

Inhibition of cell survival, viability and proliferation by dentin adhesives after direct and indirect exposure in vitro

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Abstract

Objectives The influence of dentin adhesive systems (Scotchbond Multi-Purpose, XP Bond, Xeno V, Clearfil Protect Bond, AdheSE) on cell survival, viability and proliferation was characterized after direct and indirect exposure using different cell culture techniques.

Materials and methods The primers and cured bonding parts were directly exposed to cells using cell culture inserts, and complete materials were analyzed in a dentin barrier test. Cell responses were examined in 3T3 mouse fibroblasts after 24- and 72-h exposure periods by the estimation of

total cell numbers (survival), apoptosis (viability) and cell proliferation.

Results Cell numbers were effectively reduced by the primers of AdheSE, Protect Bond, and Scotchbond Multi-Purpose as well as XP bond after direct exposure in a cell culture insert test device. Likewise, Scotchbond Multi-Purpose primer induced a rate of apoptosis (93.9%) even higher than detected with Protect Bond primer (91.6%). Cell proliferation was entirely inhibited by primers and by Xp Bond as well. The Scotchbond Multi-Purpose was most cytotoxic in a dentin barrier test device after a 24-h indirect exposure. It also increased the percentage of cells in apoptosis to 15.4% compared to untreated controls.

Conclusion Unpolymerized primers of dentin adhesives were more cytotoxic than polymerized bonding counterparts. Moreover, total etch dentin adhesives were more cytotoxic than self-etch adhesives.

Clinical relevance When dentin adhesives are used in deep cavities without a protective dentin barrier the leachable hydrophobic and hydrophilic component of dentin adhesive systems can penetrate to the pulp and may induce cytotoxic responses in pulp tissues.

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Introduction

Most of the dentin adhesives are designed to form strong bonding with dentin. The major goals of using dentin adhesives are to enhance the bonding strength between resin and the tooth structure, to increase the retention of restoration, to reduce the microleakage across the dentin–resin interface, and to scatter the occlusal stress [1].

Current dentin adhesives can be classified based on their underlying adhesion strategy as “etch and rinse” or “self-etching” adhesives. The former remove the smear layer completely via acid etching and rinsing, whilst the later incorporate the smear layer into the bonding substrate, as infiltration of resin occurs simultaneously with the self-etching process. Dental adhesives consist of methacrylates, dimethacrylates, phosphorized pentacrylates, aldehydes and organic acids. Water, acetone or alcohol is added as a solvent, and some adhesive systems also contain fillers [2]. Methacrylate-based dentin adhesives are a source of unpolymerized residual monomers which may be released into the oral cavity and may be active in adjacent oral tissues to cause adverse effects. Cytotoxic effects of current dentin adhesive systems have been reported from *in vivo* and *in vitro* studies [3, 4]. The cytotoxic effects of adhesive systems depend on the amount of unreacted resin monomers which in combination with the bonding system influences toxicity [5]. Both exposure periods and the interactions (synergism or antagonism) between the bonding components may be important parameters in determining the cytotoxicity of dentin bonding agents [6].

The common approach and principle when testing the biological behavior of materials is to start with simple *in vitro* tests mostly based on cell cultures. If these experiments and investigations of a material's efficiency deliver promising findings, then more comprehensive studies on experimental animals and usage tests (*in vivo* evaluation) will be performed. Clinical studies are the final step of this evaluation process [7]. In early tests, materials were placed in direct contact with cells in monolayer culture and cell number was used to monitor cytotoxic effects [8]. However, direct contact tests may be disadvantageous for the interpretation of cell reactions to dental materials that are directly placed on dentin. As a barrier dentin may markedly influence the response of the adjacent target dental pulp tissue [9]. If barriers between the material and cells are incorporated into an *in vitro* model, these models appear to be more appropriate for estimating the *in vivo* response. The agar diffusion test, a filter diffusion test, cell culture inserts, and dentin barrier tests are currently employed in screening assays using a physical permeable barrier between materials and target cells [10]. The main parameters for the estimation of the cytotoxicity of dental materials are to determine cell numbers, membrane permeability, intracellular metabolism or cell morphology [11]. However, when cytotoxic stimuli are severe, cells may escape from the cell cycle and undergo a programmed process of cell death called apoptosis. Apoptosis is considered an active process mediated by regulatory and effector caspases (e.g., caspase-3) and the final activation of downstream DNases [12]. Apoptotic cells can be among other means identified by flow cytometry as a sub-G1 population after being stained with propidium

iodide [13]. Viable cells are able to proliferate, synthesize new DNA and thus incorporate Bromodeoxyuridine (BrdU) in the “S” phase of the cell cycle in an experimental situation. The measurement of the amounts of the fluorometric dye in the BrdU assay is, therefore, directly correlated to the proliferation of cells [14].

Since dentin adhesive systems remain in close contact with living dental tissues over a long period of time the biocompatibility of these materials is of particular importance. Here, we used various parameters to characterize physiological cell responses [15]. The wide variety of cell types used for cytotoxicity studies of dentin bonding agents indicated the diversity of opinions on an acceptable cell culture model. Permanent mouse 3T3 fibroblasts were found sensitive and useful to test and classify the toxic effect of different dental materials. Furthermore, it was suggested that the cytotoxic effect on the mouse 3T3 cell line was somewhere between that of human pulp-derived fibroblasts. 3T3 cells were used here because cytotoxicity testing of dental adhesives does not essentially require the presence of cellular functions specific for target cells of the oral cavity. Furthermore, 3T3 cells can be easily amplified and are available in large numbers for testing, their behavior is well known, relatively consistent, and constant [4]. Moreover, this cell line is recommended by international standards (ISO 7405) [16]. The aim of the present study was to evaluate the *in vitro* effect of three self-etch and two etch and rinse bonding agents on total cell numbers as a measure of cell survival, the induction of apoptosis and the analysis of cell proliferation in exposed cell cultures. Cell morphology was assessed by scanning electron microscopy (SEM) as well. Direct exposure of cells and the use of a dentin barrier between the test materials and cell cultures for indirect exposure to mimic a relevant experimental parameter of a clinical situation were chosen for the testing in an established cell line *in vitro*.

Materials and methods

Cell culture

The 3T3 mouse fibroblast cell line obtained from the American Type Culture Collection (ATCC CRL-1658, Rockville, MD, USA) was grown in monolayers in Dulbecco's modified Eagle's medium-F12 (DMEM F12; Biological Industries, Haemek, Israel) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma, St. Louis, MO, USA), 50 units/ml penicillin and 50 µg/ml streptomycin (Sigma), 1.0 mM sodium pyruvate, and 1.5 g/l sodium bicarbonate. Cells in semi-confluent cultures were harvested using trypsin (Sigma), collected by centrifugation, and resuspended in culture medium. Viability of the cells was

tested by trypan blue exclusion, and the 3T3 fibroblast cells were then plated onto 24-well culture plates in complete medium as described below.

Test materials and cell cultures

Test materials

Three self-etch dentin adhesive systems including Xeno V, Clearfil Protect Bond, and AdheSE as well as two etch and rinse adhesive systems (Scotchbond Multi-Purpose and XP Bond), were tested in the present study. Manufacturers and individual components of the materials are presented in Table 1.

Sample preparation and cell exposure

Samples to be tested directly using cell culture inserts Adhesives (40 μ l) of various dentin adhesive systems (Scotchbond Multi-Purpose adhesive, Clearfil PB bond, AdheSE bond), XP bond and Xeno V were loaded into Teflon moulds (5×2 mm) with a micropipette under sterile conditions. The specimens were light cured for 20 s with a halogen light unit (VIP; Bisco Inc., Schaumburg, IL, USA) calibrated at 600 mW/cm². Then, the polymerized specimens were put into cell culture inserts (24-well Millicell hanging cell culture insert, 0.4 μ m PET (polyethylene terephthalate; Millipore Corporation, Billerica, MA, USA). The matching primers (40 μ l) were also loaded into inserts with a micropipette. HEMA (2-hydroxy ethyl methacrylate) (40 μ l; 8.23 mol/l) was used like the primers as a positive control material, and cell culture inserts without test materials were used as a negative control.

3T3 fibroblasts were seeded onto 24-well plates at an initial density of 5×10^5 cells/well in complete cell culture medium. The cells were incubated in a humidified air atmosphere containing 5% CO₂ at 37°C for 24 h to allow for cell adhesion. Then, culture plate inserts with test materials were placed into the wells in contact to cell culture medium.

Samples to be tested indirectly in a dentin barrier test device Human third molars with almost complete root formation were obtained following an informed consent protocol reviewed and approved by an appropriate institutional review board at the Istanbul University, The Institute of Medical Sciences. The teeth were stored in physiological saline at 4°C prior to the experiments. The teeth were embedded into a methyl methacrylate resin, and dentin slices (500±50 μ m thick) were cut from the coronal part of the molars under copious water cooling using a low speed saw (Isomet Saw; Buechler, Lake Bluff, IL, USA). The smear layer on the pulpal side was removed by etching with 50% citric acid for 30 s. Then, the dentin slices were sterilized by autoclaving. The test

was carried out using a stainless steel test apparatus as shown in Fig. 1. A dentin disc with dentinal tubules open to both sides of the apparatus was held in place between two stainless steel holders using a low-viscosity (light body) polyvinyl siloxane impression material (Zhermack Oranwash L; Zhermack Indurent Gel Badia Polesine, Rovigo, Italy). Central holes (diameter, 5 mm) in both stainless-steel holders made both sides of the dentin disc available for test materials to be applied on one side (cavity side) and cell culture medium on the opposite (Fig. 1).

Dentin adhesives were applied according to manufacturers' instructions and light cured for 20 s. Vitrebond was used as a positive control [17, 18]. The test apparatus with a dentin disc alone was used as a negative control. The dentin barrier test was performed using 24 well plates. 3T3 fibroblast cells were seeded at an initial density of 5×10^5 cells/well in complete cell culture medium. The cells were incubated in an humidified air atmosphere containing 5% CO₂ at 37°C for 24 h to allow for cell adhesion. Then, the dentin barrier test apparatuses with test materials were placed into separate wells that was not surrounded by medium. Such a device was held in place in the well by two small metal rods which allowed the culture medium to slightly contact the etched side of the dentin disc (Fig. 1). In this way, a test material was indirectly exposed to the cell culture through culture medium.

Analysis of cell responses

Determination of cell survival

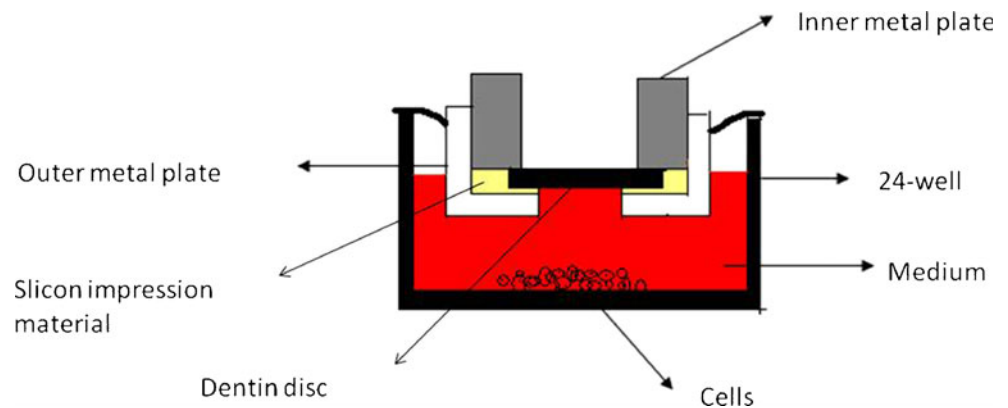
Cells were exposed to materials placed in cell culture inserts or a dentin barrier test device for 24 and 72 h. Then, cells from exposed cultures and untreated controls were collected by trypsinisation, and cell numbers indicating cell survival were counted with a hemocytometer [19]. These experiments were performed in triplicate with three replicates per material.

Analysis of apoptosis

Cells were exposed to materials placed in cell culture inserts or a dentin barrier test device for 24 and 72 h. Cells in apoptosis were identified using an Annexin V-FITC/PI staining assay (BD Pharmingen San Diego, CA, USA). One of the manifestations of apoptosis is the translocation of phosphatidylserine (PS) from the cytosolic surface to the extracellular surface of the plasma membrane. Phosphatidylserine was then detected by Annexin V staining. Briefly, cells were washed with phosphate-buffered saline (PBS) twice and resuspended in binding buffer containing 0.01 M HEPES, 0.14 mM NaCl and 2.5 mM CaCl₂. A cell suspension (1×10^5 cells in 100 μ l) in the binding buffer was

Table 1 Test materials, compounds and components

Dentin adhesive	Lot number	Manufacturer	Compound	Components
Scotchbond Multi-Purpose	6BF 2009–2006	3 M ESPE St Paul, MN, USA	Primer	Primer: water 2-Hydroxyethyl methacrylate (HEMA) copolymer of acrylic and itaconic acids
	6PN 2009–2006		Bond	Bond: bisphenol a diglycidyl ether dimethacrylate (Bis-GMA) 2-hydroxyethyl methacrylate (HEMA), Photoinitiators
Clearfil Protect Bond	00043A	Kuraray Medical Inc., Tokyo, Japan	Primer	Primer: 10-methacryloyloxydecyl hydrogen phosphate (MDP), 12-methacryloyloxydecyl pyridinium bromide (MDPB), 2-hydroxyethyl methacrylate (HEMA), hydrophilic dimethacrylate, water
	00066A		Bond	Bond: 10-methacryloyloxydecyl hydrogen phosphate (MDP), bis-phenol A diglycidyl methacrylate (Bis GMA), 2-hydroxyethyl methacrylate (HEMA), hydrophobic dimethacrylate, di-camphorquinone, <i>N,N</i> -diethanol- <i>p</i> -toluidine, silanated colloidal silica, surface treated sodium fluoride
AdheSE	K41839	Ivoclar Vivadent, Schaan, Liechtenstein	Primer	Primer: dimethacrylate, phosphonic acid acrylate, initiators and stabilizers in an aqueous solution
	K40597		Bond	Bond: HEMA, dimethacrylate, silicon dioxide, initiators and stabilizers
Xp Bond	0703002776	DENTSPLY DeTrey GmbH Konstanz, Germany		Carboxylic acid modified dimethacrylate (TCB resin); phosphoric acid modified acrylate resin (PENTA); urethane dimethacrylate (UDMA); triethylene glycol dimethacrylate (TEGDMA); 2-hydroxyethyl methacrylate (HEMA); butylated benzenediol (stabilizer); ethyl-4-dimethyl-aminobenzoate; camphorquinone; functionalized amorphous silica; <i>t</i> -butanol
				Bifunctional acrylate; acidic acrylate; functionalized phosphoric acid ester; acrylic acid; water; tertiary butanol; initiator; stabilizer
Xeno V	0801002439	DENTSPLY DeTrey GmbH Konstanz, Germany		Copolymer of acrylic and itaconic acids; water; 2-hydroxyethyl methacrylate
Vitrebond	7JB 2010–2012	3 M ESPE, St Paul, MN, USA	Liquid	
	8MF 2010–2012		Powder	Glass powder; diphenyliodonium chloride

Fig. 1 Dentin barrier test device

incubated with 5 μ l FITC-labeled Annexin V (BD Pharmingen) and PI (propidium iodide) for 15 min in the dark at room temperature. Then, the fluorescence of the cells for PI (FL-3) and Annexin V-FITC (FL-1) was measured simultaneously in a BD FACSCalibur flow cytometer and analyzed with the CellQuest software (BD Pharmingen). Data acquisition and analysis were done with CellQuest and WinMDI programs [20, 21]. The percentages of viable cells (annexin V⁻; PI⁻; lower left quadrant), cells in apoptosis (annexin V⁺; PI⁻; lower right quadrant) and necrosis (annexin V⁻; PI⁺; annexin V⁺; PI⁺; upper left and upper right quadrants) were determined. Only the percentage of cells in apoptosis (annexin V⁺; PI⁻) is presented in Figs. 4 and 5. These experiments were performed in triplicates in three independent experiments.

Analysis of cell proliferation

Cell proliferation was analyzed using a BrdU incorporation assay. After sterilization with ethanol, round cover slides were placed in 24-well plates and left under UV light overnight. Then, 7×10^4 cells/well were seeded into each well and allowed to adhere for 24 h. After that, cells were exposed to materials placed in cell culture inserts or a dentin barrier test device for 24 and 72 h. Next, cultured cells were labeled with 20 μ M 5-bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich Chemie, Steinheim, Germany) for 1 h. The slides were then fixed with ethanol for 30 min at -20°C , and subsequently rehydrated with PBS. Endogenous peroxidase activity was quenched with 0.5% H_2O_2 in methanol, and double-stranded DNA was denatured with 4 N HCl. The cells were washed with PBS, and a non-specific blocking reagent (Ultra-V-Block; Lab Vision Co., Westinghouse, CA, USA) was used to prevent non-specific binding of antibodies. The cells were then stained with a monoclonal mouse anti-BrdU antibody (1:50; Lab Vision Co., Fremont, CA, USA), and the primary antibody was detected by a biotinylated goat anti-mouse secondary antibody (Lab Vision Co., Fremont, CA, USA). After washing, peroxidase-conjugated streptavidin (Lab Vision) was applied and aminoethyl carbazole was used as a chromogen. The cells

were counterstained with Mayer's hematoxylin to augment the nuclear staining. Cells were counterstained Mayer's hematoxylin in order to discriminate 3,3-diaminobenzidine tetrahydrochloride (DAB) staining in nucleus from background and DAB unstained cells. After this, DAB staining in nucleus become more brown, DAB unstained nucleus and background became blue. BrdU labeled cells in the S-phase had red-stained nuclei. For negative controls, the cover slips of the same groups were processed with exactly the same steps, excluding the primary antibody. The BrdU-LI (the number of positively stained cells divided by the counted total number of cells) was calculated by evaluating at least 3000 cells in multiple high power fields [22]. Data were summarized from triplicates in three independent experiments.

Scanning electron microscopy

Cells grown on round cover slides as described were exposed to materials placed in cell culture inserts or a dentin barrier test device for 72 h. Then, samples were fixed and treated for evaluations using SEM. The cells were washed with PBS and fixed with 2.5% glutaraldehyde (Polysciences, Inc., Warrington, PA) in 0.1 mol/l phosphate buffer at pH 7.3 for 30 min, and 1% osmium tetroxide in 0.1 mol/l cacodylate buffer, pH 7.4, for 1 h. Next, the cells were washed with PBS, dehydrated in a graded series of ethanol and placed in 100% ethanol. Then, cells were critical-point dried, sputter coated with gold-palladium and analyzed by SEM (JSM 5200; Jeol, Tokyo, Japan). Photographs were taken at 10 kV using various magnifications and angles [23].

Statistical analysis

NCSS (Number Cruncher Statistical System) 2007&PASS 2008 Statistical Software (NCSS, Kaysville, UT, USA) was used for statistical analyses of the number of cells (cell survival), the percentage of cells in apoptosis, and cell proliferation (BrdU assay). Individual measurements were combined to mean values which were compared using the

one-way analysis of variance (ANOVA), follow-up comparisons between the groups were then carried out using Tukey's multiple comparison test. Differences between mean values were considered to be significant at $p < 0.05$. The differences between mean values calculated after the two time intervals (exposure periods) were analyzed by using Student's *t*-tests.

Results

Cell survival after exposure of cell cultures towards dentin adhesives

The total number of cells exposed the various dentin adhesive systems for 24 and 72 h was determined as a measure of cell survival. In the cell culture insert test device the primers of AdheSE, Protect Bond, and Scotchbond Multi-Purpose as well as XP bond were the most effective compounds after a 24-h exposure period (Fig. 2). These compounds were even more active than HEMA. AdheSE primer, for instance, reduced cell numbers about 9-fold compared to untreated controls. Similar results were observed after a 72-h exposure. Then, the lowest cell numbers were caused by Protect Bond primer. Noteworthy, for both time periods, the primer parts of the dentin adhesive systems caused significantly ($p < 0.05$) lower cell numbers compared with the bonding counterparts except for Protect Bond after 72 h. Xeno V was as effective as HEMA after both exposure periods (Fig. 2).

Different to the effects observed in the cell culture insert test device, the Scotchbond Multi-Purpose dentin adhesive system was the most cytotoxic material analyzed in a dentin

barrier test device after a 24-h exposure (Fig. 3). The material reduced cell numbers to about 40% compared to untreated control cultures, and was significantly more active than the positive control material (Vitrebond) included in this test system. Protect Bond, Xp Bond, Xeno V and AdheSE were in this order less cytotoxic than Scotchbond Multi-Purpose (Fig. 3). The same rank order of cytotoxic effects was detected when cell cultures were exposed for 72 h, except that Xp Bond was the most effective material (Fig. 3).

Induction of apoptosis by dentin adhesive systems

The percentage of apoptotic cells was then determined in cell cultures which survived after exposure to the various dentin adhesive systems. Scotchbond Multi-Purpose primer (93.9%) and Protect Bond Primer (91.6%) induced apoptotic cell death even higher than detected with HEMA (66.1%) in a cell culture insert test device after a 24-h exposure (Fig. 4). XP bond (75.1%) was significantly ($p < 0.05$) less active than Scotchbond Multi-Purpose primer and Protect Bond Primer. After a 72-h exposure period Xeno V (92.1%) and Scotchbond Multi-Purpose primer (91.9%) induced significantly higher apoptotic cell death rate. Here, the primer parts of the dentin adhesive systems caused a significantly ($p < 0.05$) higher rate of apoptosis than the bonding compounds only after a 24-h exposure. Scotchbond Multi-Purpose bond was the material least effective in the induction of apoptosis (3.8%), followed by AdheSE bond (4%) and Protect Bond bond (11.1%) after a 24-h exposure (Fig. 4). The activity of bonding counterparts of dentin adhesives, AdheSE primer and Xeno V in causing apoptosis was significantly ($p < 0.05$) increased after a 72-h exposure. Yet, Scotchbond Multi-Purpose

Fig. 2 Cytotoxicity of dental adhesive systems in a cell culture insert test device. Cytotoxicity was determined as the total number of cells in cultures exposed to the various dentin adhesive systems for 24 and 72 h. Bars represent mean values ($\pm$ SD) calculated from triplicates in three independent experiments ($n=9$). *Significant differences from 24-h control values, +significant differences from 72-h control values

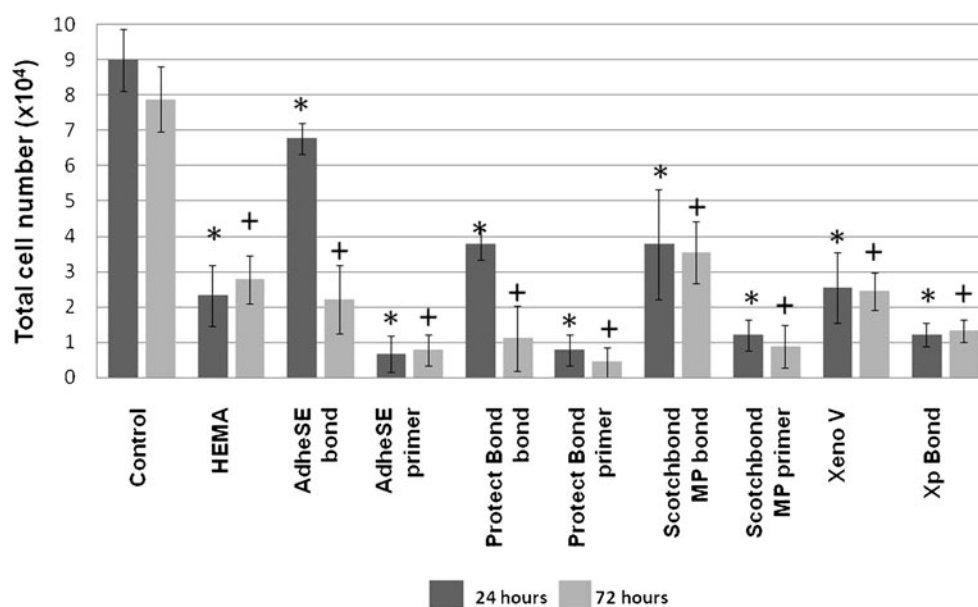
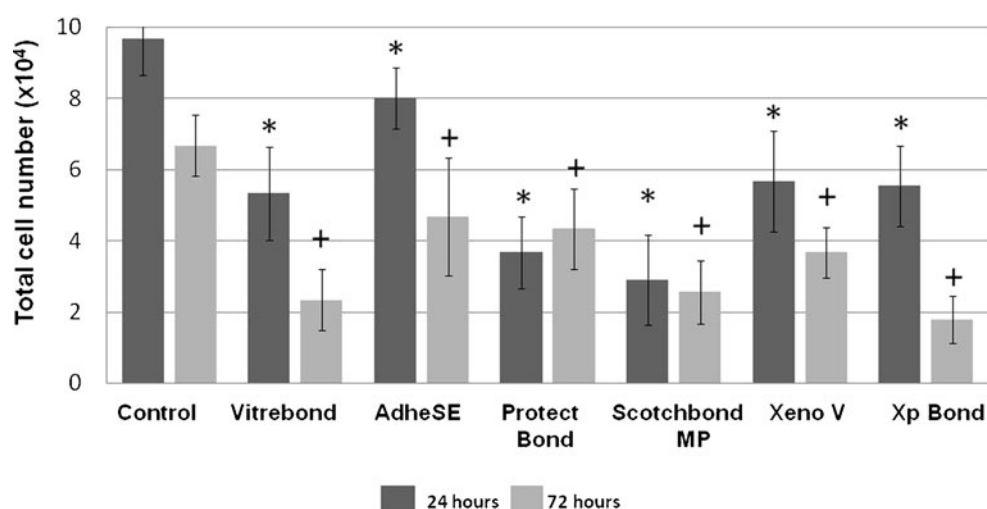


Fig. 3 Cytotoxicity of dental adhesive systems in a dentin barrier test device. Cytotoxicity was determined as the total number of cells in cultures exposed to the various dentin adhesive systems for 24 and 72 h. Bars represent mean values (+SD) calculated from triplicates in three independent experiments ($n=9$). *Significant differences from 24-h control values, + significant differences from 72-h control values



bond (17.2%) was significantly less active than the other test materials. However, Protect Bond bond (80.3%) and AdheSE primer (81%), as well as AdheSE Bond (80.9%) induced apoptosis in the same range than HEMA (58.2%) (Fig. 4).

The Protect Bond system significantly ($p<0.05$) increased the percentage of cells in apoptosis to 54.1% compared to 5.3% in untreated cell cultures in a dentin barrier test device after a 24-h exposure period (Fig. 5). Scotchbond Multi-Purpose (15.4%), XP bond (15.1%) and Xeno V (12.1%) were similarly effective, but AdheSE (8.3%) was significantly ($p<0.05$) less effective than Scotchbond Multi-Purpose and XP Bond. Vitrebond (4.9%) caused the significantly lowest rate of cells in apoptosis except for AdheSE (Fig. 5). The efficiency of the adhesive systems to cause apoptosis significantly increased after a 72-h exposure except for Protect Bond ($p<0.05$) (Fig. 5). After a 72-h exposure, Scotchbond Multi-Purpose induced a rate

of apoptotic cell death (70.7%) even higher than detected with XP Bond (60.1%) (Fig. 5)

Influence of dentin adhesives systems on cell proliferation

Cell proliferation in a cell culture insert test device was analyzed using a BrdU incorporation assay. About 19% of the cells in untreated cultures were found to be proliferating after a 24-h incubation period (Fig. 6). However, cell proliferation was completely inhibited by AdheSE primer (1.4%), Protect Bond primer (2.4%), Scotchbond Multi-Purpose primer (1.6%), and Xp Bond (2.2%). Xeno V (6.4%) was as effective as HEMA (5%), which was used as a control (Fig. 6). AdheSE bond (15.3%), Protect Bond bond (7.9%), and Scotchbond Multi-Purpose bond (8%) were significantly less effective than the corresponding primers ($p<0.05$).

Fig. 4 Apoptosis caused by dental adhesive systems in a cell culture insert test device. Apoptosis was determined as the percentage of dead cells in cultures exposed to the various dentin adhesive systems for 24 and 72 h as described in Materials and methods. Bars represent mean values (+SD) calculated from triplicates in three independent experiments ($n=9$). *Significant differences from 24-h control values, + significant differences from 72-h control values

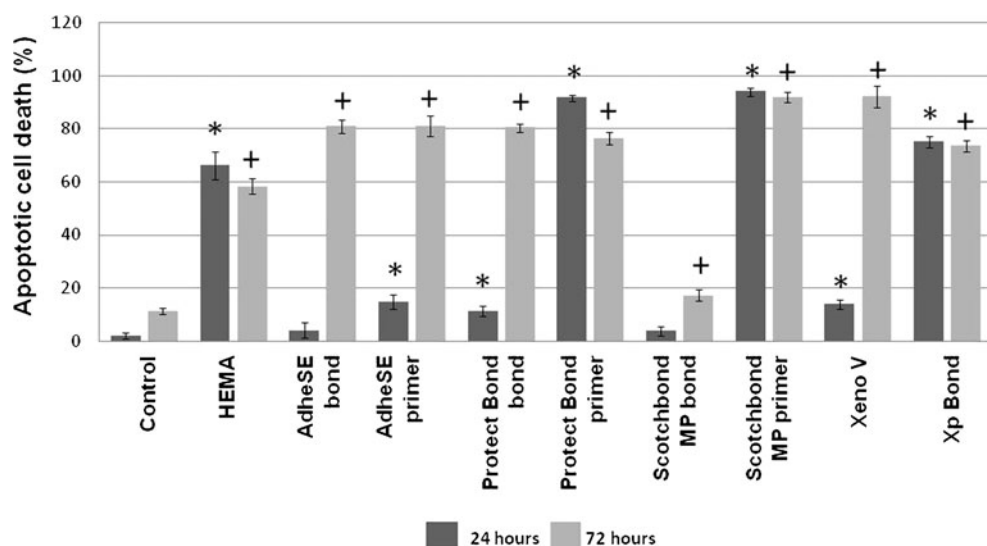
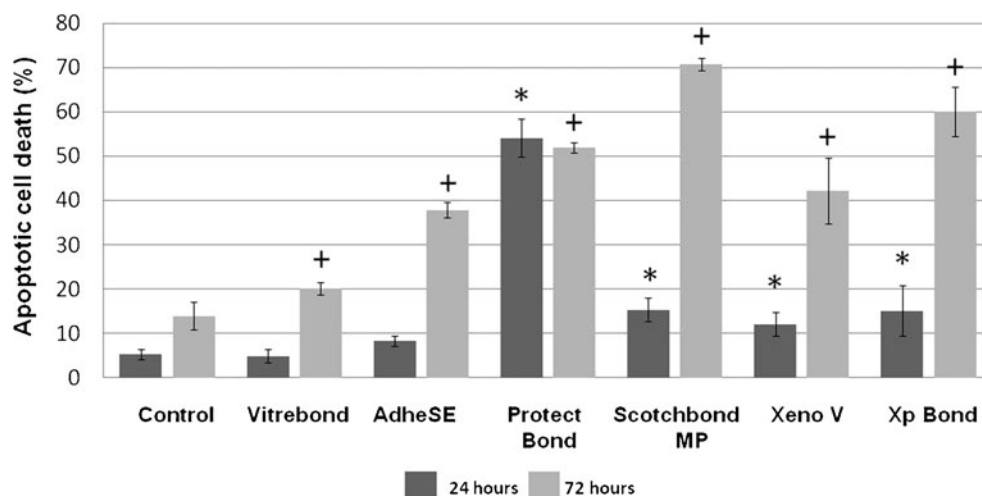


Fig. 5 Apoptosis caused by dental adhesive systems in a dentin barrier test device. Apoptosis was determined as the percentage of dead cells in cultures exposed to the various dentin adhesive systems for 24 and 72 h as described in [Materials and methods](#). Bars represent mean values (+SD) calculated from triplicates in three independent experiments ($n=9$). *Significant differences from 24-h control values, + significant differences from 72-h control values



Cell proliferation in untreated cultures significantly increased to 50% after a 72-h incubation period (Fig. 6). Yet, AdheSE primer (6.2%), Scotchbond Multi-Purpose primer (4.6%), and Xp Bond (6%) still significantly inhibited cell proliferation ($p<0.05$). Protect Bond primer (0.4%) was most effective at that time (Fig. 6). In contrast, AdheSE bond (14.4%) and Protect Bond bond (6.2%) even increased their inhibitory effect on cell proliferation after a 72-h exposure compared to untreated controls, and Scotchbond Multi-Purpose bond (23.4%) was as effective as after a 24-h exposure (Fig. 6).

Increased cell proliferation was also detected in untreated cultures in a dentin barrier test device by 23% after a 24-h exposure period (Fig. 7). Cell proliferation was again significantly inhibited by all dentin adhesive systems. Protect Bond (8.7%) was significantly more effective than AdheSE (18%), Xeno V (14.4%), Xp Bond (11.2%) and Vitrebond (11.3%), which was used as a positive control material. Since cell proliferation in untreated cultures increased by 46% after a

72-h exposure period, the inhibitory effects of all materials tested here were very similar to those observed after 24 h except for Xp Bond (Fig. 7).

Analyses of cell cultures using scanning electron microscopy

Cells grown on cover slides and exposed to the various adhesive materials were also analyzed for morphological changes by SEM. Examples of cell cultures demonstrating cytotoxicity of selected materials are presented in Fig. 8. The fibroblasts in untreated control cultures were well spread, and the flat cells appeared in close contact to the cover slide surface (Fig. 8a). However, the morphology of the cells dramatically changed after exposure to HEMA. The number of cells was considerably reduced and the few rounded cells indicated severe cytotoxic effects of the monomer (Fig. 8b). Likewise, a similar cell response was observed with Scotchbond Multi-Purpose bond, while the few cells surviving

Fig. 6 Cell proliferation in cultures exposed to dental adhesive systems in a cell culture insert test device. Cell proliferation was determined by BrdU incorporation after 24 or 72 h as described in [Materials and methods](#). Bars represent mean values (+SD) calculated from triplicates in three independent experiments ($n=9$). *Significant differences from 24-h control values, + significant differences from 72-h control values

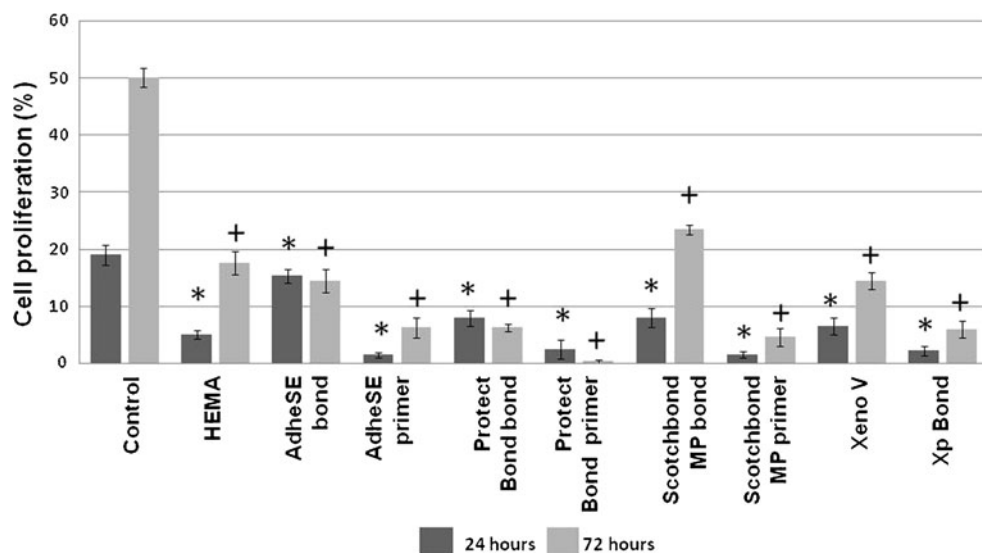
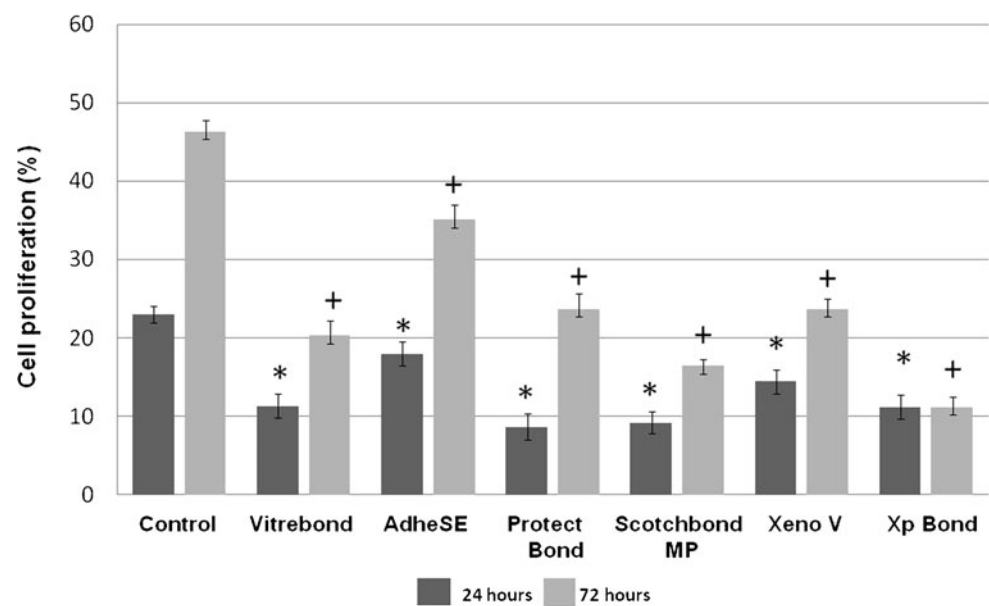


Fig. 7 Cell proliferation in cultures exposed to dental adhesive systems in a dentin barrier test device. Cell proliferation was determined by BrdU incorporation after 24 or 72 h as described in [Materials and methods](#). Bars represent mean values ($\pm$ SD) calculated from triplicates in three independent experiments ($n=9$). *Significant differences from 24-h control values, +significant differences from 72-h control values



exposure to Scotchbond Multi-Purpose primer appeared to increase in size (Fig. 8c, d). Only very few cells were left in cultures exposed to Xeno V, and structures indicating normal physiological cell functions were not detected in cultures exposed to XP Bond as well (Fig. 8e, f).

Discussion

Considering the clinical situation, the bioactivity of dentin adhesives in pulp tissues is of particular relevance. It has been discussed that the composition of dentin adhesive systems, clinical operative procedures, remaining dentin thickness, adequate lining materials and dentin permeability are factors that may influence the pulpal response to dentin adhesives [24]. The present study investigated various aspects of the cytotoxicity of currently used dental adhesive systems with different test methods.

First, this study made use of cell culture inserts which separate cells and materials. This experimental set up is suitable for the direct testing of cell responses without the prior preparation of materials extracts. The porous membrane at the bottom of the inserts allows for the passage of leaching chemicals but not for resin particles to reach the monolayer of cells beneath it. It was reported that the use of commercially available sterile cell culture inserts was convenient for extensive cytotoxic screening when compared to agar overlay and Millipore filter tests [25]. Second, the dentin barrier test may help to identify compounds that repress or amplify adverse effects of substances by reducing or increasing dentin permeability [26]. With the importance of the principle of generalizing in vitro cytotoxicity findings to the human in vivo clinical situation, this technique is recommended for use in preference to the others (ISO 7405) [16]. Adopting the use of a

dentin barrier for indirect testing of dental materials simulates the in vivo oral environment more closely, thereby helping to identify specific components of dental filling materials which may be responsible for pulpal effects through dentin, an option not available with other barrier testing methods.

Cell responses caused by dentin adhesive systems were analyzed after two exposure periods (24 and 72 h). It was reported earlier that long periods of exposure resulted in a significant increase in the cytotoxicity of components of dentin adhesives and combinations. Likewise, longer exposure periods may result in a higher incidence of synergic interactions between the various adhesive components [6]. Here, the cytotoxic effects of primers were significantly higher than cell responses caused by the bonding counterparts and other materials tested in a cell culture insert test device after a 24-h exposure. Similar results were obtained after a long exposure of 72 h except for Protect Bond. It was reported earlier that the primer part of Clearfil Protect Bond was more cytotoxic than the bonding part in a different test system. The authors suggested that the MDPB monomer in the primer part of Clearfil Protect Bond was responsible for the cytotoxic effect [27]. On the other hand, it has been discussed that this monomer has no significant deleterious effect [28]. It was assumed that cytotoxic effects of resinous dental materials depended on the concentration of leachable monomers [29]. In the present study, the primer parts of the adhesives were applied unpolymerized according to clinical situations. There is experimental evidence that polymerized dentin adhesives exhibited cytotoxicity about 2–65% lower than their unpolymerized counterparts [11]. The variation of monomer ratios in the dentin adhesive systems is probably another factor related to the different cytotoxic effects of these materials [29]. Here, the primer parts of the dentin adhesives which contain a variety of monomers were more

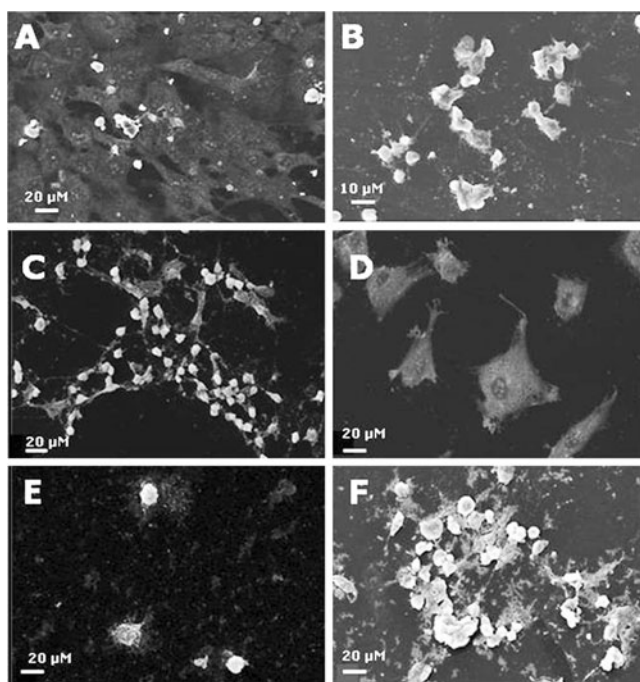


Fig. 8 Morphology of cells in cultures treated with dentin adhesives. Cultures treated with **a** negative control, **b** HEMA, **c** Scotchbond MP bond, **d** Scotchbond MP primer, **e** Xeno V, and **f** XP Bond. The cells were visualized by scanning electron microscopy (SEM)

cytotoxic than HEMA after a 72-h exposure period. It is possible that the proportions of primers or the interaction of monomers might be responsible for the relatively severe cell responses caused by the primers in the current study [6].

In the present study, the primer parts of dentin adhesives and XP Bond exhibited the highest cytotoxic effect compared to other materials. Similar to our results, XP Bond proved more cytotoxic than Clearfil Tri-S and AdheSE than previously reported [30]. This might be explained by the different ingredients of XP Bond (UDMA and TEGDMA) compared to other dentin adhesives in this study. It was reported earlier that aqueous elutes of the dentin adhesive Solobond Plus which tested severely cytotoxic contained high amounts of the monomer TEGDMA [31]. Moreover, in the present study AdheSE bond and Protect Bond bond exhibited a significant increase in cytotoxicity with increasing time periods. The differences between cytotoxic effects observed after increasing exposure periods were discussed controversy, and might be caused by the miscellaneous ingredients of different dentin adhesives. It is possible that cell cultures recover from damage caused by moderate cytotoxic agents more rapidly than from exposure to severely cytotoxic materials [4, 32, 33].

In the dentin barrier test system Scotchbond Multi-Purpose and XP Bond were observed to be most effective after 24- or 72-h exposure periods, respectively. After a 72-h exposure period, XP Bond caused significantly the lowest cell numbers except for Scotchbond Multi-Purpose and

Vitrebond. Noteworthy, Xp Bond and Scotchbond Multi-Purpose are etch-and-rinse adhesives. In agreement with the present study, it was found earlier that etch-and-rinse adhesives were more effective than self-etch adhesives. It was reported that etching removes the smear layer, enlarges the dentinal tubules at the surface and, thus, increases dentin permeability [34]. Bouillaguet et al. [35] found that the cytotoxicity of dentin adhesives decreased with increasing intervals, although there was still persistent suppression of cellular metabolism even at late intervals. Furthermore, the amounts of leachable components of dentin adhesives detected in cell culture media decreased at later intervals [35]. In the present study, it is obvious that toxic constituents of dentin adhesive systems accumulate at later time intervals, and more severe cell responses were induced after 72 h.

In this work, the primer parts of the adhesives were significantly more effective in the induction of apoptotic cell death than the bonding counterparts after direct exposure in the cell culture insert test after a 24-h exposure. A similar effect was observed only for Scotchbond Multi-Purpose primer at the end of 72 h. At this time point, apoptosis induced by Scotchbond Multi-Purpose bond was significantly lower compared to the effects caused by other test materials. The proportion of apoptotic cells for bonding counterparts, AdheSE primer and Xeno V significantly increased after a 72-h exposure. Moreover, in the dentin barrier test, a similar effect was observed except for Protect bond. Also, for the 72-h exposure period, apoptosis induced by Scotchbond Multi-Purpose and XP bond was higher compared to the effects caused by other test materials.

Similar to our findings, it was demonstrated that the unpolymerized or partially polymerized adhesive Single Bond induced apoptosis very rapidly in various cell types [36]. Furthermore, it was suggested that the apoptotic potential of Clearfil SE Bond and FL Bond was material dependent [37]. The production of reactive oxygen species (ROS) was increased in human pulp-derived cells by various dentin adhesives about 5-fold in a dose-related manner. Since the production of ROS was associated with apoptosis, it was suggested that the production of ROS in the presence of adhesives may trigger apoptotic cell death [18].

The primer parts of dentin adhesives and XP Bond were most effective in the inhibition of BrdU incorporation in the cell culture insert test indicating delayed cell proliferation after 24- and 72-h exposure periods. In the dentin barrier test, however, Protect Bond adhesive significantly inhibited cell proliferation after a 24-h exposure. Similar effects on cell proliferation were observed for etch-and-rinse adhesives after 72 h. Noteworthy, cell proliferation recovered in cell cultures exposed to dentin adhesives for 72 h except for XP Bond. Yet, a significant effect on the number of cells distributed among the various phases of the cell cycle was not observed with

AdheSE by others. Likewise, no major impact on the cell cycle was detected with self-etch adhesives, and a modest inhibition of cell proliferation might have been caused by the induction of cell death by high amounts of material. It was concluded that total etch bonding agents induced a cell cycle arrest, whereas the self-etch bonding agents did not affect the cell cycle patterns [30].

The various adverse effects of dentin adhesive systems as indicated by biochemical assays were associated with morphological alterations of exposed cells similar to results reported earlier [38]. These observations support our findings on the induction of apoptosis by dentin adhesives because pseudopod retraction, the rounding up of cells, and decreased cellular volume (pyknosis), or blebbing of the intact plasma membrane have long been known as morphological aspects considered typical for apoptosis [39].

In conclusion, various dentin adhesive systems were differentially active in the various test systems in the present study. Unpolymerized primer parts of dentin adhesives were more effective than their polymerized bonding counterparts after direct exposure to cells in the cell culture insert test, and similar effects were detected on apoptosis and cell proliferation. In the dentin barrier test device, total etch dentin adhesives were more cytotoxic than self-etch adhesives, and total etch dentin adhesives were more effective in causing cell apoptosis and inhibiting cell proliferation as well. Therefore, clinicians should consider the potential risk for pulp cells when these materials are used in deep cavities without a protective dentin barrier and when pulp cells are exposed to these materials.

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Conflict of interest The authors declare that they have no conflict of interest.

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