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Cytotoxic and mutagenic effects of dental composite materials

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Abstract

Mutagenicity of single compounds of dental resinous materials has been investigated on many occasions before, but the induction of mutagenic effects by extracts of clinically used composites is still unknown. Here, cytotoxic effects and the formation of micronuclei were determined in V79 fibroblasts after exposure to extracts of modern composite filling materials (Solitaire, Solitaire 2, Tetric Ceram, Dyract AP, Definite). For cytotoxicity testing, test specimens were aged for various time periods (0, 24, and 168 h), and V79 cells were then exposed to dilutions of the original extracts for 24, 48, and 72 h. The ranking of the cytotoxic effects of the composites according to EC50 values after a 24-h exposure period was as follows: Solitaire (most toxic) = Solitaire 2 < Tetric Ceram < Dyract AP < Definite (least toxic). Cytotoxicity was independent of the period of aging for each composite, but varied with exposure periods. The cytotoxic effect of Solitaire increased about two-fold between exposure periods of 24, 48, and 72 h, no changes were observed with Solitaire 2, and cytotoxicity of Tetric Ceram, Dyract AP, and Definite was reduced. Even eight-fold diluted original extracts of freshly mixed Solitaire test specimens increased the numbers of micronuclei about 10-fold, and Solitaire 2 was slightly less effective. The mutagenic effects of these materials were reduced in the presence of a rat liver homogenate (S9). Weak increases of the numbers of micronuclei were detected only with undiluted extracts of Tetric Ceram and Dyract AP, but Definite was not effective. Our findings suggest that mutagenic components of biologically active composite resins should be replaced by more biocompatible substances to avoid risk factors for the health of patients and dental personnel.

Keywords: Dental resin; Cytotoxicity; Mutagenicity; TEGDMA

1. Introduction

The understanding of the biocompatibility of dental composite materials has greatly improved mostly because of in vitro investigations using a great variety of experimental parameters. Acute cytotoxic effects in mammalian cell cultures were, for instance, vastly modified by the modes of sample preparation, curing conditions, aging of samples, and preparation of extracts [1–3]. Non-cured resinous materials were considered more cytotoxic than cured, and the effect of aging of test specimens on the cytotoxicity varied immensely [4]. Cytotoxicity of dental composites has been firmly attributed to the release of residual monomers because of degradation processes or incomplete polymerization of the materials [2]. More than 30 different compounds have been extracted from polymerized dental composites, and among those, major monomers, comonomers, various additives, and reaction products were detected [5,6]. It has been shown elegantly that the removal of leachable components from polymerized composites by the use of organic solvents completely decreased cytotoxicity [7].

Major monomers, comonomers, and other substances have been identified as cytotoxic compounds [8]. Because of the low amounts of compounds usually released from resin-based materials into aqueous solutions, it is obvious that effects other than acute

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cytotoxicity are of extraordinary importance for the interpretation of cell responses. Recently, the ability of some compounds to modify essential cellular functions beyond acute cytotoxic concentrations led, for instance, to the discovery of the induction of heat shock proteins to compensate for significant protein damage, modifications of the responses of cells of the immune system or cellular damage with underlying genetic effects like the induction of gene mutations or chromosomal aberrations [9–12]. The comonomer triethylene glycol dimethacrylate (TEGDMA) was identified as a mutagenic compound in mammalian cells. The substance induced gene mutations probably because of the covalent binding to DNA via Michael addition [9,13]. TEGDMA and the monofunctional monomers methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA) also increased the numbers of micronuclei in V79 cells, which indicated clastogenic activities [10]. These findings provided experimental evidence for the mutagenic potentials of single compounds of dental resinous materials. Here, we hypothesized that biologically active monomers may be released from complete dental composite materials to cause the formation of micronuclei in mammalian cells. Therefore, we first analyzed cytotoxic effects of extracts of composite test specimens. These extracts were prepared from samples, which were aged for various time intervals after curing, and cell cultures were exposed for different time periods. This information on the cytotoxicity was imperative for the finding of appropriate concentration ranges to analyze the formation of micronuclei in vitro.

2. Materials and methods

2.1. Test materials, chemicals, and reagents

Minimum essential medium Eagle (MEM), crystal violet, ethyl methane sulfonate (EMS), cyclophosphamide (CP), and Accustain® Schiff's reagent were purchased from Sigma (Taufkirchen, Germany). Fetal bovine serum (FBS), penicillin/streptomycin, and trypsin came from Life Technologies, Gibco BRL (Eggenstein, Germany). A rat liver homogenate (S9 fraction) was obtained from RCC Cytotest Cell Research (Rossdorf, Germany). The dental composite materials tested in this investigation are listed in Table 1.

Table 1
Test materials and components

Material	Manufacturer	Components
Solitaire	Heraeus Kulzer	Multiacrylate monomers Porous filler particles
Solitaire 2	Heraeus Kulzer	UDMA Multiacrylate monomers Porous filler particles Glass
Dyract AP	Dentsply DeTrey	UDMA resin TCB resin T-resin (crosslinking agent) Glass
Tetric Ceram	Vivadent Ivoclar	Bis-GMA, UDMA, TEGDMA Barium glass
Definite	Degussa	Ormocer resin/dimethacrylates Barium glass Silica Apatite

2.2. Preparation of extracts

Samples of the various resin materials were prepared in glass rings (5 mm in diameter, 2.5 mm high). The materials were light-cured for 40 s (780 mW/cm^2) from one side. The specimens were then aged in the dark for 24 or 168 h (post-curing periods) in sterile plastic dishes in an air atmosphere at 37°C and 100% humidity or immediately extracted after curing. Extracts of these samples were prepared following the recommendations of ISO 10993-12 at a ratio of 117.8 mm^2 sample surface area/ml cell culture medium. In detail, 15 samples were extracted in 5 ml cell culture medium (MEM), 10% FBS supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 30 mg HEPES (pH 7.25) at 37°C and 5% CO_2. After a 24-h extraction period, these original extracts were then serially diluted in cell culture medium prior to cytotoxicity testing or testing for the induction of micronuclei.

2.3. Gas chromatography

Specimens of the composite material Solitaire 2 were prepared as described above, and extracted immediately after curing for 24 h in water. Gas chromatographic analysis was carried out using an Agilent Technologies 6890 N Network-GC-System fitted with a split/splitless injector suitable for solid-phase microextraction (SPME) analysis, an Agilent Technologies 5973 mass selective detector (MSD), and an Agilent Technologies 7683 autosampler. Helium was used as the carrier gas with a flow rate of 1.0 ml/min. The components were separated on a 30 m × 250 μm column with a 0.25 μm film of HP-5MS stationary phase (J + W Scientific). The injector temperature was set at 250°C and operated in the splitless mode for 5 min. The column was maintained at 50°C for 5 min, then ramped to 280°C at 25°C/min and held at 280°C for 10 min. A 65 μm polydimethylsiloxane-divinylbenzene (PDMS/DVB–CAR-PDMS) SPME fiber was used. Acetanilide was used as an internal standard (tracer), and TEGDMA was identified comparing retention time and mass spectra of the extracts with that of a reference substance.

2.4. Cytotoxicity testing

V79 Chinese hamster lung fibroblasts were routinely cultivated in MEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C, and 5% CO_2. In all, 5×10^3 cells were seeded into each well of a 96-well plate and incubated for 24 h at 37°C. Then, the cell cultures were exposed to 200 μl of serial dilutions of extracts of test specimens. Discarding the exposure medium stopped the exposure of the cell cultures after 24, 48, or 72 h. Cell survival after exposure was determined using a crystal violet assay [14]. 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 (EL311, Biotek Instruments, Burlington, VT, USA). Four replicate cell cultures were exposed to serial dilutions (at least six dilutions down to 1:32) of original extracts of the materials in three independent experiments.

Dose–response curves of cell survival values (expressed as optical density readings) and serial dilutions of the materials extracts were fitted to at least 24 individual values (Table Curve^{TM} 3.10, Jandel Scientific San Rafael, USA). The dilutions of the materials extracts causing 50% cell survival (EC50 values) were calculated from these dose–response curves. Differences between median EC50 values were statistically analyzed using the Mann–Whitney U-test ($\alpha = 0.05$). In addition, the EC50 values for all materials obtained from at least three experiments with varying periods of aging (0, 24, and 168 h) and exposure periods (24, 48, and 72 h) were subjected to linear regression analyses. The slopes of the regression lines were statistically analyzed (Durbin–Watson).

2.5. Micronucleus test in vitro

V79 cells (10^5 cells) were cultivated on microscopic glass slides in 4 ml cell culture medium for 24 h at 37°C and 5% CO_2. Test specimens of all materials were extracted in cell culture medium immediately after curing as described above. Then, V79 cells were directly exposed to serial dilutions of extracts for 24 h in the absence of a metabolically active homogenate from rat liver (S9). In addition, the influence of S9 on the formation of micronuclei was analyzed after a 4-h exposure. Here, V79 cells were exposed to extracts in cell culture medium supplemented with S9-Mix (0.83 mmol/l glucose 6-phosphate, 0.67 mmol/l $NADP^+$, 0.83 mmol/l $MgCl_2$, 11.35 mmol/l KCl, 0.67 mmol/l HEPES (pH 7.2), and S9 fraction (1 mg protein/ml)). As a consequence of the addition of S9, the original extract of each test material (1.0) was diluted to 0.83. Replacing the exposure medium with fresh medium stopped the treatment of cell cultures, and the cell cultures were reincubated for 24 h. The slides were then washed in phosphate-buffered saline free of calcium and magnesium (PBS-CMF). The cells were fixed in 100% ethanol for 30 min and air-dried. Then, cells were lysed in 5 N HCl for 40 min, rinsed with demineralized water, and stained with Accustain® Schiff's reagent for 30 min at room temperature. The numbers of micronuclei were analyzed microscopically in 1000 cells/slide of three parallel cultures (slides) per concentration in three independent experiments as described elsewhere [10]. An extract of a material was considered positive if at least a three-fold increase of the numbers of micronuclei over negative controls was observed at one or more dilutions of the original extract [15]. EMS and CP (in the presence of S9) served as positive control substances [10,16].

3. Results

3.1. Cytotoxicity of extracts of dental composite materials

Results typical for the effect of the aging periods of test specimens on the cytotoxicity of the various composites are shown with extracts of Tetric Ceram test specimens (Fig. 1). In a linear regression model, the slopes of the regression lines for EC50 values of the various exposure periods (24, 48, and 72 h) were not statistically significant different from zero as calculated by Durbin–Watson statistics. Thus, these findings indicated independence of the cytotoxicity from the periods of aging of Tetric Ceram test specimens under

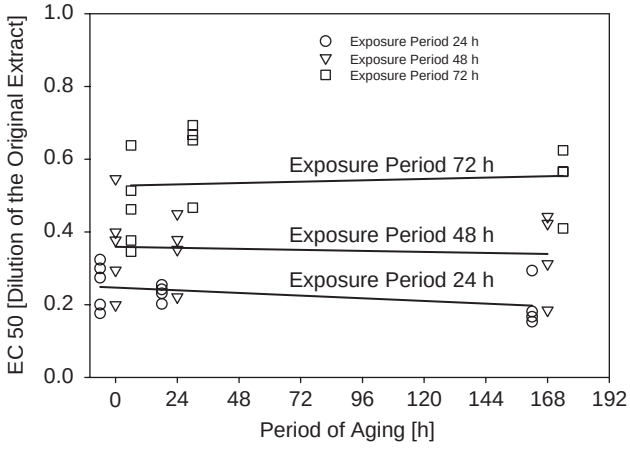

Fig. 1. Cytotoxicity of a dental composite material (Tetric Ceram) in V79 cells. The material was aged for various time periods after curing, and test specimens were extracted in cell culture medium for 24 h. The cell cultures were exposed to extracts of the material for various exposure periods (24, 48, and 72 h). EC50 values were calculated from individual dose–response curves in three independent experiments.

the current experimental conditions. Identical observations were made with all other materials (not shown). Therefore, EC50 values derived from experiments with test specimens aged for the various periods of time (0, 24, and 168 h) were combined to analyze the influence of different exposure periods on cytotoxic effects. We found that extracts of Solitaire were the most toxic media tested here (Fig. 2). After a 24-h exposure period the cell survival rates were reduced to 50% by extracts diluted more than eight-fold (factor 8.2) as calculated from dose–response curves. The cytotoxicity of Solitaire extracts even increased (factor 2) significantly between exposure periods of 24 and 48 h as well as between 24 and 72 h. Very similar effects were observed with extracts of Solitaire 2 after a 24-h exposure period; however, cytotoxicity was almost constant after long periods of exposure (Fig. 2). Since Solitaire 2 tested very toxic here, we analyzed aqueous extracts for the release of water-soluble compounds using gas chromatography–mass spectrometry (GC–MS). TEGDMA was by far the most abundant leachable detected as shown in a representative gas chromatogram (Fig. 3). A concentration of 0.66 mmol TEGDMA/l was found in undiluted extracts after a 24-h extraction period of Solitaire 2 test specimens. Extracts of Tetric Ceram were significantly less cytotoxic (factor 2) than extracts of Solitaire and Solitaire 2 after a short exposure period. In contrast to Solitaire and Solitaire 2, the cytotoxicity of extracts of Tetric Ceram significantly decreased (factor 1.6–2.4) with increasing exposure periods (Fig. 2). Cytotoxicity of extracts of Dyract AP was similar to those of Tetric Ceram; however, a significant reduction of this effect was observed after a 72-h exposure period only (Fig. 2). Extracts of Definite were only weakly cytotoxic compared to all other materials. The undiluted extracts of test specimens reduced cell viability to about 50% after a 24-h exposure, and cytotoxicity was significantly reduced after a 72-h exposure period (Fig. 2).

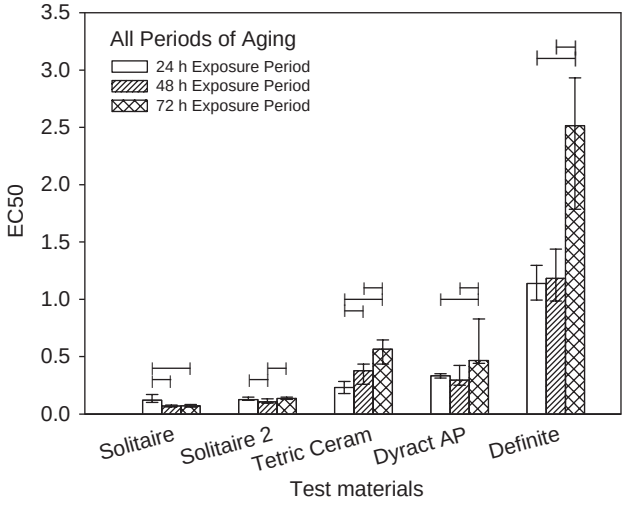

Fig. 2. Cytotoxicity of dental composites in V79 cells after various exposure periods. EC50 values (medians and 25th and 75th percentiles) for all periods of aging (0, 24, and 168 h) were combined for each exposure period (24, 48, and 72 h). EC50 values were calculated from individual dose–response curves in nine independent experiments ($n = 9$) for each material. Horizontal bars indicate statistically significant differences between EC50 values of the experimental groups.

3.2. Formation of micronuclei by extracts of dental composite materials

Reproducible and dose-related increases in micronuclei frequencies induced by diluted extracts of Solitaire and Solitaire 2 were observed after a direct exposure (without S9) for 24 h (Fig. 4). The numbers of micronuclei were increased about 10-fold by Solitaire extracts diluted by a factor of 16, and a mutagenic activity was still present with extracts diluted more than 30-fold. Extracts of Solitaire 2 were slightly less effective (factor 2) than those of Solitaire (Fig. 4). In contrast to these composites, only weak effects were observed with other resinous materials. Elevated numbers of

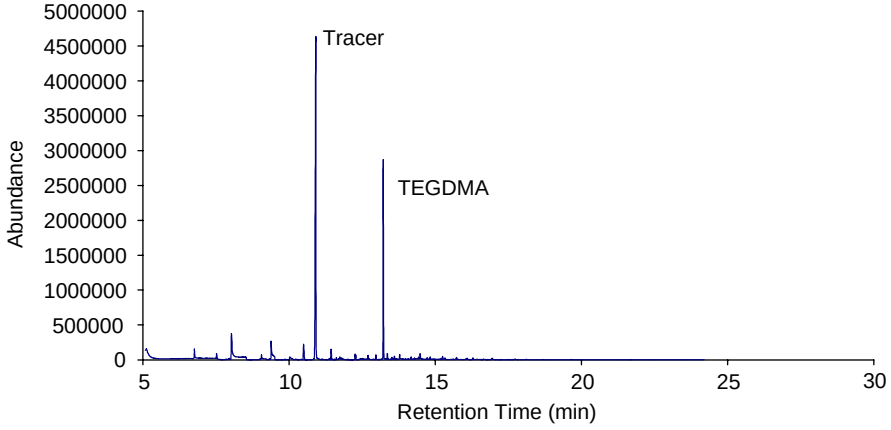

Fig. 3. Gas chromatographic profile of extracts of Solitaire 2 sampled by SPME. Gas chromatographic analysis was carried out as described in Section 2.3.

micronuclei were detected only after exposure of cells to undiluted extracts of Tetric Ceram, and Dyract caused similar effects. Extracts of Definite did not induce elevated numbers of micronuclei after a 24-h exposure period under the current experimental conditions (Fig. 4). Besides a considerable increase of the numbers of micronuclei, severe damage of V79 cell nuclei was observed in cell cultures treated with extracts of Solitaire. String like chromatin connections between separating chromosomes indicated the formation of nucleoplasmic bridges (Fig. 5). These structural abnormalities might result from chromosome breakage, which lead to the formation of chromosomes with more than one centromere [17].

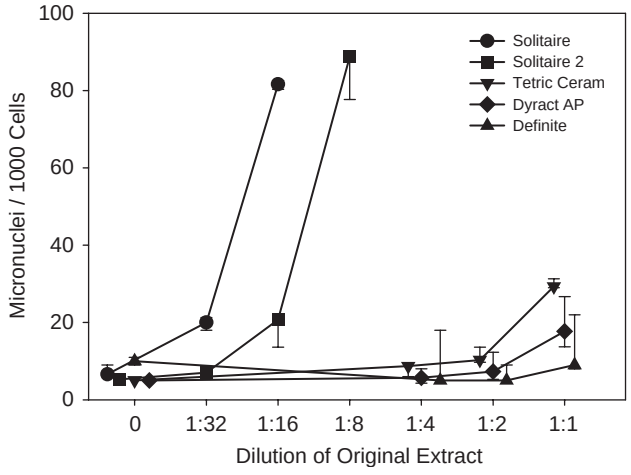

Fig. 4. Induction of micronuclei in V79 cells after exposure to extracts of dental resin materials. Original extracts (1:1) were serially diluted with cell culture medium as indicated. V79 cell cultures were exposed for 24 h, and the numbers of micronuclei (minimum, median, and maximum values) observed in three independent experiments are presented ($n = 3$).

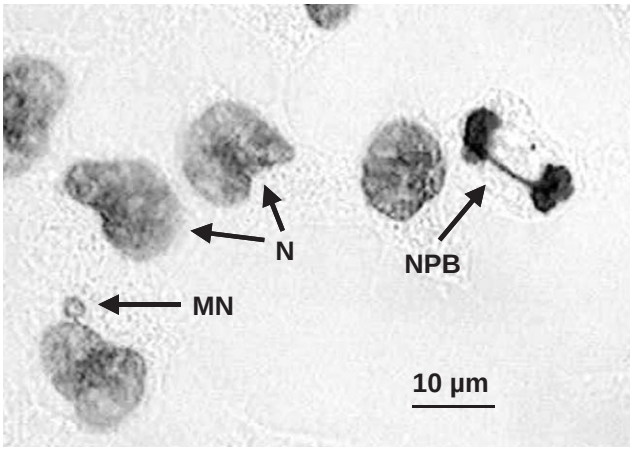

Fig. 5. Nucleoplasmic bridge formation in V79 cells after exposure extracts of Solitaire. Structures containing DNA were stained using Schiff's reagent. MN, micronucleus; NPB, nucleoplasmic bridge; N, nucleus.

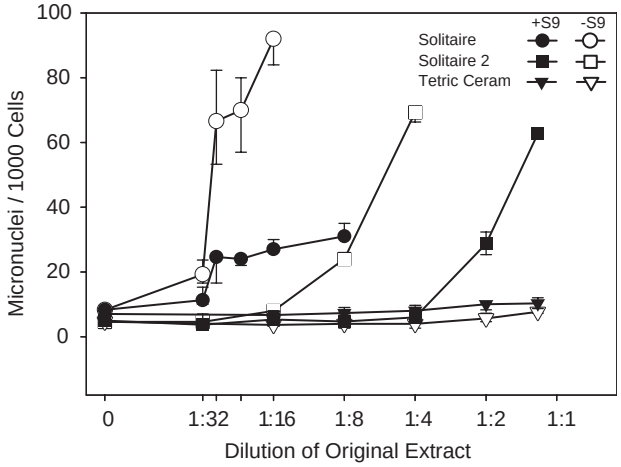

Fig. 6. Induction of micronuclei in V79 cells after exposure to extracts of Solitaire, Solitaire 2, and Tetric Ceram. Original extracts (1:1) were serially diluted with cell culture medium as indicated. V79 cell cultures were exposed for 4 h both in the presence and in the absence of a homogenate from rat liver (S9). The numbers of micronuclei (minimum, median, and maximum values) observed in three independent experiments are presented ($n = 3$).

The formation of micronuclei in V79 cells after exposure to extracts of the materials was also analyzed in the presence and absence of a liver homogenate (S9 fraction) after a short exposure period (4 h) (Fig. 6). The mutagenic potentials of the directly (without S9) active extracts of Solitaire and Solitaire 2 were reduced compared to the long exposure period, and no elevated numbers of micronuclei were detected with Dyract AP (not shown) and Tetric Ceram (Fig. 6). Moreover, the directly acting materials Solitaire and Solitaire 2 were inactivated in the presence of a S9 fraction. Inactivation of extracts of Solitaire 2 was notable in particular since the numbers of micronuclei about 10-fold higher than control values (dilution 1:4) after direct testing (without S9) were completely reduced in the presence of S9 (Fig. 6). We observed a weak but reproducible elevation of the numbers of micronuclei caused by Dyract AP (not shown) and Tetric Ceram in the presence of S9 (Fig. 6).

4. Discussion

Experimental details like the modes of sample preparation, curing conditions for mixed materials, aging of samples, and the preparation of extracts in various solvents greatly influence the cytotoxic effects of dental materials in mammalian cell cultures. Here, various periods of aging after curing of dental composite materials and the variation of exposure periods of mammalian cell cultures were analyzed for their influence on the cytotoxicity of the materials extracts. We found that test specimens of the diverse composite

materials, which were extracted for 24 h were cytotoxic in V79 cells, but the degree of cytotoxic effects varied immensely. Related to a 50% reduction of cell survival, extracts of Solitaire and Solitaire 2 were at least 10-fold more active than those of Definite after a 24-h exposure period. Tetric Ceram and Dyract were less effective than the Solitaire materials but more toxic than Definite. This overall ranking corresponds very well with previous observations on the cytotoxicity of these materials [2].

All samples were cured for the same period of time (40 s), and aging periods were extended up to 1 week. It has been discussed that an increase of the periods of aging will lead to an elevated rate of monomer conversion. Less amounts of leachable residual monomers, and a decrease of cytotoxicity because of aging of a cured material have been reported [6,18]. In the present investigation each material appeared to release the same amounts of toxic compounds after various periods of aging because an influence of the post-curing time of the materials on their toxicity was not detected. It is known that oxygen inhibited free-radical polymerization of dental polymers leads to a lower degree of monomer conversion [19]. Here, the test specimens were not covered with a polyester film during preparation, and all samples were not polished after curing. Therefore, it cannot be ruled out that a surface layer of incompletely polymerized material was formed under the current experimental conditions. The sample preparation in the present investigation does not represent an ideal processing procedure but is close to curing conditions which may occur in clinical situations where a temporary contact between the untreated material surface and the patient or dental personnel is possible.

On the other hand, only a little decrease in cytotoxicity of extracts of some compomers after aging and polishing has been observed earlier [4]. In addition, it appears that the effect of aging might depend on the chemical nature of the various materials. Reduced cytotoxicity of Solitaire, Tetric Ceram, and Dyract was not detected after aging of the materials in artificial saliva for 2 weeks but Definite was less active [2]. We also modified the time of exposure of cells to extracts in order to get insight into the capacity of the cells to compensate for toxic effects. The influence of the exposure periods on the cytotoxicity of extracts was inhomogeneous. Cytotoxicity of Solitaire was increased after a long (72 h) exposure period indicating that treated cell cultures were unable to recreate from severe cell damage. In contrast, cell survival rates increased with extended exposure periods when extracts of Dyract AP, Tetric Ceram, and Definite were tested. Increased cell survival after long exposure periods could arise because of the inactivation of the cytotoxic compounds by enzyme activities of the V79 cells. It has been reported that esterase activities were able to cleave some dimethacrylates leading to the inactivation of the chemicals [20,21]. Likewise, acrylates released from composite materials may be inactivated by binding to intracellular glutathione [22]. In addition, it cannot be ruled out that cytotoxicity was reduced because of unstable toxic compounds extracted from the materials.

There is improving knowledge of chemicals responsible for the induction of toxic effects of aqueous extracts. According to the manufacturer's information, TEGDMA is a compound of Solitaire, Solitaire 2, and Tetric Ceram, and the water-soluble monomers HEMA and TEGDMA were also identified in extracts of Dyract AP [23]. The concentration of TEGDMA, which was detected in undiluted extracts after a 24-h extraction period of Solitaire 2 test specimens in the present investigation related very well to EC50 values of TEGDMA and the concentration range detected for the induction of micronuclei [8,10].

Here, diluted extracts of Solitaire and Solitaire 2 caused a dose-related increase in the numbers of micronuclei in mammalian cells after direct exposure. The highest numbers of micronuclei were detected at concentrations close to those, which reduced cell survival to 50%. Besides the formation of micronuclei, other chromosomal abnormalities resembling structures identified as nucleoplasmic bridges gave further evidence for the induction of chromosomal breakage and rearrangements [17,24]. Similar to the induction of cytotoxic effects, a weak induction of the numbers of micronuclei was detected with undiluted extracts of Tetric Ceram and Dyract AP test specimens. So far, we can only speculate on the chemical nature of the compounds, which might be responsible for the cause of the induction of micronuclei. Some compounds of the organic matrix of dental resinous materials have been tested for the induction of gene mutations and micronuclei in mammalian cells. TEGDMA and HEMA are candidate substances. It was found earlier, that TEGDMA and HEMA induced increases of the numbers of micronuclei in V79 cells [10]. Furthermore, a concentration of 1 mmol/l TEGDMA induced numbers of micronuclei very similar to those detected with the most effective concentrations of Solitaire and Solitaire 2. The effects caused by TEGDMA were completely absent in the presence of a metabolically active homogenate from rat liver (S9) [10]. Here, the effects of Solitaire and Solitaire 2 were reduced by S9 but the active compound was not completely inactivated under the current experimental conditions. Therefore, and because TEGDMA was found here in aqueous extracts of Solitaire 2 (Fig. 3), compounds like TEGDMA or substances with related structures might be active in extracts of Solitaire, Solitaire 2, and, maybe Tetric Ceram and Dyract AP, although the presence of some other active compounds yet to be identified cannot be ruled out.

5. Conclusion

The packable composites, Solitaire and Solitaire 2, tested most cytotoxic compared with other composite resins. Most important, these materials also induced elevated numbers of micronuclei indicating the induction of chromosomal abnormalities in mammalian cells under the current experimental conditions. Compared with these materials, only weak or even no mutagenic effects were detected with Tetric Ceram, Dyract AP, and Definite. These findings with a cell culture system cannot be directly extrapolated to in vivo situations. The assessment of potential risks for patients, dental personnel, and individuals working in the development and manufacturing of monomers and composite resins should be based on more information including exposure conditions. But these analyses are beyond the limited aim of the present investigation. However, our findings suggest that it is desirable to replace cytotoxic and mutagenic components of biologically active composite resins by more biocompatible substances to avoid a potential risk for the health of patients and dental personnel.

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