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Induction of DNA strand breaks by dental composite components compared to X-ray exposure in human gingival fibroblasts

Jürgen Durner · Małgorzata Dębiak · Alexander Bürkle · Reinhard Hickel · Franz-Xaver Reichl

Abstract The toxicity of dental composites has been attributed to the release of residual monomers from polymerized resin-based composites due to degradation processes or incomplete polymerization. Some of these eluted substances have a genotoxic potential. We tested the hypothesis that realistic concentrations (and/or worst case concentrations/situations) of bisphenol-A-glycidyldimethacrylate (BisGMA), triethyleneglycol dimethacrylate (TEGDMA), 2-hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA) found in elution experiments can cause DNA strand breaks in human gingival fibroblasts (HGF). Such DNA damage was compared with that resulting from ionizing radiation coming from natural sources, dental radiography or tumor therapy. TEGDMA, HEMA and MMA did not induce DNA strand breaks at concentrations of up to 10 mM. About 24 h after incubation with 0.25 mM BisGMA, significantly more DNA strand breaks were found in HGF compared to controls. DNA strand breaks caused by 0.25 mM BisGMA, correspond to DNA strand breakage caused by irradiation with 4 Gy, only used in the high single-dose irradiation tumor therapy. But 0.25 mM BisGMA is more than 100-fold higher than that concentration found in worst case calculations. Therefore, our data did not support our hypothesis.

Keywords TEGDMA · HEMA · DNA-strand breaks · X-ray · Genotoxic potential

J. Durner · R. Hickel · F.-X. Reichl
Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestrasse 70, 80336 Munich, Germany

J. Durner (✉) · F.-X. Reichl
Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University of Munich, Nussbaumstrasse 26, 80336 Munich, Germany
e-mail: juergen.durner@med.uni-muenchen.de

M. Dębiak · A. Bürkle
Department of Biology, Molecular Toxicology Group, University of Konstanz, Jacob-Burckhardt-Str. 31, 78464 Constance, Germany

Introduction

Polymer-based dental materials are extensively used in prosthodontics as filling and adhesive materials. Composite materials consist of an inorganic and an organic component, connected by coupling agents. During light-induced hardening, the unsaturated acrylic compounds will be converted into a network of an organic and inorganic matrix. The degree of conversion is generally in the range of 55–65% (Miletic and Santini 2008). Due to the low degree of conversion, components of the composite materials can be eluted and swallowed with the saliva (Spahl et al. 1998; Uzunova et al. 2008). Elution experiments revealed that the substances bisphenol-A-glycidyldimethacrylate (BisGMA), 2-hydroxyethyl methacrylate (HEMA), triethyleneglycol dimethacrylate (TEGDMA) and methyl methacrylate (MMA) have the highest propensity to get eluted (Polydorou et al. 2007). It should be noted that these eluted substances may cause adverse local and systemic effects including allergic reactions (Goon et al. 2008). In vitro studies revealed estrogenic, mutagenic, teratogenic and genotoxic effects of composite components (Di Pietro et al. 2008; Geurtsen et al. 1998; Schweikl et al. 2006; Schwengberg et al. 2005). DNA damage caused by methacrylate monomers is of special significance due to the toxicology and biocompatibility of these substances. Under physiological situation, human oral

cells (e.g., gingival and/or pulp fibroblasts) are among the first to get in contact with eluted substances. The genotoxic potential of some dental methacrylates could be shown in a large number of investigations during the last decade (Schweikl et al. 1998, 2006). The metabolism of dental methacrylates can lead to the formation of epoxides like 2,3-epoxymethacrylic acid, which was found as an intermediate in the metabolism of methacrylic acid (Durner et al. 2009; Seiss et al. 2007). Epoxides are regarded as very toxic (predominantly carcinogenic/mutagenic) agents that can induce DNA double-strand breaks (Klaassen 1998). DNA double-strand breaks are considered as the most toxic type of DNA lesion (Mahaney et al. 2009). If they are left unrepaired they can cause cell death; if they are misrepaired they may lead to chromosomal translocations and genomic instability (Povirk 2006).

DNA strand breaks can be quantified by the 'Fluorimetric Detection of Alkaline DNA Unwinding (FADU) assay described by Birnboim and Jevcak (1981). One of us has recently described an automated, high-throughput FADU assay format that led to reduction in the numbers of cells needed and faster completion of the assay (Moreno-Villanueva et al. 2009).

It is known that ionizing radiation can also lead to DNA double-strand breaks. Based on realistic concentrations and also on worst case calculations of elutable amounts of dental methacrylates, the aim of this study was to test the hypothesis that in these concentrations the methacrylates BisGMA, TEGDMA, HEMA and MMA can cause DNA damage (single- or double-strand breaks) in human gingival fibroblasts (HGF). Such DNA damage was compared to that induced by ionizing radiation caused by natural background radiation, dental radiography and radiation in tumor therapy.

Materials and methods

Chemicals

TEGDMA, HEMA and BisGMA were purchased from ESPE (Dental AG, Seefeld, Germany), MMA from Merck (Darmstadt, Germany). Cell culture supplements including penicillin/streptomycin and trypsin/EDTA were obtained from Gibco (Eggenstein, Germany), and fetal calf serum from Biochrom AG (Berlin, Germany). TEGDMA, HEMA and MMA were directly dissolved in medium. BisGMA was dissolved in DMSO and diluted in medium (final DMSO concentration <1%).

X-ray exposure

Treatment of pulp and gingival fibroblasts for FADU experiments: For the irradiation, a X-ray generator from CHF Müller (Hamburg, Germany) was used. The dose to be delivered was applied by variation of irradiation time at a fixed dose rate. The irradiation parameters were the following: 70 keV energy, 1.25-mm aluminium filter and 9.4 mA current. The dose was measured using a PTW Universal Dosimeter UNIDOSE [D545.151.00/02] from PTW (Freiburg, Germany). About 70 µl of cell suspension (at 200 cells/µl) in a 96-well plate was irradiated on ice in a custom-made ice box (Moreno-Villanueva et al. 2009).

Human gingival fibroblasts (Primary HGF-1, American Type Culture Collection, Rockville, MD, USA), passage number 6, were cultivated in plastic cell culture plates at 37°C and 5% CO_2 in a humidified atmosphere (Reichl et al. 2008a, b). Cells were maintained in Quantum333 Medium for fibroblasts (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 50 IU/ml penicillin and 50 µg/ml streptomycin. For experiments, 0.5–1 × 10^6 cells were seeded in 6-cm cell culture plates and allowed to recover for 48 h. Then medium was replaced with fresh medium containing a test compound. Cells were incubated under standard culture conditions for 1 or 24 h. Cells were harvested with trypsin/EDTA, washed with PBS and the automated FADU assay was performed, essentially as described [17]. Briefly, cells were resuspended in suspension buffer (14 mM β-mercaptoethanol, 250 mM *meso*-inositol, 1 mM $MgCl_2$, 10 mM sodium phosphate buffer, pH 7.4) at the final cell titer of 2 × 10^5 cells/ml. About 70 µl of cell suspension was transferred to 96-well plate. About 70 µl lysis buffer (9 M Urea, 10 mM NaOH, 2.5 mM 1,2-cyclohexanedinitrilotetraacetic acid, 0.1% SDS) was added at rate of 150 µl/s, and samples were incubated for 12 min at 0°C. Pre-chilled alkaline buffer (42.5% lysis buffer, 0.2 M NaOH) was overlaid on top of the cell lysate at a rate of 10 µl/s. Alkaline unwinding was performed at 30°C for 60 min, then 140 µl neutralization buffer (1 M glucose, 14 mM β-mercaptoethanol) was added at a rate of 200 µl/s. Following incubation at 22°C for 30 min, 156 µl SybrGreen solution (1:8,333 v/v in H_2O; MoBiTec, Göttingen, Germany) was added. Samples were mixed by automatic pipetting up and down. Fluorescence was measured in a 96-well plate reader by excitation at 492 nm and emission at 520 nm. For each sample (P_x), an internal control of DNA content (T_x) was incorporated without alkaline unwinding process. Here, the unwinding was prevented by addition of neutralization buffer to samples prior to the addition of alkaline buffer. For each sample, eight P and eight T values were analyzed in parallel. Relative DNA integrity was calculated as (mean P_x/mean T_x) × 100%.

Calculations and statistics

The data are presented as means ± standard error of mean (SEM). The statistical significance ($p < 0.05$) of the

differences between the experimental groups was tested using the t test, corrected according to Bonferroni-Holm (Forst 1985).

Declaration

These experiments comply with the current laws of Germany.

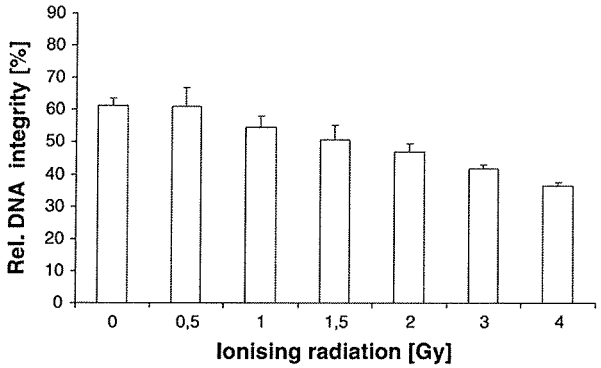

Fig. 1 Dose-dependent induction of DNA strand breaks by X-rays in gingival and pulp fibroblasts. Data are presented as means ± SEM from three independent experiments ($n = 3$)

Results

X-ray exposure

Unirradiated controls of HGF showed DNA unwinding at 38.6% (Fig. 1). X-irradiation of HGF with up to 1.5 Gy showed no significant difference in DNA strand breaks compared to controls. Irradiation with >1.5 Gy caused a significant ($p < 0.05$) increase in single-stranded DNA compared to controls. An irradiation with 4 Gy resulted in unwinding of 63.7% DNA in HGF (Fig. 1).

TEGDMA, HEMA and MMA exposure

One hour after incubation of HGF with concentrations up to 10 mM TEGDMA, HEMA or MMA, no significant differences in DNA strand breaks were found compared to controls (Fig. 2).

About 24 h after incubation of HGF with concentrations up to 10 mM TEGDMA, HEMA or MMA, no significant difference in DNA strand breaks was found compared to controls (Fig. 2).

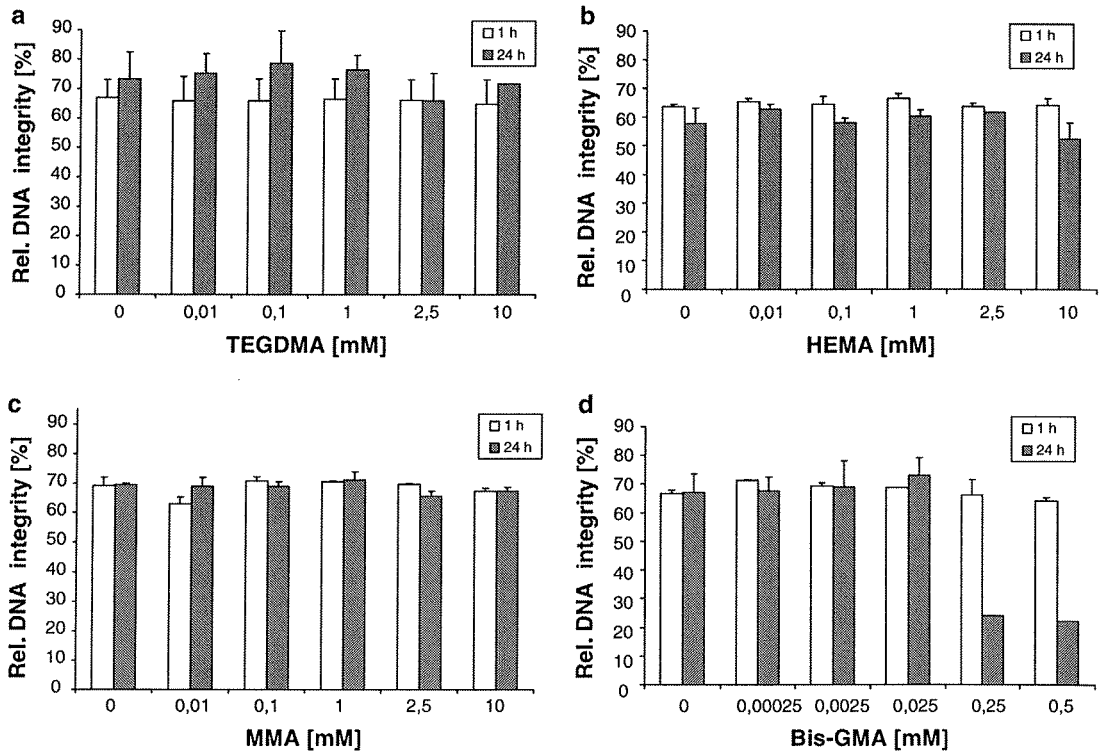

Fig. 2 HGF cells: Dose and time-dependent induction of DNA strand breaks caused by **a** triethyleneglycol dimethacrylate (TEGDMA), **b** 2-hydroxyethyl methacrylate (HEMA) **c** methyl methacrylate (MMA), and **d** bisphenol-A-glycidyl dimethacrylate (BisGMA), when incubated with human gingival fibroblasts for 1 or 24 h at 37°C. Data are presented as means ± SEM from three independent experiments ($n = 3$)

BisGMA exposure

One hour after incubation of HGF with concentrations up to 0.5 mM BisGMA, no significant ($p < 0.05$) difference on DNA strand breaks was found compared to controls (Fig. 2).

About 24 h after 0.25 mM BisGMA exposure in HGF, BisGMA caused a significant ($p < 0.05$) increase in DNA unwinding (75.8%) compared to controls (32.9%) (Fig. 2).

The following order of increased toxicity/DNA strand breaks was observed in HGF (24 h of exposure) TEGDMA $\approx$ HEMA $\approx$ MMA < BisGMA.

Discussion

Direct composite tooth-colored fillings are in widespread use and have reached a satisfactory level, aesthetically and functionally, to replace decayed or lost tooth substance. However, the biocompatibility of these materials is not fully satisfactory. It has been demonstrated that substances released from composites can enter the organism (Durner et al. 2009). Special attention was given to studies addressing the genotoxic potential of these materials, e.g., DNA strand break induction (Kleinsasser et al. 2006; Schwengberg et al. 2005).

Our experiments were performed to measure DNA strand breaks caused by the methacrylates TEGDMA, HEMA, MMA and BisGMA compared to DNA strand breaks caused by irradiation doses used in dental radiography, tumor therapy or from natural background radiation. DNA strand breaks caused by irradiation are well studied (Mahaney et al. 2009). An irradiation with 3 Gy causes in diploid cells about 1,000 single-strand breaks and 60 double-strand breaks (Mitzel-Landbeck et al. 1976). The advantage of the used modified FADU assay, compared to other assays (e.g., γ-H2AX), is the high sensitivity. In the FADU assay, the detection limit is only 47 single-strand breaks and 3 double-strand breaks (Moreno-Villanueva et al. 2009).

Genotoxicity tests measure different damage to DNA, like gene mutations (e.g., Ames-Test), cytogenic effects (e.g., chromosomal aberrations, formation of micronuclei), dominant lethal factors (genetic damage in gametes), the unscheduled DNA synthesis and DNA strand breaks (Menke et al. 2000), e.g., the somatic mutation and recombination test assay, which is based on the loss of heterozygosity in normal genes, showed, that HEMA and even BisGMA have no genotoxic effect (Arossi et al. 2010). In comparison with studies, which measured DNA strand breaks with comet assay (Kleinsasser et al. 2004) or γ-H2AX test (Urcan et al. 2010), our results, found for DNA strand breaks caused by BisGMA, were in good agreement to the results in these studies. However, in the γ-H2AX test TEGDMA caused DNA strand breaks in concentrations >0.3 mM (Urcan et al. 2010), and in the comet assay >1.0 mM (Kleinsasser et al. 2004), whereas in our study we found in HGF not more DNA strand breaks compared to controls up to 10 mM TEGDMA. In the case of comet assay, one possible explanation may be the significant differences in the preparation time (>10 h for comet assay, <2.5 h FADU assay). Moreover in the case of FADU assay, the reproducibility and the sensitivity are higher compared to comet assay (Moreno-Villanueva et al. 2009; Singh 2000).

The γ-H2AX test measures the phosphorylation of the histone H2AX after DNA strand break. In the case of γ-H2AX test, the higher DNA damage caused by TEGDMA compared to the FADU assay may be explained by an extended phosphorylation of histones (Nakamura et al. 2010; Redon et al. 2009). Histones, which are not affected, can also be phosphorylated by cytokines or reactive compounds released from stressed cells (Dickey et al. 2009). In consequence, the measurable foci (phosphorylated histones) are more as actually caused by the substance, which is called bystander effect (Burdak-Rothkamm et al. 2007).

All organisms are exposed to naturally occurring ionizing radiation. This average background radiation exposure is about 2.3 mGy per year (Elliott 2009). Besides, ionizing radiation is widely used in patients who undergo medical imaging procedures such as X-ray examination. In dental radiography, doses of 0.005 mGy are used (Brenner and Hall 2007). Moreover, in cancer therapy involving the oral cavity, e.g., for parotid gland tumors, a total irradiation dose of up to 50 Gy is used (Anand et al. 2006). Ionizing radiation can cause DNA damage by direct deposition of energy and also indirectly by ionization of water molecules to produce hydroxyl radicals that can attack DNA. Tissue stem cells are very sensitive to X-irradiation. A dose of 3 Gy leads to 90% cell death (Fliedner et al. 2002).

About 24 h after incubation of HGF with 0.25 mM BisGMA, HGF showed more DNA strand breaks compared to irradiation of HGF with 4 Gy (75.8 vs. 63.7% DNA unwinding). It should be mentioned that irradiation with 4 Gy is far away from background radiation or normal dental radiography (2.3 or 0.005 mGy, respectively) and may only be used as a single dose in some tumor treatments (Anand et al. 2006; Brenner and Hall 2007; Elliott 2009).

In a worst case calculation, the highest eluted BisGMA level into water was from the commercial composite Z100® (3 M ESPE, Seefeld, Germany), which provided about 1.5 μmol BisGMA (Reichl et al. 2008a, b). Assuming a daily saliva production of 1.0 l, a BisGMA saliva concentration of 1.5 μM may result. This concentration is more than 100-fold lower than the lowest concentration

that led to detectable DNA strand breaks upon BisGMA exposure in HGF.

Another study showed that BisGMA was eluted into 75% ethanol/25% water solution in levels of about 310 μM from 0.085 g from the commercial composite Tetric Ceram® (Ivoclar Vivadent, Schaan, Lichtenstein) (Polydorou et al. 2007). Assuming the worst case that all restorations in all teeth are replaced by Tetric Ceram® 59,000 μM may be eluted within 24 h (Issa et al. 2004). This concentration is more than 200-fold higher than that concentration with the first measured DNA strand breaks found for BisGMA exposure in HGF. This concentration may cause more DNA strand breaks than a single-dose irradiation of 4 Gy in treatment of tumor patients. However, this data, calculated from elution experiments with worst case assumptions, should be no cause for alarm for three reasons: First, normal alcoholic drinks have lower concentrations of ethanol. Second, the half-life time of 2,3-epoxymethacrylic acid (i.e., the toxic intermediate in the metabolism of dental methacrylates) is only 6.1 min (Seiss et al. 2007). Third, the contact time between ethanol and the composite and then to the oral cells is very low and therefore the elution time is much lower than 24 h as used in our experiments. It must be mentioned that 1 h after incubation of HGF with BisGMA up to 0.5 mM, no significant differences in DNA strand breaks were found compared to controls (Fig. 2).

The difference in DNA strand breaks in HGF between 1 h and 24 h after BisGMA exposure at concentration of 0.25 or 0.5 mM, respectively, may be explained by the size of the BisGMA molecule. To cause DNA strand breaks, BisGMA has to penetrate physiological barriers like cell membranes. One parameter that influences barrier penetration is the molecular size (Tehrany and Desobry 2004). The larger the molecule, the more time is needed (Tehrany and Desobry 2004). The higher toxicity (indicated as DNA strand breaks) of BisGMA compared to other (co)monomers may further be explained by the higher liposolubility of BisGMA, which may therefore enter cells more effectively compared to the other comonomers (Tehrany and Desobry 2004). Besides, it is known that BisGMA can influence the cell metabolism and can activate inflammatory markers (Chang et al. 2009). These effects may also influence the toxicity of BisGMA itself.

In our test system, data show that the dental methacrylates up to 10 mM TEGDMA, HEMA and/or MMA do not induce significantly more DNA strand breaks in HGF compared to controls. This concentration is far away from physiological situation and even from worst case calculations from elution experiments (Durner et al. 2010). About 24-h BisGMA exposure at concentrations >0.25 mM induces significant more DNA strand breaks compared to controls. This DNA damage corresponds to an ionization radiation with 4 Gy, which is used only as a single irradiation dose in tumor therapy. This BisGMA concentration will not be reached under physiological conditions. Furthermore, 0.25-mM BisGMA concentration is more than 100-fold higher than that concentration found in worst case calculations based on elutable amounts. Therefore, the results of our study do not support our hypothesis.

Conclusion

BisGMA, TEGDMA, HEMA and MMA, based on physiological situations and/or worst case calculations, did not induce more DNA strand breaks in HGF compared to controls.

DNA strand breaks caused by 0.25 mM BisGMA, correspond to the DNA strand breaks caused by irradiation with 4 Gy, a dose that is only used in a high single-dose irradiation tumor therapy.

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