Selective Influence of Dentin Thickness upon Cytotoxicity of Dentin Contacting Materials

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Abstract

The effect of three dentin contacting materials on three-dimensional cultures of pulp-derived cells was evaluated in a dentin barrier test device. The test materials (Syntac Classic, Prompt L-Pop, Vitrebond) were applied on dentin disks of different thicknesses ranging from 100 to 500 μm. After 24 h of exposure with and without perfusion of the test chamber, cell survival was evaluated using the MTT assay and related to a non-toxic control material. Syntac Classic decreased cell activity significantly ($p \leq 0.0003$), independently of the dentin thickness. For Prompt L-Pop and Vitrebond a significant influence of dentin thickness was found on the cell reaction. After exposure of the control material, photometric readings showed no dependency of the cellular reaction on dentin thickness ($p > 0.05$). It could be demonstrated that dentin acts as a barrier, decreasing the elicited cytotoxicity with increasing thickness. This effect is material related, showing little influence for the nontoxic or the glutaraldehyde containing material.

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Dentin has been shown to reduce the toxicity of certain dental materials, such as zinc oxide-eugenol (1). Permeability measurements have shown dentin to be a partial perfusion/diffusion barrier (2). Furthermore, dentin acts as an absorption medium; e.g. for eugenol or for zinc (3, 4), and it is considered to be a solid buffer, neutralizing protons derived from acids (1) that are used in self-etching dentin conditioning/bonding agents. Sclerotic dentin containing a hypermineralized surface shows even less permeability than normal dentin (5) because of partial or total occlusion of tubules. In parallel to these in vitro findings, in vivo pulp testing on experimental animals and humans has shown that restorative materials, which are in vitro cytotoxic, do not cause any significant pulp reactions when applied in class V cavities (6, 7).

On the other hand, the protective effect of dentin seems to be limited. The diffusion of small hydrophilic molecules like HEMA or TEGDMA even through sclerotic dentin has been demonstrated (8). Furthermore, in cases where bacteria are on the cavity floor beyond a filling material, pulp inflammation is observed (9), indicating the diffusion of toxic bacterial metabolic products. Based on these findings we hypothesize that the pulpal reaction towards different substances might either be attenuated or possibly intensified by the dentin, depending on the applied material and dentin thickness.

Cell cultures may be an interesting supplement to animal experimentation, because the sensitivity of such systems can be adjusted. The dentin barrier test system is an in vitro method specifically designed to mimic the clinical situation for biological testing of filling materials, and allows for a variation of parameters, like dentin thickness (10, 11). Therefore, in the present investigation, this method was used to test the above mentioned hypothesis by measuring the influence of three different dentin contacting materials upon the cell reaction when they are applied on dentin disks of varying thicknesses.

MATERIALS AND METHODS

Test Materials

The materials used for cytotoxicity testing are listed in Table 1. They were applied according to the manufacturers' instructions. A nontoxic polyvinylsiloxan impression material (President, Coltène) was used as a negative control (100% cell viability).

Cell Culture

Clonal SV 40 large T-antigen transfected cells, derived from calf dental papilla, were maintained in growth medium (MEMα, GIBCO BRL, Germany) supplemented with 20% fetal calf serum, 150 IU/ml penicillin, 150 μg/ml streptomycin, 0.125 μg/μl amphotericin B, and 0.1 mg/ml geneticin in a humidified atmosphere at 37°C in 5% CO_2. For all experiments, cells within passages 19 to 26 were used (12).

Three-dimensional cultures of SV 40 large T-antigen transfected pulp-derived cells were prepared as previously described (11). 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 into 6-well-plates with 1.25 ml of growth medium per well. The meshes were placed on the inserts and 20 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 ±

TABLE 1. Test materials

Test Material	Brand Name	Manufacturer	Lot.No.
Silicone impression material	President regular	Coltène Altstätten, Switzerland	111HK410
Resin-modified glass ionomer cement	Vitrebond	3 M ESPE Medica GmbH Borken, Germany	20000223
Dentin Adhesive	Prompt L–Pop	3 M ESPE Dental AG Seefeld, Germany	101018
Dentin Primer	Syntac classic	Vivadent Ellwangen, Germany	905113
Adhesive			916561
Bonding			903376

2 days). Culture medium (growth medium supplemented with 50 μg/ml of ascorbic acid) was changed three times a week.

Cytotoxicity Testing

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 held in place by a specific biocompatible stainless steel holder, resulting in a dentin barrier test configuration. Dentin slices of 100, 200, 300, and 500 μm thickness were cut from extracted bovine first incisors with a wheelsaw (Leitz GmbH, Germany) under constant water flow at 600 rpm and 0.6 mm/s^{-1} crosshead speed. The smear layer on the pulpal side was removed by etching with 50% citric acid for 30 s. The dentin disks were rinsed with physiological saline and sterilized by autoclaving (121°C, 25 min) as described (11). The cell culture chamber was thus separated into two compartments by the dentin disk. The three-dimensional 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/liter 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 conditions) by using the MTT assay. In further experiments, the pulpal part of the in vitro pulp chamber was perfused with cell culture medium (2 ml/h) during the incubation period (perfusion conditions).

MTT Assay

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 cells 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. 200 μl of this solution was transferred to a 96-well plate and the absorption at 540 nm (OD_{540}) was determined spectrophotometrically.

Data Analysis

The median OD_{540} of control tissue (cell cultures exposed to the polyvinylsiloxane impression material) was set to represent 100% viability. Results of cytotoxicity experiments were expressed as a percentage of matching control tissue. Each experiment was performed with nine replicates. Statistical analysis was performed applying the nonparametric Mann-Whitney test followed by applying the error rates method, thereby adjusting the significance level $\alpha = 0.05$ to $\alpha^*(k) = 1 - (1 - \alpha)^{1/k}$ (where k = number of pairwise tests to be considered).

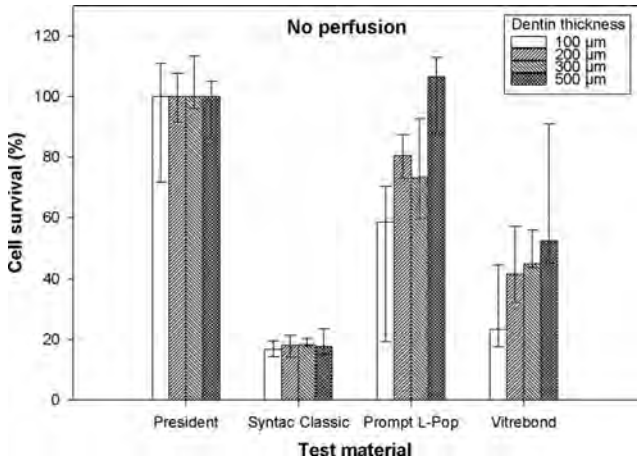

Fig 1. Cytotoxicity of test materials on three-dimensional cultures of transfected bovine pulp-derived cells. Data are expressed as percentage of the negative control cultures. The indicated values are medians, 25% and 75% percentiles (static conditions).

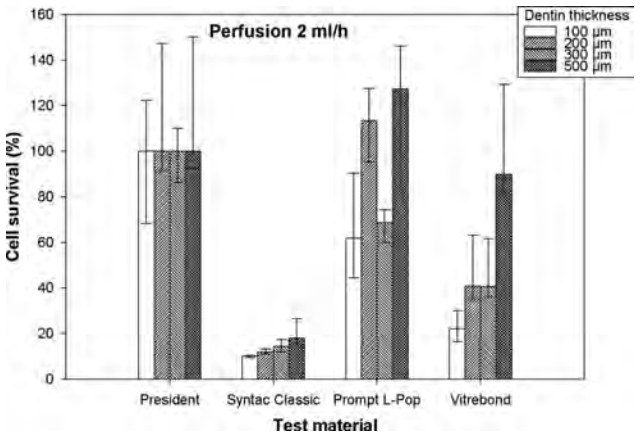

Fig 2. Cytotoxicity of test materials on three-dimensional cultures of transfected bovine pulp-derived cells. Data are expressed as percentage of the negative control cultures. The indicated values are medians, 25% and 75% percentiles (perfusion conditions).

TABLE 2. Statistical analysis of cell survival rates of different materials vs. each other on 100 μm (upper right corner) and 200 μm (lower left corner) dentin disks, and experiments under static conditions compared to perfusion conditions (diagonal bold compartments); lower left and upper right corners: left sides of compartments represent static, right sides perfusion of 2 ml/h conditions; bold diagonal compartments: on the left side results from 100 μm, and on the right side from 200 μm dentin disks.

	President	Syntac	Prompt	Vitrebond	
President	—/—	***/***	**/—	***/***	100 µm Dentin disks
Syntac	***/***	***/***	**/***	*/***	
Prompt	**/—	***/***	—/**	—/**	
Vitrebond	***/***	***/***	**/***	—/—	
	200 µm Dentin disks				static vs. 2ml/h perfusion

— p > 0.05; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

TABLE 3. Statistical analysis of cell survival rates of different materials vs. each other on 300 μm (upper right corner) and 500 μm (lower left corner) dentin disks, and experiments under static conditions compared to perfusion conditions (diagonal bold compartments); lower left and upper right corners: left sides of compartments represent static, right sides perfusion of 2 ml/h conditions; bold diagonal compartments: on the left side results from 300 μm, and on the right side from 500 μm dentin disks.

	President	Syntac	Prompt	Vitrebond	
President	—/—	***/***	**/***	***/***	300 µm Dentin disks
Syntac	***/***	*/—	***/***	***/**	
Prompt	—/—	***/***	—/—	**/*	
Vitrebond	*/—	***/***	*/—	—/—	
	500 µm Dentin disks				static vs. 2ml/h perfusion

— p > 0.05; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

RESULTS

The results of the cytotoxicity studies are summarized in Figs. 1 and 2. Tables 2 to 4 show statistical analysis of cell survival rates for the different materials, for different dentin thicknesses and for static conditions compared to perfusion of 2 ml/h. The polyvinylsiloxane impression material evoked OD_{540} readings between 0.69 and 1.12, and for 10 out of 12 comparisons of different dentin thicknesses no statistical difference of the original OD_{540} readings could be found ($p \geq 0.1223$). The OD_{540} readings for this material were set to 100%. Syntac Classic reduced cell viability to 17% and 18% on 100 to 500 μm dentin discs. It was significantly more toxic than all other test materials ($p \leq 0.023$). No significant difference of cell viability between dentin layers of different thicknesses was found ($p \geq 0.05$). Vitrebond evoked cell survival rates ranging from 23 to 53% for the dentin disks of 100 to 500 μm thickness. It was significantly more toxic than the negative control and Prompt L-Pop ($p \geq 0.0243$), which evoked cell viability rates ranging from 59% to 107%. The reaction to cytotoxicity testing both for Vitrebond and Prompt L-Pop was clearly influenced by the dimension of the dentin barrier. Significantly higher cell viability could be shown for 500 μm dentin disks compared to 100 μm disks ($p \leq 0.0092$ and $p \leq 0.0071$, respectively).

The results of the experiments with perfusion of test medium of 2 ml/h showed no statistical difference of cell viability rates either for the polyvinylsiloxane impression material between different dentin thicknesses ($p \geq 0.3099$) or between static and perfusion conditions ($p \geq 0.3767$) (Fig. 2). Cell survival rates for Syntac Classic were significantly lower under perfusion conditions (10, 12, 14, and 18% on 100–500 μm dentin disks) compared to static conditions ($p \leq 0.0118$). Vitrebond and Prompt L-Pop mostly showed no statistical difference of cell viability between static and perfusion conditions ($p > 0.05$). Interestingly, Prompt L-Pop evoked higher cell viability on 500 μm dentin disks than the nontoxic control, both for static as well as perfusion conditions.

DISCUSSION

Dentin-bonding agents normally come into close contact with dentin or even pulp tissue. The objective of this work was to investigate if and to what extent different cytotoxic reactions in the target tissue were evoked by different materials, and how they are influenced by different dentin thicknesses. Several approaches to biocompatibility testing of dentin contacting materials have been published (6, 7, 13), and animal experiments are still considered to mimic best the patient situation. However, those experiments are mostly performed on sound teeth without any predamage or inflammatory processes of the pulpal tissue because of carious lesions.

The dentin barrier test used in the present study is considered to be more sensitive than the above mentioned in vivo models, because materials like Vitrebond (7, 14) and Syntac Classic (15) are reported to be nontoxic to the pulp of sound teeth, but they evoke a toxic reaction in the dentin barrier test. This increased sensitivity may allow for a better differentiation between materials that are used on teeth with a potentially predamaged pulp. Perfusion of the cell culture department was introduced to mimic the blood flow in the dental pulp as has been described previously (11).

On the other hand, in vitro testing so far does not simulate tissue defense reactions like inflammation, immuno-induced responses of the organism or other repair mechanisms. However, it may be concluded that materials that show no reaction in the dentin barrier test have the potential to be innocuous to the pulpal tissue if other factors like bacterial invasion could be excluded.

In the present study, dentin proved to be protective for an acidic self-etching dentin bonding agent, partially protective for a light-cured glass-ionomer cement, and nonprotective for a glutaraldehyde containing dentin bonding agent. The cytotoxicity of the acidic self-etching dentin bonding agent, Prompt L-Pop, may be a result of the release of protons or because of the toxicity of the included monomers. The protective effect of dentin for the acidic self-etching dentin bonding agent may be explained, at least partially, by the buffer capacity of dentin (1). Phosphoric acid placed on dentin leads to a superficial dissolution of dentin apatite and thus the acid may become neutralized by binding of

TABLE 4. Statistical analysis of cell survival rates of various dentin thicknesses against each other

Dentin Thickness	Syntac	Prompt	Vitrebond
100 vs. 200 μm	—/*	**/**	—/**
100 vs. 300 μm	—/***	**/—	*/**
100 vs. 500 μm	—/***	***/**	**/***
200 vs. 300 μm	—/—	—/***	—/—
200 vs. 500 μm	—/**	**/—	**/—
300 vs. 500 μm	—/—	**/**	—/**

— p > 0.05; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.

Left side of compartments: static conditions, right side: perfusion 2 ml/h.

the protons to the OH^--ions and the PO_4^{3-} -ions of the apatite. Thus it is plausible that in thin dentin layers (e.g. 100 μm), the buffer capacity of dentin is soon exhausted and through dissolution the permeability of the dentin considerably increases. This will increase the toxic reaction elicited either by remaining protons or by the included monomers. For dentin thickness of e.g. 500 μm the superficial dissolution will barely change the permeability characteristics (16). Interestingly, Prompt L-Pop increased enzyme activity of the cell cultures on 300 μm and 500 μm disks to more than 100%. These results are in accordance with former experiments (17); the mechanisms yet remain without conclusive explanations. However, there was no statistical difference compared to the negative control material. The cytotoxicity of the light curing glass ionomer cement has been reported consistently in the literature (11, 14) and is thought to be a result of the release of the toxic initiator diphenyliodonium chloride DPIC1 (14). Being a water soluble substance, this initiator apparently diffuses through dentin leading to toxic reactions. No information is available on the diffusion through dentin or the adsorption characteristics of this chemical. However, it can be speculated that the initiator is bound partially to dentin and, therefore, dentin has a partially protective effect. In vivo studies though have shown no adverse pulp reaction when Vitrebond was placed in deep cavities of human teeth (7). However, these tests were performed in sound teeth without any predamage of the pulp. Thus the healthy pulp may be able to repair these toxic challenges, but it is unknown if a predamaged pulp, which may be the consequence of dentin caries, is able to respond to such a challenge in the same way.

The cytotoxicity of the glutaraldehyde containing dentin bonding agent has been reported before and may be mainly attributed to the glutaraldehyde (19) and the included monomer TEGDMA (20). Interestingly, for this material no protective effect of dentin was observed in the present study. On the other hand, this dentin bonding agent has been used for many years clinically without any obvious signs of pulp damage. Thus, one can speculate that glutaraldehyde, which is known to cross react with collagen (21), may not reach the dental pulp in sufficient quantities to elicit a toxic reaction. However, in that case, a protective effect of dentin should have occurred, because the collagen content of the dentin was not changed during the preparation procedure of the dentin disks, as was shown by measuring the protein content according to the Kjelldahl method as well as the determination of free amino groups by the TNBS-assay, before and after sterilization (unpublished data). Furthermore, it should be kept in mind that the collagen in the dentin is not apparently accessible but immured into the inorganic phase (22).

Also, one can assume that the dentin tubuli of the dentin disks in the present study contain cell culture medium because of capillary forces. The medium contained 20% serum and thus a protein fraction similar to the in vivo situation is present in the in vitro test system used in this study (23). Therefore, the speculation of a detoxification of glutaraldehyde by its passage through dentin is not supported by the data of this investigation.

The data of our in vitro study suggest that the protective properties of dentin are apparently selective and depend on the chemical nature of the dentin contacting material. Therefore, a cytotoxic material intended to be used on dentin should be tested simulating the dentin barrier. Only if then still cytotoxic, further animal experimentation should be considered to evaluate if in vivo defense mechanisms are able to counteract this challenge effectively.

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