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The induction of oxidative stress, cytotoxicity, and genotoxicity by dental adhesives

M. Demirci^a, K.-A. Hiller^b, C. Bosl^b, K. Galler^b, G. Schmalz^b, H. Schweikl^{b,*}

^a Department of Conservative Dentistry, Faculty of Dentistry, Istanbul University, Çapa, Istanbul 34390, Turkey

^b Department of Operative Dentistry and Periodontology, University of Regensburg, 93042 Regensburg, Germany

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ABSTRACT

Objectives. Polymerized dental resin materials release residual monomers that may interact with pulp tissues. We hypothesized that dental adhesives might cause cytotoxicity in pulp cells via the generation of reactive oxygen species (ROS), which may also contribute to genotoxic effects in vitro.

Methods. For cytotoxicity testing, transformed human pulp-derived cells were exposed to extracts of primers and bonding agents of Clearfil SE bond, Clearfil Protect bond, AdheSE, Prompt L-Pop, and Excite for 24 h. The cytotoxicity of the same materials was also analyzed in a dentin barrier test device using three-dimensional pulp cell cultures. The generation of ROS in monolayer cultures was measured after a 1 h exposure period by flow cytometry (FACS), and genotoxicity as indicated by the formation of micronuclei was determined in V79 cells after a 24 h exposure period.

Results. The dentin primers and bonding agents decrease cell survival in a dose-related manner. Cytotoxicity of bonding agents based on concentrations which caused 50% cell death (EC50) were ranked as follows: Excite (0.16 mg/ml) > AdheSE bond (0.30 mg/ml) > Clearfil Protect bond (0.35 mg/ml) > Clearfil SE bond (0.37 mg/ml), and Prompt L-Pop bond (0.68 mg/ml). Dentin primers were about 10-fold less effective. In contrast, no cytotoxic effects of the dental adhesives were observed in a dentin barrier test device. Yet, all dental adhesives increased the amounts of ROS about fivefold in pulp cells in a dose-related manner, and, again, the bonding agents were more efficient than the dentin primers. Finally, the number of micronuclei was increased about sixfold by extracts of the AdheSE primer.

Significance. Our results suggest that the cytotoxic potencies demonstrated by these materials might be of clinical relevance, since all dental adhesives disturbed the cellular redox state of pulp cells in monolayer cultures. As a result, the concentrations of biologically active ingredients of some of the agents may be high enough to modify pulp cell metabolism when the materials are used in deep cavities or directly contact pulp tissue.

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1. Introduction

Modern dental adhesive systems are used to improve the contact between the restorative material and the walls of the prepared cavity of the tooth. As these materials come in close

and prolonged contact with vital dentin, their influence on pulp tissue is critical. Thus, the biocompatibility of dentin bonding agents is a relevant aspect of the clinical success of these materials [1–4]. Dentin bonding agents alone proved to be cytotoxic, and it has been found that the type and

* Corresponding author. Tel.: +49 941 944 6142; fax: +49 941 944 6025.

E-mail address: helmut.schweikl@klinik.uni-regensburg.de (H. Schweikl).

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quantity of leachable components significantly influence the biological behavior of resin restorations [5–10]. Cytotoxicity of dentin bonding agents has been examined using a variety of cell lines including primary human pulp and pulp-derived cells [5–8,11–14]. Yet, the isolation of primary cells from target tissues is labor intensive and time consuming, and the resulting cell numbers are often very low compared to the almost unlimited number of cells obtained from continuous cell lines. Most important, primary cells have a limited life span. These shortcomings might be overcome by the construction of transformed primary cells with an expanded life span retaining properties of the original cells. Recently, stable cell lines were generated after transfection of primary bovine and human pulp-derived cells with coding sequences of transforming oncogenes [15–17]. Moreover, *in vitro* pulp chambers have been designed, introducing dentin as a barrier between test material and target cells. Transformed pulp-derived cells were grown as three-dimensional cultures and included in a dentin barrier test device to mimic the interactions between a target tissue, matrix proteins and filling materials that occur *in vivo* [18,19].

As a consequence of aerobic metabolism, small amounts of reactive oxygen species (ROS), are constantly generated in cells and tissues. Cellular antioxidants like glutathione act in unison to detoxify these reactive molecules, but when the balance between oxidants and antioxidants is disrupted, a condition referred to as oxidative stress exists. If oxidative stress persists, oxidative damage to lipids, proteins and nucleic acids accumulates and eventually results in biological effects ranging from the alteration of signal transduction pathways and gene expression levels to cell transformation, mutagenesis and cell death [4]. Leachables from resin-based materials such as HEMA and TEGDMA are a likely cause of cellular stress via the formation of ROS. Recently, it was shown that there was a possible link between ROS production and cytotoxic activity [20–24]. Moreover, the induction of genotoxic effects of TEGDMA and HEMA has been demonstrated *in vitro* as well, indicating DNA reactivity of the compounds. In addition, cytotoxic resin materials were shown to cause cytotoxicity and elevated numbers of micronuclei [25,26]. In the present study, we combined and analyzed various aspects of cell responses towards dental adhesives. First, we hypothesized that biologically active monomers or additives may be released from dentin bonding agents to cause cytotoxicity in pulp cell monolayers. Furthermore, the production of ROS may indicate an early onset of processes leading to cell death via apoptosis, or the generation of DNA damage as indicated by elevated numbers of micronuclei. In addition, the cytotoxicity of dentin bonding agents was also evaluated in a dentin barrier test device using three-dimensional cultures of transformed pulp-derived cells to mimic an *in vivo* situation.

2. Materials and methods

2.1. Test materials, chemicals, and reagents

The dental adhesives tested in this study are listed in Table 1. Minimal essential medium alpha (MEM α), fetal bovine serum, penicillin/streptomycin, antibiotic/antimycotic, geneticin and

crystal violet were purchased from Gibco Invitrogen (Karlruhe, Germany). Accutase was obtained from PAA Laboratories GmbH (Cölbe, Germany). 2'7'-Dichlorodihydrofluorescein diacetate (H₂DCF-DA) came from Invitrogen Molecular Probes (Karlruhe, Germany). Triethylene glycol dimethacrylate (TEGDMA) was obtained from Sigma–Aldrich (Taufkirchen, Germany).

2.2. Cytotoxicity testing using human pulp-derived cells

Clonal SV 40 large T-antigen transfected human pulp-derived cells (tHPC) were cultivated as described [17]. The cells were seeded at a density of 7.5×10^3 into each well of a 96-well plate for 24 h at 37 °C. Uncured dental adhesives (primers and bonding agents) were dissolved in pure ethanol (500 mg/ml) at room temperature, and then stock solutions were prepared in culture medium at a concentration of 10 mg/ml. Next, the stock solutions were serially diluted in culture medium, and cell cultures were then subsequently exposed to 200 μ l of culture medium containing increasing concentrations of uncured dental adhesives for 1 and 24 h. Finally, cell survival was determined using a crystal violet assay [27]. Cell cultures were washed with PBS–EDTA, fixed with 1% glutaraldehyde, and stained with crystal violet (0.02% in water). The amount of crystal violet bound to the cells was dissolved with 70% ethanol, and optical densities were measured at 600 nm in a multi-well spectrophotometer (EL312; Biotek Instruments, Burlington, VT, USA). Four replicate cell cultures were exposed to each concentration of a single material in at least four independent experiments.

Dose–response curves of cell survival (expressed as optical density readings) after a 24 h exposure period and the corresponding concentrations of materials diluted in cell culture medium (mg/ml) were fitted to the data (Table Curve 2DTM, Version 5.01; Systat Software, San Jose, CA, USA). The concentrations of the materials (mg/ml) which caused 50% cell death (EC₅₀ values) compared to cell survival in untreated controls were calculated from dose–response curves. Differences between median EC₅₀ values were statistically analyzed using the Mann–Whitney *U*-test ($\alpha = 0.05$) (SPSS, Version 13.0; SPSS, Chicago, IL, USA).

2.3. Measurement of reactive oxygen species (ROS)

The generation of ROS was measured using an oxidation-sensitive fluorescent probe 2'7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA). Intracellular esterase activity results in the formation of DCFH, a non-fluorescent compound which emits fluorescence when it is oxidized to DCF-DA [28]. Human transformed pulp-derived cells (tHPC) (2×10^5) were seeded into each well of a six-well plate and incubated for 24 h at 37 °C. Uncured dental adhesives were first dissolved in pure ethanol (500 mg/ml) and then diluted to 10 mg/ml in culture medium. Next, the cell cultures were preincubated with DCFH-DA (10 mmol/l) for 30 min at 37 °C. The cell cultures were subsequently exposed to increasing concentrations of dental adhesives in cell culture medium, and 1.5 mmol/l TEGDMA was used as a control for ROS production. One well was used for each concentration and exposure was stopped after 1 h.

Table 1 – Test materials, compounds and components

Dentin adhesive	Lot number	Manufacturer	Compound	Components
Clearfil SE bond	751AA	Kuraray Medical, Okayama, Japan	Primer	Primer: 10-methacryloyloxydecyl hydrogen phosphate (MDP), 2-hydroxyethyl methacrylate (HEMA), hydrophilic dimethacrylate, di-camphorquinone, N,N-diethanol- <i>p</i> -toluidine, water
			Bond	Bond: 10-methacryloyloxydecyl hydrogen phosphate (MDP), bis-phenol A diglycidyl methacrylate (Bis GMA), 2-hydroxyethyl methacrylate (HEMA), hydrophobic dimethacrylate, di-camphorquinone, N,N-diethanol- <i>p</i> -toluidine, silanated colloidal silica
Clearfil Protect bond	020AA	Kuraray Medical, Okayama, Japan	Primer	Primer: 10-methacryloyloxydecyl hydrogen phosphate (MDP), 12-methacryloyloxydecyl pyridinium bromide (MDPB), 2-hydroxyethyl methacrylate (HEMA), hydrophilic dimethacrylate, water
			Bond	Bond: 10-methacryloyloxydecyl hydrogen phosphate (MDP), bis-phenol A diglycidyl methacrylate (Bis GMA), 2-hydroxyethyl methacrylate (HEMA), hydrophobic dimethacrylate, di-camphorquinone, N,N-diethanol- <i>p</i> -toluidine, silanated colloidal silica, surface treated sodium fluoride
AdheSE	Primer: H25099; bond: H24960	Ivoclar Vivadent, Schaan, Liechtenstein	Primer	Primer: dimethacrylate, phosphonic acid acrylate, initiators and stabilizers in an aqueous solution
			Bond	Bond: HEMA, dimethacrylate, silicon dioxide, initiators and stabilizers
Prompt L-Pop	222786	3M ESPE Seefeld, Germany	Primer	Primer: 2-hydroxyethyl methacrylate (HEMA), polyalkenoic acid, stabilizers, water
			Bond	Bond: methacrylated phosphoric esters, Bis GMA, initiators based on camphorquinone, stabilizers
Excite	G23783	Ivoclar Vivadent, Schaan, Liechtenstein		HEMA, dimethacrylates, phosphonic acid acrylate, highly dispersed silicon dioxide, initiators and stabilizers in an alcohol solution

Then, cell cultures were detached with accutase, resuspended in culture medium, and collected by centrifugation. The cell pellet was washed three times with phosphate-buffered saline free of calcium and magnesium (CMF-PBS) and resuspended in 200 μ l CMF-PBS. Immediately after resuspension, DCF fluorescence was determined by flow cytometry (BD FACSCalibur) at an excitation wavelength of 495 nm and an emission wavelength of 530 nm (Fl-1). Mean fluorescence intensities were obtained by histogram statistics using the WinMDI program (Version 2.8). At least four independent experiments were performed with each compound. Mean fluorescence intensities were normalized to untreated control cultures (=1.0), and differences between median values were statistically analyzed using the Mann-Whitney *U*-test ($\alpha=0.05$) (SPSS, Version 13.0 SPSS; Chicago, IL, USA).

2.4. Micronucleus test in vitro

V79 Chinese hamster fibroblasts (10^5 cells) were cultivated on microscopic glass slides in 4 ml cell culture medium for 24 h at 37 °C and 5% CO₂ [25]. Uncured dentin primers and bond-

ing agents were dissolved in ethanol as described above and diluted in cell culture medium to 10 mg/ml. Then, V79 cells were exposed to increasing concentrations of the various dental adhesive systems for 24 h. After the exposure period, the cells were fixed in 100% ethanol for 30 min, air-dried, and lysed in 5N HCl for 40 min. DNA-containing structures were stained with Accustain Schiff's reagent for 30 min at room temperature. After being washed in sulfite/water (6 min) and tap water, cells were dehydrated and mounted with Entellan. The number of nuclei were determined microscopically in 1000 cells/slide of two parallel cultures (slides) per concentration. Ethyl methanesulfonate (EMS) served as a positive control substance [25]. At least four slides derived from two independent experiments were analyzed, and differences between median values were statistically analyzed using the Mann-Whitney *U*-test ($\alpha=0.05$) (SPSS, Version 13.0; SPSS, Chicago, IL, USA).

2.5. Dentin barrier test

Clonal SV 40 large T-antigen transfected cells, derived from calf dental papilla, were maintained in growth medium

(MEM α , Gibco BRL; Karlsruhe, Germany) supplemented with 20% fetal bovine serum (FBS), and three-dimensional cultures of these cells were cultivated as previously described. After an incubation period of 14 ± 2 days, these three-dimensional cultures were introduced into a dentin barrier test system [15,18,19].

Dentin slices ($500 \pm 20 \mu\text{m}$ thick) were cut from extracted bovine incisors, 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. A commercially available cell culture perfusion chamber (Minucells and Minutissue GmbH, Bad Abbach, Germany) was 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. The chambers were perfused with 0.3 ml/h assay medium (growth medium with 5.96 g/l HEPES buffer) for 24 h. After that, perfusion was switched off and test materials were introduced into the upper compartment in direct contact with the cavity side of the dentin disks. Then, the materials were cured according to the manufacturer's instructions. Subsequently, the pulpal part of the perfusion chamber containing the cell cultures was perfused with medium (2 ml/h) during an incubation period of 24 h. After an exposure period of 24 h, the cell survival in exposed three-dimensional cultures was determined by the MTT assay as described [18,19]. The median optical density values obtained from tissues exposed to a polyvinylsiloxane impression material (President regular, Coltène AG, Altstätten, Switzerland) were used as a negative-control reference (100% cell survival). The cytotoxicity of test materials was expressed as a percentage of the matching negative-control tissues. Each experiment was performed with five replicates, and each experiment was carried out at least two times. Statistical analysis was performed using the Mann-Whitney *U*-test ($\alpha = 0.05$) (SPSS, Version 13.0; SPSS, Chicago, IL, USA).

3. Results

3.1. Cytotoxicity of dental adhesives

The cytotoxicity of dentin primers and bonding agents of various self-etching dental adhesive systems and one total-etch adhesive was determined in transformed human pulp cells (tHPC) after a 24 h exposure period using the crystal violet assay. All agents tested cytotoxic but the effects varied immensely over a broad concentration range (Fig. 1). Solutions of the bonding agent of Clearfil SE bond reduced cell survival linearly between 0 and 1 mg/ml. The effects of the bonding agents of Clearfil Protect bond and AdheSE were very similar to those observed with Clearfil SE bond. The number of surviving cells was linearly decreased with increasing concentrations, and no surviving wells were detected after exposure to 1.0 mg/ml (Fig. 1). Likewise, the same low concentrations of Prompt L-Pop decreased cell survival, however, there was an increase in the mass of crystal violet stained material in wells originally treated with concentrations higher than 1 mg/ml (Fig. 1). The apparent increase in cell survival is most likely caused artificially because of the staining of fixed and dead

cells by the crystal violet dye. Solutions of the total-etch adhesive Excite were very effective in causing cell death because no remaining living cells were detected in cultures treated with concentrations of 0.5 mg/ml (Fig. 1). The cytotoxic potential of these bonding agents, based on concentrations which reduced the number of cells to 50% (EC50), were ranked as follows: Excite (0.16 mg/ml) > AdheSE bond (0.30 mg/ml) > Clearfil Protect bond (0.35 mg/ml) > Clearfil SE bond (0.37 mg/ml), and Prompt L-Pop bond (0.68 mg/ml).

The dentin primers were less cytotoxic than the bonding agents with the exception of Clearfil Protect primer. This material was as toxic as the related bonding agent (Fig. 1). In contrast, Clearfil SE primer induced a concentration-dependent decrease in cell survival at concentrations up to 5 mg/ml, and AdheSE primer and Prompt L-Pop primer were even less cytotoxic (Fig. 1). Thus, the ranking of the cytotoxic potential of dentin primers based on EC50 values was: Clearfil Protect primer (0.45 mg/ml) > Clearfil SE primer (3.0 mg/ml) > Prompt L-Pop primer (4.85 mg/ml), and AdheSE primer (>10 mg/ml).

3.2. Production of reactive oxygen species (ROS) by dental adhesives

The generation of DCF fluorescence, which is indicative of ROS production in tHPC, was determined after a short exposure period of 1 h to the dental adhesives. The monomer triethylene glycol dimethacrylate (TEGDMA) was used as a reference. A concentration of 1.5 mmol/l TEGDMA increased the amounts of ROS about threefold (Fig. 2). Similar to the analyses of the cytotoxicity described before, all adhesive agents increased the amounts of ROS in a dose-related manner. Again, however, major differences were observed between the effective concentrations, and higher amounts of dentin primers were needed to cause ROS levels similar to those elicited by the corresponding bonding agents. Solutions of the dentin primer of Clearfil SE bond increased ROS levels linearly between 0 and 2 mg/ml. A fivefold increase was detected with 2 mg/ml, and this level remained constant at higher concentrations. The Clearfil SE bond bonding agent was more effective than the primer because about 10-fold lower concentrations caused almost the same levels of ROS. The same amounts of ROS were formed in the presence of Clearfil Protect bond primer and bonding agent, but again about three- to fourfold lower concentrations of the bonding agent were required to elicit the biological response. Likewise, the bonding agents of Prompt L-Pop and AdheSE were even close to 100-fold more effective than the corresponding dentin primers. Most interestingly, almost the identical amount of 0.25 mg/ml of bonding agents of the various dental adhesives, including Excite, caused the largest increase in ROS, with only the Prompt L-Pop bonding agent being most effective at a slightly lower concentration. The reduction in ROS levels detected at higher concentrations of either primers or bonding agents of almost all dental adhesives is probably due to reduced viability. This is most obvious with the bonding agent Prompt L-Pop because ROS levels generated by concentrations higher than 0.5 mg/ml were even lower than those measured in untreated controls. Yet, no reduction in cell survival in any experimental cell cultures was detected with the crystal violet cytotoxicity assay (Fig. 2).

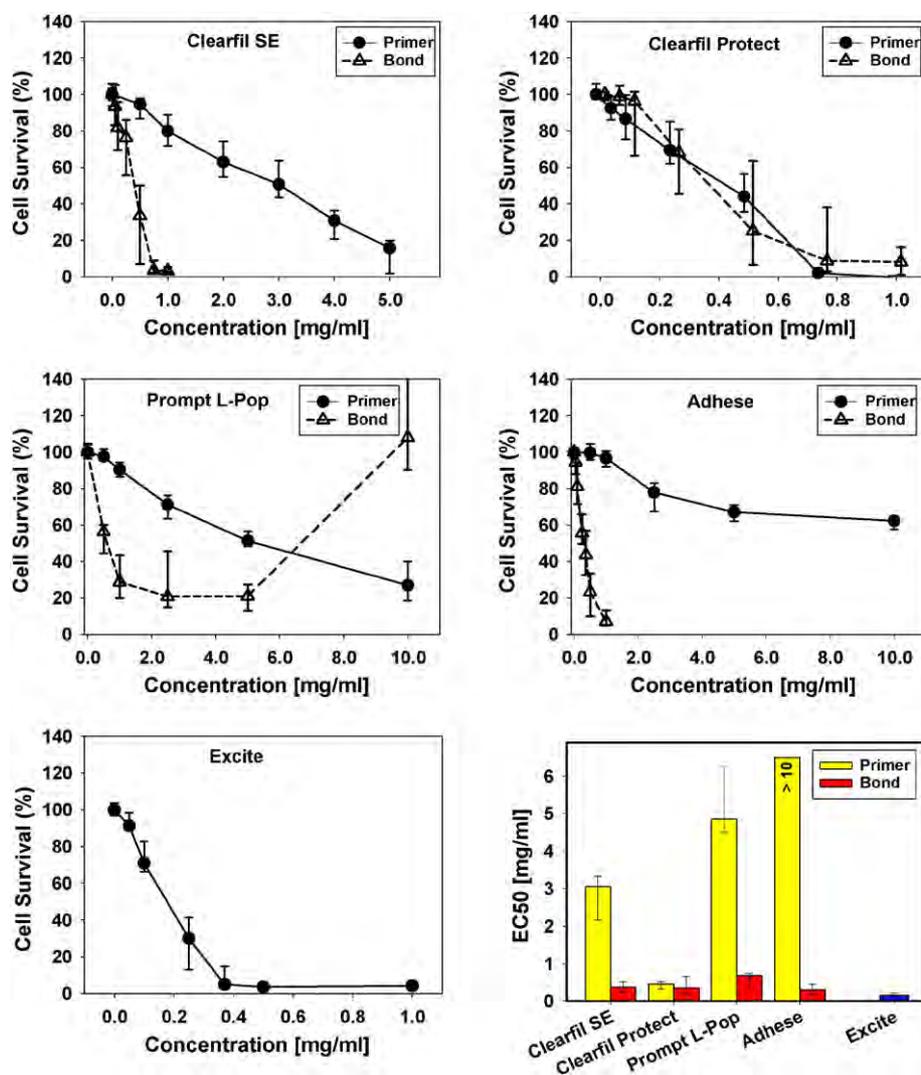


Fig. 1 – Cell survival of transformed human pulp cells after exposure to dental adhesives. Cell survival data were normalized to original optical density readings (absorbance at 600 nm) of four replicate cultures per concentration. The symbols represent medians (25th and 75th percentiles) calculated from at least four independent experiments ($n = 16–20$). Concentrations of the primers and bonding agents leading to 50% cell death (EC50 values) were calculated from fitted dose–response curves as described in Section 2. Median EC50 values plus 25th and 75th percentiles ($n = 4–5$) are presented. EC50 values of each pair of primers were statistically significant different, but such differences were not detected for each pair of the bonding agents.

3.3. Formation of micronuclei by dental adhesives

The formation of micronuclei by the compounds of the various dental adhesive systems was analyzed in V79 cell cultures (Fig. 3). No increased numbers of micronuclei were observed with solutions of the Clearfil SE bond bonding agent, Clearfil Protect bond primer and bonding agent, Prompt L-Pop primer, and Excite. Only a few materials increased the number of micronuclei in cells of treated cultures significantly compared to untreated controls (UC), and two concentrations of the positive control ethyl methanesulfonate (EMS). A slight increase was detected with Clearfil SE bond primer, and the bonding agents of Prompt L-Pop and AdheSE (Fig. 3). However, a noticeable dose-related increase was observed with AdheSE primer when 1 and 2.5 mg/ml were tested. Solutions con-

taining 2.5 mg/ml AdheSE primer increased the number of micronuclei at least sixfold compared to untreated controls. Thus, this concentration of the AdheSE primer was at least as effective as 5 mmol/l EMS (Fig. 3).

3.4. Dentin barrier test

Cytotoxicity of the dental adhesive systems was also determined in three-dimensional cell cultures introduced in a dentin barrier test device (Fig. 4). A vinyl poly siloxane material (President) and a light curing glass ionomer cement (Vitrebond) were used as a negative- and positive-control material. As expected, Vitrebond was the most toxic material, which reduced cell survival to about 50% compared with cell cultures exposed to President. However, none of the dental adhesives

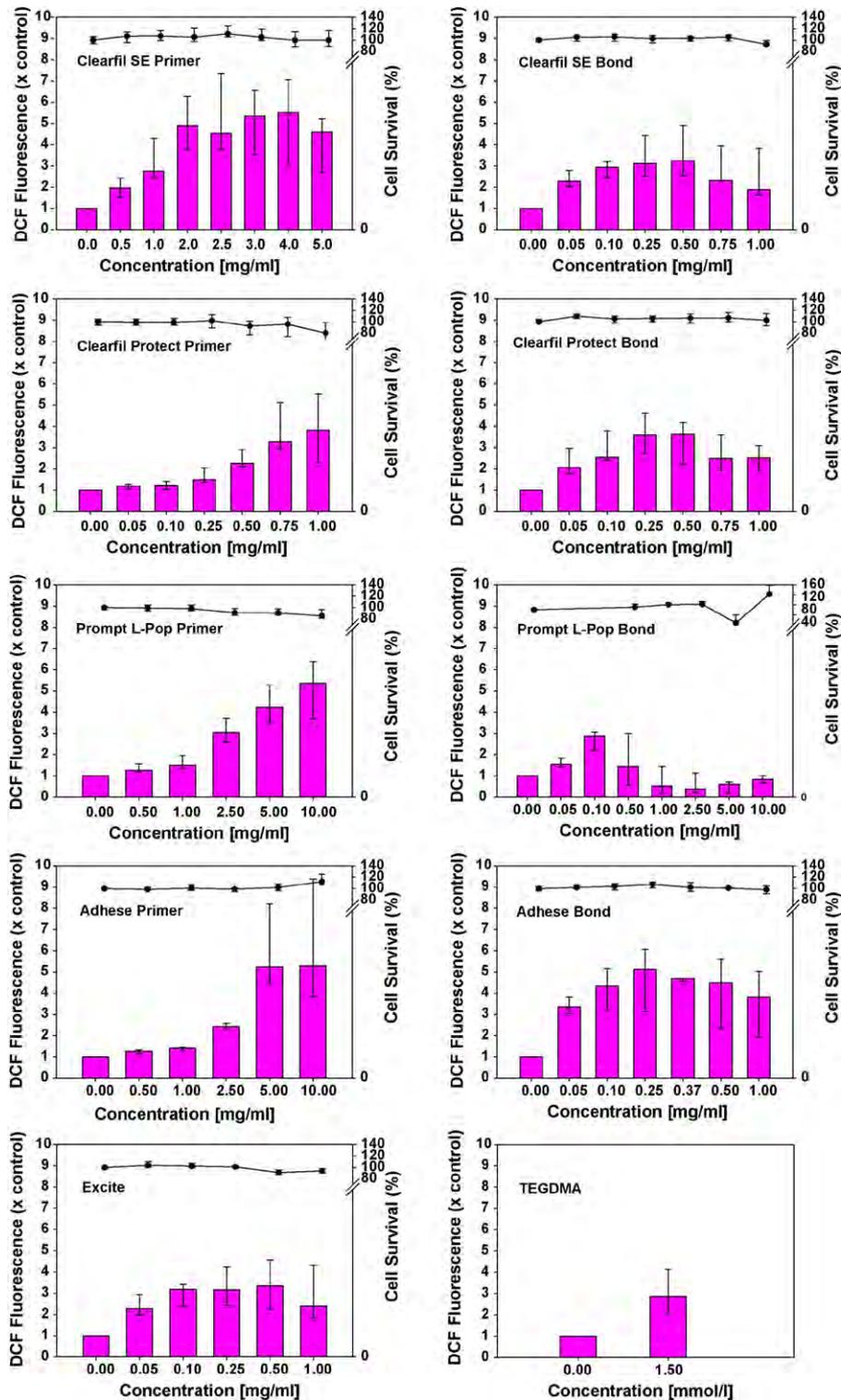


Fig. 2 – Generation of reactive oxygen species (ROS) in transformed human pulp cells after exposure to dental adhesives. The production of ROS was measured using the oxidation-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate ($H_2DCF\text{-DA}$). The cell cultures were exposed to increasing concentrations of dental adhesives in cell culture medium for 1 h, and 1.5 mmol/l TEGDMA was used as a control ($n=38$). Mean fluorescence intensities were obtained by histogram statistics using the WinMDI program (Version 2.8), and mean fluorescence intensities were normalized to untreated control cultures ($=1.0$). Bars represent medians (25th and 75th percentiles) calculated from individual histograms ($n=4\text{--}6$). Cell survival of transformed human pulp cells after a 1 h exposure to dental adhesives was normalized to original optical density readings (absorbance at 600 nm) of four replicate cultures per concentration. The symbols represent medians (25th and 75th percentiles) ($n=8\text{--}16$).

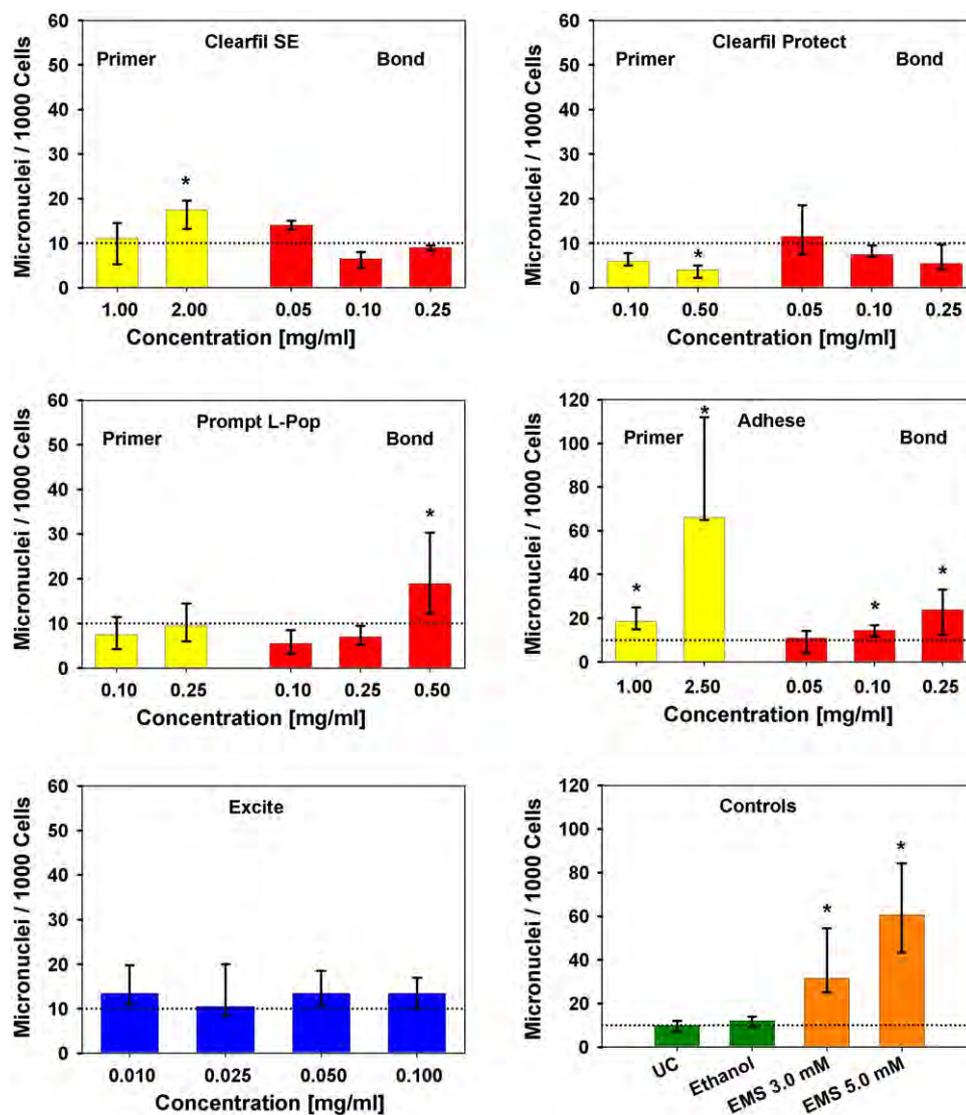


Fig. 3 – Induction of micronuclei in V79 cells after exposure to dental adhesives. The numbers of micronuclei caused by the dental adhesive compounds were obtained from four to six treated cell cultures, and bars represent medians (25th and 75th percentiles). Median numbers of micronuclei were calculated for untreated controls (UC) ($n = 36$), cultures treated with ethanol ($n = 19$), and ethyl methanesulfonate (EMS) at concentrations of 3 mmol/l ($n = 18$) and 5 mmol/l ($n = 20$). Statistically significant differences between untreated and treated cell cultures are indicated by asterisks.

significantly reduced cell survival compared with the negative control. Exposure of the cell cultures to AdheSE and Clearfil Protect bond lead to 95% and 99% cell survival. On the contrary, slightly increased cell vitality was observed with Excite (107%), with this rise being significant even after exposure to Clearfil SE bond (114%) (Fig. 4).

4. Discussion

The dental adhesives tested in the present study caused an imbalance in the cellular redox state in human pulp-derived cells after a relative short exposure period of 1 h. All adhesive agents increased the amounts of ROS as indicated by DCF fluorescence in a dose-related manner. Notably, the various dentin primers as well as bonding agents were

effective in a very similar concentration range. While the maximum amounts of ROS were generated by primers, particularly by concentrations ranging from 2 to 10 mg/ml, the bonding agents were about 3–4 up to even 100-fold more effective than their primer counterparts. Only the Clearfil Protect bond primer was effective at slightly lower concentrations than other dentin primers. The reduction in ROS levels caused by higher concentrations of the bonding agents was probably due to reduced cell viability, although a reduction in the number of cells in any treated culture was not observed with the crystal violet assay after a 1 h exposure period. Interestingly, the amount of ROS was almost equally increased by all dentin primers by a factor of 4–5 compared with untreated controls, and the bonding agents were slightly less effective in this respect. Very similar effects on ROS production were detected earlier with different adhesive systems. Extracts of

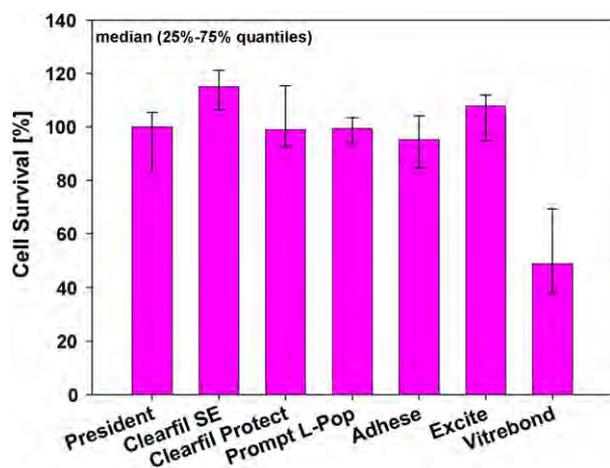


Fig. 4 – Cell survival of three-dimensional cultures in a dentin barrier test device after exposure to dental adhesives. Data are expressed as percentage of the negative-control cultures (President = silicone impression material). Cell survival rates were calculated from independent experimental cultures: President (n = 20), Clearfil SE (n = 8), Clearfil Protect (n = 10), Prompt L-Pop (n = 9), Adhese (n = 9), Excite (n = 5), and Vitrebond (n = 20). The indicated values are medians, 25th and 75th percentiles.

Scotchbond One and Optibond Solo increased the intracellular amounts of ROS as early as 15 min after stimulation, and ROS levels about 2–4.5-fold higher than controls were observed after 2 h [20].

The resin monomer TEGDMA, which was used as a reference here, increased the amounts of intracellular ROS by about 2.5-fold. These findings correlated with reports on the induction of oxidative stress caused by TEGDMA and other compounds of many resin-based dental restorative materials like HEMA or common photosensitizers [22,29–31]. From the data presented here, it is difficult to draw any conclusions concerning ROS production related to a specific compound. Yet, there is evidence that ROS generated by monomers like TEGDMA and HEMA can effectively interfere with cellular signal transduction networks regulating cell survival pathways [4]. In this way, the production of ROS by monomers has been related to the induction of cell death via apoptosis, and apoptosis was observed in cells after exposure to dental adhesives as well [4,13,32]. Hydrophilic components of dentin bonding agents, such as TEGDMA or HEMA, may diffuse from dental adhesives through dentin. Particularly in deep cavities, the monomers may possibly reach the dental pulp in concentrations that disturb the redox state of pulp cells [33–35]. The ROS-generating potential of the dental adhesives tested here was at least as high as that of 1.5 mmol/l TEGDMA, a concentrations that induced apoptosis in primary pulp cells [36]. Thus, the production of ROS by dental adhesives that was observed after a 1 h exposure period most likely triggered apoptotic pathways, although a reduction in cell survival was not detected by the crystal violet assay at that time. Consequently, the toxic potential of the materials tested here

is probably higher than estimated so far based on the data derived from conventional cytotoxicity testing. The findings that all dental adhesives tested cytotoxic based on cell survival rates after a 24 h exposure period, however, correlate with earlier reports which indicated cytotoxicity of various dental adhesives [5–13].

In our study, it was obvious that the cytotoxic potencies of the various dental adhesive agents were very similar. Similar to the generation of ROS, the concentrations of bonding agents which caused a reduction in cell survival to 50% were at least 10-fold lower than those of the dentin primers, with the exception of Clearfil Protect bond primer. According to the information which is currently available, it appears that the difference between Clearfil Protect bond primer and Clearfil SE bond primer was mostly the content of MDPB (12-methacryloyl-oxy-dodecyl pyridinium bromide) in the Clearfil Protect primer (see Table 1). MDPB is considered to be an antibacterial chemical [37]. The cytotoxic potency of MDPB was reported to be low and similar to that of other monomers used for dental materials [38]. Based on our data, we cannot rule out that MDPB is a candidate molecule that specifically increased the cytotoxic potency of the Clearfil Protect bond primer compared with Clearfil SE bond primer, as well as perhaps the other dentin primers tested here.

An in vitro dentin barrier test for cytotoxicity testing was developed to mimic a clinical situation which is better than direct cell–material contact in vitro methods, and it has the potential to, at least partially, replace animal experimentation. This goal was achieved when a dentin disc, which functioned as a barrier, a test material, and three-dimensional cultures of pulp-derived cells were combined in a cell culture perfusion chamber [18,19]. It was consistently demonstrated that dentin was an effective barrier, preventing cell damage from a great variety of materials and chemicals. For instance, cytotoxicity of dental adhesives decreased when the thickness of the dentin disc was gradually increased from 100 to 500 μm [39]. This effect was apparently selective and depended on the chemical nature of the dentin contacting material. The cytotoxicity of the resin-modified glass ionomer cement Vitrebond that was used as a positive control in the present investigation is in agreement with results from previous studies [18,19,39]. The dental adhesives, however, did not reduce cell viability in pulp-derived three-dimensional cell cultures, probably because of the protective effect of a 500 μm thick dentin disc that was placed between materials and cells. Cytotoxicity of self-etching experimental primers in human pulp cells incorporated in a simple pulp chamber was related to HEMA rather than to the antibacterial monomer MDPB [40].

Reactive oxygen species (ROS) are generally genotoxic [41]. In addition, the induction of genotoxic effects of dental resins and monomers has also been discussed as a consequence of oxidative damage originating from the generation of ROS [24,30,31]. From the results of this investigation, however, it appears as if this aspect of dental materials is more complex. There is no obvious relation between ROS production by the dental adhesives tested in the present study and genotoxicity as indicated by the formation of micronuclei. However, it cannot be ruled out that all dental adhesives which increased ROS levels, may induce DNA damage and successive gene mutations like base pair substitutions or small insertions or

deletions of base pairs not detectable by the micronucleus assay. Primers and bonding agents of Clearfil Protect bond, and the primer of Clearfil SE bond generated high levels of ROS, but a relevant formation of micronuclei was not evident. Similar observations were made with Prompt L-Pop primer and Excite. On the other hand, the generation of ROS by 2 mg/ml AdheSE primer was relatively low, but this amount of dentin primer induced a number of micronuclei more than sixfold higher than observed in untreated control cultures. Solutions of this material were at least as effective as 5 mmol/l EMS which was used as a positive control. EMS is a DNA alkylating agent, and the formation of micronuclei by this chemical was not influenced by the ROS scavenger *N*-acetylcysteine [24]. We speculate that the compound of the AdheSE primer, which caused increased numbers of micronuclei, could be a bis(acrylamide) cross-linking monomer. This chemical is indicated as a compound in the scientific documentation provided by the manufacturer, and it was also mentioned in a recent publication as an ingredient of the material [42,43]. Although bis(acrylamide) is a bifunctional molecule, it could act chemically similar to acrylamide, a chemical which can form alkylating DNA adducts and is genotoxic in vitro [44–46].

In summary, all dental adhesives analyzed in the present investigation tested cytotoxic after an exposure period of 24 h. It appeared as if the bonding agents were more effective than their primer counterparts. No cytotoxicity was detected for any of the dental adhesives tested here in a dentin barrier test device using three-dimensional cell cultures. This finding indicates that dentin may protect pulp tissue from immediate damage after the application of dental adhesives. However, the demonstrated cytotoxic potencies of the materials in monolayer cultures might be of clinical relevance. We have shown that the cellular redox state of pulp cells in these cultures was disturbed by all dentin primers and bonding agents. Thus, the concentrations of biologically active ingredients may be high enough to modify pulp cell metabolism when the materials are used in deep cavities without a protective dentin barrier and when pulp cells are exposed to these materials.

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