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Cytotoxic effects of dental bonding substances as a function of degree of conversion

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ABSTRACT

Objectives. Recently, we found that dental bonding substances tested alone or in combination with composites are far more cytotoxic than composite materials alone. These data are in line with several cytotoxicity reports but contradict *in vivo* studies showing the beneficial effects of bonding materials. The aims of the present study were to develop a preparation method for composite specimens modelling conditions in the oral cavity and to analyse the influence of bonding substances on the cytotoxicity of six different composite materials.

Methods. Cylindrical composite specimens were prepared in polyethylene blocks containing 5 mm diameter cylindrical holes (cylinder height 2 mm), covered with a polyethylene foil and light cured from one end for 40 s. In a second series of experiments, composite specimens were combined with bonding materials. Bonding was applied onto the polyethylene foil in one or two layers and light cured according to the manufacturers' instructions. Subsequently, polyethylene moulds were placed on top of the bonding materials and composites prepared as described above. After unilateral light curing from the top of the cylindrical holes, visual confirmation of adherence at the base was obtained. Specimens were added to the cultures immediately after production or after preincubation for 7 days under cell culture conditions. Specimens were incubated with L-929 fibroblasts for 72 h and cell numbers determined by flow cytometry. To evaluate the degree of conversion (DC) of bonding materials cured with and without air inhibition a third series of experiments was performed. FTIR spectroscopic measurements were made on thin-films of dentin-bonding agents, cured under both an-aerobic and aerobic conditions, to determine degree of conversion.

Results. Cytotoxicities of all six tested composites were significantly different ($p < 0.0001$) and diminished after 7 days of preincubation ($p < 0.0001$). Bonding substances had no statistically significant influence on the cytotoxicity of composite materials ($p = 0.159$). A highly significant statistical reduction in the degree of conversion for each resin cured under air inhibition conditions was documented ($p < 0.01$).

Significance. Our study demonstrates that cell culture toxicity data are highly model dependent and that internationally standardized test protocols for toxicity screening of dental materials in line with the existing standards are clearly needed to obtain comparable results.

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1. Introduction

Dental restorative treatment with resin-based materials requires placement of a bonding agent to etched, resin-primed, demineralised tooth tissue. The adhesive system is typically spread into a thin layer after application and prior to light exposure to minimize pooling, evaporate solvent carrier, and provide a minimal thickness of unfilled adhesive resin. The adhesive system is then photocured, and resin composite is incrementally placed and polymerized. Today, many 'all-in-one' adhesive systems are self-etching, combining the etching and bonding functions in a single container. These systems incorporate acidic and thus hydrophilic chemicals.

However, both the 3-step and the single-component methodologies have potential for the resulting resin–dentin bond to be less than optimal. Their application may result in formation of a linear or very low cross-linked and unfilled polymer matrix in the hybrid layer and the inhibition by atmospheric oxygen of adhesive resin polymerization in such a thin, low-viscosity bonding layer.

The degree of cytotoxicity of bonding substances varied from little or no cytotoxicity to severe cytotoxicity depending on the experimental protocol. Substances were added to cell cultures as extracts of photopolymerized bonding substances, as uncured or cured bonding materials in direct contact with the cells or on a dentin barrier or as single components.

Extracts with cell culture medium of photopolymerized bonding substances have been tested with varying experimental designs. Huang and co-workers [1,2] tested extracts from bonding agents photopolymerized on cellulose strips and showed that eluates were cytotoxic. These effects were influenced by the material and the preincubation time of bonding specimens prior to elution. The diminishing effect of preincubation varied for the different materials tested. Extracts from fresh and 16 weeks aged adhesives (disks, 5 mm in diameter by 2 mm in thickness) caused similar cytotoxicity, but failed to stimulate similar cytokine release. Leachables responsible for cytotoxicity might therefore differ from those stimulating cytokine release. When light curing was incomplete, cytotoxic effects of extracts increased [3].

In several studies, uncured bonding materials were tested in direct contact to the culture cells. Materials were tested either diluted or undiluted without light curing. Undiluted materials caused severe damage of cells [4–7], which decreased with increasing dilutions of the materials [5,6]. Uncured materials were more cytotoxic than polymerized materials [7].

In another series of studies bonding agents applied on a dentin barrier or in a simulated pulpchamber without light curing were tested. When a total-etch and a self-etch bonding system were placed on dentin discs according to the manufacturers' instructions and tested in a pulp chamber, the total etch system was more cytotoxic [8]. Schmalz et al. [9] also tested bonding substances applied on dentin disks in a pulp chamber. Low pH bonding substances did not show any cytotoxicity. It has been suggested by Meryon and Brook [10] that three dentine bonding agents tested caused severe reactions in the absence of an adequate lining *in vitro*. In this study, bonding substances were applied on dentin disks and presented to the cultured cells 20–60 s prior to light curing in indirect contact. Bouillaguet et al. [11] demonstrated the *in vitro* cytotoxicity of dentin diffusates of bonding agents, which were produced under pressure using dentin disks of human third molars. Camps et al. [12] used chambers made from extracted third molars in a cytotoxicity test.

Components of dental adhesives have been tested individually as single substances and have been shown to be cytotoxic [13–15] or to modulate cellular function [14,16].

Kaga et al. [17] compared the cytotoxicity of cured materials, uncured materials and two major components, HEMA and TEGDMA. Materials were applied in glass cylinders with indirect cell contact and HEMA contributed most to the cytotoxicity. Only HEMA and TEGDMA were detected below reported TC50 levels eluted from cured materials. Uncured bonding materials were severely cytotoxic but induced virtually no cytotoxicity when polymerized.

The primary aim of the present study was to develop a standardized protocol for specimen production resembling the conditions in tooth cavities and to relate the toxicity of adhesive systems to the toxicity of composite materials alone when the application of substances is performed in the same sequence as in the oral cavity (first bonding, then composite). The second aim was to evaluate the effect of conditions used in a previous resin-composite biocompatibility study [18]. In this, bonding substances were cured in conjunction with resin-composites in aerobic conditions where the bonding substance cure could have been affected by air inhibition. If so, the apparent biocompatibility of the composite plus bond-agent system could have been adversely affected via a reduced degree of conversion (DC) of the bonding substances, leading to a mechanism of monomer-elution into the test-cellular media.

In line with these two aims the following null hypotheses were formulated: (i) combination of composite specimens with bonding substances modelling conditions in the oral cavity has no statistically significant influence on the cytotoxicity of composite materials and (ii) cure of bonding substances with or without air inhibition does not influence the degree of conversion of bonding substances.

2. Materials and methods

2.1. *Composite and bonding materials*

Materials used in this study are listed in Table 1.

2.2. *Cytotoxicity assay*

2.2.1. *Manufacture of specimens*

Cylindrical composite specimens were prepared in polyethylene blocks containing 5 mm diameter cylindrical holes (cylinder height 2 mm), covered with a polyethylene foil (Hostaphan®, Mitsubishi Polyester Film GmbH, Wiesbaden, Germany) and light cured from one end for 40 s. In a second series of experiments, composite specimens were combined with bonding materials. Bonding was applied on a polyethy-

Table 1 – Composite and bonding materials

Material	Manufacturer	Lot number	Formulation
Etch and Prime 3.0*	Dentsply GmbH, Konstanz, Germany	0403001190	Tetra-methacryloxyethyl-pyrophosphate, HEMA, *p*-N,N-di-methyl-aminobenzoicacidethylester, camphoroquinone, BHT, ethanol, demineralised water
Prime and Bond NT*	Dentsply GmbH	0412001549	Urethandimethacrylate (R5-62-1), di- and tri-methacrylate-resins, PENTA, camphoroquinone, butylhydroxytoluole, ethyl-dimethyl aminobenzoate, cetylaminhydrofluoride, activated amorphous silica gel, acetone.
Syntac Sprint*	Ivoclar Vivadent Ets., Schaan, Liechtenstein	F67448	HEMA, methacrylate-modified polyacrylic acid, maleic acid, catalysts, stabilizers, water, acetone
Opti Bond Solo Plus*	Kerr Company, Orange, CA, USA	423755	Bis-GMA, HEMA, glycerol dimethycrylate (GDM), GPDM, fumed silicon dioxide (TS 530), fumed silicon dioxide (OX 50), Treated filler, Na_2SiF_6, ethanol
Futurabond*	VOCO GmbH, Cuxhaven, Germany	581031	Bis-GMA, TEGDMA, HEMA, acid modified methacrylate, fluoride, initiators, acetone, water.
Adper Scotchbond1 XT*	3M ESPE Dental Products, St.Paul, MN, USA	20040707	Bis-GMA, UDMA, HEMA, glycerol dimethacrylate (GDMA), modified polyacrylic acid, ethanol, water,
Adper Prompt L-Pop*	3M Espe AG	209229	Methacrylated phosphoric acid ester, initiators fluoride complexes and preservatives, water.
Admira	VOCO GmbH	008647	Bis-GMA, UDMA, TEGDMA, campherchinone, amines, filler: glass ceramics, aerosol.
Definite	Dentsply GmbH	221	Polysiloxane, dodecandioldimethacrylate, Bis-EMA, barium glass, phosphate–sulfate–apatite-mixture, silicon dioxide, amorphous, silanized (Aerosil), 2-*n*-butoxyethyl-4-dimethylaminobenzoate (BEDB), campherchinone, diphenyl(2,4,6-trimethylbenzoyl)phosphinoxide, 2-hydroxy-4-methoxy-benzophenone, di-butylhydroxytoluol (BHT), pigments in ppm range.
Filtek Z 250	3M ESPE Dental Products	20001121	Bis-GMA, UDMA, Bis-EMA, zirconia/silica filler.
Herculite XRV Enamel	Kerr Company	0-1278	Bis-GMA, TEGDMA, ethoxylated bisphenol A dimethacrylate (EBADM), 2-hydroxy-4-methoxybenzophenone (UV-9), BHT, 1,7,7-trimethylbicyclo-[2.2.1]-hepta-2,3-dione (CQ), 10-methoxy-1-sulfostilbene-3-triazolonaphthalene, sodium salt (TINOPAL), 2-(ethylhexyl)-4-(dimethylamino)benzoate (ODMAB), TS530), zinc oxide (V5500), (OX-50), γ-methacryloxypropyl-trimethoxysilane (A174), bariumaluminoborosilicate (SP345), titanium dioxide (TiO_2), pigments.
Spectrum^{TPH}	Dentsply GmbH	0011001545	Bis-GMA-adduct (adduct of 2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)-phenyl]propane with hexamethylene diisocyanate), Bis-EMA, TEGDMA, photo-initiators, stabilizers, bariumaluminoborosilicate, highly dispersed silica.
Tetric Ceram	Ivoclar Vivadent Ets.	C48674	Bis-GMA, UDMA, aliphatic dimethacrylate, bariumglass filler, silanized, ytterbiumtrifluoride (YbF3), mixed oxide, silanized, barium–aluminum–fluorosilicate glass, silanized, highly dispersed silica, silanized, additive content, catalysts and stabilizers, pigments.

* The bonding substances were all formulated with solvents, namely: water, acetone or ethanol – or mixtures of two of these solvents, as specified above.

lene foil (Hostaphan®) in one or two layers and light cured according to the manufacturers' instructions. Subsequently, polyethylene moulds were placed on top of the bonding materials and composites prepared as described above. After unilateral light curing from the top of the cylindrical holes, visual confirmation of adherence at the base was obtained. Specimens were then sterilized by UV-radiation and were added to the cultures immediately after production or after preincubation for 7 days under cell culture conditions. Specimens were incubated with L-929 fibroblasts for 72 h and cell numbers determined by flow cytometry.

All light curing materials were hardened with a Demetron Optilux curing light (Kerr company, USA; light intensity > 500 mW/cm^2).

2.2.2. *Controls*

Glass specimens with a 5 mm diameter and 2 mm height were used as controls.

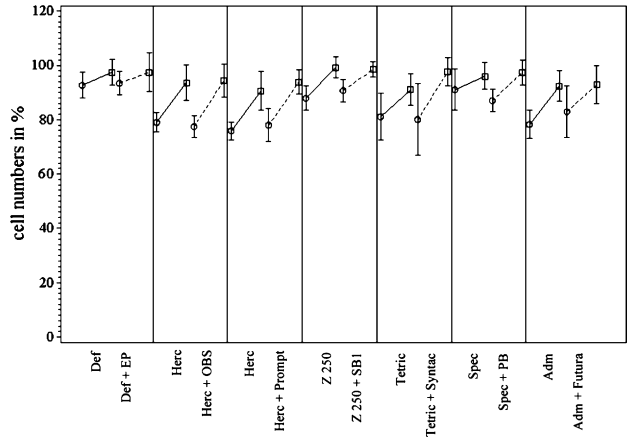

Fig. 1 – Influence of six different composite materials (Herculite XRV was tested twice) tested alone and in combination with seven different bonding materials on L-929 fibroblast cell numbers. Specimens were added to the cultures immediately after production (fresh) (○) or after preincubation for 7 days (□). Cell numbers were expressed as a percentage of controls (cultures without specimens), as described in the materials and methods. Vertical lines show the mean and standard deviations of three independent experiments performed in triplicate. Def = Definite, EP = Etch and Prime 3.0, Herc = Herculite XRV (tested alone and in combination with Optibond Solo Plus = OBS or Adper Prompt L-Pop = Prompt), Z250 = Filtek Z 250, SB1 = Scotchbond 1, Tetric = Tetric Ceram, Syntac = Syntac Sprint, Spec = Spectrum TPH, PB = Prime and Bond NT, Adm = Admira, Futura = Futurabond.

2.2.3. *Preincubation of specimens*

Specimens were either used immediately after production or preincubated in cell culture medium (one specimen in 10 ml of Dulbecco's Modified Eagle Medium (DMEM; Sigma, Germany)) at 37 °C, pH 7.2 for 7 days [19]. The culture medium was then removed and specimens used for experiments.

2.2.4. *Culture of L-929 fibroblasts*

The murine fibroblast cell line L-929 was obtained from American Type Culture Collection (ATCC, Rockville, MD). L-929 cells were cultivated in Costar 162 cm^2 flasks (Cambridge, MA) in DMEM supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 1% glutamine and 1% penicillin/streptomycin at 37 °C in a fully humidified air atmosphere containing 5% CO_2 and were passaged by trypsinization. Fibroblasts (5 ml aliquots, containing 3×10^4 cells/ml) were exposed to freshly prepared (added to cultures immediately after production) or preincubated specimens (see above) in polystyrene 6-well tissue culture plates (Costar) for 72 h at 37 °C/5% CO_2. Cells were then harvested with trypsin (2.5% in Ca^{2+} and Mg^{2+} free Hanks balanced salt solution; JRH Biosciences, KA, USA), centrifuged and resuspended in 500 μl DMEM.

2.2.5. *Flow cytometry*

Cells were counted in a volume of 500 μl DMEM over a fixed time (30 s) with a flow cytometer (FACSCalibur, Becton Dickinson, San José, CA, USA) equipped with an argon laser tuned at 488 nm. Cell numbers after exposure to test specimens were compared to controls (cultures without specimens).

2.2.6. *Statistical evaluataion of cell culture experiments*

The effects of six different composite materials tested alone or in combination with bonding substances on L-929 cell numbers were evaluated by Analysis of Variance (ANOVA). In this model, composites, bonding and preincubation time (fresh, 7 days) were included as fixed factors. The interaction between preincubation times and substances was included in the model. At each point of aging (fresh, 7 days), an ANOVA was performed to pool these 14 characteristics (six composites (Herculite XRV was tested twice), tested alone or in combination with seven different bonding materials) with the approach developed by Ryan [20,21], Einot and Gabriel [22], and Welsch [23]. Substances (seven composites tested with and without bonding) were included as fixed factors in both models. Additionally LS-means were tested for each preincubation time (fresh, 7 days), if they were significantly different from controls (100%). Multiplicity of comparisons was considered by controlling the type I experimentwise error rate with Ryan–Einot–Gabriel–Welsch–Multiple-Range-Tests. For all experiments, cell numbers in % (=cell numbers/cell numbers of control (cultures without specimens)) were used as the dependent variable.

All calculations were performed with SAS© Release 8.2.

2.3. *Spectroscopic measurement of degree of conversion*

FTIR measurements were made on thin films of the bonding agent used in conjunction with thin polyethylene sheet substrates. In cases where the bonding agent was two-component, such as Etch and Prime, the components were first mixed in accordance with manufacturers' instructions.

To measure the FTIR spectra under (i) anaerobic (oxygen-free) conditions, the bonding agents were spread into a thin film between two polyethylene sheets. To measure the spectra under (ii) air inhibition (aerobic) conditions, the bonding agents were spread onto a single sheet. This procedure was repeated three times ($n = 3$) for each bonding system.

Calculation procedures for determination of residual double bonds (% RDB) and degree of conversion (% DC) normally require the presence of a suitable internal standard. The analytic methods to determine resin cure are based upon the Beer–Lambert equation:

$$A = alc$$

where A = absorbance, a = molar absorptivity, l = path length and c = concentration.

For both cases (i) and (ii), above, absorbance spectra were measured, using 20 co-added scans at 4 cm^{-1} in transmission, on each of the un-cured specimens (Avatar FTIR, Nicolet). The specimens where then irradiated for 20 s by a QTH light source of 500 mW/cm^2 irradiance. Following irradiation, the sample spectra were re-measured in the same manner.

Applying the calculation procedure directly to the cure of methacrylate-based monomers first involves determination of the aliphatic C=C concentration of the uncured material from

Table 2 – Cell numbers of freshly prepared specimens expressed as a percentage of controls (cultures without specimens)

Substance	n	Mean	S.D.	Median
Definite and Etch and Prime 3.0	9	93.43	4.39	94.07
Definite	9	92.73	4.89	92.96
Spectrum TPH	9	91.08	7.54	91.31
Scotchbond 1	9	90.78	4.17	90.73
Filtek Z 250	9	87.93	4.4	87.33
Spectrum TPH and Prime and Bond NT	9	87.06	4.19	86.6
Admira and Futurabond	9	82.98	9.58	82.33
Tetric Ceram	9	81.16	8.63	81.58
Tetric Ceram and Syntac Sprint	9	80.1	13.21	78.42
Herculite XRV[a]	9	79.09	3.49	79.52
Admira	9	78.29	5.29	76.7
Herculite XRV and Prompt L-Pop	9	78.02	6.02	79.51
Herculite XRV and Optibond Solo Plus	9	77.49	3.95	75.86
Herculite XRV[b]	9	75.94	3.27	76.12

[a] Herculite, for comparison with Herculite and Optibond Solo Plus.
[b] Herculite, for comparison with Herculite and Prompt L-Pop.

the absorption peak occurring at 1636 cm^{-1}. After curing of the thin slice of material, the reduced concentration of aliphatic C=C is then re-determined from the reduced absorption peak.

However, the peak magnitudes – before and after curing – are not normally compared directly, but only after taking ratios with the magnitude of a reference peak which does not enter into reaction during curing. In the case of bonding agents containing aromatic groups, such as in Bis-GMA, it is convenient to take the reference aromatic vibrational peak at 1608 cm^{-1}. The percentage of residual double-bonds is then given by:

$$\%\,\mathrm{RDB} = \left(\frac{(A_{\text{aliphatic C=C}}/A_{\text{ref}})_{\text{cured}}}{(A_{\text{aliphatic C=C}}/A_{\text{ref}})_{\text{un-cured}}} \right) \times 100\%$$

and the percentage degree of conversion by:

$$\mathrm{DC} = 100 - \%\mathrm{RDB}$$

This procedure involving peak-ratios is normally considered necessary because the resin increases density during polymerization, due to shrinkage. As a result of this volume change, more remaining, unreacted C=C will be present in the beam path, resulting in conversion data which may be inappropriately low.

However, compositions that do not contain functional groups which can be used as an internal standard may be analysed by mixing in an external compound to act as a basis for determination of changes in C=C concentration, but this method is laborious.

An alternative method is to measure changes in the mass percent of methacrylate groups, although this also involves some approximations, calibrations and/or additional information [24,25].

In the present study, three of the bonding resins contained Bis-GMA, so the aromatic C···C peak at 1608 cm^{-1} could be used with the equations above.

For the remaining bonding resins, an approximate measure of the degrees of conversion was feasible, albeit with a greater uncertainty. In these cases, the simplest procedure

Table 3 – Cell numbers of 7 days preincubated specimens expressed as a percentage of controls (cultures without specimens)

Substance	n	Mean	S.D.	Median
Filtek Z 250	9	99.27	3.86	98.92
Scotchbond 1	9	98.54	2.78	98.71
Tetric Ceram and Syntac Sprint	9	97.6	5.13	97.73
Definite	9	97.46	4.84	96.33
Definite and Etch and Prime 3.0	9	97.44	7.12	94.86
Spectrum TPH and Prime and Bond NT	9	97.38	4.51	97.53
Spectrum TPH	9	96.08	4.86	96.35
Herculite XRV and Optibond Solo Plus	9	94.37	6.2	95.29
Herculite XRV and Prompt L-Pop	9	93.86	4.42	93.33
Herculite XRV[a]	9	93.68	6.59	93.92
Admira and Futurabond	9	92.86	6.94	90.85
Admira	9	92.38	5.57	93.4
Tetric Ceram	9	91.04	5.73	93.2
Herculite XRV[b]	9	90.69	7.16	89.57

[a] Herculite, for comparison with Herculite and Optibond Solo Plus.
[b] Herculite, for comparison with Herculite and Prompt L-Pop.

Table 4 – Degrees of conversion, with and without air inhibition: means and S.D.

Material	DC without air inhibition	DC with air inhibition	Reduction of DC with air inhibition in comparison to DC without air inhibition in percent
Etch and Prime 3.0.	59.4 (2.3)	37.5 (3.1)	36.9
Prime and Bond NT	67.1 (1.2)	39.3 (2.8)	41.4
Syntac Sprint	66.4 (0.9)	41.2 (1.8)	38.0
Opti Bond Solo Plus*	65.1 (1.8)	40.4 (2.9)	37.9
Futurabond*	63.4 (2.1)	35.6 (3.1)	43.8
Adper Scotchbond1 XT*	67.9 (1.3)	41.0 (2.0)	39.5
Adper Prompt L-Pop	63.4 (3.7)	33.5 (4.1)	47.2

* Denotes materials with aromatic reference groups.

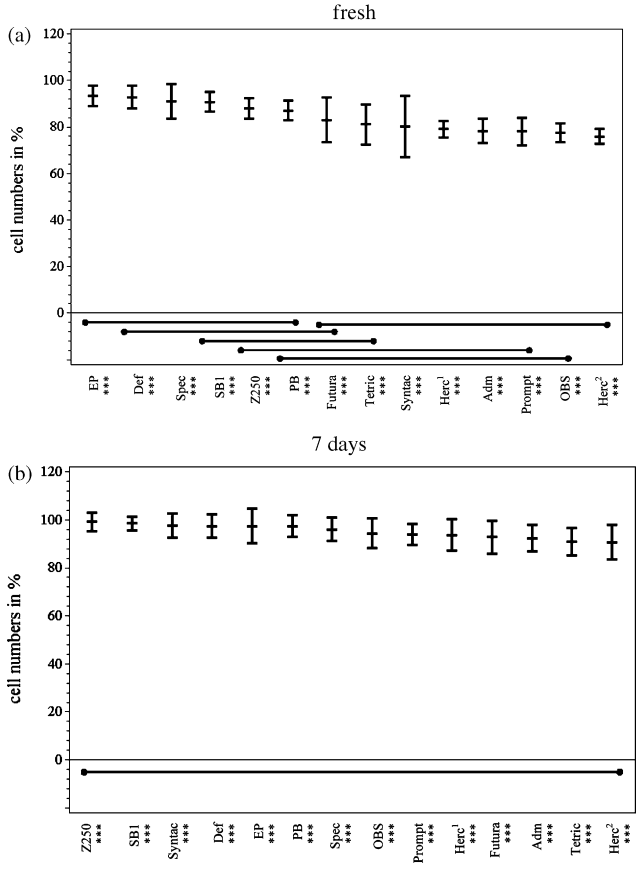

Fig. 2 – Grouping of substances with no significantly different cell toxicity: (a) freshly prepared and (b) 7 days preincubated specimens. Cell numbers were expressed as a percentage of controls (cultures without specimens), as described in the materials and methods. Vertical lines show means and standard deviations of three independent experiments, performed in triplicate; horizontal bars indicate no significant difference between substances. Stars indicate statistical significant difference from control ((*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.005$, because of multiple testing a $p < 0.005$ was considered to indicate statistical significance). Abbreviations as in Fig. 1; (1) Herculite, for comparison with Herculite and OBS and (2) Herculite, for comparison with Herculite and Prompt.

was to select one or more neighbouring spectroscopic peaks, as reference to the aliphatic C=C 1638 cm^{-1} in the expectation that these peaks were from groups not participating directly in the polymerization process.

2.4. *Statistical evaluation (measurement of degree of conversion)*

The pair-wise DC data (anaerobic/aerobic) were evaluated by Student's *t*-test, at the significance level of $p = 0.01$.

3. Results

3.1. *Cytotoxicity of bonding substances in combination with composites*

Results demonstrate that the cytotoxicity (Fig. 1, Tables 2 and 3) of all six tested composites (Table 1) was significantly different ($p < 0.0001$). Bonding substances had no statistically significant influence on the cytotoxicity of composite materials ($p = 0.159$; Null hypothesis 1 was confirmed). The results shown in Fig. 1 demonstrate that all freshly prepared materials show reduced cell numbers (for all materials, means are below 100%) compared to controls ($p < 0.005$). Rank orders concerning the degree of cytotoxicity have been established by grouping materials with no statistically significant different effects (Fig. 2). The cytotoxicity of all substances diminished after 7 days of preincubation ($p < 0.0001$).

3.2. *Degree of conversion of bonding substances with and without air inhibition*

Pairwise comparisons between each member of the groups (i) and (ii) showed a highly significant ($p < 0.01$; Null hypothesis 2 was rejected) statistical reduction in the degree of conversion for each resin cured under air inhibition conditions (Table 4).

4. Discussion

In this study, the cytotoxicity of six composite materials was tested alone or in combination with bonding substances. Our data show that all tested bonding substances had no statistically significant influence on the cytotoxicity of composite materials.

Cytotoxicity of composite materials is dependent on the release of substances (unbound free monomers and leachable components released due to degradation or erosion over time [26]) which is a function of their formulation [27]. Materials with traditional methacrylate chemistries were severely cytotoxic, whereas materials with newer chemistries or filling strategies (ormocer, silorane and transparent material) improved over time of aging in artifical salvia [27]. In our study the least cytotoxic material was also an ormocer (Definite). All other materials tested had traditional methacrylate chemistries with the exception of Admira, a modified ormocer with polar carboxylic groups. Admira was significantly more cytotoxic than Definite.

A variety of cytotoxicity data have been produced with bonding substances. Contradictory results of cell culture studies are due to different experimental designs in most cases [28]. Specimens for extracts with cell culture medium have been polymerized on cellulose strips [1,3]. In this design, bonding was applied as a thin layer comparable to the clinical situation. The inhibtion layer was not removed which resulted in severe cytotoxic effects. Evidently, uncured bonding materials are cytotoxic [4–7]. In various studies, a diffusion barrier was established between uncured bonding materials and cell cultures, which resulted in reduced or no cytotoxicity [8,9]. Kaga et al. [17] produced polymerized specimens in glass tubes covered with transparent celluloid strips which were almost non-cytotoxic. It can be assumed that these specimens lacked or had only a minimal inhibition layer.

In our previous study, bonding substances were applied on polymerized composite specimens with a brush and light cured [18] and were found to be severely cytotoxic. In the present study we established an experimental design, which mimics the situation in the oral cavity more closely. Bonding substances were applied onto polyethylene strips and light cured. Specimen moulds were then placed onto the bonding substances, filled with composite and light cured, resulting in a further polymerization of uncured bonding monomers. With this method, bonding materials did not enhance the cytotoxicity of composite materials when tested in combination. Both studies were perfomed with the same cytotoxicity test method. The reduction of mouse fibroblast cell numbers by incubation with the test substances in comparison to controls (cultures with glass specimens) was used as a measure for cytotoxicity. In the field of biocompatibility testing, methods measuring cell number or growth are generally accepted as cytotoxicity tests [29]. The comparability of cytotoxicity results obtained with L-929 mouse fibroblasts and human gingival fibroblasts has been demonstrated previously [28,30]. The main difference in the experimental setup between the present study and our previous cytotoxicity test with bonding substances [18] lies in various procedures for specimen production. Therefore, in this study the effect of these different conditions on the degree of conversion of bonding substances was investigated. The degree of conversion of bonding substances with air inhibiton, resembling to conditions used in our previous study [18] was compared to the degree of conversion of bonding substances without air inhibition. The results show clearly that air inhibition reduces the degree of conversion between 37.9% and 47.2%. These results indicate that the enhanced cytotoxicity obtained with our previous test method [18] is most likely due to unpolymerized substances in the inhibition layer. Rueggeberg and Margeson [31] have shown that an unfilled bonding resin of an earlier generation (command bonding resin, Kerr Manufacturing, Romulus, MI) had almost three times the conversion after addition and subsequent curing of the overlying composite material as that cured after blown thin with room-air. They revealed by back scatter electron imaging that the inhibited layer of the cured bonding resin was absorbed into the overlying filled composite [31]. Another explanation for the increased conversion of the bonding upon application and curing of the composite is the heat generated by the composite, which is conducted into the bonding and causes increased segmental mobility of potential reactive sites and thus higher conversion rates [31].

In conclusion, we have shown that with an experimental setting which mimics the situation in the oral cavity more closely, combined bonding/composite specimens show similar cytotoxicity to composite specimens alone. Our study further shows that standardized experimental protocols for cytotoxicity tests are clearly needed to obtain comparable results with relevance for risk assessment of dental materials in line with existing standards (prEN ISO 10993-5 [32] and ISO 7405 [33]).

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