2-Hydroxylethyl methacrylate (HEMA), a tooth restoration component, exerts its genotoxic effects in human gingival fibroblasts through methacrylic acid, an immediate product of its degradation

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Abstract HEMA (2-hydroxyethyl methacrylate), a methacrylate commonly used in dentistry, was reported to induce genotoxic effects, but their mechanism is not fully understood. HEMA may be degraded by the oral cavity esterases or through mechanical stress following the chewing process. Methacrylic acid (MAA) is the primary product of HEMA degradation. In the present work we compared cytotoxic and genotoxic effects induced by HEMA and MAA in human gingival fibroblasts (HGFs). A 6-h exposure to HEMA or MAA induced a weak decrease in the viability of HGFs. Neither HEMA nor MAA induced strand breaks in the isolated plasmid DNA, but both compounds evoked DNA damage in HGFs, as evaluated by the alkaline comet assay. Oxidative modifications to the DNA bases were monitored by the DNA repair enzymes Endo III and Fpg. DNA damage induced by HEMA and MAA was not persistent and was removed during a 120 min repair incubation. Results from the neutral comet assay indicated that both compounds induced DNA double strand breaks (DSBs) and they were confirmed by the γ-H2AX assay. Both compounds induced apoptosis and perturbed the cell cycle. Therefore, methacrylic acid, a product of HEMA degradation, may be involved in its cytotoxic and genotoxic action.

Keywords Dental restorative materials · 2-hydroxymethyl methacrylate · HEMA · Degradation · DNA damage · Cell cycle

Introduction

2-hydroxyethyl methacrylate (HEMA) is a monomer based on methacrylic acid (MAA) used to produce biomaterials such as dental adhesive and composite resins [1]. The process of polymerization, which is run in situ, is always incomplete, resulting in the presence of free monomers in the oral cavity [2]. Moreover, monomers can be released from polymer-based tooth restorations through mechanical shearing and enzymatic degradation of polymers. They may penetrate the pulp through microchannels in the dentin structure. Polymethacrylates may contain hydrolyzable ester groups at their surface and the action of esterases may release products of degradations of monomers into the oral cavity. HEMA, like many of MAA-based monomers, is an ester, so it can be targeted by esterases when in organism and can undergo degradation [3]. Efforts have been made to formulate dentin adhesives with esterase resistance, but HEMA still belongs to the most common methacrylate monomers to produce dental restoration materials [4–6]. The main product of HEMA hydrolysis is MAA, which may undergo further degradation producing several intermediates of potential biological activity (Fig. 1).

The release of methacrylate monomers into the oral cavity and the pulp provokes the question on their biological safety, because these compounds may migrate with the bloodstream into many organs and their local...
concentration may be high enough to induce adverse biological effects [2]. Genotoxicity of methacrylate monomers seems to be of a special significance due to their permanent presence in the oral cavity and pulp as well as delayed character of genotoxic effects. The genotoxicity of methacrylates used in dental practice was addressed, mainly in vitro, in several papers [2, 7–16]. It follows from those reports that methacrylates may induce DNA damage, mutations, apoptosis, cell cycle perturbation and gene expression change. However, a direct interaction between methacrylates and DNA was shown in very few studies.

We recently showed that methacrylate monomers, including HEMA, could interact with DNA of human lymphocytes, inducing single and double strand breaks and alterations to the DNA bases, including oxidative modifications [17–19]. These effects were not associated with significant changes in cell viability, but they were linked with apoptosis and cell cycle changes. Therefore, methacrylate monomers used in dentistry may induce genotoxic effects resulting in phenotypic changes. Studies on the mechanisms of these effects may help to prevent and diminish their consequences.

In searching for the mechanism underlying genotoxic effects of HEMA, one should consider the degrading action of the oral cavity esterases. A proposed degradation pathway of HEMA (Fig. 1) suggests that its most potent genotoxic intermediate is 2,3-epoxy-methacrylic acid. In fact, epoxy compounds were reported to display genotoxic properties [20]. However, the genotoxic action of MAA cannot be excluded, especially that this compound was reported to stimulate the release of tumor necrosis factor β and interleukin-6 in mouse macrophages [21, 22]. Therefore, assessment of genotoxic properties of MAA in comparison with its parent compound, HEMA, is justified. In the present work we compared genotoxic effects of HEMA and MAA in human gingival fibroblast. We investigated the ability of both compounds to induce DNA damage, apoptosis and cell cycle perturbations.

**Materials and methods**

**Chemicals**

HEMA, gradiisol and RNase A, low melting point (LMP) and normal melting point (NMP) agarose, phosphate buffered saline (PBS), DAPI (4',6-diamidino-2-phenylindole), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lectin, penicillin, streptomycin, Bradford reagent were from Sigma Chemicals (St. Louis, MO, USA). Quantum 333 medium, Dulbecco’s phosphate buffered saline (DPBS), trypsin and EDTA were from PAA Laboratories GmbH (Colbe, Germany). Methanol-free formaldehyde solution was from Thermo Fisher Scientific, Worcester, MA, USA. Mouse monoclonal anti γ-H2AX primary antibody, 1:100 dilution, anti-phospho-histone H2A.X (Ser139) clone JBW301, was obtained from Upstate (Charlottesville, VA, USA). Alexa Fluor 488 secondary antibody, 1:100 dilution, conjugated goat anti-mouse IgG was from Molecular Probes (Eugene, OR, USA). Plasmid DNA purification kit was provided by EURx (Gdansk, Poland). Cell viability, apoptosis and cell cycle kits were purchased in BD Biosciences (San Jose, CA, USA). ApoAlert Caspase Colorimetric Assay Kit was bought from Clontech Laboratories Inc (Palo Alto, CA, USA). All other chemicals were of the highest commercial grade available.
Cells

Human gingival fibroblasts (HGFs) cell line was purchased from Proviro (Berlin, Germany). The cells were grown in Quantum 333 medium containing t-glutamine and supplemented with 1% antibiotic–antimycotic solution (10,000 Units/ml penicillin, 10 mg/ml streptomycin sulphate, 25 μg/ml amphotericin B) in 75 cm² cell culture flasks to approximately 75–80% confluence and maintained in an incubator with 5% CO₂ atmosphere at 100% humidity at 37°C. After reaching confluence, the cells were washed with Dulbecco’s phosphate buffered saline, detached from the flasks by a brief treatment with 0.05% trypsin-0.02% EDTA. They were used between 5 and 8 passage. Escherichia coli, strain DH5α cells with pUC19 plasmid, were grown in a LB broth at 37°C overnight.

Cell treatment

HEMA or MAA was added from 95% water solutions to the cells in their growth medium at final concentrations from the range 1–10 mM. The control cells received only growth medium. To examine DNA damage, cell viability, apoptosis and cell cycle the cells were incubated with HEMA or MAA for 6 h at 37°C. After reaching confluence, the cells were washed with Dulbecco’s phosphate buffered saline, detached from the flasks by a brief treatment with 0.05% trypsin-0.02% EDTA. They were used between 5 and 8 passage. Escherichia coli, strain DH5α cells with pUC19 plasmid, were grown in a LB broth at 37°C overnight.

Cell viability

HGFs were washed three times with PBS and then diluted in PBS to concentration of 2.5 × 10⁵ cells/ml. For preparation of dead cells (positive control), one sample was treated with 96% ethanol for 1 min. All samples were centrifuged and cell pellets were suspended in 100 μl of 0.5 μM calcein-acetoxymethyl ester (cal AM)/10 μM propidium iodine (PI) in PBS. Cells were analyzed on a LSRII flow cytometer (Becton–Dickinson, San Jose, USA) 5 × 10⁴ cells were analyzed in each experiment repeated in triplicate.

Plasmid relaxation assay

pUC19 plasmids were exposed to UV irradiation at 35 J/m² (positive control) to check the migration of its multimeric forms (supercoiled, nicked circular and linear). UV irradiation induced strand breaks in DNA and caused the relaxation of supercoiled plasmid—one break is enough to relax one molecule of it. Structural differences between supercoiled, nicked circular and linear forms of the plasmid accounted for their different electrophoretic mobility. The ability of HEMA and MAA to damage DNA was quantified by calculating the ratio of the open circular DNA to the total amount of DNA (R). The values for supercoiled DNA were multiplied by 1.66 to correct for the decreased intercalating ability of ethidium bromide.

Comet assay

The comet assay was performed under alkaline conditions essentially according to the procedure of Singh et al. [25] with modifications as described previously [26, 27]. A freshly prepared suspension of HGFs in 0.75% LMP agarose dissolved in DPBS was spread onto microscope slides precoated with 0.5% NMP agarose. The cells were then lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After lysis, the slides were placed in an electrophoresis unit, the DNA was allowed to unwind for 40 min in the electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH > 13.

In the neutral version of the comet assay, electrophoresis was run in a buffer consisting of 100 mM Tris and 300 mM sodium acetate at pH adjusted to 9.0 by glacial acetic acid [28]. Electrophoresis was conducted for 60 min, after a 20 min equilibrium period, at electric field strength of 0.41 V/cm (50 mA) at 4°C. The slides were examined at 200× magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, Inc., San Diego, CA) equipped with a UV filter block consisting of an excitation filter (359 nm) and barrier filter (461 nm) and connected to a personal computer-based image analysis system, Lucia-Comet v. 4.51 (Laboratory Imaging, Praha, Czech Republic). Fifty images were randomly selected from each sample and the comet tail DNA was measured. Two parallel tests with aliquots of the same sample of cells were performed for a total of 100 cells. Each experiment was repeated three times. The comet tail DNA is positively correlated with the level of DNA breakage or/and alkali labile sites and is negatively correlated with the level of DNA crosslinks [23]. For the neutral version, this quantity correlates positively with DNA double strand breaks.

Oxidative damage to DNA

To compare the ability of HEMA and MAA to induce oxidative damage to DNA we used endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (Fpg), which are enzymes of the base excision DNA repair pathway. Endo III converts oxidized pyrimidines into...
strand breaks, which can be detected by the comet assay [29]. Fpg recognizes and removes mainly 7,8-dihydro-8-oxoguanine (8-oxoguanine) [30]. These enzymes recognize also a variety of other modification to the DNA bases, which cannot be detected by the comet assay in its basic form. The removing of modified bases from DNA by the enzymes leads to apurinic or apyrimidinic sites, which are subsequently cleaved by their AP-lyase activity producing a gap in the DNA strand, which can be detected by the comet assay [27, 31]. Further steps were as described in the Comet assay section. To check the ability of both enzymes in order to recognize the oxidative damage to DNA, we exposed HGFs to 20 μM hydrogen peroxide for 15 min on ice (positive control, results not shown). To express oxidative modification to the DNA bases evoked by HEMA or MAA at a particular concentration, %DNA in tail for the control (neither HEMA nor MAA) was subtracted from %DNA in tail for either chemical at this concentration.

DNA double strand breaks assays

We evaluated the ability of HEMA and MAA to induce DNA double-strand breaks (DSBs) by the neutral comet assay and the immunofluorescence assay for the phosphorylation of the H2AX histone [32]. For immunofluorescent staining, cells (1–2 × 10⁶) were washed in DPBS by centrifugation (300 × g for 5 min at room temperature), fixed by 1 ml ice-cold 1% methanol-free formaldehyde in DPBS and incubated on ice for 15 min. Cells were centrifuged (300 × g, 5 min, room temperature) and permeabilized with 80% ethanol in distilled water and kept at −20°C for 2 h until further staining. Cells were then washed three times with 1% BSA/0.2% Triton X-100/PBS (BTP) solution and stained with mouse monoclonal anti γ-H2AX primary antibody and incubated overnight at 4°C. Then, HGFs were washed three times with BTP solution and incubated with Alexa Fluor 488 secondary antibody for 1 h at room temperature in the dark. After the incubation cells were washed in BTP and counterstained with propidium iodine (PI, 5 μg/ml in DPBS in the presence of 100 μg/ml of RNase A) and incubated for 30 min at room temperature in the dark. Cells stained with Alexa Fluor 488 and PI were analyzed with LSRII flow cytometer (Becton–Dickinson Biologicals, San Jose, CA, USA) by measuring the intensity of green (530 ± 20 nm) and red (>600 nm) fluorescence of the cells. DNA content (red fluorescence of DNA-bound PI) was plotted on the x-axis and the level of γ-H2AX immunofluorescence (green fluorescence—Alexa Fluor 488) was plotted on the y-axis. Logarithmic Alexa Fluor 488 fluorescence was plotted versus linear PI fluorescence using FlowJo analysis software (TreeStar, Ashland, OR, USA). Untreated controls were used to set the threshold gating to determine the percentage of γ-H2AX positive cells. Intensity of cellular γ-H2AX immunofluorescence measured by flow cytometry is positively correlated with the level of DSBs and was used to quantify their extent [33, 34].

Apoptosis

The BD Annexin V-FITC Apoptosis Detection Kit I was used to measure apoptosis. The kit contains Annexin V conjugated to the fluorochrome FITC. After 6 h of incubation with HEMA or MAA the cells were washed in cold DPBS and resuspended in 1× binding buffer at 10⁶ cells/ml. Aliquot of 100 μl (10⁵ cells) was transferred to a 5 ml culture tube, 5 μl of Annexin V-FITC and 5 μl of PI were added, gently vortexed and incubated for 15 min at room temperature in the dark. Then, 400 μl of 1× binding buffer was added to each tube and samples were analyzed by flow cytometry. Each experiment had a negative, positive and unstained control samples. About 10,000 events were counted per sample. The apoptosis ratio was calculated as a percent of apoptotic cells in a sample.

Cell cycle

The CycleTEST PLUS DNA Reagent Kit was used to determine the DNA index (DI) and cell-cycle phase distributions. Nuclei were isolated, stained with propidium iodide and afterward analyzed on the LSRII flow cytometer according to the manufacturer instruction. The DI was calculated by dividing the mean of the relative content of the exposed G0/G1 population by the mean of the control G0/G1 population. Results were analyzed by CellFIT software.

Data analysis

The values in this study were expressed as mean ± S.E.M. from three experiments, i.e. the data from three experiments were pooled and the statistical parameters were calculated. The data obtained from cell viability were expressed as mean ± S.D, because S.E.M in this method was much more smaller than in the remaining methods and it would be not visible in the plot presenting results. All repeats of each experiment were performed the same day. The Mann–Whitney test was used to determine differences between samples with distributions departing from normality. The differences between samples with the normal distribution were evaluated by applying the Student’s t-test. Data analysis was performed using SigmaStat software (v. 3.0.0, SPSS, Chicago, USA).
Results

Cell viability

Both HEMA and MAA induced a mild, but statistically significant decrease in the viability of HGFs, reaching about 10% at the highest concentration of the chemicals, 10 mM (Fig. 2). We did not observe any difference in the cytotoxic potential of HEMA and MAA in the concentration range 0.5–5 mM, but there was a significant ($P < 0.001$) difference (3.2%) between the viability of the cells exposed to HEMA and MAA at the highest concentration of the chemicals, 10 mM. However, we do not consider this difference as biologically relevant.

DNA damage in vitro

Neither HEMA nor MAA introduced DNA breaks to isolated DNA, as evaluated by the plasmid relaxation assay, in which the ratio of the amount of open circular form of plasmid DNA to the total amount of DNA was calculated (Fig. 3). The pUC19 plasmid used in our experiment was sensitive to DNA-breaking agents as checked by UV irradiation.

DNA damage in human gingival fibroblasts

Figure 4 displays the mean percentage tail DNA of human gingival fibroblasts exposed for 6 h to HEMA or MAA and analyzed by the alkaline comet assay. Both chemicals increased tail DNA in a dose-dependent manner ($P < 0.001$ at all concentrations). DNA single- and double-strand breaks as well as alkali labile sites can be detected in this version of the comet assay. We did not observe any difference between effects induced by HEMA and MAA ($P > 0.05$).

DNA double-strand breaks

The neutral comet assay was used to detect the ability of HEMA and MAA to induce DNA double-strand breaks (DSBs). The results are displayed in Fig. 5. We observed a significant ($P < 0.05$) increase in the tail DNA at 1 mM and higher concentrations of HEMA and MAA. The effect induced by HEMA was generally greater than MAA, but this difference was not significant. The positive results obtained in the neutral comet assay, suggest the induction of DSBs by both compounds. We verified this hypothesis in the phosphorylation of H2AX histone test. The results (Fig. 6) confirm the ability of both compounds to induce DSBs. However, this time 10 mM HEMA induced significantly ($P < 0.001$) greater effect than MAA at the same concentration.

DNA repair

We analyzed the kinetics of DNA repair in human gingival fibroblasts after HEMA and MAA treatment by measuring the extent of DNA damage in the cells exposed to either chemical at 5 mM immediately after exposure as well as 30, 60, 90 and 120 min thereafter (results not shown). The cells exposed to 10 μM hydrogen peroxide (positive control) were able to recover within 45 min (results not shown). The cells exposed to HEMA or MAA at 5 mM were able to remove about 90% of the damage to their DNA within 60 min (Fig. 7) ($P < 0.001$). We did not observe any difference in the kinetics of DNA repair between by HEMA and MAA treatment.

Oxidative modifications to the DNA bases

Figure 8 presents the mean % tail DNA of human gingival fibroblasts exposed for 6 h at 37°C to 2-hydroxyethyl methacrylate (HEMA, open symbols) or methacrylic acid (MAA, closed symbols) and measured by flow cytometry with thiazole orange and propidium iodide. Displayed is the mean of three experiments of $5 \times 10^4$ measurements each, error bars denote standard deviation. ***$P < 0.001$, **$P < 0.01$ as compared with the unexposed control.
Fig. 3 DNA damage in isolated pUC19 plasmid. The picture shows three forms of the plasmid: linear (L), open circular (OC) and supercoiled (CCC) exposed to 2-hydroxyethyl methacrylate (HEMA) or methacrylic acid (MAA) at indicated concentrations or 254 nm UV at the dose rate of 0.12 J m$^{-2}$ s$^{-1}$ (positive control). The samples were electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized in UV light. The tables display the ratio of the open circular DNA to the total amount of DNA (R) of the isolated pUC19 plasmid exposed to HEMA, MAA or UV. The values for supercoiled DNA were multiplied by 1.66 to correct for the decreased intercalating ability of ethidium bromide.

| HEMA (mM)       | R      | MAA (mM)   | R      |
|-----------------|--------|------------|--------|
| 0 (control)     | 0.081  | 0 (control)| 0.012  |
| 0.5             | 0.102  | 0.5        | 0.015  |
| 1               | 0.091  | 1          | 0.018  |
| 5               | 0.130  | 5          | 0.013  |
| 10              | 0.123  | 10         | 0.013  |
| UV (positive control) | 0.998$^*$ | UV (positive control) | 0.998$^*$ |

$^*$ - p < 0.001 as compared to control

Fig. 4 DNA damage in human gingival fibroblasts exposed to 2-hydroxyethyl methacrylate (HEMA, open symbols) or methacrylic acid (MAA, closed symbols) for 6 h at 37°C. DNA damage was measured as DNA percentage in the tail in comets of alkaline version of the comet assay. The mean value for one hundred cells analyzed in each treatment in three independent experiments is displayed. Error bars represent SEM, $P < 0.001$ for all concentrations of HEMA and MAA as compared with unexposed controls.

Fig. 5 DNA damage in human gingival fibroblasts exposed to 2-hydroxyethyl methacrylate (HEMA, open symbols) or methacrylic acid (MAA, closed symbols) for 6 h at 37°C. DNA damage was measured as DNA percentage in the tail in comets of neutral version of the comet assay. The mean value for one hundred cells analyzed in each treatment in three independent experiments is displayed. Error bars represent SEM, $P < 0.001$ for 1, 5 and 10 mM concentration of HEMA and MAA as compared with unexposed controls.
DNA of the cells post-treated and untreated with Endo III in the absence of HEMA and MAA were likely to arise from the endogenous oxidative damage to the DNA bases as well as from the oxidative damage to DNA introduced during processing of the cells. We observed a significantly ($P < 0.05$ for both enzymes) greater effect induced by HEMA than by MAA.

Apoptosis

Both HEMA and MAA induced apoptosis in HGFs in a concentration-dependent manner (Fig. 9). We observed over two-fold increase in the apoptosis ratio for the highest concentration of HEMA and MAA, 10 mM. There was not a significant difference in pro-apoptotic action between HEMA and MAA ($P > 0.05$). Untreated cells were primarily Annexin V-FITC and PI negative, indicating that they were viable and did not undergo apoptosis. After incubation with either chemical, there were basically two populations of cells: cells that were viable and not undergoing apoptosis (Annexin V-FITC and PI negative) and cells undergoing apoptosis (Annexin V-FITC positive and PI negative). A minor population of cells was observed to be Annexin V-FITC and PI positive, indicating that they were in the end stage of apoptosis or already dead.

Cell cycle

In order to compare the influence of HEMA and MAA on the progression of the cell cycle of HGFs, we determined the DNA content in specific phases and checkpoints of the cycle by flow cytometry. The results clearly indicate, that there were no substantial differences in the action of both chemical on the progression of the cell cycle (Fig. 10).
general, both compounds at 0.5 mM evoked an increase in the G0/G1 cell population, accompanied by a mild decrease in the S phase cell population. Both HEMA and MAA at 1 mM had an opposite effect to that at 0.5 mM—they decreased the G0/G1 cell population and increased the S cell population. Both changes were small. We did not observe a significant change in the G2/M cell population for either compound at all concentrations.

Discussion

Methacrylate monomers used in restorative and aesthetic dentistry as well as in orthodontics are of a special concern, because they are released from their polymers into the oral cavity and the pulp, from where they can migrate with the bloodstream to virtually all parts of organism. This process can last several dozen years and the local concentration of methacrylate monomers can be high enough to induce adverse biological effects, which can accumulate with age and result in serious health problems. Therefore, work on new technologies of methacrylate-based dental materials should focus on the decrease of the degree of monomer/polymer conversion, which is currently high [4, 35]. However, monomers can be released from polymers as a consequence of mechanical stress following the chewing process and erosion. Moreover, the polymers may contain ester groups, which may be targeted by esterases present in the saliva, resulting in the degradation of monomers and releasing the products of degradation into the oral cavity. Additionally, the degradation may be induced by bacteria, especially when the composite filling is affected by polymerization shrinkage, creating a gap between the filling and dentin [36]. It is very hard to assess any immediate cytotoxic or genotoxic effect of dental fillings. The pulp seems to be the most serious target, but on the other hand, the enzymatic and mechanical releasing of monomers and products of degradation of dental fillings concerns in a higher degree the gingiva and the oral cavity epithelium. It was shown that MAA might be released from methacrylates attached in the matrix by only one terminus [37]. Therefore, methacrylate-based dental fillings and orthodontic materials may release monomers and products of their degradation by esterases into the oral cavity. Efforts to improve resistance of dental materials to esterases were made [38, 39]. Trimethylolpropane mono allyl ether dimethacrylate (TMPEDMA) is a methacrylate monomer which was added to classical HEMA/Bis-GMA mixture to increase its resistance to esterases [38]. In these studies a pronounced decrease of MAA content in TMPEDMA-modified material was observed as compared with the conventional adhesive. However, neither TMPEDMA nor any other material increasing resistance to esterases has been commonly applied in dental adhesives, so the release of monomers from them should be taken into account. Toxicity of a chemical compound interacting with living cells may depend on its degradation and/or metabolic
transformation. As a result, the toxicity of a degradation product can differ from its parent compound. Metabolic transformation (degradation) of dental composites may be caused by hydrolysis and/or enzymatic catalysis [40]. Many health concerns have arisen with respect to metabolites of methacrylates used in dentistry on demonstration of the release of monomers of Bis-GMA and several products containing the bisphenol A moiety from model dental composites [41].

We used human gingival fibroblasts as they are immediate targets in the toxic action of methacrylate monomers released from tooth restorations. Other targets are pulp cells, which can be reached by methacrylates through dentin microchannels, and endothelial cells in the oral cavity. However, micro injuries generated in the oral cavity by food or tooth brushing, may create another route of migration of the monomers into the bloodstream. That is why we investigated the action of HEMA in human peripheral blood lymphocytes in our previous work and we obtained similar results, so the degradation process of HEMA in the oral cavity and the bloodstream may be similar [17]. It was shown that unspecific esterases and saliva-derived enzymes softened the surface of methacrylate-based dental polymers by the hydrolysis of ester bonds.
Fig. 10  Cell cycle analysis in human gingival fibroblasts CCR-CM cells exposed to 2-hydroxyethyl methacrylate (HEMA) and methacrylic acid (MAA) for 6 h at 37°C. Percentage of cells in G0/G1 (black bars), S (light grey bars) and G2/M (dark grey bars) stage of the cell cycle after treatment with HEMA or MAA was presented along with histograms for each HEMA and MAA concentration. Nocodazole (Noc) was used as a positive control. Data are expressed as means of at least three independent experiments, error bars denote SD. *P < 0.05, **P < 0.001 as compared with the unexposed control.
[42]. MAA is a primary product of the degradation of HEMA by esterases and pig liver esterase (7 units/ml) was reported to decompose HEMA at a rate constant $1.3 \times 10^{-2}$ mg/(ls) [39, 43]. However, there are at least two chemical pathways in the degradation of HEMA. In the main pathway MAA is released via the valine pathway and in the other MAA is activated via epoxidation (Fig. 1) [44, 45]. Recently, it was shown that the epoxide metabolism pathway of HEMA with epoxide formation existed in human lung cancer cells A549 [46]. It was suggested that MAA might be degraded producing 2,3-epoxy methacrylic acid (2,3-EMA) and 2,3-epidoxy methacrylic acid [45]. We did not find any report concerning genotoxic action of 2,3-EMA, but some epoxy compounds are reported to be toxic [20]. Although 2,3-EMA seems to be potentially dangerous for the cell, MAA was reported to release tumor necrosis factor $\beta$ and interleukin-6 in mouse macrophages [21, 22].

Methacrylate-based restorations are prone to water penetration [47]. Water may enter the polymer matrix by diffusion into loosely cross-linked or hydrophilic domains or may be entrapped within them in the process of photopolymerization led in the moistly environment of the oral cavity. Water supports the chemical hydrolysis of ester bonds in methacrylates, which is normally expected to proceed slowly in neutral conditions of the oral cavity. However, the presence of food or some bacteria may lead to changes in pH, provoking temporary acid or base hydrolysis. After prolonged time of exposure to oral fluids, methacrylate network can become accessible to esterases cleaving ester bonds.

We observed a mild, but significant decrease in the viability of HGFs exposed to HEMA or MAA, which reached about 10% at the highest concentration of the chemicals, 10 mM (Fig. 2). Recently, Urcan et al. measured EC$_{50}$ for HEMA in HGFs to be 11.20 mM [48]. However, they applied the XTT viability assay, with a 24 h exposure to HEMA, whereas exposure time in our experiment was 6 h. If we assume a linear relationship between the viability and exposure time, which is roughly the case in our experiment, the results on viability obtained by Urcan et al. and us would be similar. We also observed a significant difference between effects exerted by HEMA and MAA at highest, 10 mM, concentration, but this difference was only about 3% and, although statistically significant, we do not consider it as biologically relevant. Methacrylate monomers can reach millimolar concentrations at least in the pulp and that is why they are used at relatively high concentrations of up to $2.5 \times 10^{-2}$ mol/l and above, in many in vitro studies [10, 14]. Recently, Nocca et al. [49] showed that the concentrations of HEMA inside 3T3 fibroblasts was 15–20 times lower than that present in the cells' medium, when added in the range 1–8 mM.

We have included the results of the plasmid relaxation experiment, because we think that they are important for the study. Comparing the results of this experiment with the results of the comet assay we drew a conclusion that HEMA had to undergo a transformation (activation) by cellular components to interact with cellular DNA. This activation might proceed according to the HEMA degradation scheme presented in Fig. 1. If so, MAA is probably not the product of such activation of HEMA, because it did not interact with DNA in the plasmid assay. Therefore, 2,3-EMA and 2,3-epidoxy methacrylic acid as well as formaldehyde are candidates to interact directly with DNA. However, this reasoning is not complete because the pathway presented in Fig. 1 does not present the complete reaction for degradation/metabolic transformation of HEMA in HGFs and the actual reaction is not known. Lack of any difference between the action of HEMA and MAA suggests that not only the activation/degradation of HEMA is required for its DNA-damaging action, but also this action may be mediated by some cellular compounds.

Both HEMA and MAA induced DNA damage resulting in a significant fragmentation of DNA in the alkaline version of the comet assay. This version, ran at pH > 13, detects single- and double DNA strand breaks (SSBs and DSBs, respectively) as well as alkali labile sites. The results obtained in the neutral version of the test suggest the ability of the chemicals to induce DSBs. However, the neutral version of the comet assay cannot be considered as the most reliable method for assessing DSBs because SSBs may interfere with measuring the breaks in the neutral comet assay. This interference occurs because the relaxation of DNA supercoils, which is essential for the picture of a comet, may occur at both neutral and alkaline pH. In other words, all positive cases in the neutral comet assay should be verified and this is the practice in our laboratory [50, 51]. Pulse field gel electrophoresis was considered as the most consistent methods for assessing DSBs [52], but nowadays the phosphorylation of the H2AX histone at serine 19 seems to be the most reliable method for doing so [53]. We verified the results obtained in the neutral comet assay by the evaluation of the phosphorylation of the H2AX variant histone with the positive outcome. The presence of $\gamma$-H2AX is widely accepted as a specific indicator for the presence of DSBs [54]. The ability of HEMA and MAA to induce DSBs is of a special concern because such DNA damage, if not repaired or misrepaired, may result in chromosome rearrangements and deletions, leading to fusion genes expressing oncogenic proteins [55]. Apart from chromosomal rearrangement, DSBs may result in the inactivation of tumor suppressor genes, activation of oncogenes and disturbing the structure of mutator genes. Aberrant products of these genes or their lack may contribute to the induction, promotion and progression of cancer transformation [56].
We observed a significant extent of DSBs in both neutral comet and phosphorylation of the H2AX histone assays. However, beside statistical significance, a question on the biological and medical relevance of the observed effect is important. The number of DSBs in a normal somatic human cell is estimated to about 50 per cell cycle and they are mostly result of the conversion of SSBs associated with normal metabolism of the cell [57]. They must be accurately repaired, as normal cells in vitro show very little sign of consequences of DSBs, including chromosom break and deletions. The ability of HEMA to induce DSBs in human gingival fibroblasts was recently observed by Urcan et al. who reported that HEMA at its EC50 concentration, 11.2 mM, induced on average 2.5 γ-H2AX-specific foci per cell [48]. This is not a great extent if we compare it with that observed in a negative control (DMSO and culture medium) — 0.5 γ-H2AX foci/cell. However, it follows from our previous considerations that even a small number of persistent DSBs may be dangerous for the cell and whole organism. Therefore, further study on the mechanism of induction of DSBs by HEMA is needed.

We showed that HEMA and MAA induced oxidative damage to DNA of HGFs. This kind of DNA damage was detected by the EndoIII and Fpg enzymes. HEMA induced slightly, but statistically significant (P < 0.05), greater extent of oxidative modifications to DNA bases than MAA. It is possible, that the total oxidative damage to DNA is greater that we observed in the experiment with DNA repair enzymes for at least three reasons. First, the enzymes we used do not recognize all possible oxidative modifications to DNA bases, although they have a wide spectrum of specificity. Moreover, they may non-specifically cleave certain modifications of the DNA bases [58]. Second, part of the total DNA damage observed in the non-modified alkaline version of comet assay, DNA strand breaks and alkali labile sites, might be also of oxidative origin and they were no longer revealed in the enzyme-modified assay. Third, oxidative damage to DNA may also affect its sugar-phosphate backbone and may not be detected by EndoIII or Fpg [59]. Our observations are in line with our previous report and other studies indicating pro-oxidative potential of HEMA and other methacrylate monomers [17, 60–62]. The results obtained in the investigation of oxidative modification of the DNA bases (Fig. 8) suggest that antioxidants can be considered to inhibit genotoxicity of HEMA/MAA. The ability of HEMA and MAA to induce oxidative modifications to the DNA bases suggests the general mechanism of cellular damage by methacrylate-based dental materials [7, 63]. Di Pietro et al. [8] showed in an in vivo study, genotoxicity of dental restorative materials. Interestingly, the genotoxicity of methacrylate composite materials was similar to that of amalgam. It was suggested that the mechanism of observed effect was based on the production of reactive oxygen species by these compounds.

DNA repair is the main reaction of the cell to DNA damage. If the cellular repair systems cannot cope with the damage, it may require a prolonged repair time resulting from the G2/M or G1/S checkpoint activation or the cell may undergo apoptosis. Therefore, the ability of HEMA and MAA to generate DNA damage may also determine the ability of these compounds to influence the cell cycle and induce apoptosis. This was observed in our study and the ability of HEMA to induce cell arrest and apoptosis was reported also in other studies [61, 64–68].

In the present study we showed that HEMA exerted similar effects on the HGFs cell cycle as its primary degradation product, MAA. It is tempting to hypothesize that both cell cycle perturbations and induction of apoptosis are consequences of the observed ability of HEMA/MAA to induce DNA damage. This is supported by the lack of a significant cytotoxic effect of HEMA/MAA, which suggests a “pure” genotoxic mechanism of the induction of apoptosis and cell cycle disturbances by these compounds. However, we cannot exclude other mechanisms. In particular, the interaction of HEMA/MAA with other cellular structures, not only DNA, should be taken into account and mitochondria seem to be the primary candidates. First, the ability of HEMA/MAA to damage DNA may concern not only nuclear but also mitochondrial DNA (mtDNA). Damage to mtDNA may induce perturbation in the overall functions of mitochondria, which, in turn may lead to induction of apoptosis mediated by apoptosis-inducing factor, a protein anchored to the inner mitochondrial membrane [69]. This may indicate direction of further research on the cyto- and genotoxicity of HEMA/MAA and other methacrylate monomers and products of their degradation.

Conclusions

HEMA may induce serious cytotoxic and genotoxic effects in human gingival fibroblasts and these effects can be assigned to its degradation product(s) rather, than to HEMA itself. Further studies are needed to determine which product(s) of HEMA degradation is responsible for biological incompatibility of HEMA and identification of this product may allow undertaking effective protective action against its harmful effects.

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Conflict of interest  The authors declare that there is not any conflict of interest.

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