were transferred to 24-well-plates and incubated until they were used for cytotoxicity experiments (14 ± 2 days). Culture medium (growth medium supplemented with 50 μg/ml of ascorbic acid) was changed three times a week.

Cytotoxicity Testing

Three-dimensional cultures were introduced into a dentin-barrier test system as described previously (16). A commercially available, cell-culture perfusion chamber (Minucells & Minutissue GmbH, Bad Abbach, Germany) made of polycarbonate with a base of 40×40 mm and a height of 36 mm was modified. The original membrane, which served as a substrate for cell growth, was replaced by a dentin disk that was held in place by a special biocompatible stainless steel holder, resulting in a dentin barrier test situation. The dentin disk (500 ± 20 μm thick) was cut from a bovine incisor, etched on one side with 50% citric acid for 30 s to remove the smear layer on the pulpal side of the dentin disk, and

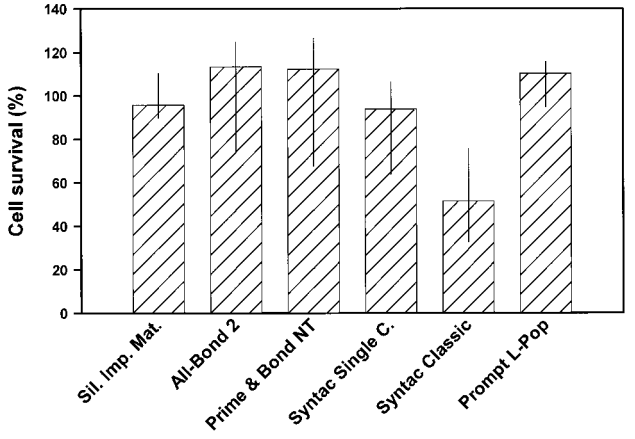

FIG 1. Cytotoxicity of dentin adhesives on three-dimensional cultures of SV40 large T-antigen transfected bovine pulp-derived cells without perfusion. Data are expressed as percentage of the negative-control cultures. The indicated values are medians, 25% and 75% percentiles. Sil. Imp. Mat. = silicone impression material.

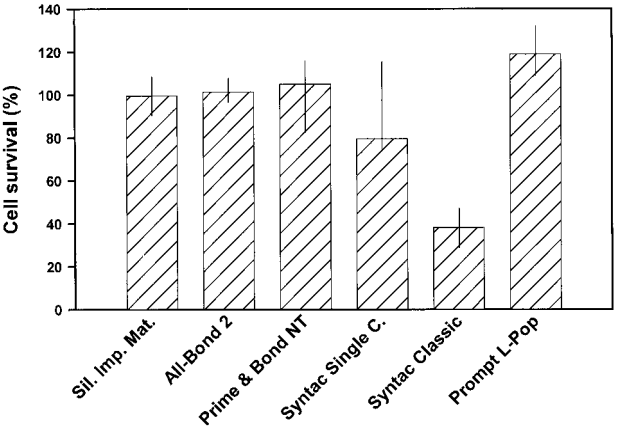

FIG 2. Cytotoxicity of dentin adhesives on three-dimensional cultures of SV40 large T-antigen transfected bovine pulp-derived cells at perfusion conditions of 0.3 ml/h. Data are expressed as percentage of the negative-control cultures. The indicated values are medians, 25% and 75% percentiles. Sil. Imp. Mat. = silicone impression material.

autoclaved as described (13). Thus, the cell culture chamber was separated into two 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 by the stainless steel holder.

All chambers were perfused with 0.3 ml of assay medium (growth medium with 5.96 g/l HEPES buffer) per hour for 24 h. Next, perfusion was switched off and test materials were introduced into the upper compartment in direct contact with the cavity side of the dentin disk. Cytotoxicity of test materials was recorded after 24 h of incubation at 37°C (static condition) by using the MTT assay. In further experiments, the pulpal part of the in vitro pulp chamber was perfused with cell-culture medium (0.3 ml/h and 2 ml/h) during the incubation period (perfusion conditions).

Cell viability of three-dimensional cultures was determined by enzyme activity (MTT assay). The tissues were removed from the pulp chambers, placed into 24-well-plates containing 1 ml of prewarmed MTT solution (0.5 mg/ml in growth medium), and incubated for 2 h at 37°C. Then, the tissues were washed two times with phosphate buffered saline. The blue formazan precipitate was extracted from the mitochondria by using 0.5 ml of dimethyl sulfoxide on a shaker at room temperature for 30 min. Two hundred μl of this solution were transferred to a 96-well-plate and the absorption at 540 nm (optical density OD540) was determined spectrophotometrically.

Each experiment was performed with five replicates. The mean OD540 of control tissues exposed to a A-silicone impression material (President regular, Coltène AG, Altstätten, Switzerland) was used as the negative control and represented 100% viability. Results of cytotoxicity experiments with test materials were then expressed as a percentage of control tissues. Each experiment was carried out three times. Statistical analysis was performed by applying the nonparametric Mann-Whitney pairwise test followed by applying the error rates method, thereby adjusting the significance level α to $\alpha^*(k) = 1 - (1-\alpha)^{1/k}$ (k = number of pairwise tests to be considered).

RESULTS

The results of the cytotoxicity studies at static conditions are summarized in Figure 1. Statistics of these experiments are shown

TABLE 3. Statistical analysis of cell survival rate in the dentin barrier test

	Sil. Imp. Mat.	All-Bond 2	Prime & Bond NT	Syntac Single Component	Syntac Classic
All-Bond 2	*				
Prime & Bond NT	*	*			
Syntac Single Component	*	+	*		
Syntac Classic	+++	+++	+++	++	
Prompt L-Pop	*	*	*	+	+++

Experiments were performed without perfusion. Sil. Imp. Mat. = silicone impression material; * $p \geq 0.05$; + $p \leq 0.05$; ++ $p \leq 0.01$; +++ $p \leq 0.001$.

TABLE 4. Statistical analysis of cell survival rate in the dentin barrier test

	Sil. Imp. Mat.	All-Bond 2	Prime & Bond NT	Syntac Single Component	Syntac Classic
All-Bond 2	*				
Prime & Bond NT	*	*			
Syntac Single Component	*	*	*		
Syntac Classic	+++	+++	+++	+++	
Prompt L-Pop	++	++	++	++	+++

Experiments were performed with perfusion (0.3 ml/h). Sil. Imp. Mat. = silicone impression material; * $p \geq 0.05$; + $p \leq 0.05$; ++ $p \leq 0.01$; +++ $p \leq 0.001$.

in Table 3. With the exception of Syntac Classic (Vivadent), no test material significantly reduced cell survival rates of three-dimensional cultures of transfected pulp-derived cells compared with the negative control ($p \geq 0.05$). Cell viability rates after application of Syntac Classic were 51.6%. The statistical difference of this survival rate from both the negative control and all other test materials was highly significant ($p \leq 0.01$). Syntac Single Component reduced cell survival rates to 93.9%, which means there was no significant difference compared with the negative control ($p \geq 0.05$) but a significant difference to All-Bond 2 and Prompt L-Pop (ESPE Dental-Medizin, Seefeld, Germany) ($p \leq 0.05$).

Cell-survival rates of cytotoxicity experiments at perfusion conditions of 0.3 ml/h are summarized in Figure 2; statistics are shown in Table 4, revealing similar results compared with static experiments. With the exception of Syntac Classic (cell survival rate: 38.2%), no test material significantly reduced cell viability compared with the negative control ($p \geq 0.05$). Syntac Single Component reduced cell survival rate to 79.6% but was not significantly more toxic than the silicone impression material or than all other test materials ($p \geq 0.05$). Application of Prompt L-Pop resulted in a viability rate (119%) that was significantly higher than that of the negative control and all other test materials ($p \leq 0.01$).

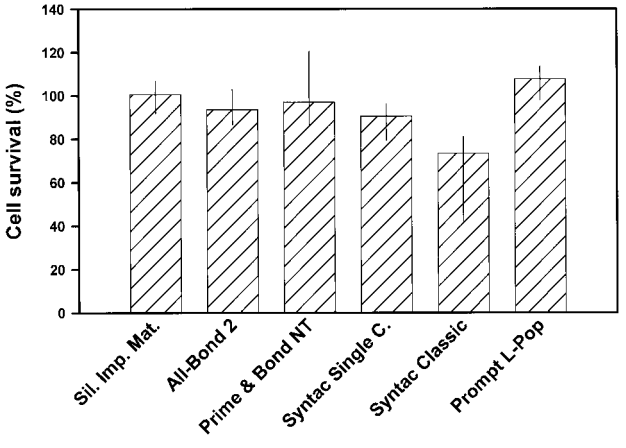

FIG. 3. Cytotoxicity of dentin adhesives on three-dimensional cultures of SV40 large T-antigen transfected bovine pulp-derived cells at perfusion conditions of 2 ml/h. Data are expressed as percentage of the negative-control cultures. The indicated values are medians, 25% and 75% percentiles. Sil. Imp. Mat. = silicone impression material.

The results of the cytotoxicity experiments at perfusion conditions of 2 ml/h are shown in Figure 3 and Table 5, revealing results similar to static conditions and to experiments at perfusion conditions of 0.3 ml/h. In these experiments, also Syntac Classic was the most toxic material and reduced viability of the three-dimensional cultures to 73.4%; which demonstrates statistically high significance compared with the negative control and to all other test materials ($p \leq 0.01$). Syntac Single Component resulted in viability rates that were significantly lower than those of the silicone impression material and Prompt L-Pop ($p \leq 0.05$). Statistical analyses of the influence of different perfusion conditions on the cytotoxicity of test materials are summarized in Table 6. Comparison of cell-viability rates of experiments performed at static conditions with those at 0.3-ml perfusion/h showed, generally, no statistically significant differences. This was also true for viability rates that resulted from experiments at static conditions compared with those from experiments at perfusion conditions of 2 ml/h ($p \geq 0.05$). Comparison of cell survival rates at 0.3-ml perfusion/h with those at 2 ml/h revealed significant differences only for the materials, Syntac Classic and Prompt L-Pop. Application of Syntac Classic evoked significantly higher cell-survival rates at perfusion conditions of 2 ml/h ($p \leq 0.001$); Prompt L-Pop had significantly lower survival rates at this perfusion condition ($p \leq 0.05$).

DISCUSSION

As the protective effect of dentin has frequently been demonstrated, recently, the concept of dentin barrier tests has been introduced in toxicity testing of dental restorative materials. Different approaches have been published (17, 18). A main problem involved in the dentin barrier test is the variability of test systems (17). Therefore, we introduced a system based mainly on commercially available components (13). Another problem of such in vitro tests is related to the cells that are used. As outlined elsewhere, stable pulp-derived cell lines with a metabolism close to odontoblasts were desirable (17, 18). Therefore, we transfected bovine pulp-derived cells with the SV 40 large T-antigen, which resulted in a stable cell line with metabolic features typical of primary pulp cells (19). A further approximation to the in vivo situation was achieved by growing these cells on nylon meshes in three-dimensional cultures (16). This experimental setup has been shown to reveal results that are in accordance with clinical experience for a series of dental cements, including zinc oxide and eugenol (16). Perfusion of the cell culture compartment was introduced to mimic

TABLE 5. Statistical analysis of cell survival rate in the dentin barrier test

	Sil. Imp. Mat.	All-Bond 2	Prime & Bond NT	Syntac Single Component	Syntac Classic
All-Bond 2	*				
Prime & Bond NT	*	*			
Syntac Single Component	+	*	*		
Syntac Classic	+++	+++	+++	++	
Prompt L-Pop	*	+	*	++	+++

Experiments were performed with perfusion (2 ml/h). Sil. Imp. Mat. = silicone impression material; * $p \geq 0.05$; + $p \leq 0.05$; ++ $p \leq 0.01$; +++ $p \leq 0.001$.

TABLE 6. Influence of perfusion conditions on the cytotoxicity of test materials

Perfusion (ml/h)	Sil. Imp. Mat.	All-Bond 2	Prime & Bond NT	Syntac Single Component	Syntac Classic	Prompt L-Pop
0–0.3	—	—	—	—	—	—
0–2	—	—	—	—	—	
0.3–2	—	—	—	—	+++	+

Sil. Imp. Mat. = silicone impression material.

the blood flow in the dental pulp, which may be responsible for the removal of toxic substances.

Animal-model, usage tests are still considered to simulate best the patient situation. However, contradictory results have been reported on direct pulp capping with dentin-bonding agents in animal experiments, with some authors reporting no damage (8) and others reporting severe reactions up to pulp necrosis (9), whereas results from studies on humans showed severe pulp damage (11).

Therefore, the model used in this study may be an interesting approach to gain further insight into the tissue reactions that are evoked by dentin-bonding agents—combining the advantages of cell-culture experiments (comparatively high degree of standardization) with an approach that includes dentin and target cells with a metabolism resembling that of pulp cells in primary culture, and thus closely simulating the patient situation.

The present data show that low pH dentin-bonding agents have no effect on pulp-derived three-dimensional cell cultures when a 0.5-mm dentin barrier was placed between material and cells. Under 0.3 ml/h perfusion conditions, the material with the lowest pH (Prompt L-Pop) even increased the enzyme activity of the cell cultures. These results, compared with the marked cytotoxicity of these substances in direct cell experiments (6), show again the protective effect of dentin. They are also in accordance with results from in vitro experiments that have shown that one-step dentin-bonding agents were less toxic in cell culture than multistep counterparts (7).

The pH measurements in nonaqueous solutions, such as Prime & Bond NT, are difficult to perform and for dentin adhesives containing water, we present the information provided by the manufacturer (Table 2).

The cytotoxic reaction of the cell cultures toward Syntac Classic may be attributed to the glutaraldehyde content of the materials (12) or due to TEGDMA (4). Our data are in accordance with results reported by other authors, who also found that Syntac Classic was more toxic than Syntac Single Component (7). Syntac Classic has been used for many years in patients, and no data on adverse pulp reactions have been reported (20). This indicates that the present test system is comparatively sensitive. On the other hand, it may be concluded that materials that show no reaction in this test system have the potential to be innocuous to the dental pulp. Generally, no influence of perfusion of the pulpal compartment of the test device on cell-culture medium was observed. This was to be expected, because most materials showed no influence on the cell cultures under static conditions. However, statistically significant changes were noted for both the most toxic material (Syntac Classic) and the most stimulating material (Prompt L-Pop). Both effects were reduced by 2 ml/h perfusion. This may be an indication that through perfusion, the relevant substances may have been removed from the culture system, thus providing an open-test system, as is the case in the patient.

In conclusion, low pH dentin-bonding agents are not cytotoxic in an in vitro pulp chamber. Damage of the dental pulp by the tested substances is unlikely to occur. The in vitro pulp chamber used in this study proved to be closer to the clinical situation than direct cell-material contact methods and has the potential to at least partially replace animal experimentation.

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