



## Genotoxicity of urethane dimethacrylate, a tooth restoration component

Tomasz Poplawski<sup>a</sup>, Katarzyna Loba<sup>a</sup>, Elzbieta Pawlowska<sup>b</sup>, Joanna Szczepanska<sup>b</sup>, Janusz Blasiak<sup>a,\*</sup>

<sup>a</sup> Department of Molecular Genetics, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland

<sup>b</sup> Department of Pediatric Dentistry, Medical University of Lodz, Pomorska 251, 92-213 Lodz, Poland

### ARTICLE INFO

#### Article history:

Received 7 July 2009

Accepted 3 December 2009

Available online 11 December 2009

#### Keywords:

Urethane dimethacrylate

UDMA

DNA damage

DNA repair

Comet assay

Apoptosis

Cell cycle

### ABSTRACT

Urethane dimethacrylate (UDMA) is used in dental restorative materials in its polymeric form. However, the process of polymerization is usually incomplete and the monomers of UDMA can diffuse into the oral cavity and the pulp, reaching millimolar concentrations. In the present work we showed that UDMA at 0.1 and 1.0 mM decreased the viability of and induced DNA damage in lymphocytes in a concentration dependent manner, but it did not affect a plasmid DNA *in vitro*. UDMA at 1 mM induced apoptosis in lymphocytes. The lymphocytes exposed to UDMA were able to repair their DNA within 60 min. Analysis with DNA repair enzymes Endo III and Fpg showed that UDMA induced mainly oxidative DNA lesions. Vitamin C and chitosan decreased genotoxic effect of UDMA. Our results show that monomers of UDMA may exert pronounced cyto- and genotoxic effects in human lymphocytes and chitosan can be considered as a protection against such effects.

© 2009 Elsevier Ltd. All rights reserved.

### 1. Introduction

Methacrylate resin-based materials are commonly used in restorative dentistry. They are in the form of polymers in the restoration and the process of polymerization takes place in the tooth. This process is usually incomplete and a considerable amount of monomers, up to 25% of the total, may be unbound to the tooth restoration (Geurtsen, 1998; Spahl et al., 1998). These monomers may be released into the oral cavity and the pulp, via microchannels present in the dentine, from where they can penetrate virtually every organ through the bloodstream. Methacrylate polymers present in the tooth may be degraded to monomers and oligomers as a consequence of mechanical stress following the chewing process. Also enzymatic degradation of polymers by the substance contained in the saliva may be another source of methacrylate monomers. The local concentration of monomers may be in the millimolar range, high enough to induce adverse biological effects (Schweikl et al., 2006). Some of the methacrylate monomers, due to their small size, can penetrate the body through standard protective gloves and masks, so not only patients but dental personnel can be exposed to these substances. There is a growing body of evidence suggesting the involvement of methacrylate monomers in systemic or local effects like allergic reactions, body toxicity, cytotoxicity, estrogenicity, and mutagenicity (see Schweikl et al., 2006 for review).

Urethane dimethacrylate (UDMA, Fig. 1) belongs to the most common methacrylate resin monomers used in the synthesis of dental composites. It is used as an alternative to bisphenol A-diglycidyl dimethacrylate (bis-GMA), because it has a reduced viscosity, increased filler loading and greater stiffness due to the elasticity of the urethane linkages (Beatty et al., 1993). Tensile strength, flexural strength, and modulus of elasticity of components containing UDMA are better as compared to composites prepared from bis-GMA and other aromatic-based materials (Asmussen, 1990). However, the urethane-based polymers absorb significantly more water than the aromatic-based materials. Absorptions of aqueous solvents may cause passive and enzymatic hydrolytic degradation of the polymer matrix (Ferracane, 2006). This process did not only change mechanical properties of urethane-based polymers, but also initiated the release of unbound monomers and degradation by-products into the oral environment. Thus, the question about the biological safety of UDMA is justified, especially that some adverse biological effects associated with UDMA have been demonstrated. A disturbance was shown in the cell cycle in epidermal oral carcinoma cells and human foreskin fibroblasts after incubation with UDMA (Nassiri et al., 1994). In another study UDMA impaired the function of pulpal immunocompetent cells in rats (Jontell et al., 1995). UDMA released from dental materials was reported to reach a toxic level in human oral cells to induce their necrosis (Reichl et al., 2006). Series of *in vitro* studies showed that UDMA could induce cell death, but the mechanism of UDMA cytotoxicity remains unknown (Al-Hiyasat et al., 2005; Mohsen et al., 1998; Ratanasathien et al., 1995; Reichl et al., 2006; Yoshii, 1997). The cytotoxicity of a compound may be

\* Corresponding author. Tel.: +48 42 635 43 34; fax: +48 42 635 44 84.  
E-mail address: [jblasiak@biol.uni.lodz.pl](mailto:jblasiak@biol.uni.lodz.pl) (J. Blasiak).

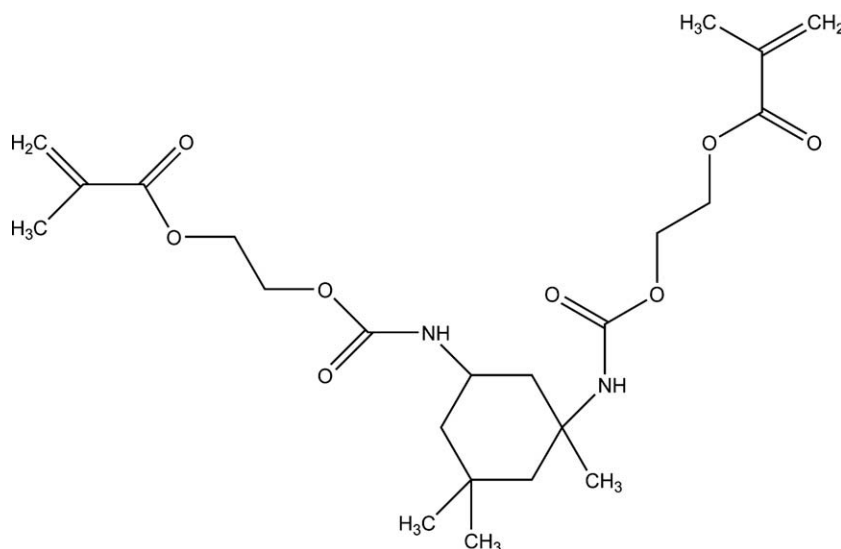


Fig. 1. Molecular structure of urethane dimethacrylate (UDMA).

underlined by its genotoxicity, but data on the latter are still scarce and controversial. Schweikl et al. (1998) showed that that UDMA did not display mutagenic activity in the Ames test, but it showed the ability to induce micronuclei (Schweikl et al., 2001). UDMA-induced DNA damage evaluated *en masse* by the comet assay (Kleinsasser et al., 2006, 2004), but thus far the molecular mechanisms have not been elucidated. In the present work we investigated the genotoxic and cytotoxic effect of UDMA in human peripheral blood lymphocytes and cancer cells by measuring cell viability, DNA damage and repair, cell cycle and apoptosis. We applied two modes of incubation with the chemical: short (1 h) and long (24 h) to distinguish between immediate and prolonged effects of UDMA exposure.

## 2. Materials and methods

### 2.1. Chemicals

UDMA (CAS-N 72869-86-4) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). This chemical is used for the production of composite materials by several dental companies (Polydorou et al., 2009a). Low melting point (LMP) and normal melting point (NMP) agarose, phosphate buffered saline (PBS), DAPI (4,6-diamidino-2-phenylindole), dimethyl sulfoxide (DMSO) and methylglycol chitosan were purchased also from Sigma–Aldrich Co. (St. Louis, MO, USA). Plasmid DNA isolation kit was provided by EURx (Gdansk, Poland). Cell viability, apoptosis and cell-cycle kits were purchased in BD Biosciences (San Jose, CA, USA). Fetal bovine serum (FBS), lectin, penicillin, streptomycin, RPMI medium and lymphocyte separation medium LSM1077 were from PAA (Pasching, Austria). All other chemicals were of the highest commercial grade available.

### 2.2. Cells

Human lymphocytes were isolated from the peripheral blood obtained from young (23–25 years), healthy, non-smoking donors by centrifugation in a density gradient of LSM1077 (30 min, 1200g, 25 °C). The cells were pooled from 3 to 5 donors, depending on the experiment. We used human alveolar basal epithelial A549 cells for cell-cycle analysis. The cells were obtained from Prof. M. Soszynski of Department of Molecular Biophysics, Univer-

sity of Lodz, Poland. A549 cells were grown in DMEM medium with L-glutamine supplemented with 10% FBS, 100 U/ml penicillin and 100 g/ml streptomycin in a 5% CO<sub>2</sub> atmosphere at 37 °C. *Escherichia coli* cells, strain DH5 $\alpha$  with pUC19 plasmid, were grown in a LB broth with penicillin at 37 °C overnight.

### 2.3. Cell treatment

UDMA at final concentrations 100–1000  $\mu$ M was added to the cells in RPMI 1640 from its 1 M solution in DMSO. The final concentration of DMSO in the samples did not exceed 0.05%, which did not influence the processes under study. The control cells received only the growth medium and DMSO at 0.05%. To examine DNA damage and cell viability, the cells were incubated with the chemical for 1 and 24 h at 37 °C. Each experiment included a positive control, which was hydrogen peroxide applied at 20  $\mu$ M for 15 min on ice producing a pronounced DNA damage.

### 2.4. Cell viability

Cells were washed three times with PBS and then diluted in PBS to concentration of  $2.5 \times 10^5$  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  $\mu$ l of 0.5  $\mu$ M calcein-acetoxymethyl ester (cal AM)/10  $\mu$ M propidium iodine (PI) in PBS. Cells were gently shaken and incubated with the dyes for 30 min at 37 °C in a tissue culture incubator and then analyzed on a LSRII (Becton Dickinson, San Jose, USA) flow cytometer equipped with 488 nm laser excitation and BD FACS Diva software v 4.1.2.  $5 \times 10^4$  cells were analyzed in each experiment repeated in triplicate.

### 2.5. Plasmid relaxation assay

pUC19 plasmids were isolated from DH5 $\alpha$  *E. coli* cells with Genematrix Plasmid Miniprep DNA Purification Kit (EURx, Gdansk, Poland), according to the manufacturer's instruction. Plasmids were exposed to UV irradiation (254 nm) at 150 J/m<sup>2</sup> (positive control) to check the migration of its multimeric forms (supercoiled, nicked circular and linear). UV irradiation was performed at 4 °C with UVC lamp (NeoLab, Heidelberg, Germany) emitting UV light at 254 nm at a dose rate of 0.12 J/s/m<sup>2</sup>. UV light induced strand breaks of DNA and altered plasmid electrophoretic pattern.

Plasmid samples at 250 ng/l were subjected to 1% agarose gel electrophoresis carried out in  $0.5 \times$  TBE (Tris–Borate–EDTA) buffer. The gel was stained with ethidium bromide (0.5 mg/ml) and scanned by a CCD camera under ultraviolet light. Densitometry analysis was performed with the GeneTools by Syngene (Cambridge, UK) software. The ability of UDMA 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 (Kainthla and Zewail-Foote, 2008).

### 2.6. Comet assay

The comet assay was performed under alkaline conditions essentially according to the procedure of Singh et al. (1988) with modifications (Klaude et al., 1996) as described previously (Poplawski et al., 2009). Suspension of cells in 0.75% low-melting-point agarose dissolved in PBS was spread onto microscope slides pre-coated 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 electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was conducted at 4 °C (the temperature of the running buffer did not exceed 12 °C) for 20 min at an electric field strength of 0.73 V/cm (290 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 µg/ml DAPI and covered with cover slips. To prevent additional DNA damage, all the steps described above were conducted under dimmed light or in the dark. 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 (Singh and Stephens, 1997). Electrophoresis in the neutral version was conducted for 60 min, after a 20 min equilibrium period, at electric field strength of 0.41 V/cm (500 mA) at 4 °C.

The slides with comets 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). A hundred comets was randomly selected from each sample and the percentage of DNA in the tail of comet was measured. Each experiment was repeated three times. The percentage of DNA in the tail (% tail DNA) was analyzed. It is positively correlated with the level of DNA breakage or/and alkali-labile sites in the cell and is negatively correlated with the level of DNA crosslinks (Tice et al., 2000). For the neutral version, this quantity correlates positively with DNA double-strand breaks. The mean value of the % tail DNA in a particular sample was taken as an index of the DNA damage in this sample.

### 2.7. Oxidative damage to DNA

To assess the role of oxidative damage to DNA in the genotoxicity of UDMA, we employed DNA repair enzymes endonuclease III (Endo III) and formamidopyrimidine–DNA glycosylase (Fpg). Endo III converts oxidized pyrimidines into strand breaks, which can be detected by the comet assay (Collins et al., 1993). Fpg recognizes and removes 7,8-dihydro-8-oxoguanine (8-oxoguanine), the imidazole ring-opened purines 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-Gua) and 4,6-diamino-5-formamidopyrimidine (Fapy-Ade) as well as small amounts of 7,8-dihydro-8-oxo-adenine (8-oxoadenine) (Krokan et al., 1997). The removal of specific modified bases from DNA by this enzyme leads to apurinic or apyrimidinic sites, which are subsequently cleaved by its AP-lyase activity

producing a gap in the DNA strand, which can be detected by the comet assay. After the incubation with UDMA and lysis in 2.5 M NaCl, 0.1 mM EDTA, 10 mM Tris–HCl, 1% Triton X-100, pH 10.0, for 1 h at 4 °C, the slides from the comet assay were washed three times in the enzyme buffer containing 40 mM HEPES–KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0 and drained, and the agarose was covered with 50 µl of the enzyme buffer or the enzyme at 1 µg/ml in buffer, sealed with a cover glass and incubated for 30 min at 37 °C (Tudek et al., 1998). Further steps were done as described in the Section 2.6. Lymphocytes exposed to 20 µM hydrogen peroxide for 10 min on ice serve as a positive control. Since the enzymatic buffer induced a DNA damage, the values of the %DNA in tail in the buffer were only subtracted from the value for the enzyme with buffer. To express oxidative modification to the DNA bases evoked by UDMA at a particular concentration, %DNA in tail for the control (no UDMA) was subtracted from %DNA in tail for UDMA at this concentration.

### 2.8. Pulsed field gel electrophoresis

Pulsed field gel electrophoresis was performed as described previously (Poplawski et al., 2009). Briefly, the cells were exposed to UDMA, mixed with agarose and after solidifying were incubated overnight with proteinase K. Electrophoresis ran for 24 h at 6 V/cm and the time-pulse gradient was 1–500 s for 60 s and 1500–1600 s for 120 s at 14 °C. Chromosomes of the yeast *Saccharomyces cerevisiae* (Bio-Rad) were used as molecular weight standards. DNA double-strand breaks were measured as the fraction of activity released (FAR; ratio of DNA released in an electrophoretic lane and total DNA) (Foray et al., 1999).

### 2.9. Apoptosis

The BD Annexin V-FITC Apoptosis Detection Kit I was used to measure apoptosis. The kit contains Annexin V conjugated to the fluoro-chrome FITC that has affinity to the phosphatidylserine, which is transferred through cell membrane in the earlier stages of apoptosis. Propidium iodine was used to distinguish early apoptotic cells from cells undergo late apoptosis or necrosis. Cells that are viable are Annexin V-FITC and PI negative, cells that are in early apoptosis are Annexin-FITC positive and PI negative, cells that are in late apoptosis are both Annexin-FITC and PI positive, cells already dead are only PI positive. After 6 h of incubation with UDMA cells were washed in cold PBS and resuspended in  $1 \times$  binding buffer at  $10^6$  cells/ml. Five microlitre of Annexin V-FITC and 5 µl of PI were added to an aliquot of 100 µl ( $10^5$  cells) of cells suspension, gently mixed by pipetting and incubated for 30 min at room temperature in the dark. Next, 400 µl of  $1 \times$  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.

### 2.10. Cell cycle

In the cell-cycle experiment, we replaced lymphocytes with highly proliferating human cancer cells. 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 iodine 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 FlowJo software (v. 7.2.4).

### 2.11. DNA repair

To examine DNA repair, the cells after the 1 h pre-treatment with 0.75 mM UDMA at 37 °C were washed and resuspended in a fresh, UDMA-free RPMI 1640 medium preheated to 37 °C. Aliquots of the suspension were taken immediately and 30, 60, 90, and 120 min later. Placing the samples in an ice bath stopped the repair activity of cells. Cells exposed to 20  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 15 min served as a positive control, whereas lymphocytes incubated in fresh pure medium only were negative controls.

### 2.12. Data analysis

The values in this study were expressed as mean  $\pm$  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  $\pm$  S.D. 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).

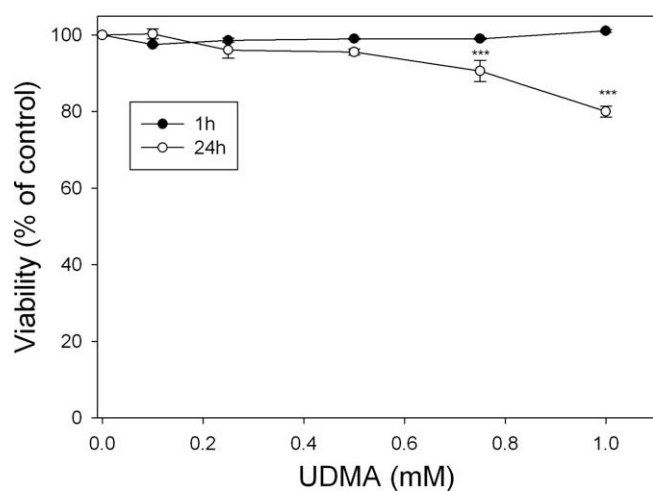
## 3. Results

### 3.1. Cell viability

UDMA decreased the viability of the cells in a dose dependent manner, with the relative viability of about 80% of the control at UDMA concentrations, 0.75 mM and 1 mM ( $p < 0.001$ ) after 24 h of incubation (Fig. 2). No significant changes in the viability was observed after 1 h incubation.

### 3.2. DNA damage in vitro

The analysis of the conformation of the plasmid DNA exposed to UDMA at various concentrations is displayed in Table 1 and Fig. 3. The densitometric analysis showed no changes in the optical density of any plasmid conformation at any concentration of the chemical. The plasmid irradiated with UV (positive control) displayed a decrease in the density of supercoiled form and an in-



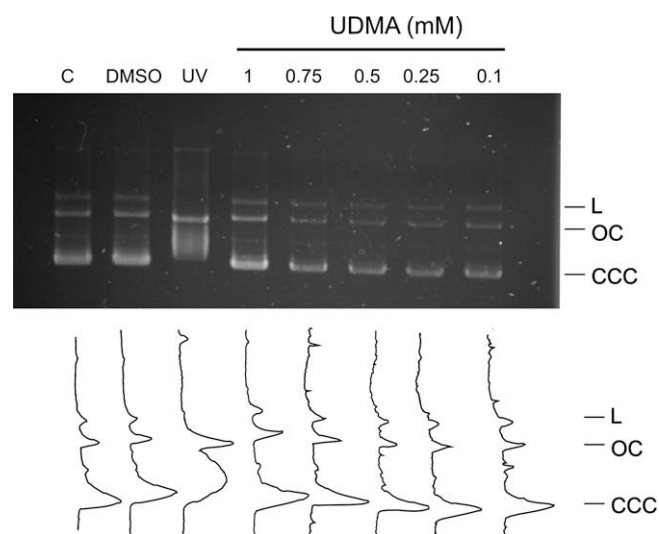
**Fig. 2.** Cell viability of human peripheral blood lymphocytes after 1 h (black symbols) and 24 h (white symbols) incubation with the urethane dimethacrylate (UDMA) measured by flow cytometry with calcein-acetoxymethyl ester and propidium iodide. Displayed is the mean of three experiments of  $5 \times 10^4$  measurements each, error bars denote standard deviation, \*\*\* $p < 0.001$  as compared with the unexposed control.

**Table 1**

The ratio of open circular DNA to the total amount of DNA (*R*) of isolated pUC19 plasmid exposed to urethane dimethacrylate (UDMA). The *R* values for supercoiled DNA were multiplied by 1.66 to correct for the decreased intercalating ability of ethidium bromide.

UDMA (mM)	<i>R</i>
0 (control)	0.125
0 (DMSO)	0.130
UV (positive control)	0.341*
0.1	0.129
0.25	0.129
0.5	0.137
0.75	0.160
1	0.145

\*  $p < 0.001$  as compared to control.

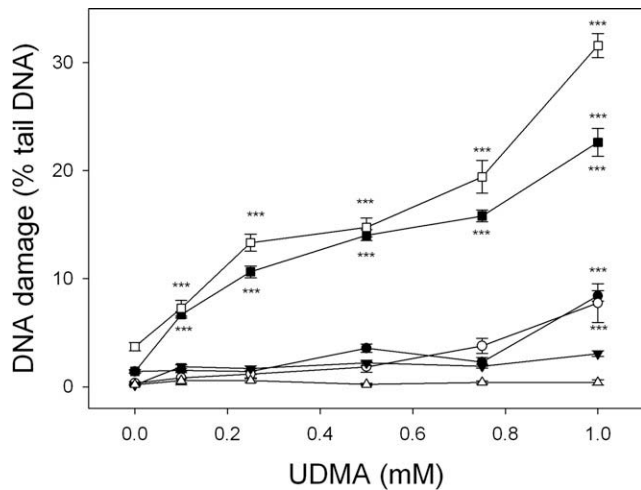


**Fig. 3.** Agarose gel electrophoresis of pUC19 plasmid exposed to urethane dimethacrylate UDMA at 0.1; 0.25; 0.5; 0.75 and 1 mM or UV radiation at the dose rate of  $0.12 \text{ J m}^{-2} \text{ s}^{-1}$  as compared with unexposed control (C) and UDMA solvent (DMSO). The dependence of the optical density on the distance of migration is also displayed. L, denotes the linear form of the plasmid; OC, open circular; CCC, covalently closed circular.

crease in the density of the two remaining forms. Therefore, UDMA may not have an ability to interact directly with DNA.

### 3.3. DNA damage in human lymphocytes

Fig. 4 displays the mean percentage tail DNA of lymphocytes exposed for 1 or 24 h to UDMA and analyzed by the comet assay in alkaline, pH 12.1 or neutral version. In the alkaline version, UDMA increased tail DNA in a concentration-dependent manner ( $p < 0.001$  for both kind of incubations at all UDMA concentrations). Single and double DNA strand breaks as well as alkali-labile sites can be detected in this version of the comet assay. Because the extent of DNA damage in the neutral and pH 12.1 versions of the comet assay was much smaller than in alkaline one, we conclude that UDMA induced rather alkali-labile sites than strand breaks. However, the extent of the tail DNA for neutral version was significantly different from the control. Therefore, a small number of DNA double-strand breaks could be induced by UDMA. To verify this speculation we performed pulsed field gel electrophoresis, which is probably more specific method for assessment DSBs than comet assay. Using this technique we checked that UDMA did not induce DSBs (results not shown).



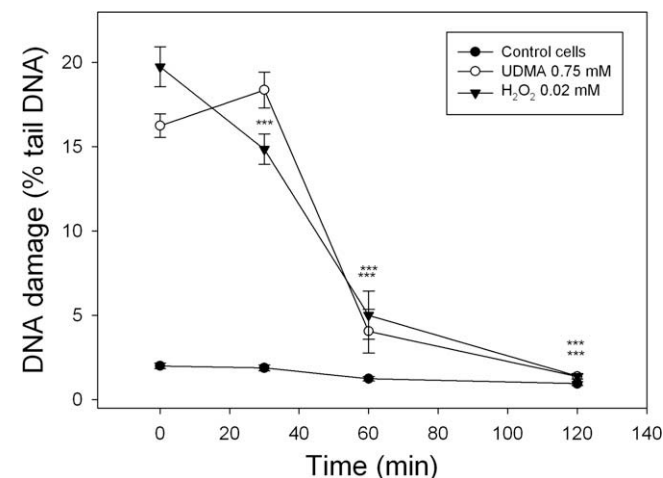
**Fig. 4.** DNA damage in human lymphocytes exposed to (UDMA) for 1 h (black) or 24 h (white) at 37 °C measured as percentage in the tail DNA in comets of alkaline version (square), neutral (triangle) and pH 12.1 (circles) of the comet assay. The mean value for 100 cells analyzed in each treatment in three independent experiments is displayed, and error bars represent S.E.M.

#### 3.4. DNA repair

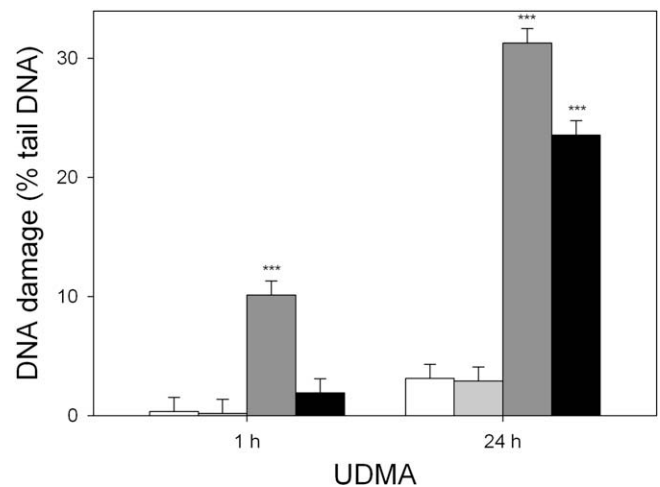
We analyzed the kinetics of DNA repair in human lymphocytes after UDMA treatment by measuring DNA damage in the cells exposed to UDMA at 0.75 mM immediately after the exposure as well as 30, 60 and 120 thereafter (Fig. 5). The comet tail DNA of the control cells was constant and of a low value, indicating that preparation and subsequent processing of the lymphocytes did not introduce any significant damage to their DNA. The cells exposed to UDMA were able to partially recover after 60 min and fully recover after 120 min ( $p < 0.001$ ).

#### 3.5. Oxidative modifications to the DNA bases

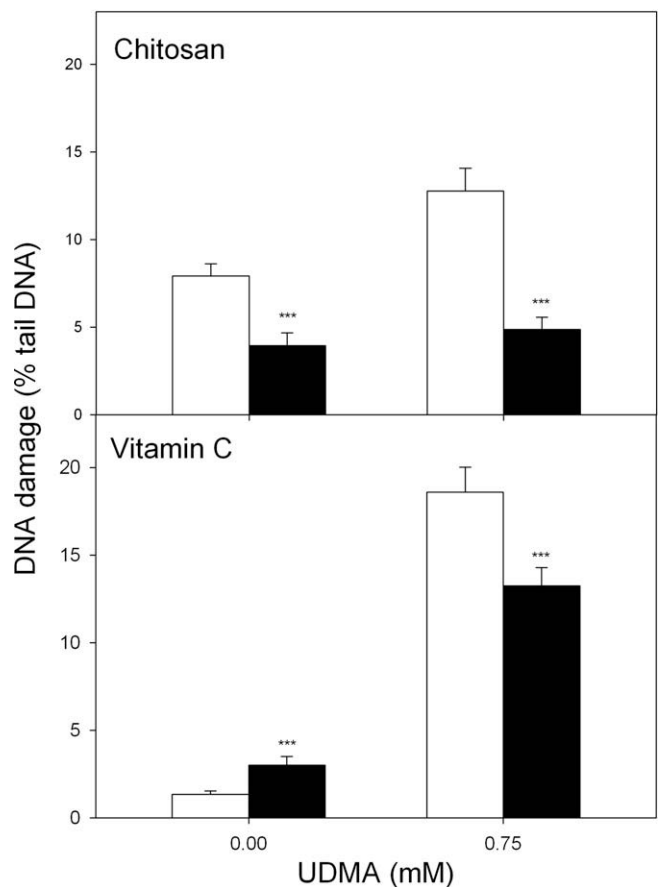
Fig. 6 presents the mean % tail DNA of human lymphocytes exposed for 1 h and 24 h at 37 °C to UDMA at 0.75 mM, lysed and



**Fig. 5.** Time course of DNA repair in human lymphocytes exposed for urethane dimethacrylate (UDMA) for 1 h at 0.75 mM (white circles) and treated with hydrogen peroxide at 20  $\mu$ M for 15 min on ice (black triangles) compared with untreated control (black circles). After the exposure, the cells were washed and incubated in a UDMA-free medium at 37 °C. The number of cells in each time-interval was 100. The figure shows mean results from three independent experiments. Error bars denote S.E.M.



**Fig. 6.** Oxidative modifications to the DNA bases evoked by urethane dimethacrylate (UDMA) at 0.75 mM measured as percentage of DNA in the tail in alkaline comet assay with formamidopyrimidine-DNA glycosylase (Fpg, dark grey bars) and endonucleases III (Endo III, black bars) after 1 h or 24 h incubation. White and light grey bars represent percentage of DNA in the tail in alkaline comet assay with Fpg and Endo III for control cells (without UDMA), respectively. The value of comet tail DNA in the presence of either enzyme for UDMA was reduced by the value obtained in comet assay without any enzyme and the value for enzymatic buffer only. The number of cells analyzed was 100. The results are the mean of three independent experiments. Error bars denote S.E.M.



**Fig. 7.** DNA damage measured as the mean comet tail DNA of human lymphocytes exposed for 1 h at 37 °C to urethane dimethacrylate (UDMA) in the absence (empty bars) or in the presence (black bars) of 0.2% methylglycol chitosan (chitosan) or 50  $\mu$ M vitamin C (vitamin C). The number of cells in each treatment was 100. The figure shows mean results from three independent experiments. Error bars denote S.E.M.

post-treated with Fpg or EndoIII, reduced by mean % tail DNA for cells incubated only with enzymatic buffer. As a result, we analyzed only these modifications to the DNA bases, which were not recognized in the non-modified version of the comet assay. Lymphocytes exposed to UDMA for 24 h and treated with either enzyme showed greater % tail DNA than those untreated with any enzyme ( $p < 0.001$ ). Lymphocytes exposed to UDMA for 1 h and treated with Fpg showed greater % tail DNA than those untreated with enzyme ( $p < 0.001$ ). This suggests that oxidative modifications to the DNA bases play a role in the genotoxic action of UDMA. The extent of DNA damage increased with the prolonged time of incubation.

### 3.6. Effect of vitamin C and methylglycol chitosan on the DNA-damaging action of UDMA

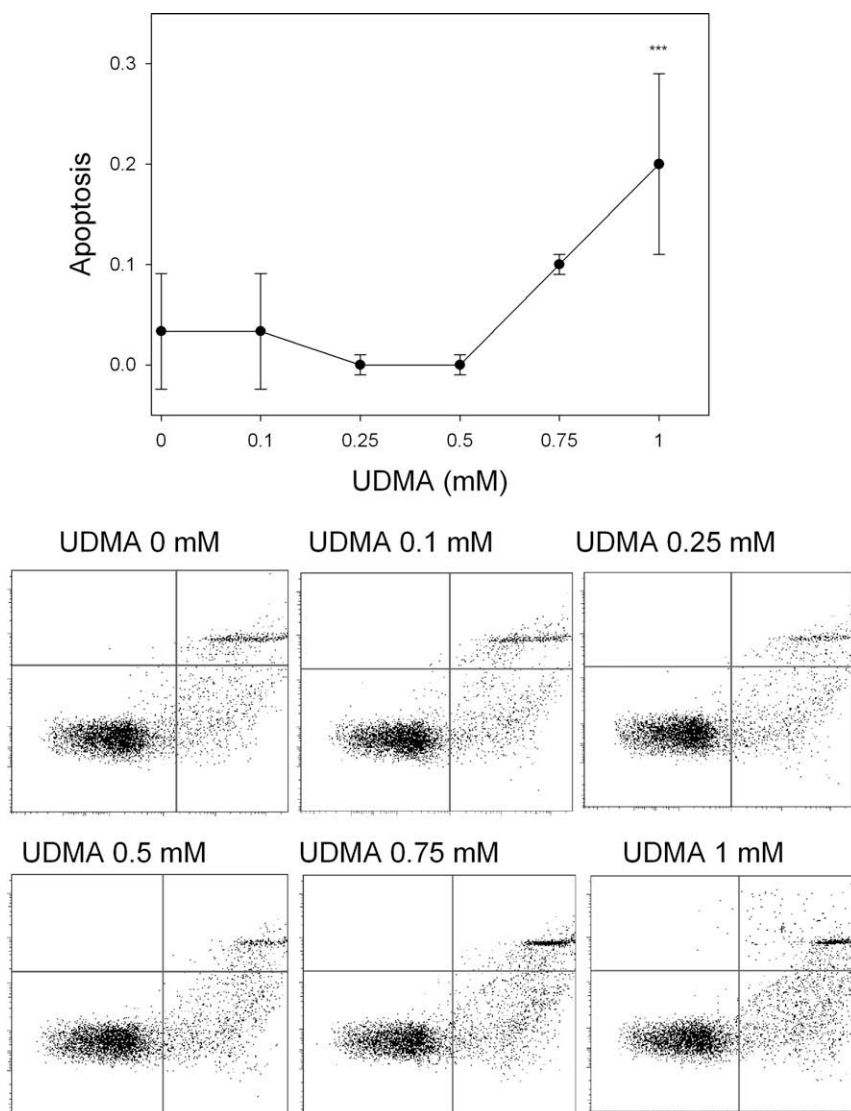
Fig. 7 shows the mean percentage tail DNA for lymphocytes treated with UDMA in the presence or in the absence of 50  $\mu\text{M}$  vitamin C or 0.2% methylglycol chitosan. Both agents significantly decreased the extent of DNA damage induced by UDMA ( $p < 0.01$ ).

### 3.7. Apoptosis

UDMA-induced apoptosis in human lymphocytes at 1 mM (Fig. 8). After a 6 h incubation with UDMA at 0.1, 0.25, 0.5 and 0.75 mM the cells were Annexin V-FITC and PI negative, so they were viable and not undergoing apoptosis. After incubation with UDMA at 1 mM, there were 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). Apoptosis was expressed as a ratio of the number of early and late apoptotic cells to the number of cells with no measurable apoptosis. We observed an increase in apoptosis in the lymphocytes at 0.75 mM UDMA, but this increase was not statistically significant ( $p = 0.117$ ).

### 3.8. Cell cycle

In order to determine whether UDMA had an influence on the progression of cell cycle, human alveolar basal epithelial A549 cancer cells were exposed 24 h to UDMA at increasing concentrations.



**Fig. 8.** Apoptosis of human lymphocytes exposed to urethane dimethacrylate (UDMA). Apoptosis was assessed by flow cytometry with Annexin V-FITC/propidium iodide (PI). Displayed is the mean of three experiments of  $5 \times 10^4$  measurements each, error bars denote standard deviation. The apoptosis was expressed as a percentage of early and late apoptotic cells.

UDMA did not influence the cell cycle of A549 cells (results not shown).

#### 4. Discussion

In the present study we evaluated the ability of UDMA to damage DNA in normal human lymphocytes. We applied the chemical at millimolar concentration range, since monomers eluted from dental-composite materials can be found in this range in human tissues (Bouillaguet et al., 1996; Cetinguc et al., 2007; Noda et al., 2002; Polydorou et al., 2009b). Such concentrations are high enough to induce cyto- and genotoxic effects not only in the pulp cells, but almost all human cells when UDMA penetrates human body through the bloodstream. We observed a significant DNA-damaging effect of UDMA and these results are in agreement with previous reports suggesting genotoxic potential of UDMA monomer (Kleinsasser et al., 2006, 2004).

We incubated cells with UDMA for 6 h in the apoptosis study, because a longer time of incubation might yield false-positive results, as stated by the producer of the apoptosis kit. We observed the ability of UDMA to induce apoptosis, which is in accordance with the reports published so far on apoptotic properties of dental methacrylate monomers (Eckhardt et al., 2009; Lee et al., 2006; Poplawski et al., 2009; Schweikl et al., 2006). Comparing the results for the DNA-damaging and pro-apoptotic activity of UDMA (Figs. 4 and 8, respectively) suggests that only a small part of DNA lesions observed at the highest concentration of UDMA (1 mM) might arise from the induction of apoptosis in human lymphocytes. Inability of UDMA at 1 mM to induce DNA double-strand breaks, as we checked by pulsed field gel electrophoresis, confirm this suggestion. The extent of DNA lesions induced by apoptosis is too small to be detected by neutral version of comet assay, but it is visible in alkaline version due its sensitivity (one double-strand break is equal to two single strand breaks). Unfortunately, we have no possibility to separate DNA lesions caused by apoptosis from another DNA lesions observed in alkaline comet assay, so we can only speculate about their level. The observed DNA lesions could arise by starting up DNA fragmentation, which is a feature for apoptosis. We can consider at least two pathways that could cause apoptosis in cells incubated with UDMA: UDMA may act as a genotoxic carcinogen inducing DNA lesions that impairs DNA metabolism, culminating in the induction of cell death by apoptosis. The another pathway may follow from biodegradation of UDMA. This process might result in a mitochondrial dysfunction and, therefore, could initiate mitochondria-dependent apoptotic pathway that involves pro- and antiapoptotic protein binding, the release of cytochrome c, and transcription-independent p53 signaling, leading to cell death.

UDMA in our study did not induce DNA strand breaks directly in isolated DNA, which suggests that the chemical required cellular activation to display the DNA-damaging properties. The mechanism of metabolic transformation of UDMA is not clear and there were no reports on this topic. It was only shown that UDMA cytotoxicity seemed to be caused by alterations of glucose metabolism arising from mitochondrial dysfunction (Nocca et al., 2009) or from oxidative stress (Schweikl et al., 2008). UDMA has a similar chemical structure to other methacrylate dental monomers. We suppose that its biodegradation may occurred in the same manner than other monomers, including chemical hydrolysis by agents that stimulate the secretion of saliva changes in pH, enzymatic degradation by hydrolysis, or oxidation (Santerre et al., 2001). Main intermediates produced under two metabolic pathways for the degradation of monomers are methacrylic acid and epoxides (Reichl et al., 2002). The latter are particularly important because they are toxic and possibly mutagenic and carcinogenic agents.

We observed a substantial difference between the alkaline version of the comet assay and the neutral or pH 12.1 versions of the test, suggesting that UDMA could predominantly produce alkali-labile sites and not strand breaks in DNA. The alkali-labile sites may arise as a result of the action of reactive oxygen species generated during the degradation of UDMA, because it was shown that other structurally similar monomer, TEGDMA also used in dental resin-based materials, stimulated reactive oxygen species production in human fibroblasts and human monocytic leukemia THP-1 cells (Schweikl et al., 2008; Eckhardt et al., 2009). Our result strongly supports this hypothesis. We used two enzymes that recognized independently oxidative modifications to the DNA bases. This is a well-established, direct method to detect oxidative DNA damage. Endo III recognizes preferentially oxidized pyrimidines, whereas Fpg preferentially recognizes oxidized purines. Both enzymes are involved in the initial stages of the base excision repair to remove specific modified bases from DNA. As a result of their action is the formation of an apurinic or apyrimidinic site (AP-site), which is consequently cut producing a gap in the DNA strand (David-Cordonnier et al., 2000). The amount of the oxidative DNA lesions increased with UDMA incubation time. These data indicated that DNA lesions evoked by UDMA could accumulate in time as long as UDMA is present. Because UDMA may diffuse into the tooth pulp or the gingiva continuously, the cells may not manage with a great amount of DNA lesions and undergo apoptosis or cancer transformation.

The results obtained are in agreement with other reports suggesting that oxidative mechanisms may underline cyto- and genotoxic activity of methacrylates. Triethylene glycol dimethacrylate (TEGDMA) may contribute to the oxidative stress in human fibroblast (Schweikl et al., 2008). It is a serious threat for cells because it is connected with drastic depletion of cellular glutathione which serves as the main cell detoxicant agent (Stanislawski et al., 2003). The negative effect of methacrylates action on human cells can be decreased by antioxidants (Stanislawski et al., 2003; Walther et al., 2004). We observed that vitamin C decreased the extent of DNA damage induced by UDMA in human lymphocytes. These results might be taken into consideration in the clinical practice, as the vitamin can easily be supplemented in the diet. We also observed a pronounced protective effect of methylglycol chitosan on DNA lesions generated by UDMA. Chitosan is often used in dental practice due its antimicrobial and water sorbent activity that helps to keep stability of dental-composite materials. Result obtained in our work strongly suggests another chitosan favorable property. In light of the role of oxidative lesions to DNA bases in UDMA genotoxicity and the similarity in the protective effects of chitosan and vitamin C, we suggest that chitosan displayed an antioxidant action in our experiment. Recently Liu et al. (2009) showed that chitosan oligosaccharides had a protective effect against hydrogen peroxide and lowered oxidative stress. However, the mechanism of action of chitosan on DNA lesions evoked by UDMA remains unknown, but this subject is worth further research as the compound, in contrary to vitamin C, may be permanently fixed to the tooth structure.

At 0 mM UDMA the extent of DNA damage in the presence of chitosan did not differ from that in the presence of vitamin C (Fig. 7,  $p = 0.312$ ). We observed a significant ( $p < 0.001$ ) difference between initial levels of DNA damage in these two groups and it might follow from the inter-individual differences in the donor groups enrolled in these experiments. We have not aimed to compare directly the protective action of chitosan and that is why we have used different control groups.

Results obtained in our work showed that Fpg produced significant more DNA breaks than Endo III, suggesting that majority of DNA lesions induced by UDMA are oxidized purines. This is not surprising, because oxidation potentials of DNA bases that

determine the level of oxidation are different for different bases. Guanine is most easily oxidized among all the DNA bases, because the oxidation potential of it is lower than remaining three bases (Burrows and Muller, 1998; Steenken and Jovanovic, 1997). Another important factor to determine the susceptibility of DNA bases to oxidative attacks is the distribution of electrons involved in the chemical reaction of oxidation. A large part of electrons with the highest energy is located on the 5'-G of GG sequence in B-form of double-stranded DNA (Sugiyama and Saito, 1996; Saito et al., 1998), and thus guanine is most likely to be oxidized.

UDMA could be also involved in other than oxidative mechanism of DNA damage. Apart from oxidized purines, the Fpg protein also releases a ring-opened N-7 guanine adduct from DNA (Tudek et al., 1998). Such DNA lesions may arise in a consequence of the action of DNA methylation agents (Li et al., 1997) and subsequent alkaline treatment (Graves et al., 1992). Alkaline condition occurs in the comet assay technique, thus DNA methylation can be involved in the genotoxic action of UDMA. As UDMA possesses a methyl group in its structure, we can speculate that the chemical can be demethylated inside the cell and the methyl group can be transferred onto DNA bases, but a precise mechanism underlying this process needs further study.

As a consequence of DNA damage, the progression of mammalian cells through the normal cell cycle may be stopped. This cell-cycle arrest may be needed for the activation of DNA repair pathways or apoptosis, when the cell is not able to repair the damage to its DNA. Since we showed genotoxic activity of UDMA in mammalian cells, we hypothesize that it may induce a cell-cycle delay. We used the cell-cycle experiments in human alveolar basal epithelial A549 cells instead of human lymphocytes, because the latter in our experiment were not sensitive to nocodazole used as a positive control. UDMA did not evoke any significant changes in the cell cycle of the human cancer cells. This is rather consistent with the remaining data obtained in this research. UDMA did not induce DNA double-strand breaks, which are the most serious DNA damage and which can stimulate cell-cycle arrest in the G2/M checkpoint (see Houtgraaf et al., 2006 for review). This was confirmed by pulse field gel electrophoresis, which is probably the most reliable method for assessing DNA double-strand breaks. Such disturbances in the cell cycle may be also evoked by a massive DNA damage other than double-strand breaks – single strand breaks, base modifications, bulky adducts and others, which the DNA repair machinery is not able to deal with. However, we did not observed an enormously high level of DNA damage in this experiment (Fig. 4), since lymphocytes repaired the UDMA-induced damage during an 1 h incubation (Fig. 5). In summary, we have shown that UDMA may induce a broad spectrum of cyto- and genotoxic effects in human cells and that chitosan can be considered to decrease the risk associated with the use of UDMA.

## 5. Conflict of interest

The authors declare no conflicts of interest.

## Acknowledgements

This work was supported by the Ministry of Science and Higher Education, Grant No. N N403 188134. The authors thank Ms. Monika Kicinska for her help in editing the manuscript.

## References

- Al-Hiyasat, A.S., Darmani, H., Milhem, M.M., 2005. Cytotoxicity evaluation of dental resin composites and their flowable derivatives. *Clinical Oral Investigation* 9, 21–25.
- Asmussen, A.P., 1990. Mechanical properties of heat treated restorative resins for use in the inlay/onlay technique. *European Journal of Oral Sciences* 98, 564–567.
- Beatty, M.L.S., Moore, B.K., Phillips, R.W., Roberts, T.A., 1993. Effect of crosslinking agent content, monomer functionality, and repeat unit chemistry on properties of unfilled resins. *Journal of Biomedical Materials Research* 27, 403–413.
- Bouillaguet, S., Wataha, J.C., Hanks, C.T., Ciucchi, B., Holz, J., 1996. In vitro cytotoxicity and dentin permeability of HEMA. *Journal of Endodontics* 22, 244–248.
- Burrows, C.J., Muller, J.G., 1998. Oxidative nucleobase modifications leading to strand scission. *Chemical Reviews* 98, 1109–1151.
- Cetinguc, A., Olmez, S., Vural, N., 2007. HEMA diffusion from dentin bonding agents in young and old primary molars in vitro. *Dental Materials* 23, 302–307.
- Collins, A.R., Duthie, S.J., Dobson, V.L., 1993. Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis* 14, 1733–1735.
- David-Cordonnier, M.H., Laval, J., O'Neill, P., 2000. Clustered DNA damage, influence on damage excision by XRS5 nuclear extracts and *Escherichia coli* Nth and Fpg proteins. *The Journal of Biological Chemistry* 275, 11865–11873.
- Eckhardt, A., Gerstmayr, N., Hiller, K.A., Bolay, C., Waha, C., Spagnuolo, G., Camargo, C., Schmalz, G., Schweikl, H., 2009. TEGDMA-induced oxidative DNA damage and activation of ATM and MAP kinases. *Biomaterials* 30, 2006–2014.
- Ferracane, J.L., 2006. Hygroscopic and hydrolytic effects in dental polymer networks. *Dental Materials* 22, 211–222.
- Foray, N., Arlett, C.F., Malaise, E.P., 1999. Underestimation of the small residual damage when measuring DNA double-strand breaks (DSB): is the repair of radiation-induced DSB complete? *International Journal of Radiation Biology* 75, 1589–1595.
- Geurtsen, W., 1998. Substances released from dental resin composites and glass ionomer cements. *European Journal of Oral Sciences* 106, 687–695.
- Graves, R., Laval, J., Pegg, A.E., 1992. Sequence specificity of DNA repair by *Escherichia coli* Fpg protein. *Carcinogenesis* 13, 1455–1459.
- Houtgraaf, J.H., Versmissen, J., van der Giessen, W.J., 2006. A concise review of DNA damage checkpoints and repair in mammalian cells. *Cardiovascular Revascularization Medicine* 7, 165–172.
- Jontell, M., Hanks, C.T., Bratel, J., Bergenholtz, G., 1995. Effects of unpolymerized resin components on the function of accessory cells derived from the rat incisor pulp. *Journal of Dental Research* 74, 1162–1167.
- Kainthla, R., Zewail-Foote, M., 2008. Oxidative DNA damage following photoexcitation of daunomycin: direct role of oxygen. *Journal of Photochemistry and Photobiology* 198, 200–204.
- Klaude, M., Eriksson, S., Nygren, J., Ahnstrom, G., 1996. The comet assay: mechanisms and technical considerations. *Mutation Research* 363, 89–96.
- Kleinsasser, N.H., Schmid, K., Sassen, A.W., Harreus, U.A., Staudenmaier, R., Folwaczny, M., Glas, J., Reichl, F.X., 2006. Cytotoxic and genotoxic effects of resin monomers in human salivary gland tissue and lymphocytes as assessed by the single cell microgel electrophoresis (comet) assay. *Biomaterials* 27, 1762–1770.
- Kleinsasser, N.H., Wallner, B.C., Harreus, U.A., Kleinjung, T., Folwaczny, M., Hickel, R., Kehe, K., Reichl, F.X., 2004. Genotoxicity and cytotoxicity of dental materials in human lymphocytes as assessed by the single cell microgel electrophoresis (comet) assay. *Journal of Dentistry* 32, 229–234.
- Krokan, H.E., Standal, R., Slupphaug, G., 1997. DNA glycosylases in the base excision repair of DNA. *Biochemical Journal* 325 (Pt. 1), 1–16.
- Lee, D.H., Lim, B.S., Lee, Y.K., Ahn, S.J., Yang, H.C., 2006. Involvement of oxidative stress in mutagenicity and apoptosis caused by dental resin monomers in cell cultures. *Dental Materials* 22, 1086–1092.
- Stanislawski, M.L., Bourd, K., Soheili-Majid, E., Goldberg, M., Pèrianin, A., 2003. TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. *Journal of Biomedical Materials Research* 66A, 476–482.
- Li, Q., Laval, J., Ludlum, D., 1997. Fpg protein releases a ring-opened N-7 guanine adduct from DNA that has been modified by sulfur mustard. *Carcinogenesis* 18, 1035–1038.
- Liu, H.T., Li, W.M., Xu, G., Li, X.Y., Bai, X.F., Wei, P., Yu, C., Du, Y.G., 2009. Chitosan oligosaccharides attenuate hydrogen peroxide-induced stress injury in human umbilical vein endothelial cells. *Pharmacological Research* 59, 167–175.
- Mohsen, N.M., Craig, R.G., Hanks, C.T., 1998. Cytotoxicity of urethane dimethacrylate composites before and after aging and leaching. *Journal of Biomedical Materials Research* 39, 252–260.
- Nassiri, M.R., Hanks, C.T., Cameron, M.J., Strawn, S.E., Craig, R.G., 1994. Application of flow cytometry to determine the cytotoxicity of urethane dimethacrylate in human cells. *Journal of Biomedical Materials Research* 28, 153–158.
- Nocca, G., Martorana, G.E., De Sole, P., De Palma, F., Callà, C., Corsale, P., Antenucci, M., Gambarini, G., Chimenti, C., Giardina, B., Lupi, A., 2009. Effects of 1,4-butanediol dimethacrylate and urethane dimethacrylate on HL-60 cell metabolism. *European Journal of Oral Science* 117, 175–181.
- Noda, M., Wataha, J.C., Kaga, M., Lockwood, P.E., Volkmann, K.R., Sano, H., 2002. Components of dental adhesives modulate heat shock protein 72 expression in heat-stressed THP-1 human monocytes at sublethal concentrations. *Journal of Dental Research* 81, 265–269.
- Polydorou, O., König, A., Hellwig, E., Kümmerer, K., 2009a. Urethane dimethacrylate: a molecule that may cause confusion in dental research. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials* 91, 1–4.

- Polydorou, O., Konig, A., Hellwig, E., Kummerer, K., 2009b. Long-term release of monomers from modern dental-composite materials. *European Journal of Oral Sciences* 117, 68–75.
- Poplawski, T., Pawlowska, E., Wisniewska-Jarosinska, M., Ksiazek, D., Wozniak, K., Szczepanska, J., Blasiak, J., 2009. Cytotoxicity and genotoxicity of glycidyl methacrylate. *Chemico Biological Interactions* 180, 69–70.
- Ratanasathien, S., Wataha, J.C., Hanks, C.T., Dennison, J.B., 1995. Cytotoxic interactive effects of dentin bonding components on mouse fibroblasts. *Journal of Dental Research* 74, 1602–1606.
- Reichl, F.X., Durner, J., Hickel, R., Spahl, W., Kehe, K., Walther, U., Gempel, K., Liebl, B., Kunzelmann, K.H., Hume, W., 2002. Uptake, clearance and metabolism of TEGDMA in guinea pigs. *Dental Materials* 18, 581–589.
- Reichl, F.X., Esters, M., Simon, S., Seiss, M., Kehe, K., Kleinsasser, N., Folwaczny, M., Glas, J., Hickel, R., 2006. Cell death effects of resin-based dental material compounds and mercurials in human gingival fibroblasts. *Archives of Toxicology* 80, 370–377.
- Saito, I., Nakamura, T., Nakatani, K., Yoshioka, Y., Yamaguchi, K., Sugiyama, H., 1998. Mapping of the hot spots for DNA damage by one-electron oxidation: efficacy of GG doublets and GGG triplets as a trap in long-range hole migration. *Journal of American Chemical Society* 120, 12686–12687.
- Santerre, J.P., Shajii, L., Leung, B.W., 2001. Relation of dental composite formulations to their degradation and the release of hydrolyzed polymeric-resin-derived products. *Critical Reviews in Oral Biology* 12, 136–151.
- Schweikl, H., Hiller, K.A., Eckhardt, A., Bolay, C., Spagnuolo, G., Stempf, T., Schmalz, G., 2008. Differential gene expression involved in oxidative stress response caused by triethylene glycol dimethacrylate. *Biomaterials* 29, 1377–1387.
- Schweikl, H., Schmalz, G., Rackebandt, K., 1998. The mutagenic activity of unpolymerized resin monomers in *Salmonella typhimurium* and V79 cells. *Mutation Research* 415, 119–130.
- Schweikl, H., Schmalz, G., Spruss, T., 2001. The induction of micronuclei in vitro by unpolymerized resin monomers. *Journal of Dental Research* 80, 1615–1620.
- Schweikl, H., Spagnuolo, G., Schmalz, G., 2006. Genetic and cellular toxicology of dental resin monomers. *Journal of Dental Research* 85, 870–877.
- Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantification of low levels of DNA damage in individual cells. *Experimental Cell Research* 17, 84–191.
- Singh, N.P., Stephens, R.E., 1997. Microgel electrophoresis: sensitivity, mechanisms, and DNA electrostretching. *Mutation Research* 383, 167–175.
- Spahl, W., Budzikiewicz, H., Geurtsen, W., 1998. Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry. *Journal of Dentistry* 26, 137–145.
- Steenken, S., Jovanovic, S., 1997. How easily oxidizable is DNA? One-electron reduction potentials of adenosine and guanosine radicals in aqueous solution. *Journal of American Chemical Society* 119, 617–618.
- Sugiyama, H., Saito, I., 1996. Theoretical studies of GG-specific photocleavage of DNA via electron transfer: significant lowering of ionization potential and 5'-localization of HOMO of stacked GG bases in B-form DNA. *Journal of American Chemical Society* 118, 7063–7068.
- Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis* 35, 206–221.
- Tudek, B., Van Zeeland, A.A., Kusmierek, J.T., Laval, J., 1998. Activity of *Escherichia coli* DNA-glycosylases on DNA damaged by methylating and ethylating agents and influence of 3-substituted adenine derivatives. *Mutation Research* 407, 169–176.
- Walther, U.I., Siagian, I.I., Walther, S.C., Reichl, F.X., Hickel, R., 2004. Antioxidative vitamins decrease cytotoxicity of HEMA and TEGDMA in cultured cell lines. *Archives of Oral Biology* 49, 125–131.
- Yoshii, E., 1997. Cytotoxic effects of acrylates and methacrylates: relationships of monomer structures and cytotoxicity. *Journal of Biomedical Materials Research* 37, 517–524.