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An In Vitro Pulp Chamber with Three-Dimensional Cell Cultures

Gottfried Schmalz, DMD, PhD, DDS, Uta Schuster, PhD, Katja Nuetzel, DDS, and Helmut Schweikl, PhD

To better simulate the in vivo situation, a three-dimensional fibroblast cell culture was introduced into an in vitro pulp chamber model. The system was evaluated by testing a series of dental filling materials. After a 24-h exposure with (0.3 or 5 ml/h) and without perfusion of the pulp chamber, the tissues were subjected to a routine MTT assay. Zinc phosphate cement, conventional glass ionomer cements, a silicone impression material, and zinc oxide-eugenol did not influence cell viability, compared with untreated controls; but, a light-curing glass ionomer cement significantly reduced cell survival. Perfusion of the chambers did not significantly influence the results, but perfusion conditions of 5 ml/h lead to a general decrease of cell vitality. The three-dimensional cell culture system in an in vitro pulp chamber seems to be a substantial improvement, because zinc oxide-eugenol does not evoke a cellular reaction (as is the case in vivo), and the test system is sensitive enough to detect other toxicants.

According to national and international regulations, dental filling materials have to be evaluated for biocompatibility before being applied on patients (ANSI-ADA-document no. 41, ISO 7405) (1, 2). For this purpose, animal experiments and cell culture tests are available. Animal experiments for cytotoxicity testing of dental materials are time-consuming, expensive, and subject to extensive public discussions. Cell culture methods, on the other hand, are better standardized and reproducible. They are fast and easy to perform at relatively low costs (3). However, due to the in vitro test situation, extrapolation of test results to patients is problematic. This was shown with the material zinc oxide-eugenol, which in cell culture experiments evoked strongly toxic reactions (4), but did not damage dental pulp tissue when applied on the intact dentin (5).

Therefore, special cell culture methods have been developed recently for testing dental filling materials introducing dentin as a barrier between test material and target cells. Basically, two approaches were described: dentin slices from either the third human molars or pressed dentin powder chips were used (6–8). Both methods have apparent technical disadvantages: appropriate human dentin is only available in limited amounts, large differences in hydraulic conductance between different human dentin slices were reported (9), and pressed dentin chips do not correspond favorably to the anatomical structure of natural dentin. Furthermore, the target cells were usually grown on the bottom of the culture flasks, resulting in unwelcome dilution effects by the nutrition medium.

We described an alternative technique that should overcome these difficulties: mouse fibroblasts were seeded directly on bovine dentin disks that were obtained in a standardized way as described previously (10). The device is mainly based on commercially available components for constructing an artificial pulp chamber, fulfilling a basic requirement for standardized testing techniques. An additional advantage of this artificial pulp chamber was regarded to be the possibility of perfusing the pulpal part with nutrition medium, thus simulating in vivo pulpal blood flow. However, even with this test protocol, zinc oxide-eugenol still evoked a strong toxic cell reaction (10). It was hypothesized that the use of an unphysiological cell monolayer in these in vitro test system, which does not simulate the three-dimensional structure of the dental pulp tissue, may be the reason for this still existing discrepancy between the in vitro test and the in vivo situation.

Three-dimensional cell cultures have been used for cytotoxicity testing of cosmetic products and have been made commercially available (11, 12). These cell culture models consist of several layers of normally dividing, metabolically active fibroblasts. The fibroblasts are derived from neonatal human foreskin and seeded on nylon meshes where they attach and grow. The fibroblasts naturally secrete their own extracellular matrix molecules (i.e. collagen fibrils, fibronectin, and both sulfated and nonsulfated glycosaminoglycans) (11). Previous studies on cytotoxicity of various chemicals showed excellent intralaboratory reproducibility with good agreement of the test results to existing in vivo data from animal experiments (12).

In the present study, we introduced such a three-dimensional cell culture into the in vitro pulp chamber described previously. The sensitivity of the three-dimensional cell culture system was tested using different concentrations of phenol. The suitability of this approach was evaluated by testing the cytotoxicity of a series of dental filling materials, which had been used in previous tests with monolayers of a permanent cell line (L929-mouse fibroblasts). The test was performed under static conditions or under perfusion conditions, whereby the pulpal part of the artificial pulp chamber was perfused with cell culture medium.

TABLE 1. Test materials

Test Material	Brand Name	Manufacturer	Batch No.
Control: cotton pellet soaked with culture medium			
Silicone impression material	President regular	Coltene AG (Altstätten, Switzerland)	C 1784
Light-curing glass ionomer cement	Vitrebond	3M Medica GmbH (Borken, Germany)	1995 0 720
Zinc phosphate cement	Harvard Cement	Richter & Hoffmann Harvard-Dental GmbH (Berlin, Germany)	136 (powder) 099 (liquid)
Conventional glass ionomer cements	Ketac-Fil	Espe GmbH & Co KG (Seefeld, Germany)	007
	Ketac-Silver	Espe GmbH & Co KG (Seefeld, Germany)	534
Zinc oxide-eugenol cement		Eugenol: Merck-Suchard (Hohenbrunn, Germany)	818455
		Zinc oxide: E. Wasserfuhr GmbH (Bonn, Germany)	34920.027 A5

MATERIALS AND METHODS

Cell Culture

The three-dimensional nylon mesh culture system for fibroblasts (Skin^2 model ZK1000), originally developed for testing cosmetic products and described previously (11, 12), was obtained from Advanced Tissue Sciences (La Jolla, CA). On arrival, the tissue cultures were removed from the shipping agar, cleaned of residual agar with growth medium (Dulbecco's Minimal Essential Medium, DMEM) supplemented with 10% fetal calf serum (FCS), and transferred to 48-well tissue culture plates. The cultures were maintained in growth medium in a humidified atmosphere at 37°C and 5% CO_2 until used in cytotoxicity experiments.

Test Materials

The materials used for cytotoxicity testing are listed in Table 1. They were mixed according to the manufacturer's instructions. Zinc phosphate cement was mixed in two different powder/liquid ratios (1:1 and 2:1, wt/wt). Zinc oxide-eugenol cement was mixed in a powder/liquid ratio of 4.5:1 (wt/wt). Each material was tested 15 times; cotton pellets soaked in culture medium were used as negative controls (100% cell survival).

The sensitivity of the three-dimensional cell culture system was tested in 48-well tissue culture plates using different concentrations of phenol (0.05%, 0.1%, 0.5%, 1%, 2% (vol/vol)) in assay medium (DMEM supplemented with 2% FCS). Furthermore, 200 µl of phenol (2%, 4%, 6%, 8%, and 10% (vol/vol)) in assay medium were applied to the "cavity" side of the in vitro pulp chambers (without perfusion) by means of cotton pellets. Cell cultures fed with assay medium served as negative controls (100% cell survival). After a 24-h incubation, vitality of the tissue cultures was determined using the MTT assay.

In Vitro Pulp Chamber

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 that served as a substrate for cell growth was replaced by a dentin disk held in place by a special biocompatible stainless-steel holder, resulting in a dentin barrier test situation. The dentin disk (500 µm thick) was cut from a bovine incisor, etched on one side with 50% citric acid for 30 sec, and autoclaved as described (10). 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 (DMEM supplemented with 2% FCS) per h 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). In further experiments, the pulpal part of the in vitro pulp chamber was perfused with cell culture medium (0.3 ml/h and 5 ml/h) during the incubation period (perfusion condition).

Quantification of Cell Reaction

Cell viability of exposed tissues was determined by enzyme activity (MTT assay) after 24 h. 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 DMEM) and incubated for 2 h at 37°C. Then the tissues were washed two times with phosphate-buffered saline (PBS). The blue formazan precipitate was extracted from the mitochondria using 0.5 ml isopropanol on a shaker at room temperature for 30 min. Two hundred microliters of this solution were transferred to a 96-well plate and the absorption at 540 nm (OD_{540}) was determined spectrophotometrically. The

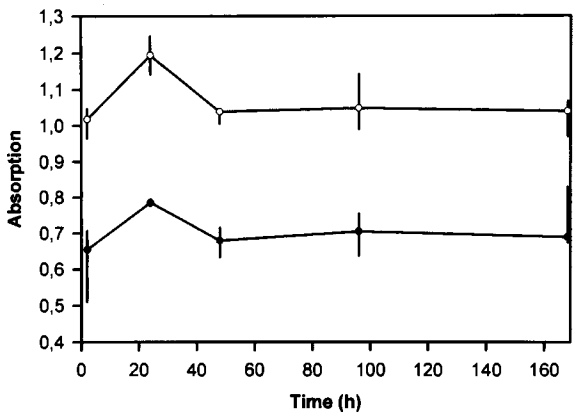

FIG 1. Growth kinetics of skin^2 tissue cultures. Growth curve of cell cultures grown in 48-well plates is indicated by *open circles*. Growth curve of cell cultures grown in perfusion chambers is indicated by *closed circles*. The indicated values are medians, minima, and maxima from triplicates.

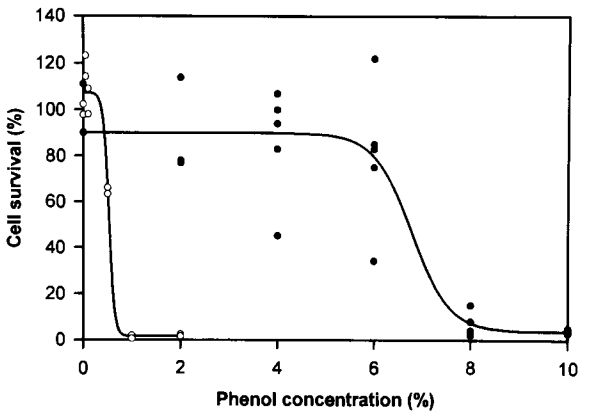

FIG 2. Dose-response curve of skin^2 fibroblasts to phenol. Cell cultures in 48-well plates and in perfusion chambers (with 500 μm dentin slices) were exposed to different concentrations of phenol. The dose-response curve of cells in 48-well plates is indicated by *open circles*; the dose-response curve of cells in perfusion chambers is indicated by *closed circles*. The indicated values are single values.

mean optical density (OD_{540}) of the negative (untreated) control tissues was set to represent 100% viability. Results were expressed as a percentage of untreated control.

Statistics

Statistical analysis was performed 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

Growth Kinetics of Tissue Cultures

Cell growth of the three-dimensional fibroblast cell cultures was monitored in triplicate for up to 7 days in 48-well tissue culture plates and in perfusion chambers (0.3 ml/h), respectively, using the MTT assay. Growth curves revealed similar kinetics of cells growing in 48-well plates and in perfusion chambers (Fig. 1) indicating that perfusion conditions of 0.3 ml/h do not disturb normal growth of the fibroblasts. The generally lower OD values resulting from cell cultures grown in perfusion chambers are due to experimental conditions: the edge of the tissue cultures is crushed by the stainless-steel holder holding in place the tissue cultures and the dentin slices.

Cytotoxicity of Phenol

Figure 2 reveals a dose-response curve of the three-dimensional tissue cultures to phenol, indicating a concentration dependent decrease of cell survival both in 48-well plates and in perfusion chambers. The 50% toxic concentration value (TC_{50}) of cultures grown in 48-well tissue culture plates is 0.53% phenol. The dentin barrier in the perfusion chambers shifts the TC_{50} value to 6.7% phenol, thus demonstrating the protective effect of dentin against this toxic substance.

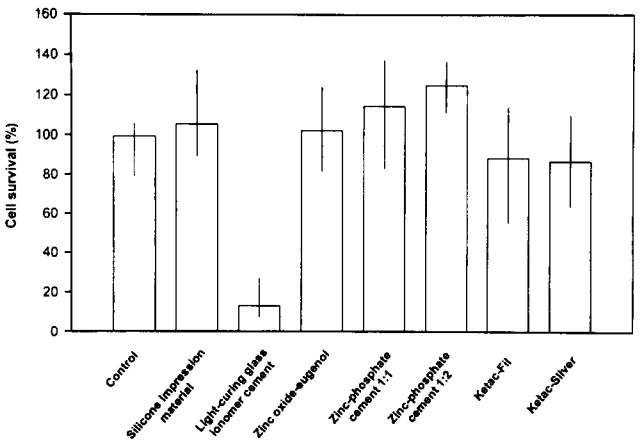

FIG 3. Summary of cell survival (MTT assay) without perfusion. Skin^2 fibroblasts were exposed to dental materials for 24 h. Data are expressed as the percentage of untreated control cultures; the indicated values are medians, 25% and 75% percentiles.

Cytotoxicity of Test Materials without Perfusion

The results of the cytotoxicity experiments at static conditions are summarized in Fig. 3. Statistics of these experiments are shown in Table 2. Zinc phosphate cement had no cytotoxic effects on the three-dimensional tissues at both mixing ratios. Cell viability rates after exposure to zinc phosphate cement at a powder/liquid ratio of 1:1 were 114%; which means no statistical significance compared with the negative control ($p \geq 0.05$). The same material with a mixing ratio of 1:2 evoked similar results with cell viability rates of 125%. Conventional glass ionomer cements (Ketac-Fil and Ketac-Silver) reduced the survival rate of cells to 89% and 87%, respectively, but were not significantly different from untreated control cultures ($p \geq 0.05$). Application of the light-curing glass ionomer cement (Vitrebond) caused considerable cell damage. Only 15% of the cells survived after a 24-h exposure. The statistical difference of this survival rate from both the negative control and all other materials was highly significant ($p \leq 0.001$). Zinc oxide-eugenol cement did not influence the vitality of the cultures

TABLE 2. Statistics of experiments without perfusion

	Control	Sil. Imp. Mat.	L.-C. GIC	ZnOE	ZPC 1:1	ZPC 1:2	Ketac-Fil
Sil. Imp. Mat.	–						
L.-C. GIC	+++	+++					
ZnOE	–	–	+++				
ZPC 1:1	–	–	+++	–			
ZPC 1:2	+	–	+++	–	–		
Ketac-Fil	–	–	+++	–	–	++	
Ketac-Silver	–	+	+++	–	–	++	–

–, $p \geq 0.05$; +, $p \leq 0.05$; ++, $p \leq 0.01$; +++, $p \leq 0.001$. Sil. Imp. Mat., silicone impression material; L.-C. GIC, light-curing glass ionomer cement; ZnOE, zinc oxide-eugenol cement; ZPC, zinc phosphate cement.

TABLE 3. Statistics of experiments with perfusion (0.3 ml/h)

	Control	Sil. Imp. Mat.	L.-C. GIC	ZnOE	ZPC 1:1	ZPC 1:2	Ketac-Fil
Sil. Imp. Mat.	++						
L.-C. GIC	+++	+++					
ZnOE	–	–	+++				
ZPC 1:1	–	–	+++	–			
ZPC 1:2	–	–	+++	–	–		
Ketac-Fil	–	++	+++	–	–	–	
Ketac-Silver	–	+	+++	–	–	–	–

–, $p \geq 0.05$; +, $p \leq 0.05$; ++, $p \leq 0.01$; +++, $p \leq 0.001$. Sil. Imp. Mat., silicone impression material; L.-C. GIC, light-curing glass ionomer cement; ZnOE, zinc oxide-eugenol cement; ZPC, zinc phosphate cement.

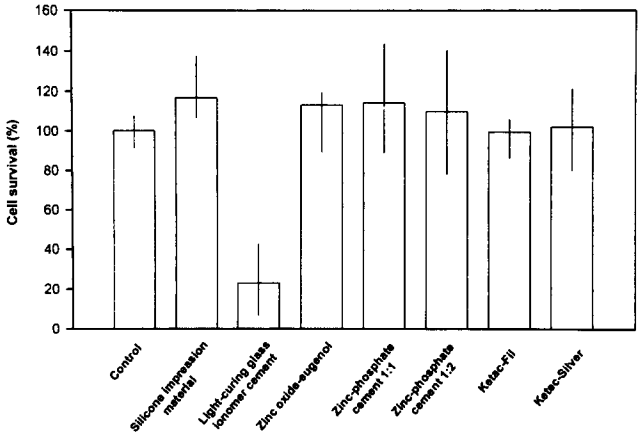

FIG 4. Summary of cell survival (MTT assay) with 0.3 ml perfusion/h. Skin^2 fibroblasts were exposed to dental materials for 24 h. Data are expressed as percentage of untreated control cultures; the indicated values are medians, 25% and 75% percentiles.

(102% cell survival). Also, the silicone impression material President was a nontoxic material and did not reduce viability of the cultures (105% cell survival).

Cytotoxicity of Test Materials after Perfusion Conditions of 0.3 ml/h

Cell survival rates of cytotoxicity experiments at perfusion conditions of 0.3 ml/h are summarized in Fig. 4; statistics are shown in Table 3, showing similar results compared with static experiments. With the exception of the light-curing glass ionomer cement, no test material significantly reduced the viability rate of the three-dimensional cell cultures in comparison with untreated controls. The light-curing glass ionomer cement, however, caused survival rates (23%) that were significantly lower than those of the negative controls and all other materials ($p \leq 0.001$).

Cytotoxicity of Test Materials after Perfusion Conditions of 5 ml/h

The results of the cytotoxicity studies at perfusion conditions of 5 ml/h are summarized in Fig. 5 and in Table 4, showing that all experiments, with the exception of Ketac-Silver (87% cell survival), resulted in significantly lower survival rates, compared with the negative control groups ($p \leq 0.05$).

Influence of Different Perfusion Conditions on the Cytotoxicity of Test Materials

Statistical analysis of the influence of different perfusion conditions on the cytotoxicity of materials is summarized in Table 5. Comparison of cell viability rates of experiments performed at static conditions with those at 0.3 ml perfusion/h showed no statistically significant differences ($p \geq 0.05$). Comparison of cell viability rates at static conditions with those at 5 ml perfusion/h showed decreased cell survival rates at perfusion conditions for some materials (silicone impression material, zinc phosphate cement in both mixing ratios). Cell survival rates of experiments performed at different perfusion conditions indicated increased cell damage at 5 ml perfusion/h, compared with 0.3 ml perfusion/h.

DISCUSSION

A three-dimensional cell culture system, originally developed for cytotoxicity testing of cosmetic products, was introduced into a commercially available perfusion chamber modified for a dentin barrier test system. The sensitivity of the three-dimensional cell culture system was tested using different concentrations of phenol.

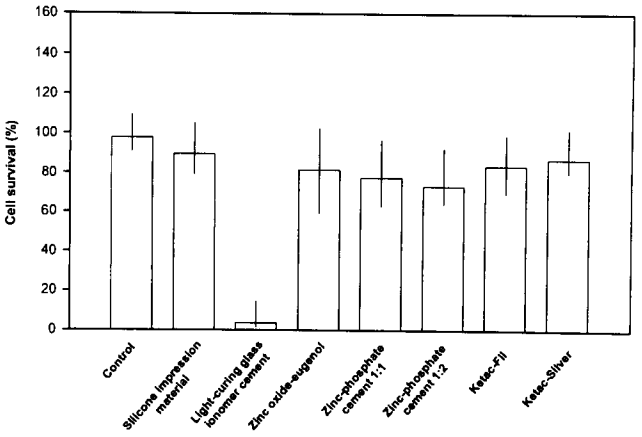

FIG 5. Summary of cell survival (MTT assay) with 5 ml perfusion/h. Skin^2 fibroblasts were exposed to dental materials for 24 h. Data are expressed as percentage of untreated control cultures; the indicated values are medians, 25% and 75% percentiles.

The dentin barrier test system was evaluated by testing a series of dental filling materials and by comparing the results with those obtained from previous studies with monolayer cultures of L929-mouse fibroblasts. Furthermore, the influence of different perfusion conditions (without perfusion, 0.3 ml/h, and 5 ml/h), simulating the pulpal blood flow, was tested.

Dose-response curves of the three-dimensional fibroblast cell cultures to phenol show a concentration dependent decrease of cell survival both in 48-well tissue culture plates and in perfusion chambers with a dentin barrier. TC_{50} of cultures grown in 48-well-plates is 0.53% phenol. Dose-response curves of L929-monolayer cells revealed a TC_{50} value of 0.09% phenol (unpublished observations). This difference might be due to a several-fold higher cell density in the three-dimensional tissue culture, compared with the monolayer. Studies of Wataha et al. (13), who investigated the cytotoxicity of metal ions on Balb/c 3T3 monolayer cells, showed higher TC_{50} values for copper ions with increasing cell density. Furthermore, in the three-dimensional cultures, primary cells are used. Studies investigating the cytotoxicity of metal cations on two cell lines (L929-mouse fibroblasts and HaK kidney cells) and on primary gingival fibroblast cells revealed that primary cells basically show the same rank order of toxicity as continuous cell lines, but at higher concentrations of test materials (14).

The TC_{50} value of cultures grown in perfusion chambers with a dentin barrier is 6.7% phenol, thus demonstrating the protective effect of dentin against phenol. These findings are in accordance with previous studies revealing that the cytotoxic effect of varying phenol concentrations to L929-fibroblast monolayers is a function of the thickness of the intermediate dentin slice (15). In these studies, the TC_{50} value was 0.6% phenol using a 500 μm dentin slice, again indicating the lower sensitivity of three-dimensional cell cultures.

Comparison of the results at different perfusion conditions shows similar cytotoxicity of test materials at static experiments and experiments with 0.3 ml perfusion/h. Only the toxicity of the light-curing glass ionomer cement moderately decreased at perfusion conditions of 0.3 ml/h. However, perfusion of the chamber with 5 ml medium/h did not further decrease its toxicity. Perfusion conditions of 5 ml/h may lead generally to an increased cell death or cell disruption. The reason for that may be a strong mechanical irritation of the cells. Perfusion conditions of both 0.3 and 5 ml/h were chosen according to the literature that describes a pulpal blood flow of 20 to 82.4 ml/min/100 g (16, 17). This corresponds to a perfusion of 0.6 to 5 ml/h/pulp, assuming a pulpal wet weight of 50 to 100 mg. Minuth et al. (18) recommend perfusion conditions of 1 ml/h for optimal differentiation of kidney cells.

The lack of toxicity of zinc phosphate cement and the silicone impression material in the present study is in accordance with our previous findings with L929-fibroblasts and correlates well with in vitro data from the literature (4, 10). Also, the application of zinc phosphate cement on monkey teeth confirmed that a possible pulp reaction caused by zinc phosphate cement is of a mild nature (5).

Cytotoxicity testing of the conventional glass ionomer cements using monolayer cultures of L929-fibroblasts revealed consider-

TABLE 4. Statistics of experiments with perfusion (5 ml/h)

	Control	Sil. Imp. Mat.	L.-C. GIC	ZnOE	ZPC 1:1	ZPC 1:2	Ketac-Fil
Sil. Imp. Mat.	−						
L.-C. GIC	+++	+++					
ZnOE	+	−	+++				
ZPC 1:1	++	−	+++	−			
ZPC 1:2	++	+	+++	−	−		
Ketac-Fil	+	−	+++	−	−	−	
Ketac-Silver	−	−	+++	−	−	−	−

−, $p \geq 0.05$; +, $p \leq 0.05$; ++, $p \leq 0.01$; +++, $p \leq 0.001$. Sil. Imp. Mat., silicone impression material; L.-C. GIC, light-curing glass ionomer cement; ZnOE, zinc oxide-eugenol cement; ZPC, zinc phosphate cement.

TABLE 5. Influence of perfusion conditions on the cytotoxicity of test materials

Perfusion	Control	Sil. Imp. Mat.	L.-C. GIC	ZnOE	ZPC 1:1	ZPC 1:2	Ketac-Fil	Ketac-Silver
0–0.3	−	−	−	−	−	−	−	−
0–5	−	+	−	−	+	+++	−	−
0.3–5	−	+++	++	++	++	+	+	−

−, $p \geq 0.05$; +, $p \leq 0.05$; ++, $p \leq 0.01$; +++, $p \leq 0.001$. Sil. Imp. Mat., silicone impression material; L.-C. GIC, light-curing glass ionomer cement; ZnOE, zinc oxide-eugenol cement; ZPC, zinc phosphate cement.

able cell damage after 24 h (10). In the present study, neither Ketac-Fil nor Ketac-Silver had any cytotoxic effect using three-dimensional cell cultures, thus correlating well with animal studies and clinical trials (5).

The high cytotoxicity of the light-curing glass ionomer cement shown herein is in agreement with results from agar diffusion tests and millipore filter tests (4), but contradictory to results from usage tests (19). It was postulated that the cytotoxic effects are due to 2-hydroxyethyl-methacrylate, one component of the light-curing glass ionomer cement tested (20). According to the manufacturer (3M Medica GmbH, Borken, Germany), Vitrebond is not indicated for direct pulp capping. If a pulp exposure occurs, covering of the exposure with a calcium hydroxide material is recommended. Whereas the light-curing glass ionomer cement caused complete cell death in our previous studies using L929-monolayer cells (0% cell survival) (10), 15% of the three-dimensional fibroblast cultures survived after a 24-h exposure to this material under static conditions. Cell viability increased to 23% in experiments with perfusion conditions of 0.3 ml/h, thus potentially better simulating the in vivo situation.

The high toxicity of zinc oxide-eugenol cement observed with L929-monolayer cultures in previous studies is in accordance with results from other cell culture experiments (4), but in disagreement with results from pulp studies on experimental animals (5). In the present study, application of zinc oxide-eugenol evoked no cytotoxic reaction on the three-dimensional tissue cultures. Therefore, we could prove the cytotoxicity reducing effect of dentin in combination with a three-dimensional cell culture for zinc oxide-eugenol in the commercially available in vitro pulp chamber. The finding suggests that the use of three-dimensional cell cultures in commercially available dentin barrier test systems is an essential improvement in the in vitro pulp chamber methodology.

Drs. Schmalz, Schuster, Nuetzel, and Schweikl are affiliated with the Department of Operative Dentistry and Periodontology, University of Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany. Address requests for reprints to Dr. Gottfried Schmalz, Department of Operative Dentistry and Periodontology, University of Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany.

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