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# Induction of DNA double-strand breaks in primary gingival fibroblasts by exposure to dental resin composites

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ABSTRACT

Dental resin composites and their reactive monomers/co-monomers have been shown to elicit cytotoxic responses in human gingival fibroblasts (HGF), and their metabolic radical intermediates have the potential to attack the DNA backbone, which may induce DNA double-strand breaks (DSBs). In this study we have tested the cytotoxicity and induction of DSBs by the most common composite resin monomers/co-monomers: BisGMA, HEMA, TEGDMA, and UDMA in gingival fibroblasts using the sensitive γ-H2AX DNA repair focus assay. Our results show increasing monomer cytotoxicities in the order of BisGMA > UDMA > TEGDMA > HEMA, an order that was also observed for their capacity to induce DSBs. BisGMA at the $EC_{50}$ concentration of 0.09 mM evoked the highest rate of γ-H2AX foci-formation that was 11-fold higher DNA DSBs as compared to the negative controls that ranged between 0.25 and 0.5 γ-H2AX foci/HGF cell. Our results for the first time show that exposure to dental resin monomers can induce DSBs in primary human oral cavity cells, which underscores their genotoxic capacity.



## 1. Introduction

There is a permanent rise in employment of dental resin composites in restorative dentistry since 1990s, while there are concerns about their biocompatibility and biochemical stability in both patients and dentists (for review see [1]). Moreover, there is an increasing incidence of oral cavity carcinoma in many parts of the world, for instance an estimated 405,000 new cases of oral cancer worldwide, while 66,650 in the countries of European Union (EU) per year were diagnosed up to 2006 which translates to an increase of 51% [2]. However, the etiology of oral cancer remains unclear. Thus, besides the major risk factors such as smoking and drinking, there has been intensive research on the detection of tumor initiation potential and long-lasting effects by hazardous xenobiotics [1].

Dental resin composites, which consist of organic polymers with inorganic fillers have the potential to elicit genotoxic effects. The most commonly used monomers/co-monomers are: bisphenol-A-glycidylmethacrylate (BisGMA), hydroxyethylene methacrylate (HEMA), triethyleneglycoldimethacrylate (TEGDMA), and urethanedimethacrylate (UDMA), which are used as bonding resins and direct filling materials, e.g. in cements and dentin adhesives, and as sealing agents for inlays, crowns, and orthodontic brackets [1].

Unreacted monomers/co-monomers can be released from dental composites during insertion and even after polymerization by means of both physical and chemical processes, directly into the oral cavity [3,4] or via dentin microchannels into the pulp where they may reach millimolar concentrations [5,6]. In the pulp they can damage the resident cells and/or migrate further into the blood-stream [6]. Additionally, leaching monomers/co-monomers can, diluted by saliva, enter the digestive tract [7]. After cellular absorption of monomers/co-monomers, they can form radical intermediates that can be metabolised to epoxy compounds as detected by microsome assay [8]. Epoxy intermediates have, via radical formation, the potential to attack biomolecules among which the DNA is a critical target (e.g., the N7-position of guanine) [9]. Such DNA modification may increase the toxicity in exposed cells and may induce mutagenic/cancerogenic effects [1,10].

In addition, monomers/co-monomers have the potential to increase the levels of reactive oxygen species (ROS) [11]. ROS are known mediators of signaling cascades but elevated levels of ROS can disrupt the cellular redox balance, resulting in oxidative DNA damage and apoptosis in mammalian cells [1,11]. Along this line, ROS attack of DNA might induce adverse toxic effects in the affected cells and organisms like mutagenicity and genotoxicity

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[10,11] as well as embryo toxicity and teratogenicity [12]. Furthermore, dental resin composites and their leached reactive monomers/co-monomers can induce cytotoxic responses in human gingival fibroblasts (HGF) leading to reduced viability, plasma membrane damage, DNA fragmentation, and increased cell death [1,13]. Processes that may induce mutations and cancer, when the compounds reach adequate concentration [14]. Previous *in vitro* studies estimated that diffusion through the human dentin layer may lead to concentrations of leached monomers/co-monomers in the millimolar range, since 1.5–8 mM HEMA levels were noted in dentin [5]. TEGDMA levels of up to 4 mM were observed in the pulp [6] and may be high enough to induce significant harmful effects, as 5 mM TEGDMA can efficiently induce apoptosis *in vitro* [15].

Genotoxic effects of BisGMA, HEMA, TEGDMA, and UDMA have been demonstrated using the DNA single-strand break (SSB)-specific Comet assay in human lymphocytes and human salivary gland tissue cells [10,16]. Likewise, glycidylmethacrylate (GMA) was observed to elicit cytotoxic and genotoxic effects in human peripheral blood lymphocytes and cancer cells as detected by the SSB-specific Comet assay, the Annexin V apoptosis test and DNA double-strand break (DSB)-specific pulsed field gel electrophoresis [14]. Since the radical intermediates formed by the monomers/co-monomers noted above can attack DNA on both strands this may lead to DSB induction in HGFs and other exposed cells.

Here, we tested the genotoxic action of the most common monomers/co-monomers: BisGMA, HEMA, TEGDMA, and UDMA in HGFs using a γ-H2AX DSB assay that detects the phosphorylated (γ) histone H2AX molecules that form in chromatin surrounding DSBs [17,18]. Additionally, we investigated cytotoxicity, in order to determine the cytotoxic effects of these monomers/co-monomers in primary HGFs, which in the physiological situation are highly exposed to monomers/co-monomers after release from composites into the human oral cavity [19].

## 2. Materials and methods

### 2.1. Chemicals

The monomers/co-monomers triethyleneglycoldimethacrylate (TEGDMA; CAS-No. 109-16-0), bisphenol-A-glycidylmethacrylate (BisGMA; CAS-No. 1565-94-2), hydroxyethylene methacrylate (HEMA; CAS-No. 868-77-9), and urethanedimethacrylate (UDMA; CAS-No. 72869-86-4) were obtained from Evonik Röhm (Essen, Germany).

HEMA and TEGDMA were directly dissolved in medium. BisGMA and UDMA were dissolved in dimethyl sulfoxide (DMSO, 99% purity; Merck, Darmstadt, Germany) and diluted with medium (final DMSO concentration: <1%). Control cells received either DMSO (<1%) in medium, or 1 mM hydrogen peroxide ($H_2O_2$; VWR International, Darmstadt, Germany).

### 2.2. Cell culture and drug treatment

The human gingival fibroblasts (HGFs, Cat–No.:1210412) were obtained from Provitro, Cell-Lining (Berlin, Germany). The HGFs (passage 8) were grown on 175 $cm^2$ cell culture flasks to approximately 75–85% confluence and maintained in an incubator with 5% $CO_2$ atmosphere at 100% humidity and 37 °C. Quantum 333 medium supplemented with L-glutamine and 1% antibiotic/antimycotic solution (10,000 Units/ml penicillin, 25 mg/ml streptomycin sulphate, 25 μg/ml amphotericinB; PAA Laboratories GmbH, Cölbe, Germany) was used to culture HGFs. After reaching confluence the cells were washed with Dulbecco's phosphate buffered saline (PAA Laboratories), detached from the flasks by a brief treatment with trypsin/EDTA (PAA Laboratories).

### 2.3. XTT-based viability assay

We used the XTT-based cell viability assay to determine the half-maximum-effect concentration ($EC_{50}$) values for the investigated resin compounds in HGFs. HGFs at a concentration of 20,000 cells/well were seeded into a 96-well microtiter plate in 100 μl of medium, followed by incubation for 24 h. After removal of medium, the cells were treated with medium containing BisGMA (0.002–0.2 mM), HEMA (0.01–100 mM), TEGDMA (0.01–2.5 mM), and UDMA (0.02–2 mM) followed by incubation for 24 h. Control cells received either medium only or medium + DMSO (final DMSO concentration: <1%.). As a further negative control the cells were exposed to 1% Triton X-100 [20]. After incubation for 20 h, the cell monolayers were washed and a mixture of XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent (in RPMI without Phenol red) and electron-coupling reagent (PMS [*N*-methyldibenzopyrazine methyl sulphate] in phosphate buffered saline) was added as recommended by the supplier (cell proliferation kit II; Roche Diagnostics GmbH Penzberg, Germany) 4 h before photometric analysis. This assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells [20]. Therefore, this conversion only occurs in viable cells. The formazan formation was quantified spectrophotometrically at 450 nm (reference wavelength 670 nm) using a microtiter plate reader (Victor 3; Perkin Elmer Las GmbH Jügesheim, Germany). All experiments were repeated five times.

### 2.4. γ-H2AX immunofluorescence

We investigated DSB formation in HGF cultures unexposed and exposed to dental resin compounds by the γ-H2AX focus assay, which is a direct marker for DSBs [17,18,21]. For this microscopic assay 12 mm round cover slips (Carl Roth GmbH, Karlsruhe, Germany) were cleaned in 1 N HCl and distributed into a 24-well plate. HGFs were seeded at $7 \times 10^4$ cells/mL medium in each well followed by overnight incubation at 37 °C. The cells were exposed to medium containing the resins in the following concentrations: BisGMA (0.09; 0.03; 0.009 mM), HEMA (11.2; 3.7; 1.12 mM), TEGDMA (3.6; 1.2; 0.36 mM), and UDMA (0.1; 0.03; 0.01 mM) for 6 h. Since resin monomers/co-monomers released from dental restorative materials, like HEMA and TEGDMA, may reach millimolar concentrations in the pulp [6,22], we used three concentrations based on the $EC_{50}$ values in the millimolar range (1×, $^1/_3$× and $^1/_{10}$× $EC_{50}$). Negative control cells received either medium only (control for HEMA, TEGDMA) or medium with DMSO (control for BisGMA, UDMA) at a final concentration of <1% for 6 h. Positive control cells received 1 mM $H_2O_2$ in medium for 15 min.

For immunofluorescent staining, cells were first washed 2 × 5 min with PBS, fixed by adding 0.5 ml ice-cold 4% paraformaldehyde in PBS for 10 min at 4 °C, washed with cold PBS (4 °C) for 4 × 2 min, and permeabilized for 10 min with 0.5 ml of triton-citrate buffer (0.1% sodium citrate, 0.1% Triton X-100) at 4 °C. After washing 4 × 2 min with PBS, cells were blocked for 20 min with 4 drops of serum-free blocking buffer (Dako, Hamburg, Germany) per well at RT. Thereafter, cells were incubated with mouse monoclonal anti γ-H2AX (Millipore, Billerica, MA, USA) at 1:1300 dilution in antibody diluent (0.3 ml per well) (Dako, Hamburg, Germany) at 4 °C overnight. After 4 × 5 min washes with PBS at 4 °C, cells were incubated with FluoroLink Cy3-labelled goat anti-mouse secondary antibody (GE Healthcare, Munich, Germany) at a dilution of 1:1300 in antibody diluent (0.3 ml per well) for 1 h at RT in the dark. Cells were then washed 2 × 5 min in PBS, and rinsed 5 min with deionized water at RT.

Finally, the cover slips were each placed on 0.2 μl of a mixture of 1 ml Prolong antifade (Invitrogen, Karlsruhe, Germany) and 5 μl DAPI (Invitrogen) on a glass slide (76 × 26 mm; Carl Roth, Karlsruhe, Germany).

### 2.5. Image acquisition

HGFs were investigated using a Zeiss Axioplan 2 imaging fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a motorized filter wheel and appropriate filters for excitation of red, green and blue fluorescence. Images were obtained using a 63× and a 100× Plan-Neofluar oil immersion objective (Zeiss) and the ISIS fluorescence imaging system (MetaSystems, Altlussheim, Germany).

### 2.6. Data analysis

The values performed from the XTT-based viability assay were calculated as percentage of the 100% controls using Graph Pad Prism 4 (Graph Pad Software Inc., San Diego, USA), where they were plotted on a concentration log-scale and the range of the maximum slope was derived. Half-maximum-effect substance concentration at the maximum slope was revealed as $EC_{50}$. The $EC_{50}$ values were obtained as half-maximum-effect concentrations from the fitted curves. Data are presented as means ± standard error of the mean (s.e.m., $n = 5$). The statistical significance ($p < 0.05$) of the differences between the experimental groups was checked using the *t*-test, corrected according to the Bonferroni–Holm modification preferred by [23].

For quantitative analysis of the γ-H2AX test, foci were counted by the same investigator (E.U.) by eye down the fluorescence microscopic using a 100× objective. Disrupted cells were excluded from analysis. Cell counting was performed until at least 40 cells containing at least one focus were reached [21,24] and each experiment was performed at least 3 times. The mean number of cells scored and the standard deviation (SD) were calculated. Values were compared using the Student's *t*-test ($p < 0.001$).