Aus der Klinik und Poliklinik für Zahnerhaltung und Parodontologie

der Ludwig-Maximilians-Universität München

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Induction and repair of DNA double-strand breaks by dental materials in human gingival fibroblasts

Dissertation

zum Erwerb des Doktorgrades der Zahnmedizin

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

vorgelegt von

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2017

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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Tag der mündlichen Prüfung: 07.12.2017

Meinen lieben Eltern und meiner Familie

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Table of contents

1 Abbreviations

HGFs	Human gingival Fibroblasts
DNA-DSBs	DNA double-strand breaks
ChKM	Walkhoff Solution
2-CP	2-Chlorophenol
3-CP	3-Chlorophenol
4-CP	4-Chlorophenol
MMA	Methyl methacrylate
EGDMA	Ethylene glycol dimethacrylate
TEGDMA	Triethylene glycol dimethacrylate
ROS	Reactive oxygen species
Asc	Ascorbic acid
ACC	*N*-acetylcystine
HEMA	2-hydroxyethylmethacrylate
PMMA	Poly-methyl methacrylate
Bis-GMA	Bisphenol-A-glycidyl methacrylate
UDMA	Urethan dimethacrylate
GDMA	Glycerol dimethacrylate
DMSO	Dimethyl sulfoxide
EC_{50}	Half-maximum effect concentration
PBS	Phosphate-buffered saline
GSH	Glutathione
XTT	Tetrazolium salt
ATM	Serine-protein kinase ATM

2 Publication list

2.1 Publications for Cumulative Dissertation

- Shehata M, Durner J, Thiessen D, Shirin M, Lottner S, Van Landuyt K, Furche S, Hickel R, Reichl FX. 2012. Induction of DNA double-strand breaks by monochlorophenol isomers and ChKM in human gingival fibroblasts. Arch Toxicol. 86(9):1423-1429.

- Lottner S, Shehata M, Hickel R, Reichl FX, Durner J. 2013. Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to methacrylate based monomers. Dent Mater. 29(9):991-998.

2.2 Further Publications

- Van Landuyt KL, Geebelen B, Shehata M, Furche SL, Durner J, Van Meerbeek B, Hickel R, Reichl FX. 2012. No evidence for DNA double-strand breaks caused by endodontic sealers. J Endod. 38(5):636-641.

- Reichl FX, Lohle J, Seiss M, Furche S, Shehata MM, Hickel R, Muller M, Dranert M, Durner J. 2012. Elution of TEGDMA and HEMA from polymerized resin-based bonding systems. Dent Mater. 28(11):1120-1125.

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- Furche S, Hickel R, Reichl FX, van Landuyt K, Shehata M, Durner J. 2013. Quantification of elutable substances from methacrylate based sealers and their cytotoxicity effect on human gingival fibroblasts. Dent Mater. 29(6):618-625.

- Shehata M, Durner J, Eldenez A, Van Landuyt K, Styllou P, Rothmund L, Hickel R, Scherthan H, Geurtsen W, Kaina B. Carell T, Reichl FX. 2013. Cytotoxicity and induction of DNA double-strand breaks by components leached from dental composites in primary human gingival fibroblasts. Dent Mater. 29(9):971-979.

- Rothmund L, Shehata M, Van Landuyt KL, Schweikl H, Carell T, Geurtsen W, Hellwig E, Hickel R, Reichl FX, Hogg C. 2015. Release and protein binding of components from resin based composites in native saliva and other extraction media. Dent Mater. 31(5):496-504.

- Eldeniz AU, Shehata M, Hogg C, Reichl FX. 2016. DNA double-strand breaks caused by new and contemporary endodontic sealers. Int Endod J. 49(12):1141-1151.

3 Confirmation of Co-authors

The confirmation of co-authors is submitted separately.

4 Introduction

4.1 Induction of DNA double-strand breaks by monochlorophenol isomers and Walkhoff solution (ChKM) in human gingiva fibroblasts

Endodontic therapy plays an important role in the preservation of teeth function [1]. Disinfection of root canals is considered to be an important step during endodontic treatment [2, 3]. Phenol has been traditionally used in dental treatment as a sedative for the pulp or as disinfectant for carious cavity and root canal [4]. However, phenol is regarded to be a mutagenic and carcinogenic agent, therefore, its use in dental practice is restricted [4, 5]. Monochlorophenols are derivatives of phenol, which are still used in dental practice. They are more active antiseptics/disinfectants than phenol, which makes them good disinfectants for root canals [6]. Monochlorophenols exist in three isomers: 2-CP, 3-CP and 4-CP, with 4-CP is considered most effective antiseptic compound [3, 4, 7-9]. Chlorophenols represent a wide group of substances with different toxicities [10]. In chlorophenols, the molecule phenol is chlorinated up to five-fold. Monochlorophenols have a higher antibacterial, antiseptic and disinfectant potential compared to other disinfectants or phenol [6, 11]. The use of monochlorophenols is rather controversial because of the high toxicity and mutagenicity of higher substituted chlorophenols [4, 8, 10]. Walkhoff (ChKM) solution is a Monochlorophenol-containing disinfectant. ChKM solution contains monochlorophenol compound 4-CP, camphor and menthol. In literature, the use of ChKM solution is controversially discussed because of possible (DNA)-toxicity of the ingredient 4-CP [11]. However, it is unknown whether ChKM can induce DNA damage in human oral cells.

In the first study of the dissertation titled “induction of DNA double-strand breaks (DSBs) by ChKM and monochlorophenol compounds”, 2-CP, 3-CP and 4-CP were tested in

human gingival fibroblasts (HGFs), DNA DSBs (foci) induced in HGFs to monochlorophenols or ChKM were investigated using the γ-H2AX DNA focus assay; Shehata M, Durner J, Thiessen D, Shirin M, Lottner S, Van Landuyt K, Furche S, Hickel R, Reichl FX. Induction of DNA double-strand breaks by monochlorophenol isomers and ChKM in human gingival fibroblasts. Arch Toxicol, 2012;86:1423-9.

4.2 Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to methacrylate-based monomers

Resin based dental (co)monomers are widely used in contemporary dental restorative materials. The conversion of (co)monomers can be induced by light and/or by autopolymerisation. However, incomplete polymerization means that (co)monomers and additives can diffuse into the oral cavity or into the pulp [12-14]. These released substances can then enter the bloodstream [15]. Moreover, the methacrylates such as MMA, EGDMA and TEGDMA were identified in the air of dental technicians' workplaces [16]. (Co)monomers and additives released from resin-containing products can cause various adverse effects such as allergic contact dermatitis and bronchial asthma [17, 18]. *In vitro* studies have shown that some dental methacrylates can cause cytotoxic, estrogenic and mutagenic reactions [19-21]. Thereby, reactive oxygen species (ROS) and epoxides play an important role in the metabolism of dental methacrylates [22-24]. During the metabolism of these xenobiotics (*e.g.* MMA, TEGDMA) the amount of ROS and epoxides increases while the amounts of (physiological) radical scavengers, such as glutathione or vitamin C, decrease. Most epoxides as well as ROS are regarded as highly toxic agents reacting with different cellular molecules and cellular structures such as DNA [22, 25]. In this context, the number of different cancers of the oral mucosa is increasing in adults of 45 years and older with a simultaneous decrease in tobacco and

alcohol consumption [26]. DNA DSBs caused by mutagenic agents like epoxides and ROS are considered the most toxic type of DNA lesions [27]. If they are left unrepaired they can cause cell death and, if they are misrepaired they may lead to chromosomal translocations and genomic instability [28]. Using the γ-H2AX-assay, a previous study has shown that methacrylate based dental monomers can induce DNA DSBs in HGF [29]. H2AX, a protein from the H2A family and a component of the histone octamer in nucleosomes, can be phosphorylated by different kinases to γ-H2AX. This phosphorylation recruits and localizes DNA repair proteins at the foci [30]. The foci represent DNA DSBs and can be used as a biomarker for DNA damage. A labeled antibody against γ-H2AX can be used to label the foci, which can then be visualized using an immunofluorescence [29]. Many studies have dealt with the toxicity of (co)monomers and other substances from dental resins. Little is known about how to prevent cell damage. In some studies, it could be demonstrated that, the addition of antioxidant substances such as the vitamins C (Asc) and E or ACC can reduce the cytotoxic effects of dental monomers such as TEGDMA, HEMA or PMMA [31-34]. It is not known whether antioxidants lead to a reduction of DNA DSBs in human oral cells. Human oral cells (*e.g.* gingival and/or pulp fibroblasts) in this physiological situation are among the first to come into contact with eluted substances.

The aim of the second study of the dissertation was therefore to test the hypothesis that the antioxidants Asc or ACC can reduce the number of DNA DSBs caused by the dental (co)monomers Bis-GMA, UDMA, GDMA and EGDMA in HGF. The number of foci of DNA DSBs were investigated using γ-H2AX focus-assay;

Lottner S, Shehata M, Hickel R, Reichl FX, Durner J. Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to methacrylate based monomers. Dent Mater.2013;29(9):991-998.

5 Material und Method

5.1 Induction of DNA double-strand breaks by monochlorophenol isomers and ChKM in human gingiva fibroblasts

5.1.1 XTT-test

A XTT–based cell viability assay was used to determine the half-maximum effect concentrations (EC_{50}) for monochlorophenol compounds 2-CP, 3-CP, 4-CP and ChKM in HGFs. Negative control cells received either medium only, or medium + DMSO. Positive control cells received 1 mM H_2O_2 + medium, or 1 mM H_2O_2 + medium + DMSO for 10 min. The formazan formation was quantified spectrophotometrically using a microtiter plate reader (Victor 3, Perkin Elmer Las, Jügesheim, Germany). XTT-assay method is described in detail in the first study of the dissertation [35].

5.1.2 γ-H2AX-test

DNA DSBs formation was tested in HGFs by the γ-H2AX DNA focus assay. HGFs were exposed to medium containing substances in the following concentrations (corresponding to EC_{50}, 1/3 EC_{50} and 1/10 EC_{50} values, received from the XTT-assay). Negative control cells received either medium alone, or medium + DMSO. Positive control cells received 1 mM H_2O_2 + medium, or 1 mM H_2O_2 + medium + DMSO for 10 min. Cells were incubated with mouse monoclonal anti-γ-H2AX and subsequently stained with FluoroLink Cy3-labeled goat anti-mouse secondary antibody. HGFs were investigated using a Zeiss Axioplan 2 imaging fluorescence microscope (Zeiss). DNA-DBs (foci) were counted and cell counting was performed until at least 80 cells. Cells containing 40 or more foci will be counted as multi-foci cell. γ-H2AX DNA focus assay method is described in detail in the first study of the current dissertation (Shehata et al. 2012).

5.2 Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to methacrylate based monomers

DNA DSBs formation in Asc, ACC, UDMA, GDMA, EGDMA and Bis-GMA were tested in HGFs using γ-H2AX DNA focus assay. The cells were exposed to medium containing the tested (co)monomers or the antioxidants in three concentrations based on EC_{50} results from XTT Cytotoxicity test (1/10 x,1/3 x,1 x EC_{50}). The antioxidant concentrations were based on cytotoxicity experiments in our group [29, 31]. Negative control cells only received medium. While the cells for the positive control received 1000 µM H_2O_2 in medium.

The cells were not preincubated with antioxidants so as to simulate physiological conditions during the filling of a cavity. DNA DSBs formation was determined in HGFs unexposed and exposed to dental resin compounds by the γ-H2AX DNA focus assay.

Since resin (co)monomers, like HEMA and TEGDMA, released from dental restorative materials, may reach millimolar concentrations in the pulp [12, 36], three concentrations were used based on the EC_{50} values from XTT-experiments on HGF from our group in the millimolar range (1×, 1/3× and 1/10× EC_{50}) [29].

The details are shown in the second study of the current dissertation (lottner et al. 2013) The results are shown as means (SD). The statistical significance ($p < 0.05$) of the differences between the experimental groups was tested using the *t*-test, corrected according to Bonferroni-Holm [38].

6 Results

6.1 Induction of DNA double-strand breaks by monochlorophenol isomers and ChKM in human gingiva fibroblasts

6.1.1 XTT-test

ChKM solution was the most toxic, compared to all other investigated compounds. Significant ($p < 0.05$) increase in toxicity of compounds was found as follows: camphor < 2-CP < menthol < 3-CP < 4-CP < ChKM.

6.1.2 γ-H2AX-test

In negative control, an average of 3 DNA DSBs foci each were found. In positive control, 35 DNA DSBs foci each were found. When HGF were exposed to the EC_{50} of monochlorophenols or ChKM, following DNA DSBs foci-rate were found: 3-CP 18 foci, 4-CP 19 foci, 2-CP 20 foci and ChKM 21 foci. The highest rates of DNA DSBs foci were found when HGFs were exposed to the EC_{50} of each substance, compared to their corresponding 1/3 EC_{50} or 1/10 EC_{50}. About 20 DNA-DNA DSBs foci per cell were found when HGFs were exposed to the substances (concentration in parenthesis): 2-CP (4 mM), 3-CP (2.3 mM), 4-CP (2.1 mM) or ChKM (corresponding to 1.5 mM 4-CP). About 22 % of the cells contained multi-foci when HGFs were exposed to substances with the EC_{50} (in parenthesis): 2-CP (4 mM), 4-CP (2.1 mM), or ChKM (corresponding to 1.5 mM 4-CP). Only 13 % of the cells contained multi-foci when HGFs were exposed to 3-CP with EC_{50} of 2.3 mM.

Also, see data and graphic illustration in detail in the first study of the current dissertation (Shehata et al. 2012)

6.2 Effects of antioxidants on DNA double-strand breaks in human gingiva fibroblasts exposed to methacrylate based monomers

6.2.1 H2AX-assay with antioxidants

None of the antioxidants tested showed a significant reduction the number of foci/cell compared with the negative controls, when incubated with the antioxidants Asc or ACC with HGF irrespective of their concentration. At a concentration of 500 µM, the antioxidant Asc induced significant ($p < 0.05$) more DNA DSBs in HGF compared to the controls. 500 µM Asc induced approximately a three-fold increase in the number of foci/cell compared to the controls. The number of multi foci cell increased with the concentration of Asc. The antioxidant ACC (at all tested concentration 50–500 µM) showed no significant ($p > 0.05$) increased induction of DNA DSBs in HGF compared to the controls. For the following experiments Asc was used in a concentration of 100 µM and ACC in a concentration of 500 µM.

6.2.2 γ-H2AX-assay with (co)monomers alone and in combination with antioxidants

H_2O_2 in a concentration of 1000 µM induced 23 foci/cell, HGF incubated in medium had 1 foci/cell.

Bis-GMA:

The addition of 100 µ M Asc to 90 µM Bis-GMA significantly reduced the number of foci/cell from 4 to 1 in HGF. The addition of 500 µM ACC to 90 µM Bis-GMA significantly reduced the number of foci/cell from 4 to 1 in HGF. All the tested Bis-GMA concentrations showed no increase in the number of multi foci cells when compared to the negative controls.

UDMA:

The addition of 100 µM Asc to 100 µM UDMA significantly reduced the number of foci/cell from 2 to 1 in HGF. The addition of 500 µM ACC to 33.5 or 100 µM UDMA, respectively, significantly reduced the number of foci/cell. All the tested UDMA concentrations showed no increase in the number of multi foci cells when compared with the negative controls.

EGDMA:

The addition of 100 µM Asc to 272 or 906.7 or 2720 µM EGDMA significantly reduced the number of foci/cell in HGF. The addition of 500 µM ACC to 272 or 906.7 or 2720 µ M EGDMA significantly reduced the number of foci/cell in HGF. 2720 µM EGDMA induces 4% multi focus cells (negative controls: 0.35%). The addition of 100 µM Asc or 500 µM ACC caused the percentage of multi foci cells to decrease, but was not statistically different from the percentage of multi foci cells in negative controls.

GDMA:

The addition of 100 µM Asc to 250 or 2500 µM GDMA significantly reduced the number of foci/cell in HGF. The addition of 500 µM ACC to 2500 µM GDMA significantly reduces the number of foci/cell in HGF. 2500 µM GDMA induces 1.96% multi focus cells (negative controls: 0.35%). By addition of 100 µM Asc or 500 µM ACC the percentage of multi foci cells decreased, but was not statistically different from the percentage of multi foci cells in negative controls.

Also, see the graphic illustration in detail in the second study of the current dissertation (Lottner et al. 2013)

7 Summary / Synopsis

7.1 Synopsis

Phenol has been traditionally used in dental treatment, it is regarded as a mutagenic and carcinogenic agent [4, 5], its use in dental practice is now therefore restricted. Monochlorophenols are derivatives of phenol, which are still used clinically as root canal disinfectants. ChKM solution contains the monochlorophenol isomer 4-CP and camphor as active ingredients for root canal disinfection.

In the first study of the dissertation, the induction of DNA DSBs by ChKM and monochlorophenol compounds (2-CP, 3-CP, 4-CP) was tested in human gingival fibroblasts (HGFs). DNA DSBs (foci) induced in HGFs were investigated using the γ-H2AX DNA focus assay.

(Co)monomers in dental composites such as MMA, Bis-GMA, UDMA, GDMA and EGDMA can be released due to incomplete polymerization and diffuse into the oral cavity or into the pulp [12, 14]. (Co)monomers from dental resin composites have a cytotoxic and genotoxic potential [29]. In previous studies, it has been demonstrated that antioxidants can decrease the cytotoxicity of dental (co)monomers [31, 34]. In the second study of the dissertation, the hypothesis was tested if the antioxidants Asc or ACC can reduce the number of DNA DSBs caused by the dental methacrylate-based monomers Bis-GMA, UDMA, GDMA and EGDMA in HGF. The number of foci of DNA DSBs were investigated using γ-H2AX DNA focus assay.

7.1.1 Induction of DNA double-strand breaks by monochlorophenol isomers and ChKM in human gingiva fibroblasts

Induction of DNA DSBs in HGFs by monochlorophenols or ChKM were investigated using the γ-H2AX DNA focus assay. In the γ-H2AX DNA focus assay, foci represent DNA DSBs [39]. ChKM solution, containing 4-CP and camphor showed a higher toxicity compared to 4-CP solution solely. This may be explained by the additive toxic effect of camphor. These data are in agreement with the findings of another study which showed that camphor can increase the cytotoxicity of phenolic compounds, even in other cell lines [40]. It has been described that the reduced cell proliferation may be related to altered cell cycle progression and cell viability, as chlorinated phenols can induce oxidative stress [40, 41]. In the first study of the dissertation, we found that 2-CP, 3-CP, 4-CP and ChKM can induce DNA DSBs in HGFs through the activation of the kinase ATM by its phosphorylation, which explains the generation of DNA DSBs foci as a consequence of massive DNA DSBs formation, because one of the early responses to DNA DSBs is the ATM-dependent phosphorylation of the histone H2AX at the C-terminal Ser 139 [40, 42]. It was found that γ-H2AX foci were readily discernible in HGFs nuclei by immunofluorescence using phosphohistone γ-H2AX-specific antibodies. Enumeration of γ-H2AX foci revealed that 2-CP, 3-CP, 4-CP and ChKM can induce significantly higher DNA-DSBs-specific γ-H2AX foci, compared to the negative control, but significantly lower rates compared to the positive control. A significant ranking in DNA toxicity (DSBs) of the tested compounds results in following order 2-CP <3-CP <4-CP < ChKM. It is interesting that the identical toxicity ranking of compounds was found in both XTT-test and γ-H2AX-test, although, the XTT-test measures the activity of intramitochondrial dehydrogenases while the γ-H2AX-test measures the induction of DNA-DSBs. This is also valid for the additive toxic effect of the combination of camphor

and 4-CP in the ChKM solution for both test systems. Similar results were obtained regarding the formation of multi-foci cells (cells in which more than 40 foci were found), when HGFs were exposed to the same concentrations (1/10 x,1/3 x,1 x EC_{50}) of compounds, with the only exception for 3-CP. Significantly lower rate of multi-foci cells was found with 3-CP, compared to 2-CP, 4-CP or the ChKM solution for all tested concentration. It can be hypothesized that DNA damage caused by 3-CP is more efficiently repairable by either specific DNA repair mechanisms, compared to DNA damage caused by 2-CP, 4-CP and ChKM or the nature and/or quality (and not quantity) of DNA damage, which may lead to different DNA toxicities (and different types of repair) among the investigated monochlorophenols. As it was described for different DNA damages (and repair) caused by cis-platins [43, 44]. Monochlorophenols and ChKM solution are used as a local intracanal disinfectants in endodontic therapy, applied by cotton pellets into pulp chamber and can diffuse into root canals [2], it could last up to 4 weeks, which indicates that the periapical tissues may be exposed for relatively long periods. In the first study of the dissertation, *in vitro,* DNA damage in HGFs was found at 6-h exposure time. The concentrations of 4-CP and camphor in the ChKM solution in dental practice can even reach higher values, compared to the concentrations we used in the γ-H2AX test. Moreover, DNA damage was already found at much lower concentrations. However, *in vitro* systems represent ‘‘closed’’ steady state systems, while in the human physiological situation, an ‘‘open’’ system is available with blood, enzymes and possibility for distribution, metabolism and elimination, which may lead to reduced compound toxicity. It is unknown whether in the human physiological situation the DNA damage, caused by these substances, can lead to an increase in the degeneration of human oral cells. Additional studies addressing the nature of DNA lesions elicited by dental monochlorophenol compounds and their repair are required to better estimate their genotoxic potential.

7.1.2 Effects of antioxidants on DNA double-strand breaks in human gingiva fibroblasts exposed to methaclylate monomers

The conversion of (co)monomers in dental composites can be induced by light and/or by auto-polymerization. However, incomplete Polymerization of (Co)monomers lead to release of residual (co)monomers into the oral cavity or into the pulp [12, 14]. Oxidative induction of DNA DSBs in HGFs after incubation with Bis-GMA was shown by Blasiak et al. [45]. Antioxidants are radical scavenging, forming adducts with the radicals or acting as a reducing agent due to its low redox potential [46]. Some studies have shown that the addition of antioxidants such as vitamins C (Asc) or E, ACC or uric acid to the cell culture medium can reduce cytotoxicity [31, 33, 34]. In the second study of the dissertation, it was demonstrated that the addition of the antioxidants Asc (100 µM) or ACC (500 µM) could reduce the number of induced DNA DSBs in methacrylate-based (co)monomers (Bis-GMA, UDMA, GDMA and EGDMA) in HGF *in vitro*. The antioxidant nature of Asc not only affects the metabolism of xenobiotics, but also physiological relevant redox reaction which play an important role in DNA replication and protein biosynthesis. The antioxidant nature of Asc in a concentration of 100 µM can also reduce the number of DNA DSBs during incubation with different methacrylate based dental monomers. This shows good correlation with other studies that have measured the cell protective effects after addition of Asc to cells incubated with TEGDMA or HEMA [31]. It is possible that Asc reacts with the ROS or epoxides during the metabolism of the methacrylate derivates and thereby prevents cellular and DNA damage. In contrast to Asc, ACC showed no dose dependent induction of DNA DSBs when compared with the negative control, up to the maximum tested concentration of 500 µM. ACC is closely linked to GSH synthesis and the regeneration cycle. GSH is a thiol-containing

antioxidant, which prevents damage to important cellular components caused by ROS such as free radicals and peroxides. The regeneration of GSH through ACC may be one reason for its cell protective effect. The reduction of intracellular GSH level after incubation with methacrylate based monomers is well known [47]. The elevation of intracellular GSH levels and that antioxidative properties of ACC could explain the reduction of DNA DSBs formation after incubation of HGF with (co)monomers Bis-GMA, UDMA, EGDMA and GDMA. It was shown that antioxidants not only reduce the cytotoxicity but also the genotoxicity. One explanation is the reduction of ROS and epoxides during metabolism of (co)monomers. Therefore, the question arises, is it useful to add antioxidants to the matrix of composites to reduce cell damage and inflammatory response? The protective effect may be superimposed by the fact that antioxidants may be interfere in the polymerization process by scavenging the free radicals necessary for building up long polymer chains and a three-dimensional polymer network. Apart from the reduction of the mechanical and physical properties, the monomer polymer conversion can be reduced, leading to an increase of unreacted (co)monomers and other additives. The higher the amount of unreacted and elutable substance, the lower the biocompatibility of the material. the second study of the dissertation supports the hypothesis that the addition of the antioxidants Asc and ACC can reduce the number of DNA DSBs *in vitro*.

7.2 Zusammenfasssung / Syopsis

7.2.1 Synopsis

Phenol wurde traditionell in der zahnärztlichen Behandlung eingesetzt. Inzwischen ist sein mutagenes und karzinogenes Potenzial bekannt [4, 5] und daher seine Verwendung in der zahnärztlichen Praxis untersagt. Monochlorphenole sind Phenol-Derivate, die immer noch klinisch als Wurzelkanal-Desinfektionsmittel verwendet werden. ChKM-Lösung enthält das Monochlorphenol-Isomer 4-CP und Campher als Wirkstoffe zur Wurzelkanal-Desinfektion.

In der ersten Studie der Dissertation wurde die Induktion von DNA DSBs durch ChKM und Monochlorphenolverbindungen (2-CP, 3-CP, 4-CP) in HGFs getestet. DNA DSBs (Foci), die in HGFs induziert wurden, wurden unter Verwendung des γ-H2AX-DNA-Fokus-Assays untersucht.

(Co)monomere in Dentalkompositen wie MMA, Bis-GMA, UDMA, GDMA und EGDMA können aufgrund unvollständiger Polymerisation freigesetzt und in die Mundhöhle oder über die Pulpa aufgenommen werden [12, 14]. Methacrylate haben ein zytotoxisches und genotoxisches Potential [29]. Frühere Studien zeigten, dass Antioxidantien die Zytotoxizität von solchen dentalen (Co)monomeren verringern können [31, 34].

In der zweiten Studie der Dissertation wurde die Hypothese überprüft, ob die Antioxidantien Asc oder ACC die Anzahl der DNA DSBs reduzieren können, die durch die Dentalmethacrylat-basierten Monomere Bis-GMA, UDMA, GDMA und EGDMA in HGF verursacht werden. Die Anzahl der Foci von DNA DSBs wurde unter Verwendung des γ-H2AX-DNA-Fokus-Assay untersucht.

7.2.2 Induktion von DNA-Doppelstrangbrüchen durch Monochlorphenol-Isomere und ChKM in menschlichen Gingiva-Fibroblasten

Die Induktion von DNA DSBs in HGFs durch Monochlorphenole oder ChKM wurde unter Verwendung des γ-H2AX-DNA-Fokus-Assays untersucht. Im γ-H2AX-DNA-Fokus-Assay stellen Foci DNA DSBs dar [39]. ChKM-Lösung, die 4-CP und Campher enthält, zeigte eine höhere Toxizität im Vergleich zu 4-CP allein. Dies kann durch den additiv toxischen Effekt von Kampfer erklärt werden. Diese Daten stimmen mit den Ergebnissen einer anderen Studie überein, die zeigte, dass Kampfer die Zytotoxizität von phenolischen Verbindungen auch in anderen Zelllinien erhöhen kann [40]. Des weiteren wurde beschrieben, dass chlorierte Phenole oxidativen Stress induzieren können und so eine reduzierte Zellproliferation mit einer veränderten Zellzyklusprogression und Zelllebensfähigkeit in Zusammenhang stehen kann [40, 41]. In der ersten Studie der Dissertation wurde festgestellt, dass 2-CP, 3-CP, 4-CP und ChKM DNA DSBs in HGFs durch die Aktivierung des Kinase-ATM durch Phosphorylierung induzieren können, die die Entstehung von DNA-DSB-Foci als Folge der massiven DNA-DSB-Bildung erklärt, da eine der ersten Reaktionen auf DNA DSBs die ATM-abhängige Phosphorylierung des Histons H2AX am C-terminalen Ser 139 ist [40, 42]. Es wurde festgestellt, dass γ-H2AX-Foci in HGFs-Kernen unter Verwendung von Phosphohiston-γ-H2AX-spezifischen Antikörpern in der Immunfluoreszenz leicht erkennbar waren. Die Auszählung von γ-H2AX-Foci zeigte, dass 2-CP, 3-CP, 4-CP und ChKM signifikant höhere DNA-DSBs-spezifische γ-H2AX-Foci im Vergleich zur negativ Kontrolle induzieren können, aber deutlich niedrigere Raten im Vergleich zur positiv Kontrolle. Eine signifikante Rangfolge in der DNA-Toxizität (DSBs) der getesteten Verbindungen ergibt die folgende Reihenfolge 2-CP <3-CP <4-CP <ChKM. Es ist interessant, dass eine identische Toxizität der Verbindungen sowohl im XTT-Test als auch im γ-H2AX-Test gefunden wurde, obwohl der XTT-Test die Aktivität intramitochondrialer

Dehydrogenasen misst, im Gegensatz zum γ-H2AX-Test, der die Induktion von DNA DSBs misst. Dies gilt auch für den additiv toxischen Effekt der Kombination von Kampfer und 4-CP in der ChKM-Lösung für beide Testsysteme. Ähnliche Ergebnisse wurden hinsichtlich der Bildung von Multi-Foci-Zellen (Zellen, in denen mehr als 40 Foci gefunden wurden) erhalten, wenn HGFs den gleichen Konzentrationen (1/10 x, 1/3 x, 1 x EC50) von Verbindungen ausgesetzt wurden, mit Ausnahme von 3-CP. Eine signifikant niedrigere Rate von Multi-Foci-Zellen wurde mit 3-CP gefunden, verglichen mit 2-CP, 4-CP oder der ChKM-Lösung für alle getesteten Konzentrationen. Es wird vermutet, dass die durch 3-CP verursachte DNA-Schädigung durch spezifische DNA-Reparaturmechanismen effizienter reparierbar ist, verglichen mit DNA-Schäden, die durch 2-CP, 4-CP und ChKM verursacht wurden, oder die Art und/oder Qualität (und nicht die Quantität) von DNA-Schäden, die zu verschiedenen DNA-Toxizitäten (und verschiedenen Arten von Reparaturen) unter den untersuchten Monochlorphenolen führen können. Wie es für verschiedene DNA-Schäden (und Reparatur) beschrieben wurde, die durch cis-platine verursacht wurden [43, 44]. Monochlorphenole und ChKM-Lösung werden als lokale intracanal-Desinfektionsmittel in der endodontischen Therapie eingesetzt. Unter Anwendung von Baumwollpellets in der Pulpenkammer können diese in die Wurzelkanäle diffundieren [2]. Eine Anwendung kann bis zu 4 Wochen dauern, was eine Exposition des periapikalen Gewebe für einen längeren Zeitraum bedeuten kann. In der ersten Studie der Dissertation wurden in vitro DNA-Schäden in HGFs bereits nach 6-h-Expositionszeit gefunden. Die Konzentrationen von 4-CP und Kampfer in der ChKM-Lösung in der Praxis können sogar höhere Werte erreichen, verglichen mit den Konzentrationen, die wir im γ-H2AX-Test verwendeten. Darüber hinaus wurde eine DNA-Schädigung bereits bei viel niedrigeren Konzentrationen gefunden. In vitro-Systeme repräsentieren jedoch "geschlossene" steady state Systeme, während in der menschlichen physiologischen Situation ein

"offenes" System mit Blut, Enzymen und Verteilungsmöglichkeit, Metabolismus und Eliminierung zur Verfügung steht, was zu einer verminderten Toxizität einer Verbindung führen kann. Bis jetzt ist nicht bekannt, ob in der menschlichen physiologischen Situation die durch diese Substanzen verursachte DNA-Schädigung zu einer Zunahme der Degeneration menschlicher oraler Zellen führen kann. Zusätzliche Studien, die die Natur von DNA-Läsionen betreffen, die durch dentale Monochlorphenolverbindungen hervorgerufen werden, und ihre Reparatur sind erforderlich, um ihr genotoxisches Potential besser abzuschätzen.

7.2.3 Effekte von Antioxidantien auf DNA-Doppelstrangbrüche in menschlichen Gingiva-Fibroblasten, die Methaclylat-Monomeren ausgesetzt sind

Die Umwandlung von (Co)monomeren in Dentalkompositen kann durch Licht und/oder durch Autopolymerisation induziert werden. Unvollständige Polymerisation von (Co)monomeren führt zur Freisetzung von Rest-(Co)monomeren in die Mundhöhle oder in die Pulpa [12, 14]. Die oxidative Induktion von DNA DSBs in HGFs nach Inkubation mit Bis-GMA wurde von Blasiak et al. gezeigt [45]. Antioxidantien sind Radikalfänger, die Addukte mit Radikalen bilden können oder als Reduktionsmittel aufgrund geringem Redoxpotentials agieren können [46]. Einige Studien haben gezeigt, dass die Zugabe von Antioxidantien wie Vitaminen C (Asc) oder E, ACC oder Harnsäure zum Zellkulturmedium die Cytotoxizität verringern kann [31, 33, 34]. In der zweiten Studie der Dissertation, Es wurde gezeigt, dass die Zugabe der Antioxidantien Asc (100 µM) oder ACC (500 µM) die Anzahl der induzierten DNA DSBs in Methacrylat-basierten (Co) -Monomeren (Bis-GMA, UDMA, GDMA und EGDMA) in HGF *In vitro* reduzieren könnte. Der antioxidative Charakter von Asc wirkt sich nicht nur auf den Metabolismus von Xenobiotika, sondern auch auf physiologisch relevante Redoxreaktionen aus, die bei der DNA-Replikation und der Proteinbiosynthese eine wichtige Rolle spielen. Der

antioxidative Charakter von Asc in einer Konzentration von 100 µM kann auch die Anzahl der DNA DSBs während der Inkubation mit verschiedenen Dentalmonomeren auf Methacrylatbasis reduzieren. Dies zeigt eine gute Korrelation mit anderen Studien, die den Zell protektiven Effekt nach Zugabe von Asc zu Zellen, die mit TEGDMA oder HEMA inkubiert wurden, gemessen haben [31]. Es ist möglich, dass Asc mit dem ROS oder den Epoxiden während des Metabolismus der Methacrylatderivate reagiert und dadurch Zell- und DNA-Schäden verhindert. Im Gegensatz zu Asc zeigte ACC keine dosisabhängige Induktion von DNA DSBs im Vergleich zur Negativkontrolle bis zur maximal getesteten Konzentration von 500 µM. ACC ist eng mit der GSH-Synthese und dem Regenerationszyklus verknüpft. GSH ist ein Thiol-haltiges Antioxidans, das Schäden an wichtigen zellulären Komponenten, die durch ROS verursacht werden, wie freie Radikale und Peroxide, verhindert. Die Regeneration von GSH durch ACC kann ein Grund für seine zellschützende Wirkung sein. Die Reduktion des intrazellulären GSH-Spiegels nach Inkubation mit Monomeren auf Methacrylatbasis ist bekannt [47]. Die Erhöhung des intrazellulären GSH-Spiegels und die antioxidativen Eigenschaften von ACC könnten die Reduktion der DNA-DSBs-Bildung nach Inkubation von HGFs mit den (Co)monomeren Bis-GMA, UDMA, EGDMA und GDMA erklären. Es wurde gezeigt, dass Antioxidantien nicht nur die Zytotoxizität, sondern auch die Genotoxizität reduzieren. Eine Erklärung ist die Reduktion von ROS und Epoxiden während des Stoffwechsels von (Co)monomeren. Daher ist die Frage, ist es sinnvoll, Antioxidantien in die Matrix von Composites einzubringen, um Zellschäden und entzündliche Reaktion zu reduzieren? Die Schutzwirkung kann dadurch überlagert werden, dass Antioxidantien in den Polymerisationsvorgang eingreifen können, indem die freien Radikale, die für den Aufbau langer Polymerketten und dem dreidimensionales Polymernetzwerk erforderlich sind, abgefangen werden. Abgesehen von der Verringerung der mechanischen und physikalischen Eigenschaften könnte die

Monomerpolymerumwandlung reduziert werden, was zu einer Erhöhung der nicht umgesetzten (Co)Monomere und anderer Additive führen würde. Je höher die Menge an nicht umgesetzter und eluierbarer Substanz ist, desto geringer ist die Biokompatibilität des Materials. Die zweite Studie der Dissertation unterstützt die Hypothese, dass die Zugabe der Antioxidantien Asc und ACC die Anzahl der DNA DSBs *in vitro* reduzieren kann.

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 the first publication (see page 9), 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.

In the second publication (see page 9), I am listed as second author. I have carried out main analytical part, sample preparation, data analysis and parts of paper work.

9 Publication I

Shehata M, Durner J, Thiessen D, Shirin M, Lottner S, Van Landuyt K, Furche S, Hickel R, Reichl FX. 2012. Induction of DNA double-strand breaks by monochlorophenol isomers and ChKM in human gingival fibroblasts. Arch Toxicol. 86(9):1423-1429

Arch Toxicol (2012) 86:1423–1429
DOI 10.1007/s00204-012-0861-z

REPRODUCTIVE TOXICOLOGY

Induction of DNA double-strand breaks by monochlorophenol isomers and ChKM in human gingival fibroblasts

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Received: 14 September 2011 / Accepted: 16 November 2011 / Published online: 22 May 2012

Abstract Phenol has been traditionally used in dental treatment as a sedative for the pulp or as disinfectant for carious cavity and root canal. However, phenol is regarded as a mutagenic and carcinogenic agent and its use in dental practice is now therefore restricted. Monochlorophenols are derivatives of phenol, which are still used clinically as root canal disinfectants, they are even more active antiseptics/disinfectants than phenol, and the so-called Walkhoff (ChKM) solution makes use of monochlorophenol for root canal disinfection. Ingredients in the ChKM solution are the monochlorophenol compound 4-chlorophenol (4-CP), camphor, and menthol. In literature, the use of the ChKM solution is controversial because of a possible DNA toxicity of the ingredient 4-CP. However, it is unknown whether ChKM can really induce DNA damage in human oral cells. In this study, the induction of DNA double-strand breaks (DSBs) by ChKM and monochlorophenol compounds (2-chlorophenol, 2-CP; 3-chlorophenol, 3-CP; and 4-chlorophenol, 4-CP) was tested in human gingival fibroblasts (HGFs). DNA DSBs (foci) induced in HGFs unexposed and exposed to monochlorophenols or ChKM solution were investigated using the γ-H2AX DNA focus assay, which is a direct marker for DSBs. DSBs result in the ATM-dependent phosphorylation of the histone H2AX. When cells were exposed to medium or medium + DMSO (1 %) (negative controls), an average of 3 foci per cell were found. In positive control cells (H_2O_2 + medium, or H_2O_2 + medium + DMSO (1 %), an average of 35 foci each were found. About 20 DSB foci per cell were found, when HGFs were exposed to 2-CP (4 mM), 3-CP (2.3 mM), 4-CP (2.1 mM), or ChKM (corresponding to 1.5 mM 4-CP). Our results show increasing DNA toxicities in the order of 2-CP < 3-CP < 4-CP < ChKM solution. An additive DNA toxicity was found for 4-CP in combination with camphor in the ChKM solution, compared to the 4-CP alone. No significant differences regarding multi-foci cells (cells that contain more than 40 foci) were found when HGFs were exposed to the EC_{50} concentrations (given in parenthesis) of ChKM (1.5 mM), 4-CP (2.1 mM), or 2-CP (4 mM). Significantly fewer multi-foci cells were found when HGFs were exposed to the EC_{50} concentration (given in parenthesis) of 3-CP (2.3 mM), compared to the EC_{50} concentrations of ChKM, 4-CP, or 2-CP. Monochlorophenol compounds and/or ChKM solution can induce DSBs in primary human oral (cavity) cells, which underscores their genotoxic capacity.

Keywords Monochlorophenols · Walkhoff solution · Human gingival fibroblasts · DNA DSBs · γ-H2AX

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Introduction

Endodontic therapy plays an important role in the preservation and function of the teeth (Dimitriu et al. 2009). The aim of successful root canal treatment is to remove the

inflammatory and necrotic pulp tissue as well as elimination of pathogenic microorganisms. Endodontic environment provides a selective habitat for the establishment of a mixed, predominantly anaerobic bacteria flora (Nair 2004). Therefore, disinfection of the root canals is considered to be an important step during endodontic treatment (Waltimo et al. 2005; Llamas et al. 1997). Phenol has traditionally been used in the dental treatment as sedative for the pulp or as disinfectant for carious cavities and root canals. However, phenol is regarded as a mutagenic and carcinogenic agent (Borzelleca et al. 1985; Kasugai et al. 1991); nowadays, use of phenol in dental practice is restricted. Monochlorophenols are derivatives of phenol, which are still used in dental practice. They are even more active antiseptics/disinfectants than phenol, which makes them good disinfectants for root canals (Violich and Chandler 2010). A monochlorophenol containing disinfectant is the so-called Walkhoff (ChKM) solution. Ingredients in the ChKM solution are the monochlorophenol compound 4-chlorophenol (4-CP), camphor, and menthol. Chlorophenols represent a wide group of substances with different toxicities (Solyanikova and Golovleva 2004). In chlorophenols, the molecule phenol is chlorinated one to fivefolds up. Monochlorophenols have a higher antibacterial, antiseptic, and disinfectant potential compared to other disinfectants or phenol (Violich and Chandler 2010; Da Silva et al. 2007) Monochlorophenols exist in three isomers: 2-chlorophenol (2-CP; orthochlorophenol), 3-chlorophenol (3-CP, metachlorophenol), and 4-CP (parachlorophenol), with 4-CP as the most effective compound (Llamas et al. 1997; Farrell and Quilty 1999; Cooper and Jones 2008; Gulcan et al. 2008; Borzelleca et al. 1985). The use of monochlorophenols is rather controversial because of the high toxicity and mutagenicity of other chlorophenols, as for example pentachlorophenol (PCP). PCP has been well documented as a mutagenic and carcinogenic agent (Borzelleca et al. 1985; Harrison and Madonia 1971; Cooper and Jones 2008). PCP was used as a potent fungicide until 1970 when its use was prohibited in the EU. Even though PCP was never used in clinical dentistry, some authors compare the toxicity of monochlorophenols with other higher substituted chlorophenols (Cooper and Jones 2008). In literature, the use of ChKM solution is controversial because of "possible" (DNA)-toxicity of the ingredient monochlorophenol compound 4-CP (Da Silva et al. 2007). However, it is unknown whether ChKM can really induce DNA damage(s) in human oral cells. In this study, the induction of DNA double-strand breaks (DSBs) by ChKM and monochlorophenol compounds (2-CP, 3-CP, 4-CP) was tested in human gingival fibroblasts (HGFs). DSBs (foci) induction in HGFs unexposed and exposed to monochlorophenols or ChKM were investigated using the γ-H2AX DNA focus assay, which is a direct marker for DSBs. In the γ-H2AX assay, foci represent DNA double-strand breaks, which can induce ATM-dependent phosphorylation of the histone H2AX (Sedelnikova et al. 2002).

Materials and methods

Chemicals

2-CP, 3-CP, 4-CP, and DMSO were obtained from Sigma-Aldrich (St. Louis, MO, USA), ChKM solution from Adolf Haupt&Co (Würzburg, Germany, Ch.B. 73044), and H_2O_2 from VWR International (Darmstadt, Germany). All chemicals and reagents were of the highest purity available.

Cell culture and drug treatment

The human gingival fibroblasts (HGFs, Cat–No.:1210412) were obtained from Provitro, Cell-Lining (Berlin, Germany). The HGFs (passage 9) were grown on 175-cm^2 cell culture flasks to approximately 75–85 % confluence and maintained in an incubator with 5 % CO_2 atmosphere at 100 % humidity and 37 °C. Quantum 333 medium supplemented with L-glutamine and 1 % antibiotic/antimycotic solution (10,000 U/ml penicillin, 25 mg/ml streptomycin sulfate, 25 mg/ml amphotericin B; PAA Laboratories, Cölbe, Germany) was used to culture HGFs. After reaching confluence, the cells were washed with Dulbecco's phosphate-buffered saline (PAA Laboratories) detached from the flasks by a brief treatment with trypsin/EDTA (PAA Laboratories).

XTT-based viability assay

Tetrazolium salt (XTT)–based cell viability assay was used, according to the method described in earlier studies (Urcan et al. 2010), to determine the half-maximum effect concentrations (EC_{50}) for monochlorophenol compounds and ChKM in HGFs. HGFs at 20,000 cells/well were seeded into a 96-well microtiter plate in 1 ml of medium, and then the cells were incubated for 24 h. After removal of medium, the cells were treated with medium containing 2-CP (0.1–30 mM), 3-CP (0.1–30 mM), and 4-CP (0.1–30 mM), or ChKM (0.1–30 mM, corresponding to 4-CP), Control cells received either medium alone, medium + DMSO (final DMSO concentration: 1 %), or 1 % Triton X-100. After incubation for 20 h, the cell monolayers were washed and a mixture of tetrazolium salt XTT (sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent, in RPMI 1640, without phenol red and

electron-coupling reagent (PMS [*N*-methyldibenzopyrazine methyl sulfate] in phosphate-buffered saline) was added as recommended by the supplier (cell proliferation kit II; Roche Diagnostics Penzberg, Germany) 4 h before photometric analysis. The formazan formation was quantified spectrophotometrically at 450 nm (reference wavelength 670 nm) using a microtiter plate reader (Victor 3, Perkin Elmer Las, Jügesheim, Germany). All experiments were repeated five times.

γ-H2AX immunofluorescence

DNA DSBs formation was tested in HGFs unexposed and exposed to monochlorophenol compounds and ChKM by the γ-H2AX focus assay method, which is a direct marker for DSBs. For this microscopic assay, 12-mm round cover slips (Carl Roth, Karlsruhe, Germany) were cleaned in 1 N HCl and distributed into a 24-well plate. In each well medium, HGFs were seeded at 7×10^4 cells/ml and followed by overnight incubation at 37 °C. HGFs were exposed to medium containing substances in the following concentrations (corresponding to EC_{50}, 1/3 EC_{50} and 1/10 EC_{50} values, received from the XTT assay): 2-CP (3,960, 1,320, and 396 μM), 3-CP (2,300, 767, and 230 μM), 4-CP (2,100, 700, and 210 μM), and ChKM solution (1,540, 513, and 154 μM; corresponding to 4-CP in the ChKM solution) for 6 h. Negative control cells received either medium alone, or medium + DMSO (final DMSO concentration: 1 %). Positive control cells received 1 mM H_2O_2 + medium, or 1 mM H2O2 + medium + DMSO (final DMSO concentration: 1 %) for 10 min. For immunofluorescent staining, cells were first washed 2 × 5 min with PBS, fixed by adding 0.5 ml of 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 15 min with 0.5 ml of triton-citrate buffer (0.1 % sodium citrate, 0.1 % Triton X-100) at 4 °C. After washing 4 × 2 min with PBS, cells were blocked for 20 min with 0.2 ml of serum-free blocking buffer (Dako, Hamburg, Germany) per well at 25 °C. Thereafter, cells were incubated with mouse monoclonal anti-γ-H2AX (Millipore, Billerica, MA, USA) at 1:1,300 dilution in antibody diluent (0.3 ml per well) (Dako) at 4 °C overnight. After 4 × 5 min washes with PBS at 4 °C, cells were incubated with FluoroLink Cy3-labeled goat anti-mouse secondary antibody (GE Healthcare, Munich, Germany) at a dilution of 1:1,300 in antibody diluent (0.3 ml per well) for 1 h at 25 °C in the dark. HGFs were then washed 2 × 5 min in PBS and rinsed 5 min with deionized water at 25 °C. Finally, the cover slips were each placed on 0.2 ml of a mixture of 2 ml Prolong antifade and DAPI (Invitrogen) (76 × 26 mm; Carl Roth, Invitrogen, Karlsruhe, Germany) on a glass slide.

Image acquisition

HGFs were investigated using a Zeiss Axioplan 2 imaging fluorescence microscope (Zeiss, Göttingen, Germany) equipped with a motorized filter wheel and appropriate filters for excitation of red, green, and blue fluorescence. Images were obtained using a 63× and a 100× Plan-Neofluar oil immersion objective (Zeiss) and the ISIS fluorescence imaging system (MetaSystems, Altlussheim, Germany).

Data analysis

XTT test

The values calculated from the XTT-based viability assay were calculated as percentage of the 100 % control values, using Graph Pad Prism 4 (Graph Pad Software Inc., San Diego, USA), where they were plotted on a concentration log-scale and range of the maximum slope were comprised. Half-maximum-effect substance concentration at the maximum slope was revealed as EC_{50}. The EC_{50} values were obtained as half-maximum-effect concentrations from the fitted curves. Data are presented as means ± standard error of the mean (SEM). Each experiment was repeated five times. The statistical significance ($p < 0.05$) of the differences between the experimental groups was checked using the Student's *t* test, corrected according to Bonferroni–Holm (Forst 1985).

γ-H2AX test

For quantitative analysis of the γ-H2AX test, DSBs (foci) were counted by the same investigator by eye down the fluorescence microscopic using a 100× objective. Disrupted cells were excluded from the analysis. Cell counting was performed until at least 80 cells were reached. Each experiment was repeated three times. The mean number of cells was scored and the standard error of the mean was calculated. Values were compared using the Student's *t* test ($p < 0.05$). If one cell contains 40 or more foci, it will be counted as multi-foci cell (Urcan et al. 2010).

Results

XTT test

EC_{50} values of compounds were found (mM; mean ± SEM; $n = 5$) and given in (Table 1). ChKM solution was the most toxic compound (-solution), compared to the other compounds. Significant ($p < 0.05$) increase in toxicity of

Table 1 EC_{50} values (mM; mean ± SEM, $n = 5$) and relative toxicities of compounds. XTT-based cell viability assay was used to determine the half-maximum effect concentrations (EC_{50}) for substances in HGF

	EC_{50} ± SEM (mM)	Relative toxicity
Camphor	23.80 ± 0.51	1.0
2-CP[a]	3.96 ± 0.11	6.0
Menthol[b]	3.20 ± 0.25	7.4
3-CP[c]	2.30 ± 0.10	10.3
4-CP[d]	2.10 ± 0.10	11.3
ChKM[e]	1.54 ± 0.11	15.5

[a] Significantly different to camphor

[b] Significantly different to 2-CP and camphor

[c] Significantly different to menthol, 2-CP, and camphor

[d] Significantly different to 3-CP, menthol, 2-CP, and camphor

[e] Significantly different to 4-CP, 3-CP, menthol, 2-CP, and camphor

compounds was found as follows: camphor < 2-CP < menthol < 3-CP < 4-CP < ChKM. Relative toxicities are given in Table 1.

γ-H2AX test

In "negative" control cells (cells with medium only, or with medium + DMSO), 3 DSB foci each were found (Fig. 1). In "positive" control cells (H_2O_2 + medium or H_2O_2 + medium + DMSO), 35 DSB foci each were found (Fig. 1). When HGF were exposed to the EC_{50} concentrations of monochlorophenols or ChKM, following DSB foci were found: 3-CP 18 foci, 4-CP 19 foci, 2-CP 20 foci, and ChKM 21 foci (Fig. 1). The highest rates of DSB foci were found when HGFs were exposed to the EC_{50} concentration of each substance, compared to their corresponding 1/3 EC_{50}-, or 1/10 EC_{50} concentration (Fig. 1). About 20 DSB foci per cell were found when HGFs were exposed to the substances (concentration in parenthesis): 2-CP (4 mM), 3-CP (2.3 mM), 4-CP (2.1 mM), or ChKM (corresponding to 1.5 mM 4-CP) (Fig. 1). About 22 % of the cells contained multi-foci when HGFs were exposed to substances with the EC_{50} concentration (in parenthesis): 2-CP (4 mM), 4-CP (2.1 mM), or ChKM (corresponding to 1.5 mM 4-CP). Only 13 % of the cells contained multi-foci when HGFs were exposed to 3-CP with EC_{50} concentration of 2.3 mM (Table 2).

Discussion

Disinfection of the root canal is considered to be an important step during endodontic treatment. Monochlorophenols are still used as intracanal disinfectants. In the cytotoxicity test experiment using the XTT test, we have found that 2-CP, 3-CP, 4-CP, and ChKM can significantly reduce cell viability in HGFs in which higher toxicity was found for ChKM solution (the so-called Walkhoff solution), compared to the conventional single 4-CP solution. ChKM includes 4-CP, camphor, and menthol. In the XTT, ChKM reveals 15-fold more toxic than camphor and about 1.5-fold more toxic than 4-CP (Table 1). The higher toxicity of ChKM solution, compared to the single 4-CP solution, may be explained by the additive toxic effect of camphor. This additive toxic effect was evaluated using the isobologram method described in previous studies, (Berenbaum 1985; Nirmalakhandan et al. 1994). These data

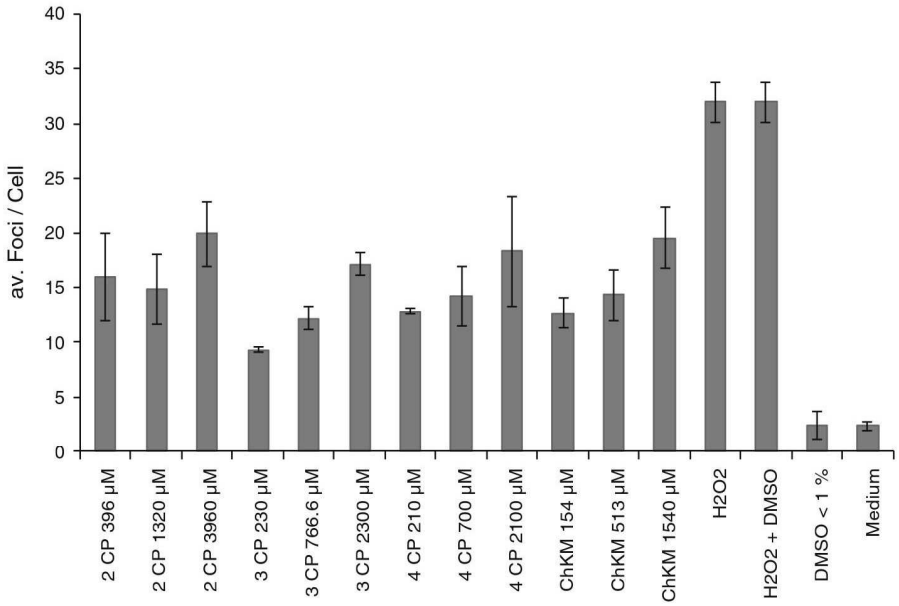

Fig. 1 Average γ-H2AX foci formation per cell, exposed to 2-CP, 3-CP, 4-CP, or ChKM. "Positive" control cells received H_2O_2 + medium or H_2O_2 + medium + DMSO (1 %). "Negative" control cells received medium only or medium + DMSO (1 %) (mM; mean ± SEM, $n = 3$)

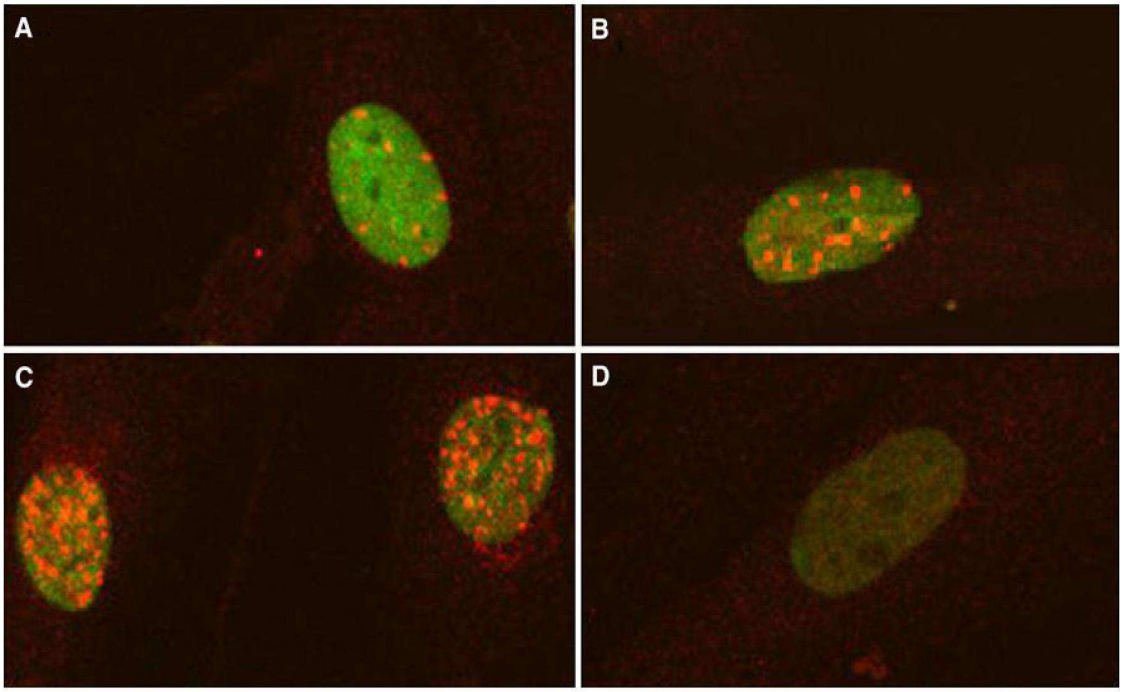

Fig. 2 Representative images of immunocytochemistry staining for H2AX phosphorylation in HGFs exposed to EC_{50} values (in *parenthesis*) of compounds: 4-CP (2.1 mM) with 8 foci (**a**); ChKM (1.5 mM) with 15 foci (**b**); "Positive" control cells (with medium + H_2O_2) with multi-foci (**c**); and "Negative" control cells (with medium alone) with 0–3 focus (i) (**d**). Syber Green (*green*) is a marker for DNA and stains the whole nucleus of the cell, γ-H2AX-specific foci reproduced in *orange* (color figure online)

are in agreement with the data published by other author who has found that camphor can increase the cytotoxicity of phenolic compounds even in other cell lines (Soekanto et al. 1996). It has been described that the reduced cell proliferation may be related to altered cell cycle progression and cell viability, as chlorinated phenols can induce oxidative stress (Soekanto et al. 1996; Zhao et al. 1995). In our study, we have found that 2-CP, 3-CP, 4-CP, and ChKM can induce DNA DSBs in HGFs. This indicates that 2-CP, 3-CP, 4-CP, and ChKM lead to the activation of the kinase ATM by its phosphorylation, which is explained by the generation of DNA DSBs as a consequence of massive DNA DSB formation, because one of the early responses to DSBs is the ATM-dependent phosphorylation of the histone H2AX at the C-terminal Ser 139 (Sedelnikova et al. 2002; Lobrich et al. 2005). It was found that γ-H2AX foci were readily discernible in HGFs nuclei by immunofluorescence using phosphohistone γ-H2AX-specific antibodies (Fig. 2). Microscopic enumeration of γ-H2AX foci revealed that 2-CP, 3-CP, 4-CP, and ChKM can induce significantly higher DSB-specific γ-H2AX foci, compared to the "negative" controls (e.g., with medium alone), but at significantly lower rates compared to the "positive" control H_2O_2 (Fig. 1). About 20 DSB foci were found per cell when HGFs were exposed to 2-CP (4 mM), 3-CP (2.3 mM), 4-CP (2.1 mM), or ChKM solution (corresponding to 1.5 mM 4-CP). Following is the significant ranking in DNA toxicity (DSBs) of the tested compounds indicating that 2-CP < 3-CP < 4-CP < ChKM. It is interesting that the identical toxicity ranking of compounds was

Table 2 Formation of multi-DSB foci (in %) in the DNA by corresponding concentrations of the substance

	2-CP			3-CP			4-CP			ChKM solution		
	1/10 EC_{50}	1/3 EC_{50}	EC_{50}	1/10 EC_{50}	1/3 EC_{50}	EC_{50}	1/10 EC_{50}	1/3 EC_{50}	EC_{50}	1/10 EC_{50}	1/3 EC_{50}	EC_{50}
Concentration (mM)	0.39	1.32	3.96	0.23	0.80	2.30	0.21	0.70	2.10	0.15	0.51	1.54
Multi-foci cells in %	6.80[a]	7.14[b]	21.70[c]	3.30	3.83	13.34	8.34[a]	7.26[b]	21.49[c]	4.97[a]	8.57[b]	20.59[c]
SEM	0.15	0.15	0.43	0.10	0.09	0.27	0.16	0.18	0.42	0.12	0.11	0.38

Data represent the mean values ± SEM, (mM; $n = 3$)

[a] Significantly ($p < 0.05$) different to the corresponding 1/10 EC_{50} value of 3-CP

[b] Significantly ($p < 0.05$) different to the corresponding 1/3 EC_{50} values of 3-CP

[c] Significantly ($p < 0.05$) different to the corresponding EC_{50} value of 3-CP

found in both XTT test and γ-H2AX test, although, the XTT test measures the activity of intramitochondrial dehydrogenases while the γ-H2AX test measures the induction of DNA DSBs. This is also valid for the additive toxic effect of the combination of camphor + 4-CP in the ChKM solution for both test systems. Similar results were obtained regarding the formation of multi-foci cells (cells in which more than 40 foci were found), when HGFs were exposed to the same concentrations of compounds as described above for the foci formations, with the only exception for 3-CP. It is unclear why a significantly lower rate of multi-foci cells was found with 3-CP, compared to 2-CP, 4-CP, or the ChKM solution for each concentration (see Table 2). It can be hypothesized that DNA damage caused by 3-CP is more efficiently repairable by either specific DNA repair mechanisms, compared to DNA damage caused by 2-CP, 4-CP, and ChKM or the nature and/or quality (and not quantity) of DNA damage, which may lead to different DNA toxicities (and different types of repair) among the investigated monochlorophenols, as it was described for different DNA damages (and repair) caused by cisplatins (Alt et al. 2007; Schorr et al. 2010). Monochlorophenols and ChKM solution are used as a local intracanal disinfectants in endodontic therapy, applied by cotton pellets into pulp chamber and can diffuse into root canals (Waltimo et al. 2005); it could last up to 4 weeks, which indicates that the periapical tissues may be exposed for relatively long periods. In our studies, DNA damage was found at much lower time, already at 6-h exposure. The concentrations of 4-CP and camphor in the ChKM solution (see 2.1 chemical section) commonly used in dental clinics can even reach higher values (4-CP 2140 mM and camphor 4740 mM), compared to the concentrations we used in the γH2AX test. Moreover, DNA damage was already found at much lower concentrations. However, in vitro systems represent "closed" steady state systems, while in the human physiological situation, an "open" system is available with blood, enzymes, and possibility for distribution, metabolism and elimination, which may lead to reduced compound toxicity. It is unknown whether in the human physiological situation the DNA damage, caused by these substances, can lead to an increase in the degeneration of human oral cells. Additional studies addressing the nature of DNA lesions elicited by dental monochlorophenol compounds and their repair are required to better estimate their genotoxic potential.

Conclusion

Monochlorophenol compounds and/or ChKM, as used as endodontic disinfectants, can induce DSBs in primary human oral (cavity) cells, which underscore their genotoxic capacity.

Acknowledgments We gratefully acknowledge the technical assistance of Stefan Schulz.

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10 Publication II

Lottner S, Shehata M, Hickel R, Reichl FX, Durner J. 2013. Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to methacrylate based monomers. Dent Mater. 29(9):991-998.

DENTAL MATERIALS 29 (2013) 991–998

Available online at www.sciencedirect.com

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Effects of antioxidants on DNA-double strand breaks in human gingival fibroblasts exposed to methacrylate based monomers

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ARTICLE INFO

Article history:
Received 23 October 2012
Received in revised form
7 March 2013
Accepted 3 July 2013

Keywords:
Dental composite
BisGMA
UDMA
EGDMA
DNA double-strand breaks, DSBs
Antioxidants
Ascorbic acid
N-acetylcysteine
γ-H2AX assay
Reactive oxygen species, ROS

ABSTRACT

Objective. (Co)monomers from dental resin composites have cytotoxic and genotoxic potential. In previous studies it has been demonstrated that antioxidants can decrease the cytotoxicity of various dental (co)monomers. In this study the effects of the antioxidants N-acetylcysteine (ACC) and ascorbic acid (Asc) on the number of DNA double-strand breaks (DSBs) in human gingiva fibroblasts (HGFs) were tested.

Methods. HGF was incubated with the (co)monomers bisphenol-A-glycidyl methacrylate (BisGMA), urethandimethacrylate (UDMA), ethylene glycol dimethacrylate (EGDMA) or 1,3-glyceroldimethacrylate (GDMA) with and without addition of antioxidants ACC and Asc. DNA-DSBs were determined using the γ-H2AX assay.

Results. Asc induced at 500 μM significant more DNA-DSBs in HGFs compared with controls (4.92 (1.28) *vs.* 1.62 (0.67); foci/cell mean (standard deviation), $n = 3$). Most DNA-DSBs were found after incubation of HGFs with 90 μM BisGMA (4.05 (0.56)) and 2720 μM EGDMA (5.36 (1.59)). The addition of 100 μM Asc or 500 μM ACC leaded to a statistical significant reduction of DNA-DSBs in HGFs for all tested (co)monomers. After incubation of HGFs with 2720 μM EGDMA and 500 μM ACC the foci/cell decrease from 5.36 (1.59) to 1.9 (1.17) (controls: 1.12 (0.24)). After incubation of HGFs with 90 μM BisGMA and 100 μM Asc the foci/cell decrease from 4.05 (0.56) to 1.96 (0.59) (controls: 1.12 (0.24)).

Significance. All tested (co)monomers can induce DNA-DSBs but addition of antioxidants (Asc or ACC) leads to reduction of DNA-DSBs.

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http://dx.doi.org/10.1016/j.dental.2013.07.005

1. Introduction

Methacrylate based dental (co)monomers are widely used in contemporary dental restorative materials. The conversion of (co)monomers can be induced by light and/or by autopolymerisation. However the polymerization is incomplete meaning that (co)monomers and additives can diffuse into the oral cavity or into the pulp. From here the released substances can enter the organism *via* the bloodstream [1]. Moreover the methacrylates methyl methacrylate (MMA), ethylene glycol dimethacrylate (EGDMA), and triethylene glycol dimethacrylate (TEGDMA) were identified in the air of dental technicians' workplaces [2].

(Co)monomers and additives released from resin-containing products can cause in persons, who are exposed to these substances, various adverse effects such as allergic contact dermatitis and bronchial asthma [3,4]. *In vitro* studies have also shown that some dental methacrylates can cause cytotoxic, estrogenic and mutagenic reactions [5–7]. Thereby reactive oxygen species (ROS) and epoxides play an important role in the metabolism of dental methacrylates [8–10]. During the metabolism of these xenobiotics (*e.g.* MMA, TEGDMA) the amount of ROS and epoxides increases while the amounts of (physiological) radical scavengers such as glutathione or vitamin C decrease.

Most epoxides as well as ROS are regarded as very toxic agents reacting with different cellular molecules and cellular structures such as desoxyribonucleic acid (DNA) [8,11].

In this context it is interesting to note that the number of different cancers of the oral mucosa is increasing in adults of 45 years and older with a simultaneous decrease in tobacco and alcohol consumption [12]. Different factors such as human papillomaviruses (HPV), xenobiotics from different sources and their metabolic products are discussed [13].

DNA double-strand breaks (DNA-DSBs) caused by mutagenic agents like epoxides and ROS are considered as the most toxic type of DNA lesion [14]. If they are left unrepaired they can cause cell death; if they are misrepaired they may lead to chromosomal translocations and genomic instability [15]. Using the γ-H2AX-assay, a previous study has shown that methacrylate based dental monomers can induce DNA-DSB in HGF [16]. H2AX, a protein from the H2A family and a component of the histone octamer in nucleosomes, can be phosphorylated by different kinases to γ-H2AX. This phosphorylation recruits and localizes DNA repair proteins at the foci [17]. The foci represent DNA-DSBs and can be used as a biomarker for DNA damage. A labeled antibody against γ-H2AX can be used to label the foci which can then be visualized using an immunofluorescence microscope (for visualization of foci see [16]).

Many studies have dealt with the toxicology of (co)monomers and other substances from dental resins. Less is known about how they prevent cell damage. In some studies it could be demonstrated that the addition of antioxidant substances such as the vitamins C (ascorbic acid, Asc) and E or *N*-acetylcysteine (ACC) can reduce the cytotoxic effects of dental monomers such as TEGDMA, 2-hydroxyethyl methacrylate (HEMA) or poly-methyl methacrylate (PMMA) [18–21]. It is not known whether antioxidants lead to a reduction of DNA-DSB in human oral cells. Human oral cells (*e.g.*, gingival and/or pulp fibroblasts) in this physiological situation are among the first to come into contact with eluted substances.

The aim of this study was therefore to test the hypothesis that the antioxidants Asc or ACC can reduce the number of DNA-DSBs caused by the dental methacrylates BisGMA, UDMA, GDMA and EGDMA in HGF. The number of foci of DNA-DSBs were investigated using γ-H2AX focus assay, which is a direct marker for DNA-DSBs.

2. Methods

2.1. Chemicals

UDMA, GDMA and BisGMA were obtained from Evonik Röhm (Essen, Germany). EGDMA, Asc and ACC were obtained from Sigma–Aldrich (St. Louis, MO, USA). All solvents and reagent products were obtained from Merck, Darmstadt, Germany. Asc, ACC, UDMA, GDMA and EGDMA were directly dissolved in medium, whilst BisGMA was first dissolved in DMSO (Sigma–Aldrich, St. Louis, USA) and then diluted with medium (final DMSO concentration: <1%; from prior experiments it was known that this DMSO concentration did not cause more foci to appear in the cells as compared with the controls). 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 grown in 175 cm^2 cell culture flasks to approximately 75–85% confluence and maintained in an incubator with 5% CO_2 atmosphere at 37 °C with 100% humidity. Quantum 333 with L-glutamine and 1% antibiotic/antimycotic solution (10,000 U/ml penicillin, 25 mg/ml streptomycin sulphate, 25 mg/ml amphotericin B; PAA Laboratories, Cölbe, Germany) were used to culture HGFs. The cell cultures were washed with Dulbecco's phosphate buffered saline (PBS; PAA Laboratories) without calcium and magnesium. After reaching confluence the cells were washed with Dulbecco's PBS, detached from the flasks by a brief treatment with trypsin/EDTA (PAA Laboratories).

2.3. γ-H2AX immunofluorescence

DNA-DSB formation was determined in HGFs unexposed and exposed to dental resin compounds by the 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 7×10^4 cells/ml in each well with medium, followed by overnight incubation at 37 °C. The cells were exposed to medium containing the resins or the antioxidants in the following concentrations: BisGMA (9; 30; 90 μM), EGDMA (272; 906.7; 2720 μM), GDMA (250; 833.3; 2500 μM), UDMA (10; 33.5; 100 μM), Asc (50; 100; 200; 500 μM) and ACC (50; 100; 200; 500 μM) for 6 h. The antioxidant concentrations were based on cytotoxicity experiments in our group [16,18]. The cells were not preincubated with antioxidants so as to simulate physiological conditions during the filling of a cavity.

Table 1 – Number of induced foci per cell caused by ascorbic acid (Asc). HGF were incubated with Asc in different concentrations for 6 h and the number of foci were determined with γ-H2AX assay. Data are presented as mean (standard deviation (SD)); $n = 3$; p-value (Student's t-test) and the percentage of multi foci (>40 foci) cells.

Asc (μM)	Foci/Cell (SD)	p-Value	Percentage of multi foci cells
500	4.92 (1.28)	0.02	3.83
200	2.10 (0.34)	0.33	1.09
100	1.35 (1.58)	0.8	0.76
50	0.94 (0.53)	0.24	0
Negative control	1.62 (0.67)	–	0
Positive control	16.15 (3.6)	0.002	8.86

Since resin (co)monomers, like HEMA and TEGDMA, released from dental restorative materials, may reach millimolar concentrations in the pulp [22,23], three concentrations were used based on the EC_{50} values from XTT-experiments on HGF from our group in the millimolar range (1×, 1/3× and 1/10× EC_{50}) [16]. Negative control cells only received medium for 6 h. While the cells for the positive control received 1000 μM H_2O_2 in medium for 15 min. These concentrations and incubation times were based on the results of cytotoxicity experiments performed on our group [16].

For immunofluorescent staining, cells were first washed 2 × 5 min with PBS, fixed by adding 0.5 ml ice-cold 4% paraformaldehyde in PBS for 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 4 × 5 min with PBS, cells were blocked for 20 min with four drops of serum-free blocking buffer (Dako, Hamburg, Germany) per well at 25 °C. Thereafter, cells were incubated with the primary antibody mouse monoclonal anti γ-H2AX (Millipore, Billerica, MA, USA) at 1:1300 dilution in antibody diluent (0.3 ml per well; Dako) at 4 °C overnight. After 4 × 5 min washes with PBS at 4 °C, 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 3 × 5 min in PBS and 1 × 5 min in TAE and after that cells were incubated with CyBR green at a dilution of 1:50,000 in TAE for 15 min. CyBr green is an immune fluorescence staining, that dye the nuclear. Cells were then washed 2 × 5 min in PBS and 2 × 5 min with deionized water. Finally, the cover slips were each placed on 0.1 ml of 1 ml Prolong antifade gold (Invitrogen, Karlsruhe, Germany) on a glass slide (76 mm × 26 mm; Carl Roth).

2.4. *Image acquisition*

HGFs were investigated using a Zeiss CLSM imaging fluorescence microscope (Zeiss, Göttingen, Germany) equipped with a motorized filterwheel and appropriate filters for excitation of red, green and blue fluorescence. Images were obtained using a 63× and a 100× Plan-Neofluar oil immersion objective (Zeiss) and the fluorescence imaging system LSM Image Browser (Zeiss). The foci/cell were counted. If the number of foci is >40 per cell the cell was counted as multi foci cell.

2.5. *Calculations and statistics*

The results are shown as means (SD). The statistical significance ($p < 0.05$) of the differences between the experimental groups was tested using the t-test, corrected according to Bonferroni-Holm [24].

3. Results

3.1. *γ-H2AX-assay with antioxidants*

None of the antioxidants tested when incubated with the antixoidants Asc or ACC with HGF showed a significant reduction the number of foci/cell when comparison with the negative controls, irrespective of their concentration.

At a concentration of 500 μM, the antioxidant Asc induced statistically significant ($p < 0.05$) more DNA-DSBs in HGF when compared with the controls (Table 1; 4.92 (1.28) *vs.* 1.62 (0.67)). 500 μM Asc induced approximately a three-fold increase in the number of foci/cell compared to the controls. The number of multi foci cell increased with the concentration of Asc.

The antioxidant ACC (at all tested concentrations 50–500 μM) showed no statistically significant ($p > 0.05$) increased induction of DNA-DSBs in HGF when compared with to the controls (Table 2).

For the further experiments Asc was used in a concentration of 100 μM and ACC in a concentration of 500 μM.

3.2. *γ-H2AX-assay with (co)monomers alone and in combination with antioxidants*

H_2O_2 in a concentration of 1000 μM induced 23.25 (1.73) foci/cell, HGF incubated in medium had 1.39 (0.4) foci/cell.

BisGMA:

The addition of 100 μM Asc to 90 μM BisGMA significantly reduced the number of foci/cell from 4.05 (0.56) to 1.96 (0.59) in HGF (Table 3). The addition of 500 μM ACC to 90 μM BisGMA significantly reduced the number of foci/cell from 4.05 (0.56) to 1.92 (0.14) in HGF (Table 3). All the tested BisGMA concentrations showed no increase in the number of multi foci cells when compared with the negative controls.

UDMA:

The addition of 100 μM Asc to 100 μM UDMA significantly reduced the number of foci/cell from 2.50 (0.37) to 1.64 (0.25) in HGF (Table 4). The addition of 500 μM ACC to 33.5 or 100 μM UDMA, respectively, significantly reduced the number of foci/cell (Table 4). All the tested UDMA concentrations

Table 2 – Number of induced foci per cell caused by acetylcysteine (ACC). HGF were incubated with ACC in different concentrations for 6 h and the number of foci were determined with γ-H2AX assay. Data are presented as mean (standard deviation (SD)); $n = 3$; p-value (Student's t-test) and the percentage of multi foci (>40 foci) per cell.

ACC (μM)	Foci/Cell (SD)	p-Value	Percentage of multi foci cells
500	2.17 (1.14)	0.51	0.75
200	1.3 (1.16)	0.70	0
100	1.52 (0.9)	0.89	0
50	1.47 (0.88)	0.82	0
Negative control	1.62 (0.67)	–	0
Positive control	16.15 (3.6)	0.002	8.86

Table 3 – Number of induced foci per cell caused by bisphenol-A-glycidyl methacrylat (BisGMA), BisGMA/ascorbic acid (Asc) or BisGMA/acetylcysteine (ACC), respectively. HGF were incubated with different concentrations of BisGMA (without antioxidant) or different concentrations of BisGMA and 100 μM Asc or different concentrations of BisGMA and 500 μM ACC for 6 h and the number of foci were determined with γ-H2AX assay. Data are presented as mean (standard deviation (SD)), $n = 3$.

BisGMA (μM)	Foci/Cell		
	Without antioxidant	With Asc (100 μM)	With ACC (500 μM)
90	4.05 (0.56*)	1.96 (0.59**)	1.92 (0.14**)
30	2.12 (0.54*)	1.77 (0.86)	1.24 (0.51)
9	2.13 (0.98)	1.32 (0.68)	0.98 (0.31)
Negative control	1.39 (0.4)	1.59 (0.59)	1.12 (0.24)
Positive control	23.25 (1.73)	23.25 (1.73)	23.25 (1.73)

* Statistical significant increase ($p < 0.05$) of the number of foci/cell compared with negative controls.
** Statistical significant reduction ($p < 0.05$) of the number of foci/cell with antioxidant compared with the number of foci/cell without antioxidant at the same BisGMA concentration.

Table 4 – Number of induced foci per cell caused by urethandimethacrylat (UDMA), UDMA/ascorbic acid (Asc) or UDMA/acetylcysteine (ACC), respectively. HGF were incubated with different concentrations of UDMA (without antioxidant) or different concentrations of UDMA and 100 μM Asc or different concentrations of UDMA and 500 μM ACC for 6 h and the number of foci were determined with γ-H2AX assay. Data are presented as mean (standard deviation (SD)), $n = 3$.

UDMA (μM)	Foci/Cell		
	Without antioxidant	With Asc (100 μM)	With ACC (500 μM)
100	2.5 (0.37*)	1.64 (0.25**)	1.31 (0.4**)
33.5	2.21 (0.38*)	1.39 (0.58)	1.16 (0.11**)
10	1.56 (0.7)	1.34 (0.66)	0.69 (0.11)
Negative control	1.39 (0.4)	1.59 (0.59)	1.12 (0.24)
Positive control	23.25 (1.73)	23.25 (1.73)	23.25 (1.73)

* Statistical significant increase ($p < 0.05$) of the number of foci/cell compared with negative controls.
** Statistical significant reduction ($p < 0.05$) of the number of foci/cell with antioxidant compared with the number of foci/cell without antioxidant at the same UDMA concentration.

showed no increase in the number of multi foci cells when compared with the negative controls.

EGDMA:

The addition of 100 μM Asc to 272 or 906.7 or 2720 μM EGDMA significantly reduced the number of foci/cell in HGF (Table 5). The addition of 500 μM ACC to 272 or 906.7 or 2720 μM EGDMA significantly reduced the number of foci/cell in HGF (Table 5). 2720 μM EGDMA induces 4.56% multi focus cells (negative controls: 0.35%). The addition of 100 μM Asc or 500 μM ACC caused the percentage of multi foci cells to decrease, but was not statistically different from the percentage of multi foci cells in negative controls.

GDMA:

The addition of 100 μM Asc to 250 or 2500 μM GDMA significantly reduced the number of foci/cell in HGF (Table 6). The addition of 500 μM ACC to 2500 μM GDMA significantly reduces the number of foci/cell in HGF (Table 6). 2500 μM GDMA induces 1.96% multi focus cells (negative controls: 0.35%). By addition of 100 μM Asc or 500 μM ACC the percentage of multi foci cells decreased, but was not statistically different from the percentage of multi foci cells in negative controls.

4. Discussion

The release of unpolymerized (co)monomers and additives has been shown and studied *in vitro* [25]. A further release of dental resins may be due to the degradation of methacrylate based polymers to monomers and oligomers as a consequence of mechanical stress following *e.g.* the chewing process. Enzymatic degradation of polymers by the enzymes contained in

Table 5 – Number of induced foci per cell caused by ethyleneglykoldimethacrylat (EGDMA), EGDMA/ascorbic acid (Asc) or EGDMA/acetylcysteine (ACC), respectively. HGF were incubated with different concentrations of EGDMA (without antioxidant) or different concentrations of EGDMA and 100 μM Asc or different concentrations of EGDMA and 500 μM ACC for 6 h and the number of foci were determined with γ-H2AX assay. Data are presented as mean (standard deviation (SD)), $n = 3$.

EGDMA (μM)	Foci/Cell		
	Without antioxidant	With Asc (100 μM)	With ACC (500 μM)
2720	5.36 (1.59*)	2.6 (0.57**)	1.9 (1.17**)
906.7	3.1 (0.24*)	0.74 (0.42**)	1.26 (0.69**)
272	1.95 (0.37)	0.58 (0.06**)	0.97 (0.34**)
Negative control	1.39 (0.4)	1.59 (0.59)	1.12 (0.24)
Positive control	23.25 (1.73)	23.25 (1.73)	23.25 (1.73)

* Statistical significant increase ($p < 0.05$) of the number of foci/cell compared with negative controls.
** Statistical significant reduction ($p < 0.05$) of the number of foci/cell with antioxidant compared with the number of foci/cell without antioxidant at the same EGDMA concentration.

the saliva can be another source of methacrylates. Released (co)monomers can be metabolized to carbon dioxide *in vitro* and *in vivo* [26]. Epoxides such 2,3-epoxy methacrylic acid and ROS therefore play an important role [27]. Oxidative induction of DNA-DSBs in HGFs after incubation with BisGMA was shown by Blasiak et al. [28]. Firstly it is important to determine what toxicological reactions occur after incubation of the cells with methacrylate based monomers and secondly to protect patients from this possible damage.

The general mechanisms of antioxidants are radical scavenging, formation of adducts with the radical or it acts as a reducing agent due to its low redox potential [29]. Some studies have shown that the addition of antioxidants such as vitamins C or E, ACC or uric acid to the cell culture medium can reduce cytotoxicity [18,20,21]. Our study demonstrated that the addition of the antioxidants Asc (100 μM) or ACC (500 μM) could reduce the number of DNA-DSBs in HGF. The concentration of the antioxidant to be added should be determined in prior experiments due to the possibility of (geno)toxic reactions in the cells caused by the antioxidant itself, such as that demonstrated with Asc (Table 1).

The highest tested Asc concentration of 500 μM, which corresponds to 88 mg/l, induced significantly more DNA-DSBs when compared with the negative controls. The reference value for Asc in plasma is in the range of 5–15 mg/l (28.4–255.5 μM). Some studies have shown that even by an intake of 2500 mg/d Asc the plasma level will not exceed 85 μM [30]. In rare cases plasma concentrations of 500 μM or higher can be reached *e.g.* temporary after intravenous application. Because of the high water solubility of Asc and easy renal excretion an accumulation is unlikely *in vivo*, whereas *in vitro* Asc can act during the whole incubation time [31].

One explanation for the significant increase in the number of foci/cell, as well as the percentage of multi foci cells in HGF when exposed to 500 μM Asc as compared to the negative controls may be the formation of H_2O_2 [32]. *In vitro* studies have shown that pharmacological doses of Asc used in cancer therapy can produce concentrations in excess of 25 μM H_2O_2, which may be responsible for cell death in the cancer cells [32]. It cannot be excluded that in our experiment H_2O_2 was produced, resulting in an increase of foci formation in HGFs.

The antioxidant nature of Asc not only affects the metabolism of xenobiotics, but also physiological relevant redox reactions which play an important role in DNA replication and protein biosynthesis. An interference in this processes may lead to cellular and DNA damage [33]. Both these reasons could also explain our finding that at Asc concentration of above 200 μM more multi foci cells were found compared with the negative controls.

Table 6 – Number of induced foci per cell caused by 1,3-glyceroldimethacrylat (GDMA), GDMA/ascorbic acid (Asc) or GDMA/acetylcysteine (ACC), respectively. HGF were incubated with different concentrations of GDMA (without antioxidant) or different concentrations of GDMA and 100 μM Asc or different concentrations of GDMA and 500 μM ACC for 6 h and the number of foci were determined with γ-H2AX assay. Data are presented as mean (standard deviation (SD)), $n = 3$.

GDMA (μM)	Foci/Cell		
	Without antioxidant	With Asc (100 μM)	With ACC (500 μM)
2500	2.57 (0.49*)	1.24 (0.5**)	0.96 (0.74**)
833.3	2.02 (0.83)	1.21 (0.56)	1.32 (0.31)
250	1.93 (0.38)	0.82 (0.41**)	1.06 (0.55**)
Negative control	1.39 (0.4)	1.59 (0.59)	1.12 (0.24)
Positive control	23.25 (1.73)	23.25 (1.73)	23.25 (1.73)

* Statistical significant increase ($p < 0.05$) of the number of foci/cell compared with negative controls.
** Statistical significant reduction ($p < 0.05$) of the number of foci/cell with antioxidant compared with the number of foci/cell without antioxidant at the same GDMA concentration.

The antioxidant nature of Asc in a concentration of 100 μM can also reduce the number of DNA-DSBs during incubation with different methacrylate based dental monomers (Tables 3–6). This shows good correlation with other studies that have measured the cell protective effects after addition of Asc to cells incubated with TEGDMA or HEMA [18]. It is possible that Asc reacts with the ROS or epoxides during the metabolism of the methacrylate derivates and thereby prevents cellular and DNA damage.

In contrast to Asc, ACC showed no dose depend induction of DNA-DSBs when compared with the negative controls up, to the maximum tested concentration of 500 μM. ACC is closely linked to glutathione (GSH) synthesis and the regeneration cycle. GSH is a thiol-containing antioxidant, which prevents damage to important cellular components caused by ROS such as free radicals and peroxides. Moreover GSH is involved in many physiological processes such as signal transduction, gene expression, apoptosis, protein glutathionylation, and nitric oxide (NO) metabolism [34]. The regeneration of GSH through ACC may be one reason for its cell protective effect.

The reduction of intracellular GSH level after incubation with methacrylate based monomers is well known [35]. It is also known that GSH plays an important role in the metabolism of methacrylate based monomers ROS and epoxides. It is possible that GSH acts as radical scavenger *i.e.* by formation of the disulfide GSSG or by direct reaction with the reactive species (adduct formation). To increase the cell protective state it is important to elevate the GSH level *e.g.* by using ACC [36]. GSH can also act as an antioxidant. The elevation of intracellular GSH levels and that antioxidative properties of ACC could explain the reduction of DNA-DSBs formation after incubation of HGF with the (co)monomers BisGMA, UDMA, EGDMA and GDMA.

The comonomer EGDMA has a chemical structure comparable to that of TEGDMA. It is used in composites as well as in prostheses. From EGDMA it is known that it can cause allergic reactions, especially by dental technicians [37]. The guinea pig maximization test showed for EGDMA a moderate to strong sensitizing potential [38]. The amount of elutable EGDMA from a 100 mg specimen of Tetric Evo Ceram® (Ivoclar Vivadent, Ellwangen, Germany) in ethanol/water (3:1) was 102.2 μM [39]. Assuming the worst case situation, that all teeth are replaced by Tetric Evo Ceram® (32 teeth; about 0.5 g per tooth/filling ≈16 g Tetric Evo Ceram®), and with a daily saliva production of about 1 l[40]) a EGDMA concentration of about 16.35 mM could result. This is about 18 times higher than the EC_{50} value known for HGF in XTT-test and about 6 times higher than the highest tested concentration of 2720 μM. At both concentrations significantly more DNA-DSBs and multi foci cells were found compared with the negative controls. However this data, calculated from elution experiments for human worst case situations, should be no cause for alarm, for two reasons: First, to elute most of the non polymerized (co)monomers and additives, water or artificial saliva proved to be less effective than ethanol/water 3:1 which was recommended by the United States Food and Drug Administration (US FDA) as a food/oral simulating liquid of clinical relevance [41]. Therefore higher elutable amounts compared with the physiological conditions (saliva elution) are to be expected. Second, the dilution by beverages is not considered in this worst case calculation.

The (co)monomers BisGMA, UDMA and EGDMA induce at their EC_{50} value from XTT-test significantly more foci/cell when compared with the foci/cell from the negative controls. The concentrations used for BisGMA and UDMA are obviously lower when compared with EGDMA. This finding correlates well with other cell culture experiments, where BisGMA induced cytotoxic damage at low concentrations. From degradation of BisGMA it is known that it releases methacrylates and in contrast to other (co)monomers also bisphenol A and bisphenol A, linked degradation products which can be a health risk to humans [42]. Besides the metabolic products, other factors such as electrical charge, molecular weight, chemical structure and lipophilicity also influence the cytotoxicity of a substance [43].

From elution experiments it is known that basic monomers like BisGMA and UDMA were elutable in lower amounts when compared with the (co) monomers like EGDMA, TEGDMA, GDMA or HEMA, *e.g.* HEMA leaching from dentin adhesives may reach concentrations from 1.5 to 8 mM [44]. Therefore the high concentrations needed to induce cytotoxic or inflammatory reactions in cells after incubation with (co)monomers are not necessarily an all-clear signal for cellular damage.

It was shown that antioxidants not only reduce the cytotoxicity but also the genotoxicity. One explanation is the reduction of ROS and epoxides during metabolism of (co)monomers. Therefore the question arises is it useful to add antioxidants to the matrix of composites to reduce cell damage and inflammatory response? The protective effect may be superimposed by the fact that antioxidants may be interfere in the polymerization process by scavenging the free radicals necessary for building up long polymer chains and a three dimensional polymer network. Apart from the reduction of the mechanical and physical properties, the monomer polymer conversion can be reduced, leading to an increase of unreacted (co)monomers and other additives. The higher the amount of unreacted and elutable substance, the lower the biocompatibility of the material.

Our study supports the hypothesis that the addition of the antioxidants Asc and ACC can reduce the number of DNA-DSBs *in vitro*.

5. Conclusions

DNA damage can be caused by different ways *e.g.* oxidative damage or formation of DNA intercalation products. The fact that antioxidants Asc and ACC reduce the number of DNA-DSBs *in vitro* suggests that ROS and epoxides are mainly responsible for the genotoxicity of the dental methacrylates and not the interaction with the DNA by covalently/electrostatically binding or DNA intercalation.

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12 Acknowledgements

I would like to express my sincere gratitude to Prof. Dr. Dr. Franz-Xaver Reichl for the continuous support of my PhD study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time.

I would also like to thank Dr. Christof Högg for his scientific support.