



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 EC₅₀ 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.

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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 bloodstream [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/carcinogenic 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 cm² cell culture flasks to approximately 75–85% confluence and maintained in an incubator with 5% CO₂ 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 (EC₅₀) 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*-methylidibenzopyrazine 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 7×10^4 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 EC₅₀ values in the millimolar range ($1 \times$, $1/3 \times$ and $1/10 \times$ EC₅₀). 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 H₂O₂ 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 EC₅₀. The EC₅₀ 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 ($p < 0.001$).

Table 1
EC₅₀ values (mmol/L) and relative toxicities of HEMA, TEGDMA, UDMA and BisGMA as determined by XTT viability assay.

Substance	EC ₅₀	Relative toxicity
HEMA	11.20 (0.60)	1
TEGDMA	3.60 (0.20) ^a	3
UDMA	0.10 (0.04) ^{a,b}	112
BisGMA	0.09 (0.01) ^{a,b}	124

The EC₅₀ values of the tested monomers/co-monomers and their relative toxicities in HGFs. Values are represented as mean (±s.e.m.; n = 5).

^a significantly (p < 0.05) different to HEMA.

^b significantly (p < 0.05) different to TEGDMA.

3. Results & discussion

3.1. Exposure with resin monomers reduces cell viability

It was found that incubation with all compounds reduced cell viability in HGFs significantly (p < 0.05). The highest EC₅₀ value was found for HEMA after 24 h of exposure (EC₅₀ 11.20 ± 0.60; mean [mmol/L] ± s.e.m.; n = 5; Table 1). HEMA displayed a 124-fold lower toxicity in comparison to BisGMA that was significantly (p < 0.05) higher toxic compared to UDMA, TEGDMA, and HEMA (Table 1). These EC₅₀ values match closely with previous experiments [13]. The reduced cell proliferation may relate to altered cell cycle progression and cell viability by, e.g. resin monomer-induced oxidative stress and the subsequent activation of cell signaling cascades [11].

3.2. Dental resin composites induce DNA double-strand breaks

Recent work has indicated that the exposure of cells to BisGMA, TEGDMA, UDMA, or HEMA induces genomic damage leading to the

activation of the DNA damage responsive kinase ATM through its phosphorylation at Ser 1981 as observed by flow cytometry [11]. This is likely caused by the generation of DNA DSBs as a consequence of massive ssDNA break formation [10,14]. One of the early responses to DSBs is the ATM-dependent phosphorylation of histone H2AX at the C terminal Ser 139, then denoted γ-H2AX [18]. γ-H2AX immunofluorescent staining discloses microscopically visible foci at DSB sites enabling DSBs to be quantified [17,21].

Here we used the sensitive γ-H2AX focus assay to monitor whether DSBs are formed in HGFs after 6 h exposure to BisGMA, TEGDMA, UDMA, and HEMA. It was found that γ-H2AX foci were readily discernible in HGF nuclei by immunofluorescence using phospho-histone (γ) H2AX-specific antibodies (Fig. 1). Microscopic enumeration of γ-H2AX foci revealed that BisGMA, UDMA, TEGDMA and HEMA treatment induced DSB-specific γ-H2AX foci rates significantly above background values (Fig. 2; p < 0.001, Student's t-test). Six hours of exposure with BisGMA at 0.009 mM; UDMA at 0.03 mM or TEGDMA at 0.36 mM evoked similar rates of γ-H2AX foci-formation in HGFs, TEGDMA-induced foci rate at 1.2 mM was 2-fold higher than in cells treated with 1.12 mM HEMA (Fig. 2). Furthermore, the yield of the average number of induced foci/cell was positively correlated with increasing compound concentration for all substances (Fig. 2). The negative controls DMSO and medium cultures displayed 0.5–0.25 γ-H2AX foci/cell.

It was observed that BisGMA at 0.09 mM induced 2-fold higher DSBs rate as compared to UDMA at a similar concentration (0.1 mM) (Fig. 2). This DSB-forming effect may result from the significant ssDNA break induction capacity of BisGMA as observed in human lymphocytes [10]. BisGMA also displays a higher liposolubility than UDMA, which may lead to more effective cell penetration as compared to other monomers/co-monomers [25].

Likewise, inducing ~2-fold higher rates of DSBs with 1.2 mM TEGDMA relative to HEMA at 1.12 mM may be linked to TEGDMA's

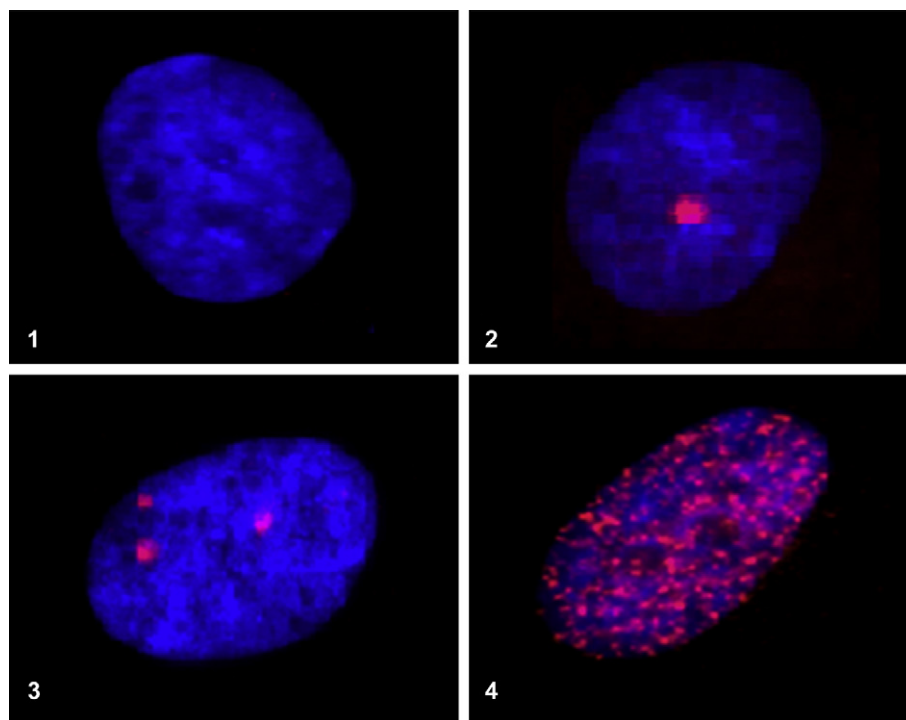


Fig. 1. Representative images of immunofluorescent staining for H2AX phosphorylation (red) in HGFs after a 6 h exposure to BisGMA, TEGDMA, UDMA, or HEMA, compared to control cells. While DAPI (blue) is a marker for DNA and stains the whole nucleus of a cell, the red γ-H2AX-specific foci reproduce as pink due to color overlay. Magnification in the original 1000×. (1) A nucleus of HGFs without foci, as is typically seen in most untreated cells. (2) A nucleus of HGFs with one focus, which can occur in treated cells (in this case with HEMA). (3) A nucleus of HGFs displaying three foci (in this case after TEGDMA treatment). (4) A nucleus of HGFs with more than 30 foci, induced by H₂O₂ treatment.

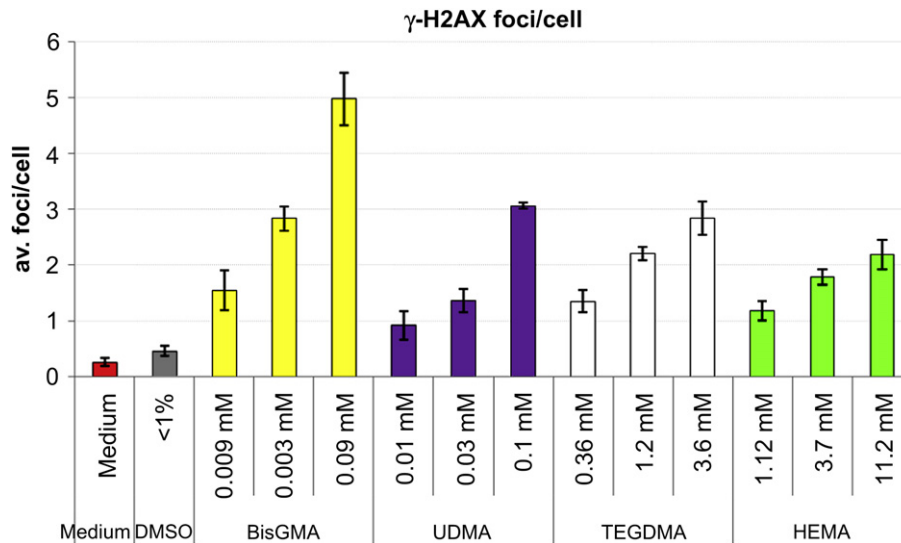


Fig. 2. Average of induced γ -H2AX foci per cell in HGFs elicited by a 6 h exposure to BisGMA, TEGDMA, UDMA, or HEMA. Negative control cells for HEMA and TEGDMA received medium, for BisGMA and UDMA they received DMSO at a final concentration of <1%. All results are the mean of least three independent experiments and error bars show the standard deviation.

efficiency to cause lipid peroxidation *in vitro* [26] and/or its ability to interact with the lipid bilayer of cell membranes in a surfactant-like manner [27], with both membrane effects being able to induce cell death [28].

The genotoxicity experiments presented here make it attractive to speculate about a risk assessment; we hypothesize in a worst-case scenario of 32 teeth that are filled with max. 0.5 g composite each involving BisGMA, the concentration of the theoretically released BisGMA could be up to 1.12 mg “free” BisGMA that affect the HGFs, which corresponds approximately 0.002 mmol/L. The suggestion is based on 0.07% of used BisGMA that is directly released from the commercial composite Z100[®] into the oral cavity [3] and the average portion of monomer/co-monomer amount in composites of ~10%, which equals to a total of 1.6 g in an oral cavity with 32 treated teeth, as supposed above. If we consider the daily saliva production as 1.0 L/d depending on drinking volume, sports and other factors [29], a BisGMA saliva concentration may account for 0.002 mM. This concentration is 4.5-fold lower than the BisGMA concentration that was able to increase the DNA DSBs after a 6 h exposure in cultured HGFs of our experiments. However, BisGMA elution into the saliva has been found to be non-linear with time and to remain at a high level for 7–28 days after polymerization [4]. This underscores the potential adverse effects of the monomers/co-monomers of dental materials, since they can be released into the oral cavity in a long period of time.

Additionally, organic solvents like ethanol, chloroform, and toluol can increase the elution of the monomers. While 50% of the leachable monomers are eluted within 3 h in water, 75% are eluted into ethanol–water mixture, within the same time span [30]. Along this line, 0.085 g of the commercial composite Tetric Ceram[®] polymerized for 20 s was shown to release BisGMA to levels of about 0.31 mM after exposure to a 75% ethanol aqueous solution [4]. In a worst-case assumption, if 32 teeth are replaced with 0.5 g per tooth Tetric Ceram[®], a total concentration of 59 mM BisGMA could release into an ethanol–water mixture. However, these assumed data should trigger no alarm, since normal alcoholic drinks contain significantly lower concentrations of ethanol and the contact time between the ethanol and the composite during a sip will be short-lived and restricted to a small contact surface.

Since our results show that dental resin monomers/co-monomers can induce severe DNA damage further studies

addressing the nature of DNA lesions and their repair elicited by dental monomers/co-monomers compounds are required to further estimate their carcinogenic potential.

4. Conclusions

Our investigation for the first time reveals the induction of DNA double-strand breaks by dental resin materials in human primary gingival fibroblasts exposed to EC₅₀ concentrations for a relatively short period. This directly demonstrates the genotoxic potential of resin monomers.

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Appendix

Figures with essential colour discrimination. Figs. 1 and 2 in this article may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.11.065.

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