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A Commercially Available Cell Culture Device Modified for Dentin Barrier Tests

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The suitability of a dentin barrier test based on a commercially available cell culture chamber was evaluated by testing the cytotoxicity of dental cements. The two chambers of the culture device as produced are separated by a membrane. This was replaced by a bovine dentin disk (500 μm thick). Mouse fibroblasts were grown on the "pulpal" side of the dentin for 24 h; test materials were then placed into the "cavity" side of the upper chamber. The number of viable cells was determined after 24 h. After exposure to zinc phosphate cement at a powder/liquid ratio of 2:1, ~100% of cells survived. A ratio of 1:1 yielded 81% survival. Only 24% and 28% of the cells survived after exposure to Ketac Fil and Ketac Silver, respectively. The light-curing glass ionomer cement (Vitrebond) and zinc oxide-eugenol killed all cells. These results agree with those obtained from a previous study, wherein the dentin barrier test device was constructed in our laboratory.

Artificial pulp chambers for cytotoxicity testing of dental materials differ in constructions (1–4). Although, the rationale is the same, the different set-ups have only a few aspects in common. The common factor is that they include dentin as a diffusion or adsorption barrier between the test materials, and the cells whose response to test materials is recorded. Meryon (1) showed that dentin powder reduced the cytotoxicity of zinc oxide-eugenol, depending on the thickness of the pressed dentin disks. Hume (2) used a different approach with tooth crowns from extracted human third molars as a "natural" barrier between test materials and cell culture medium. Aliquots of the culture medium were collected after a certain time of exposure and analyzed for cytotoxicity in a separate reaction. He found a good correlation between cytotoxic effects and clinical data. A sophisticated system was introduced by Hanks et al. (3). Dentin disks from crowns of third human molars separated the test materials from the test cell system. It was reported that, depending on the dentin thickness, dentin disks reduced the diffusion into the artificial pulp chamber to clinically relevant concentrations. Phenol was shown to be a suitable positive control substance and can be used to compare results from different laboratories (4). A similar dentin barrier test device was introduced by Schmalz and Schweikl (5) using bovine dentin disks. It was shown that the influence of different phenol concentrations depended on the concentration of the toxicant and on the thickness of the dentin disk between material and cells (5).

All designs developed so far differ both in size of the device and the materials used and are not commercially available. In most cases, handling of these pulp chambers is complicated. Consequently, the described devices hardly meet the criteria to recommend them as test chambers in standard protocols. In the present study, we used a commercially available cell culture chamber that was originally designed for different cell culture experiments (6). In this perfusion chamber, the original membrane, that served as a substrate for cell growth, was replaced by a dentin disk held in place by a steel holder, resulting in a dentin barrier test method. This test device is therefore based on a commercially available device that is considered to be an important prerequisite of a standard test (7). The objective of this study was to test the cytotoxicity of several dental materials with this device and to compare the results with those we found recently using an artificial pulp chamber constructed in our laboratory (8).

MATERIALS AND METHODS

Test Materials

The test materials are listed in Table 1. The zinc phosphate cement was mixed in two different powder:liquid ratios (1:1 and 2:1, w/w). Zinc oxide-eugenol was mixed in a powder:liquid ratio of 4.5:1 (w/w). A nontoxic vinyl-silicone (President) was used as a negative control material, and a 1.5% aqueous solution of phenol was used as the positive control substance (5).

In Vitro Dentin Barrier Test Device

The commercially available cell culture device (Minucells/Minutissue, Bad Abbach, Germany) made of polycarbonate with a base of 40 × 40 mm and a height of 36 mm was modified. A membrane in the original perfusion chamber was replaced by a dentin disk as a substrate for cell growth (Fig. 1). The dentin disk was cut from a bovine incisor (500 μm thick), etched on one side with 50% citric acid for 30 s, and autoclaved as described (5). The disk was placed into the cell culture chamber by means of a special biocompatible stainless steel holder. Thus, the cell culture chamber

TABLE 1. Test materials

Test Material	Brand Name	Manufacturer	Batch No.
Zinc phosphate cement	Harvard	Richter & Hoffmann Berlin, Germany	Powder 180 Liquid 559
Conventional glass ionomer cement	Ketac-Fil	Espe Seefeld, Germany	016C29W218D
Conventional glass ionomer cement	Ketac-Silver	Espe Seefeld, Germany	360E11W106D
Light-cured glass ionomer cement	Vitrebond	3M Medica GmbH Borken, Germany	91DO1
Zinc oxide-eugenol		Caelo Caesar & Lorentz Hilden, Germany	90265058
Negative control	President	Coltene AG Altstätten, Switzerland	9204 151
Positive control	Phenol	Merck Darmstadt, Germany	

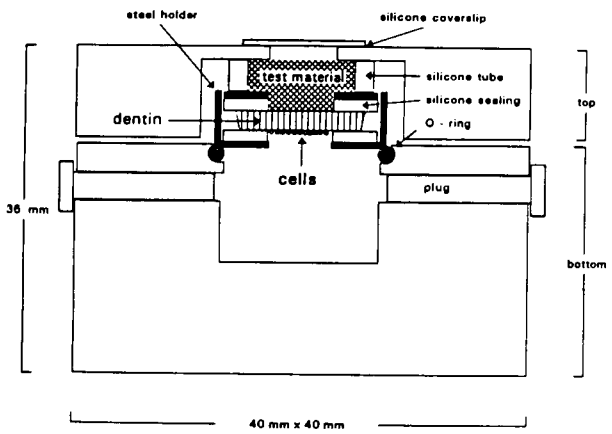

FIG 1. Diagram of the modified commercially available test device. A dentin disk is placed between two silicone reals in a stainless steel holder. Cells are grown on the "pulpal" side of the dentin disk, and test materials are applied on the opposite ("cavity") side of the disk.

was separated into two compartments by the dentin disk. Cells were grown on the etched side of the dentin disk facing the "pulpal" part of the device (lower chamber) as described herein. The test materials were introduced into a silicone tube in the upper chamber in direct contact with the "cavity" side of the dentin disk. The top and the bottom of the chamber were held together by metal clips.

Cytotoxicity Testing of Dental Cements

L-929 mouse fibroblasts (ATCC CCL1), cultivated in Basal Medium Eagle (supplemented with 5% newborn calf serum), were seeded in the lower chamber ("pulpal" side) of the test device (~130 cells/mm^2). A cotton pellet soaked with cell culture medium was placed in the upper chamber ("cavity" side). This set-up was incubated upside down at 37°C in an air atmosphere containing 5% CO_2. After 24 h, the cotton pellet was replaced by a test material (Table 1); the cells were exposed to the material in an incubator (37°C, 5% CO_2) for 24 h. Viable cells on the dentin disk were stained with fluorescein diacetate and counted (5).

Ten replicate cultures were tested for each of the test materials and for the positive (1.5% phenol, 20 μl) and negative controls (vinyl-silicone) in one experiment (5). Duplicate experiments were performed. The cell numbers of the experimental cultures with test materials were expressed relative to the cell numbers of the negative control and the positive control cultures (percentage of cell survival). These data were used for statistical analyses with the Mann-Whitney U test for comparing medians (9). The level of significance was set to $\alpha = 0.05$. Regression analysis was conducted to compare these data with those obtained from experiments using a pulp chamber constructed in our laboratory (8).

RESULTS

The results of the cytotoxicity experiments using a dentin barrier test device, based mainly on a commercially available perfusion chamber, are summarized in Fig. 2. The zinc phosphate cement at a mixing ratio of 2:1 powder:liquid had no cytotoxic effect on the cells grown on the "pulpal" side of the bovine dentin disk (Fig. 2). The same material with a mixing ratio of 1:1 was weakly cytotoxic, because only 81% of the originally seeded cells survived after 24 h of exposure to the material (Fig. 2); the difference between these two survival rates was not statistically significant (Table 2), and no significant difference from the negative control (vinyl-silicone) could be observed (Table 3).

The two conventional glass ionomer cements caused considerable cell damage, compared with the effect of the zinc phosphate cement. Only 28% of the cells survived after exposure to Ketac Silver, and Ketac Fil reduced cell numbers to 24% of the negative control values (Fig. 2). The differences of these survival rates from both the negative and the positive controls were highly significant (Table 3). With the light-curing glass ionomer cement and the zinc oxide-eugenol, no cells survived after 24-h exposure (Fig. 2); these

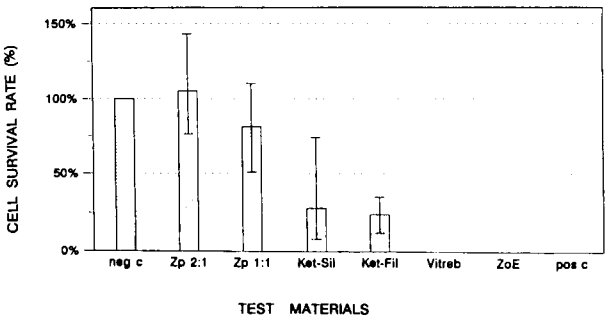

FIG 2. Cytotoxicity of dental cements. Cell numbers of the experimental cultures that were exposed to the dental cements were normalized to the cell numbers of negative (vinyl-silicone = 100%) and positive (1.5% phenol = 0%) control cell cultures. Results are shown as the relative medians with 25% and 75% quantiles of ten replicate cell cultures. *neg c*, negative control; *Zp*, zinc phosphate cement; *Ket-Sil*, Ketac-Silver; *Ket-Fil*, Ketac-Fil; *Vitreb*, Vitrebond; *ZoE*, zinc oxide-eugenol; *pos c*, positive control.

TABLE 2. Statistical analysis of the cytotoxicity values illustrated in Fig. 2

	Zp 2:1	Zp 1:1	Ket-Sil	Ket-Fil	Vitreb	ZOE
Zp 2:1		NS*	†	†	†	†
Zp 1:1			†	†	†	†
Ket-Sil				NS	†	†
Ket-Fil					†	†
Vitreb						NS
ZOE						

Zp, zinc phosphate cement; Ket-Sil, Ketac-Silver; Ket-Fil, Ketac-Fil; Vitreb, Vitrebond; ZOE, zinc oxide-eugenol.

* NS, notes the absence of significance ($p > 0.05$).

† $p \leq 0.001$.

TABLE 3. Statistical analysis of the cytotoxicity values of the test materials compared with control substances

	Negative Control	Positive Control
Zp 2:1	NS*	†
Zp 1:1	NS	†
Ket-Sil	†	†
Ket-Fil	†	†
Vitreb	†	NS
ZOE	†	NS

Zp, zinc phosphate cement; Ket-Sil, Ketac-Silver; Ket-Fil, Ketac-Fil; Vitreb, Vitrebond; ZOE, zinc oxide-eugenol; negative control, vinyl-silicone; positive control, 1.5% phenol solution.

* NS, notes the absence of significance ($p > 0.05$).

† $p \leq 0.001$.

values were not significantly different from those obtained with a phenol solution, which was used as the positive control (Table 3). The zinc phosphate cement at both mixing ratios was significantly less cytotoxic than both conventional glass ionomer cements; the light-curing glass ionomer cement and the zinc oxide-eugenol were the most toxic materials tested (Table 2).

The survival rates of the mouse fibroblasts in the present study were compared with the cytotoxicity data of a previous study (8), in which we tested the same materials in a dentin barrier test device constructed in our laboratory. The regression analysis showed an excellent correlation between the two sets of data ($r = 0.99$) interestingly, about two times more cells survived in the commercially available chamber (Fig. 3).

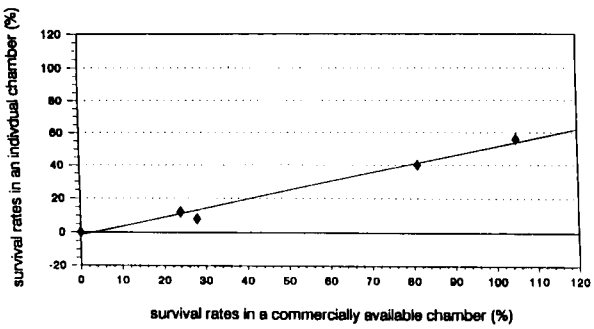

FIG 3. Regression analysis on the cytotoxicity data derived from the two different dentin barrier test methods. *Abscissa* represents survival rates of cells in the modified commercially available cell culture device. *Ordinate* represents the survival rates of cells in an individually constructed dentin barrier test device (8). *Line* is the calculated regression line ($y = -1.66 + 0.53\,x$; $r = 0.99$; $p \leq 0.001$).

DISCUSSION

The suitability of a commercially available cell culture perfusion chamber as a dentin barrier test device was characterized by a set of dental materials with known toxic potentials. The low toxicity of zinc phosphate cement in the present study is in accordance with reports from the literature (10). In addition, the influence of the mixing ratios on the cytotoxicity of the material correlates very well with our previous findings in a simple dentin barrier test (8). The cytotoxicity of the conventional glass ionomer cements is in accordance with other findings using cell culture tests for freshly mixed materials (12), but it is in contradiction to animal studies and clinical trials (11, 13). The different biological reactions may be explained by the high sensitivity of the glass ionomer cement toward fluids interfering with the setting process (14). In the present study, the fluid in the upper chamber of the test device was removed by means of a cotton pellet before insertion of the test material. However, air blasting was avoided because we observed that air blasting resulted in a decrease of living cells in the pulp chamber (unpublished observations).

The high cytotoxicity of the light-curing glass ionomer cement shown herein is in agreement with results from implantation tests (15), but contradictory to results from usage tests (16). However, few reports exist on the biological properties of these materials so far. The high toxicity observed with the zinc oxide-eugenol cement is in accordance with results from other cell culture experiments (17), but in disagreement with the results from pulp studies on experimental animals (11) and on humans (18). The reason for this disagreement may be the insufficient simulation of the in vivo situation in the dentin barrier test.

The good correlation of the cell survival rates in the dentin barrier tests published previously with those of the commercially available system in the present study suggests that the effects of the test materials are identical in both devices. The higher survival rates of cells in the modified commercially available chamber may be caused by the larger volume of the "pulp chamber" (450 μl), compared with the individually constructed device (150 μl) and the smaller surface area of test materials covering the dentin disk, or a combination of both. The covered surface area of the dentin disk in the commercially available chamber is 12.6 mm^2 (4 mm in diameter), whereas 19.6 mm^2 (5 mm in diameter) are covered in the homemade chamber. The influence of the surface area on the

cytotoxicity of various dental materials has been shown previously (19).

Considering the need for a standard protocol for a dentin barrier test, the method using a modified commercially available test device may be advantageous because it is independent of the variations of individual designs. It can therefore be recommended for further studies, with the potential of being included in standard testing programs. Most importantly, this test device is a modified perfusion chamber. Perfusion of the bottom part of the chamber with culture medium could mimic the natural blood flow in the pulp and overcome the static in vitro situation of the various dentin barrier test devices presently in use. This approach and other modifications of the test method are currently being investigated to simulate better in vivo conditions and to improve the correlation between cytotoxicity data obtained from in vitro dentin barrier tests and usage tests.

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