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# Cytotoxicity and DNA double-strand breaks induced by dental composite eluates and the effects of antioxidants on dental resin co-monomer epoxy metabolites

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Meiner lieben Frau und meiner Familie

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# 1 Abbreviations

| | |
|---|---|
| **Asc** | Ascorbic acid |
| **BHT** | 2,6-Di-*t*-butyl-4-methyl phenol |
| **CQ** | Camphorquinone |
| **CSA** | Champhoric acid anhydride |
| **DC** | Degree of conversion |
| **DDHT** | Diethyl-2,5-dihydroxytrephthalate |
| **DMABEE** | 4-Dimethylaminobenzoic acid ethyl ester |
| **DMEM** | Dulbecco's modified eagle medium |
| **DNA-DSBs** | DNA double-strand breaks |
| **$EC_{50}$** | Half-maximum effect concentration |
| **EGDMA** | Ethylene glycol dimethacrylate |
| **EMPA** | 2,3-Epoxy-2-methylpropionic acid |
| **EMPME** | 2,3-Epoxy-2-methyl-propionicacid-methylester |
| **FCS** | Fetal calf serum |
| **GC/MS** | Gas chromatography/mass spectrometry |
| **GSH** | Glutathione |
| **HEMA** | 2-Hydroxyethyl methacrylate |
| **HGFs** | Human gingival fibroblasts |
| **HMBP** | 2-Hydroxy-4-methoxy-benzophenone |
| **HPMA** | Hydroxypropyl methacrylate |
| **MA** | Methacrylic acid |
| **NAC** | N-acetylcystine |
| **PBS** | Phosphate-buffered saline |
| **ROS** | Reactive oxygen species |
| **TEGDMA** | Tetraetheleneglycol dimethacrylate |
| **TinP** | 2(2′-Hydroxy-5′-methylphenyl) benzotriazol |
| **TMPTMA** | Trimethylolpropane trimethacrylate |
| **XTT** | Tetrazolium salt |

# 2 Publication list

## 2.1 Publication for Cumulative Dissertation

Yang Y, Reichl FX, Shi J, He X, Hickel R, Högg C. *Cytotoxicity and DNA double-strand breaks in human gingival fibroblasts exposed to eluates of dental composites*. Dent Mater. 2017 In press, https://doi.org/10.1016/j.dental.2017.10.002

Yang Y, He X, Shi J, Hickel R, Reichl FX, Högg C. *Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to dental resin co-monomer epoxy metabolites.* Dent Mater. 2017; 33(4), 418-426.

## 2.2 Further Publications

He X, Reichl FX, Wang Y, Michalke B, Milz S, Yang Y, Stolper P, Lindemaier G, Graw M, Hickel R, Högg C. *Analysis of titanium and other metals in human jawbones with dental implants – A case series study*. Dent Mater. 2016; 32:1042-51.

Schuster L, Reichl FX, Rothmund L, He X, Yang Y, Van Landuyt KL, Kehe K, Polydorou O, Hickel R, Högg C. *Effect of Opalescence (R) bleaching gels on the elution of bulk-fill composite components.* Dent Mater. 2016; 32:127-35.

Rothmund L, Reichl FX, Hickel R, Styllou P, Styllou M, Kehe K, Yang Y, Högg, C. *Effect of layer thickness on the elution of bulk-fill composite components.* Dent Mater. 2017; 33(1), 54-62.

# 3 Confirmation of Co-authors

The confirmation of co-authors is submitted separately.

# 4 Introduction

Light-cured composite resins consist of methacrylate resin matrix, additives and inorganic fillers [1]. The polymerization of dental composites is incomplete and residual co-monomers and additives can leach [2-5]. Many factors such as the light density, curing time and distance between light source and dental composite, etc. can affect DC [6]. The lower the DC of a composite the more composite components can be released [7]. Released (co)monomers and additives may penetrate to pulp via dentinal tubules, and affect the activity of dental pulp cells or enter the intestine by swallowing, then further reaching the circulatory system and organs [8-10]. Additionally, it was shown that methacrylates can cause allergic reactions such as asthma and contact dermatitis [11].

The residual co-monomers TEGDMA and HEMA can leach from incompletely polymerized composite resins [8]. Our previous studies investigated the uptake, distribution and elimination of TEGDMA and HEMA by means of radiolabelled $^{14}$C-TEGDMA and $^{14}$C-HEMA in guinea pigs [12, 13], as a result, the formation of MA, a metabolisation intermediate of TEGDMA and HEMA, was described [12, 13]. MA can be metabolized by two different pathways [14], and it was speculated that EMPME might be formed in epoxide pathway [15]. Additionally, it has been demonstrated that MA can also be oxidized to epoxy metabolite EMPA [15-17]. In a previous study, it was shown that $^{14}$C-TEGDMA and $^{14}$C-HEMA are mainly metabolized via epoxide pathway in A549 cells [18], moreover, the formation of EMPA in human oral cells has also been demonstrated [16].

A previous study revealed the cytotoxicity of 35 dental composite monomers and additives in human primary fibroblast cultures [19]. In addition, the mutagenicity, embryo toxicity and teratogenicity caused by released (co)monomers were also demonstrated [15]. However, most studies were performed using single composite components [20-22], therefore less data for cytotoxicity and no data for DSBs induction are available for composite eluates consisting of multiple components. In comparison with single-component experiments, study with qualified and quantified eluates may

reflect a situation closer to physiology. In the present study, therefore, cytotoxicity and DNA-DSBs induction in HGFs were investigated with dental composite eluates. The multiple composition of eluates was qualified and quantified.

This work was illustrated in the following publication: Yang Y, Reichl FX, Shi J, He X, Hickel R, Högg C. *Cytotoxicity and DNA double-strand breaks in human gingival fibroblasts exposed to eluates of dental composites*. Dent Mater. 2017 In press, https://doi.org/10.1016/j.dental.2017.10.002

In a previous study, the toxicity of EMPME and EMPA was investigated by means of a modified fluorescent stem-cell test on the embryonic stem cells of mice; as a result, embryotoxic effect and teratogenic effect were observed for EMPME and EMPA respectively [15]. The epoxy compounds are considered highly reactive molecules and toxic agents [17] which can lead to cell death if unrepaired; if they are misrepaired, chromosomal translocations and genomic instability may occur [23]. It has been demonstrated that TEGDMA and HEMA can cause DNA-DSBs [24, 25], and the addition of antioxidants, such as Asc or NAC, can reduce the cytotoxic effects and DNA-DSBs [20, 26, 27]. However, in comparison with the precursors, TEGDMA, HEMA and the intermediate MA, whether the epoxy metabolites can induce more DNA-DSBs, and whether antioxidants can lead to the reduction of DNA-DSBs in the presence of co-monomer epoxy metabolites are still unknown. Therefore, in this study, the effects of Asc and NAC on the epoxide-induced DNA-DSBs in HGFs were investigated.

This work was illustrated in the following publication: Yang Y, He X, Shi J, Hickel R, Reichl FX, Högg C. *Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to dental resin co-monomer epoxy metabolites.* Dent Mater. 2017; 33(4), 418-426.

# 5 Materials and Methods

## 5.1 Cytotoxicity and DNA double-strand breaks in human gingival fibroblasts exposed to eluates of dental composites

The composites Esthet.X® HD (Dentsply, Caulk, USA), Venus® (Heraeus Kulzer, Hanau,

Germany), X-tra fil® (VOCO GmbH, Cuxhaven, Germany), CLEARFIL™ AP-X (Kuraray Europe GmbH, Hattersheim am Main, Germany), Admira® Fusion (VOCO GmbH, Cuxhaven, Germany) and QuiXfil® (DENTSPLY DeTrey GmbH, Konstanz, Germany) were polymerized and immersed into DMEM for 72h. Subsequently, HGFs were incubated with the corresponding composite eluates. The cell viability of HGFs was obtained from an XTT-based assay. DNA-DSBs were determined using a γ-H2AX immunofluorescence assay. The qualification and quantification of eluates were performed by GC/MS.

### 5.2 Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to dental resin co-monomer epoxy metabolites

MA, EMPME were obtained from Provitro GmbH (Berlin, Germany); EMPA was synthesized by oxidation of MA, according to the method described by Yao and Richardson [28]. $EC_{50}$ Values were obtained from an XTT-based viability assay in which the HGFs (Passage 10, Provitro GmbH, Berlin, Germany) were treated with medium containing MA (1-100 mM), EMPME (0.5-12 mM) and EMPA (0.01-10 mM), respectively, followed by incubation for 24 h. A γ-H2AX immunofluorescence assay was performed to determine the DNA-DSBs. The cells were exposed for 6 h to medium containing MA (15.64; 5.21; 1.56 mM), EMPME (2.58; 0.86; 0.26 mM), and EMPA (1.72; 0.57; 0.17 mM), respectively, or the antioxidants alone; the concentrations of antioxidants tested alone were Asc (50; 100; 200; 500 μM) and NAC (50; 100; 200; 500 μM). The concentrations of antioxidants to be added to MA, EMPME, EMPA for γ-H2AX assay were: Asc (50; 100; 200 μM) and NAC (50; 100; 200; 500 μM).

# 6 Results

## 6.1 Cytotoxicity and DNA double-strand breaks in human gingival fibroblasts exposed to eluates of dental composites

### 6.1.1 XTT assay

No significant difference ($p>0.05$) of cell viability was found in eluates of investigated composites.

### 6.1.2 γ-H2AX assay

The eluates of Esthet.X® HD and Venus® induced significant ($p<0.05$) higher number of DSBs-foci (0.43 ± 0.05 and 0.39 ± 0.04 foci/cell), compared to control. The eluates of X-tra fil®, CLEARFIL™ AP-X, Admira® Fusion and QuiXfil® showed no significant differences ($p>0.05$) in the number of DSBs-foci, compared to control.

### 6.1.3 GC/MS analysis

A total of 12 substances were detected from investigated composite eluates.

In the eluates of Esthet.X® HD, TEGDMA, HEMA, EGDMA, CQ, DMABEE, HMBT and CSA were detected. The highest concentrations of EGDMA (3.18 μM) and HMBP (11.20 μM) were found.

In the eluates of Venus®, TEGDMA, CQ, DMABEE, HMBT, DDHT and CSA were detected. DDHT was only found for Venus®. The highest concentrations of TEGDMA (1080.23 μM), CQ (9.69 μM) and CSA (5.68 μM) were found.

In the eluates of X-tra fil®, TEGDMA, HEMA, CQ, DMABEE, BHT, and CSA were detected.

In the eluates of CLEARFIL™ AP-X, TEGDMA, CQ and CSA were detected.

In the eluates of Admira® Fusion, CQ, DMABEE, BHT, TinP and CSA were detected. TinP was only found for Admira® Fusion.

In the eluates of QuiXfil®, TEGDMA, HEMA, HPMA, CQ, DMABEE, BHT, HMBP, TMPTMA and CSA were detected. HPMA and TMPTMA were only found for

QuiXfil®. The highest concentrations of HEMA (110.46 μM) and BHT (1.10 μM) were found.

## 6.2 Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to dental resin co-monomer epoxy metabolites

### 6.2.1 XTT assay

HGFs showed a dose-dependent loss of viability after exposure to MA, EMPME or EMPA for 24 h. The lowest $EC_{50}$ value was found for EMPA ($EC_{50}$: 1.72 mM). The cytotoxicity could be ranked in the following order: EMPA>EMPME>MA.

### 6.2.2 γ-H2AX assay with antioxidants

No significant reduction of DSBs-foci/cell was found for Asc and NAC at all concentrations, compared to the negative control.

Asc (500 μM) induced significantly more DSBs-foci/cell (0.75±0.08) in HGFs compared to control (0.39±0.08). NAC at all concentrations showed no significant induction of DSBs-foci compared to control.

### 6.2.3 γ-H2AX assay with MA, EMPME and EMPA, respectively, in the presence/ absence of antioxidants

MA:

At concentrations of 15.64 mM ($EC_{50}$) and 5.21 mM ($1/3EC_{50}$), MA induced 1.76±0.19 and 1.63±0.12 DSBs-foci/cell, respectively. The addition of Asc (50-200 μM) or NAC (50-500 μM) to 15.64 mM and 5.21 mM MA significantly reduced the number of foci/cell compared to exposure with MA alone. The concentration of 1.56 mM ($1/10EC_{50}$) MA showed no significant increase in the number of foci/cell in HGFs. No significant DSBs-foci reduction was found with the addition of Asc (50-200 μM) or NAC (50-500 μM) to 1.56 mM MA.

EMPME:

At a concentration of 2.58 mM ($EC_{50}$), EMPME induced a 5-fold higher number of DSBs-foci/cell (6.15±0.34) in HGFs, compared to control. When HGFs were exposed to 2.58 mM EMPME, with the addition of Asc or NAC, the number of foci/cell was significantly reduced compared to exposure with 2.58 mM EMPME alone. The addition of NAC (50-500 μM) to 2.58 mM EMPME significantly reduced the number of foci/cell compared to Asc (50-200 μM). No significant difference in foci induction was found when HGFs were exposed to 0.86 mM ($1/3EC_{50}$) and 0.26 mM ($1/10EC_{50}$) EMPME. No significant DSBs-foci reduction was found with the addition of Asc (50-200 μM) or NAC (50-500 μM) to 0.86 mM and 0.26 mM EMPME. Micronuclei could be observed at 2.58 mM EMPME.

EMPA:

The concentration of 1.72 mM ($EC_{50}$) EMPA induced a 20-fold higher number of DSBs-foci/cell (9.90±0.90), and 0.57 mM ($1/3EC_{50}$) EMPA induced a 6-fold higher number of foci/cell (3.00±0.20), compared to control. At concentrations of 1.72 mM and 0.57 mM, DSBs-foci reduction was noted in the presence of Asc (50-200 μM) or NAC (50-500 μM), while the addition of NAC (50-500 μM) significantly reduced the number of foci/cell compared to Asc (50-200 μM). The most reduction could be found with 1.72 mM EMPA, the presence of NAC (50;100;200;500 μM) induced a 15-fold, 17-fold, 14-fold and 14-fold lower number of foci/cell, respectively. The concentration of 0.17 mM ($1/10EC_{50}$) EMPA showed no significant increase in DSBs-foci. No significant DSBs-foci reduction was found with the addition of Asc (50-200 μM) or NAC (50-500 μM) to 0.17mM EMPA.

# 7 Synopsis/Zusammenfassung

## 7.1 Synopsis

Methacrylate-based dental resins are frequently used in a clinical context because of their aesthetic properties and physical performance. (co)monomers and additives were

found to be eluted from dental composites after polymerization [2-5]. It has been reported that released (co)monomers and additives can cause cytotoxicity, mutagenicity, embryo toxicity and teratogenicity [15, 19]. However, former studies were performed using single composite components [20-22], therefore less data for cytotoxicity and no data for DSBs induction are available for composite eluates consisting of multiple components. Moreover, the co-monomers TEGDMA and HEMA can be metabolized to intermediate MA [14] which can be further metabolised to related epoxy metabolite EMPA [15-17]. Additionally, in this process, it's speculated that another epoxy metabolite EMPME, may also be formed [15]. Epoxy compounds are considered to be highly mutagenic and carcinogenic agents [10, 17]. It was found that the addition of antioxidants Asc or NAC, can reduce the cytotoxicity and DNA-DSBs of dental resin co-monomers [20, 26, 27]. But effects of antioxidants on DNA-DSBs in the presence of co-monomer epoxy metabolites are not known.

In the first study, HGFs were exposed to dental composite eluates consisting of multiple components to investigate cytotoxicity and induction of DNA-DSBs by using an XTT and a γ-H2AX assay respectively. In comparison with single-component experiments, this study, may reflect a situation closer to physiology. The multiple composition of eluates was qualified and quantified using GC/MS.

In the second study, HGFs were incubated with MA, EMPME and EMPA respectively, in the presence or absence of Asc or NAC. $EC_{50}$ Values were obtained from an XTT-based viability assay. DNA-DSBs were determined using a γ-H2AX assay.

### 7.1.1 Cytotoxicity and DNA double-strand breaks in human gingival fibroblasts exposed to eluates of dental composites

In the investigated composite eluates, additives such as BHT, CQ, DMABEE were found. but the concentration detected were far below than that can cause cell toxicity based on previous studies [19, 29].

In the present study, the highest concentration of TEGDMA was found in the eluates of Venus® and Esthet.X® HD (1080 μM and 1019 μM). Our previous study found that a single exposure with TEGDMA at concentrations of 1200 μM (1/3 $EC_{50}$) and 360 μM

(1/10 $EC_{50}$) induces 7-fold and 4-fold higher number of DSBs-foci compared to negative control [21]. In the present study, however, the concentrations of TEGDMA in the eluates of Venus® and Esthet.X® HD, which are close to that of 1/3 $EC_{50}$ [21], only induced 2-fold higher number of DSBs-foci compared to negative control; and no significant DNA-DSBs induction was observed neither in the eluates of X-tra fil® nor CLEARFIL™ AP-X, where the concentrations of TEGDMA (494 μM and 479 μM) are higher than that of 360 μM (1/10 $EC_{50}$ [21]). In summery, in the present study, on the one hand, the concentrations of TEGDMA may play a dominant role in inducing DNA-DSBs in the investigated composite eluates, on the other, the composite eluates containing multiple components induced lower rates of DSBs compared to the single exposure with TEGDMA [21]. The reduced rates of DSBs may be attributed to the addition of 10% FCS to DMEM during XTT and γ-H2AX assays, which can lead to protein binding of (co)monomers and additives [30, 31], resulting less (co)monomers and additives available to induce DNA-DSBs.

In addition, interactive effects among multiple components in the eluates may also reduce the toxicity: It was shown that an interactive effect is found for multiple dental components acting at specific concentrations and time conditions [32]. Ratanasathien et al. demonstrated that antagonistic effect plays a dominant role after 24h culture when exposed to two different dental (co)monomers simultaneously [33]. Therefore, it can be speculated that, in comparison with a single exposure with TEGDMA, when HGFs are exposed to the eluates of Esthet.X® HD, Venus®, X-tra fil® and CLEARFIL™ AP-X, the multiple components eluted from composite may lead to an antagonistic effect, consequently reduce the rates of DNA- DSBs. This may also explain that no significant cytotoxicity was found in all investigated eluates in XTT assay.

### 7.1.2 Cytotoxicity and DNA-DSBs induced by dental co-monomer intermediate and epoxy metabolites

In this study, the relative cytotoxicity of EMPA and EMPME was 9-fold and 6-fold higher than that of precursor MA respectively. A γ-H2AX assay showed that EMPME and EMPA gave rise to more severe damage in formation of DNA-DSBs compared to

MA. The explanation may be that epoxides EMPME and EMPA are highly reactive and unstable molecules and, therefore, may exert higher toxicity.

A previous study investigated the DNA-DSBs induced by TEGDMA at 3.6 mM ($EC_{50}$) and 1.2 mM ($1/3EC_{50}$), and HEMA at 11.2 mM ($EC_{50}$) and 3.7 mM ($1/3EC_{50}$) [24]. In comparison with these former results, current study showed higher rates of DSBs-foci at 2.58 mM ($EC_{50}$) EMPME, 1.72 mM ($EC_{50}$) EMPA and 0.57 mM ($1/3EC_{50}$) EMPA. These data indicate that epoxy metabolites can cause more severe DNA damage than their metabolic precursors TEGDMA and HEMA, even at a lower concentration. Similarly, it has been demonstrated that the DNA damage induced by acrylamide [34], is possibly triggered by its epoxy metabolite glycidamide [35]. Due to the epoxy structural similarity of the EMPME and EMPA to glycidamide, it can be assumed that, when HGFs are exposed to co-monomers TEGDMA and HEMA, the formed epoxy metabolites EMPME and EMPA, may be involved in DNA-DSBs induction.

### 7.1.3 Effects of antioxidants on DNA-DSBs induced by dental co-monomer intermediate and epoxy metabolites

Our data show that when HGFs were exposed to MA (15.64 and 5.21 mM), EMPA (1.72 and 0.57 mM) or EMPME (2.58 mM), significant DNA-DSBs induction was found. An addition of Asc or NAC significantly reduced the number of DNA-DSBs. These results are in line with other studies [20, 36]. Asc is regarded as a radical scavenger which can act as a anti-genotoxic agent [27, 37], and it was found that the presence of Asc can prevent the formation of DNA adducts [38]. NAC is known as a thiol-containing antioxidant and protects cellular components by reducing cellular ROS level [39]. Previous studies have shown that NAC can reduce the cytotoxicity and genotoxicity of methacrylate-based dental co-monomers [20, 27]. In the present study, after the addition of Asc or NAC to MA, EMPA or EMPME, NAC leads to more prominent reduction of DNA-DSBs compared to Asc. This may be attributed by the formation of endogenous ROS, triggered by Asc, leading to the depletion of the GSH level [40]. While on the the contrary, NAC increases the GSH level which contributes to protect DNA from damage caused by oxidative effects and DNA-adducts formation [39, 41]. Therefore, NAC is

considered as a preferable antioxidant to Asc in terms of DNA damage caused by dental (co)monomers, as well as by their metabolites.

## 7.2 Zusammenfassung

Methacrylat-basierte dentale Kunststofffüllungen werden aufgrund ihrer ästhetischen Eigenschaften und physikalischen Eigenschaften häufig in einem klinischen Kontext verwendet. Solche Dentalkomposite können (Co)monomere und Additive freisetzen [2-5], die Zytotoxizität, Mutagenität, Embryotoxizität und Teratogenität indizieren können [15, 19]. In bisherigen Studien wurden nur einzelne Komposit-Inhaltsstoffe [20-22] untersucht, daher sind für Komposit-Eluate, die mehrere Komposit-Inhaltsstoffe enthalten nur wenige Daten zur Zytotoxizität und keine Daten für DNA-DSB-Induktion verfügbar. Darüber hinaus können die Comonomere TEGDMA und HEMA zu dem Intermediat MA metabolisiert werden [14], welches weiter zum Epoxymetaboliten EMPA metabolisieren kann [15-17]. Des Weiteren wurde die Bildung eines weiteren Epoxymetabolit EMPME diskutiert [15]. Epoxidverbindungen gelten als stark mutagen und krebserregend [10, 17]. Indessen wurde festgestellt, dass die Zugabe von Antioxidantien Asc oder NAC die Zytotoxizität und DNA-DSBs von dentalen Comonomeren reduzieren kann [20, 26, 27]. Die Wirkungen von Antioxidantien auf DNA-DSBs in Gegenwart von Comonomer-Epoxymetaboliten sind jedoch bisher nicht bekannt.

In der ersten Studie wurden HGFs dentalen Kompositeluaten, die mehrere Komposit-Inhaltsstoffe enthalten ausgesetzt, um die Zytotoxizität und Induktion von DNA-DSBs mittels eines XTT-bzw. eines γ-H2AX-Assays zu untersuchen. Im Vergleich zu Experimenten mit einzelnen Komposit-Inhaltsstoffen kann die aktuelle Studie eine physiologische Situation widerspiegeln. Die Zusammensetzung der Eluate wurde mittels GC/MS qualifiziert und quantifiziert.

In der zweiten Studie wurden HGFs mit MA, EMPME bzw. EMPA in Gegenwart oder Abwesenheit der Antioxidantien Asc oder NAC inkubiert. Die $EC_{50}$-Werte wurden mit einem XTT-basierenden Viabilitäts assay ermittelt. DNA-DSBs wurden unter Verwendung eines γ-H2AX-Assays bestimmt.

### 7.2.1 Zytotoxizität und DNA-DSBs in humanen Gingiva Fibroblasten nach Exposition mit dentalen Kompositen Eluaten

In den Eluaten der untersuchten Komposite konnten Additive wie BHT, CQ, DMABEE detektiert werden, allerdings lagen alle nachgewiesenen Konzentrationen weit unter den Grenzwerten, welche laut früheren Studien Zelltoxizität verursachen können [19, 29].

In der vorliegenden Studie wurden die höchsten Konzentrationen von TEGDMA in den Eluaten von Venus® und Esthet.X® HD (1080 μM und 1019 μM) gefunden. Unsere frühere Studie zeigte, dass eine Einzel-Exposition mit TEGDMA bei Konzentrationen von 1200 μM ($1/3EC_{50}$) und 360 μM ($1/10EC_{50}$) eine 7-fach und 4-fach höhere Anzahl von DSBs-Foci im Vergleich zur Negativkontrolle induzierte [21]. In der vorliegenden Studie induzierten jedoch die Konzentrationen von TEGDMA in den Eluaten von Venus® und Esthet.X® HD, die nahe an $1/3EC_{50}$ [21] liegen, nur eine 2-fach höhere Anzahl von DSBs-Foci im Vergleich zur negativen Kontrolle; des Weiteren wurden keine signifikanten DNA-DSB-Induktionen, weder für die Eluate von X-tra fil® noch für die von CLEARFIL™ AP-X beobachtet, obwohl in diesen Eluaten die Konzentrationen von TEGDMA (494 μM und 479 μM) höher waren als 360 μM ($1/10EC_{50}$ [21]). Also könnten in der vorliegenden Arbeit einerseits die Konzentrationen von TEGDMA eine dominante Rolle bei der Induktion von DNA-DSBs in den untersuchten Kompositeluaten spielen, andererseits induzierten die Kompositeluate, die mehrere Komponenten enthielten, niedrigere DSB-Raten im Vergleich zu einer Einzel-Exposition mit TEGDMA [21]. Reduzierte DSB-Raten können der Zugabe von 10% FCS zu DMEM im XTT- und γ-H2AX-Assay zugeschrieben werden, da (Co)monomere und Additive durch Proteinbindung gebunden werden können [30, 31], und so weniger (Co)monomeren und Additive zur Induktion von DNA-DSBs zur Verfügung stehen.

Zusätzlich können interaktive Effekte zwischen mehreren Komponenten in den Eluaten die Toxizität reduzieren: So wurde bereits ein interaktiver Effekt zwischen mehreren dentalen Einzel-Komponenten untereinander bei bestimmten Konzentrationen und Zeitbedingungen beschrieben [32]. Des Weiteren zeigten Ratanasathien et al., dass der antagonistische Effekt in einer 24h Kultur eine dominante Rolle spielt, wenn diese gleichzeitig zwei verschiedenen dentalen (Co)monomeren ausgesetzt wird [33]. Daher

kann angenommen werden, dass im Vergleich zu einer Einzel-Exposition von HGFs mit TEGDMA, die verschiedenen Komponenten in den jeweiligen Eluat von Esthet.X® HD, Venus®, X-tra fil® oder CLEARFIL™ AP-X antagonisieren könnten und folglich sich die DNA-DSB-Rate verringert. Dies könnte auch erklären, dass in allen untersuchten Eluaten im XTT-Assay keine signifikante Zytotoxizität gefunden wurde.

### 7.2.2 Zytotoxizität und Induktion von DNA-DSBs durch dentale Comonomer Intermediate und Epoxy-Metabolite

In dieser Studie wurde eine 9-fach bzw. 6-fach höhere relative Zytotoxizität von EMPA und EMPME als bei deren Precursor MA festgestellt. Der γ-H2AX-Assay zeigte, dass EMPME und EMPA im Vergleich zu MA erheblich mehr DNA-DSBs verursachten. Eine Erklärung dafür könnte sein, dass die Epoxide EMPME und EMPA hochreaktive und instabile Moleküle sind und daher eine höhere Toxizität ausüben können.

Eine frühere Studie untersuchte die durch TEGDMA induzierten DNA-DSBs bei 3,6 mM ($EC_{50}$) und 1,2 mM ($1/3EC_{50}$), und HEMA bei 11,2 mM ($EC_{50}$) und 3,7 mM ($1/3EC_{50}$) [24]. Im Vergleich zu diesen früheren Ergebnissen zeigt die aktuelle Studie höhere Raten von DSBs-Foci bei 2,58 mM ($EC_{50}$) EMPE, 1,72 mM ($EC_{50}$) EMPA und 0,57 mM ($1/3EC_{50}$) EMPA. Diese Daten weisen darauf hin, dass Epoxymetaboliten sogar bei niedrigeren Konzentrationen einen schwereren DNA-Schaden als ihre metabolischen Precursor TEGDMA und HEMA verursachen können. Gleichermaßen konnte gezeigt werden, dass der durch Acrylamid [34] induzierte DNA-Schaden möglicherweise durch seinen Epoxymetaboliten Glycidamid ausgelöst wird [35]. Aufgrund der epoxy-ähnlichen Struktur von EMPME und EMPA zu Glycidamid kann angenommen werden, dass bei einer Exposition von HGFs mit den Comonomeren TEGDMA und HEMA die gebildeten Epoxymetaboliten EMPME und EMPA an der DNA-DSB-Induktion beteiligt sein können.

### 7.2.3 Effekt von Antioxidantien auf durch dentale Comonomer-Intermediate und Epoxy-Metabolite induzierte DNA-DSBs

Unsere Daten zeigten, dass MA (15,64 and 5,21 mM), EMPA (1,72 and 0,57 mM) oder EMPME (2,58 mM) in HGFs signifikante DNA-DSBs induzieren. Die Zugabe von Asc oder NAC reduzierte die Anzahl von DNA-DSBs signifikant. Diese Ergebnisse werden durch anderen Studien bestätigt [20, 36]. Asc ist ein Radikalfänger, der als antigentoxisches Agens wirken kann [27, 37] und die Bildung von DNA-Addukten verhindern kann [38]. NAC ist ein Thiol-haltiges Antioxidans, das zelluläre Komponenten durch Verringerung des zellulären ROS-Spiegels schützen kann [39]. So konnte in früheren Studien gezeigt werden, dass NAC die Zytotoxizität und Genotoxizität von Methacrylat-basierten (Co)monomeren reduzieren kann [20, 27]. In der vorliegenden Studie führte die Zugabe von NAC bei der Exposition von HGFs mit MA, EMPA oder EMPME zu einer deutlichen Reduktion der DSBs-DSBs gegenüber einer Asc Beimischung. Dies könnte der durch Asc ausgelösten Bildung von endogenen ROS zugeschrieben werden, was zu einer Abnahme des GSH-Spiegels führt [40]. Im Gegensatz dazu erhöht NAC den GSH-Spiegel, was dazu beiträgt, die DNA vor Schäden durch oxidative Effekte und Bildung von DNA-Addukten zu schützen [39, 41]. Im Hinblick auf DNA-Schäden, die durch dentale (Co)monomere sowie durch ihre Metaboliten verursacht werden ist als Antioxidationsmittel NAC gegenüber Asc vorzuziehen.

# 8 The share of participation in the presented work

The share of each author is deducted from the sequence of the listed authors and co-authors.

In both publications (see 9 and 10), I am listed as first author. I have accomplished the main part of practical work, conducted the whole evaluation, statistical analysis and written the whole publication on my own.

# 9 Publication I

Yang Y, Reichl FX, Shi J, He X, Hickel R, Högg C. *Cytotoxicity and DNA double-strand breaks in human gingival fibroblasts exposed to eluates of dental composites*. Dent Mater. 2017 In press, https://doi.org/10.1016/j.dental.2017.10.002

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# Cytotoxicity and DNA double-strand breaks in human gingival fibroblasts exposed to eluates of dental composites

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ABSTRACT

*Objective.* Previously, single composite components were used to study cytotoxicity and induction of DNA double-strand breaks (DNA-DSBs) of dental composite resins. In the present study, cytotoxicity and induction of DNA-DSBs in human gingival fibroblasts (HGFs) were investigated with dental composite eluates consisting of multiple components. The eluates were qualified and quantified.

*Methods.* The composites Esthet.X® HD, Venus®, X-tra fil®, CLEARFIL™ AP-X, Admira® Fusion and QuiXfil® were polymerized and immersed into Dulbecco's modified Eagle's medium (DMEM) for 72 h. Subsequently, HGFs were incubated with the corresponding composite eluates. The cell viability of HGFs was obtained from an XTT assay. DNA-DSBs were determined using a γ-H2AX assay. The qualification and quantification of eluates were performed by gas chromatography/mass spectrometry (GC/MS).

*Results.* HGFs exposed to the eluates of all investigated composites showed no significant loss of cell viability, compared to negative control. Significant DNA-DSBs induction could be found in HGFs exposed to the eluates of Esthet.X® HD (0.43 ± 0.05 foci/cell) and Venus® (0.39 ± 0.04 foci/cell), compared to control (0.22 ± 0.03 foci/cell). A total of 12 substances were detected from the investigated composite eluates. Five of them were methacrylates: tetraethyleneglycol dimethacrylate (TEGDMA), 2-hydroxyethyl methacrylate (HEMA), hydroxypropyl methacrylate (HPMA), ethyleneglycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TMPTMA). The highest concentration of HEMA (110.5 μM), HPMA (86.08 μM) and TMPTMA (4.50 μM) was detected in the eluates of QuiXfil®. The highest concentration of TEGDMA was 1080 μM in Venus® eluates and the highest concentration of EGDMA was 3.18 μM in Esthet.X® HD eluates.

*Significance.* Significant DNA-DSBs induction can be found in HGFs exposed to the eluates of Esthet.X® HD and Venus®. The interactive effects among released (co)monomers and

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additives may influence the cytotoxicity and induction of DNA-DSBs, compared to exposure with single composite component.



## 1. Introduction

Light-cured composite resins consist of (co)monomers and additives like photoinitiators, coinitiators, photostabilizers, inhibitors and inorganic fillers [1]. The polymerization of dental composites is incomplete [2]. Previous studies revealed that (co)monomers and additives can be eluted from dental composites [2–5]. The degree of conversion (DC) depends on many factors such as the light density, curing time and distance between light source and dental composite, as well as the composition and shade of the dental material [6]. The lower the DC of a composite the more composite components can be eluted [7]. (Co)monomers and additives may penetrate to pulp via dentinal tubules, then affect the activity of dental pulp cells or enter the intestine by swallowing, subsequently reaching the circulatory system and organs [8–10]. Additionally, the (co)monomers (methacrylates) can cause allergic reactions such as asthma and contact dermatitis [11].

Geurtsen et al. investigated 35 dental resin composite monomers and additives in human primary fibroblast cultures, in which, the cytotoxicity of (co)monomers and additives was revealed [12]. The mutagenicity, embryo toxicity and teratogenicity caused by released (co)monomers were also reported [13]. Moreover, it was shown that TEGDMA and HEMA can be metabolized to epoxy compound 2,3-epoxy-2-methylpropionic acid (EMPA) [14], and the formation of another epoxide, 2,3-epoxy-2-methylpropionicacid-methylester (EMPME), was postulated [13]. The formation of epoxide in human oral cells (for example, human gingival fibroblasts (HGFs) and human pulp fibroblasts) has been demonstrated [15]. In our previous study, EMPME and EMPA were not only found to induce cytotoxicity, but also to induce higher rates of DNA double-strand breaks (DNA-DSBs) in HGFs, compared to their metabolic precursors, TEGDMA and HEMA [16,17]. DNA-DSBs are considered as the most toxic type of DNA lesion [18].

To date, studies on cytotoxicity and DNA-DSBs concerning dental composite resins have dealt with the effects of single composite components [16,18,19]. However, less data for cytotoxicity and no data for induction of DSBs are available for composite eluates consisting of multiple components. Experiments with qualified and quantified eluates may reflect a situation closer to physiology, compared to single-component experiments. Therefore, in the present study, cytotoxicity and induction of DNA-DSBs in HGFs were investigated with dental composite eluates. The multiple composition of eluates was qualified and quantified.

In the null hypothesis, it is assumed that composite eluates do not induce cytotoxicity and DNA-DSBs in HGFs.

## 2. Methods

The investigated composites including manufacturers' data are listed in Table 1. The six types of investigated composites

**Table 1 – Investigated dental materials, manufacturers, LOT numbers, types, and polymerization times; composition of each material based on manufacturer's data.**

| Product name | Type | Manufacturer | LOT | Composition of materials based on manufacturer's data | Polymerization time |
|---|---|---|---|---|---|
| Esthet.X® HD | Micro-hybrid | Dentsply, Caulk, USA | 160523 | Bisphenol A-glycidyl methacrylate (Bis-GMA), ethoxylated bisphenol-A dimethacrylate (BisEMA), TEGDMA, CQ, photoinitiator, stabilizer, pigments | 20 s |
| Venus® | Micro-hybrid | Heraeus Kulzer, Hanau, Germany | 010504A | Bis-GMA, TEGDMA and contains 58.7% filler (by volume), such as Barium Aluminium Fluoride glass; Highly dispersive Silicon Dioxide | 20 s |
| X-tra fil® | Multi-hybrid | VOCO GmbH, Cuxhaven, Germany | 010106 | Bis-GMA, urethane dimethacrylate (UDMA), TEGDMA | 10 s |
| CLEARFIL™ AP-X | Micro-hybrid | Kuraray Europe GmbH, Hattersheim am Main, Germany | A50079 | Bis-GMA, TEGDMA; silanated barium glass filler, silanated silica filler, silanated colloidal silica | 20 s |
| Admira® Fusion | Nano-hybrid Ormocer® | VOCO GmbH, Cuxhaven, Germany | 1648518 | ORMOCER® | 20 s |
| QuiXfil® | Micro-hybrid | DENTSPLY DeTrey GmbH, Konstanz, Germany | 1605000136 | UDMA, TEGDMA, Di- and trimethacrylate resins, carboxylic acid modified dimethacrylate resin, BHT, silanated strontium aluminium sodium fluoride phosphate silicate glass | 10 s |

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represent materials of various categories like micro-hybrid, nano-hybrid and multi-hybrid. In addition, the investigated composites were selected because former elution studies and preliminary tests have shown various composition and relatively high amounts of methacrylates and additives (e.g. TEGDMA, DMABEE) [5,20–22].

### 2.1. Sample preparation

Composite samples (Table 1) were prepared by placing the uncured dental composite into a polytetrafluoroethylene (PTFE) ring (10 mm diameter and 2 mm thickness) placed on a plastic matrix strip (Frasaco, Tettnang, Germany). The surface area of each sample was 219.8 $mm^2$ (approximately 300 mg each). Then the uncured composite was polymerized using a LED-lamp (Elipar STM 10® high intensity halogen light, 1200 $mW/cm^2$, 3 M ESPE, Seefeld, Germany), according to the instructions of the manufacturers (Table 1). The light intensity of the LED-lamp was controlled with Demetron® Radiometer (Kerr, USA) and was always between 1100 and 1200 $mW/cm^2$. The top surface of the composite sample was not covered with a plastic strip during polymerization, in order to create a worst-case scenario [23]. For each investigated composite, 2 groups with 4 samples each ($n = 4$) were prepared: (1) eluates for XTT and γ-H2AX assays; (2) eluates for GC/MS analysis.

After sample preparation, they were transferred into brown glass vials (Macherey-Nagel, Düren, Germany) and 879 μl fetal calf serum (FCS)-free DMEM (PAN-Biotech, Aidenbach, Germany) was added. As internal standard, caffeine (CF) (0.01 mg/ml) was added to group 2. All samples were incubated for 72 h at 37 °C in the dark. The ratio of the sample surface area to the volume of the solution was approximately 2.5 $cm^2$/ml, which is within the 0.5–6.0 $cm^2$/ml range recommended by ISO [24] and a previous study [23].

The eluates of group 1 were collected in a volume of 800 ml/sample, sterile-filtered (Millipore 0.22 mm) and performed by XTT and γ-H2AX assays.

For GC/MS analysis, the eluates of group 2 were collected in a volume of 100 μl/sample and previously extracted one time with 100 μl ethyl acetate (LC-MS-Grade, ROTISOLV® ≥99.9%, Roth, Karlsruhe, Germany) (1:1 v/v). To optimize layer separation, the samples were centrifuged at 2800 rpm for 10 min [4]. 1 μl each was analyzed by GC/MS.

### 2.2. Cell culture

HGFs were obtained from Provitro GmbH (Berlin, Germany). The HGFs (passage 8) were cultured in the same manner as described in our previous study [17].

### 2.3. XTT-based viability assay

An XTT-based cell viability assay was used to determine the viability of HGFs. This assay was performed according to our previous study [16]. The cells were treated with composite-eluates in DMEM (group 1), in this process, 10% FCS was added, followed by incubation for 24 h at 37 °C, with 5% $CO_2$ and 100% humidity. Control cells received medium only; as negative control the cells were treated with 1% Triton X-100 [16]. The optical density (OD) was determined spectrophotometrically at 450 nm (reference wavelength 670 nm), using a microplate reader (MULTISKAN FC; Thermo Fisher Scientific (Shanghai) Instruments Co. Ltd, China). Four independent experiments were performed (n = 4), each time in triplicate. The cell viability was calculated according to the following equation:

$$\text{Cell Viability}(\%) = \frac{\text{OD of test group}}{\text{OD of control group}} \times 100 \quad (1)$$

### 2.4. γ-H2AX immunofluorescence

DNA-DSBs formation was determined in HGFs by γ-H2AX assay. 12 mm round cover slips (Carl Roth, Karlsruhe, Germany) were cleaned in 1 N HCl and distributed into a 24-well plate. HGFs were seeded at $7 \times 10^4$ cells/ml in each well with the medium, followed by overnight incubation at 37 °C. The cells were exposed for 6 h to composite eluates in DMEM (group 1), with addition of 10% FCS. Normally an exposure time at 1, 4, 6 or 24 h is used for γ-H2AX assay [25–27]. According to preliminary tests on HGFs exposed to composite eluates, the DSBs-foci in captured images were faint and difficult to evaluate at exposure times less than 6 h. Exposure times longer than 6 h generally caused massive loss of cells along with distorted nuclei of residual cells. Therefore, in the present study, HGFs were exposed to composite eluates for 6 h to obtain distinct and bright DSBs-foci. This was also described in our former studies [16,17,19]. Negative control cells received the medium for 6 h. Positive control cells received 1 mM $H_2O_2$ (Sigma-Aldrich, Steinheim, Germany) in the medium for 15 min. Immunofluorescent staining was performed according to our previous study [17]. Four independent experiments were performed (n = 4).

### 2.5. Image acquisition

For investigation of HGFs, a Zeiss CLSM imaging fluorescence microscope (Zeiss, Göttingen, Germany), equipped with a motorized filter wheel and appropriate filters for excitation of red (wavelength: 594 nm) and green (wavelength: 488 nm) fluorescence, was used. Images were obtained using a 63× and a 100× Plan-Neofluar oil-immersion objective (Zeiss) and the fluorescence-imaging system LSM Image Browser (Zeiss).

### 2.6. GC/MS analysis

The analysis of the eluates was performed on a Finnigan Trace GC ultra gas chromatograph connected to a DSQ mass spectrometer (Thermo Electron, Dreieich, Germany). A J&W VF-5ms capillary column (length 30 m, inner diameter 0.25 mm; coating 0.25 m; Agilent, Böblingen, Germany) was used as the capillary column for gas chromatographic separation. Helium 5.0 was used as carrier gas at a constant flow rate of 1 ml/min. The temperature of the transfer line was 250 °C. For sample analysis 1 μl each was injected in splitless mode (splitless time 1 min, split flow 50 ml/min). For capillary transfer the programmable temperature vaporizing (PTV) inlet was heated from 30 °C to 320 °C (14.5 °C/s) and finally held for 5 min at this temperature. The GC oven was initially heated isothermally at 50 °C for 2 min, then increased to 280 °C (25 °C/min) and finally remained for 5 min at this temperature. The mass

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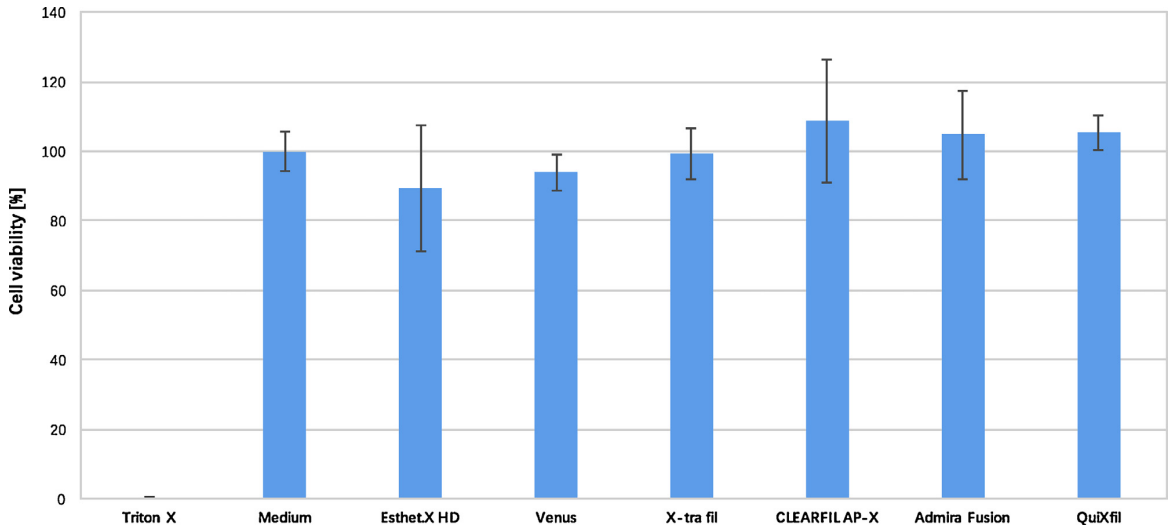


**Fig. 1 – HGFs viability in XTT assay after incubation with the eluates of investigated composites (Table 1) for 24 h. Control cells received medium only, negative control cells were treated with 1% Triton X-100. Data are expressed as percentage of control (Eq. (1)) and represent mean ± SD (n = 4).**

spectrometer (MS) was operated in the electron impact mode (EI) at 70 eV (ion source temperature: 240 °C). Samples were recorded in full scan mode (*m*/*z* 50-600).

Identification of the relevant compounds was achieved by comparing their mass spectra and retention times to the corresponding reference standards. For each reference standard compound a calibration was performed. The quantity of an identified analyte was calculated by correlating its characteristic mass peak area to the corresponding precompiled calibration curve (internal standard caffeine). Four independent experiments were performed (n = 4).

### 2.7. *Data analysis*

The values of XTT assay were calculated as percentage of the controls using Graph Pad Prism 4 (Graph Pad Software Inc., San Diego, USA). Data are shown as mean ± standard deviation (SD) (n = 4), each performed in triplicate.

In the γ-H2AX assay, the DSBs-foci/cell were counted by the same investigator, using the fluorescence microscopic with a 100× objective. Data are shown as mean ± standard error of the mean (SEM) (n = 4).

The statistical significance ($p < 0.05$) of the differences in XTT and γ-H2AX assays was determined using the Student's *t*-test, corrected according to Bonferroni–Holm [28].

GC/MS results are presented as mean ± SD (n = 4).

## 3. Result

### 3.1. *XTT assay*

HGFs exposed to the eluates of the investigated composites (Table 1) showed no significant ($p > 0.05$) difference of cell viability, compared to control (Fig. 1).

**Table 2 – Detected eluted composite components.**

| Compound abbreviation | Compound |
|---|---|
| HEMA | 2-Hydroxyethyl methacrylate |
| HPMA | Hydroxypropyl methacrylate |
| EGDMA | Ethylene glycol dimethacrylate |
| TEGDMA | Tetraethyleneglycol dimethacrylate |
| TMPTMA | Trimethylolpropane trimethacrylate |
| CQ | Camphorquinone |
| DMABEE | 4-Dimethylaminobenzoic acid ethyl ester |
| BHT | 2,6-Di-t-butyl-4-methyl phenol |
| HMBP | 2-Hydroxy-4-methoxy-benzophenone |
| TinP | 2(2′-Hydroxy-5′-methylphenyl) benzotriazol |
| DDHT | Diethyl-2,5-dihydroxytrephthalate |
| CSA | Champhoric acid anhydride |

### 3.2. *γ-H2AX assay*

$H_2O_2$ (1 mM) induced 10.13 ± 1.75 DSBs-foci/cell in the positive control. Medium induced 0.22 ± 0.03 DSBs-foci/cell in the negative control (Table 3).

The eluates of Esthet.X® HD and Venus® induced significantly ($p < 0.05$) higher number of DSBs-foci (0.43 ± 0.05 and 0.39 ± 0.04 foci/cell), compared to control. The other eluates (X-tra fil®; CLEARFIL™ AP-X; Admira® Fusion; QuiXfil®) showed no significant differences ($p > 0.05$) in the number of DSBs-foci, compared to control (Table 3).

The representative images of immunofluorescent staining for γ-H2AX are shown in Fig. 2

### 3.3. *GC/MS analysis*

A total of 12 substances (Table 2) were detected from investigated composite eluates. The quantification of eluted components is shown in Table 4.

In the eluates of Esthet.X® HD, TEGDMA, HEMA, EGDMA, CQ, DMABEE, BHT, HMBP and CSA were detected. EGDMA was

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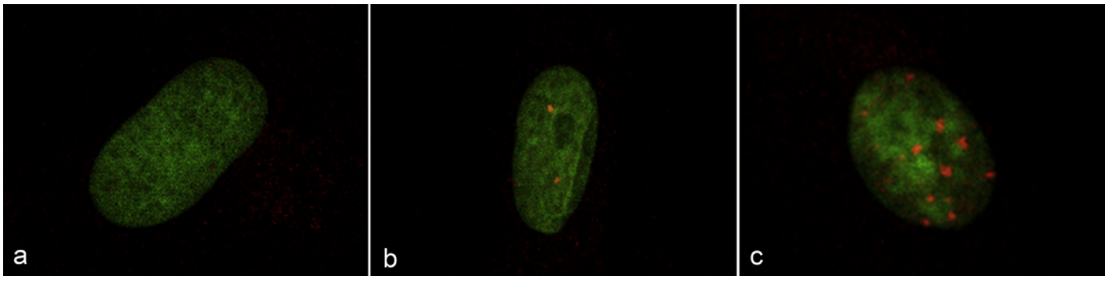


**Fig. 2 – Representative images of immunofluorescent staining for H2AX phosphorylation (orange) in HGFs, after exposure to different substances compared to control cells. Sybr green (green) is a marker for DNA and stains the whole nucleus of the cell. (a) A nucleus of HGFs without foci, as typically seen in untreated cells with medium only (negative control). (b) A nucleus of HGFs with two foci, which can occur in treated cells (in this case, with the eluate of Esthet.X® HD). (c) A nucleus of HGFs with eleven foci induced by $H_2O_2$ (positive control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

**Table 3 – Average of induced γ-H2AX DSBs-foci/cell in HGFs elicited by a 6 h exposure to the DMEM eluates of investigated composites. Negative control cells received medium only. Data are expressed as mean ± SEM (n = 4).**

| Foci/Cell (SEM) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Esthet.X® HD | Venus® | X-tra fil® | CLEARFIL™ AP-X | Admira® Fusion | QuiXfil® | Medium | $H_2O_2$ |
| 0.43 (0.05)* | 0.39 (0.04)* | 0.26 (0.04) | 0.28 (0.04) | 0.20 (0.02) | 0.23 (0.02) | 0.22 (0.03) | 10.13 (1.75) |

* Significantly different ($p < 0.05$) to negative control (medium).

**Table 4 – Qualification and quantification of substances in the DMEM eluates of investigated composites (Table 1). Data are presented as mean ± SD [μM] (n = 4).**

| mean (SD) [μM] | Esthet.X® HD | Venus® | X-tra fil® | CLEARFIL™ AP-X | Admira® Fusion | QuiXfil® |
|---|---|---|---|---|---|---|
| HEMA | 2.53 (1.07) | – | 71.65 (3.37) | – | – | 110.46 (6.92) |
| HPMA | – | – | – | – | – | 86.08 (1.59) |
| EGDMA | 3.18 (1.41) | – | – | – | – | – |
| TEGDMA | 1019.30 (262.78) | 1080.23 (128.25) | 494.37 (43.58) | 478.60 (1.65) | – | 328.95 (29.79) |
| TMPTMA | – | – | – | – | – | 4.50 (0.81) |
| CQ | 7.98 (4.42) | 9.69 (1.69) | 4.99 (1.38) | 3.05 (0.97) | 4.64 (3.98) | 4.82 (0.80) |
| DMABEE | 11.19 (1.06) | 0.06 (0.01) | 35.38 (1.65) | – | 21.28 (2.73) | 54.98 (1.89) |
| BHT | 0.07 (0.01) | – | 0.12 (0.03) | – | 0.13 (0.07) | 1.10 (0.31) |
| HMBP | 11.20 (3.67) | 6.18 (2.17) | – | – | – | 1.80 (0.15) |
| TinP | – | – | – | – | 8.15 (5.55) | – |
| DDHT | – | 0.59 (0.13) | – | – | – | – |
| CSA | 2.14 (0.06) | 5.68 (2.40) | 3.90 (0.56) | 5.28 (0.97) | 4.50 (2.66) | 5.47 (0.44) |

only found for Esthet.X® HD. The highest concentration of HMBP (11.20 μM) was found for Esthet.X® HD, compared to all investigated composite eluates.

In the eluates of Venus®, TEGDMA, CQ, DMABEE, HMBP, DDHT and CSA were detected. DDHT was only found for Venus®. The highest concentrations of TEGDMA (1080.23 μM), CQ (9.69 μM) and CSA (5.68 μM) were found for Venus®, compared to all investigated composite eluates.

In the eluates of QuiXfil®, TEGDMA, HEMA, HPMA, CQ, DMABEE, BHT, HMBP, TMPTMA and CSA were detected. HPMA and TMPTMA were only found for QuiXfil®. The highest concentrations of HEMA (110.46 μM) and BHT (1.10 μM) were found for QuiXfil®, compared to all investigated composite eluates.

In the eluates of X-tra fil®, TEGDMA, HEMA, CQ, DMABEE, BHT and CSA were detected.

In the eluates of CLEARFIL™ AP-X, TEGDMA, CQ and CSA were detected.

In the eluates of Admira® Fusion, CQ, DMABEE, BHT, TinP and CSA were detected. TinP was only found for Admira® Fusion.

## 4. Discussion

In the present study, the XTT and γ-H2AX assays were performed to investigate the cytotoxicity and genotoxicity of dental composite eluates. Generally distilled water, saliva, ethanol, methanol, etc. are used to perform dental composite elution [2,29–31]. Recent studies showed that DMEM is a comparable elution medium to saliva and representative for oral environment [20,30]. Moreover, previous studies investigated cytotoxicity and DNA-DSBs induction using only single composite components [16,18,19]. Therefore, DMEM as elution medium combined with qualification and quantification of

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multiple composition of eluates, may reflect a situation closer to physiology, compared to single-component experiments. In the present study, the released composite components were qualified and quantified in DMEM to achieve the utmost relevance in HGFs incubation with investigated composite eluates in XTT and γ-H2AX assays.

Additives were detected in all eluates of investigated composites. In a previous study, BHT ($EC_{50}$: 170 μM) was noted as the most cytotoxic additive among the tested initiators, coinitiators, inhibitors and photostabilizers [12]. In the present study, the highest concentration of BHT was found for QuiXfil® (1 μM), which is more than 100-fold lower compared to cytotoxic concentration of BHT cited above [12]. The photoinitiator CQ is considered as an allergen [32], which was detected in all investigated eluates. It was shown that CQ induces DNA damage and increases intracellular reactive oxygen species at concentrations >50 μM in HGFs [33]. In our study the highest concentration of CQ (9.7 μM) was found in the eluate of Venus®, which is 5-fold lower than toxic concentration of CQ cited above [33]. DMABEE can induce cell apoptosis and necrosis [34]. In the present study, the highest concentration of DMABEE (55 μM) was measured for QuiXfil®. This is 22-fold lower than the cytotoxic concentration of 1.2 mM, described in HGFs [12]. In summary, regarding the single-component toxicity, the concentrations of above discussed additives were always lower than corresponding toxic concentrations from previous studies [12,33]. Concerning multiple-component toxicity, results for all investigated eluates of present study showed no cytotoxicity. Therefore, there is no evidence that additives increase cytotoxicity in multiple-component eluates.

HEMA was detected in the eluates of Esthet.X® HD, X-tra fil® and QuiXfil®. However, HEMA is not listed in the manufacturers' data. HEMA is described as a degradation product from urethanedimethacrylate (UDMA) during GC/MS analysis procedure [35]. But impurities of composite components (e.g. UDMA) are also possible [4]. Therefore, the source of HEMA is unknown. It was shown that HEMA-induced apoptosis is a response to DNA damage [36]. In the current study, the highest concentration of HEMA was measured for QuiXfil® at 110 μM. In previous studies cytotoxic concentration at 2.4 mM [12] and genotoxic concentration at 1.1 mM for HEMA were found in HGFs [16]. In summary, regarding the single-component toxicity, the concentrations of HEMA detected in the present study were far below cited cytotoxic and genotoxic concentrations [12,16]. Concerning multiple-component toxicity, this is in line with the cytotoxicity results of HEMA-containing eluates (Esthet.X® HD, X-tra fil® and QuiXfil®). Nevertheless, among HEMA-containing eluates, only Esthet.X® HD induced significant DSBs with the lowest concentration of HEMA (2.5 μM), but QuiXfil® with a 44-fold higher concentration of HEMA showed no significant induction of DSBs. Therefore, there is no evidence that HEMA increases cytotoxicity and DNA-DSBs in multiple-component eluates.

In the present study, the highest concentration of TEGDMA was found in the eluates of Venus® and Esthet.X® HD (1080 μM and 1019 μM). This is in an agreement with a previous study, where a concentration of 1448 μM TEGDMA was detected for Esthet.X after 24 h elution in DMEM [20]. Our previous study revealed that a single exposure with TEGDMA at concentrations of 1200 μM (1/3 $EC_{50}$) and 360 μM (1/10 $EC_{50}$) induces 7-fold and 4-fold higher number of DSBs-foci compared to negative control [16]. In the present study, however, the concentrations of TEGDMA in the eluates of Venus® and Esthet.X® HD, which are close to that of 1/3 $EC_{50}$ [16], only induced 2-fold higher number of DSBs-foci compared to negative control; and no significant DNA-DSBs induction was observed neither in the eluates of X-tra fil® nor CLEARFIL™ AP-X, where the concentrations of TEGDMA (494 μM and 479 μM) measured are higher than that of 360 μM (1/10 $EC_{50}$ [16]). In summery, in the present study, on the one hand, the concentrations of TEGDMA may play a dominant role in inducing DNA-DSBs in the investigated composite eluates, on the other, the composite eluates containing multiple components induced lower rates of DSBs compared to the single exposure with TEGDMA [16]. The reduced rates of DSBs may be attributed to the addition of 10% FCS to DMEM during XTT and γ-H2AX assays, which can lead to protein binding of (co)monomers and additives [20,31], as a result, there are less (co)monomers and additives available to induce DNA-DSBs.

In addition, interactive effects among multiple components in the eluates may also reduce the toxicity: It was shown that an interactive effect is found for multiple dental components acting at specific concentrations and time conditions [37]. Ratanasathien et al. demonstrated that antagonistic effect plays a dominant role after 24 h when mouse fibroblasts are exposed to a mixture of two different dental (co)monomers [38]. Therefore, it can be assumed that, when HGFs are exposed to the eluates of Esthet.X® HD, Venus®, X-tra fil® and CLEARFIL™ AP-X, the multiple components eluted from composite may lead to an antagonistic effect, consequently reduce the rates of DNA-DSBs, compared to a single exposure with TEGDMA.

In the XTT assay, no significant cytotoxicity was found in all investigated eluates. In the eluates of X-tra fil®, CLEARFIL™ AP-X and QuiXfil®, the detected concentrations of TEGDMA were lower than 0.5 mM. This is in agreement with a previous study, reporting a single incubation of TEGDMA at concentrations up to 0.5 mM does not reduce the viability of HGFs [39]. However, in the eluates of Esthet.X® HD and Venus®, the detected concentrations of TEGDMA were higher than 1 mM. This is inconsistent with the findings of Mavrogonatou et al., reporting a viability of 77.9% for HGFs exposed to a single incubation of TEGDMA at 0.5–1 mM [39]. The differences may be due to the use of different methodologies. Particularly, in the present study, the cytotoxicity of eluates containing multiple components instead of single component (TEGDMA), was investigated. Likewise, protein binding and antagonistic effect as described above, may also explain the results of XTT assay in the present study.

However, it must be noted, that significant higher number of DNA-DSBs induced by the eluates of Esthet.X® HD and Venus® should trigger no alarm. In the present study, a worst-case scenario for maximum release of components was created, using samples with surface area of 220 $mm^2$, and with the presence of oxygen inhibition layer, based on previous studies [23,40,41]. The surface area of our sample is 4 times larger than that of typical restorations (52 $mm^2$) [40]. It has been demonstrated that a larger surface area of the sample

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increases the release of components [41]. Besides, the presence of oxygen inhibition layer also contributes to a increased amount of released components [41]. However, in a clinical situation, the exposed surface area is limited and the oxygen inhibition layer will be removed by grinding and polishing [42].

It must be taken into account that in a physiological situation, the amounts of components can also be reduced by the effects of protein binding in saliva [20,31]. Additionally, interactive effects among multiple components may also influence the toxicity. This is quite important, particularly, for the concerns of safety and potential hazards of materials after dental resin restoration.

The null hypothesis is rejected because some dental composite eluates can induce DNA-DSBs in HGFs, but no cytotoxic effect was found.

## 5. Conclusion

Significant DNA-DSBs induction can be found in HGFs exposed to the eluates of Esthet.X® HD and Venus®. The interactive effects among released (co)monomers and additives may influence the cytotoxicity and induction of DNA-DSBs, compared to exposure with single composite component.

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# 10 Publication II

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# Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to dental resin co-monomer epoxy metabolites

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ABSTRACT

*Objective.* Eluted dental resin co-monomers can be metabolized to intermediate methacrylic acid (MA) and, further, to epoxy metabolites. Antioxidants have been studied previously, with the intention of decreasing the DNA double-strand breaks (DNA-DSBs) in human gingival fibroblasts (HGFs). In this study, the effects of the antioxidants, ascorbic acid (Asc) and N-acetylcysteine (NAC), were investigated on co-monomer metabolite-induced DNA-DSBs.

*Methods.* HGFs were incubated with MA, 2,3-epoxy-2-methyl-propionicacid-methylester (EMPME) and 2,3-epoxy-2-methylpropionic acid (EMPA), respectively, in the presence or absence of antioxidants (Asc or NAC). $EC_{50}$ Values were obtained from an XTT-based viability assay. DNA-DSBs were determined using a γ-H2AX assay.

*Results.* The cytotoxicity of the compounds could be ranked in the following order (mean ± SEM; n = 4): EMPA > EMPME > MA. The average number of DSBs-foci/cell induced by each substance at $EC_{50}$-concentration could be ranked in the following order (mean ± SD; n = 4): EMPA > EMPME > MA. EMPA (1.72 mM) and EMPME (2.58 mM) induced the highest number of DSBs-foci, that is 21-fold and 13-fold, respectively, compared to control (0.48 ± 0.08 foci/cell). The addition of Asc (50; 100; 200 μM) or NAC (50; 100; 200; 500 μM) to MA (15.64; 5.21 mM), EMPME (2.58 mM), and EMPA (1.72; 0.57 mM) significantly reduced the number of foci/cell in HGFs. The highest reduction could be found in HGFs with 1.72 mM EMPA, the addition of NAC (50; 100; 200; 500 μM) induced a 15-fold, 17-fold, 14-fold and 14-fold lower number of DSBs-foci/cell, respectively.

*Significance.* Dental co-monomer epoxy metabolites, EMPME and EMPA, can induce DNA-DSBs. The addition of antioxidants (Asc or NAC) leads to reduction of DNA-DSBs, and NAC leads to more prominent reduction of DNA-DSBs compared to Asc.



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## 1. Introduction

The unpolymerized co-monomers triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) can be released from incompletely polymerized composite resins [1], and thereby affect the activity of dental pulp cells or enter the intestine by swallowing, subsequently reaching the circulatory system and organs [1,2]. Our previous studies have demonstrated the uptake, distribution and elimination of radiolabeled $^{14}$C-TEGDMA and $^{14}$C-HEMA in guinea pigs [3,4]. As a result, the metabolism of TEGDMA and HEMA was postulated, and the formation of methacrylic acid (MA), a metabolisation intermediate of TEGDMA and HEMA, was described [3,4]. MA can be metabolized by two different pathways [5]. In one pathway (epoxide pathway), it was suggested that 2,3-epoxy-2-methyl-propionicacid-methylester (EMPME) might be formed [6]. Additionally, the C-C-double bond of MA can be oxidized, consequently, the epoxy metabolite, 2,3-epoxy-2-methylpropionic acid (EMPA) can be formed [6–8]. In this process, hydrogen peroxide is involved as chemical catalyst [9], and cytochrome P450 2E1 (CYP2E1) also plays an important role [7]. In a previous study, it was shown that $^{14}$C-TEGDMA and $^{14}$C-HEMA are mainly metabolized via epoxide pathway in A549 cells [10], and the formation of EMPA in human oral cells (for example, human gingival fibroblasts (HGF) and human pulp fibroblasts (HPF)) has also been demonstrated [7].

In a previous study, the toxicology of EMPME and EMPA was investigated by the use of a modified fluorescent stem-cell test; as a result, the teratogenic effect was observed for EMPA, and an embryotoxic effect was observed for EMPME on the embryonic stem cells of mice [6]. A similar genotoxicity of epoxides was also found in glycidamide, the epoxy metabolite of acrylamide, which is commonly present in fried food [11], is highly reactive toward DNA by formation of covalent adducts on the N7-position of guanine, N3-position of adenine and N1-position of deoxyadenosine [12]. Since the glycidamide has an epoxy structure similar to those of EMPME and EMPA, it is likely that they will lead to a similar genotoxicity. Since the DNA damage can lead to carcinogenic and mutagenic effects [13], the epoxides are considered to be highly reactive molecules and toxic agents [8]. If they are left unrepaired, they can lead to cell death; chromosomal translocations and genomic instability may occur if they are misrepaired [14].

Many studies have dealt with the toxicology of co-monomers such as TEGDMA and HEMA, which can induce DNA-DSBs [15,16]. Schweikl et al. demonstrated that HEMA-induced apoptosis is a response to DNA damage [17]. However, in comparison with the precursors, TEGDMA, HEMA and the intermediate MA, whether the epoxy metabolites can induce more DNA-DSBs is still unknown. In this study, therefore, the effect of the co-monomer epoxy metabolites, EMPME and EMPA, on the DNA-DSBs, was investigated. In some studies, it has been demonstrated that the addition of antioxidants, such as Asc or NAC, can reduce the cytotoxic effects and DNA-DSBs of dental resin co-monomers [18–20]. It is not known whether antioxidants can lead to the reduction of DNA-DSBs in the presence of co-monomer epoxy metabolites. Therefore, in this study, the effects of Asc and NAC on the epoxide-induced DNA-DSBs in HGFs were also investigated.

## 2. Methods

### 2.1. Chemicals

EMPME and MA were obtained from Sigma–Aldrich (Weinheim, Germany). EMPA was synthesized by oxidation of MA, according to the method described by Yao and Richardson [9]. For the determination of cytotoxic effects, a cell-proliferation kit II from Roche Diagnostics (Mannheim, Germany) was used. Asc was purchased from Sigma–Aldrich (St. Louis, MO, USA), NAC was obtained from Alfa Aesar GmbH (Karlsruhe, Germany). Hydrogen peroxide ($H_2O_2$) was obtained from Sigma–Aldrich (Steinheim, Germany). MA, EMPME, EMPA, Asc and NAC were dissolved directly in the medium. All chemicals and reagents were of the highest purity available.

### 2.2. Cell culture

HGFs were obtained from Provitro GmbH (Berlin, Germany). The HGFs (passage 10) were cultured as described in our former study [15].

### 2.3. XTT-based viability assay

An XTT-based cell viability assay was used to determine the half-maximum effect concentration ($EC_{50}$) values for the investigated substances in HGFs. This assay was performed according to our previous study [15]. The cells were treated with medium containing MA (1–100 mM), EMPME (0.5–12 mM) and EMPA (0.01–10 mM), respectively, followed by incubation for 24 h. The formazan formation was quantified spectrophotometrically at 450 nm (reference wavelength 670 nm), using a microplate reader (MULTISKAN FC; Thermo Fisher Scientific (Shanghai) Instruments Co., Ltd., China). Four independent experiments were performed, each time in triplicate.

### 2.4. γ-H2AX immunofluorescence

DNA-DSBs formation was determined in HGFs by γ-H2AX assay, as described in our previous study [15]. In the following the procedure and modifications for the present study is outlined:

12 mm round cover slips (Carl Roth, Karlsruhe, Germany) were cleaned in 1 N HCl and distributed into a 24-well plate. HGFs were seeded at $7 \times 10^4$ cells/ml in each well with the medium, followed by overnight incubation at 37 °C. The cells were exposed for 6 h to medium containing the MA, EMPME, and EMPA, respectively, or the antioxidants alone; the concentrations of MA, EMPME and EMPA are determined by $EC_{50}$, $1/3EC_{50}$ and $1/10EC_{50}$, based on the XTT values: MA (15.64; 5.21; 1.56 mM), EMPME (2.58; 0.86; 0.26 mM), EMPA (1.72; 0.57; 0.17 mM), the concentrations of antioxidants tested alone were Asc (50; 100; 200; 500 μM) and NAC (50; 100; 200; 500 μM); these concentrations were based on a previous study [19]. Considering toxicity caused by 500 μM Asc from our result, the concentrations of antioxidants to be added to MA, EMPME, EMPA for γ-H2AX assay were: Asc (50; 100; 200 μM) and NAC (50; 100; 200; 500 μM). Negative control cells received the medium for 6 h. Positive control cells received 1 mM $H_2O_2$ in

the medium for 15 min. For immunofluorescent staining, cells were first washed 2 × 5 min with PBS, and were fixed by adding 0.5 ml ice-cold 4% paraformaldehyde in PBS for 5 min at 4 °C, washed with cold PBS (4 °C) for 4 × 2 min, and permeabilized for 10 min with 0.5 ml of triton–citrate buffer (0.1% sodium citrate, 0.1% Triton X-100) at 4 °C. After washing for 4 × 5 min with PBS, the cells were blocked for 20 min with four drops of serum-free blocking buffer (Dako, Hamburg, Germany) per well, at 25 °C. Thereafter, the cells were incubated with the primary antibody mouse monoclonal anti γ-H2AX (Millipore, Billerica, MA, USA) at a dilution of 1:1300 in antibody diluent (0.3 ml per well; Dako), at 4 °C overnight. After 4 × 5 min washes with PBS at 4 °C, the cells were incubated with FluoroLink Cy3-labeled goat anti-mouse secondary antibody (GE Healthcare, Munich, Germany) at a dilution of 1:1300 in antibody diluent (0.3 ml per well) for 2 h, at 25 °C, in the dark. Cells were then washed for 3 × 5 min in PBS, thereafter, cells were incubated with CyBR green at a dilution of 1:50000 in Tris-acetate-EDTA (TAE) buffer, for 15 min. Cells were then washed for 2 × 5 min in PBS and 2 × 5 min with deionized water. Finally, the cover slips were each placed on 20 µl of 1 ml Prolong antifade gold (Invitrogen, Karlsruhe, Germany) on a glass slide (76 mm × 26 mm; Carl Roth). Four independent experiments were performed.

### 2.5. *Image acquisition*

HGFs were investigated using a Zeiss CLSM imaging fluorescence microscope (Zeiss, Göttingen, Germany), equipped with a motorized filter wheel and appropriate filters for excitation of red (wavelength: 594 nm) and green (wavelength: 488 nm) fluorescence. Images were obtained using a 63 × and a 100 × Plan-Neofluar oil-immersion objective (Zeiss) and the fluorescence-imaging system LSM Image Browser (Zeiss).

### 2.6. *Data analysis*

The values of XTT assay were calculated as percentage of the controls using Graph Pad Prism 4 (Graph Pad Software Inc., San Diego, USA). Values were plotted on a concentration log-scale and the range of the maximum slope was derived. $EC_{50}$ values were obtained as half-maximum-effect concentrations from the fitted curves. Data are shown as means ± standard error of the mean (SEM) of four independent experiments (n = 4), each performed in triplicate.

In the γ-H2AX test, the DSBs-foci/cell were counted by the same investigator, using the fluorescence microscopic with a 100× objective. Data are shown as means ± standard deviation (SD) of four independent experiments (n = 4).

The statistical significance ($p < 0.05$) of the differences between the experimental groups was compared using the Student's *t*-test, corrected according to Bonferroni–Holm [21].

## 3. Result

### 3.1. *XTT assay*

HGFs showed a dose-dependent loss of viability after exposure to MA, EMPME or EMPA for 24 h. The lowest $EC_{50}$ value was found for EMPA ($EC_{50}$: 1.72 mM) (Table 1). The $EC_{50}$ value of MA was about 6-fold higher than that of EMPME, and 9-fold higher than that of EMPA. The cytotoxicity could be ranked in the following order: EMPA > EMPME > MA. The relative toxicities are given in (Table 1).

**Table 1 – The $EC_{50}$ values (mM; mean ± SEM; n = 4) of the tested substances and the relative toxicity in HGFs as determined by XTT viability assay.**

| Substance | $EC_{50}$ ± SEM [mM] | Relative toxicity |
|---|---|---|
| MA | 15.64 ± 1.1 | 1 |
| EMPME[a] | 2.58 ± 0.3 | 6 |
| EMPA[b] | 1.72 ± 0.4 | 9 |

[a,b]Significantly different ($p < 0.01$) to MA.

**Table 2 – Number of induced DSBs-foci per cell caused by different concentrations of Asc or NAC. HGFs were incubated with Asc (50–500 µM) or NAC (50–500 µM) for 6 h, respectively. The number of foci was determined with γ-H2AX assay. Data are presented as mean ± SD, n = 4.**

| Antioxidant | Foci/cell ± SD | | | |
|---|---|---|---|---|
| | 500 µM | 200 µM | 100 µM | 50 µM |
| Asc | 0.75 ± 0.08* | 0.51 ± 0.12 | 0.39 ± 0.07 | 0.46 ± 0.17 |
| NAC | 0.48 ± 0.12 | 0.40 ± 0.07 | 0.45 ± 0.10 | 0.48 ± 0.13 |
| Negative control | 0.39 ± 0.08 | | | |
| Positive control | 11.76 ± 1.92 | | | |

* Significantly different ($p < 0.05$) to negative control.

### 3.2. *γ-H2AX assay with antioxidants*

Asc and NAC, at all concentrations tested, showed no significant reduction of the number of DSBs-foci/cell compared to the negative control.

Asc (500 µM) induced significantly more DSBs-foci/cell (0.75 ± 0.08) in HGFs compared to control (0.39 ± 0.08) (Table 2). NAC at all concentrations showed no significant induction of DSBs-foci in HGFs compared to control (Table 2).

### 3.3. *γ-H2AX assay with MA, EMPME and EMPA, respectively, in the presence/absence of antioxidants*

In the positive control, $H_2O_2$ (1 mM) induced 15.88 ± 1.75 DSBs-foci/cell. In the negative control, 0.48 ± 0.08 DSBs-foci/cell was found.

#### 3.3.1. MA

At concentrations of 15.64 mM ($EC_{50}$) and 5.21 mM ($1/3EC_{50}$), MA induced 1.76 ± 0.19 and 1.63 ± 0.12 DSBs-foci/cell, respectively. The addition of Asc (50–200 µM) or NAC (50–500 µM) to 15.64 mM and 5.21 mM MA significantly reduced the number of foci/cell compared to exposure with MA alone (Fig. 2). The concentration of 1.56 mM ($1/10EC_{50}$) MA showed no significant increase in the number of foci/cell in HGFs (Fig. 1). No significant DSBs-foci reduction was found with the addition of Asc (50–200 µM) or NAC (50–500 µM) to 1.56 mM MA (Fig. 2).

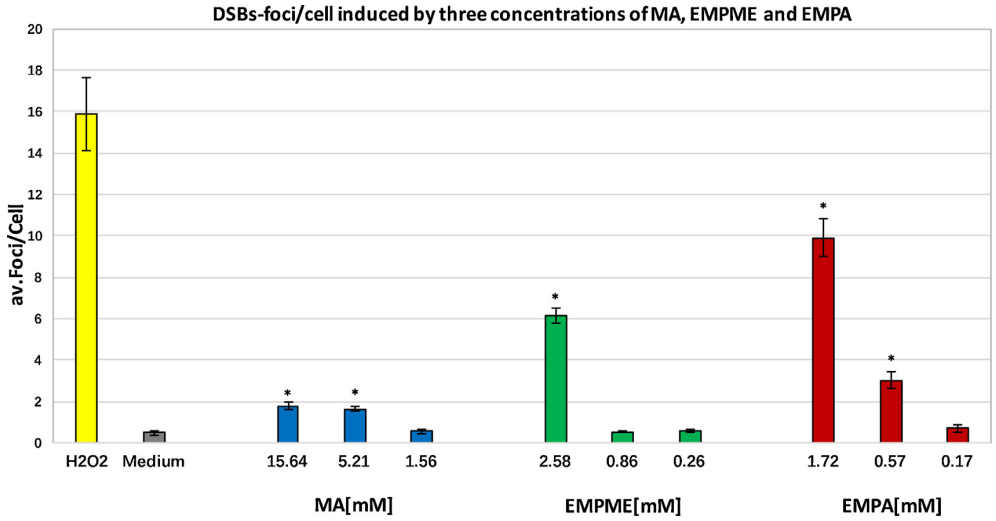


**Fig. 1 – Average of induced γ-H2AX DSBs-foci/cell in HGFs elicited by a 6 h exposure to MA, EMPME and EMPA at $EC_{50}$, $1/3EC_{50}$ and $1/10EC_{50}$ concentrations. Negative control cells received the medium only. All results are expressed as the mean of four independent experiments; error bars represent the standard deviation. Values were compared using the Student's t-test. (*Statistically significant ($p < 0.01$) induction of the number of foci/cell compared to control).**

### 3.3.2. *EMPME*

At a concentration of 2.58 mM ($EC_{50}$), EMPME induced a 5-fold higher number of DSBs-foci/cell (6.15 ± 0.34) in HGFs, compared to control (Fig. 1). When HGFs were exposed to 2.58 mM EMPME, with the addition of Asc or NAC, the number of foci/cell was significantly reduced compared to exposure with 2.58 mM EMPME alone (Fig. 3). The addition of NAC (50–500 μM) to 2.58 mM EMPME significantly reduced the number of foci/cell compared to Asc (50–200 μM). No significant difference in foci induction was found when HGFs were exposed to 0.86 mM ($1/3EC_{50}$) and 0.26 mM ($1/10EC_{50}$) EMPME (Fig. 1). No significant DSBs-foci reduction was found with the addition of Asc (50–200 μM) or NAC (50–500 μM) to 0.86 mM and 0.26 mM EMPME (Fig. 3). Micronuclei could be observed at 2.58 mM EMPME (Fig. 5)

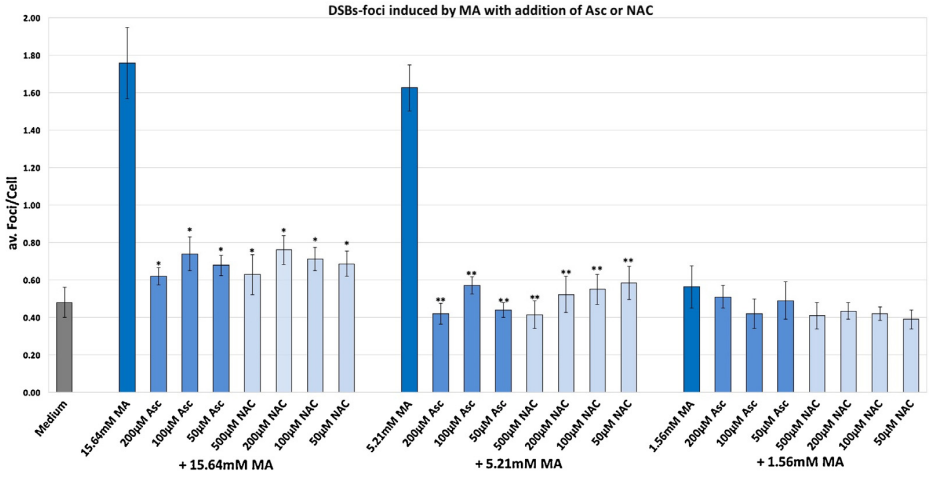


**Fig. 2 – Average of induced γ-H2AX DSBs-foci/cell in HGFs elicited by a 6 h exposure to MA at $EC_{50}$, $1/3EC_{50}$ and $1/10EC_{50}$ concentrations, with addition of Asc (50–200 μM) or NAC (50–500 μM), respectively. Negative control cells received the medium only. All results are expressed as the mean of four independent experiments; error bars represent the standard deviation. Values were compared using the Student's t-test. (*Statistically significant ($p < 0.01$) reduction of the number of foci/cell compared to 15.64 mM ($EC_{50}$) MA, without adding Asc or NAC). (**Statistically significant ($p < 0.01$) reduction of the number of foci/cell compared to 5.21 mM ($1/3EC_{50}$) MA, without adding Asc or NAC).**

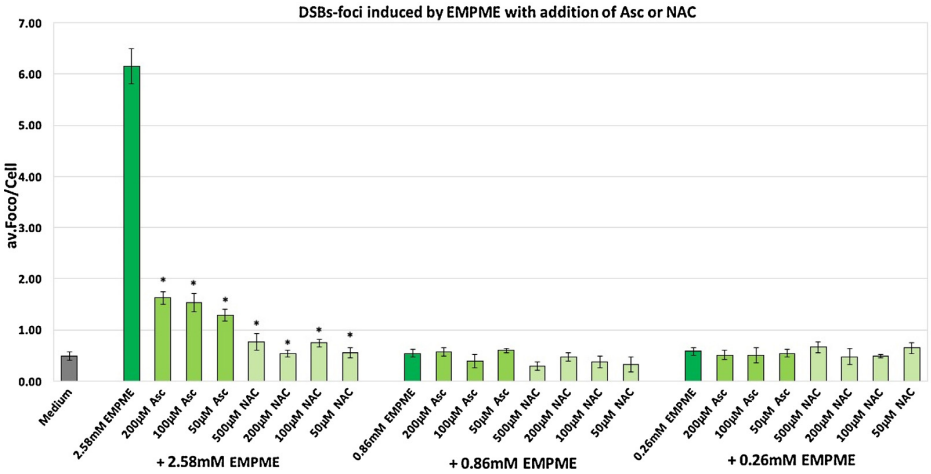


**Fig. 3 – Average of induced γ-H2AX DSBs-foci/cell in HGFs elicited by a 6 h exposure to EMPME at $EC_{50}$, $1/3EC_{50}$ and $1/10EC_{50}$ concentrations with addition of Asc (50–200 μM) or NAC (50–500 μM) respectively. Negative control cells received the medium only. All results are expressed as the mean of four independent experiments; error bars represent the standard deviation. Values were compared using the Student's t-test. (*Statistically significant ($p < 0.01$) reduction of the number of foci/cell compared to 2.58 mM ($EC_{50}$) EMPME without adding Asc or NAC).**

### 3.3.3. EMPA

The concentration of 1.72 mM ($EC_{50}$) EMPA induced a 20-fold higher number of DSBs-foci/cell (9.90 ± 0.90), and 0.57 mM ($1/3EC_{50}$) EMPA induced a 6-fold higher number of foci/cell (3.00 ± 0.20), compared to control (Fig. 4). At concentrations of 1.72 mM and 0.57 mM, DSBs-foci reduction was noted in the presence of Asc (50–200 μM) or NAC (50–500 μM), while the addition of NAC (50–500 μM) significantly reduced the number of foci/cell compared to Asc (50–200 μM). The most reduction could be found with 1.72 mM EMPA, the presence of NAC (50;100;200;500 μM) induced a 15-fold, 17-fold, 14-fold and 14-fold lower number of foci/cell, respectively. The concentration of 0.17 mM ($1/10EC_{50}$) EMPA showed no significant increase in DSBs-foci (Fig. 1). No significant DSBs-foci reduction was found with the addition of Asc (50–200 μM) or NAC (50–500 μM) to 0.17 mM EMPA (Fig. 4)

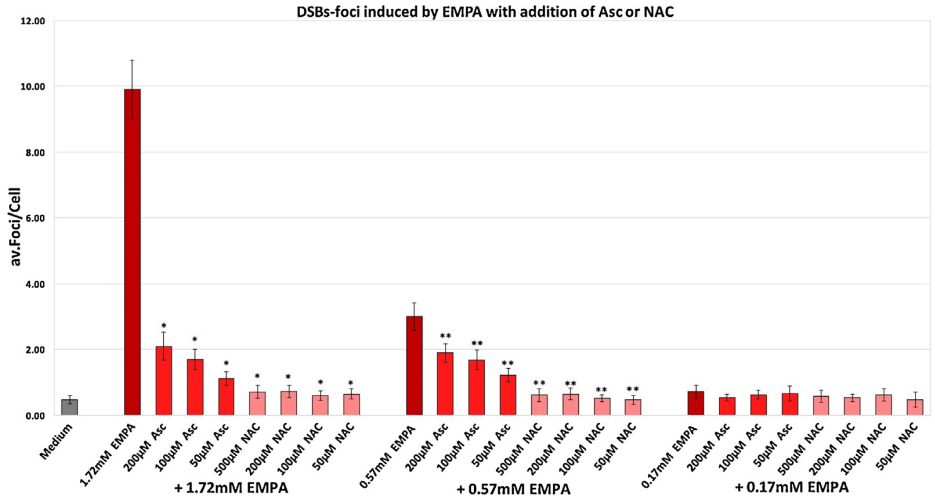


**Fig. 4 – Average of induced γ-H2AX DSBs-foci/cell in HGFs elicited by a 6 h exposure to EMPA at $EC_{50}$, $1/3EC_{50}$ and $1/10EC_{50}$ concentrations with addition of Asc (50–200 μM) or NAC (50–500 μM) respectively. Negative control cells received the medium only. All results are expressed as the mean of four independent experiments; error bars represent the standard deviation. Values were compared using the Student's t-test. (*Statistically significant ($p < 0.01$) reduction of the number of foci/cell compared to 1.72 mM ($EC_{50}$) EMPA without adding Asc or NAC). (**Statistically significant ($p < 0.01$) reduction of the number of foci/cell compared to 0.57 mM ($1/3EC_{50}$) EMPA without adding Asc or NAC).**

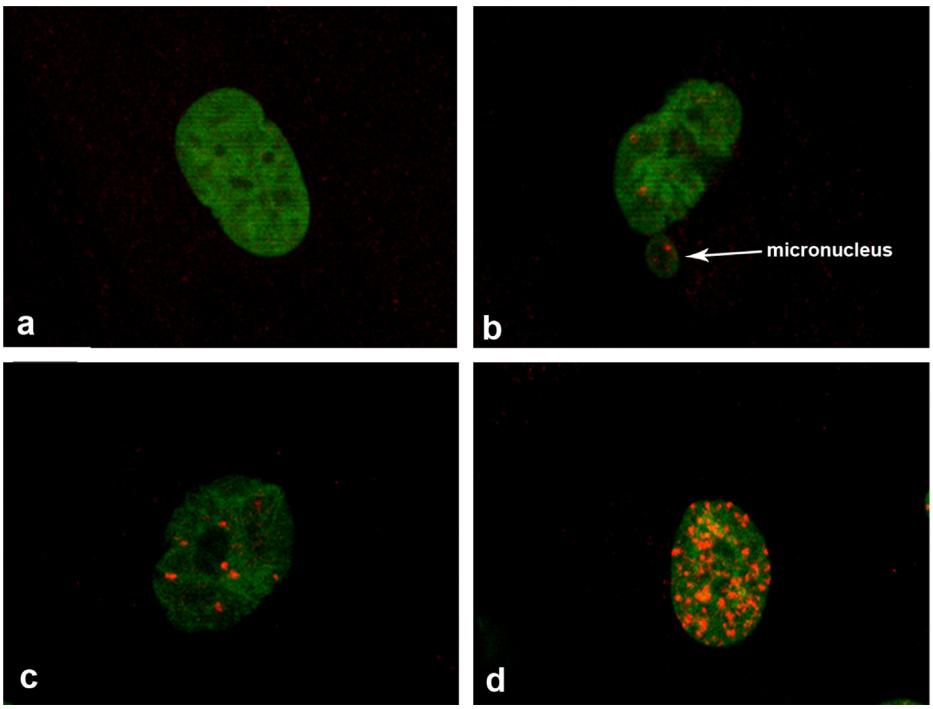


**Fig. 5 – Representative images of immunofluorescent staining for H2AX phosphorylation (orange) in HGFs, after exposure to different substances compared to control cells. Sybr green (green) is a marker for DNA and stains the whole nucleus of the cell. (a) A nucleus of HGFs without foci, as typically seen in untreated cells (in this case with medium). (b) A nucleus of HGFs with two foci, which can occur in treated cells (in this case, with 2.58 mM ($EC_{50}$) EMPME); the arrow shows the presence of micronucleus (MN). (c) A nucleus of HGFs displaying nine foci (in this case, with 1.72 mM ($EC_{50}$) EMPA). (d) A nucleus of HGFs with more than forty foci induced by $H_2O_2$.**

## 4. Discussion

The co-monomers, TEGDMA and HEMA, can be eluted from unpolymerized dental composite resins and can be metabolized to intermediate MA [5]. The biological conversion of MA to its related epoxy metabolite EMPA has been described [6–8]. Additionally, in this process, another epoxy metabolite, EMPME, may be formed [6]. Epoxides are considered to be highly mutagenic and carcinogenic agents [2,8].

In this study, the relative toxicity of the EMPA was 9-fold higher than that of MA, and that of EMPME was 6-fold higher than MA. In addition to cytotoxicity, a γ-H2AX assay was performed to test the genotoxicity of MA, EMPME and EMPA. The present study showed that the epoxy compounds EMPME and EMPA gave rise to more severe damage to DNA-DSBs compared to MA. The explanation may be that the epoxy metabolites EMPME and EMPA are highly reactive and unstable molecules and, therefore, may exert higher toxicity. It was shown that MA may play a key role in DNA-cell-binding assay [22], and, therefore, it is very likely that its related epoxy metabolites are able to induce higher toxic effects, such as DNA-DSBs, compared to MA. In contrast to EMPME, a higher cytotoxicity and more DNA-DSBs were observed for EMPA in this study. It has been shown that acids can lower the pH conditions of cell medium leading to an increased cytotoxicity and DNA damage in many cell lines [23,24]. Therefore, in the present study, EMPA (carboxylic acid) may also decrease the pH condition, resulting in a higher cytotoxicity and more DSBs induction.

A previous study investigated the DNA-DSBs induced by co-monomers TEGDMA at 3.6 mM ($EC_{50}$) and 1.2 mM (1/3$EC_{50}$), and HEMA at 11.2 mM ($EC_{50}$) and 3.7 mM (1/3$EC_{50}$) concentrations [15]. In comparison with the former results, higher rates of DSBs-foci were found in the current study, at 2.58 mM ($EC_{50}$) EMPME, 1.72 mM ($EC_{50}$) EMPA and 0.57 mM (1/3$EC_{50}$) EMPA. These data indicate that epoxy metabolites can cause more severe DNA damage than their metabolic precursors, TEGDMA and HEMA, even at a lower concentration. Moreover, this also raises an interesting question: is the DNA damage really caused by the co-monomers, TEGDMA and HEMA, or is it actually induced by their epoxy metabolites, EMPME and EMPA. It has been demonstrated that the DNA damage induced by acrylamide (for example, in fried food) [11], is possibly triggered by its epoxy metabolite glycidamide [25]. Due to the epoxy structural similarity of the EMPME and EMPA to glycidamide, it is very likely that, when HGFs are exposed to co-monomers, TEGDMA and HEMA, the EMPME and EMPA formed are involved in DNA-DSBs induction.

Further, the current study examined whether the antioxidants Asc and NAC can reduce MA-induced and epoxides-induced DNA-DSBs formation in HGFs. Our data showed that,

when HGFs were exposed to MA (15.64 and 5.21 mM), EMPA (1.72 and 0.57 mM) and EMPME (2.58 mM), the number of DSBs-foci/cell was significantly decreased due to Asc and NAC. These results are in agreement with other studies [19,26]. Since Asc and NAC are regarded as radical scavengers, and adducts can also be formed with radicals, which then reduce the toxicity [27]. It was reported that the cytotoxicity was reduced by the addition of Asc or NAC to the cell-culture medium [18,20,28]. NAC has been shown to reduce DNA deletions in ATM-deficient mice [29], and orally administered mixtures of antioxidants, including Asc and NAC, have been shown to reduce ionizing radiation-induced DSBs [30].

In the present study, the highest concentration of tested Asc was 500 μM, but it induced significantly more DSBs-foci compared to the negative control. One explanation for this phenomenon may be linked to Asc's ability to induce reactive oxygen species (ROS) and oxidative stress [31]. Additionally, in our study, it is also possible that Asc (500 μM) induced the formation of $H_2O_2$, which has been described in previous study [32], leading to the increase of DNA-DSBs in HGFs. Conversely, the antigenotoxic role of Asc has been demonstrated [20,33], and it has also been shown that the presence of Asc can prevent the formation of DNA adducts [34]; this is in an agreement with present study that Asc (50–200 μM) had a protective effect on HGFs by reducing the DNA-DSBs. The protective mechanism regulated by Asc at a lower concentration is, therefore, more dominant than toxicity caused by xenobiotic and Asc itself.

In contrast with Asc, NAC showed no significant induction of DNA-DSBs when compared with the negative control, even at the highest concentration of 500 μM. NAC is known as a thiol-containing antioxidant and protects cellular components by alleviating damage caused by ROS [29]. Previous studies have shown that NAC can reduce the cytotoxicity and genotoxicity of methacrylate-based dental co-monomers [19,20]. Although the mechanism of NAC's reducing the genotoxicity of epoxides is still unknown, in this process, a protective mechanism related to glutathione (GSH) synthesis is considered to play a key role. It was reported that, a reduction of intercellular GSH level with increased formation of ROS was observed when cells were exposed to resin monomers [35]. Additionally, the addition of NAC to glycidamide increased the GSH content of hepatocytes [27]. In our study, HGFs were exposed to EMPME (2.58 mM) and EMPA (1.72 and 0.57 mM) in the presence of NAC (50–500 μM), the number of DSBs-foci/cell was significantly reduced. This protective effect of NAC for epoxide-induced DSBs is in agreement with a previous study showing that NAC can play a protective role against acrylamide-induced DNA damage, which may due to the reduction of the genotoxicity of its related epoxide glycidamide [36]. Epoxide-induced DNA adducts have previously been demonstrated [12], wherein the addition of NAC can reduce DNA-adduct formation [29]; however, the cause of the formation of EMPME and EMPA-induced adducts is still unclear, which should be addressed in further studies.

The results of this study show that NAC (50–500 μM) leads to prominent reduction in DSBs-foci compared to Asc (50–200 μM). This may be explained by the formation of endogenous ROS, triggered by Asc, leading to the depletion of the GSH level [37], while the contrary effect, caused by NAC, increases the GSH level, which protects DNA from oxidative damage and formation of DNA-adducts [12,29]. NAC is, therefore, considered to be a preferable antioxidant to Asc in terms of DNA damage caused by dental resin (co)monomers, as well as by their metabolites.

Epoxides were reported to induce micronucleus (MN) which is closely related to DNA-DSBs or unrepaired DNA breaks [38,39]. This corresponds to the findings in our study that MN can be observed at 2.58 mM EMPME; the presence of MN in our study indicates that gene mutation may have occurred in HGFs after exposure to EMPME.

The genotoxicity of epoxide presented in this study makes it necessary to perform a rough risk assessment. We assume a worst-case scenario, that 32 teeth are filled with TEGDMA-containing composite resin. According to average estimate of volume of some typical restorations [40], a max. 0.2 g composite resin per tooth is calculated. The average portion of TEGDMA in a dental composite is about 10%, and maximum 10% of TEGDMA is actually released in a methanol–water mixture within 84 h [41]. Theoretically, therefore, an amount of 38.4 mg MA can be formed, according to the calculation method described by Seiss et al. [8]. If we consider a daily saliva production of approximately 1 L [42], and the conversion rate of MA to EMPA is about 5% [8], then a concentration of 60 μM EMPA could result. This value is far below $1/10EC_{50}$ concentration of EMPA. However, these data, calculated from an elution experiment in a human worst-case situation, should trigger no alarm, since saliva is less effective in eluting unpolymerized co-monomer than a methanol solution. Furthermore, clinically, it is unrealistic to fill 32 teeth with 0.2 g each simultaneously; therefore, the eluted TEGDMA cannot reach such a high concentration, consequently the amount of epoxides formed in biological systems can be extremely lower than that obtained by worst-case scenario.

The present study supports the hypothesis that the co-monomer epoxy metabolites, EMPME and EMPA, can induce DNA-DSBs. The addition of the antioxidants (Asc or NAC) can reduce the DNA-DSBs induced by EMPME and EMPA.

## 5. Conclusion

The dental co-monomer epoxy metabolites, EMPME and EMPA, are more cytotoxic, which can also induce more DNA-DSBs, compared to their precursor MA. The addition of antioxidants (Asc or NAC) to EMPME and EMPA can reduce the number of DNA-DSBs foci. NAC exhibits a superior protective effect compared to Asc.

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