Kumulative Habilitationsschrift

Aus der Poliklinik für Zahnerhaltung und Parodontologie

Direktor Prof. Dr. Reinhard Hickel

Habilitationsschrift zum Thema:

„**Analytik, Toxikologie und Toxizität von freigesetzten Inhaltsstoffen aus dentalen Materialen**“

vorgelegt

von

Dr. rer. biol. hum. Christof Högg

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INHALTSVERZEICHNIS

Abkürzungsverzeichnis

AGP	Humanes α_1-acid glycoprotein
Asc	Ascorbinsäure
BDDMA	1,4-Butylenglykoldimethacrylat
BHT	2,6-Di-tert-butyl-4-methylphenol
Bis-EMA	Bisphenol-A-Dimethacrylat
Bis-GMA	Bisphenol-A-glycidylmethacrylat
BPA	Bisphenol A
CQ	Campherquinon
CyHEMA	Cyclohexylmethacrylat
CYP2E1	Cytochrom P450 2E1
DC	Polymerisationsgrad („Degree of conversion“)
DCHP	Dicyclohexylphthalat
DDDMA	1,10-Decandioldimethacrylat
DEDHTP	Diethyl-2,5-dihydroxyterephthalat
DEGDMA	Diethylenglycoldimethacrylat
DMABEE	4-N,N-Dimethylaminobenzoesäurebutylethoxyester
DMEM	„Dulbecco's modified Eagle's medium“
DNA-DSBs	DNA-Doppelstrangbrüche
DODDMA	1,12-Dodekandioldimethacrylat
EC_{50}	mittlere effektive Konzentration
EGDMA	Ethylenglykoldimethacrylat
EMPA	2,3-Epoxy-2-Methylpropionsäure
EMPME	2,3-Epoxy-2-methyl-propionsäuremethylester
FTIR	„Fourier transform infrared spectroscopy“
GC/MS	„Gas chromatography/mass spectrometry“
Gew.-%	Gewichtsprozent
GMA	Glycidylmethacrylat
HEMA	2-Hydroxyethylmethacrylat
HGF	Humane Gingivafibroblasten
HMBP	2-Hydroxy-4-methoxybenzophenon
HPF	Humane Pulpafibroblasten
HPLC/DAD	„High-performance liquid chromatography/diode array detection“
HPLC/FLD	„High-performance liquid chromatography/fluorescence detection“
HPMA	Hydroxypropylmethacrylat
HSA	Humanserumalbumin

MMA	Methylmethacrylat
NAC	N-Acetylcystein
PF 15%	Opalescence Bleichgel PF 15%
PF 35%	Opalescence Bleichgel PF 35%
PMGDM	Pyromellitic dianhydride glycerol dimethacrylate
PMMA	Polymethylmethacrylat
TEGDMA	Triethylenglycoldimethacrylat
THFMA	Tetrahydrofurfurylmethacrylat
TMPTMA	Trimethylolpropantrimethacrylat
TPGDA	Tripropylenglycoldiacrylat
UDMA	Urethandimethacrylat

1. Einleitung

Zahnärztliche Komposite auf Methacrylatbasis werden aufgrund ihrer ästhetischen und physikalischen Eigenschaften häufig verwendet und bestehen aus anorganischen Füllstoffen und einer organischen Matrix. Sie werden als Fissurenversiegler oder Befestigungskomposite für indirekte Restaurationen, überwiegend jedoch als Füllungsmaterialien verwendet [1]. Materialien auf Methacrylatbasis werden auch in der 3D-Drucktechnik zum Erstellen von Kronen, Aufbissschienen, Zahnersatz und chirurgischen Schablonen (guided implant sugery) eingesetzt [2-4]. Als (Co)Monomere werden meist (Di)-Methacrylate verwendet, die in Basismonomere (z.B. Bisphenol-A-glycidylmethacrylat (Bis-GMA), Urethandimethacrylat (UDMA)) und (Co)Monomere (z.B. Triethylenglycoldimethacrylat (TEGDMA), 2-Hydroxyethylmethacrylat (HEMA)) unterteilt werden. Letztere werden den Kompositen zugesetzt, um eine bessere Verarbeitung der viskosen Basismonomere zu ermöglichen [5, 6]. Dentinadhäsive bestehen ebenfalls aus Monomeren und werden als Haftvermittler bei direkten und indirekten Restaurationen verwendet.

Die Monomer-Polymer-Konversion bei der Aushärtung dieser Werkstoffe ist nie vollständig, so dass nicht-polymerisierte Restmonomere und Additive freigesetzt und in den Organismus aufgenommen werden können [1, 7]. Nicht vernetzte (Co)Monomere können durch Speichelzutritt aber auch z.B. durch Nahrung bzw. Getränke (z.B. hochprozentige Alkohole) aus der Füllung ausgelaugt werden [8] und dann verschluckt werden oder über den Dentinliquor durch das Dentin zum Zahnnerv (Pulpa) diffundieren [9, 10].

Nach der Freisetzung in die Mundhöhle können die ausgewaschenen (Co)monomerverbindungen und Additive in unmittelbaren Kontakt mit den Zellen der hoch proliferativen Mundschleimhaut (z.B. humane Gingivafibroblasten (HGF)) und/oder durch das Dentin diffundieren und in Kontakt mit den vitalen Zellen des Zahnmarks (z.B. humane Pulpafibroblasten (HPF)) treten [11]. Im Weichgewebe des Zahnmarks können freigesetzte (Co)Monomere und Additive außerdem in die systemische Blutzirkulation übertreten. Neben Abrasion, Verschleiß und Elution werden freigesetzte (Co)Monomere und Additive aus Kompositfüllungen nach dem Verschlucken im Darm nahezu vollständig resorbiert [12-15]. Abradierte Partikel mit einer Größe bis zu 100 µm können inhaliert und über die Lunge in die Blutbahn gelangen. Beim Verblasen von Dentinadhäsiven ohne Kofferdamm können (Co)Monomere und Additive auf die Mundschleimhaut gelangen und dort resorbiert werden. Beträchtliche Mengen werden dabei auch inhaliert und in der Lunge resorbiert [16]. Auch zahnärztliches Personal ist während dem Formen und Polieren von Kompositrestaurationen oder dem Entfernen von alten Kompositrestaurationen aerosolisiertem Kompositstaub ausgesetzt [17-19].

Darüber hinaus wird auch von allergischen Reaktionen wie Asthma und Kontaktdermatitis,

verursacht durch Methacrylate und Additive berichtet [20]. So können durch den Hautkontakt mit unpolymerisierten Kompositpasten Allergien ausgelöst werden [21]. Handschuhe bilden hierbei nur einen geringen Schutz [22].

In Zellkulturversuchen konnten für einige der industriell verwendeten Acrylatverbindungen signifikante mutagene Effekte gezeigt werden [23, 24]. Auch für mehrere (Co)Monomere wie TEGDMA, HEMA, MMA, BisGMA, Glycidylmethacrylat (GMA) zahnärztlicher Füllungswerkstoffe können, wie in in-vitro-Studien gezeigt wurde, negative Auswirkungen wie Mutagenität, Teratogenität, Genotoxizität, Zytotoxizität und östrogene Aktivität in Zellen der Mundhöhle hervorrufen [25-31]. Unter anderem konnte in vier verschiedenen *Salmonella typhimurium*-Stämmen mit Hilfe des Ames-Tests für subtoxische Konzentrationen des monofunktionellen Monomers GMA genetische Veränderungen gefunden werden, die durch die Anwesenheit einer Mikrosomenfraktion im Kulturmedium verstärkt wurden [32]. Darüber hinaus wurden auch in einem eukaryontischen Testmodell mit Hilfe von Lungenfibroblasten des Hamsters (V79/HPRT-Test) neben GMA insbesondere auch für TEGDMA und Methylmethacrylat (MMA) mutagene Veränderungen der DNA nachgewiesen [32, 33]. Nach den Ergebnissen weiterer Studien wurde postuliert, dass den mutagenen Effekten von TEGDMA möglicherweise die Deletion größerer DNA-Sequenzen sowie deren Transposition auf benachbarte DNA-Regionen zu Grunde liegen [30]. Auch für die molekular größeren Methacrylate Bis-GMA und Urethandimethacrylat (UDMA) konnten an HeLa-Zellkulturen genotoxische Effekte gezeigt werden [34]. Schließlich konnten auch durch MMA in Lymphomzellen der Maus (L5178Y/TK) ausgeprägte mutagene Effekte ausgelöst werden [35]. Später zeigten Studien, dass die Antioxidantien Ascorbinsäure (Asc) bzw. N-Acetylcystein (NAC) die Zytotoxizität und Genotoxizität von Methacrylatbasierten (Co)Monomeren reduzieren können [36-38].

Aus dentalen Kompositen freigesetzte Inhaltsstoffe können bereits intraoral zu toxischen Xenobiotika umgewandelt werden. Aber auch sowohl pulmonal als auch intestinal aufgenommene (Co)Monomere aus Kompositen können nach der Resorption im Organismus metabolisiert werden. Die aus freigesetzten (Co)Monomeren gebildeten Zwischenprodukte können die Funktion von Proteinen und Genom beeinträchtigen. Unsere früheren Studien haben die Aufnahme, Verteilung und Eliminierung von radioaktiv markiertem ^{14}C-TEGDMA und ^{14}C-HEMA bei Meerschweinchen gezeigt [13, 39]. TEGDMA und HEMA werden zu Methacrylsäure (MA) und darauffolgend hauptsächlich über den sogenannten Epoxyweg metabolisiert [13, 39-41]. Im Epoxyweg kann der Epoxy-Metabolit 2,3-Epoxy-2-methylpropionsäure (EMPA) gebildet werden [29, 41-43]. Des Weiteren ist sehr wahrscheinlich, dass in vivo 2,3-Epoxy-2-methyl-propionsäuremethylester (EMPME) gebildet werden kann [29]. Epoxide gelten als hochreaktive Moleküle und mutagene/carcinogene Substanzen [43]. So ergaben Untersuchungen zur Toxikologie von EMPME und EMPA mit

Hilfe eines modifizierten Fluoreszenz-Stammzelltests dass EMPA eine teratogene Wirkung und EMPME eine embryotoxische Wirkung auf die embryonalen Stammzellen von Mäusen hat [29].

Hinsichtlich der Freisetzung von Inhaltsstoffen aus dentalen Kompositen und der Toxikologie dieser freigesetzten Inhaltsstoffe stellten sich folgende Fragen:

1. Welchen Einfluss hat die Schichtdicke auf die Freisetzung von Inhaltsstoffen aus dentalen Bulk-Fill Kompositen?

2. Können freigesetzte Inhaltsstoffe aus dentalen Kompositen an Proteine in nativem Speichel binden und welchen Einfluss hat dies auf die Toxizität?

3. Welchen Einfluss haben Bleachinggele auf die Freisetzung von Inhaltsstoffen aus konventionellen dentalen Kompositen und Bulk-Fill Kompositen?

4. Welche Inhaltsstoffe werden aus 3D-gedruckten Aufbissschienenmaterialen im Vergleich zu gefrästen und konventionellen Materialien freigesetzt?

5. Welchen Einfluss haben Antioxidantien auf Comonomer-Epoxy-Metaboliten-induzierte DNA-Doppelstrangbrüche in humanen Gingivafibroblasten?

6. Welche Zytotoxizität und Induktion von DNA-Doppelstrangbrüchen in humanen Gingivafibroblasten ergeben sich durch Exposition mit Eluaten aus dentalen Kompositen im Vergleich zu der Exposition mit Einzelkomponenten?

7. Welchen Einfluss haben Antioxidantien als neue Komponente in dentalen Kompositen auf die Freisetzung von Inhaltsstoffen und den Polymerisationsgrad?

2. Methoden und Ergebnisse (Publikationen)

2.1. Freisetzung von Inhaltsstoffen aus dentalen Kompositen

2.1.1. Einfluss der Schichtdicke auf die Freisetzung von Inhaltsstoffen aus dentalen Bulk-Fill Kompositen [44]

Für Schichtdicken > 2 mm wurde bisher die sogenannte Inkrementschichttechnik bei posterioren Kompositrestaurationen mit einer maximalen Schichtdicke von 2 mm angewendet, um (Co-)Monomere ausreichend umzusetzen. Die Entwicklungen von Bulk-Fill Kompositen versprechen eine Beschleunigung des Restaurationsprozesses bei denen mindestens 4 mm dicke Inkremente in einem Schritt ausgehärtet werden können. Ziel der vorliegenden Studie war es, den Einfluss der Schichtdicke auf die Elution von Komponenten aus Bulk-Fill-Kompositen zu untersuchen. Die Komposite ELS Bulkfill, SDR Bulkfill und Venus Bulkfill wurden nach den Anweisungen der Hersteller polymerisiert. Für jedes Komposit wurden drei Gruppen mit je vier Proben (n=4) vorbereitet: 1. Proben mit einer Schichtdicke von 2 mm; 2. Proben mit einer Schichtdicke von 4 mm (gemäß Herstellerangaben) und 3. Proben mit einer Schichtdicke von 6 mm (entgegen der Herstellerempfehlung). Die Proben wurden 24 h und 7d lang in Methanol und Wasser eluiert. Die resultierenden Eluate wurden mittels „gas chromatography/mass spectrometry“ (GC/MS) analysiert. Insgesamt wurden 11 verschiedene eluierbare Substanzen aus den untersuchten Kompositen identifiziert. Folgende Methacrylate zeigten eine Zunahme der Elution bei höherer Schichtdicke: TEGDMA (SDR Bulkfill, Venus Bulkfill), EGDMA (Venus Bulkfill). Es wurde kein signifikanter Unterschied in der Elution von HEMA in Abhängigkeit von der Schichtdicke gefunden. Die höchste TEGDMA-Konzentration betrug 146 µg/ml für SDR Bulkfill bei einer Schichtdicke von 6 mm nach 7d in Wasser. Die höchste gemessene HEMA-Konzentration von 108 µg/ml wurde im Methanol-Eluat von Venus BulkFill nach 7d bei einer Schichtdicke von 6 mm festgestellt. Eine gemäß Hersteller nicht freigegebene Schichtdicke von 6 mm kann im Vergleich zur Elution bei einer Schichtdicke von 2 und 4 mm zu einer erhöhten Elution einiger Bulk-Fill Kompositinhaltsstoffen führen. Daher sollten die Herstellerangaben strikt befolgt werden.

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Effect of layer thickness on the elution of bulk-fill composite components

Lena Rothmund[a,b], *Franz-Xaver Reichl*[a,b], *Reinhard Hickel*[a], *Panorea Styllou*[a,b], *Marianthi Styllou*[a,b], *Kai Kehe*[b,c], *Yang Yang*[a,b], *Christof Högg*[a,b,*]

[a] *Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany*

[b] *Walther-Straub-Institute of Pharmacology and Toxicology, Nussbaumstr. 26, 80336 Munich, Germany*

[c] *Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstr. 11, 80937 Munich, Germany*

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ABSTRACT

Objective. An increment layering technique in a thickness of 2 mm or less has been the standard to sufficiently convert (co)monomers. Bulk fill resin composites were developed to accelerate the restoration process by enabling up to 4 mm thick increments to be cured in a single step. The aim of the present study is to investigate the effect of layer thickness on the elution of components from bulk fill composites.

Methods. The composites ELS Bulk fill, SDR Bulk fill and Venus Bulkfill were polymerized according to the instruction of the manufacturers. For each composite three groups with four samples each ($n=4$) were prepared: (1) samples with a layer thickness of 2 mm; (2) samples with a layer thickness of 4 mm and (3) samples with a layer thickness of 6 mm. The samples were eluted in methanol and water for 24 h and 7 d. The eluates were analyzed by gas chromatography/mass spectrometry (GC/MS).

Results. A total of 11 different elutable substances have been identified from the investigated composites. Following methacrylates showed an increase of elution at a higher layer thickness: TEGDMA (SDR Bulk fill, Venus Bulk fill), EGDMA (Venus Bulk fill). There was no significant difference in the elution of HEMA regarding the layer thickness. The highest concentration of TEGDMA was 146 μg/mL for SDR Bulk fill at a layer thickness of 6 mm after 7 d in water. The highest HEMA concentration measured at 108 μg/mL was detected in the methanol eluate of Venus Bulk fill after 7 d with a layer thickness of 6 mm.

Significance. A layer thickness of 4 mm or more can lead to an increased elution of some bulk fill components, compared to the elution at a layer thickness of 2 mm.

* *Corresponding author at:* Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany. Tel.: +49 89 2180 73809; fax: +49 89 2180 73817.
E-mail address: christof.hoegg@lrz.uni-muenchen.de (C. Högg).

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1. Introduction

In the last decade the use of resin based composites (RBCs) has increased tremendously. RBCs, consisting of a number of (co)monomers and additives, belong to the most commonly used filling materials. Due to the incomplete (co)monomer-polymer conversion, a release of the unpolymerized (co)monomers from the dental composite is described [1,2]. There are many *in vitro* studies on the toxicity and biocompatibility, which have shown that some of the eluted (co)monomers and additives even have estrogenic, mutagenic, teratogenic and genotoxic effects [3–6]. Previous *in vivo* studies have demonstrated that HEMA, TEGDMA and BisGMA can be metabolized to the expoxy compound 2,3-epoxymethacrylic acid in hepatic microsomes [7–9]. Epoxides are regarded as mutagenic and carcinogenic agents [10–12].

The final degree of conversion (DC) depends mainly on intrinsic factors such as the chemical structure of the (co)monomer and photo initiator concentration and extrinsic factors such as polymerization conditions and curing modes [13,14]. The energy of the light emitted from a light curing unit decreases drastically when transmitted through a rinsing composite [15]. Thus far, an increment layering technique in a thickness of 2 mm or less has been the standard to sufficiently convert (co)monomers [16].

A new category of RBCs, bulk-fill resin composites, has been introduced over the past few years. They were developed to accelerate the restoration process by enabling up to 4 mm thick increments to be cured in a single step, thereby skipping the time-consuming layering process. The manufacturers explain that the higher depth of cure of the bulk-fill resin composites is due to the more potent initiator system or/and higher translucency. Studies have already been performed on the mechanical properties of bulk-fill composites [17–22]. Thus, for example, for cuspal deflection [22], the marginal integrity of a filling [20,21], just as for its cure depth [21] better results of bulk-fill composites, compared to composites which are added in the incremental technique were detected. However, also adverse results were found compared to conventional composites such as the conversion rate, for bulk-fill composites [23]. A conversion rate >55% for bulk-fill composites is still in the clinically acceptable range but it is still less than for conventional composites [23].

It was shown that the elution of bulk-fill composites is comparable to that of conventional materials despite their increased layer thickness of 4 mm [24] and amount of eluted (co)monomers increases with elution time [25,26].

However, there are no data available to what extent a layer thickness of up to 6 mm, in comparison to a layer thickness of 2 and 4 mm, has an effect on the amount of elutable components from bulk fill composites. The aim of the present study is therefore to clarify the effect of layer thickness on the elution of components from bulk fill composites. In the null hypothesis it is assumed that a variation of layer thickness does not have an influence on the concentration of eluted substances from bulk fill composites.

2. Materials and methods

The tested composites including manufacturers' data are listed in Table 1.

2.1. Preparation of samples

Composites (Table 1) were polymerized exactly according to instruction of the manufacturer. For each composite three groups with four samples each ($n=4$) were prepared: (1) samples with a layer thickness of 2 mm; (2) samples with a layer thickness of 4 mm and (3) samples with a layer thickness of 6 mm. For the preparation of the samples, polytetrafluoroethylene (PTFE) rings with a diameter of 6 mm were used. The PTFE rings were filled with uncured dental material, covered with plastic strips (Frasaco, Tettnang, Germany) to prevent the formation of an oxygen inhibition layer and were finally polymerized with a LED-lamp (Elipar S^{TM}10® high intensity halogen light, 1200 mW/cm^2, 3 M ESPE, Seefeld, Germany) in accordance with the manufacturer's instructions (Table 1). The curing unit was directly applied on the sample's surface. The light intensity of the LED-lamp was controlled with Demetron® Radiometer (Kerr, USA) and was always between 1100 and 1200 mW/cm^2. Samples had approximately a volume of 56.6, 113.1 and 169.7 mm^3, and surface area of 94.3, 132.0 and 169.7 mm^2 at a layer thickness of 2, 4 and 6 mm, respectively.

Subsequently, samples were incubated (face up) in brown glass vails (Macherey-Nagel, Düren, Germany) with 1 ml of methanol (GC Ultra Grade, RATISOLV® ≥99.9%, Roth, Karlsruhe, Germany) or 1 ml water (LC-MS-Grade, ROTISOLV®, Roth, Karlsruhe, Germany) and stored in the dark at 37 °C and analyzed after 1 d and 7 d by gas chromatography/mass spectrometry (GC/MS) [27]. 100 μl of the water eluates were 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 [28].

As internal standard caffeine (CF) solution (0.01 mg/ml) (HPLC ≥99.0%, Sigma Aldrich, St. Louis, United States) was added.

2.2. Analytical procedure

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 μm; 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 five 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 five min at this temperature. The mass spectrometer (MS) was operated

Table 1 – Investigated dental materials, manufacturer and lot numbers; composition of each material based on manufacturer's data; curing time recommended by manufacturer.

Product name	Type	Manufacturer	LOT	Composition of materials based on manufacturer's data	Polymerization time
ELS Bulk fill	Bulk-Fill composite	Saremco, Rebstein, Switzerland	C297	Barium glass, silanised, ytterbium trifluoride (YbF_3), bisphenol A glycidylmethacrylate (BisGMA), ethoxylated bisphenol A dimethacrylate (BisEMA), catalysts, inhibitors, additives	20 s
SDR Bulk fill	Bulk-Fill composite	Dentsply, Konstanz, Germany	1410000302	Barium-alumino-fluoro-borosilicate glass, Strontium alumino-fluoro-silicate glass, modified urethane dimethacrylate resin, ethoxylated bisphenol A dimethacrylate (BisEMA), triethyleneglycol dimethacrylate (TEGDMA), camphorquinone (CQ) Photoinitiator, Photoaccelerator, Butylated hydroxyl toluene (BHT), UV Stabilizer, Titanium dioxide, Iron oxide pigments, fluorescing agent	20 s
Venus Bulkfill	Bulk-Fill composite	Heraeus Kulzer, Hanau, Germany	010106	Urethane dimethacrylate (UDMA), ethoxylated bisphenol A dimethacrylate (BisEMA), approximately 65% w/w and 38% vol inorganic fillers, such as Ba-Al-F silicate glass, YbF_3 and SiO_2.	20 s

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).

2.3. *Calculations and statistics*

The results are presented as means ± standard deviation (SD). The statistical significance ($p < 0.05$) of the differences between the experimental groups was analyzed by one-way ANOVA and the post hoc test (Tukey's HSD test) [29].

3. Results

A total of 11 different elutable substances (Table 2) have been identified from the investigated composites (Table 1). The quantification and significant differences are shown in Figs. 1–3.

3.1. *ELS® Bulk Fill (Fig. 1)*

In the eluates (methanol and water) of ELS® Bulk Fill the composite components CQ, BHT and HMBP were detected.

Table 2 – Detected eluted composite components.

Compound abbreviation	Compound
BEMA	Benzyl methacrylate
EGDMA	Ethylene glycol dimethacrylate
HEMA	Hydroxyethyl methacrylate
HPMA	Hydroxypropyl methacrylate
TEGDMA	Triethylene glycol dimethacrylate
BHT	2,6-Di-t-butyl-4-methyl phenol
CQ	Camphorquinone
CSA	Champhoric acid anhydride
DMABEE	4-N,N-dimethylaminobenzoic acid butyl ethoxy ester
HMBP	2-Hydroxy-4-methoxybenzophenone
DDHT	Diethyl-2,5-dihydroxytrephthalate

3.2. *SDR Bulk Fill (Fig. 2)*

In the methanol eluates of SDR Bulk Fill CQ, BHT, TEGDMA, HMBP, HPMA, HEMA, CSA, DMABEE, BEMA and DDHT were detected. After 24 h elution in methanol a layer thickness of 6 mm resulted in a significant higher TEGDMA elution (6 mm: 72.8 μg/mL) compared to a layer thickness of 4 mm (60.4 μg/mL) and a layer thickness of 2 mm (57.1 μg/mL), respectively. After 7 d elution in methanol a layer thickness of 6 mm resulted in a significant higher TEGDMA elution (6 mm: 88.1 μg/mL) compared to a layer thickness of 4 mm (65.5 μg/mL) and a layer thickness of 2 mm (64.3 μg/mL), respectively.

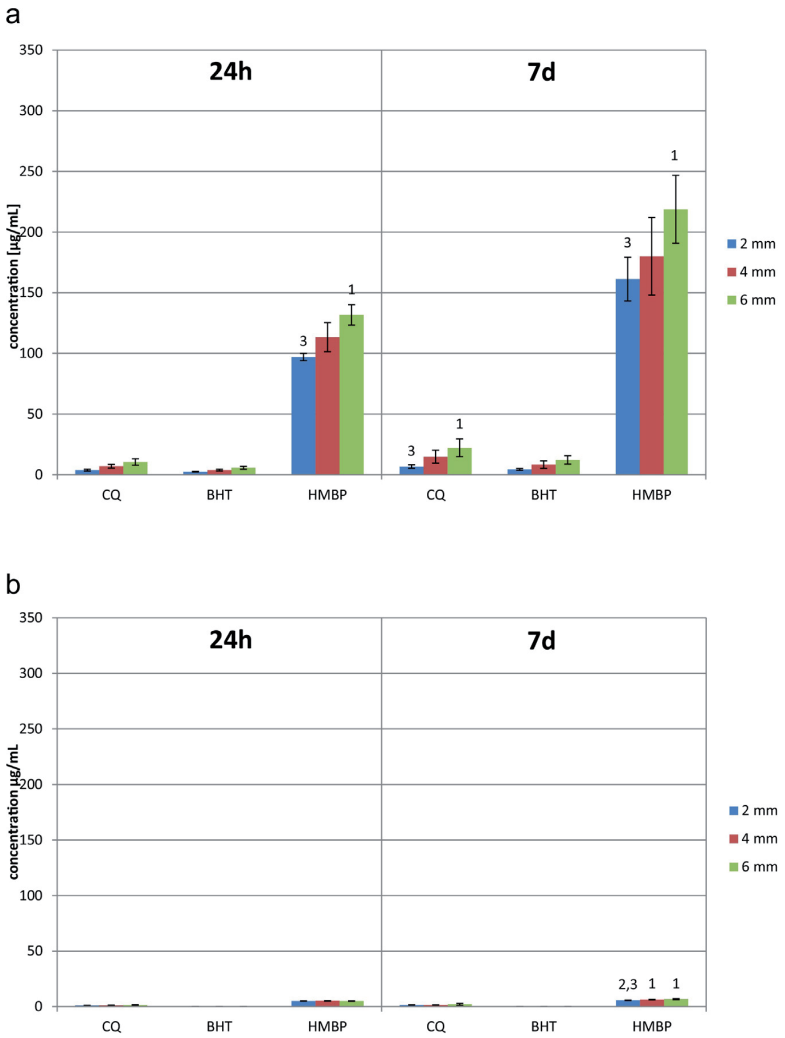

Fig. 1 – ELS bulk fill 24 h, 7 d elution in (a) methanol and (b) water. 1 = significantly ($p < 0.05$) different from 2 mm; 2 = significantly ($p < 0.05$) different from 4 mm; 3 = significantly ($p < 0.05$) different from 6 mm.

In the water eluates of SDR Bulk Fill CQ, BHT, TEGDMA, HMBP, HPMA, CSA, DMABEE and DDHT were detected. After 24 h elution in water a layer thickness of 6 mm resulted in a significant higher TEGDMA elution (6 mm: 134.9 µg/mL) compared to a layer thickness of 4 mm and a layer thickness of 2 mm, respectively. A layer thickness of 4 mm (103.0 µg/mL; 24 h; water) resulted in a significant higher TEGDMA elution compared to a layer thickness of 2 mm (70.5 µg/mL; 24 h; water). After 7 d elution in water a layer thickness of 2 mm resulted in a significant lower TEGDMA elution (2 mm: 82.9 µg/mL) compared to a layer thickness of 4 mm (124.9 µg/mL) and a layer thickness of 6 mm (146.2 µg/mL), respectively.

3.3. *Venus Bulkfill* (Fig. 3)

In the methanol eluates of Venus Bulkfill HEMA, EGDMA, CQ, CSA, BHT, TEGDMA, DDHT and HMBP were detected. After 24 h elution in methanol a layer thickness of 6 mm (7.1 µg/mL) resulted in a significant higher EGDMA elution compared to a layer thickness of 2 mm (2.6 µg/mL). TEGDMA elution at a layer thickness of 2 mm resulted in a significant lower elution (2 mm: 9.1 µg/mL; 24 h; methanol) compared to a layer thickness of 6 mm (11.6 µg/mL; 24 h; methanol). After 7 d elution in methanol a layer thickness of 6 mm resulted in a significant higher EGDMA elution (6 mm: 8.3 µg/mL) compared to a layer thickness of 4 mm (4.3 µg/mL) and a layer thickness of 2 mm (3.7 µg/mL), respectively. TEGDMA elution at a layer thickness of 6 mm (12.0 µg/mL; 7 d; methanol) resulted in a significant higher elution compared to a layer thickness of 2 mm (9.6 µg/mL; 7 d; methanol).

In the water eluates of Venus Bulkfill CQ, CSA, BHT, TEGDMA and HMBP were detected. After 24 h elution in water a layer thickness of 6 mm (7.8 µg/mL) resulted in a significant higher TEGDMA elution compared to a layer thickness of 2 mm

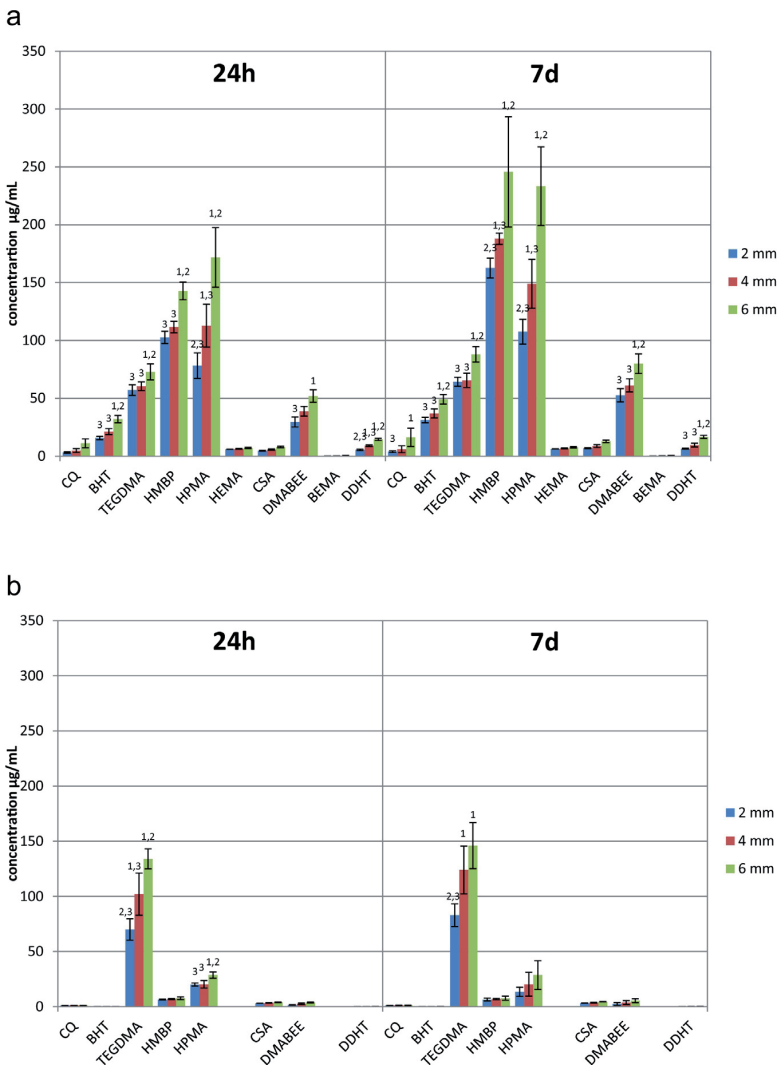

Fig. 2 – SDR bulk fill 24 h, 7 d elution in (a) methanol and (b) water. 1 = significantly ($p < 0.05$) different from 2 mm; 2 = significantly ($p < 0.05$) different from 4 mm; 3 = significantly ($p < 0.05$) different from 6 mm.

(6.5 µg/mL). After 7 d elution in water a layer thickness of 6 mm resulted in a significant higher TEGDMA elution (6 mm: 7.14 µg/mL) compared to a layer thickness of 4 mm (6.4 µg/mL) and a layer thickness of 2 mm (6.4 µg/mL), respectively.

4. Discussion

In this study the effect of layer thickness on the elution of components from dental bulk fill composites was investigated.

According to manufactures' data investigated bulk fill flowable composites are used as liners and bases and have to be coated/capped with a conventional composite. Therefore exposure to oral environment of released composite components may be of minor relevance. Nevertheless, released components may penetrate through the dentinal tubuli to the pulp [30,31] and there affect the vitality and regenerative ability of the pulp [32]. Furthermore in the present study the samples were eluted both in methanol as well as in water because water allows the utmost physiological comparison to dentinal fluid and human saliva [28,33].

In present study eluates were analyzed by GC/MS. Long chained methacrylates such as urethane dimethacrylate (UDMA) and ethoxylated bisphenol A dimethacrylate (BisEMA) can undergo discrimination in the injector of GC [34–36]. For example UDMA can decompose to minor amounts of HEMA [34,35]. Current results showed amounts of HEMA in the eluates of SDR Bulkfill and Venus Bulkfill. As analyzing eluates of polymerized composites and also impurity of starting material (e.g. UDMA, BisEMA) of the composite is possible, source of HEMA is not clear. Therefore UDMA and BisEMA were not quantified by degradation products in the present study. Only

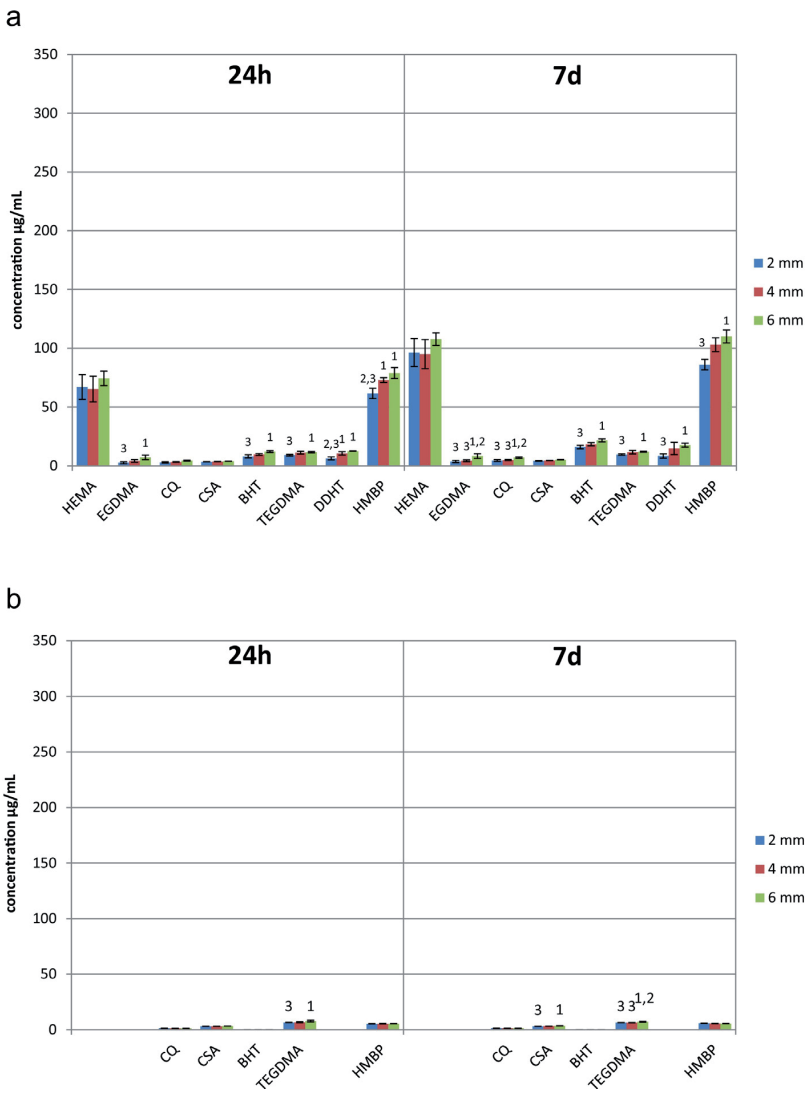

Fig. 3 – Venus bulk fill 24 h, 7 d elution in (a) methanol and (b) water. 1 = significantly ($p < 0.05$) different from 2 mm; 2 = significantly ($p < 0.05$) different from 4 mm; 3 = significantly ($p < 0.05$) different from 6 mm.

minor amount of HEMA after discrimination of UDMA are detectable [34,35]. In the present study relatively high amounts of HEMA were found in the eluates of SDR Bulkfill and Venus Bulkfill, despite the fact that according to manufacturers' data, HEMA is not part of composition of both composites. Therefore source of HEMA is unknown.

In the eluates of ELS Bulkfill no smaller (co)monomers such as HEMA (or TEGDMA) were detected by GC/MS. For example a decomposition of UDMA to minor amounts of HEMA was described [34,35]. Therefore our assumption is that only marginal amounts of long chained methacrylates e.g. BisEMA and/or UDMA may be eluted from the composite ELS Bulkfill®.

The extent and rate of elution of components from composites is dependent upon several factors: the DC of (co)monomers, the composition and solubility characteristics of the extraction solvent and the size and chemical characteristics of the leachable species [37]. Till now, an incremental layering technique has been the standard procedure in direct posterior composite restorations to reduce polymerization shrinkage and achieve adequate depth of cure as well as reducing the elution of (co)monomers and additives [16]. On the other hand, bulk-fill composites guarantee a sufficient polymerization depth at increments up to 4 mm strength [38], which can be attributed to an increased translucence through reduced filling material content with simultaneously increased filling particle size [39]. The aforementioned properties allow a quick one-incremental technique to fill a complete cavity for bulk-fill composites in many cases [17]. However, polymerization of dental composites is incomplete. The lower the conversion rate of a composite, the more

residual (co)monomers can be eluted [40]. These elutable (co)monomers (methacrylates) can result in allergic reactions [41], such as asthma, allergic rhinoconjunctivitis or contact dermatitis [42]. A conversion rate of > 55% for bulk-fill composites is still in the clinically acceptable range but it is less than for conventional composites [23]. In previous studies (co)monomers and additives indicated concentration-dependent cytotoxic effects, such as increased cell death, damage of the plasma membrane or an increased content of released lactate dehydrogenase [43–45].

In the present study also the elution of a layer thickness of 6 mm was investigated as a worst-case situation. In a previous study relation of cure depth to degree of conversion for a conventional composite was investigated [46]. Based on the lamp energy of present study (22–24 J/cm^2) a conversion rate of about >55% at a curing depth of 6 mm for conventional composite of above cited study [46] can be estimated. Considering an increased translucence through reduced filling material content with simultaneously increased filling particle size for bulk-fill composites [39], bulk-fill samples with a layer thickness of 6 mm of the present study should be sufficiently cured. Nevertheless other studies showed a significant lower the at the bottom surface compared to top surface for bulk-fill composites at a layer thickness of 4 mm [47,48], but no correlation between DC and (co)monomer release was found at neither top nor bottom surface [47]. However there is no evidence for a correlation between DC and (co)monomer release, because elution mechanism is also related to the molecular weight and hydrophobicity of (co)monomers as well as the filler content [48] and consequently depended on investigated material and the final network characteristics of the resin-matrix [24,47,49].

TEGDMA is a (co)monomer frequently used in composites due to its low viscosity and ability to enrich the organic resin matrix of composites with a maximum of inorganic filler particles [50]. TEGDMA was detected in the eluates of SDR Bulkfill and Venus Bulkfill. The highest eluted TEGDMA concentration in the present study is 146 μg/mL for SDR at a layer thickness of 6 mm after 7 d in water. This concentration is significantly higher than the concentration in the eluate generated at a layer thickness of 2 mm (83 μg/mL). The cytotoxic concentration for TEGDMA is 1058 μg/mL for human mucous membrane cells [51]. Although there is a significant increase in the elution of TEGDMA from SDR Bulkfill® at a layer thickness of 6 mm compared to a layer thickness of 2 mm, this value is almost 10 times lower than the cited cytotoxic concentration.

For the composites Venus Bulkfill and SDR Bulkfill®, the layer thickness did not influence the amount of eluted HEMA significantly. HEMA is used in dental composites as a (co)monomer of the organic resin matrix due to its hydrophilic application. HEMA could be detected in the eluates from SDR Bulkfill® and Venus Bulkfill®. It is known that HEMA can cause cytotoxic and genotoxic effects [52]. Besides, HEMA can induce a higher concentration of reactive oxygen species (ROS) at 390 μg/mL [53,54]. The highest HEMA concentration measured at 108 μg/mL was detected in the methanol eluate of Venus Bulk fill® after 7 d at a layer thickness of 6 mm. In previous studies cytotoxic concentrations for HEMA at 312 μg/mL in human pulp fibroblasts [3] and at 1548 μg/mL in human gingival fibroblasts [51] were found. The HEMA concentrations detected in our study are therefore far below those cytotoxic concentrations [3,51].

The photoinitiator CQ is a component released from all investigated bulkfill composites. CQ is considered as a powerful allergen [55], which can also cause oxidative stress and DNA damage [56]. After 7 d, the highest CQ concentration was measured in the methanol eluates of ELS Bulkfill® at 22 μg/mL. This concentration was significant higher than the concentration of the eluates at a layer thickness of 2 mm (6 μg/mL). Only for methanol eluates an effect of layer thickness on the amount of elutable composite components could be observed, in contrast to the water eluates. Previous studies indicated a significant concentration-dependent increase of intracellular ROS and DNA damage in hGF starting at a concentration of 8.3 μg/mL [56]. The concentration detected in our study is almost three times higher than this value. A previous study of our group showed that CQ is not detectable in native human saliva [28]. Based on these results, toxic effects are not to be expected in the human physiological situation.

For the composite SDR Bulkfill®, after 24 h and 7 d in methanol, a layer thickness of 6 mm resulted in a significant higher release of DMABEE compared to a layer thickness of 4 mm and 2 mm, respectively. In the water eluates the layer thickness did not influence the release of DMABEE significantly. DMABEE is a coinitiator used in composites to accelerate the breakdown of initiators into radicals and thereby the polymerization [57]. The highest value for DMABEE (78 μg/mL) was measured in the methanol eluates of SDR Bulkfill® after 7 d at a layer thickness of 6 mm in the present study. This is almost three times less than the cytotoxic concentration of 237 μg/mL, which is described for human mucous membrane cells [3].

HMBP is added to dental composites as a UV-absorbing compound to reduce the amount of discoloration in the resin of the final composite [58]. After 7 d, the highest concentration of HMBP was detected in the methanol eluate of SDR Bulkfill® at a layer thickness of 6 mm (246 μg/mL). A layer thickness of 6 mm resulted in a significant higher HMBP elution compared to a layer thickness of 4 mm (188 μg/mL) and a layer thickness of 2 mm (163 μg/mL), respectively. HMBP showed estrogenic effects in human embryonic kidney fibroblast cells. HMBP showed a higher relative luciferase activity than the negative controls at 228 μg/mL [59]. The concentration of HMBP in the methanol eluates was almost 30 times higher than the concentration of the corresponding water eluates. Based on this result, toxic effects are not to be expected in the human physiological situation.

It must be taken into account that a double layer thickness does not lead to twice as much elution values. But our results showed that the elution values of methacrylates increase with the surface area. This is in accordance with many previous studies [60–64].

The results of the present study showed that the manufacturer's instructions should be followed strictly, because in many cases a layer thickness of 6 mm (worst-case situation) resulted in a higher amount of eluted bulk fill composite components. This may lead to higher uptake in patients.

The null hypothesis is rejected because a variation of layer thickness can lead to different releases of components from bulk-fill composites.

5. Conclusion

A layer thickness of 4 mm or more can lead to an increased elution of some bulk fill components, compared to the elution at a layer thickness of 2 mm.

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2.1.2. Freisetzung und Proteinbindung von Inhaltsstoffen aus dentalen Kompositen in nativem Speichel und anderen Extraktionsmedien [45]

Unpolymerisierte (Co-)Monomere und Additive können aus Methacrylat-basierenden Kompositen freigesetzt werden und in den menschlichen Organismus gelangen. In der vorliegenden Studie wurde die Bindung von freisetzbaren Kompositinhaltsstoffen an Speichelproteine und Plasmaproteine untersucht. Die Komposite Admira flow, Venus Diamond flow, Filtek Supreme XTE flow, Tetric EvoCeram und Tetric EvoFlow wurden gemäß den Anweisungen des Herstellers polymerisiert. Anschließend wurden die resultierenden Proben (n=4) in nativem Speichel, proteinfreiem Speichel (künstlicher Speichel), Wasser und Ethylacetat bei 37 °C für 24 h bzw. 72 h inkubiert und die Eluate mittels GC/MS analysiert. Zur Bestimmung der Bindung an Speichelproteine wurden die Konzentrationen der im nativen Speichel nachgewiesenen (Co-)Monomere und Additive mit den entsprechenden Konzentrationen der in proteinfreiem Speichel, Wasser bzw. Ethylacetat nachgewiesenen (Co-)Monomere und Additive verglichen. Zur Bewertung der Affinität von TEGDMA, EGDMA, DEGDMA, PMGDM, BPA und DCHP zu Humanserumalbumin (HSA) und humanem α_1-acid glycoprotein (AGP) wurde ein Plasmaproteinbindungstest durchgeführt. Die statistische Signifikanz ($p < 0,05$) der Unterschiede zwischen den Versuchsgruppen wurde mit Hilfe der Einweg-Varianzanalyse (ANOVA) und der anschließenden Tukey-Analyse geprüft. Die Konzentrationen von TEGDMA, GMA und CyHEMA, die im nativen Speichel freigesetzt wurden waren signifikant niedriger als die Konzentration, die in proteinfreiem Speichel bzw. Wasser freigesetzt wurden (Admira flow: Konzentration von TEGDMA nach 72 h: 0,08 mmol/L (nativer Speichel), 0,34 mmol/L (proteinfreier Speichel), 0,39 mmol/L (Wasser)). Die im nativen Speichel freigesetzten Konzentrationen von HEMA, EGDMA, DDDMA und CQ lagen im Vergleich zu den anderen Extraktionsmedien unter der Nachweisgrenze. Die untersuchten Methacrylate wiesen eine Proteinbindung an HSA + AGP zwischen 82 und 85% auf. Die Proteinbindung von DCHP lag bei 96,6% und die von BPA bei 95,2%. Somit spiegeln künstlicher Speichel bzw. Wasser als Extraktionsmedia nicht die tatsächliche physiologische Situation im Körper wider. Speichel- und Plasmaproteine können (Co-)Monomere und Additive binden und dadurch zu einer geringeren Bioverfügbarkeit von freigesetzten Kompositinhaltsstoffen in vivo als bisher angenommen führen.

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Release and protein binding of components from resin based composites in native saliva and other extraction media

Lena Rothmund[a,b,*], *Mostafa Shehata*[a,b], *Kirsten L. Van Landuyt*[c], *Helmut Schweikl*[d], *Thomas Carell*[e], *Werner Geurtsen*[f], *Elmar Hellwig*[g], *Reinhard Hickel*[a], *Franz-Xaver Reichl*[a,b], *Christof Högg*[a,b]

[a] *Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany*
[b] *Walther-Straub-Institute of Pharmacology and Toxicology, Nussbaumstr. 26, 80336 Munich, Germany*
[c] *KU Leuven Biomat, Department of Oral Health Sciences, KU Leuven, Kapucijnenvoer 7, B-3000 Leuven, Belgium*
[d] *Department of Operative Dentistry and Periodontology, University of Regensburg Medical Centre, 93042 Regensburg, Germany*
[e] *Department of Organic Chemistry, Ludwig-Maximilians-University of Munich, Butenandtstr. 5-13, House F, 81377 Munich, Germany*
[f] *Department of Conservative Dentistry and Periodontology, Medical University Hannover, 30623 Hannover, Germany*
[g] *Department of Operative Dentistry and Periodontology, University Medical Center Freiburg, Dental School and Hospital, Hugstetter Straße 55, 79106 Freiburg i. Br., Germany*

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ABSTRACT

Objectives. Unpolymerized (co)monomers and additives can be released from resin based composites (RBCs) and can enter the human organism. In this study, the binding of ingredients from composites to salivary proteins and plasma proteins was investigated.

Methods. The composites investigated were Admira® flow, Venus® Diamond flow, Filtek™ Supreme XTE flow, Tetric EvoCeram®, Tetric EvoFlow®. The samples ($n = 4$) were polymerized according to the instructions of the manufacturer of RBCs. The samples were immersed into native saliva, protein-free saliva (artificial saliva), water and ethyl acetate, and incubated at 37 °C for 24 h or 72 h. The eluates were analyzed by gas chromatography/mass spectrometry. To determine the binding to salivary proteins, the concentration of (co)monomers and additives detected in native saliva was compared to the concentration of (co)monomers and additives detected in protein-free saliva, water and ethyl acetate respectively.

To assess the affinity of TEGDMA, EGDMA, DEGDMA, PMGDMA, BPA, and DCHP to human serum albumin (HSA) and human α_1-acid glycoprotein (AGP), a plasma protein binding assay (ABNOVA, Transil XL PPB Prediction Kit TMP-0212-2096) was performed.

The statistical significance ($p < 0.05$) of the difference between the experimental groups was tested using the one-way-analysis of variance (ANOVA), followed by Tukey's analysis.

Results. The concentration of TEGDMA, GMA and CyHEMA released in native saliva was significantly lower than the concentration released in protein-free saliva or water (Admira®

* *Corresponding author*: Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany. Tel.: +49 89 2180 73842; fax: +49 89 2180 73841.
E-mail address: lena.rothmund@gmx.de (L. Rothmund).

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flow: concentration of TEGDMA after 72 h: 0.08 mmol/L (native saliva), 0.34 mmol/L (protein-free saliva), 0.39 mmol/L (water)). The concentrations of HEMA, EGDMA, DDDMA and CQ released in native saliva remained even below the detection limit, compared to the other extraction media.

Protein binding of the tested methacrylates to HSA + AGP was 82–85%, the binding of DCHP was 96.6%, and the binding of BPA was 95.2%.

Significance. Artificial saliva or water as extraction medium does not reflect the real physiological situation in the body. Salivary and plasma proteins may bind (co)monomers and additives and may thereby contribute to a lower bioavailability of leachables from RBCs in vivo than previously thought.

1. Introduction

In the last decade the use of resin based composites (RBCs) has increased tremendously. RBCs, consisting of a number of (co)monomers and additives, belong to the most commonly used filling materials. Due to the incomplete (co)monomer-polymer conversion, a release of the unpolymerized (co)monomers from the dental composite is possible [1,2]. There are many in vitro studies on the toxicity and biocompatibility, which have shown that some of the eluted (co)monomers and additives even have estrogenic, mutagenic, teratogenic and genotoxic effects [3–6]. Previous in vivo studies have demonstrated that HEMA, TEGDMA and BisGMA can be metabolized to the expoxy compound 2,3-epoxymethacrylic acid in hepatic microsomes [7–9]. Epoxides are regarded as mutagenic and carcinogenic agents [10–12].

A previous study has shown that the amount of eluted (co)monomers and their recovery rate is dependent on the medium used for extraction. Thus, the recovery rate of TEGDMA by HPLC analysis is lower if a cell culture medium with fetal calf serum is used, which contains a high amount of proteins [13].

To assess the toxicity and biocompatibility of dental composites, it is not only important to know which (co)monomers and additives are released and in what quantity, but whether these substances may also bind to proteins. This protein binding could lead to a reduced bioavailability in vivo [14]. Only the free, non-protein-bound fraction of an active agent may exert pharmacological or toxicological effects, penetrate cell membranes and can be eliminated [14].

Proteins are present in a concentration of 2.5 g/L in saliva and they have a decisive influence on the properties of saliva [15]. Overall, saliva contains more than 1000 proteins with a molecular weight from 40 to 1000 kDa and are almost exclusively glycoproteins [16]. In quantity, the mucins dominate, which occur in two basic forms in saliva. Mucins are generally classified in high molecular weight proteins (MUC5B, former MG1) and low molecular weight proteins (MUC7, former MG2) [16]. Biologically active and antibacterial salivary proteins are: agglutinin, lactoferrin, lysozyme, peroxidases, histidine-rich proteins (histatins), defensins and cystatins [17,18].

The aim of this study was to examine the binding of releasable ingredients from the investigated RBCs to the above-mentioned proteins. For this purpose native saliva (with proteins) was used as an extraction medium for the elution experiments in addition to protein-free saliva, water or ethyl acetate.

The concentration of (co)monomers and additives released in native saliva was compared to the concentration of (co)monomers and additives released in protein-free saliva, water and ethyl acetate, respectively. (Co)monomers and additives can also be swallowed and can enter the blood and finally the whole organism by absorption via the intestinal tract [19]. In addition to the knowledge of the binding of (co)monomers to salivary proteins, the binding to plasma proteins is also important to assess the toxicity of dental materials. Therefore, the binding of some methacrylates and additives to specific plasma proteins was also examined. The null hypotheses tested was that the recovery rate of (co)monomers and additives via GC–MS would not be influenced by salivary or plasma proteins.

2. Materials and methods

2.1. Chemicals

All chemicals and reagent products were obtained from Merck, Darmstadt, Germany and were of highest purity available. RBCs tested in the elution study are shown in Table 1. Four flowable composites and Tetric Evo Ceram® (a nano-hybrid composite) were investigated. The flowable composites were chosen because preliminary tests have shown, that the tested flowables can elute more (co)monomers which were considered more relevant for our study.

To assess the plasma protein binding to human serum albumin (HSA) + α_1-glycoprotein (AGP), a protein binding assay (Transil XL PPB Prediction Kit TMP-0212-2096) from ABNOVA (Taipei, Taiwan) was performed.

All substances detected in the elution study and tested in the protein binding assay with abbreviations are shown in Table 2.

2.2. Preparation of samples

From each of the five light-curable RBCs (Table 1), 32 samples ($n = 4$) of approximately 100 mg (thickness of 2 mm; diameter of 5 mm; surface of the cylinder of 70.65 mm^2) were prepared according to standard procedure as follows. For the

Table 1 – Investigated dental materials, manufacturer and lot numbers; composition of each material based on manufacturer's data; curing time recommended by manufacturer.

Composite; Manufacturer; [LOT in parenthesis]	Composition of material based on manufacturer's data	Curing time recommended by manufacturer
Admira Flow; VOCO GmbH, Cuxhaven, Germany; [1232535]	N/A	40 s for shade A3 in layers of max. 2 mm
Venus® Diamond Flow; Heraeus Kulzer GmbH; Hanau; Germany; [010100]	UDMA, Bis-EMA, Ba-Al-F silicate glass, YbF3; SiO_2	20 s for shade A3 in layers of max. 2 mm
Filtek™ Supreme XTE Flow; 3 M ESPE, Seefeld, Germany; [N336909]	Bis-GMA, TEGDMA and Bis-EMA, silanized ceramic; silanized silicic acid; dimethacrylate-polymer (functionalized); zirconium oxide powder glass (silanized), additives	20 s for shade A3 in layers of max. 2 mm
Tetric Ceram®; Ivoclar Vivadent, Ellwangen, Germany; [R09964]	Bis-GMA, UDMA, and Bis-EMA, barium glass, ytterbium trifluoride, mixed oxide, prepolymer, additives, catalysts, stabilizers, pigments	20 s for shade A3 in layers of max. 2 mm
Tetric Flow®; Ivoclar Vivadent, Ellwangen, Germany; [R54703]	Bis-GMA, UDMA and DDDMA, barium glass, ytterbium trifluoride, mixed oxide,highly dispersed silicon dioxide, copolymer, additives, catalysts, stabilizers, pigments	20 s for shade A3 in layers of max. 2 mm

Abbreviations: Bis-GMA: bisphenol-A-glycidylmethacrylate; TEGDMA: triethylene glycol dimethacrylate; Bis-EMA: ethoxylated bisphenol-A-dimethacrylate; UDMA: urethane dimethacrylate; DDDMA, 1,10-decanediol dimethacrylate.
N/A: no data available.

Table 2 – Abbreviations of compounds found in RBCs and tested in protein-binding assay; Limit of detection (mmol/L).

Compound Abbreviation	Compound	Limit of detection [mmol/L]
CyHEMA (comonomer)	Cyclohexylmethacrylate	0.001
DDDMA (comonomer)	1,10-decandiol dimethacrylate	0.0005
DEGDMA (comonomer)	Diethylene glycol dimethacrylate	0.004
EGDMA (comonomer)	Ethylene glycol dimethacrylate	0.004
GMA (comonomer)	Glycidyl methacrylate	0.003
HEMA (comonomer)	Hydroxyethyl methacrylate	0.001
PMGMA (comonomer)	Pentamethylene glycol methacrylate	0.0005
TEGDMA (comonomer)	Triethylene glycol dimethacrylate	0.001
BPA (decomposition product of monomers)	Bisphenol A	0.00008
BHT (inhibitor, antioxidant)	2,6-Di-t-butyl-4-methyl phenol	0.00005
CQ (photoinitiator)	Camphorquinone	0.0006
DMABEE (coinitiatior)	4-N,N-dimethylaminobenzoic acid butyl ethoxy ester	0.0003
DCHP (softener)	Dicyclo hexyl phthalate	0.0004
DEDHTP (softener)	Diethyl-2,5-dihydroxyterephthalate	0.0002

preparation of the samples, polytetrafluoroethylene (PTFE) rings were used. The PTFE rings were filled with uncured dental material, covered with plastic strips (Frasaco, Tettnang, Germany) to prevent the formation of an oxygen inhibition layer and were finally polymerized with a LED-lamp (Elipar S^{TM}10® high intensity halogen light, 1200 mW/cm^2, 3 M ESPE, Seefeld, Germany) in accordance with the manufacturer's instructions (Table 1). The curing unit was directly applied on the sample's surface. The light intensity was controlled with Demetron® Radiometer (Kerr, USA) and was always between 1100 and 1200 mW/cm^2.

Directly after curing, the samples were immersed into 500 μL of native saliva, protein-free saliva, water (LC–MS-Grade, ROTISOLV®, Roth, Karlsruhe, Germany) and ethyl acetate (LC–MS-Grade, ROTISOLV® ≥ 99.9%, Roth, Karlsruhe, Germany), respectively. The samples were stored in the absence of light in brown GC-vials (Macherey-Nagel, Düren, Germany) at 37 °C for 24 h and 72 h, respectively.

Native saliva was collected from three volunteers as described in previous studies [20]: the volunteers were asked not to swallow and spat into a 50 mL Falcon-Tube. The saliva was immediately used as extraction medium without prior freezing. To produce protein-free saliva as extraction medium, native saliva was centrifuged for 45 min by using Amicon filter (Cut-off 3 kDa) (Amicon Ultra-4 Centrifugal Filter Devices, Merck, Darmstadt, Germany). Ethyl acetate was used as a slightly polar and aprotic solvent since it dissolves even higher lipophilic ingredients from dental composites.

2.2.1. *Preparation of samples in protein-free saliva and water*

After 24 h and 72 h, 10 μL of an aqueous caffeine solution (0.01 mg/mL) was added as internal standard to determine the relative quantities of substances released from RCBs (see Section 2.3) and the sample was mixed for 30 s. The resulting eluate was transferred to 15 mL tubes and extracted with

200 μL of ethyl acetate. To optimize layer separation, the samples were centrifuged at 2800 rpm for 10 min. A total of 100 μL of the organic layer was analyzed by gas chromatography/mass spectrometry (GC–MS).

2.2.2. *Preparation of samples in native saliva*

The internal standard was added according to Section 2.2.1. The eluate was subsequently transferred into the Amicon filter and was centrifuged for 45 min at 4000 rpm. The filtrate was then extracted with 200 μL of ethyl acetate and centrifuged at 2800 rpm for 10 min. A total of 100 μL of the organic layer was analyzed by GC–MS.

2.2.3. *Preparation of samples in ethyl acetate*

After 24 h and 72 h, a caffeine solution (0.01 mg/mL) in ethyl acetate was added as internal standard and the sample was mixed. Afterwards, 100 μL of the eluate was analyzed by GC–MS.

2.3. *Analytical procedure*

The analysis of the eluates was performed on a Finnigan Trace GC ultra gas chromatograph connected to a Dual-Stage Quadrupole (DSQ) mass spectrometer (Thermo Electron, Dreieich, Germany). A Factor Four® capillary column (length 25 m, inner diameter 0.25 mm; coating 0.25 μm; Varian, Darmstadt, Germany) was used as the capillary column for GC. The GC oven was heated from 50 °C (2 min isotherm) to 280 °C (5 min isotherm) with a rate of 25 °C/min, and 1 μL of the solution was injected with a split ratio of 1:30. Helium 5.0 was used as carrier gas at a constant flow rate of 1 mL/min. The temperature of the split–splitless injector, as well as of the direct coupling to the mass spectrometer, was 250 °C. MS was operated in the electron ionization mode (EI, 70 eV), ion source was operated at 240 °C; only positive ions were scanned. The performed scan ran over the range *m/z* 50–600 at a scan rate of 4 scan/s for scans operated in full scan mode in order to qualify the analytes.

The results were referred to an internal caffeine standard (0.01 mg/mL caffeine = 100%), which allows to determine the relative quantities of substances released from various resin-based materials as described in previous studies [21,22]. All eluates were analyzed five times ($n = 5$). Values of integrated areas of the relevant signals of the compounds were compared to the corresponding reference standards and normalized by means of the caffeine standard. Identification of the various substances was achieved by comparing their mass spectra with those of reference standards, the NIST/EPA library and literature data [23]. Detection limit for each compound is listed in Table 2.

2.4. *Plasma protein binding assay*

To determine the plasma protein binding of TEGDMA, EGDMA, DEGDMA, PMGDM, BPA and DCHP to HSA + AGP (mixed in each well at a physiological ratio of 24:1), a protein binding assay (ABNOVA, Transil® XL PPB Prediction Kit TMP 0212-2096) was performed. As a positive control propranolol hydrochloride was used due to its high protein binding and as a negative control caffeine was chosen due to its low protein binding properties [24,25].

The assay was performed exactly according to the manufacturer's instructions. Plasma protein binding was determined by incubating a fixed concentration of the above mentioned substances with varying concentrations of the plasma proteins HSA and AGP. The compounds tested were dissolved in dimethyl sulfoxide (DMSO) and a stock solution was prepared (80 μM). The final DMSO concentration was 2%. The frozen plate was thawed at 25 °C for 3 h and then centrifuged for 5 s at 750 g. Fifteen μL from a stock solution with 80 μM of the test compound were added, thus retaining the final assay concentration at 80 μM. The substances were incubated for 12 min on a plate shaker at 1000 rpm. After incubation, the plate was centrifuged for 10 min at 750 g. The supernatant (150 μL) was removed and extracted with 100 μL of ethyl acetate and then analyzed by GC–MS.

2.4.1. *Derivatization of propranolol hydrochloride with pentafluorobenzoyl chloride*

In order to analyze the positive control propranolol of the protein binding assay by GC–MS, the process of derivatization was necessary. For derivatization of propranolol, pentafluorobenzoyl chloride was used. First, the propranolol dissolved in wells was transferred into the corresponding base by 10% NaOH aq. Afterwards the free base was extracted with 100 μL of dichloromethane. The propranolol base was derivatized in the presence of trimethylamine with pentafluorobenzoyl chloride. Ten μL of the propranolol base were mixed with 150 μL of the derivatization reagent and incubated at 25 °C for 2 h. After 2 h the sample was reduced under a gentle stream of N_2 to dryness. A total of 100 μL of dichloromethane was added and analyzed by GC–MS [26].

2.5. *Calculations and statistics*

The results are presented as the mean ± standard deviation (SD). The statistical significance ($\alpha = 0.05$) of the differences between the experimental groups was tested using the one-way-ANOVA, followed by Tukey's analysis [13].

3. Results

3.1. *Elution test*

Since there were only few significant differences in the amount of (co)monomers and additives released from RBCs between 24 and 72 h, only the 72 h results are shown in graphic form (Figs. 1–5).

Significant differences between 24 and 72 h:

- Admira® flow: DMABEE in the ethyl acetate eluate after 72 h (0.09 mmol/L) significantly higher than after 24 h (below limit of detection).
- Venus® Diamond flow: TEGDMA in the protein-free saliva eluate after 72 h (0.05 mmol/L) significantly higher than after 24 h (0.03 mmol/L). TEGDMA in the water eluate after 72 h (0.05 mmol/L) significantly higher than after 24 h (0.03 mmol/L).

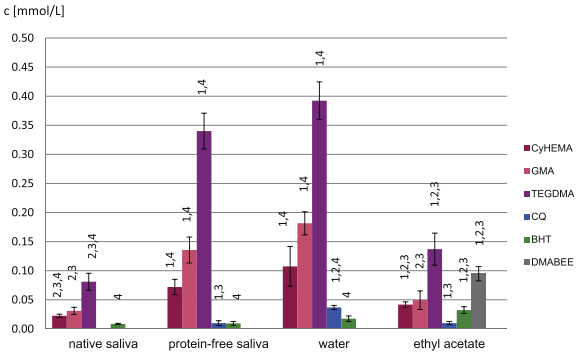

Fig. 1 – Admira® flow 72 h Elution. 1 = significantly ($p < 0.05$) different from native saliva; 2 = significantly ($p < 0.05$) different from protein-free saliva; 3 = significantly ($p < 0.05$) different from water; 4 = significantly ($p < 0.05$) different from ethyl acetate.

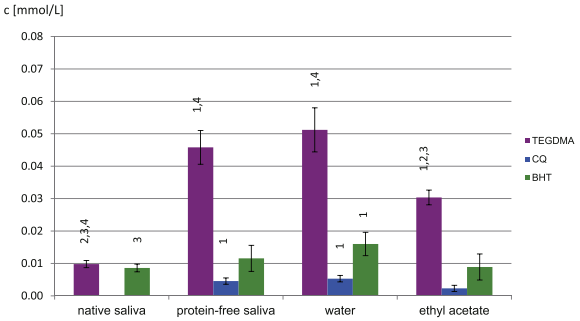

Fig. 2 – Venus® Diamond flow 72 h Elution. 1 = significantly ($p < 0.05$) different from native saliva; 2 = significantly ($p < 0.05$) different from protein-free saliva; 3 = significantly ($p < 0.05$) different from water; 4 = significantly ($p < 0.05$) different from ethyl acetate.

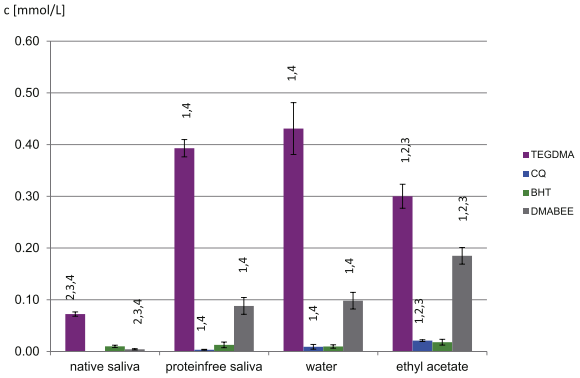

Fig. 3 – Filtek™ supreme XTE flow 72 h Elution. 1 = significantly ($p < 0.05$) different from native saliva; 2 = significantly ($p < 0.05$) different from protein-free saliva; 3 = significantly ($p < 0.05$) different from water; 4 = significantly ($p < 0.05$) different from ethyl acetate.

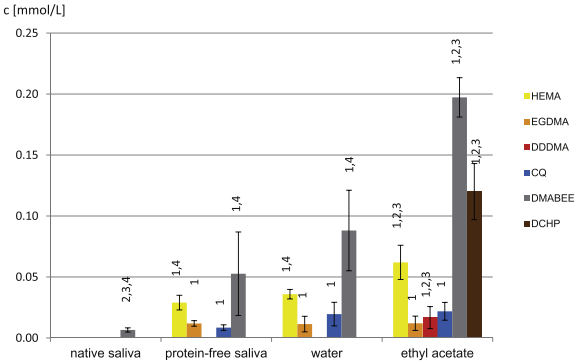

Fig. 4 – Tetric EvoCeram® 72 h Elution. 1 = significantly ($p < 0.05$) different from native saliva; 2 = significantly ($p < 0.05$) different from protein-free saliva; 3 = significantly ($p < 0.05$) different from water; 4 = significantly ($p < 0.05$) different from ethyl acetate.

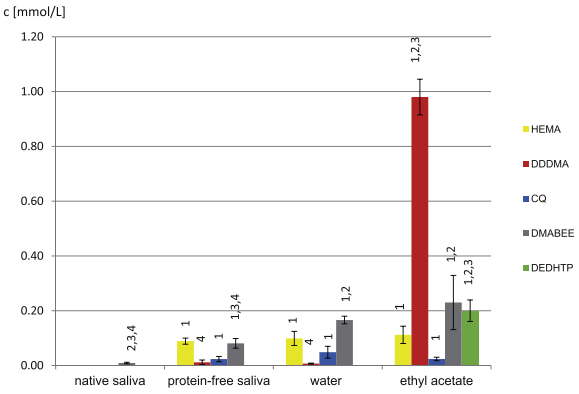

Fig. 5 – Tetric EvoFlow® 72 h Elution. 1 = significantly ($p < 0.05$) different from native saliva; 2 = significantly ($p < 0.05$) different from protein-free saliva; 3 = significantly ($p < 0.05$) different from water; 4 = significantly ($p < 0.05$) different from ethyl acetate.

- Tetric EvoFlow®: DMABEE in the protein-free saliva eluate after 24 h (0.10 mmol/L) significantly higher than after 72 h (0.08 mmol/L). DMABEE in the water eluate after 72 h (0.17 mmol/L) significantly higher than after 24 h (0.09 mmol/L).

3.1.1. *Release of ingredients from Admira® flow (Fig. 1)*

The analysis of all eluates showed a release of CyHEMA, GMA, TEGDMA and BHT. CQ was not detectable in the eluates generated with native saliva.

The concentrations of CyHEMA, GMA and TEGDMA released in native saliva were significantly lower compared to the concentrations of CyHEMA, GMA and TEGDMA released in protein-free saliva or water. The amount of TEGDMA released in native saliva (after 72 h: 0.08 mmol/L) was significantly lower than the amount of TEGDMA released in protein-free saliva

(after 72 h: 0.34 mmol/L) or water (after 72 h: 0.39 mmol/L). No significant differences could be detected between the eluates generated with protein-free saliva or water (exception: amount of CQ released in water after 72 h was significantly higher than in protein-free saliva).

3.1.2. *Release of ingredients from Venus® Diamond flow (Fig. 2)*

The amount of TEGDMA released in native saliva (after 72 h: 0.01 mmol/L) was significantly lower compared to the amount of TEGDMA released in protein-free saliva (after 72 h: 0.05 mmol/L) or water (after 72 h: 0.05 mmol/L).

CQ could not be detected in native saliva. The concentration of TEGDMA, CQ or BHT released in protein-free saliva did not differ significantly from the concentration released in water.

3.1.3. *Release of ingredients from Filtek™ Supreme XTE flow (Fig. 3)*

The analysis of the eluates showed a release of TEGDMA, CQ, BHT and DMABEE. CQ could not be detected in the eluates generated with native saliva. The highest concentration of TEGDMA was released in water (0.43 mmol/L) after 72 h. This value was significantly higher than the amount of TEGDMA released in native saliva (0.07 mmol/L). The amount of TEGDMA, CQ, BHT and DMABEE released in protein-free saliva did not differ significantly from the amount of TEGDMA, CQ, BHT and DMABEE released in water.

3.1.4. *Release of ingredients from Tetric EvoCeram® (Fig. 4)*

In the eluates generated with native saliva, only DMABEE could be detected. The concentration of HEMA, EGDMA, CQ and DMABEE released in protein-free saliva did not differ significantly from the concentration released in water. DDDMA (after 72 h: 0.02 mmol/L) and DCHP (after 72 h: 0.12 mmol/L) could only be detected in the eluates generated with ethyl acetate.

3.1.5. *Release of ingredients from Tetric EvoFlow® (Fig. 5)*

In the eluates generated with native saliva, only DMABEE could be detected. The concentration of HEMA, DDDMA, CQ and DMABEE released in protein-free saliva did not differ significantly from the concentration released in water. The highest concentration of DDDMA was detected after 72 h in the eluates generated with ethyl acetate (0.98 mmol/L). DEDHTP could only be detected in the ethyl acetate eluate (after 72 h: 0.2 mmol/L).

3.2. *Binding of methacrylates and additives to plasma proteins*

The results of the plasma-protein binding assay are shown in Table 3. The highest binding to HSA + AGP was detected for DCHP at 96.6%. The lowest binding to HSA + AGP was detected for PMGDM at 82.0%.

Table 3 – Plasma-protein binding of BPA, DEGDMA, EGDMA, PMGDMA TEGDMA and DCHP to HSA and AGP (%, mean ± SD, *n* = 3).

Protein binding to HSA und AGP	[%]
BPA	95.2 ± 0.46
DEGDMA	83.5 ± 1.38
EGDMA	84.7 ± 1.59
PMGDMA	82.0 ± 2.11
TEGDMA	82.5 ± 1.79
DCHP	96.6 ± 2.39
Propranolol hydrochloride (positive control)	93.6 ± 0.11
Caffeine (negative control)	40.6 ± 7.90

4. Discussion

Various factors may have an influence on the release of (co)monomers from RBCs. It was shown in previous studies that the amount released depends substantially on the extraction medium used, in addition to mechanical stress (due to chewing), the degree of polymerization of the composite and the porosity of the material [27,28]. The influence of the extraction medium can be explained by the Nernst partition coefficient, which was determined for numerous leachables from RBCs by Durner et al. [29]. According to the Nernst partition coefficient, water and protein-free saliva eluted almost the same amount of (co)monomers and additives in our study, highly lipophilic compounds like DDDMA were mostly eluted by ethyl acetate. This result is consistent with an earlier study [30], which demonstrated a very similar ability of artificial saliva and water to elute (co)monomers and additives. However, neither water nor artificial saliva reflects the real physiological situation in the oral cavity. In the present study, therefore, the effect of native saliva on the elution of (co)monomers and additives was evaluated. In order to obtain knowledge about the influence of the extraction medium, native saliva (containing proteins), protein-free saliva, water or ethyl acetate were used. The present elution study demonstrates that the amount of detectable (co)monomers and additives strongly depends on the extraction medium that was used. Each one of the five tested composites showed a large difference between native saliva, in which only very small amounts of (co)monomers and additives could be detected and the three other extraction media (protein-free saliva, water, and ethyl acetate).

For example, it was observed for Admira® flow that the maximum concentration of TEGDMA measured in the physiological situation (in native saliva) at 0.08 mmol/L was significantly lower – by about a factor of five – than the concentration of TEGDMA in the water eluate (0.39 mmol/L) or protein-free saliva eluate (0.34 mmol/L). For TEGDMA, the same effect of native saliva and protein-free saliva or water could be observed in Venus® Diamond flow and Filtek™ supreme XTE flow. Furthermore, it was noticed that CQ and HEMA were not detectable in native saliva compared to protein-free saliva and water.

The reason for the low recovery rate of (co)momomers might be based on the composition of native saliva. Native saliva contains various salts up to 3% and glycoproteins

(mainly mucins) [16], which may bind (co)monomers and additives. There a few studies investigating (co)monomers and additives in human saliva [31–33], but there is no study which compares the extraction media water, protein-free saliva and native saliva with regard to the binding of (co)monomers and additives to salivary proteins. Only for TEGDMA, it was demonstrated in a previous study that the recovery rate in a serum-containing cell culture medium was much lower, compared to the recovery rate of TEGDMA in a serum-free cell culture medium, distilled water, saline or artificial saliva [13]. Thereupon, an interaction of TEGDMA with the ingredients of the serum-containing cell culture medium was postulated. The low recovery rate of (co)monomers and additives in native saliva, which was observed in this study, compared to the higher recovery rate of (co) monomers and additives in protein-free saliva and water, emphasizes that it is important to choose the right extraction medium for the elution. Both for the evaluation of the toxicity and for a reasonable risk assessment of the (co)monomers and additives, it is therefore important to know in what quantities these substances are released from the composite and in what concentrations they are present in native human saliva. In previous studies (co)monomers and additives indicated concentration-dependent cytotoxic effects, such as increased cell death, damage of the plasma membrane or an increased content of released lactate dehydrogenase [34–36].

TEGDMA was detected in the present study in the eluates of the composites Filtek™ supreme XTE flow, Venus® Diamond flow and Admira® flow. The highest concentration of TEGDMA was found in the water eluate of Filtek™ supreme XTE flow after 72 h (0.43 mmol/L) and was almost ten times lower than the known cytotoxic concentration for human gingival fibroblasts (HGF) (3.7 mmol/L) [37].

TEGDMA and HEMA are the most commonly used comonomers in dental composites. HEMA could be detected in the eluates from Tetric EvoFlow® and Tetric EvoCeram®. It is known that HEMA can cause cytotoxic and genotoxic effects [38]. Besides, HEMA can induce a higher concentration of reactive oxygen species (ROS) at 3 mmol/L [39,40]. The highest HEMA concentration measured at 0.18 mmol/L was detected in the ethyl acetate eluate of Tetric EvoFlow® after 24 h. In previous studies cytotoxic concentrations for HEMA at 2.4 mmol/L in human pulp fibroblasts [3] and at 11.9 mmol/L in human gingival fibroblasts [37] were found. The HEMA concentrations detected in all extraction media in our study are therefore far below those cytotoxic concentrations, described by Geurtsen et al. [3] and Reichl et al. [37].

The photoinitiator CQ is a component of all the composites examined. In the present study CQ was found in the eluates generated with protein-free saliva, water and ethyl acetate. In the native saliva eluates, CQ remained below the detection limit. CQ is considered a powerful allergen [41], which can also cause oxidative stress and DNA damage [42]. The highest CQ concentration was measured in the water eluates of Tetric EvoFlow® after 72 h at 0.05 mmol/L. Previous studies indicated a significant concentration-dependent increase of intracellular ROS and DNA damage in hGF starting at a concentration of 0.05 mmol/L [42]. Since the concentrations of CQ in all eluates with native saliva were below the detection limit (0.0006 mmol/L), the physiological concentration in the oral cavity is therefore likely to remain – by about a factor of 80 – below the genotoxic concentration.

In order to improve the degree of polymerization of a composite, co-initiators such as DMABEE are usually added. DMABEE can induce apoptosis in human monoblastoid cells and necrosis at 10 μM [43]. The highest DMABEE concentration (0.23 mmol/L) was detected in the ethyl acetate eluate of Tetric EvoFlow® after 72 h. This is five times lower than the stated cytotoxic concentration for human oral mucosa cells (1.23 mmol/L) [3] and 20-fold higher than the minimum apoptotic or necrotic concentration [43]. This finding however, should not be cause for concern, as the concentration in the physiological situation (native saliva: 0.009 mmol/L) does not reach the apoptotic or necrotic concentration.

Plasma protein binding of methacrylates and additives to HSA and AGP

(Co)monomers can be released from dental materials into saliva and they can enter the systemic circulation by absorption via the intestinal tract [19]. Additionally, (co)monomers from composites and adhesives may diffuse through dentin into the pulp and become bioavailable for metabolism [44]. In hepatic microsomes TEGDMA can be metabolized in vivo to 2,3-epoxymethacrylic acid [9]. The formation of the toxic 2,3-epoxymethacrylic acid as intermediate in the metabolism of dental materials could not only be observed in hepatic microsomes but also in cells of the oral mucosa (gingival and pulp fibroblasts) [7,45]. Epoxides are regarded as cytotoxic and mutagenic substances [46–48].

Only the free, non-protein-bound fraction of active substances can exert pharmacological or toxicological effects, penetrate cell membranes, and can be eliminated [14]. Considering the results of the present plasma protein binding assay, only approximately 18% of TEGDMA would be available for the metabolism to highly reactive epoxides, due to the binding to HSA or AGP (binding of the tested methacrylates to HSA and AGP was 82–85%). The values determined for the negative control caffeine and the positive control propranolol were in accordance with the known values from the literature (caffeine: 41%; propranolol: 94 %) [24,25].

The binding of active ingredients or contaminants to proteins (plasma proteins, transport proteins) is usually reversible [49]. Due to the high reactivity of methacrylates the formation of covalent bonds (e.g. with thiol groups of proteins) might also be possible [50]. Due to their alpha-beta-unsaturated carbonylic acid moiety, methacrylates represent Michael acceptors which can easily react with bionucleophiles. This was already described by the reaction of Michael acceptors with glutathione [51]. The protein binding of methacrylates could contribute to a reduced exposure to epoxides, as the parent compounds may be available only at lower levels for epoxidation in liver or gingival cells, or could be converted even slower to the corresponding epoxide. Epoxide hydrolases and glutathione S-transferases can be found ubiquitous in all body tissues. They convert epoxides to the corresponding non-toxic dihydrodiols and glutathione-conjugates [52]. Therefore, they could potentially lead to lower concentrations of epoxides and thus contribute to a lower exposition of these toxic

intermediates. The results of the present study showed that (co)monomers and additives are bound to proteins in both the oral cavity and in the plasma. The null hypotheses tested in our study is therefore rejected. In the physiological situation the local exposure to components from RBCs is therefore likely to be lower than previously thought.

5. Conclusion

The concentration of (co)monomers and additives released into native saliva is significantly lower than the concentration released in protein-free saliva or water. This could result in a lower exposure of (co)monomers and additives in the physiological situation than previously thought.

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2.1.3. Einfluss von Bleachinggelen auf die Freisetzung von Inhaltsstoffen aus konventionellen dentalen Kompositen und Bulk-Fill Kompositen [46, 47]

Bleichbehandlungen können sich auf das Polymernetzwerk von Zahnkompositen auswirken. In den beiden vorliegenden Studien wurde der Einfluss verschiedener Bleachinggele auf die Freisetzung von Kompositinhaltsstoffen untersucht. Die konventionellen Komposite Tetric EvoCeram, CLEARFIL AP-X, Tetric EvoFlow, Filtek Supreme XT, Ceram X mono+, Admira und Filtek Silorane, und Bulk-Fill Komposite Tetric EvoCeram Bulk Fill, QuiXFill und X-tra Fill wurden gemäß den Anweisungen des Herstellers polymerisiert und mit den Bleichgelen Opalescence PF 15% (PF 15%) 5 h und PF 35% (PF 35%) 30 min lang behandelt. Anschließend wurden die Proben jeweils 24 h und 7d in Methanol und Wasser inkubiert und die resultierenden Eluate mittels GC/MS analysiert. Ungebleichte Kompositproben dienten als Kontrollgruppe. Bei den konventionellen Kompositen wurden nach der Bleichbehandlung insgesamt 16 verschiedene eluierbare Substanzen identifiziert. Darunter befanden sich 6 Methacrylate: 1,10-Decandioldimethacrylat (DDDMA), 1,12-Dodekandioldimethacrylat (DODDMA), Ethylenglycoldimethacrylat (EGDMA), 2-Hydroxyethylmethacrylat (HEMA), Triethylenglycoldimethacrylat (TEGDMA) und Urethandimethacrylat (UDMA). Im Vergleich zu den ungebleichten Kontrollen zeigten die Komposite Tetric EvoCeram, CLEARFIL AP-X und Tetric EvoFlow nach der Bleichbehandlung eine geringere Elution von UDMA, TEGDMA und HEMA. Eine erhöhte Elution von UDMA, DMABEE, BPA und TEGDMA wurde für die Komposite Filte Supreme XT, Ceram X mono+, Admira und Filtek Silorane nach der Bleichbehandlung im Vergleich zu den ungebleichten Kontrollen festgestellt. Bei den untersuchten Bulk-Fill Kompositen wurden nach der Bleichbehandlung insgesamt 7 verschiedene eluierbare Substanzen identifiziert. Drei davon waren Methacrylate: HEMA, TEGDMA und Trimethylolpropantrimethacrylat (TMPTMA). Im Vergleich zu den ungebleichten Kontrollen wurde nach der PF-15%-Behandlung ein Anstieg der Elution der folgenden Verbindungen festgestellt: HEMA (Tetric EvoCeram Bulk Fill), TEGDMA (QuiXFill, X-tra fil) und 4-N,N-Dimethylaminobenzoesäurebutylethoxyester (DMABEE) (Tetric EvoCeram Bulk Fill, QuiXFill, X-tra Fill). Die folgenden Verbindungen zeigten eine Verringerung der Elution nach PF 35%-Behandlung im Vergleich zu den Kontrollen: TEGDMA (QuiXFill) und DMABEE (Tetric EvoCeram Bulk Fill). Zusammenfassend können Bleichbehandlungen bei konventionellen und Bulk-Fill Kompositen sowohl zu einer verringerten als auch zu einer erhöhten Elution von Inhaltsstoffen im Vergleich zur unbehandelten Kontrolle führen.

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Effect of Opalescence® bleaching gels on the elution of dental composite components

Lena Schuster[a,b], *Lena Rothmund*[a,b], *Xiuli He*[a,b], *Kirsten L. Van Landuyt*[c], *Helmut Schweikl*[d], *Elmar Hellwig*[e], *Thomas Carell*[f], *Reinhard Hickel*[a], *Franz-Xaver Reichl*[a,b], *Christof Högg*[a,b,*]

[a] *Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany*
[b] *Walther-Straub-Institute of Pharmacology and Toxicology, Nussbaumstr. 26, 80336 Munich, Germany*
[c] *KU Leuven Biomat, Department of Oral Health Sciences KU Leuven, Kapucijnenvoer 7, 3000 Leuven, Belgium*
[d] *Department of Operative Dentistry and Periodontology, University Hospital Regensburg, University of Regensburg, 93042 Regensburg, Germany*
[e] *Department of Operative Dentistry and Periodontology, Albert-Ludwigs-University, 79106 Freiburg, Germany*
[f] *Department of Organic Chemistry, Ludwig-Maximilians-University of Munich, Butenandtstr. 5-13, House F, 81377 Munich, Germany*

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ABSTRACT

Objectives. Bleaching treatments can affect on the polymer network of dental composites. This study was performed to evaluate the influence of different bleaching treatments on the elution of composite components.

Methods. The composites Tetric EvoCeram®, CLEARFIL™ AP-X, Tetric EvoFlow®, Filtek™ Supreme XT, Ceram X® mono+, Admira and Filtek™ Silorane were treated with the bleaching gels Opalescence PF 15% (PF 15%) for 5 h and PF 35% (PF 35%) for 30 min and then stored in methanol and water for 24 h and 7 d. The eluates were analyzed by gas chromatography/mass spectrometry (GC/MS). Unbleached specimens were used as control group.

Results. A total of 16 different elutable substances have been identified from the investigated composites after bleaching-treatment. Six of them were methacrylates: 1,10-decandioldimethacrylate (DDDMA), 1,12-dodekandioldimethacrylate (DODDMA), ethylenglycoldimethacrylate (EGDMA), 2-hydroxyethylmethacrylate (HEMA), triethylenglycoldimethacrylate (TEGDMA) and urethandimethacrylate (UDMA). Compared with the unbleached controls the composites Tetric EvoCeram®, CLEARFIL™ AP-X and Tetric EvoFlow® showed a reduced elution of UDMA, TEGDMA and HEMA after bleaching-treatment. Compared with the unbleached controls an increase elution of UDMA, DMABEE, BPA and TEGDMA for the composites Filtek™ Supreme XT, Ceram X® mono+, Admira and Filtek™ Silorane after bleaching-treatment has been detected. The highest concentration of UDMA was 0.01 mmol/l (Tetric EvoCeram®, water, 24 h, controls), the highest concentration of TEGDMA was 0.28 mmol/l (CLEARFIL™ AP-X, water, 7 d, controls), the highest concentration of HEMA was 0.74 mmol/l (Tetric EvoFlow®, methanol, 7 d, PF 35%), the highest

* *Corresponding author at*: Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany. Tel.: +49 89 2180 73809; fax: +49 89 7095 73817.
E-mail address: christof.hoegg@lrz.uni-muenchen.de (C. Högg).

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concentration of DMABEE was 0.10 mmol/l (Ceram X® mono+, water, 7 d, PF 35%) and the highest concentration of BPA was 0.01 mmol/l (Admira, methanol, 7 d, controls).
Significance. Bleaching treatments can lead to a reduced or an increased elution of substances from the dental composites.

1. Introduction

The consciousness of asthetic appearance in society is increasing: lots of patients are not satisfied with the color of their teeth [1]. There are various possibilities of whitening teeth, so personal esthetic expectations of the patient can be fulfilled [2]. Bleaching methods are based on carbamide- or hydrogen peroxide gel: (1) over-the-counter products (maximum 10% peroxides); (2) home bleaching methods (15% peroxides); (3) in-office-bleaching methods (35% peroxides) and finally chairside-bleaching methods (38% peroxides) [3,4]. Bleaching trays are only used with the home bleaching and the in-office-bleaching method [5].

The use of resin based composites are rapidly evolving and patients are more aware of, and demanding of, esthetic tooth-colored restorations [6]. So in the past various studies were carried out to examine the reaction of dental composites on bleaching products: thereby the microhardness [7–10] and the surface texture [8,11–13] were tested. Further studies were carried out whether bleaching treatment have an influence on color of dental composites [14–16].

Patients undergoing bleaching treatment often have insufficient restorations which have to be replaced before starting the bleaching procedure preventing the contact of bleaching gel with the pulp which could result in pulp damage such as apoptosis of human dental pulp cells or irritation of the tooth [17].

Our earlier studies showed that bleaching treatments can affect the elution of monomers and other substances from dental composites [18–20]. (Co)monomers (methacrylates) besides initiators, stabilizers, additives and pigments are part of the organic resin matrix of unpolymerized composites [21]. The polymerization of composites is incomplete: the lower the conversion rate of a composite the more residual (co)monomers can be eluted [22]. Elutable residual (co)monomers (methacrylates) can cause allergic reactions [23] such as asthma, rhinoconjunctivitis allergica or contact dermatitis [24].

Numerous studies concerning the monomer elution from dental composites have been carried out so far [25–27]. Less data are available about the influence on the amount of elutable substances from dental composites after previous bleaching procedures. Bleaching treatments can have an effect on the three-dimensional polymer network of dental composites [19]. The aim of this study was to find out whether different bleaching treatments affect the time-related elution of components from various dental composites.

2. Materials and methods

The tested composites including manufacturers' data and the bleaching gels are listed in Tables 1 and 2.

2.1. Preparation of samples

For sample preparation the method of our earlier study [19] has been improved: the bleaching treatment of the samples has been performed excluding daylight, because light sources additionally can activate peroxides of bleaching gels. Thus the bleaching process could be accelerated [28].

The sample preparation is described in detail: about 100 mg of each unpolymerized composite (Table 1) was inserted in one increment in a Teflon ring (6 mm diameter, thickness 1.9 mm = surface 92.36 mm^3) placed on a plastic matrix strip (Frasaco, Tettnang, Germany). After covering samples with another plastic matrix strip, samples were polymerized according to instruction of manufacturer (Table 1) by using a dental manual light-curing unit (Elipar S10, 3 M ESPE, Seefeld, Germany) which was directly placed on the matrix strip. The light intensity of the LED lamp (1200 mW/cm^2) was checked using Demetron® Radiometer (Kerr, USA).

For bleaching procedure two concentrations of bleaching gel were used exactly according to the instructions of the manufacturer (Table 2): Opalescence® PF 15% Tooth Whitening Systems (PF 15%) (home bleaching procedure) and Opalescence® PF 35% Tooth Whitening Systems (PF 35%) (in-office-bleaching procedure). For each composite (Table 1) 3 groups with 4 samples each ($n = 4$) were prepared: (1) samples bleached with PF 15% for 5 h; (2) samples bleached with PF 35% for 30 min and (3) unbleached samples: control group. During bleaching process the samples were stored excluding daylight.

After bleaching process the bleaching gel was removed: (1) roughly remove by spatula; (2) carefully remove by absolute dry cotton sticks.

Subsequently samples were incubated in brown glass vails (Macherey-Nagel, Düren, Germany) with 1 ml of methanol (GC Ultra Grade, RATISOLV® ≥99.9%, Roth, Karlsruhe, Germany) at 37 °C and analyzed after 1 d and 7 d by GC/MS [19]. As internal standard caffeine (CF) solution (0.01 mg/ml) (HPLC ≥ 99.0%, Sigma–Aldrich, St. Louis, United States) was added.

Water (LC–MS-Grade, ROTISOLV®, Roth, Karlsruhe, Germany) elutions were carried out as outlined above. For GC/MS analysis water samples were extracted with ethyl acetate (1:1, v/v) (LC–MS-Grade, ROTISOLV® ≥99.9%, Roth, Karlsruhe, Germany).

Table 1 – Investigated dental materials, manufacturer, LOT numbers and type; composition of each material based on manufacturer's data.

Product name	Manufacturer	LOT	Type	Composition of materials based on manufacturer's data	Polymerization time based on manufacturer's data
Tetric EvoCeram®	Ivoclar Vivadent AG, Schaan, Liechtenstein	R09964	Nano-hybrid composite	Bis-GMA, UDMA, Bis-EMA; barium-glass, YbF3, mixed oxide, pre-polymerized fillers	10 s
CLEARFIL™ AP-X	Kuraray Europe GmbH, Hattersheim am Main, Germany	01436A	Micro-hybrid composite	Bis-GMA, TEGDMA; silanated barium glass filler, silanated silica filler, silanated colloidal silica	20 s
Tetric EvoFlow®	Ivoclar Vivadent AG, Schaan, Liechtenstein	R54703	Flowable nano-hybrid composite	Bis-GMA, UDMA, DDDMA; barium-glass, YbF3, silicon dioxide, mixed oxide, copolymer	10 s
Filtek ™ Supreme XTE	3 M ESPE, Seefeld, Germany	N408477	Nanocomposite	Bis-GMA, UDMA, TEGDMA, PEGDMA, Bis-EMA; ZrO_2–SiO_2 cluster SiO_2 and ZrO_2 nanofiller	20 s
Ceram X® mono+	DENTSPLY DeTrey GmbH, Konstanz, Germany	1205000769	Universal Nano-Ceramic Restorative	Methacrylate modified polysiloxane, dimethacrylate resin; barium-aluminum-borosilicate glass, silicon dioxide nano filler	10 s
Admira	VOCO GmbH, Cuxhaven, Germany	1229069	Ormocer®	Ormocer, Bis-GMA, UDMA	40 s
Filtek™ Silorane	3 M ESPE, Seefeld, Germany	N383597	Silorane	Hydrophobic resin matrix; fine quartz particles and radiopaque yttrium fluoride	20 s

Bis-EMA: bisphenol A polyetheylene glycol dimethacrylate; Bis-GMA: bisphenol-A diglycidyl dimethacrylate; DDDMA: dekandioldimethacrylate; PEGDMA: poly(ethylene glycol) dimethacrylate; TEGDMA: triethyleneglycol dimethacrylate; UDMA: urethane dimethacrylate.

2.2. *Analytical procedure*

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 Factor Four® capillary column (length 25 m, inner diameter 0.25 mm; coating 0.25 μm; Varian, Darmstadt, Germany) was used as the capillary column for GC. The GC oven was heated from 50 °C (2 min isotherm) to 280 °C (5 min isotherm) with a rate of 25 °C/min, and 1 μl of the solution was injected with a split ratio of 1:30. Helium 5.0 was used as carrier gas at a constant flow rate of 1 ml/min. The temperature of the split–splitless injector, as well as of the direct coupling to the mass spectrometer, was 250 °C. MS was operated in the electron ionization mode (EI, 70 eV), ion source was operated at 240 °C; only positive ions were scanned. The performed scan ran over the range *m/z* 50–600 at a scan rate of 4 scan/s for scans operated in full scan mode in order to qualify the analytes.

The results were referred to an internal caffeine standard (0.1 mg/caffeine = 100%), which allows to determine the relative quantities of substances released from various resin-based materials. All eluates were analyzed five times. The integration of the chromatograms was carried out over the base peak or other characteristic mass peaks of the compounds, and the results were normalized by means of the internal CF standard. Identification of the various substances was achieved by comparing their mass spectra with those of reference compounds, the NIST/EPA library, literature data,

Table 2 – Bleaching materials tested, manufacturers, LOT-numbers.

Product name	Composition of bleaching agent (wt.%) based on manufacturer's data	Abbreviation	Bleaching time based on manufacturer's data	Manufacturer	LOT
Opalescence® tooth whitening systems PF 15%	Carbamide peroxide <25; poly acrylic acid <10; sodium hydroxide <5; sodium fluoride 0.25	PF 15%	4–6 h	Ultradent® Products, Inc., S. South Jordan, UT, USA	B7B15
Opalescence® tooth whitening systems PF 35%	Carbamide peroxide 20; hydrogen peroxide <10; sodium hydroxide <5; sodium fluoride 0.25	PF 35%	30 min	Ultradent® Products, Inc., S. South Jordan, UT, USA	B74MV

Table 3 – Detected eluted substances from the composites treated with the bleaching gels.

Compound abbreviation	Compound
BHT	2,6-Di-t-butyl-4-methylphenol
BPA	Bisphenol-A
BPE	Phenylbenzoat
DCHP	Dicyclohexylphtalat
DDDMA	1,10-Decandioldimethacrylate
DDHT	Diethyl-2,5-dihydroxyterephthalat
DMABEE	4-N,N-dimethylaminobenzoesäureethylester
DODDMA	1,12-dodekandioldimethacrylate
EGDMA	Ethylenglycoldimethacrylate
HEMA	2-Hydroxyethylmethacrylate
HMBP	2-Hydroxy-4-methoxybenzophenon
HQME	Hydrochinone methyl ether
TEGDMA	Triethylenglycoldimethacrylate
TinP	2(2′-Hydroxy-5′-methylphenyl)benzotriazol, Tinuvin P
Tin 328	Tinuvin 328, 2(2′-hydroxy-3′,5′-di-tert-amylphenyl)benzotriazol
UDMA	Urethan dimethacrylate

and/or by a chemical analysis of their fragmentation pattern [29].

Bis-GMA and Bis-EMA were analyzed by LC/MS using the method described in previous study [30]. Detected values for Bis-GMA and Bis-EMA of investigated dental composites were under detection limit.

2.3. Calculations and statistics

The results are presented as means ± standard deviation (SD). The statistical significance ($p < 0.05$) of the differences between the experimental groups was analyzed by one-way ANOVA and the post hoc Test (Tukey's HSD test) [31].

3. Results

A total of 16 different elutable substances (Table 3) have been identified from the investigated composites (Table 1) after bleaching-treatment (Table 2). The quantification and significant differences are shown in Figs. 1–7. In the following only the relevant substances of the eluates of the tested composites such as methacrylates are listed:

3.1. *Tetric EvoCeram®* (Fig. 1)

Methanol 7 d:

The PF 15%-treatment resulted in a significant lower UDMA elution (PF 15%:170.2 μmol/l) compared to the PF 35%-treatment (272.4 μmol/l).

Water 24 h:

The PF 15%-treatment resulted in a significant lower UDMA elution (PF 15%: 6.6 μmol/l) compared to the controls (9.7 μmol/l).

3.2. *CLEARFIL™ AP-X* (Fig. 2)

Methanol 7 d:

The PF 15%-treatment as well as the PF 35%-treatment resulted in a significant lower TEGDMA elution (PF 15%: 69.5 μmol/l; PF 35%: 60.8 μmol/l) compared to the controls (287.1 μmol/l).

Water 7 d:

The PF 15%-treatment resulted in a significant lower TEGDMA elution (PF 15%: 221.5 μmol/l) compared to the controls (280.5 μmol/l).

3.3. *Tetric EvoFlow®* (Fig. 3)

Methanol 24 h:

The PF 15%-treatment resulted in a significant lower HEMA elution (PF 15%: 199.0 μmol/l) compared to the controls (492.4 μmol/l).

Methanol 7 d:

The PF 15%-treatment resulted in a significant lower HEMA elution (PF 15%: 488.5 μmol/l) compared to the PF 35%-treatment (735.9 μmol/l).

3.4. *Filtek™ Supreme XTE* (Fig. 4)

Methanol 24 h:

The PF 15%-treatment resulted in a significant higher UDMA elution (PF 15%: 38.2 μmol/l) compared to the controls (21.8 μmol/l).

3.5. *Ceram X® mono+* (Fig. 5)

Methanol 24 h:

The PF 15%-treatment resulted in a significant higher UDMA elution (PF 15%: 7.8 μmol/l) compared to the controls (1.1 μmol/l) as well as to the PF 35%-treatment (3.5 μmol/l).

Water 7 d:

The PF 15%-treatment as well as the PF 35%-treatment resulted in a significant higher DMABEE elution (PF 15%: 78.0 μmol/l; PF 35%: 99.1 μmol/l) compared to the controls (30.5 μmol/l).

3.6. *Admira* (Fig. 6)

Methanol 24 h:

The PF 15%-treatment resulted in a significant higher BPA elution (PF 15%: 8.2 μmol/l) compared to the controls (3.3 μmol/l).

Water 24 h:

The PF 15%-treatment resulted in a significant higher TEGDMA elution (PF 15%: 106.0 μmol/l) compared to the controls (67.3 μmol/l) as well as to the PF35%-treatment (69.1 μmol/l).

3.7. *Filtek™ Silorane* (Fig. 7)

Methanol 24 h:

The PF 15%-treatment resulted in a significant higher BPA elution (PF 15%: 5.0 μmol/l) compared to the controls (1.9 μmol/l) as well as to the PF 35%-treatment (2.1 μmol/l).

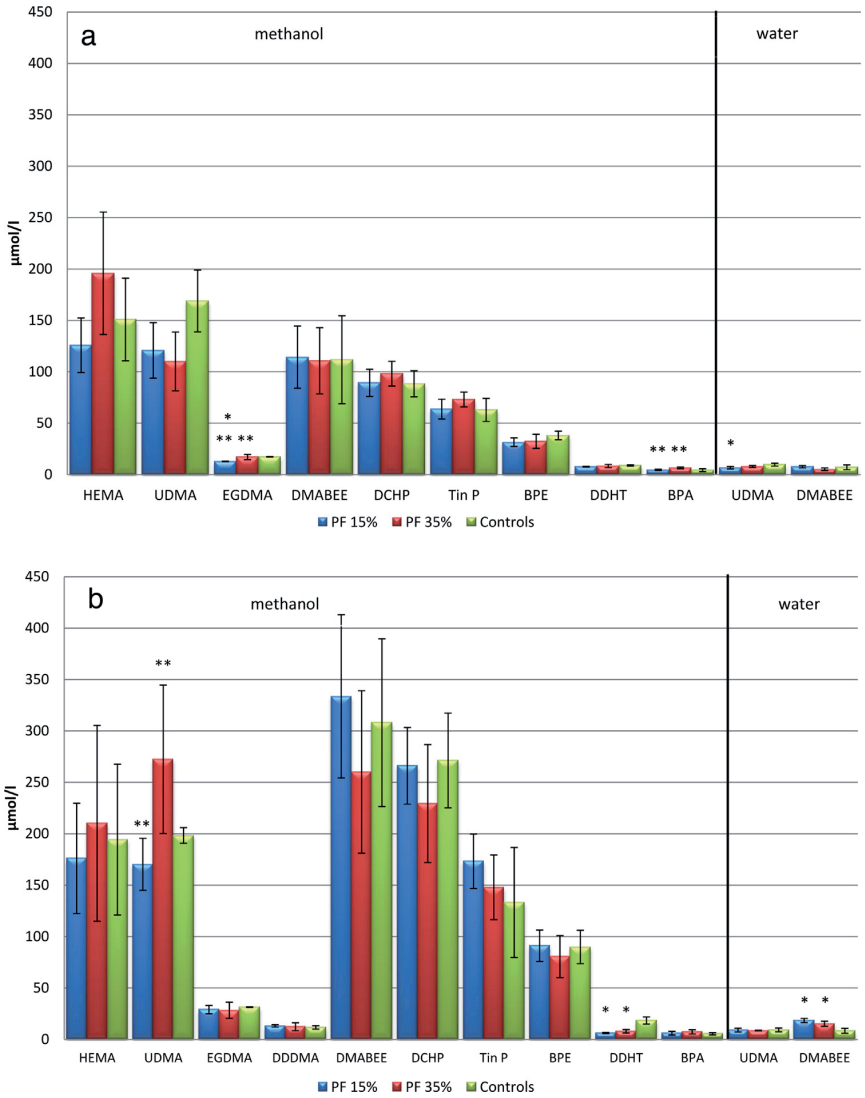

Fig. 1 – Tetric® Evo Ceram 24 h (a) and 7 d (b) elution in methanol and water. * Significantly different ($p < 0.05$) to control. ** PF 15% treated samples significantly different ($p < 0.05$) to PF 35% treated samples.

Methanol 7 d:

The PF 15%-treatment resulted in a significant higher BPA elution (PF 15%: 0.6 µmol/l) compared to the controls (0.2 µmol/l) as well as to the PF 35%-treatment (0.2 µmol/l).

4. Discussion

In this study the influence of bleaching-procedures on the elution of substances from dental composites was investigated.

In earlier own studies composites such as Tetric EvoCeram®, Tetric EvoFlow® and Filtek™ Supreme XTE have been treated with the bleaching gels PF 15% and PF 35% in order to analyze their methanolic eluates [19]. In the present study not only the mentioned composites but also Admira, Ceram X® mono+, CLEARFIL™ AP-X and Filtek™ Silorane have been treated with both bleaching gels PF 15% and PF 35%. In addition to methanol all samples have been eluted in water to allow the utmost physiological comparison to human saliva [21]. Furthermore water is almost similar to dentin fluid; thus eluted substances can penetrate the dentin tubuli and finally enter the pulp [32,33]. Here the vitality and regenerative capacity of the dentin–pulp complex could be impaired [34]. Additionally in comparison to the methanol eluates of the composites such as Tetric EvoCeram®, Tetric EvoFlow® and Filtek™ Supreme XTE of our earlier study [19] present results show different concentrations of elutable substances: for example in our earlier study [19] Tetric EvoFlow® LOT M61775 (24 h, PF 15%) samples released 1/2 less DMABEE compared to the present study from Tetric EvoFlow® LOT

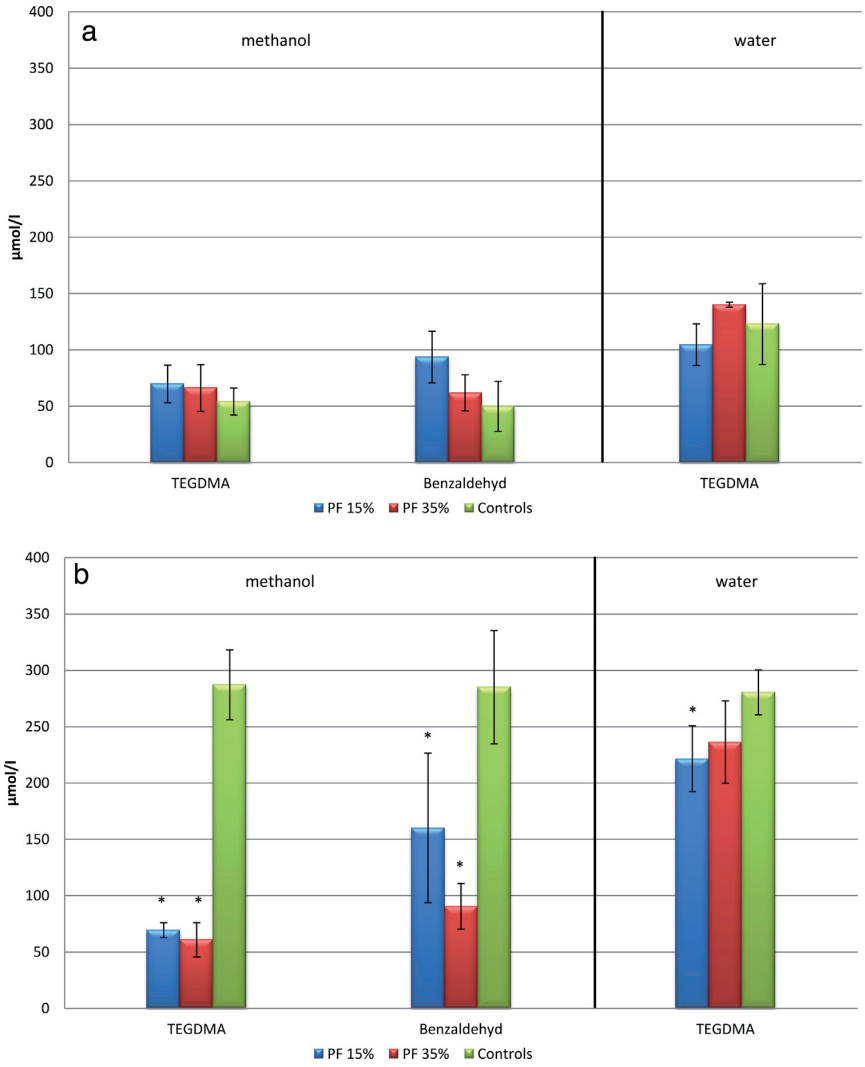

Fig. 2 – CLEARFIL® AP-X 24 h (a) and 7 d (b) elution in methanol and water. * Significantly different ($p < 0.05$) to control.

R54703. Due to the variations in the released amount of substances from composites with different batch numbers (LOT numbers) it might be deduced that the concentration of some substances have obviously been changed by the manufacturers.

4.1. *Reduced elution caused by bleaching treatments compared to the controls*

Concerning the composite Tetric EvoCeram® after 24 h in water the PF 15%-treatment resulted in a 1/3 less elution of UDMA compared to the controls. Manufacturers often use UDMA as a basic monomer in composites to ensure a better durability, biocompatibility and less shrinkage of the composite [35]. The cytotoxic concentration of UDMA for human oral mucous membrane cells is 0.27 mmol/l [36].

The highest elution value in this study for UDMA (0.01 mmol/l) (Tetric EvoCeram®, water, 24 h, controls) is about 30 times below cited cytotoxic concentration.

Concerning the composite CLEARFIL™ AP-X after 7 d in water the PF 15%-treatment resulted in a 1/5 less release of TEGDMA compared to the controls. After 7 d in methanol both the PF 15%- and PF 35%-treatment showed about 1/4 of TEGDMA release compared to the controls. TEGDMA is a comonomer used in composites due to its low viscosity and ability to enrich the organic resin matrix of composites with a maximum of inorganic filler particles [35]. According to literature [36] the cytotoxic concentration for TEGDMA is 3.7 mmol/l in human oral mucous membrane cells. In this study the highest amount of TEGDMA was found for the controls of CLEARFIL™ AP-X after 7 d in water (0.28 mmol/l). This value is 13 times lower than the cited cytotoxic concentration.

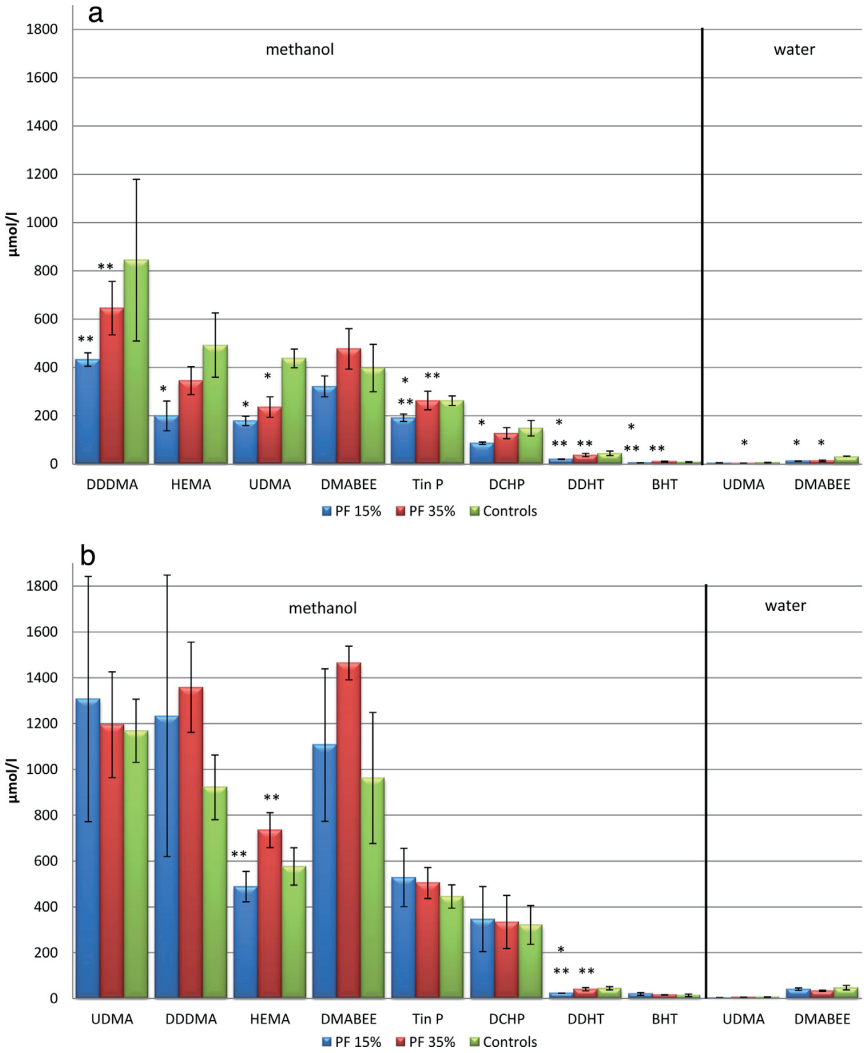

Fig. 3 – Tetric EvoFlow® 24 h (a) and 7 d (b) elution in methanol and water. * Significantly different ($p < 0.05$) to control. ** PF 15% treated samples significantly different ($p < 0.05$) to PF 35% treated samples.

Concerning the composite Tetric EvoFlow® after 24 h in methanol the PF 15%-treatment resulted in a 3/5 less elution of HEMA compared to the controls. Due to its hydrophilic properties HEMA is used as comonomer of the organic resin matrix in dental composites. According to previous studies oxidative stress and apoptosis are effects caused by HEMA [37–39]. In this study the highest HEMA concentration (0.74 mmol/l) was measured in the methanol eluates of the composite Tetric EvoFlow® (PF 35%; 7 d). HEMA has been only detected in the methanol eluate. Because the HEMA concentration in water eluates was below the detection limit (0.14 µmol/l). This concentration remains about a factor of 21,000 below the concentration for oxidative stress to human gingival fibroblasts (HEMA = 3 mmol/l) [38] and about a factor of 71,000 below the apoptosis of human primary pulp fibroblasts (HEMA = 10 mmol/l) [39].

The mechanism of hydrogen peroxide and carbamide peroxide bleaching is not fully understood. Additionally unspecified oxidation processes can occur [40]. Especially long-chained molecules like UDMA and Bis-GMA can be degraded by interaction with peroxides [18]. This could explain the reduced release of UDMA, TEGDMA and HEMA caused by bleaching treatments compared with the controls of the above mentioned composites.

4.2. *Reduced elution caused by PF 15%-treatment compared to PF 35%-treatment*

Concerning the composite Tetric EvoCeram® after 7 d in methanol the PF 15%-treatment resulted in a 1/3 lower elution of UDMA compared to the PF 35%-treatment. The composite Tetric EvoFlow® after 7 d in methanol and PF 15%-treatment

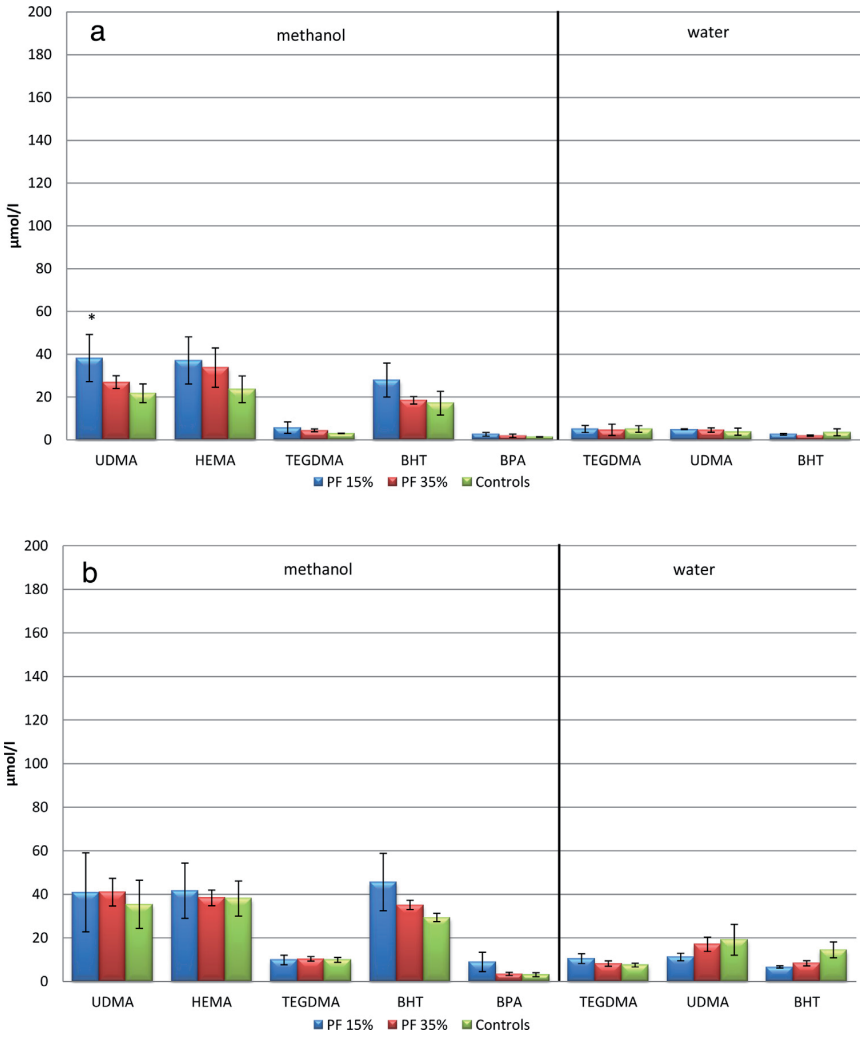

Fig. 4 – Filtek ™ Supreme XT 24 h (a) and 7 d (b) elution in methanol and water. * Significantly different ($p < 0.05$) to control.

also showed a 1/3 lower elution of HEMA compared to the PF 35%-treatment. The oxidation of peroxides within the bleaching process is unspecific [40] and therefore not cleared in detail till now. Nevertheless it was shown that peroxides can modify the microhardness of the composite surface [41,42]. In addition Marson et al. reported that penetration of hydrogen peroxide into the dental structure is time-dependent [43]. Based on the use of bleaching-gels according to application instructions of the manufacturer, these findings and the results of composites mentioned above give rise to the following assumption: longer interaction time (5 h) of PF 15% may cause degradation respectively oxidation of elutable substances, if peroxides penetrate through the composite surface into lower layers. Using PF 35% with a shorter interaction time of 30 min this effect would not occur to the same extent. Obviously it is not the concentration of peroxides of the bleaching gel but the different exposure time of both bleaching gels which determines the minor elution (7 d, methanol) of UDMA and HEMA when treated with PF 15% compared to PF 35%.

4.3. *Increased elution caused by bleaching treatments compared to the controls*

Concerning the composite Filtek™ Supreme XT after 24 h in methanol the PF 15%-treatment resulted in a 2-fold higher elution of UDMA compared to the controls. Concerning the composite Ceram X® mono+ after 7 d in water the PF 15%-treatment and the PF 35%-treatment resulted in a about 3 times higher amount of DMABEE compared to the controls. DMABEE is a coinitiator used in composites to accelerate the breakdown of initiators into radicals and therefore the polymerization [44]. The highest DMABEE concentration was detected in the water eluates (0.10 mmol/l) for the composite Ceram X® mono+ after 7 d treated with PF 35%. This value is

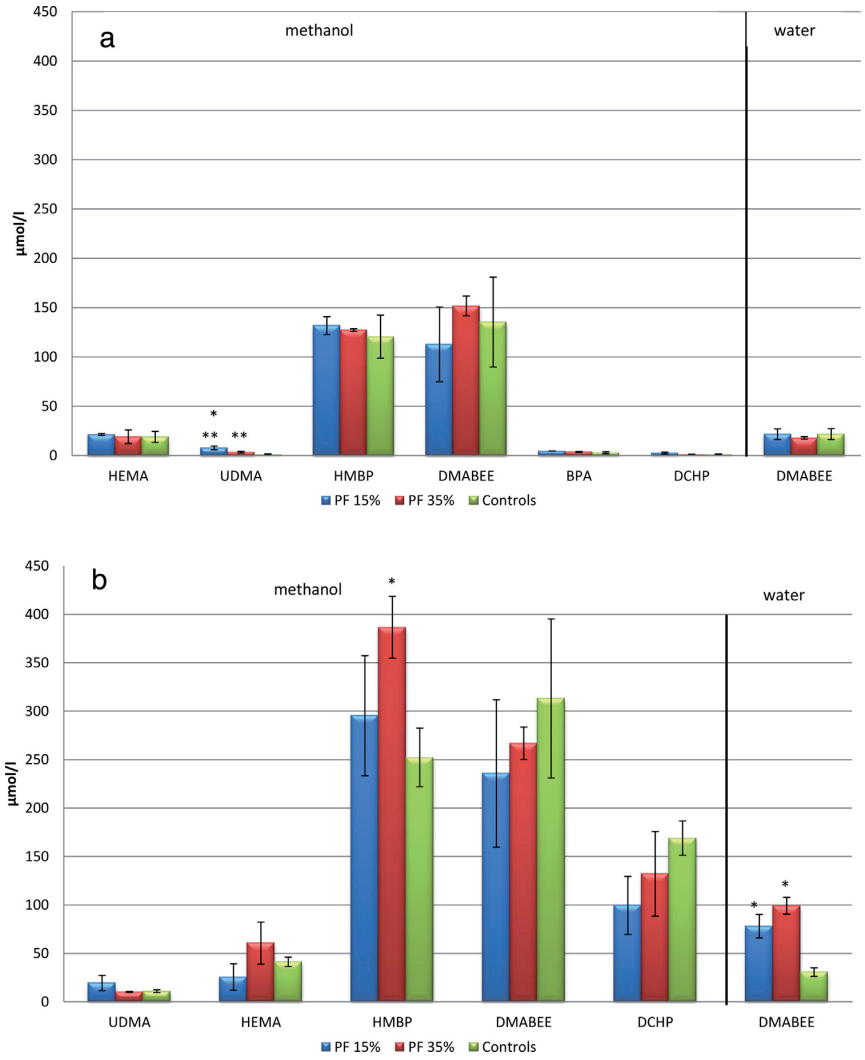

Fig. 5 – Ceram X® mono+ 24 h (a) and 7 d (b) elution in methanol and water. * Significantly different ($p < 0.05$) to control. ** PF 15% treated samples significantly different ($p < 0.05$) to PF 35% treated samples.

12 times lower than the cytotoxic concentration of human oral mucous cells (1.23 mmol/l) [45].

Concerning the composites Admira and Filtek™ Silorane after 24 h in methanol the PF 15%-treatment resulted in a 3/5 higher elution of BPA compared to the controls. Usually manufactures do not add BPA to composites but it can either be found as impurity from synthesis process of Bis-GMA and Bis-EMA or formed in course of their degradation [46]. According to manufacturers' data (Table 1) the composite Admira contains Bis-GMA, while there is no information for Filtek™ Silorane concerning the resin matrix. Recent studies reported that there is a possible connection between Bis-GMA based dental fillings and sudden psychosocial disorders in childhood [47]. Moreover it has been shown that BPA has very similar effect compared to estrogen: at a concentration of 0.01 mmol/l more chaperone proteins are expressed in human cells expressing human estrogen receptor α [48]. The level of chaperone regulates the stress-induced cell activity, viability and mortality [48]. In the present study the highest BPA concentration (0.01 mmol/l) was found in the controls of the methanol eluates for Admira after 7 d. BPA has been only detected in the methanol eluates and remains below the detection limit (0.02 µmol/l) in the water eluates. Thus regarding the BPA EC_{50} for human placenta choriocarcinoma cells given in the literature with 0.1 mmol/l [49] and the value concerning the enhanced expression of chaperones with 0.01 mmol/l [48] the concentration in the present study is about 5000- and 500-times lower.

Our previous study showed that bleaching processes can lead to an increased concentration of e.g. DMABEE and BPA from the composite Filtek™ Supreme [19]. Additionally the results of the bleached composites mentioned above show an

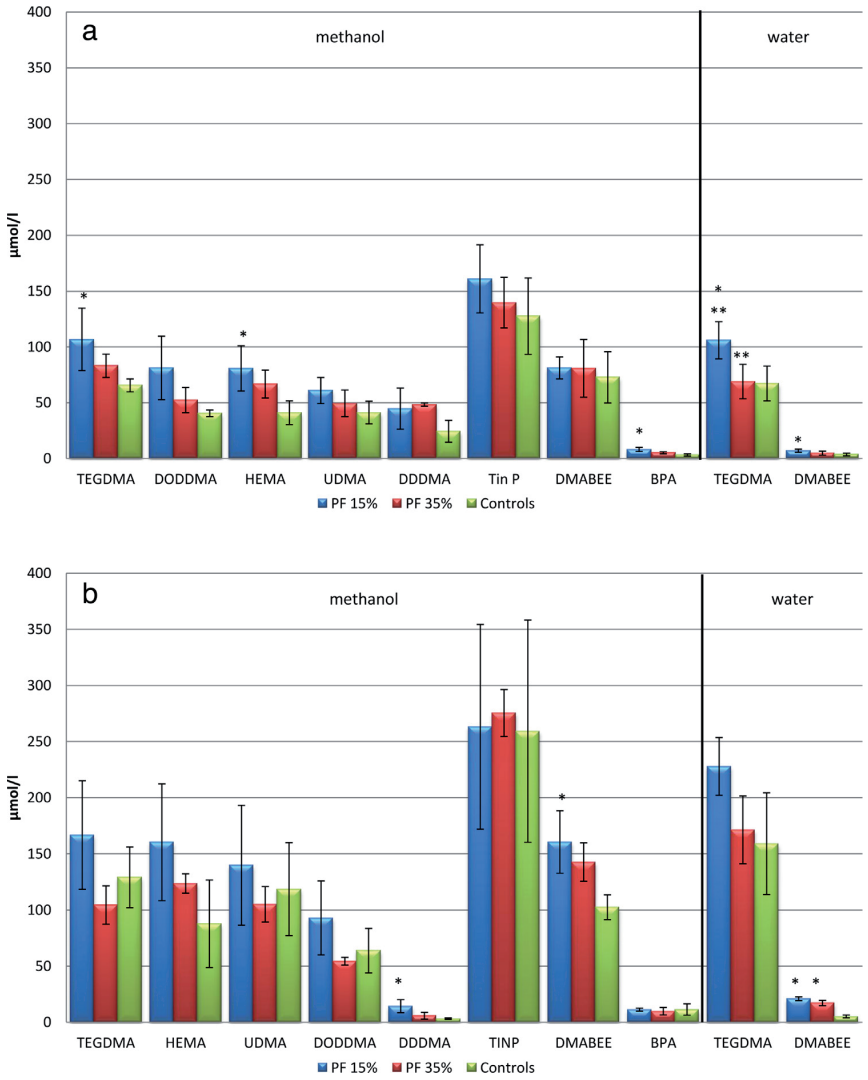

Fig. 6 – Admira 24 h (a) and 7 d (b) elution in methanol and water. * Significantly different ($p < 0.05$) to control. ** PF 15% treated samples significantly different ($p < 0.05$) to PF 35% treated samples.

increased concentration of released UDMA compared to the controls.

4.4. *Increased elution by PF 15%-treatment compared to the PF 35%-treatment*

Concerning the composite Ceram X® mono+ after 24 h in methanol the PF 15%-treatment resulted in a 2-fold higher elution of UDMA compared to the PF 35%-treatment. The same applies to the composite Admira after 24 h in water, which released 1/3 more of TEGDMA after PF 15%-treatment compared to the PF 35%-treatment. After 7 d in methanol the composite Filtek™ Silorane showed also 1/3 higher release of BPA after the PF 15%-treatment compared to the PF 35%-treatment. Thus these findings suggest that the exposure time of the bleaching gels (PF 15%: 5 h, PF 35%: 30 min), other than the different concentrations of peroxides in the bleaching gels, cause an increased release of UDMA, TEGDMA and BPA for the above mentioned composites after bleaching-treatment. Also in this case the effect of peroxides on the micro-hardness of the composite surface [41,42] and the time-dependent penetration of hydrogen peroxide into the dental structure [43] seems to be the determining factor. In our previous study the increased elution of methacrylates caused by the effect of peroxides in deeper layers of the composite has already been described [19]. Presented results of the above mentioned composites showed also an increased elution of the substances UDMA, TEGDMA and BPA after

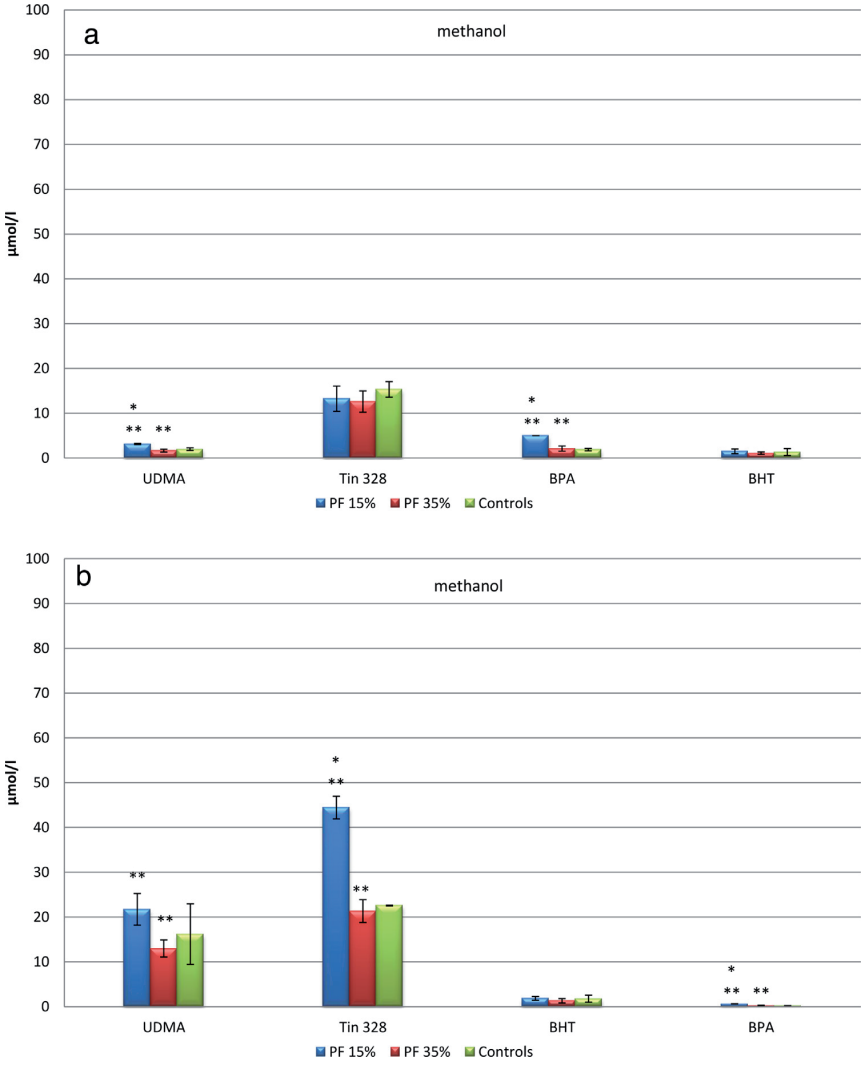

Fig. 7 – Filtek™ Silorane 24 h (a) and 7 d (b) elution in methanol. * Significantly different ($p < 0.05$) to control. ** PF 15% treated samples significantly different ($p < 0.05$) to PF 35% treated samples.

the PF 15%-treatment compared to the PF 35%-treatment. Thus these results confirm findings of our previous study [19].

In 2004 Wattanapayungkul et al. [42] postulated that the roughening of the surface of each composite is dependent on the bleaching treatment; this is not only caused by differences in resin matrix components but also by the different filler sizes within the composite subtypes. This result is also confirmed by Polydorou et al. [12]: PF 15%-treatment showed a significant change in the surface texture (loss of resin parts and surface cracks) for the composite Tetric® EvoFlow; concerning Filtek™ Supreme XT or Tetric® EvoCeram after polishing and PF 15%-treatment no changes in the surface were found. This assumption is confirmed by our results. Referring to Tetric® EvoCeram, CLEARFIL® AP-X and Tetric® EvoFlow after bleaching treatment a reduction of elution amounts were detected. Whereas the bleaching treatment of Filtek™ Supreme XT, Ceram X® mono+, Admira and Filtek™ Silorane caused an increase of elution mounts.

Methacrylates are classified as potent allergens [23]. Since 1990 dentists and patients have shown an increasing number of allergic reactions to methacrylates [50–52]. Among all investigated composites only for Filtek™ Silorane and Ceram X® mono+ methacrylates could be detected, only in the methanol eluates. This applies to the PF 15%-treatment, the PF 35%-treatment and to the controls. Whereas no methacrylates could be detected in the physiological situation of the water eluate concerning Filtek™ Silorane and Ceram X® mono+. Thus in case of a patient suffering from methacrylate allergy Filtek™ Silorane and Ceram X® mono+ should be preferred

to the other investigated composites whenever a composite restoration is necessary.

5. Conclusion

Depending on the dental material, bleaching treatments can either lead to a reduced or an increased elution of substances from the dental composites. Those composites in which a decrease of eluted methacrylates was detected (Tetric EvoCeram®, CLEARFIL™ AP-X, Tetric EvoFlow®) should be preferred in case of composite restorations with patients suffering from methacrylate allergy.

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Effect of Opalescence® bleaching gels on the elution of bulk-fill composite components

Lena Schuster[a,b], *Franz-Xaver Reichl*[a,b], *Lena Rothmund*[a,b], *Xiuli He*[a,b], *Yang Yang*[a,b], *Kirsten L. Van Landuyt*[c], *Kai Kehe*[d,e], *Olga Polydorou*[f], *Reinhard Hickel*[a], *Christof Högg*[a,b,*]

[a] *Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany*
[b] *Walther-Straub-Institute of Pharmacology and Toxicology, Nussbaumstr. 26, 80336 Munich, Germany*
[c] *KU Leuven Biomat, Department of Oral Health Sciences, KU Leuven, Kapucijnenvoer 7, B-3000 Leuven, Belgium*
[d] *Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstr. 11, 80937 Munich, Germany*
[e] *Walther Straub Institute of Pharmacology and Toxicology, Ludwig Maximilians University of Munich, Goethestraße 33, 80336 Munich, Germany*
[f] *Department of Operative Dentistry and Periodontology, University Medical Center Freiburg, Dental, School and Hospital, Hugstetterstraße 55, 79106 Freiburg, Germany*

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ABSTRACT

Objectives. Bleaching treatments can affect release of components from conventional composites. In this continuing study the influence of two different bleaching gels on the elution of bulk-fill composite components was investigated.

Methods. The composites Tetric EvoCeram® Bulk Fill, QuiXFil™ and X-tra fil were treated with the bleaching gels Opalescence PF 15% (PF 15%) for 5 h and PF 35% (PF 35%) for 30 min and then stored in methanol and water for 24 h and 7d. The eluates were analyzed by gas chromatography/mass spectrometry (GC/MS). Unbleached specimens were used as control group.

Results. A total of 7 different elutable substances have been identified from the investigated composites after bleaching-treatment. Three of them were methacrylates: 2-hydroxyethyl methacrylate (HEMA), triethylene glycol dimethacrylate (TEGDMA) and trimethylolpropane trimethacrylate (TMPTMA). Compared to the unbleached controls an increase in elution after PF 15%-treatment of following compounds was found: HEMA (Tetric EvoCeram® Bulk Fill), TEGDMA (QuiXFil™, X-tra fil) and 4-N,N-dimethylaminobenzoic acid butyl ethoxy ester (DMABEE) (Tetric EvoCeram® Bulk Fill, QuiXFil™, X-tra fil). Following compounds showed a reduction in elution after PF 35%-treatment compared to controls: TEGDMA (QuiXFil™) and DMABEE (Tetric EvoCeram® Bulk Fill). The highest concentration of HEMA was 0.22 mmol/l (Tetric EvoCeram® Bulk Fill, methanol, 7d, PF 15%), the highest concentration of TEGDMA was

* *Corresponding author at*: Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany. Tel.: +49 89 2180 73809; fax: +49 89 7095 73817.
E-mail address: christof.hoegg@lrz.uni-muenchen.de (C. Högg).

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0.3 mmol/l (X-tra fil, water, 7d, PF 15%) and the highest concentration of DMABEE was 0.05 mmol/l (QuiXFil™, water, 7d, PF 35%).

Significance. PF 15% and PF 35% can lead to reduced and/or increased elution of some bulk-fill components, compared to unbleached bulk-fill composites.

1. Introduction

The aesthetic demands on our society are continuously growing. White teeth are also a crucial factor for an appealing appearance. In addition to veneers and tooth-colored crowns, for example, bleaching the teeth is the most common procedure to aesthetically enhance them [1]. For this purpose, there are four different methods which work with carbamide and/or hydrogen peroxide gel. (1) In-office bleaching methods (35% peroxides), (2) chairside-bleaching methods (38% peroxides) [2], (3) home-bleaching methods (15% peroxides) [3] and (4) over-the-counter products (maximum 10% peroxides). In order to apply the bleaching gel, bleaching trays are only used for the home-bleaching method and the in-office bleaching method [4]. Carbamide peroxide decomposes during the bleaching process upon contact with saliva in hydrogen peroxide and urea, which in turn decomposes into ammonia and carbon dioxide. Hydrogen peroxide, disassociates into oxygen and water [5,6]. However, both in the chairside- and in the in-office bleaching method, hypersensitivity in the teeth [7] and damage to the pulp cells [8] can be noted due to the use of high-percentage (38% or 35%) carbamide or hydrogen peroxide gels. Through a reduction in the percentage content of the bleaching gel, as in the home bleaching methods (15% peroxides), a reduction in the hypersensitivities can be achieved [9].

However, if insufficient restorations are detected in a patient before a bleaching process, they must be swapped out in each case before starting the bleaching process because damage to the pulp cells can be caused upon contact of the bleaching gels with the pulp [10]. For this purpose, different composite materials are taken into account such as bulk-fill or other conventional composites. The latter are used in the incremental technique, i.e. integrated into maximum 2 mm thick layers [11], to ensure, according to the manufacturer, the complete polymerization of the composite. On the other hand, bulk-fill composites guarantee a sufficient polymerization depth at increments up to 4 mm strength [12], which can be attributed to an increased translucence through reduced filling material content with simultaneously increased filling particle size [13]. The aforementioned properties allow a quick one-incremental technique to fill a complete cavity for bulk-fill composites in many cases [14].

Studies have already been performed on the mechanical properties of bulk-fill composites [14–16]. Thus, for example, for cuspal deflection [17], the marginal integrity of a filling [11,18], just as for its cure depth [11] better results of bulk-fill composites compared to composites which are added in the incremental technique were detected. However, also adverse results were found compared to conventional composites such as the conversion rate, for bulk-fill composites [19]. At >55%, conversion rate for bulk-fill composites is still in the clinically acceptable range but it is still less than for conventional composites [19].

In the past, some composite materials were already studied in terms of their reaction to a bleaching process. For example, changes in terms of surface structure [6,20,21] and microhardness [22,23] are tested. In our own studies it could be shown that the bleaching processes also affect the release of monomers (methacrylates) and other components from dental composites [24–27]. In addition to initiators, stabilizers, additives and pigments, monomers (methacrylates) are also components of the organic resin matrix of non-polymerized composites [28]. However, polymerization of dental composites is incomplete. The lower the conversion rate of a composite, the more residual monomers can be eluted [29]. These elutable monomers (methacrylates) can result in allergic reactions [30], such as asthma, allergic rhinoconjunctivitis or contact dermatitis [31].

In our own earlier studies [24–27], the effect of the bleaching gels Opalescence® PF 15% (PF 15%) and Opalescence® PF 35% (PF 35%) on the release of components from conventional composites was investigated. The aim of this continuing study is the investigation of the effect of PF 15% and PF 35% on the time-dependent elution of composite components from various bulk-fill composites.

In the null hypothesis, it is assumed that PF 15% and PF 35% have no effect on the release of components from bulk-fill composites compared to the release of components without PF 15% or PF 35% treatment.

2. Materials and methods

The tested composites including manufacturers' data and the bleaching gels are listed in Tables 1 and 2.

2.1. *Preparation of samples*

The preparation of samples as well as the bleaching treatment has been performed according to our earlier study [27]. Composites (Table 1) were polymerized and bleached (Table 2) exactly according to instruction of manufacturer. For each composite (Table 1) 3 groups with 4 samples each ($n = 4$) were prepared: 1. samples bleached with PF 15% for 5 h; 2. samples bleached with PF 35% for 30 min and 3. unbleached samples: Control group. QuiXFil™ and X-tra fil were used in universal shade and Tetric EvoCeram® Bulk Fill in IVA (universal A-shade).

Subsequently samples were incubated in brown glass vails (Macherey-Nagel, Düren, Germany) with 1 ml of methanol (GC Ultra Grade, RATISOLV® ≥ 99.9%, Roth, Karlsruhe, Germany) at

Table 1 – Investigated dental materials, manufacturer, lot numbers and type; composition of each material based on manufacturer's data.

Product name	Type	Manufacturer	LOT	Composition of materials based on manufacturer's data	Polymerization time
Tetric EvoCeram® Bulk Fill	Bulk-fill composite	Ivoclar Vivadent AG, Schaan, Liechtenstein	P84129	Bis-GMA, UDMA, Bis-EMA, Barium-glass, YbF3, mixed oxide, pre-polymerized fillers	10 s
QuiXFil™	Bulk-fill composite	DENTSPLY DeTrey GmbH, Konstanz, Germany	12090000634	UDMA, TEGDMA, Di- and trimethacrylate resins, carboxylic acid modified dimethacrylate resin, BHT, Silanated strontium aluminium sodium fluoride phosphate silicate glass	10 s
X-tra fil	Bulk-fill composite	VOCO GmbH, Cuxhaven, Germany	1245232	Bis-GMA, UDMA, TEGDMA	10 s

Abbreviations of manufacturer's data: Bis-EMA, bisphenol A polyetheylene glycol dimethacrylate; Bis-GMA, bisphenol-A diglycidyl dimethacrylate; UDMA, urethane dimethacrylate.

Table 2 – Bleaching materials tested, manufacturers, LOT-number.

Product name	Composition of bleaching agent (wt.%)	Abbreviation used in the text	Stated bleaching time by manufacturer	Manufacturer	LOT
Opalescence® tooth whitening systems PF 15%	Carbamide peroxide <25; Poly acrylic acid <10; Sodium hydroxide <5; Sodium fluoride 0.25	PF 15%	4–6 h	Ultradent® Products, Inc., S. South Jordan, UT, USA	B7B15
Opalescence® tooth whitening systems PF 35%	Carbamide peroxide 20; Hydrogen peroxide <10; Sodium hydroxide <5; Sodium fluoride 0.25	PF 35%	30 min	Ultradent® Products, Inc., S. South Jordan, UT, USA	B74MV

37 °C and analyzed after 1 d and 7 d by GC/MS [24]. As internal standard caffeine (CF) solution (0.01 mg/ml) (HPLC ≥ 99.0%, Sigma–Aldrich, St. Louis, United States) was added.

Water (LC–MS-Grade, ROTISOLV®, Roth, Karlsruhe, Germany) elutions were carried out as outlined above. For GC/MS analysis water samples were extracted with ethyl acetate (1:1 v/v) (LC–MS-Grade, ROTISOLV® ≥ 99.9%, Roth, Karlsruhe, Germany).

2.2. *Analytical procedure*

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 Factor Four® capillary column (length 25 m, inner diameter 0.25 mm; coating 0.25 μm; Varian, Darmstadt, Germany) was used as the capillary column for GC. The GC oven was heated from 50 °C (2 min isotherm) to 280 °C (5 min isotherm) with a rate of 25 °C/min, and 1 μl of the solution was injected with a split ratio of 1:30. Helium 5.0 was used as carrier gas at a constant flow rate of 1 ml/min. The temperature of the split–splitless injector, as well as of the direct coupling to the mass spectrometer, was 250 °C. MS was operated in the electron ionization mode (EI, 70 eV), ion source was operated at 240 °C; only positive ions were scanned. The performed scan ran over the range *m/z* 50–600 at a scan rate of 4 scan/s for scans operated in full scan mode in order to qualify the analytes.

The results were referred to an internal caffeine standard (0.1 mg/caffeine = 100%), which allows to determine the relative quantities of substances released from various resin-based materials. All eluates were analyzed five times. The integration of the chromatograms was carried out over the base peak or other characteristic mass peaks of the compounds, and the results were normalized by means of the internal CF standard. Identification of the various substances was achieved by comparing their mass spectra with those of reference compounds, the NIST/EPA library, literature data, and/or by a chemical analysis of their fragmentation pattern [32].

2.3. *Calculations and statistics*

The results are presented as means ± standard deviation (SD). The statistical significance ($p < 0.05$) of the differences between

Table 3 – Detected eluted substances from the composites treated with the bleaching gels.

BHT	2,6-Di-t-butyl-4-methyl phenol
DCHP	Dicyclohexyl phthalate
DMABEE	4-N,N-Dimethylaminobenzoic acid butyl ethoxy ester
HEMA	2-Hydroxyethyl methacrylate
TEGDMA	Triethylene glycol dimethacrylate
Tin P	Tinuvin P, 2(2′-hydroxy-5′-methylphenyl) benzotriazol
TMPTMA	Trimethylolpropane trimethacrylate

the experimental groups was analyzed by one-way ANOVA and the post hoc test (Tukey's HSD test) [33].

3. Results

A total of 7 different elutable substances (Table 3) have been identified from the investigated composites (Table 1) after bleaching-treatment (Table 2). The quantification and significant differences are shown in Figs. 1–3. In the following only the relevant substances of the eluates of the tested composites such as methacrylates are listed:

3.1. *Tetric EvoCeram® bulk fill (Fig. 1)*

Methanol 24 h:

The PF 15%-treatment resulted in a significant higher HEMA elution (PF 15%: 192.7 μmol/l) compared to the controls (106.1 μmol/l) as well as to the PF 35%-treatment (89.5 μmol/l).

The PF 15%-treatment resulted in a significant higher DMABEE elution (PF 15%: 87.1 μmol/l) compared to the controls (65.7 μmol/l) as well as to the PF 35%-treatment (47 μmol/l).

Methanol 7d:

The PF 15%-treatment resulted in a significant higher HEMA elution (PF 15%: 224.9 μmol/l) compared to the controls (152.9 μmol/l) as well as to the PF 35%-treatment (83.1 μmol/l).

The PF 15%-treatment resulted in a significant higher DMABEE elution (PF 15%: 209.5 μmol/l) compared to the PF 35%-treatment (84.8 μmol/l). The PF 35%-treatment resulted in a significant lower DMABEE elution compared to the controls (169.5 μmol/l).

Water 24 h:

The PF 15%-treatment resulted in a significant higher DMABEE elution (PF 15%: 11.8 μmol/l) compared to the PF 35%-treatment (8.6 μmol/l).

Water 7d:

The PF 15%-treatment resulted in a significant higher DMABEE elution (PF 15%: 31.1 μmol/l) compared to the PF 35%-treatment (17.3 μmol/l).

3.2. *QuiXFil™ (Fig. 2)*

Methanol 24 h:

The PF 15%-treatment resulted in a significant higher DMABEE elution (PF 15%: 148.1 μmol/l) compared to the controls (81.0 μmol/l).

Methanol 7d:

The PF 15%-treatment resulted in a significant higher TEGDMA elution (PF 15%: 42.3 μmol/l) compared to the controls (30.3 μmol/l) as well as to the PF 35%-treatment (31.0 μmol/l).

The PF 15%-treatment resulted in a significant higher DMABEE elution (PF 15%: 240.9 μmol/l) compared to the controls (156.9 μmol/l) as well as to the PF 35%-treatment (171.3 μmol/l).

Water 24 h:

The PF 35%-treatment resulted in a significant lower TEGDMA elution (PF 35%: 28.0 μmol/l) compared to the controls (60.7 μmol/l).

3.3. *X-tra fil (Fig. 3)*

Methanol 24 h:

The PF 15%-treatment resulted in a significant higher DMABEE elution (PF 15%: 41.4 μmol/l) compared to the controls (22.4 μmol/l).

Water 7d:

The PF 15%-treatment resulted in a significant higher TEGDMA elution (PF 15%: 295.4 μmol/l) compared to the controls (193.8 μmol/l) as well as to the PF 35%-treatment (192.3 μmol/l).

4. Discussion

In this study the influence of the bleaching gels PF 15% and PF 35% on the elution of components from dental bulk-fill composites was investigated.

Flowable bulk-fill composites were not selected for present study because they always have to be covered with a layer from conventional composites [15] and therefore are not in direct contact with the bleach gel.

For elution, the samples were transferred both in methanol as well as in water because water allows the utmost physiological comparison to human saliva [28]. Furthermore water is almost similar to dentinal fluid; thus, released components can penetrate through the dentinal tubuli to the pulp [34,35] and there affect the vitality and regenerative ability of the pulp [36].

4.1. *Increased elution by PF 15%-treatment compared to the controls*

For the composite Tetric EvoCeram® Bulk Fill, after 24 h in methanol with PF 15%-treatment, almost twice as much HEMA was detected compared to the controls. HEMA is used in dental composites as a comonomer of the organic resin matrix due to its hydrophilic application. In earlier studies, effects caused by HEMA such as oxidative stress and apoptosis were determined [37–39]. The highest value for HEMA in the present study can be found for Tetric EvoCeram® Bulk Fill (PF 15%; 7d) in methanol with 0.22 mmol/l. HEMA has been only detected in the methanol eluate. Because the HEMA concentration in water eluates was below the detection limit (0.14 μmol/l), this concentration remains about a factor of 21,000 below the concentration, which is described to indicate oxidative stress in human gingival fibroblasts (HEMA = 3 mmol/l) [38] and about a factor of 71,000 below that concentration, which can induce apoptosis in human primary pulp fibroblasts (HEMA = 10 mmol/l) [39].

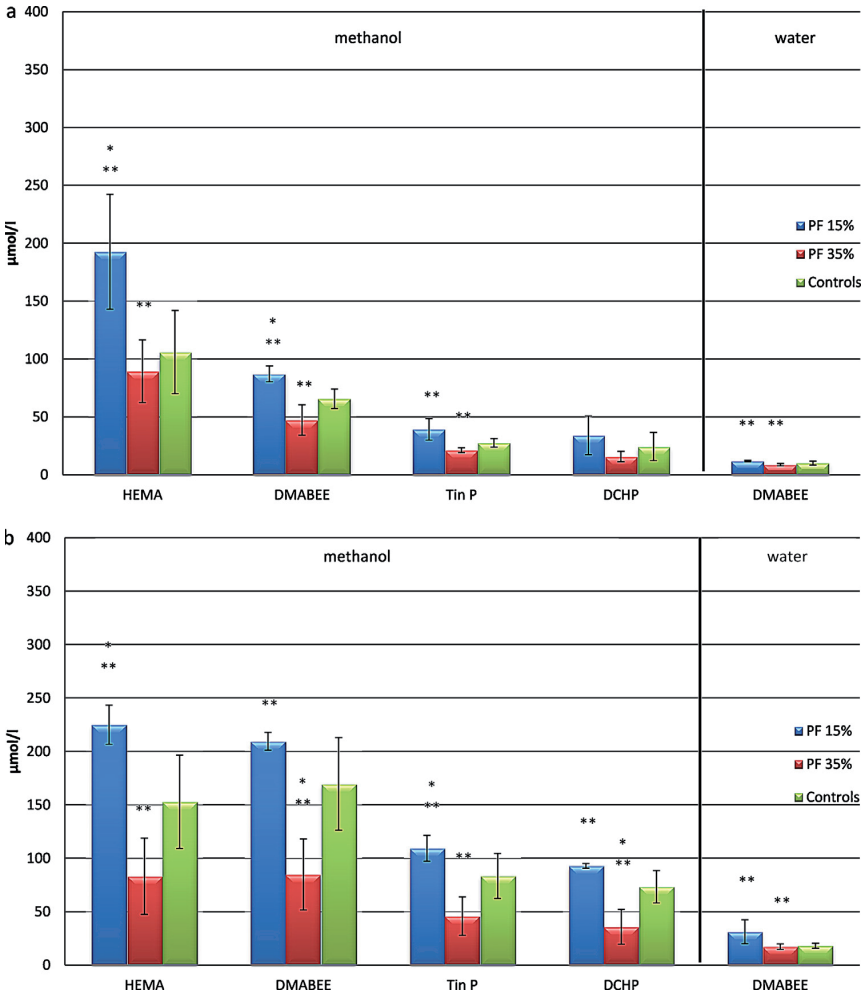

Fig. 1 – Tetric EvoCeram® Bulk Fill 24 h (a) and 7d (b) elution in methanol and water. *Significantly different ($p < 0.05$) to control. **PF 15% treated samples significantly different ($p < 0.05$) to PF 35% treated samples.

After 24 h in methanol, the composite QuiXFil™ has almost twice the DMABEE release with PF 15%-treatment compared to the controls. DMABEE is a coinitiator used in composites to accelerate the breakdown of initiators into radicals and thereby the polymerization [40]. The highest value measured in the present study for DMABEE in water is 0.05 mmol/l for QuiXFil™ (7d; PF 35%). This is almost 25 times less than the cytotoxic concentration of 1.23 mmol/l, which is described for human mucous membrane cells [41].

For the composite X-tra fil, after 7d in water and PF 15%-treatment, 1/3 more TEGDMA could be detected compared to the controls. TEGDMA is a comonomer used in composites due to its low viscosity and ability to enrich the organic resin matrix of composites with a maximum of inorganic filler particles [42]. The cytotoxic concentration for TEGDMA is 3.7 mmol/l for human mucous membrane cells [43]. The highest eluted TEGDMA concentration in the present study is 0.3 mmol/l for X-tra fil after 7d in water and PF 15%-treatment. Thus, this is around 12 times less compared to the cited cytotoxic concentration.

Our previous study showed that bleaching process can lead to an increased release of components such as TEGDMA, HEMA and DMABEE compared to controls for the conventional composites Admira, Ceram X® mono+, Tetric Flow®, Filtek™ Supreme XT and Tetric EvoCeram® [24,27]. Oxidation through peroxides in the bleaching process is non-specific [44] and is therefore not clarified in detail. However, changes in the micro-hardness of the composite surface by peroxides could be determined [45,46]. Thus, it is possible that the polymer network of the composite surface is damaged by carbamide/hydrogen peroxide contained in the bleaching gel and thus an increased release of the components can be detected [24]. This could also explain the increased release of the components HEMA, DMABEE and TEGDMA of the aforementioned bulk-fill composites Tetric EvoCeram® Bulk Fill, QuiXFil™ and X-tra fil in the current study.

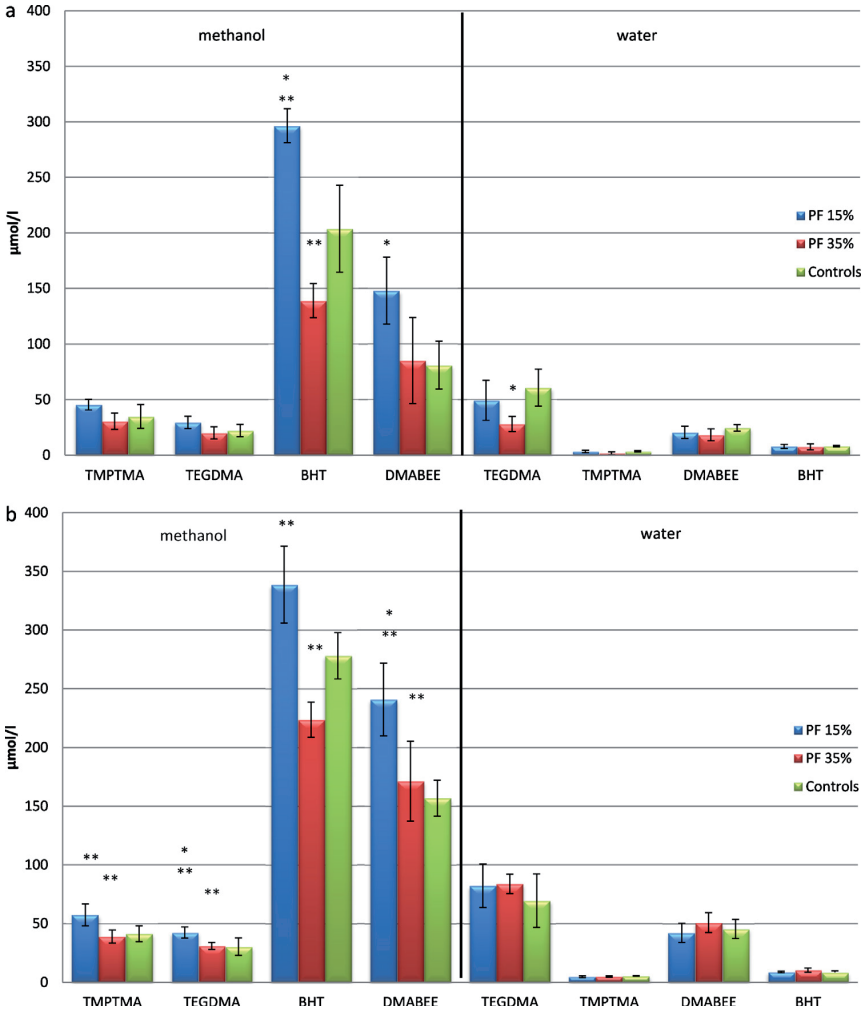

Fig. 2 – QuiXFil™ 24 h (a) and 7d (b) elution in methanol and water. *Significantly different ($p < 0.05$) to control. **PF 15% treated samples significantly different ($p < 0.05$) to PF 35% treated samples.

4.2. *Reduced elution by PF 35%-treatment compared to the controls*

For the composite Tetric EvoCeram® Bulk Fill, after 7d in methanol with PF 35% treatment, 2 times less DMABEE was detected compared to the controls. After 24 h in water, the composite QuiXFil™ shows approximately one half less TEGMA release with PF 35%-treatment compared to the controls. Only for X-tra fil, PF 35%-treatment had no effect on the release of components compared to the control.

The mechanism of hydrogen peroxide and carbamide peroxide bleaching is not yet completely understood because non-specific oxidation processes occur [44]. Especially long-chain molecules such as TEGDMA and Bis-GMA or other eluted components may undergo concomitant oxidative decomposition from peroxides [26]. This could explain the reduced release of DMABEE and TEGDMA in the bleaching process with PF 35% compared to the controls of the aforementioned bulk-fill composites Tetric EvoCeram® Bulk Fill and QuiXFil™ in the present study.

4.3. *Increased elution by PF 15%-treatment compared to PF 35%-treatment*

For the composites Tetric EvoCeram® Bulk Fill (24 h und 7d) and QuiXFil™ (7d) treated with PF 15%, all substances eluted in methanol (Figs. 1 and 2) have significantly higher concentrations compared to PF 35%-treatment. Thus, e.g. for Tetric EvoCeram® Bulk Fill after 7d in methanol (Fig. 1b) 2.5 times higher values could be determined for all eluted substances through PF 15%-treatment compared to PF 35%-treatment. After 7d the composite X-tra fil showed in water (PF 15%) (Fig. 3b) a 1.5 times higher concentration only for TEGDMA compared to PF 35%-treatment. These findings suggest that longer exposure time (PF 15%: 5 h, PF 35%: 30 min), other than different concentrations of peroxides in the bleaching gels,

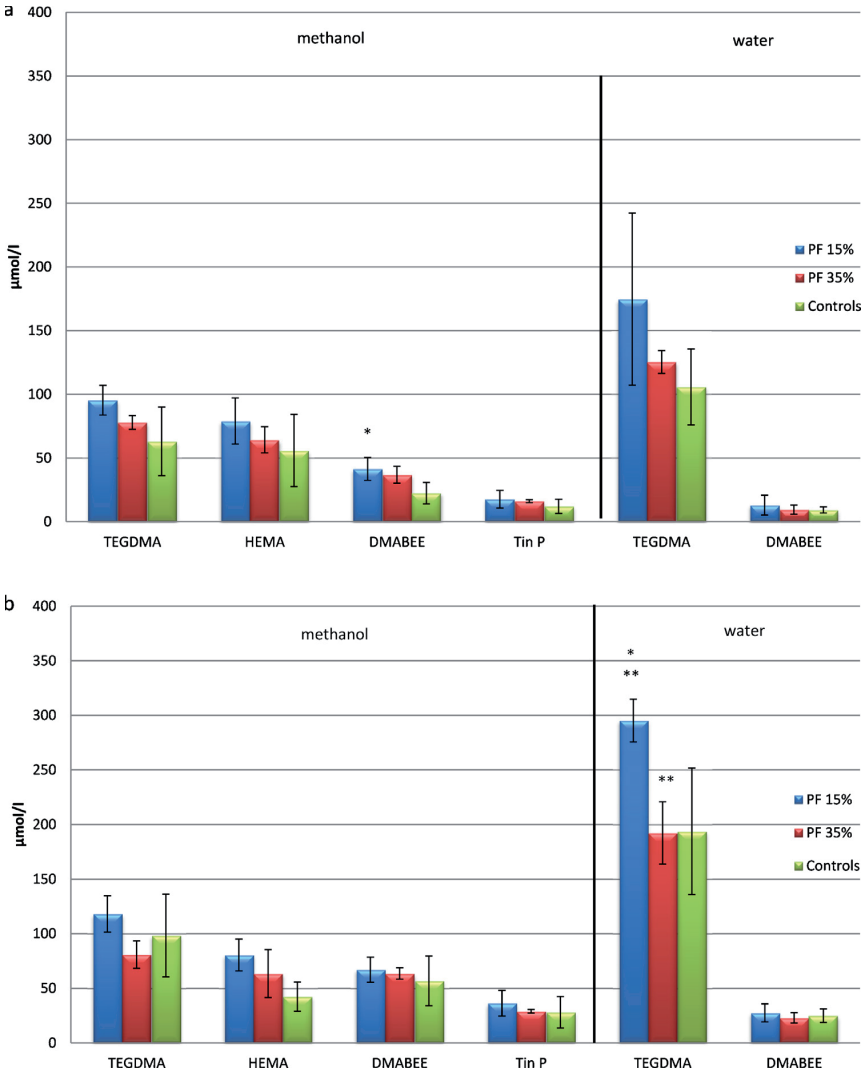

Fig. 3 – X-tra fil 24 h (a) and 7d (b) elution in methanol and water. *Significantly different ($p < 0.05$) to control. **PF 15% treated samples significantly different ($p < 0.05$) to PF 35% treated samples.

cause an increased release of components for investigated composites after bleaching-treatment. Also in this case the effect of peroxides on the micro-hardness of the composite surface [45,46] and in addition, the time-dependent penetration of hydrogen peroxide into the dental structure [47] seems to be the determining factor. In our previous studies the increased elution of methacrylates caused by the effect of peroxides in deeper layers of composite has already been described [24,27]. These facts are also supported by our results for the investigated bulk fill composites with PF 15%-treatment compared to PF 35%-treatment.

Previously, for some conventional composite, Polydorou et al. [6] found significant changes in surface texture after bleaching process. Thus it was assumed that these effects are caused by dissolution of the adhesive bond between filler materials and organic matrix [6]. Additionally Wattanapayungkul et al. [46] postulated that roughening of the surface of each composite is dependent on bleaching treatment; this effect is not only caused by differences in resin matrix components but also by the different filler sizes within the composite subtypes. In comparison to conventional composites, bulk-fill composites have less filler material content with simultaneous increased filling particle size, resulting in increased translucence [13]. In our earlier study, effect of PF 15% and PF 35% on elution of conventional composites was investigated [27]: for example elution values of HEMA in Tertic Evo Ceram® (Ivoclar Vivadent AG) showed no significant differences after PF 15% treatment compared to control (24 h and 7d; methanol) [27]. In the present study the bulk fill composite Tetric Evo Ceram® Bulk Fill of the same manufacturer and with same ingredients (according to manufacture's data) was investigated: for HEMA about two times higher values

were found for PF 15% treatment compared to controls (24 h, methanol). Therefore lager filling particle size may influence elution values of bulk fill composites treated with bleaching gels compared to conventional composites with smaller filling particle size.

Generally in our early study [27] it has been shown for conventional composites that elution by effect of bleaching is dependent on composition of each single composite. This is also confirmed in present study for investigated bulk-fill composites. Therefore the strongest effect through a bleaching process shows Tetric EvoCeram® Bulk Fill, followed by QuiXFil™ and X-tra fil (weakest effect) for PF 15% and also for PF 35%. Consequently, within the investigated bulk-fill composites the results vary in concentration depending on composition of each dental material.

The null hypothesis is rejected because PF 15% and PF 35% leads to different releases of components from bulk-fill composites, compared to the release of unbleached bulk-fill composites.

5. Conclusion

PF 15% and PF 35% can lead to reduced and/or increased elution of some bulk-fill components, compared to unbleached bulk-fill composites.

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2.1.4. Elutionsverhalten von 3D-gedruckten, gefrästen und konventionellen Aufbissschienen auf Methacrylatharzbasis [4]

Die Freisetzung von unpolymerisierten (Co-)Monomeren und Additiven aus Materialien auf Methacrylharzbasis kann negative Auswirkungen wie Mutagenität, Teratogenität, Genotoxizität, Zytotoxizität und östrogene Aktivität verursachen. Ziel der vorliegenden Studie war es, die Freisetzung von Inhaltsstoffen und das zytotoxische Potenzial von PMMA-basierten Aufbissschienenmaterialien unter Berücksichtigung physiologischer Schienengrößen zu untersuchen. Untersucht wurden drei Materialien, die für die additive (3D-Druck), subtraktive (Fräsen) und konventionelle (Pulver und Flüssigkeit) Herstellung verwendet werden. Von jedem Schienenmaterial (SHERAprint-ortho plus (additiv), SHERAeco-disc PM20 (subtraktiv) und SHERAORTHOMER (konventionell)) wurden 16 (n=4) Probenkörper (6 mm Durchmesser und 2 mm Höhe) einer bei der Herstellung von Aufbissschienen typischen Oberflächenbehandlung unterzogen. Anschließend wurden die Proben sowohl in Wasser als auch in Methanol 24 bzw. 72 h lang bei 37 °C eluiert und mittels GC/MS analysiert. Mit den Methacrylaten Tetrahydrofurfurylmethacrylat (THFMA), 1,4-Butylenglykoldimethacrylat- (BDDMA) und Tripropylenglykoldiacrylat (TPGDA) wurde ein XTT-basierter Zellviability-assay mit humanen Gingivafibroblasten (HGFs) durchgeführt. Zur Projektion der Ergebnisse auf eine durchschnittliche physiologische Schienengröße in einem „Worst-Case“-Szenario wurden Unter- und Oberkieferaufbissschienen hergestellt und vermessen. Für SHERAeco-disc PM20 und SHERAORTHOMER wurden keine Inhaltstoffe in der Wasser-Elution detektiert. SHERAprint-ortho plus eluierte die höchste THFMA-Konzentration von 7,47 µmol/l nach 72 h in Wasser. Sechs (Co-)Monomere und fünf Additive wurden in den Methanol-Eluaten aller drei getesteten Materialien nachgewiesen. Die XTT-basierten Zellviabilitätstests ergaben eine EC_{50} von 3006 µmol/l für THFMA, 2569,5 µmol/l für BDDMA und 596,7 µmol/l für TPGDA. Im Lösungsmittel Methanol überstiegen insgesamt die freigesetzten Komponenten aus den untersuchten Schienenmaterialien die zytotoxischen Konzentrationen in HGFs, die für ein „Worst-Case“-Szenario in physiologischer Schienengröße berechnet wurden. In den Wassereluaten konnte nur das Methacrylat THFMA aus SHERAprint-ortho plus in Konzentrationen unterhalb zytotoxischer Werte in HGFs bestimmt werden. Derzeit sind industriell vorgefertigte Polymerrohlinge für die subtraktive Fertigung (SHERAeco-disc PM20) in Bezug auf die Biokompatibilität überlegen. Neu entwickelte Werkstoffe für die additive Fertigung scheinen eine komplexere Zusammensetzung aufzuweisen und damit das Potenzial für eine Elution einer größeren Anzahl von (Co)Monomeren und Additiven zu haben.

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Elution behavior of a 3D-printed, milled and conventional resin-based occlusal splint material

Lennart Wedekind[a], *Jan-Frederik Güth*[a,d,*], *Josef Schweiger*[a], *Maximilian Kollmuss*[b], *Franz-Xaver Reichl*[b,c], *Daniel Edelhoff*[a], *Christof Högg*[b,c]

[a] *Department of Prosthetic Dentistry, University Hospital, LMU Munich, Germany*
[b] *Department of Conservative Dentistry and Periodontology, University Hospital, LMU Munich, Germany*
[c] *Walther Straub Institute of Pharmacology and Toxicology, Faculty of Medicine, LMU Munich, Germany*
[d] *Department of Prosthodontics, Center for Dentistry and Oral Medicine (Carolinum), Goethe-University Frankfurt am Main*

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ABSTRACT

Objective. The elution of unpolymerized (co-)monomers and additives from methacrylic resin-based materials like polymethyl methacrylate (PMMA) can cause adverse side effects, such as mutagenicity, teratogenicity, genotoxicity, cytotoxicity and estrogenic activity.

The aim of this study was to quantify the release and the cytotoxicity of residual (co-)monomers and additives from PMMA-based splint materials under consideration of real splint sizes. Three different materials used for additive (3D printing), subtractive (milling) and conventional (powder and liquid) manufacturing were examined.

Methods. The splint materials SHERAprint-ortho plus (additive), SHERAeco-disc PM20 (subtractive) and SHERAORTHOMER (conventional) were analysed. 16 (n = 4) sample discs of each material (6 mm diameter and 2 mm height) were polished on the circular and one cross-section area and then eluted in both distilled water and methanol. The discs were incubated at 37 °C for 24 h or 72 h and subsequently analysed by gas chromatography/mass spectrometry (GC/MS) for specifying and quantifying released compounds. XTT-based cell viability assays with human gingival fibroblasts (HGFs) were performed for Tetrahydrofurfuryl methacrylate (THFMA), 1,4-Butylene glycol dimethacrylate (BDDMA) and Tripropylenglycol diacrylate (TPGDA). In order to project the disc size to actual splint sizes in a worst-case scenario, lower and upper jaw occlusal splints were designed and volumes and surfaces were measured.

Results. For SHERAeco-disc PM20 and for SHERAORTHOMER no elution was determined in water. SHERAprint-ortho plus eluted the highest THFMA concentration of 7.47 μmol/l ±2,77 μmol/l after 72 h in water. Six (co-)monomers and five additives were detected in the methanol eluates of all three materials tested. The XTT-based cell viability assays resulted in a EC_{50} of 3006 ± 408 μmol/l for THFMA, 2569.5 ± 308 μmol/l for BDDMA and 596.7 ± 88 μmol/l for TPGDA.

* *Corresponding author at*: Department of Prosthodontics, Center for Dentistry and Oral Medicine (Carolinum), Goethe-University Frankfurt am Man, Theodor-Stern-Kai 7, 60596 Frankfurt am Main, Germany.
E-mail address: gueth@med.uni-frankfurt.de (J.-F. Güth).
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Significance. With the solvent methanol, released components from the investigated splint materials exceeded cytotoxic concentrations in HGFs calculated for a worst-case scenario in splint size. In the water eluates only the methacrylate THFMA could be determined from SHERAprint-ortho plus in concentrations below cytotoxic levels in HGFs.

1. Introduction

In the recent years, additive manufacturing (3D printing) has gained extensive attention in the field of dentistry. The fabrication of crowns, dentures and occlusal splints are few of the many applications of 3D-printing in dentistry. The increasing digital transformation has been gradually replacing conventional techniques, which commonly make use of polymethyl methacrylate (PMMA), a resin-based material. PMMA features advantages such as high aesthetics and good chemical and physical properties, while being relatively low in costs. Computer-aided design and computer-aided manufacturing (CAD/CAM) is based on either subtractive (milling or grinding of prefabricated material blocks) or additive (3D printing) methods. Subtractive manufacturing is not new to the dental discipline and reached the dental industry in the late 1980s. Therefore, milling materials have been extensively studied and have proven to be superior to conventionally processed materials regarding their physical properties [1–4]. 3D printing is a newer technology, which utilises liquid materials as opposed to industrially prefabricated milling blocks. As these novel materials developed for 3D printing require a particular composition, there is little knowledge about their biocompatibility and other properties so far [5].

An unfavourable effect of all methacrylate-based materials is the incomplete conversion of (co-)monomers to polymers, even under ideal polymerisation conditions. The degree of conversion (DC) is mainly influenced by parameters of the manufacturing process such as monomer to polymer ratio, polymerization time and temperature and, in case of photo-polymerization, light density and curing time [6,7]. The incomplete conversion of monomers entails a potential risk for a release of unbound residual (co-)monomers, additives and reaction products [8–11]. Furthermore, parts of the unreacted components may stay enclosed within the three-dimensional chain network and are therefore initially non-extractable [12]. Degradation processes through mechanical action [13], chemical interaction in the oral environment such as salivary [14] and bacterial enzymes [15] and hydrolysis [16] may lead to an increased elution of resin components.

In vitro studies concluded that (co-)monomers like triethylene glycol dimethacrylate (TEGDMA), 2-hydroxyethyl methacrylate (HEMA), methyl methacrylate (MMA) and extractable additives can induce negative effects like mutagenicity, teratogenicity, genotoxicity, cytotoxicity and estrogenic activity in cells of the oral cavity [17–21]. Further DNA damage may be supported by the degradation and metabolization of HEMA and TEGDMA to into epoxides such as 2,3-epoxymethacrylic acid (2,3-EMA). These epoxides are suspected to react with desoxyribonucleic acid (DNA), which can lead to DNA double-strand breaks (DNA-DSBs) [22,23]. Another way for DNA damage is oxidative stress resulting from the binding of methacrylate to the cell antioxidant and the radical scavenger glutathione (GSH) with subsequent depletion of GSH and increase of reactive oxygen species (ROS) [23–25].

As there are many studies reporting elution data for conventional and prefabricated milling materials, very few investigations can be found for additively processed materials [26]. This in-vitro study aims to investigate the component elution and the cytotoxic potential of PMMA-based occlusal splint materials under consideration of real splint sizes. The three methacrylate-based materials used in this study are commercially available for the additive (3D printing), subtractive (milling) and conventional (powder and liquid for injection molding) production of occlusal splints.

2. Materials and methods

2.1. Splint materials

The properties of investigated materials are shown in Table 1.

2.2. Preparation of specimens

Cylinders with a diameter of 6 mm and a height of 20 mm of each test material were fabricated according to the manufacturer's specifications.

2.2.1. Additive manufacturing (3D printing): SHERAprint-ortho plus

Cylinders for additive (3D) print technology were designed with CAD-software (Geomagic Design X, 3D Systems, Rock Hill, South Carolina, USA) and printed with the SHERAeco-print 30 (SHERA, Lemförde, Germany) in Digital Light Processing (DLP) technique with Force-Feedback-Technology in a layer thickness of 50 μm. According to the manufacturer, an angulation of 20° to the building platform was set. After the printing process, excessive material and the supporting structure were removed through a gentle wipe with SHERAclear-p and paper tissues. The printed material was cleaned twice in an ultrasonic bath with SHERAultra-p for three minutes; in between, excessive and dissolved material was blown off by compressed air. A postpolymerization step is implemented with the SHERAflash- light plus through two times 2000 flashes of light and under exclusion of oxygen by a constant flow of inert gas (nitrogen, 1 bar).

2.2.2. Subtractive manufacturing: SHERAeco-disc PM20

Cylinders for subtractive manufacturing were designed with CAD-software (Geomagic Design X, 3D Systems, Rock Hill,

Table 1 – Investigated splint materials, manufacturer, LOT numbers and material composition based on manufacturer data.

Product name	Manufacturer	LOT	Composition of materials based on manufacturer's data
SHERAprint-ortho plus	SHERA® Werkstoff-Technologie GmbH & Co. KG, Lemförde, Germany	11317	Acrylated resin, tetrahydrofurfuryl methacrylate (THFMA), Alipathic urethane acrylate, (1-methyl-1,2-ethanediyl)bis[oxy(methyl-2,1-ethanediyl)]diacrylate (TPGDA), diphenyl (2,4,6- trimethylbenzoyl)phosphine oxide
SHERAeco-disc PM20		89360	Polymethyl methacrylate (PMMA), dimethylacrylate Colour pigments
SHERAORTHOMER		41565 (powder) 35608 (liquid)	Powder: Polymethyl methacrylates (PMMA), benzoyl peroxide Colour pigments Liquid: Methylmethacrylate (MMA), 1,4-butanediol dimethacrylate (BDDMA), N,N-Bis(2-hydroxyethyl)-*p*-toluidin (DHEPT), 2(2′-hydroxy-5′-methylphenyl)benzotriacol (TinP), hydroquinone monomethyl ether, hydroquinone, colour pigments Catalysts

South Carolina, USA) and milled from industrially prefabricated SHERAeco-disc PM20 blanksby Ceramill Motion 2 (Ammann Girrbach GmbH, Pforzheim, Germany).

2.2.3. *Conventional manufacturing: SHERAORTHOMER*

Cylinders for conventional manufacturing were manufactured with polymer powder and monomer liquid in the dough technique. In accordance with the manufacturer's information, the mixing ratio was 2:1 weight proportion polymer powder to monomer liquid. Powder and liquid were mixed in a ceramic mixing cup with a metal spatula and subsequently poured into a polytetrafluorethylen (PTFE)-tube (Reichelt Chemietechnik, Heidelberg, Germany). For the polymerisation process, the PTFE-tubes were then placed in a pressure pot at 45 °C under 3 bar pressure for 10 min.

2.2.4. *Finalization of specimens*

The prepared cylinders of all materials were then sliced into circular discs through serial cuts under water cooling by a Secotom 50 (Struers, Augsburg, Germany). The circular surface and one cross-section area of the discs were high gloss polished according to a set polishing protocol as follows: grinding with corundum sandpaper 350 μm then 400 μm, rubberize with 3-stage polishers for acrylics from Komet, REF 9603, 9641, 9644 (Komet Dental, Gebr. Brasseler GmbH & Co. KG, Lemgo, Germany), pro-polishing with goat hair brush white, Polirapid 48 mm, REF 52766 (Nordwest Dental GmbH & Co. KG, Münster, Germany) and pumice powder (Benzer-Dental AG, Zürich, Switzerland), high-gloss polishing with Abraso Star Glaze, high luster polishing paste, REF 52000163 (Bredent, Senden, Germany) and cotton cloth (calico) fine disc, Polirapid, 100 mm, REF 60103 (Nordwest Dental GmbH & Co. KG, Münster, Germany), cleaning with steam jet and ultrasonic bath. The second cross-section area of each disc remained unpolished. 16 fin. l test specimens of each material measured 6 mm in diameter and 2 mm in height, which results in a specimen surface of 94.2 mm^2 and a volume of 56.5 mm^3.

2.3. *Sample incubation*

The specimens were incubated in tightly sealed brown glass vails (Macherey-Nagel, Düren, Germany) with 1 ml methanol (GC Ultra Grade, ROTISOLV® ≥99.9%, Roth, Karlsruhe, Germany) or 1 ml water (LC–MS-Grade, ROTISOLV®, Roth, Karlsruhe, Germany) at 37 °C. After 24 h and 72 h, eluates were analysed by GC/MS. Standard caffeine (CF) solution (0.01 mg/mL) (HPLC ≥ 99.0%, Sigma–Aldrich, St. Louis, United States) was added as an internal standard to enable the determination of relative amounts of eluted substances. Water samples need to be extracted with ethyl acetate (1:1, v/v) (LC–MS-Grade, ROTISOLV® ≥99.9%, Roth, Karlsruhe, Germany) for the analysis with GC/MS [27].

2.4. *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-5 ms capillary column (length 30 m, inner diameter 0.25 mm, coating 0.25 μm; 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 at this temperature for 5 min. The GC oven was initially heated isothermally at 50 °C for 2 min, then increased to 280 °C (25 °C/min) and finally held at this temperature for 5 min. The mass 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).

Table 2 – Detected compounds, compound function, molecular weight and CAS number.

Compound abbreviation	Compound name	Compound function	Molecular weight (g/mol)	CAS-number
BDDMA	1,4 Butylene glycol dimethacrylate	(Co-)monomer	226.27	2082-81-7
BHT	2,6-Di-tert-butyl-4-methylphenon	Inhibitor, antioxidant	220.35	128-37-0
BP	Biphenyl	Initiator	154.21	92-52-4
BPE	Phenyl benzoate	Initiator	198.22	93-99-2
DCHP	Dicyclohexylphtalat	Plasticizer	330.42	84-61-7
EGDMA	Ethylene glycol dimethacrylate	(Co-)monomer	198.22	97-90-5
HEMA	2-Hydroxyethyl methacrylate	(Co-)monomer	130.14	868-77-9
HPMA	3-Hydroxyprophyl methacrylate	(Co-)monomer	144.17	27813-02-1
MMA	Methyl methacrylate	(Co-)monomer	100.12	80-62-6
THFMA	Tetrahydrofurfuryl methacrylate	(Co-)monomer	170.21	2455-24-5
TinP	2-(2′-Hydroxy-5′-methylphenyl)benzotriazol, Tinuvin P	Photo stabilizer	225.25	2440-22-4

2.5. *XTT-based viability assay*

XTT-based viability assays in HGFs were performed for those acrylates that were part of the composition of materials (Table 1) without existing data regarding their EC_{50} in HGFs.

2.5.1. *Chemicals*

Tetrahydrofurfuryl methacrylate (THFMA), 1,4-Butanediol dimethacrylate (BDDMA) and Tri(propylene glycol) diacrylate (TPGDA) were obtained from Sigma-Aldrich (Munich, Germany). The substances were dissolved in dimethyl sulfoxide (DMSO, 99% purity; Merck, Darmstadt, Germany) and diluted with medium (final DMSO concentration: <1%). Control cells were exposed to Triton X (1%; Sigma-Aldrich, Munich, Germany) in medium.

2.5.2. *Cell culture for the XTT-based viability assay*

HGFs(Cat.–No.:1210412) were obtained from Provitro GmbH (Berlin, Germany). The HGFs were cultured as described in our former study [28].

2.5.3. *XTT-based viability assay*

An XTT-based cell viability assay was used to define the half-maximum effect concentration (EC_{50}) for the investigated substances in HGFs. This assay was performed as described in our former study [28]. The cells were treated with Dulbecco's modified eagle's medium (DMEM) (PAN Biotech, Aidenbach, Germany) containing THFMA (0.01–10 mM), BDDMA (0.1–30 mM) and TPGDA (0.1–30 mM) respectively, followed by incubation at 37 °C, with 5% CO_2 and 100% humidity for 24 h. Control cells received medium only; as a negative control the cells were exposed to 1% Triton X-100. The formazan formation was quantified by determination of the optical density (OD) with a spectrophotometer at 450 nm (reference wavelength 670 nm), using a microplate reader (MULTISKAN FC; Thermo Fisher Scientific, Waltham, MA). Three independent experiments were performed, each time in triplicate. The cell viability was calculated according to the following equation:

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

2.6. *Splint measurement*

A standard working model series AG-3 (Frasanco GmbH, Tettnang, Germany) for maxilla and mandible was scanned with ZIRKONZAHN.Scan® (Zirkonzahn S.R.L., Gais, Italy) and served for the design of upper and lower jaw Michigan splints with ZIRKONZAHN.Modellier® software (Zirkonzahn S.R.L., Gais, Italy). Splint thickness was configured to 1.5 mm with a minimum of 1 mm.

The splint dimensions were subsequently measured by metrology software (Geomagic Control X, 3D Systems, Rock Hill, South Carolina, USA).

2.7. *Data analysis*

The GC/MS and XTT-based cell viability assay results are presented as mean ± standard deviation (SD).

3. Results

3.1. *GC/MS results*

A total of 11 eluted substances (Table 2) were determined in the three materials tested, whereof six are (meth)acrylates.

3.1.1. *SHERAprint-ortho plus*

The solvent methanol eluted the (co-)monomers MMA, HEMA, HPMA, THFMA and EGDMA as well as the inhibitor BHT and the photo stabilizer TinP (Table 3).

In the solvent water an elution of the (co-)monomer THFMA was determined (Table 3).

3.1.2. *SHERAeco-disc PM20*

In methanol an elution of the (co-)monomer MMA as well as the initiator components BP and BPE were determined (Table 3).

No (meth)acrylates or additives were determined in the water eluates.

Table 3 – Qualification and quantification of substances in methanol and water eluates of tested materials after 24 h and 72 h. Data are presented as mean ± SD [µM].

SHERAprint-ortho plus (additive manufacturing)		
Compound	Methanol	
Mean ± SD [µM]	24 h	72 h
MMA	66.09	70.32
±	16.74	7.45
HEMA	238.98	378.98
±	33.02	37.12
HPMA	57.16	108.14
±	9.89	15.76
THFMA	100.83	131.92
±	10.82	3.08
EGDMA	1.36	3.30
±	0.21	2.53
BHT	0.12	0.37
±	0.04	0.10
TinP	1.08	1.34
±	0.92	1.45
Compound	Water	
Mean ± SD [µM]	24 h	72 h
THFMA	2.03	7.47
±	0.22	2.77
SHERAeco-disc PM20 (subtractive manufacturing)		
Compound	Methanol	
Mean ± SD [µM]	24 h	72 h
MMA	2383.28	3513.53
±	237.62	226.98
BP	30.94	55.83
±	2.90	1.92
BPE	957.21	1676.58
±	109.87	76.96
SHERAORTHOMER (conventional manufacturing)		
Compound	Methanol	
Mean ± SD [µM]	24 h	72 h
MMA	8173.80	8768.23
±	963.08	148.65
BP	2.90	3.10
±	0.28	0.28
BDDMA	148.32	161.00
±	48.37	52.13
BPE	434.82	481.70
±	20.52	25.77
TinP	368.05	440.16
±	31.15	20.14
DCHP	3.14	5.48
±	0.65	1.08

3.1.3. *SHERAORTHOMER*

In the methanol eluates the (co-)monomers MMA and BDDMA, the photo stabilizer TinP, the softener DCHP and the initiator components BP and BPE could be determined (Table 3).

No (meth)acrylates or additives were determined in the water eluates.

3.2. *XTT assay results*

The EC_{50} values of THFMA, BDDMA and TPGDA in HGFs were determined and a relative toxicity was calculated according to those values (Table 4).

Table 4 – EC_{50} values (mean ± SD) and relative toxicity of THFMA, BDDMA and TPGDA in HGFs.

mean ± SD [µM]	EC_{50}	Relative toxicity
THFMA	3006 ± 408	1
BDDMA	2569.5 ± 308	1.2
TPGDA	596.7 ± 88	5.0

3.3. *Splint measurement results*

The measurements resulted in a surface of 5128.6 mm^2 and a volume of 3217.9 mm^3 for an upper jaw occlusal splint, and a surface of 5153.9 mm^2 and a volume of 3198.9 mm^3 for a lower jaw occlusal splint (Fig. 1). The mean between upper and lower jaw splint is 5141.25 mm^2 (surface) and 3208.4 mm^3 (volume).

4. Discussion

As a high biocompatibility and therefore health safety of dental materials is of crucial significance for patients as well as practitioners. This in-vitro study focuses on the elution characteristics of three different methacrylic resin-based PMMA-materials for dental splint fabrication in additive (3D printing), subtractive (milling) and conventional manufacturing (powder and liquid) techniques.

Since being classified as a Class IIa medical device according to the European Medical Device Directive (guideline 93/42/EEC), SHERAprint-ortho plus, which was developed for the relatively new technique of additive manufacturing, is authorised for a continuous or repeated intraoral use for up to 30 days and was therefore chosen as a test material in this study. For the well-established methods of subtractive and conventional manufacturing, SHERAeco- disc PM20 (subtractive) and the conventional cold cure material SHERAORTHOMER were chosen (all test materials from SHERA®, Lemförde, Germany).

The measurements were performed with GC which is recommended as the method of choice for the determination of MMA [29] and has been used by different authors [17,30–32]. The combination with a highly compatible mass spectrometry enables the disclosure of detailed structural information and allows a precise determination of eluted compounds [33]. Based on the results of this study, GC/MS can be designated as an effective method to determine elution characteristics of PMMA.

The elution periods of 24 h and 72 h were already used in our previous study on elution characteristics of composites [34]. Other studies additionally measured the elution over a period of 7 days [35,36]. According to different authors, the major proportion of (co-)monomers and additives usually elutes within the first 24 h [34,37]. In this study, the 24 h results always were lower than the 72 h results.

In accordance to previous studies with methacrylic resin-based materials [34,38], in the present study also specimen disk dimensions of 6 mm in diameter and 2 mm in height were used. All investigated specimens underwent the same surface treatment. In approach to the manufacturing of dental splints, where the inner side is not submitted to post processing procedures like polishing because of an otherwise impending loss

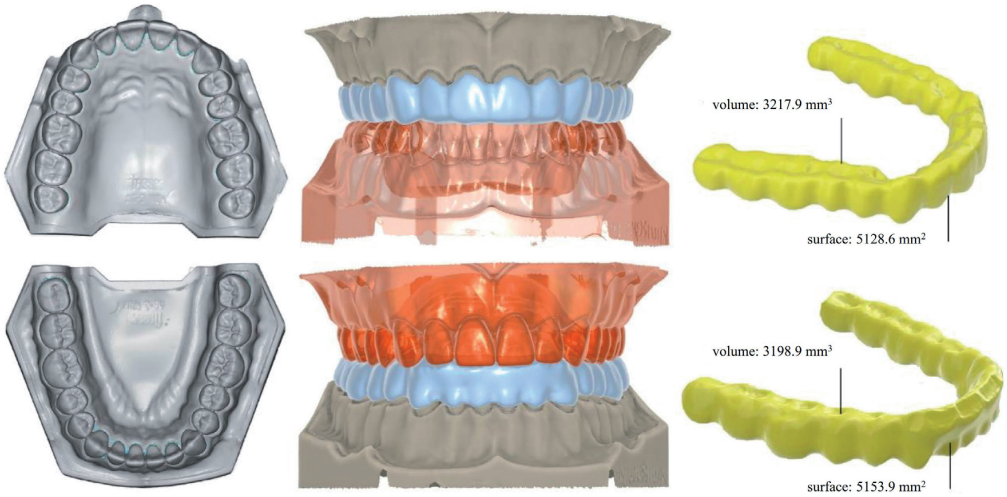

Fig. 1 – CAD of uper and lower jaw splints for volume and surface area determination.

of fit, one specimen cross section area remained unpolished. This is relevant since it could be shown that polishing diminishes residual monomer elution [39,40].

The number and quantity of eluted compounds and their detection rate is highly conditional to the solvent medium; consequently solvents should be chosen pursuant to requirements [41,42]. In the present study, distilled water and methanol were chosen as solvents. Water is considered most similar to human saliva and dentin fluid and therefore designated as a relevant solvent to allow the physiological comparison to intraoral conditions [43,44]. Organic solvents like methanol have a much better solubility of organic matrix components and deeper matrix penetration [45]. The (unintentional) breakdown of (co-)monomers into smaller proportions is much less with methanol then with other strong organic solvents like acetone and enables the elution of a maximum proportion of residual compounds [38,46] and therefore reflects the worst-case situation of released compounds.

Since no data were found on occlusal splint volume or surface, we designed and measured a Michigan splint for upper and lower jaw. This allowed the upscaling of our results to average splint size for a worst-case scenario in splint size. According to present results, the mean splint is 56.8 times higher in volume and 54.6 times higher in surface than the investigated specimen. However, elution characteristics and mechanism of methacrylic resin-based material depend on the molecular weight, hydrophobicity, filler content, investigated material and the final network characteristics of the resin-matrix [47–51]. For a worst-case scenario calculation, a splint/specimen-factor of 55.7 (mean of volume and surface factor) was used to calculate maximal possible elution in splint size. As a major part of the oral cavity is lined with HGFs, they partly get in direct contact to the splint material. Therefore, in the following, their EC_{50} was used to assess the cytotoxic potential of the projected elution data in splint size.

MMA as the main component of PMMA was found in the solvent methanol of all three tested materials. In a previous study, the EC_{50} for MMA in HGFs was determined at 70 mmol/l [52]. In the methanol eluates, the highest MMA-concentration was found in SHERAORTHOMER with 8768 μmol/l after 72 h. When projected to splint dimensions, in a worst-case scenario MMA could reach a concentration of 488378 μmol/l after 72 h in methanol, which would exceed the EC_{50} cited above in HGFs about 7 times. The second highest MMA concentration was found in SHERAeco-disc PM20 with 3514 μmol/l after 72 h. Extrapolated to splint dimensions, MMA could elute in concentrations of up to 195729 μmol/l within 72 h in methanol, exceeding the EC_{50} cited above in HGFs by a factor of about 3. The least MMA concentration was found in SHERAprint-ortho plus with 70 μmol/l after 72 h. Referred to splint dimensions in a worst-case scenario, the MMA concentration in methanol could reach 3899 μmol/l after 72 h and would therefore be under cytotoxic concentrations by a factor of about 18. In the water eluates of all three investigated materials however, no MMA could be detected. Since water eluates provides the highest possible approximation to dentinal fluid and human saliva [34,53], no cytotoxic effects of MMA on the physiological situation of humans are to be expected.

The monofunctional methacrylate THFMA is a listed component in the manufacturer's data of SHERAprint- ortho plus (Table 1) only and accordingly was only detected in its eluates. For example, THFMA can be used as a diluent to lower material viscosity [54]. In the present study cytotoxicity for THFMA in HGFs was determined by XTT viability assay with an EC_{50} of 3006 μmol/l. The highest THFMA concentration in this study was found in the methanol eluates of SHERAprint-ortho plus after 72 h with a value of 132 μmol/l. In a splint sized worst-case scenario, THFMA concentration in methanol would be 7352 μmol/l within 72 h surpassing the EC_{50} in HGFs by a factor of about 2. However, highest THFMA concentration in water eluates of SHERAprint- ortho plus was measured after 72 h with a value of 7 μmol/l. Projected to splint dimensions

THFMA concentration (416 μmol/l), would be about 7 times lower than determined cytotoxic concentration and 18 times lower than the concentration of the corresponding methanol eluates. Based on these results, cytotoxic effects are not to be expected from eluting THFMA in the human physiological situation.

The acrylate HEMA has only been found in the methanol eluates of SHERAprint-ortho plus. HEMA is hydrophilic, reduces surface tension and is therefore often used as a primer component in dentin-bonding systems [55]. HEMA is not listed as a component of the investigated materials by the manufacturers. HEMA is described as a possible degradation product of the long-chained Urethan dimethacrylate (UDMA). UDMA was described to disintegrate into HEMA in the injector during GC/MS-analysis [56,57]. Additionally, HEMA is described as a possible impurity of methacrylic resin-based material components (e.g. UDMA) [27]. Therefore, the source of HEMA in our eluates is unknown. In concentrations of 1 mmol/l, HEMA induces inflammatory effects and malfunction of the cellular redox balance by excessive formation of reactive oxygen species (ROS) [25]. According to Urcan et al. [28], the EC_{50} of HEMA in HGFs is 11 mmol/l. The highest HEMA concentration found in SHERAprint-ortho plus was 379 μmol/l after 72 h in methanol. In a worst-case scenario in splint size, HEMA-elution could be 21110 μmol/l, surpassing the EC_{50} values by Urcan et al. by a factor of about 2. In the water eluates, no HEMA could be detected, therefore no cytotoxic effects are to be expected in the human physiological situation.

The difunctional methacrylate EGDMA has a high allergic potential [58] and frequent cross-reaction with HEMA [59]. It is utilised as a cross linker in linear polymers and has been detected exclusively in SHERAprint-ortho plus in methanol with a maximum concentration of 3 μmol/l after 72 h. The EC_{50} in HGFs is 0.46 mmol/l [60]. In a worst-case scenario in splint size, EGDMA could reach a concentration of 184 μmol/l within 72 h which is about 3 times lower than the cytotoxic dose.

The methacrylate BDDMA could only be identified in SHERAORTHOMER with the solvent methanol. Nocca et al. [61] stated that 0.4 mmol/l BDDMA decreased cell proliferation by 80% and significantly raised cell death rate in a human leukaemia HL-60 cell line. In the present study XTT-based viability assay for BDDMA in HGFs resulted in an EC_{50} of 2570 μmol/l. The highest BDDMA concentration found in SHERAORTHOMER was 161 μmol/l after 72 h. In a worst-case scenario in splint size the solvent methanol leads to a possible elution of 8968 μmol/l within 72 h. This would be about 3 times higher than the EC_{50}. Since there was no elution registered in all the water samples, toxic effects are not to be expected from eluting BDDMA in the human physiological situation.

Noteworthy, TPGDA, a multifunctional acrylate that acts as a diluent and cross-linker in ultraviolet curable resins, is listed by the manufacturer as a component of SHERAprint-ortho plus, but could not be detected at all. This may be due to its cross-linking characteristics and a high conversion rate. It is described as a moderate sensitizer by Björker [62]. The EC_{50} of TPGDA in HGFs is 597 μmol/l, which means a relative toxicity of 5 to THFMA. TPGDA is therefore five times more cytotoxic than THFMA.

Besides (meth)acrylates, the inhibitor BHT, one of the most cytotoxic additives investigated by Geurtsen et al. [60], was found in SHERAprint-ortho plus in methanol but not in water. It could be shown that on oral administration BHT can be hepatotoxic in rats [63]. BHT might trigger allergic rhinitis and asthma [64]. The highest concentration found in this study was 0.4 μmol/l after 72 h in methanol. The EC_{50} in HGFs is 170 μmol/l [60]. In a worst-case scenario in splint dimensions, BHT could be released in methanol in concentrations of 21 μmol/l within 72 h. A cytotoxic concentration for HGFs would still be about 8 times higher.

Deductively it can be summarized that in approximation to potential cytotoxic effects in a worst-case-scenario in splint size the solvent methanol eluted compounds in concentrations partially exceeding cytotoxic concentrations in HGFs. These results should not necessarily be interpreted as alarming, since methanol is not comparable to saliva. The water samples, allowing the utmost comparison to saliva, showed no elution of any investigated components for SHERAORTHOMER and SHERAeco-disc PM20, for SHERAprint-ortho plus the methacrylate THFMA eluted in concentrations that turned out to be about 7 times below the deducted EC_{50} in HGFs. It can therefore be assumed that under normal physiological intraoral conditions no undesirable cytotoxic effects from neither one of the tested splint materials are to be expected. Rothmund et al. determined a significantly lower (co-)monomer release in native saliva compared to protein-free saliva and water [34]. A binding of (co-)monomers and additives to salivary proteins could effectuate a lower bioavailability and therefore lower cytotoxic potential in vivo [34]. Additionally, it was shown that big differences in the MMA concentration between whole saliva (40 μg/mL) and the salivary film on palatal appliances (180 μg/mL) can occur [30]. Consequently, in physiological situation substances could accumulate in particular on unpolished inner fitting surfaces where saliva flow rate is low. Therefore, the concentration could be conditional to the specific location, resulting in a higher cytotoxic potential.

Noticeable is also the considerably higher number of different methacrylate types that could be detected in the eluates from SHERAprint-ortho plus (five) compared to SHERAORTHOMER (two) and SHERAeco-disc PM20 (one). The material composition of the additive material SHERAprint-ortho plus may be more complex due to higher material requirements in the processing chain in terms of printability or the fact of its being provided in a premixed form. It is important to note that a single-compound's cytotoxicity is not necessarily adding up in a multi-component exposure. Complex interactions and antagonistic effects between different compounds can sometimes even lead to a lower cytotoxicity in multi-component exposure [18,65,66]. Furthermore, oral bacteria might have an impact on the cytotoxicity of (co-)monomers. When HGFs were co-cultured with *Streptococcus mitis* strains and HEMA (3 mM) was added, a cross-protection with beneficial effects for both with a decrease of HGFs' cell deaths was observed [67].

Nevertheless, numerous (meth)acrylates and additives are strong sensitizers and can cause undesirable effects like triggering and formation of allergies and cross-reactions [59,68]. If an allergy is already known and confirmed by patch-testing,

special attention should be paid to the use of appropriate materials.

For the moment, industrially prefabricated polymer blanks for subtractive manufacturing (SHERAeco-disc PM20) may be superior in terms of biocompatibility. Newly developed materials for additive manufacturing seem to have a more complex composition and therefore the potential for an elution of a higher number of (co-)monomers and additives.

5. Conclusion

With the solvent methanol, released components from the investigated splint materials exceeded cytotoxic concentrations in HGFs calculated for a worst-case scenario in splint size. In the water eluates only the methacrylate THFMA could be determined from SHERAprint-ortho plus in concentrations below cytotoxic levels in HGFs. Therefore, in the physiological (water/saliva) situation health risk is of minor relevance.

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2.2. Toxikologie der freigesetzten Inhaltsstoffe aus dentalen Kompositen

2.2.1. Einfluss von Antioxidantien auf Comonomer-Epoxy-Metaboliten-induzierte DNA-Doppelstrangbrüche in humanen Gingivafibroblasten [48]

Aus Kompositmaterialien freigesetzte (Co)Monomere können über das Zwischenprodukt Methacrylsäure (MA) zu Epoxy-Verbindungen metabolisiert werden. Des Weiteren konnte gezeigt werden, dass Antioxidantien die zytotoxische Wirkung und DNA-Doppelstrangbrüche (DNA-DSBs) von (Co)Monomeren reduzieren können. In der vorliegenden Studie wurde der Einfluss der Antioxidantien Ascorbinsäure (Asc) und N-Acetylcystein (NAC) auf durch (Co)Monomer-Epoxy-Metaboliten induzierte DNA-DSBs untersucht. Dazu wurden HGFs mit MA, 2,3-Epoxy-2-Methylpropionsäure-Methylester (EMPME) bzw. 2,3-Epoxy-2-Methylpropionsäure (EMPA) in Anwesenheit bzw. Abwesenheit von Asc bzw. NAC inkubiert. Die Zytotoxizität wurde mit einem XTT-basierten Zellviability-assay ermittelt und die DNA-DSBs mit einem γ-H2AX-Assay bestimmt. Für die untersuchten Substanzen ergab sich folgende relative Reihenfolge der Zytotoxizität: EMPA > EMPME > MA. Die durchschnittliche Anzahl der DSBs-Foci/Zelle, die von der jeweiligen Substanz bei der EC_{50} induziert wurde, konnte in der folgenden Reihenfolge geordnet werden: EMPA > EMPME > MA. EMPA (1,72 mM) und EMPME (2,58 mM) induzierten die höchste Anzahl von DSBs-Foci/Zelle, d.h. das 21-fache bzw. 13-fache im Vergleich zur Kontrolle. Die Zugabe von Asc (50; 100; 200 µM) bzw. NAC (50; 100; 200; 500 µM) zur Inkubation mit MA (15,64 und 5,21 mM), EMPME (2,58 mM) und EMPA (1,72 und 0,57 mM) reduzierte die Anzahl der DSBs-Foci/Zelle in HGFs signifikant. Die größte Reduktion wurde für 1,72 mM EMPA gefunden: Die Zugabe von NAC (50; 100; 200; 500 µM) führte zu einer 15-fachen, 17-fachen, 14-fachen bzw. 14-fachen Verringerung der Anzahl der DSBs-Foci/Zelle. Die Comonomer-Epoxy-Metaboliten EMPME und EMPA weisen im Vergleich zu ihrem metabolischen Vorläufer, MA eine höhere Zytotoxizität auf und induzieren mehr DNA-DSBs. Die Antioxidantien Asc bzw. NAC führen zu einer Verringerung der Anzahl von DNA-DSBs, wobei NAC im Vergleich zu Asc insgesamt zu einer niedrigeren Anzahl von DNA-DSBs führte.

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

Yang Yang[a,b], *Xiuli He*[a,b], *Jianwei Shi*[c], *Reinhard Hickel*[a], *Franz-Xaver Reichl*[a,b], *Christof Högg*[a,b,*]

[a] *Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany*

[b] *Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University of Munich, Nußbaumstr. 26, 80336 Munich, Germany*

[c] *Department of Orthodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany*

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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.

* *Corresponding author at:* Department of Operative/Restorative Dentistry, Periodontology and Pedodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany. Fax: +49 89 7095 73817.
E-mail address: Christof.hoegg@lrz.uni-muenchen.de (C. Högg).

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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 7×10^4 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).

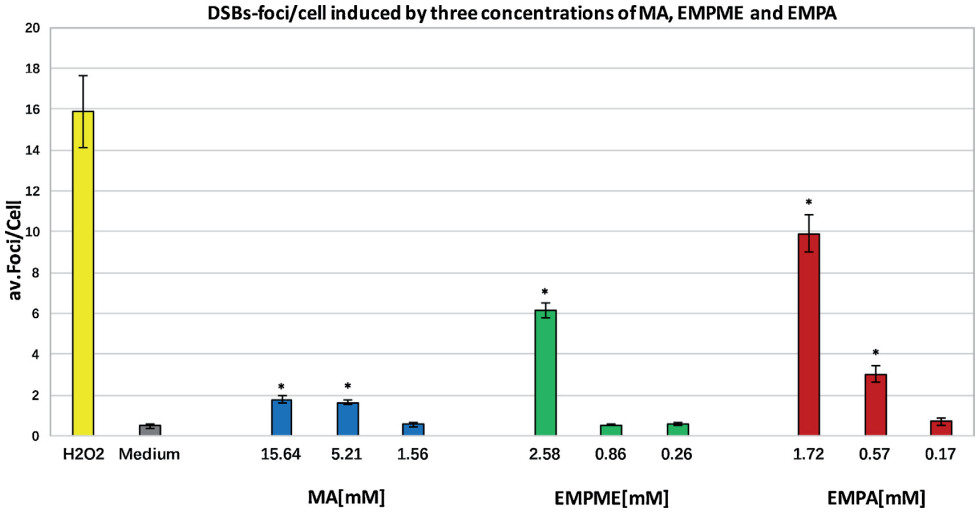

Fig. 1 – Average of induced γ-H2AX DSBs-foci/cell in HGFs elicited by a 6 h exposure to MA, EMPME and EMPA at EC_{50}, $1/3EC_{50}$ and $1/10EC_{50}$ concentrations. Negative control cells received the medium only. All results are expressed as the mean of four independent experiments; error bars represent 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)

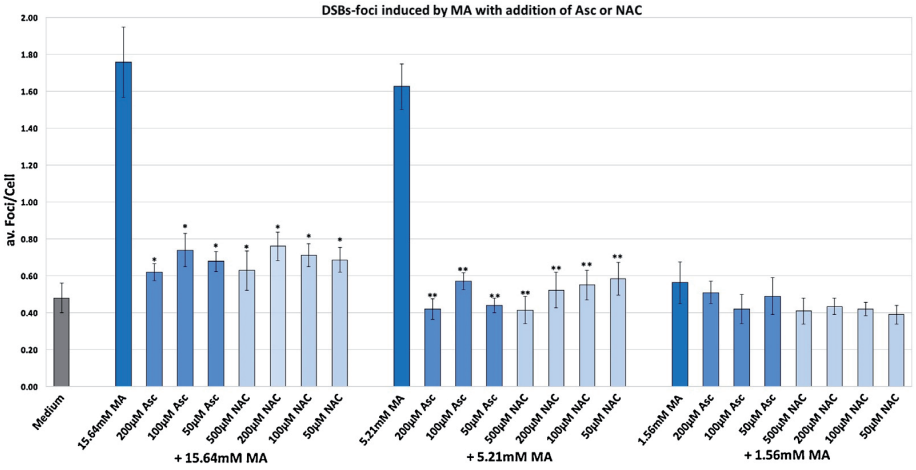

Fig. 2 – Average of induced γ-H2AX DSBs-foci/cell in HGFs elicited by a 6 h exposure to MA at EC_{50}, $1/3EC_{50}$ and $1/10EC_{50}$ concentrations, with addition of Asc (50–200 μM) or NAC (50–500 μM), respectively. Negative control cells received the medium only. All results are expressed as the mean 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).**

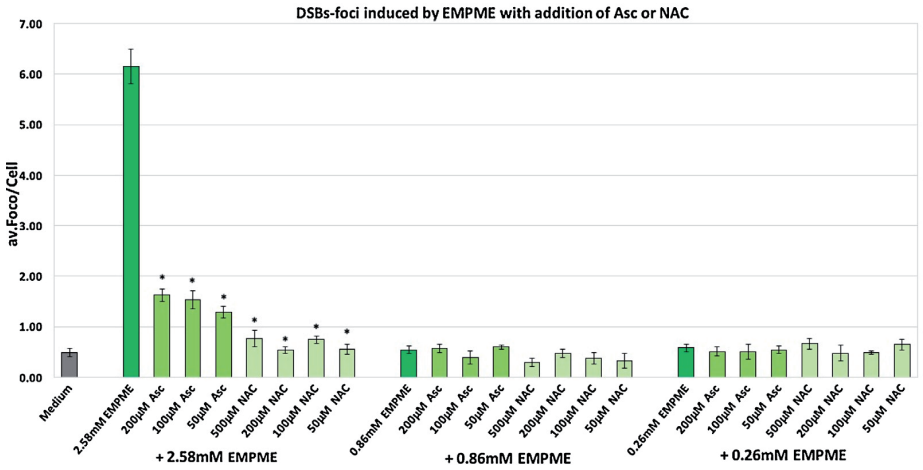

Fig. 3 – Average of induced γ-H2AX DSBs-foci/cell in HGFs elicited by a 6 h exposure to EMPME at EC_{50}, $1/3EC_{50}$ and $1/10EC_{50}$ concentrations with addition of Asc (50–200 μM) or NAC (50–500 μM) respectively. Negative control cells received the medium only. All results are expressed as the mean 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)

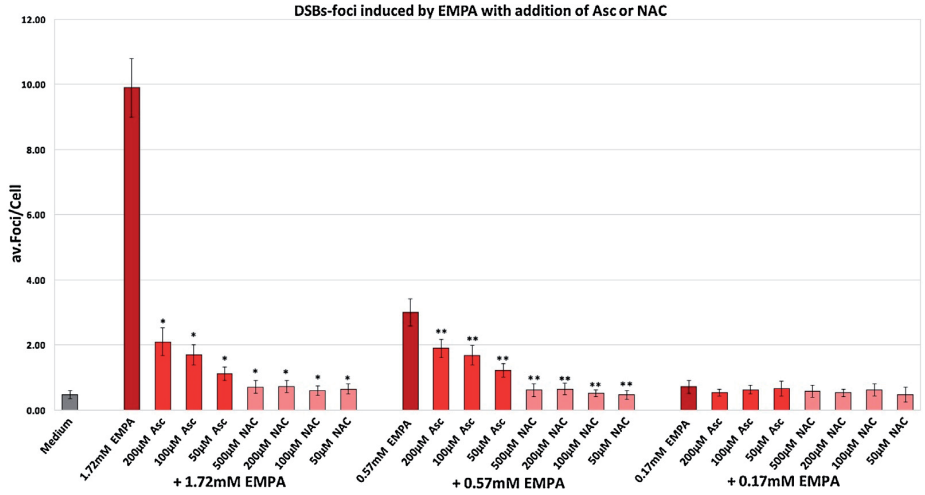

Fig. 4 – Average of induced γ-H2AX DSBs-foci/cell in HGFs elicited by a 6 h exposure to EMPA at EC_{50}, $1/3EC_{50}$ and $1/10EC_{50}$ concentrations with addition of Asc (50–200 μM) or NAC (50–500 μM) respectively. Negative control cells received the medium only. All results are expressed as the mean 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).**

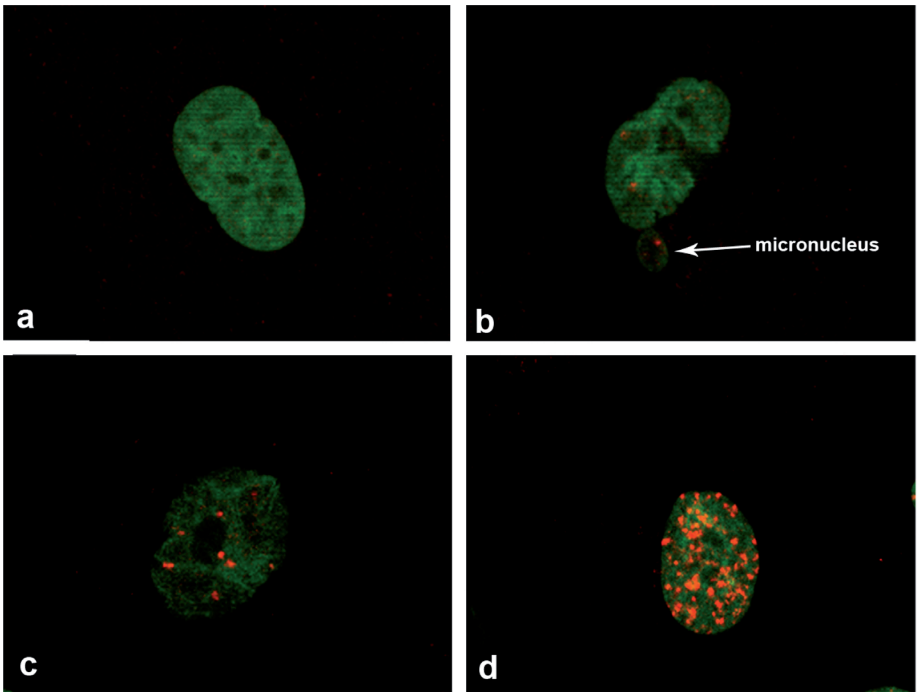

Fig. 5 – Representative images of immunofluorescent staining for H2AX phosphorylation (orange) in HGFs, after exposure to different substances compared to control cells. Sybr green (green) is a marker for DNA and stains the whole nucleus of the cell. (a) A nucleus of HGFs without foci, as typically seen 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 ($1/3EC_{50}$), and HEMA at 11.2 mM (EC_{50}) and 3.7 mM ($1/3EC_{50}$) 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 ($1/3EC_{50}$) 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. 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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2.2.2. Zytotoxizität und Induktion von DNA-Doppelstrangbrüche in humanen Gingivafibroblasten bei Exposition mit Eluaten aus dentalen Kompositen [26]

Bisher wurden einzelne Kompositinhaltsstoffe zur Untersuchung der Zytotoxizität und der Induktion von DNA-Doppelstrangbrüchen (DNA-DSBs) von dentale Kompositmaterialien verwendet. In der vorliegenden Studie wurden die Zytotoxizität und die Induktion von DNA-DSBs in HGFs mit Komposit-Eluaten, die aus mehreren Inhaltsstoffen bestehen untersucht. Die aus den untersuchten Kompositen freigesetzten Inhaltsstoffe wurden mittels GC/MS qualifiziert und quantifiziert. Die Kompositmaterialien EsthetX HD, Venus, X-tra fil, CLEARFIL AP-X, Admira Fusion und QuiXfil wurden gemäß Herstellerangaben polymerisiert und 72 h in Dulbecco's modified Eagle's medium (DMEM) eluiert. Anschließend wurden HGFs mit den entsprechenden Komposit-Eluaten inkubiert. Zur Bestimmung der Zytotoxizität und Gentoxizität der untersuchten Komposit-Eluate wurden XTT- und γ-H2AX-Tests durchgeführt. HGFs, die den Eluaten der untersuchten Komposite ausgesetzt waren, zeigten im Vergleich zur Negativkontrolle (nur DMEM) keinen signifikanten Einfluss auf Zellviabilität. Allerdings induzierten die Eluate von Esthet.X HD (0,43 Foci/Zelle) und Venus (0,39 Foci/Zelle) in HGFs, im Vergleich zur Kontrolle (0,22 Foci/Zelle) eine signifikant höhere Anzahl an DNA-DSBs. In den untersuchten Komposit-Eluaten wurden insgesamt 12 Substanzen nachgewiesen. Fünf davon waren Methacrylate: Tetraethylenglykoldimethacrylat (TEGDMA), 2-Hydroxyethylmethacrylat (HEMA), Hydroxypropylmethacrylat (HPMA), Ethylenglykoldimethacrylat (EGDMA) und Trimethylolpropantrimethacrylat (TMPTMA). Die höchste Konzentration von HEMA (110,5 µM), HPMA (86,08 µM) und TMPTMA (4,50 µM) wurde in den Eluaten von QuiXfil, die höchste Konzentration von TEGDMA (1080 µM) in den Venus-Eluaten und die höchste Konzentration von EGDMA (3,18 µM) in den Esthet.X HD-Eluaten nachgewiesen. Die Exposition von HGFs mit den Eluaten von Esthet.X HD und Venus zeigten eine signifikante Induktion von DNA-DSBs. In allen untersuchten Eluaten wurde keine signifikante Zytotoxizität festgestellt. Interaktive Effekte zwischen den freigesetzten (Co)Monomeren und Additiven können die Zytotoxizität und die Induktion von DNA-DSBs im Vergleich zur Exposition mit Einzelkomponenten positiv beeinflussen.

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

Yang Yang[a,b], *Franz-Xaver Reichl*[a,b], *Jianwei Shi*[c], *Xiuli He*[a,b], *Reinhard Hickel*[a], *Christof Högg*[a,b,*]

[a] *Department of Conservative Dentistry and Periodontology, University Hospital, LMU Munich, Germany*
[b] *Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University of Munich, Nußbaumstr. 26, 80336 Munich, Germany*
[c] *Department of Orthodontics, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany*

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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

* *Corresponding author at:* Department of Conservative Dentistry and Periodontology, University Hospital, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany. Fax: +49 89 7095 73817.
E-mail address: christof.hoegg@lrz.uni-muenchen.de (C. Högg).
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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

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 7×10^4 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

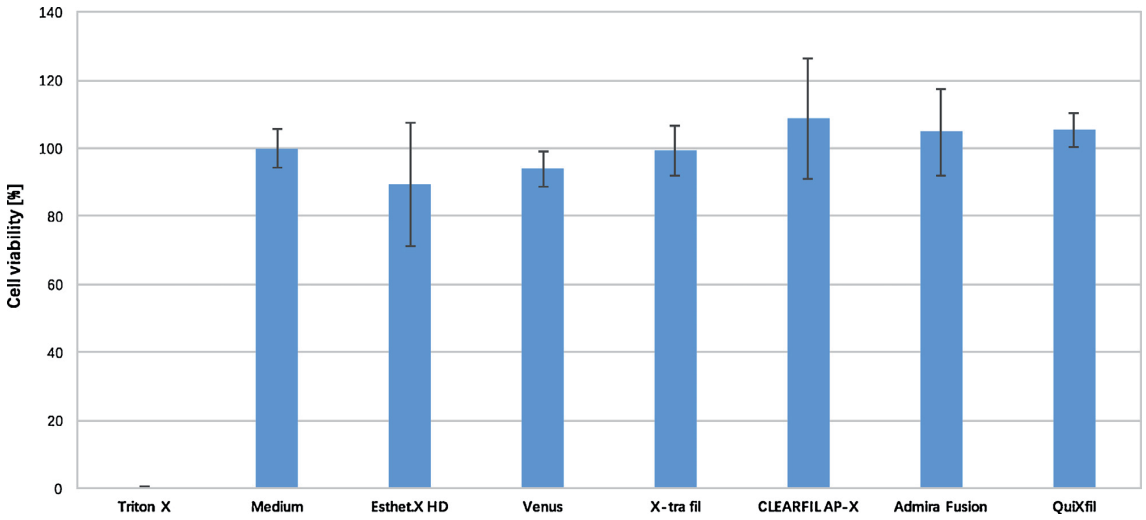

Fig. 1 – HGFs viability in XTT assay after incubation with the eluates of investigated composites (Table 1) for 24 h. Control cells received medium only, negative control cells were treated with 1% Triton X-100. Data are expressed as percentage of control (Eq. (1)) and represent mean ± SD (n = 4).

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

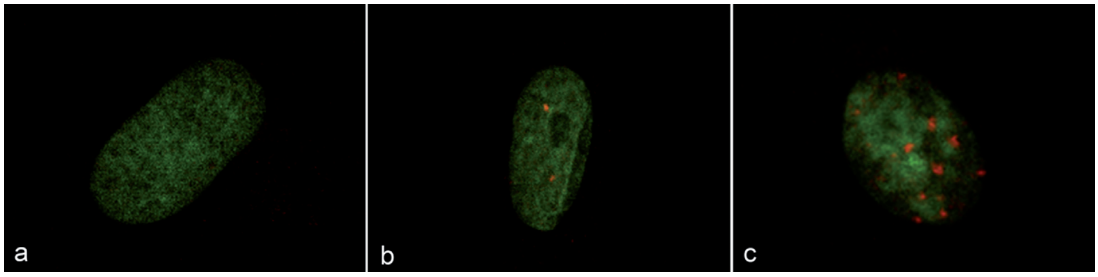

Fig. 2 – Representative images of immunofluorescent staining for H2AX phosphorylation (orange) in HGFs, after exposure to different substances compared to control cells. Sybr green (green) is a marker for DNA and stains the whole nucleus of the cell. (a) A nucleus of HGFs without foci, as typically seen 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

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

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.

Acknowledgments

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2.2.3. Einfluss von Antioxidantien, als neue Komponente in dentalen Kompositen auf die Freisetzung von Inhaltsstoffen und den Polymerisationsgrad [49]

Die Antioxidantien Ascorbinsäure (Asc) und N-Acetylcystein (NAC) können in vitro die Genotoxizität dentaler (Co)Monomere und deren Epoxymetaboliten reduzieren. In der vorliegenden Studie wurden Asc bzw. NAC als neue/zusätzliche Kompositkomponente in lichthärtenden Kompositmaterialien hinsichtlich ihrer Auswirkungen auf den Polymerisationsgrad (DC) und die Elution von Kompositinhaltsstoffen untersucht. Zusätzlich wurde die Freisetzung von Asc bzw. NAC bestimmt. Asc bzw. NAC (1, 0,1 und 0,01 Gew.-%) wurde experimentell in die drei lichthärtende Kompositmaterialien Venus, Grandio und Filtek supreme XTE Komposite eingebracht und gemäß den Anweisungen der Hersteller polymerisiert. Die Proben wurden in Methanol und Wasser eluiert. Für jedes Komposit-Antioxidans-Gemisch und Elutionsmedium wurden jeweils vier Proben (n = 4) hergestellt. Die Eluate wurden mittels GC/MS, high-performance liquid chromatography/diode array detection“ (HPLC/DAD) und „high-performance liquid chromatography/fluorescence detection“ (HPLC/FLD) analysiert. Der DC der Komposit-Antioxidans-Gemische wurde in Echtzeit mittels „Fourier transform infrared spectroscopy“ (FTIR) gemessen. Die höchsten Konzentrationen von eluiertem Asc waren 313,98 µM (Venus-1 Gew.-% Asc; 1 d; Methanol) und 245,34 µM (FiltekTM Supreme XTE-1 Gew.-% Asc; 5 min; Wasser). Die höchsten Konzentrationen an eluiertem NAC waren 42,99 µM (1 d; FiltekTM Supreme XTE-1 Gew.-% NAC; 1 d; Methanol) und 108,11 µM (FiltekTM Supreme XTE-1 Gew.-% NAC; 7 d; Wasser). Die Elution von Triethylenglykoldimethacrylat (TEGDMA) war bei Venus-1 Gew.-% Asc und Grandio-1 Gew.-% Asc (1 d und 7 d Methanol/Wasser) im Vergleich zur Kontrolle signifikant erhöht. Kein signifikanter Unterschied wurde für die TEGDMA-Elution in Filtek Supreme XTE-1 Gew.-% Asc/NAC gefunden. Der DC wurde im Vergleich zur Kontrolle (Komposit ohne Antioxidationsmittel) in Grandio und Filtek supreme XTE nach 1, 0,1 und 0,01 Gew.-% Asc-Zugabe und in Venus nach 1 und 0,1 Gew.-% Asc-Zugabe signifikant verringert. Bei den Komposit-NAC-Mischungen war nur der DC von Grandio-1 Gew.-% NAC signifikant reduziert. Die experimentelle Einmischung von 1 Gew.-% NAC als neue Kompositkomponente in Filtek Supreme XTE hatte keine Auswirkungen auf den DC, die Elution von Kompositinhaltsstoffen und setzte eine effektive Konzentration an Antioxidans frei, um die Toxizität von (Co)Monomeren und deren Metaboliten zu verringern. Daher stellt Filtek Supreme XTE-1 Gew.-% NAC eine vorteilhafte Mischung dar.

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Antioxidants as a novel dental resin-composite component: Effect on elution and degree of conversion

Yang Yang[a,b,c], *Franz-Xaver Reichl*[a,c], *Nicoleta Ilie*[a], *Jianwei Shi*[b,d], *Julia Dhein*[a,c], *Reinhard Hickel*[a], *Christof Högg*[a,c,*]

[a] *Department of Conservative Dentistry and Periodontology, University Hospital, LMU Munich, Germany*
[b] *Key Laboratory of Oral Medicine, Guangzhou Institute of Oral Disease, Stomatology Hospital of Guangzhou Medical University, Guangzhou 510140, China*
[c] *Walther Straub Institute of Pharmacology and Toxicology, Faculty of Medicine, LMU Munich, Germany*
[d] *Department of Orthodontics and Dentofacial Orthopedics, University Hospital, LMU Munich, Germany*

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ABSTRACT

Objective. Ascorbic acid (Asc) and N-acetylcysteine (NAC) were reported to reduce genotoxicity induced by dental (co)monomers and their epoxy metabolites. The aim of the present study was to investigate Asc or NAC as novel components in light-curable methacrylate based dental composites regarding their effects on degree of conversion (DC) and elution of composite components. Additionally, the release of Asc or NAC was determined.

Methods. Asc or NAC (1, 0.1, 0.01 or 0 wt%) was experimentally incorporated into the composites Venus®, Grandio® and Filtek™ Supreme XTE and polymerized according to the instruction of manufacturers. The samples were elussted in methanol and water. For each composite-antioxidant mixture and elution medium four samples (n = 4) were prepared. The eluates were analyzed by gas chromatography/mass spectrometry (GC/MS), high-performance liquid chromatography/ ultraviolett/diode array detection (HPLC/UV/DAD) and high-performance liquid chromatography/fluorescence detection (HPLC/FLD). DC of composite-antioxidant mixtures was measured in real-time with Fourier transform infrared spectroscopy (FTIR).

Results. The highest concentrations of eluted Asc were 313.98 μM (Venus®-1 wt% Asc; 1 day; methanol) and 245.34 μM (Filtek™ Supreme XTE-1 wt% Asc; 5 min; water). The highest concentrations of eluted NAC were 42.99 μM (1 day; Filtek™ Supreme XTE-1 wt% NAC; 1 day; methanol) and 108.11 μM (Filtek™ Supreme XTE-1 wt% NAC; 7 day; water). Triethylene glycol dimethacrylate (TEGDMA) elution was significantly increased in Venus®-1 wt% Asc and Grandio®-1 wt% Asc (1 day and 7 day methanol/water), compared to control. No significant difference was found for TEGDMA elution in Filtek™ Supreme XTE-1 wt% Asc/NAC. DC was significantly decreased compared to control (= composite without antioxidant) in Grandio® and Filtek™ Supreme XTE after 1, 0.1 and 0.01 wt% Asc incorporation and in Venus® after 1 and 0.1 wt% Asc incorporation. For composite-NAC mixtures, only DC of Grandio®-1 wt% NAC was significantly reduced.

* *Corresponding author at*: Department of Conservative Dentistry and Periodontology University Hospital, LMU Munich, Goethestr. 70, Munich 80336, Germany.
E-mail address: christof.hoegg@lrz.uni-muenchen.de (C. Högg).
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Significance. Incorporation of NAC (1 wt%), as a novel composite component, into Filtek™ Supreme XTE, had no effect on DC and composite component elution, and supplies sufficient amount of antioxidant which may reduce toxicity. Therefore, it represents a beneficial mixture.

1. Introduction

Methacrylate-based dental resins are frequently used in a clinical context because of their reliable aesthetic properties and physical performance. Light-cured resin composites consist of different (co)monomers and additives [1]. The polymerization of light-cured resin composites is incomplete and the residual (co)monomers and additives can be leachable [2]. They may contact 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 [3,4]. Additionally, allergic reactions such as asthma and contact dermatitis caused by methacrylates are also reported [5].

Previous studies have demonstrated that the (co)monomers TEGDMA and HEMA can be metabolized to the intermediate methacrylic acid (MA), and further to the epoxy metabolites 2,3-epoxy-2-methyl-propionicacid-methylester (EMPME) and 2,3-epoxy-2-methylpropionic acid (EMPA) [6–9]. These epoxy compounds are considered to be highly reactive molecules and regarded as mutagenic and carcinogenic agents [4,9–12]. Accordingly, teratogenic/embryotoxic effects were observed for EMPA and EMPME in the embryonic stem cells of mice [12]. Furthermore, EMPME and EMPA were reported to elicit severe cytotoxicity and higher rates of DNA double-strand breaks (DNA-DSBs) in human gingival fibroblasts (HGFs), compared to TEGDMA or HEMA addition alone [13,14]. DNA-DSBs can lead to carcinogenic and mutagenic effects [15].

Asc and NAC are regarded as radical scavengers [16]. It was shown that Asc and NAC can reduce the cytotoxicity induced by TEGDMA and HEMA [17,18]. Our previous studies have demonstrated that the addition of Asc or NAC to cell culture medium can reduce genotoxicity of dental (co)monomers and their epoxy metabolites, EMPA and EMPME [14,19,20]. Another study has reported that orally administered mixtures of antioxidants, including Asc and NAC, can reduce ionizing radiation-induced DSBs [21].

Recently, studies on incorporation of NAC into self-polymerizing poly-methyl methacrylate (PMMA)-based dental resin (two component system; typically used in prosthetic dentistry) resulted in a decrease of degree of conversion (DC), compared to untreated PMMA [22].

Till now, no data are available for the effect on DC and elution of composite components after incorporation of Asc or NAC into light-curable methacrylate based dental composites. Therefore, in the present study, Asc or NAC, as a novel composite component in light-curable methacrylate based dental composites, was investigated regarding their effects on DC and elution of composite components. Additionally, the release of Asc or NAC from new mixtures was determined.

2. Methods

The investigated resin-composites including manufacturers' data are listed in Table 1.

2.1. *Sample preparation*

2 g of each resin-composite (Table 1) were prepared by placing the uncured material on a glass plate in the dark, followed by addition of 1 wt% (20 mg), 0.1 wt% (2 mg), 0.01 wt% (0.2 mg) or 0 wt% (0 mg; control group) grounded fine powder of Asc (Sigma-Aldrich, China) or NAC (Sigma-Aldrich, China). Asc or NAC was experimentally incorporated into the uncured composite with a dental spatula for best possible homogenous distribution.

For high-performance liquid chromatography/ultraviolett/diode array detection (HPLC/UV/DAD) –, high-performance liquid chromatography/fluorescencedetection (HPLC/FLD) – and gaschromatography/massspectroscopy (GC/MS)-analyses corresponding composite-antioxidant mixture was polymerized in a Teflon mold (5 mm diameter and 2 mm thickness), placed on a plastic matrix strip (Frasaco, Tettnang, Germany). The uncured mixture was polymerized using a LED-light curing unit (LCU) (Elipar STM 10® high performance LED, 1200 mW/cm^2, 3M ESPE, Seefeld, Germany), according to the instructions of the manufacturers (Table 1). The irradiance of the LED-LCU was controlled with a radiometer (Demetron®, Kerr, USA) and was always between 1100 and 1200 mW/cm^2. The top surface of the composite sample was covered with a plastic strip during polymerization. After sample preparation, samples were transferred into brown glass vials (Macherey-Nagel, Düren, Germany) and 1 ml Methanol (GC Ultra Grade, RATISOLV® ≥99.9%, Roth, Karlsruhe, Germany) or 1 ml water (LC–MS-Grade, ROTISOLV®, Roth, Karlsruhe, Germany) was added. As internal standard (GC/MS analysis) caffeine (CF) solution (0.01 mg/ml) (HPLC ≥99.0%, Sigma-Aldrich, St. Louis, United States) was added. All samples were incubated at 37 °C in the dark. Quadruplets were prepared for each composite-antioxidant mixture and elution medium.

For GC/MS analysis the eluates were measured after incubation for 1 day and 7 day. Water samples were previously extracted with 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 [23].

Measurements of Asc and NAC are not appropriate on GC/MS mode due to strong hydrophilic character and poor evaporability. Therefore, two different HPLC methods were applied:

Table 1 – Investigated dental materials, manufacturers, LOT numbers, types, shades, 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	Shade	Polymerization time
Venus®	Micro-hybrid	Heraeus Kulzer, Hanau, Germany	010504A	Bis-GMA, TEGDMA and contains 587 % filler (by volume), such as Barium Aluminium Fluoride glass; Highly dispersive Silicon Dioxide	A 2	20 s
Grandio®	Nano-hybrid	VOCO GmbH, Cuxhaven, Germany	1650433	Bis-GMA, TEGDMA	A 2	20 s
Filtek™ Supreme XTE	Nano-hybrid	3 M ESPE, Seefeld, Germany	N642628	Bis-GMA, UDMA, TEGDMA, PEGDMA, Bis-EMA; ZrO_2–SiO_2 cluster SiO_2 and ZrO_2 nanofiller	A 2	20 s

The concentrations of Asc in eluates of composite-Asc mixtures were quantified by HPLC/DAD/UV after incubation for 5 min, 1 day and 7 day.

The concentrations of NAC in eluates of composite-NAC mixtures were quantified by HPLC/FLD after incubation for 5 min, 1 day and 7 day.

2.2. *HPLC/UV/DAD analysis*

Eluates of composite-Asc mixtures were analyzed on a LaChrom HPLC/UV/DAD system (Merck, VWR, Darmstadt, Germany). Separations were carried out using a 5 μm, 100 × 4.6 mm Hypersil GOLD AX column and a 5 μm, 10 × 4 mm Hypersil GOLD AX guard column (Thermo Fisher Scientific, Dreieich, Germany). The column was operated at a flow rate of 0.5 ml/min with an isocratic mobile phase for 25 min, consisting of 65% acetonitrile (Merck, Darmstardt, Germany) and 35% 100 nM ammonium acetate buffer (pH 6.8) (Merck, Darmstardt, Germany). The chromatograms were recorded at 267 nm wavelength. The injection volume was 100, 200 or 400 μl.

Identification of Asc was achieved by comparing the retention time and UV-spectrum to an Asc reference standard. A calibration was performed by correlating peak areas of five different reference standard Asc concentrations (10, 20, 100, 200, 400 μM). Quantity of Asc from analyzed samples was calculated accordingly.

2.3. *HPLC/FLD analysis*

To quantify the concentration of NAC in the eluates of composite-NAC mixtures, a derivatization procedure was performed according to Ercal et al. [24] with modifications as described in the following: 100 μl sample eluate was added to 300 μl of a 1 mM N-(1-pyrenyl) maleimide (NPM) (Sigma-Aldrich, India) in Acetonitrile (LC–MS Grade, Roth, Karlsruhe, Germany) and incubated 23 °C for 5 min to form NPM-NAC adduct. To stop the reaction 5 μl of 2 M HCl was added. The resulting solution was analyzed on a LaChrom Elite HPLC/FLD system (VWR, Darmstadt, Germany). Separations were carried out using a 5 μm, 250 mm × 4 mm LiChrospher 60 RP-18 SelectB column and a 5 μm, 4 mm × 4 mm LiChroCart guard column filled with LiChrospher 100 RP-18 (Merck, Darmstadt, Germany). The column was operated at a flow rate of 0.7 ml/min with a gradient using water (HPLC-grade, Millipore, VWR, Darmstadt) with 1‰ Acetic acid (100%, Merck, Darmstadt, Germany)/1‰ Phosphoric acid (85%, Merck, Darmstadt, Germany) and Acetonitrile. Starting at 48%, Acetonitrile was linearly increased over 8 min up to 52% and held for another 12 min. Within 1 min, the eluent was changed to 55% Acetonitrile, held for 14 min, and returned to 48% Acetonitrile within 1 min. The column was reconditioned for at least 10 min. The chromatograms were recorded at an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The injection volume was 50 μl.

Identification of NPM-NAC adduct was achieved by comparing the retention time to NPM-derivatized NAC reference standard. A calibration was performed by correlating peak areas of five different derivatized reference standard NAC concentrations at 50, 125, 250, 500, 1000 nM. Quantity of NAC from analyzed samples was calculated accordingly.

2.4. *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-5 ms 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 five 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 five min at this temperature. The mass 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).

2.5. *Degree of conversion (DC)*

The measurements of the DC (n = 6) were performed in real time with an FTIR-Spectrometer with an attenuated total reflectance (ATR) accessory (Nexus, Thermo Nicolet, Madison, USA). The corresponding unpolymerized composite-antioxidant mixture (see Section 2.1) was put directly on the diamond ATR crystal in a Teflon mold (5 mm diameter and 2 mm thickness). The uncured mixture was polymerized using the same LED-LCU with same exposure time mentioned above. The FTIR spectra were recorded in real time for 5 min on the lower surface of the samples. DC was calculated by assessing the variation in peak height ratio of the absorbance intensities of methacrylate carbon to carbon (C—C) double bond peak at 1634 cm^{-1} by employing the aromatic C—C double bond peak at 1608 cm^{-1} as an internal standard during polymerization of the uncured material using Eq. (1).

$$DC_{peak}\% = \left(1 - \frac{\left(1634cm^{-1}/1608cm^{-1}\right)_{peak\ \ height\ \ after\ \ curing}}{\left(1634cm^{-1}/1608cm^{-1}\right)_{peak\ \ height\ \ before\ \ curing}}\right) \times 100 \quad (1)$$

2.6. *Calculations and statistics*

The results are presented as means (standard deviation, SD). The statistical significances ($p < 0.05$) of the differences in the experimental groups were asssnalyzed by one-way ANOVA and the post hoc test (Tukey's HSD test) [25].

3. Results

The results of HPLC, GC/MS and DC are shown in Fig. 1, and Tables 3–6. The samples of Venus® were not measurable after incorporation of 1 wt% NAC due to polymerization without photoinitiation. Therefore no data for HPLC, GC/MS and DC results of Venus®-1 wt% NAC are available.

3.1. *HPLC analysis*

Concentrations of Asc and NAC, released from composite-antioxidant mixtures are shown in Table 3.

3.1.1. *HPLC/UV/DAD*

Asc calibration on HPLC/UV/DAD resulted in a linear calibration curve (Eq. (2)) ($R^2 = 0.999$; limit of quantification (LOQ) = 2.05 μM)

$$y = 40704x - 5613282 \quad (2)$$

Table 2 – Detected eluted composite components.

Compound abbreviation	Compound
HEMA	2-Hydroxyethyl methacrylate
TEGDMA	Triethylene glycol dimethacrylate
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

For composite-Asc mixtures, Asc was detected in methanol and water eluates of all three investigated composites containing 1 wt% and 0.1 wt% Asc. While the concentrations in the eluates of composites containing 0.01 wt% were always lower than LOQ. The concentration of Asc (Venus®-1 wt% Asc) increased from 185.05 μM (5 min; methanol) to 313.98 μM (1 day; methanol) which was the highest concentration of eluted Asc in this study. While in Grandio®-Asc and Filtek™ Supreme XTE-Asc mixtures, the concentrations of eluted Asc decreased with increasing elution time (methanol and water). The highest concentration of eluted Asc in water eluate was 245.34 μM (5 min; Filtek™ Supreme XTE-1 wt% Asc).

3.1.2. *HPLC/FLD*

NAC calibration on HPLC/FLD resulted in a linear calibration curve (Eq. (3)) ($R^2 = 0.999$; LOQ = 0.03 μM)

$$y = 31708x + 5290208 \quad (3)$$

For composite-NAC mixtures, NAC was detected in methanol and water eluates of Grandio® and Filtek™ Supreme XTE containing 1, 0.1 and 0.01 wt% NAC, and Venus® containing 0.1 and 0.01 wt% NAC. The highest concentration of eluted NAC in methanol was 42.99 μM (1 d; Filtek™ Supreme XTE-1 wt% NAC). The highest concentration of eluted NAC in water (108.11 μM) was found for Filtek™ Supreme XTE-1 wt% NAC after 7 day elution, where the eluted concentrations of NAC increased. A NAC increase with increasing elution time was also found for Filtek™ Supreme XTE-0.1 wt% NAC in water.

3.2. *GC/MS analysis*

A total of nine released composite components (Table 2) were detected from investigated composites.

3.2.1. *Composite-Asc mixture*

3.2.1.1. *Venus®-Asc mixture (Table 4).* For Venus®-Asc misxture, CQ, CSA, BHT, TEGDMA, DDHT and HMBP were detected. TEGDMA elution was significantly increased in 1, 0.1 and 0.01 wt% of incorporated Asc after 1 and 7 day elution in methanol and water, compared to control.

3.2.1.2. *Grandio®-Asc mixture (Table 5).* For Grandio®-Asc mixture, HEMA, CQ, CSA, BHT, DMABEE, TEGDMA and TinP were detected. A significant decrease of elution was found for HEMA in 1, 0.1 and 0.01 wt% of incorporated Asc after 1

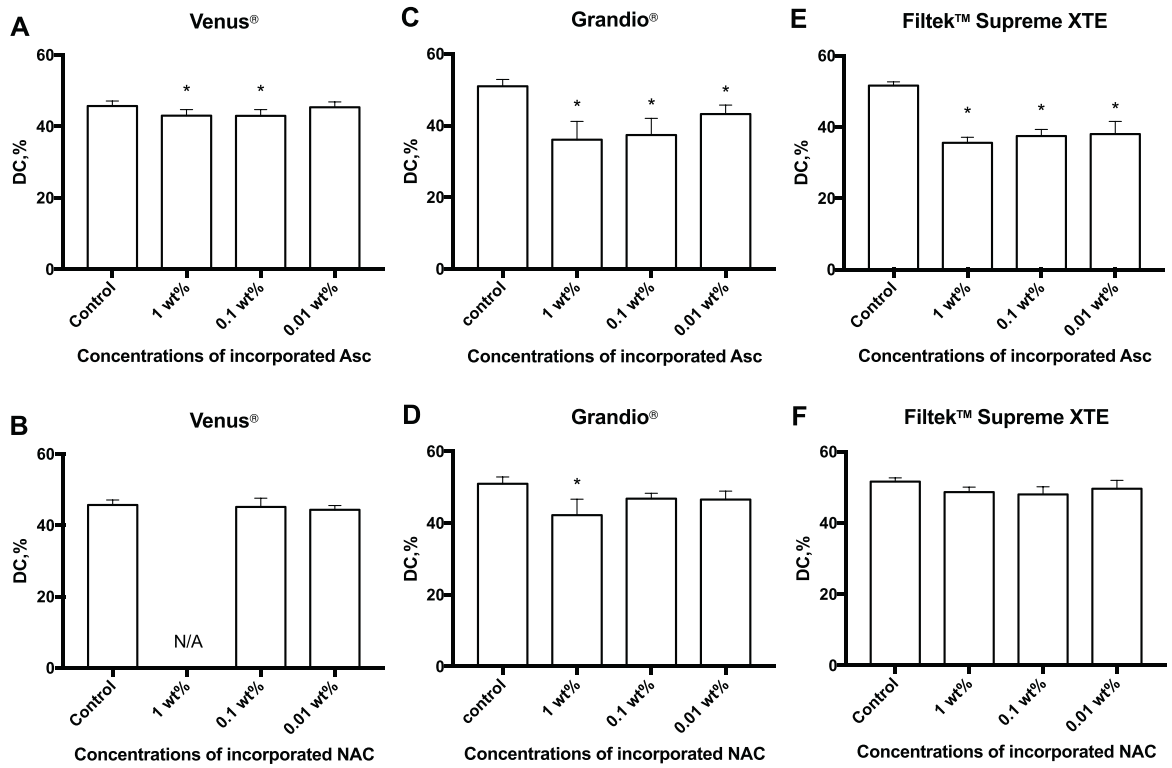

Fig. 1 – Degree of conversion of composite-antioxidant mixture. (A) Venus® -Asc (0.01, 0.1 and 1 wt%). (B) Venus® -NAC (0.01, 0.1 and 1 wt%). (C) Grandio® -Asc (0.01, 0.1 and 1 wt%). (D) Grandio® -NAC (0.01, 0.1 and 1 wt%). (E) Filtek Supreme XTE® -Asc (0.01, 0.1 and 1 wt%). (F) Filtek™ Supreme XTE-NAC (0.01, 0.1 and 1 wt%). Data are presented as mean ± SD, n = 6, * $p < 0.05$. N/A: no data available.

Table 3 – Elution of incorporated Asc or NAC from composite-antioxidant mixture after 5 min, 1 day and 7 day in methanol and water. Data are presented as mean (SD), n = 4. LOQ: limit of quantification. N/A: no data available.

Elution of Asc or NAC; mean (SD); μM		Venus®		Grandio®		Filtek™ Supreme XTE	
	Methanol	Water	Methanol	Water	Methanol	Water	
1 wt% Asc	5 min	185.05 (18.71)	119.46 (28.46)	195.51 (53.98)	132.69 (26.86)	286.39 (70.58)	245.34 (69.02)
	1 day	313.98 (17.80)	15.72 (5.95)	109.16 (44.70)	10.67 (3.23)	< LOQ	156.38 (50.67)
	7 day	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	18.39 (6.69)
0.1 wt% Asc	5 min	12.09 (3.71)	6.02 (1.42)	11.32 (4.73)	9.15 (1.45)	15.24 (5.57)	13.98 (3.31)
	1 day	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	7.04 (3.04)
	7 day	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
0.01 wt% Asc	5 min	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
	1 day	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
	7 day	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
1 wt% NAC	5 min	N/A	N/A	17.87 (1.67)	48.60 (6.34)	27.43 (8.33)	35.28 (9.41)
	1 day	N/A	N/A	6.72 (0.93)	35.69 (4.28)	42.99 (2.73)	89.39 (19.98)
	7 day	N/A	N/A	2.03 (0.47)	42.25 (5.36)	27.63 (6.87)	108.11 (16.14)
0.1 wt% NAC	5 min	7.20 (1.02)	2.05 (0.46)	5.59 (3.73)	2.18 (0.07)	3.90 (1.87)	2.61 (0.85)
	1 day	24.25 (3.20)	0.33 (0.13)	1.88 (0.68)	0.16 (0.04)	6.95 (3.35)	6.09 (1.53)
	7 day	4.45 (0.72)	0.19 (0.06)	0.45 (0.18)	0.11 (0.03)	4.08 (2.14)	8.20 (1.22)
0.01 wt% NAC	5 min	0.84 (0.14)	0.25 (0.04)	0.52 (0.16)	1.68 (0.91)	0.67 (0.35)	0.47 (0.22)
	1 day	0.20 (0.09)	0.18 (0.11)	< LOQ	< LOQ	< LOQ	< LOQ
	7 day	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

Table 4 – Qualification and quantification of substances eluted from Venus® incorporated with 1, 0.1 and 0.01 wt% Asc or NAC for 1 and 7 day in methanol and water. Data are presented as mean (SD), n = 4.

TblColHeadVenus®; mean (SD); μM	1 day in methanol				7 day in methanol			
	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc
CQ	16.42 (4.43)	19.18 (2.24)[b,c]	10.74 (1.98)[d]	10.23 (1.13)[a,d]	18.37 (3.39)	29.77 (4.29)[a,b,c]	13.68 (1.88)[d]	11.41 (1.40)[a,d]
CSA	9.52 (2.12)	3.70 (0.19)[a,b]	5.69 (0.61)[a]	6.54 (0.88)[a,d]	13.49 (2.18)	5.16 (0.37)[a,c]	10.08 (1.62)[a,b,d]	6.72 (0.74)[a,c]
BHT	2.89 (0.71)	2.92 (0.17)[b,c]	1.27 (0.11)[a,d]	1.19 (0.30)[a,d]	4.30 (0.58)	3.86 (0.45)[b,c]	2.14 (0.27)[a,d]	1.60 (0.35)[a,d]
TEGDMA	630.41 (34.93)	818.51 (44.49)[a]	831.40 (9.63)[a]	752.48 (83.52)[a]	652.86 (23.78)	1147.78 (41.93)[a,c]	1006.23 (86.75)[a,d]	1074.71 (40.55)[a]
DDHT	46.22 (9.65)	57.09 (1.80)[b,c]	45.00 (2.20)[d]	37.75 (2.95)[d]	34.87 (10.04)	92.76 (3.04)[a,b,c]	58.99 (2.55)[a,b,d]	20.05 (1.97)[a,c,d]
HMBP	184.05 (31.82)	179.54 (10.12)[b]	147.54 (10.75)	133.14 (10.77)[a,d]	286.46 (40.89)	324.70 (8.27)[b,c]	263.75 (18.47)[d]	243.58 (12.11)[d]
	1 day in water				7 day in water			
	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc
CQ	1.52 (0.17)	2.07 (0.60)[c]	0.86 (0.19)[d]	1.39 (0.21)	2.92 (0.59)	3.76 (0.57)[b,c]	1.31 (0.21)[a,d]	0.98 (0.24)[a,d]
CSA	0.90 (0.13)	0.43 (0.09)[a]	0.54 (0.13)[a]	0.41 (0.04)[a]	1.24 (0.24)	0.61 (0.03)[a]	0.71 (0.17)[a]	0.72 (0.20)[a]
TEGDMA	148.87 (13.07)	191.00 (16.20)[a]	173.18 (9.80)[a]	182.66 (2.15)[a]	138.39 (23.73)	260.30 (9.97)[a,b,c]	224.84 (10.01)[a,d]	215.11 (16.83)[a,d]
	1 day in methanol				7 day in methanol			
	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC
CQ	16.42 (4.43)	N/A	29.29 (0.94))[a,b]	15.32 (1.71)[c]	18.37 (3.39)	N/A	48.47 (3.78))[a,b]	24.93 (2.07)[c]
CSA	9.52 (2.12)	N/A	7.48 (0.62)	6.20 (0.63)[a]	13.49 (2.18)	N/A	13.01 (1.48)	10.30 (1.01)
BHT	2.89 (0.71)	N/A	5.61 (0.27))[a,b]	2.68 (0.11)[c]	4.30 (0.58)	N/A	9.03 (0.59)	4.41 (0.37)
TEGDMA	630.41 (34.93)	N/A	754.83 (40.41))[a,b]	548.13 (56.88)[c]	652.86 (23.78)	N/A	769.44 (26.02))[a,b]	620.63 (31.78)[c]
DDHT	46.22 (9.65)	N/A	65.97 (1.43))[a,b]	40.33 (1.46)[c]	34.87 (10.04)	N/A	80.67 (4.02))[a,b]	34.66 (1.17)[c]
HMBP	184.05 (31.82)	N/A	238.98 (13.98))[a,b]	159.85 (1.97)[c]	286.46 (40.89)	N/A	348.96 (25.12))[a,b]	277.87 (11.71)[c]
	1 day in water				7 day in water			
	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC
CQ	1.52 (0.17)	N/A	2.55 (0.51))[a,b]	1.85 (0.15)[c]	2.92 (0.59)	N/A	4.67 (1.19)[a,b]	2.75 (0.36)[c]
CSA	0.90 (0.13)	N/A	1.51 (0.16)[a,b]	1.06 (0.05)[c]	1.24 (0.24)	N/A	1.74 (0.22)[a]	1.83 (0.18)[a]
TEGDMA	148.87 (13.07)	N/A	171.54 (6.91))[a,b]	141.77 (1.85)[c]	138.39 (23.73)	N/A	165.70 (8.98)	153.81 (4.59)

N/A: no data available.
[a] Significantly different to control.
[b] Significantly different to 0.01 wt% group.
[c] Significantly different to 0.1 wt% group.
[d] Significantly different to 1 wt% group, $p < 0.05$).

Table 5 – Qualification and quantification of substances eluted from Grandio® incorporated with 1, 0.1 and 0.01 wt% Asc or NAC for 1 and 7 day in methanol and water. Data are presented as mean (SD), n = 4.

Grandio®; mean (SD); μM	1 day in methanol				7 day in methanol			
	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc
HEMA	13.25 (2.20)	4.53 (0.92)[a,b,c]	0.09 (0.05)[a,d]	0.08 (0.05)[a,d]	19.29 (2.45)	3.77 (0.81)[a,b,c]	0.43 (0.05)[a,d]	0.28 (0.14)[a,d]
CQ	5.57 (0.85)	20.69 (0.80)[a,b,c]	11.08 (4.57)[d]	8.55 (2.48)[d]	6.10 (0.71)	27.41 (1.38)[a,b,c]	12.38 (0.33)[a,d]	12.04 (3.27)[a,d]
CSA	2.12 (0.16)	2.76 (0.52)[c]	3.68 (0.54)[d]	4.56 (1.41)[a]	2.69 (0.19)	3.91 (1.06)	7.38 (2.73)[a]	4.85 (1.68)
BHT	9.13 (1.21)	20.24 (5.69)[a,b,c]	11.04 (2.06)[d]	10.67 (3.88)[d]	13.30 (1.26)	12.60 (1.82)	19.73 (8.75)	15.27 (5.25)
DMABEE	0.82 (0.22)	13.45 (2.40)[a,b,c]	6.60 (4.25)[a,d]	5.15 (1.42)[d]	1.78 (0.27)	19.74 (1.32)[a,b]	12.23 (8.69)[a]	9.37 (3.68)[d]
TEGDMA	198.13 (61.39)	708.12 (184.02)[a]	474.56 (104.78)	546.38 (150.28)[a]	326.05 (14.99)	672.21 (97.48)[a,b,c]	416.16 (36.98)[d]	472.84 (135.65)[d]
TinP	10.78 (7.57)	37.65 (9.98)[a,c]	18.42 (4.43)[d]	22.54 (7.13)	26.05 (3.14)	61.47 (17.35)[a,b,c]	26.54 (6.93)[d]	36.56 (12.71)[d]
	1 day in water				7 day in water			
	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc
HEMA	1.40 (0.16)	0.03 (0.02)[a]	0.03 (0.01)[a]	0.03 (0.02)[a]	0.69 (0.11)	0.04 (0.03)[a]	0.02 (0.00)[a]	0.03 (0.03)[a]
CQ	1.28 (0.34)	5.24 (2.18)[a,b,c]	1.43 (0.49)[d]	1.84 (1.22)[d]	1.34 (0.39)	7.86 (1.99)[a,b,c]	0.95 (0.62)[d]	2.13 (1.56)[d]
CSA	0.76 (0.24)	1.28 (0.10)	1.09 (0.52)	0.80 (0.20)	1.12 (0.24)	1.40 (0.41)[c]	2.03 (0.06)[a,b,d]	1.38 (0.33)[c]
TEGDMA	91.41 (13.55)	167.68 (35.97)[a,b,c]	115.21 (13.05)[d]	119.49 (12.53)[d]	99.47 (9.58)	185.45 (32.56)[a,b,c]	116.25 (22.84)[d]	109.22 (29.41)[d]
	1 day in methanol				7 day in methanol			
	Control	1 wt%NAC	0.1 wt% NAC	0.01 wt% NAC	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC
HEMA	13.25 (2.20)	13.32 (1.48)[c]	18.17 (0.84)[a,d]	15.79 (1.08)	19.29 (2.45)	15.37 (1.34)[c]	28.17 (12.02)[d]	19.24 (0.99)
CQ	5.57 (0.85)	11.45 (2.05)[a]	9.31 (0.27)[a]	9.59 (2.37)[a]	6.10 (0.71)	15.16 (2.07)[a]	15.20 (0.13)[a]	15.93 (3.24)[a]
CSA	2.12 (0.16)	0.79 (0.18)[a,b]	0.78 (0.17)[a,b]	1.95 (0.52)[c,d]	2.69 (0.19)	1.14 (0.13)[a,b]	1.30 (0.37)[a,b]	3.19 (0.32)[c,d]
BHT	9.13 (1.21)	7.21 (1.34)	12.45 (5.65)	9.53 (1.67)	13.30 (1.26)	8.03 (1.06)[a,b,c]	14.52 (0.91)[d]	14.98 (1.74)[d]
DMABEE	0.82 (0.22)	12.73 (2.09)[a,b,c]	6.95 (0.44)[a,d]	6.40 (1.40)[a,d]	1.78 (0.27)	18.86 (1.37)[a,b,c]	11.51 (0.51)[a,d]	9.11 (1.43)[a,d]
TEGDMA	198.13 (61.39)	216.25 (56.33)	272.27 (6.89)	266.42 (44.75)	326.05 (14.99)	238.80 (12.04)[a,b,c]	309.12 (5.06)[d]	299.37 (16.99)[d]
TinP	10.78 (7.57)	14.66 (4.23)[c]	24.51 (0.05)[a,d]	23.02 (1.19)[a]	26.05 (3.14)	25.88 (1.88)[b,c]	40.74 (2.13)[a,d]	34.42 (2.23)[a,d]
	1 day in water				7 day in water			
	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC
HEMA	1.40 (0.16)	1.73 (0.14)[a,b,c]	1.40 (0.17)[d]	1.33 (0.06)[d]	0.69 (0.11)	2.19 (0.33)[a,b,c]	1.11 (0.27)[d]	1.44 (0.27)[a,d]
CQ	1.28 (0.34)	3.37 (1.07)[a,b,c]	1.38 (0.39)[d]	1.62 (0.96)[d]	1.34 (0.39)	6.82 (1.77)[a,b,c]	3.23 (0.86)[d]	3.47 (1.30)[d]
CSA	0.76 (0.24)	0.58 (0.12)	0.90 (0.16)[d]	0.37 (0.10)[a,c]	1.12 (0.24)	0.57 (0.18)[a]	0.85 (0.14)[b]	0.44 (0.07)[a,c]
TEGDMA	91.41 (13.55)	144.00 (5.98)[a,b,c]	111.17 (8.41)[a,d]	105.39 (6.65)[d]	99.47 (9.58)	125.49 (6.22)[a,b]	105.89 (17.08)	96.56 (11.01)[d]

[a] Significantly different to control.
[b] Significantly different to 0.01 wt% group.
[c] Significantly different to 0.1 wt% group.
[d] Significantly different to 1 wt% group, $p < 0.05$).

Table 6 – Qualification and quantification of substances eluted from Filtek™ Supreme XTE incorporated with 1, 0.1 and 0.01 wt% Asc or NAC for 1 and 7 day in methanol and water. Data are presented as mean (SD), n = 4.

Filtek™ Supreme XTE; mean (SD); μM	1 day in methanol				7 day in methanol			
	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc
CQ	8.52 (0.21)	7.67 (2.17)	6.69 (1.21)	6.73 (0.26)	8.28 (0.25)	9.99 (1.07)	8.76 (3.24)	8.98 (0.97)
CSA	16.06 (0.02)	1.54 (0.40)[a,b]	1.45 (0.39) [a,b]	0.75 (0.13)[a,c,d]	16.00 (0.03)	2.83 (2.46) [a]	0.93 (0.22) [a]	0.48 (0.07) [a]
BHT	7.11 (0.32)	23.13 (8.54)[a,b,c]	11.72 (1.16)[d]	10.96 (0.72)[d]	10.76 (0.58)	32.60 (23.03)	13.61 (3.76)	13.98 (1.29)
TEGDMA	39.94 (4.04)	27.16 (8.17)	35.60 (9.60)	34.73 (4.73)	40.46 (4.42)	46.41 (4.16)	42.78 (10.13)	33.85 (2.21)
	1 day in water				7 day in water			
	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc	Control	1 wt% Asc	0.1 wt% Asc	0.01 wt% Asc
CQ	2.67 (0.11)	3.00 (1.77)[c]	1.04 (0.18)[d]	1.49 (0.25)	2.71 (0.10)	4.70 (2.41)[c]	0.55 (0.16)[d]	2.24 (0.88)
CSA	2.61 (0.07)	1.18 (0.42)[a]	1.13 (0.27)[a]	0.75 (0.46)[a]	2.53 (0.08)	1.59 (0.50)[a,b]	0.84 (0.15)[a]	0.49 (0.25)[a,d]
TEGDMA	6.60 (0.30)	5.99 (1.59)[b,c]	13.43 (3.35)[a,d]	10.17 (1.07)[d]	4.09 (0.03)	12.81 (5.56)[a]	11.31 (4.76)	9.61 (1.82)
	1 day in methanol				7 day in methanol			
	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC
CQ	8.52 (0.21)	8.82 (0.27)[c]	9.81 (0.60)[a,d]	9.14 (0.25)	8.28 (0.25)	8.38 (0.16)[b,c]	9.53 (0.48)[a,d]	9.07 (0.17)[a,d]
CSA	16.06 (0.02)	16.00 (0.02)	16.06 (0.03)	16.02 (0.01)	16.00 (0.03)	15.98 (0.03)	16.01 (0.03)	15.98 (0.01)
BHT	7.11 (0.32)	6.15 (0.37)[c]	8.94 (0.58)[a,b,d]	6.58 (0.71)[c]	10s.76 (0.58)	8.94 (0.29)[a],[3]	12.66 (1.36)[a,b,d]	10.17 (0.56)[c]
TEGDMA	39.94 (4.04)	35.93 (2.27)	43.75 (3.80)	39.16 (4.95)	40.46 (4.42)	36.52 (2.61)[c]	44.88 (4.24)[d]	40.44 (4.28)
	1 day in water				7 day in water			
	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC	Control	1 wt% NAC	0.1 wt% NAC	0.01 wt% NAC
CQ	2.67 (0.11)	2.64 (0.14)	2.48 (0.04)	2.56 (0.03)	2.71 (0.10)	2.62 (1.31)	2.47 (0.04)	2.64 (0.05)
CSA	2.61 (0.07)	2.57 (0.03)	2.65 (0.09)	2.65 (0.07)	2.53 (0.08)	2.53 (1.26)	2.51 (0.03)	2.61 (0.08)
TEGDMA	6.60 (0.30)	6.66 (1.31)	7.48 (0.61)	7.22 (0.66)	4.09 (0.03)	5.08 (2.66)	7.42 (0.71)[a]	4.78 (0.20)

[a] Significantly different to control.
[b] Significantly different to 0.01 wt% group.
[c] Significantly different to 0.1 wt% group.
[d] Significantly different to 1 wt% group, $p < 0.05$).

and 7 day elution in methanol and water, compared to control. TEGDMA elution was significantly increased compared to control in 1 wt% Asc incorporation after 1 and 7 day elution in methanol and water; and 0.01 wt% Asc incorporation after 1 day methanol elution.

3.2.1.3. *Filtek™ Supreme XTE-Asc* (*Table 6*). For Filtek™ Supreme XTE-Asc mixture, CQ, CSA, BHT and TEGDMA were detected. TEGDMA elution was significantly increased for 0.1 wt% Asc incorporation after 1 day elution in water; and for 1 wt% Asc incorporation after 7 day elution in water, compared to control.

3.2.2. *Composite-NAC mixture*

3.2.2.1. *Venus®-NAC mixture* (*Table 4*). For Venus®-NAC mixture, CQ, CSA, BHT, TEGDMA, DDHT and HMBP were detected. TEGDMA elution was significantly increased for 0.1 wt% NAC incorporation after 1 day elution in water and 1 and 7 day in methanol, compared to control.

A significantly increased TEGDMA elution was found 0.1 wt%, compared to control.

3.2.2.2. *Grandio®-NAC mixture* (*Table 5*). For Grandio®-NAC mixture, HEMA, CQ, CSA, BHT, DMABEE, TEGDMA and TinP were detected. HEMA elution was significantly increased for 0.1% NAC incorporation after 1 day in methanol, 1 wt% NAC incorporation after 1 day and 7 day in water and 0.01 wt% NAC incorporation after 7 day in water, compared to control. A significant decrease of TEGDMA elution was found for 1 wt% of incorporated NAC after 7 day elution in methanol, compared to control. TEGDMA elution was significantly increased compared to control for 1 and 0.1 wt% NAC incorporation after 1 day elution in water; and for 1 wt% NAC incorporation after 7 day elution in water.

3.2.2.3. *Filtek™ Supreme XTE-NAC mixture* (*Table 6*). For Filtek™ Supreme XTE-NAC mixture, CQ, CSA, BHT and TEGDMA were detected. TEGDMA elution was significantly increased for 0.1 wt% NAC incorporation after 7 day elution in water, compared to control.

3.3. DC

For composite-Asc mixture, DCs of Grandio® and Filtek™ Supreme XTE with 1, 0.1 and 0.01 wt% Asc were significantly decreased (Fig. 1C, E), compared to control. DCs of Venus® with 1 and 0.1 wt% Asc were significantly decreased (Fig. 1A), compared to control.

For composite-NAC mixture, DC of Grandio® with 1 wt% NAC was significantly decreased (Fig. 1D), compared to control. No significant DC change was found for Filtek™ Supreme XTE with 1, 0.1 and 0.01 wt% NAC (Fig. 1F), compared to control.

4. Discussion

The effects of Asc or NAC after incorporation (1, 0.1 and 0.01 wt%) into three different composites (Venus®, Grandio® and Filtek™ Supreme XTE) were determined on the DC, on the elution of composite components and on the release of Asc or NAC.

These investigated composites were selected because previous studies have shown various compositions and high releases of methacrylates and additives [26–28].

From all investigated composites only Venus® samples were not measurable after incorporation of 1 wt% NAC. These results indicate that only for Venus® the incorporation of 1 wt% NAC may lead to an unstable composite mixture which is able to polymerize without photoinitiation.

4.1. *Effect of Asc/NAC on DC*

Photoinitiated polymerization occurs by a chain reaction between the free radicals formed by the photoinitiating system and the monomers [29]. CQ, a photoinitiator, has been widely used for light-cured dental composite [29]. Photopolymerization is initiated by CQ/amine photoinitiating system. In this process, aminoalkyl radical, a key radical initiating polymerization, was produced via photoinduced electron transfer [29]. In addition, a direct hydrogen atom abstraction of triplet state of CQ on the monomer may also form reactive radicals [29]. Hence, the significantly reduced DC after incorporation of Asc or NAC into composites in this study might be due to these antioxidants which can scavenge initiating radicals [30], consequently suppressing the initiating of chain polymerization. Additionally, the efficiency of photopolymerization also depends on the steric structure of the amine-derived radicals, which must approach the reactive unsaturated bond in a monomer [31]. Therefore the incorporation of Asc or NAC might interfere the diffusion of electrons, which impedes the formation of exciplex [32] or further polymerization.

A significant lower DC was found for all investigated composites containing 1, 0.1 and 0.01 wt% Asc (except for Venus®-0.01 wt% Asc), compared to control. While for NAC incorporation, no significant change of DC was detected for Venus® and Grandio® containing 0.1 wt% and 0.01 wt% NAC, and for Filtek™ Supreme XTE with 1 wt% NAC. This indicates that Asc has a stronger influence on DC than NAC. In addition to a stronger ability of scavenging radicals and interfering the diffusion of electrons, another explanation might be that Asc possesses chain-breaking properties by inhibiting free-radical-mediated chain reactions [33].

4.2. *Elution of composite components and effect of Asc/NAC*

For the GC/MS analysis, methanol and water were used as elution media for 1 day and 7 day elution experiments according to our previous studies [23,26].

TEGDMA is a (co)monomer frequently used in composites to enrich the organic resin matrix of composites with a maximum of inorganic filler particles [34]. A significant increase of eluted composite components (e.g. TEGDMA), compared to control, can cause adverse effects [13,35]. Our previous study revealed that an exposure with TEGDMA at concentrations of 360 μM induces 4-fold higher number of DNA-DSBs-foci in HGFs compared to negative control [13]. TEGDMA was detected in the eluates of all investigated composites. The highest TEGDMA concentration in methanol

eluates was 1148 μM (Venus® -1 wt% Asc, 7 day). This is about 3 times higher than the cited genotoxic concentration. However, methanol eluates represent the maximal elutable concentration of (co)monomers and additives [37]. However, water eluates allow the utmost physiological comparison to dentinal fluid and human saliva [27,38]. In comparison, in this study highest concentration of TEGDMA in water was 260 μM (Venus® -1 wt% Asc, 7 day). This is 1.4 times lower than the cited genotoxic concentration.

HEMA is used in dental composites as a (co)monomer of the organic resin matrix due to its hydrophilic application. In previous studies for HEMA a genotoxic concentration at 1100 μM was found in HGFs [13]. HEMA was detected only in the eluates of Grandio. The highest HEMA concentration with 28 μM was detected in the methanol eluate of Grandio-0.1 wt% NAC after 7 day. This is 39 times lower than the cited genotoxic concentration.

Incorporation of Asc into Venus® (1, 0.1 and 0.01 wt%, 1 day/7 day) and Grandio® (1 wt%, 1 day/7 day), and incorporation of NAC into Venus® (0.1 wt%, 1 day) led to a significant increase of TEGDMA in methanol and water eluates, compared to control. These results indicate that the incorporation of Asc or NAC into some composites can promote the release of TEGDMA and accordingly may induce adverse (side) effects (e.g. methacrylate allergy, cytotoxic/genotoxic effects [13,35]).

However, our data also showed that the incorporation of Asc or NAC at specific wt% did not influence TEGDMA release (e.g. Filtek™ Supreme XTE-1, 0.1 and 0.01 wt% Asc or NAC 1 and 7 day in methanol). In Grandio® a significant reduced release of TEGDMA after incorporation of 1 wt% NAC (7 day, methanol) and a significant reduced release of HEMA after incorporation of Asc (1, 0.1, 0.01 wt%, methanol / water, 1 day/7day) could be detected. These results might be explained by the interaction of Asc or NAC with components of the composite and therefore less HEMA or TEGDMA is elutable as a positive effect.

Our previous study demonstrated that a lower DC is accompanied by a higher amount of eluted composite components [40] as a negative effect. However, molecular weight and hydrophobicity of (co)monomers as well as the filler content play also a role on elution mechanism. Therefore, the correlation between DC and component elution remains to be further illustrated [41,42]. In the present study, no relation between DC and the elution of composite components after the incorporation of Asc or NAC at any wt% could be found.

4.3. *Release of Asc or NAC from composite-antioxidant mixtures*

Asc can diminish DNA lesion by scavenging reactive species directly and reducing their formation, or preventing proteins that repair DNA against radical attack [43]. NAC can scavenge free radicals through thiol side-chain directly as well as by simultaneous increase of intracellular glutathione (GSH) content [44]. Additionally, evidence shows that NAC reduces the availability of free dental resin monomers by reacting with the methacrylic group through Michael-type addition [45,46]. Both Asc and NAC were reported to prevent the formation of DNA adducts [47,48].

In our previous studies cytotoxicity and genotoxicity of dental (co)monomers (e.g. TEGDMA) and their epoxy metabolites (e.g. EMPA) have been found [13,14]. Furthermore, we reported that cell culture medium eluates of dental composites containing total elutable components also induced DNA-DSBs [28]. Recently, we demonstrated that the presence of Asc or NAC significantly reduced cytotoxicity and genotoxicity induced by dental (co)monomers and their epoxy metabolites [14,17,19,20]. Concentrations higher than 50 μM Asc or NAC caused a significant reduction of DNA-DSBs induced by dental (co)monomer intermediates and their epoxy metabolites, compared to control in HGFs [14]. Therefore these concentrations play a key role in the reduction of cell toxicity.

Based on this 50 μM Asc/NAC [14], the present study was designed for the incorporation of NAC or Asc starting with 0.01 wt%, which corresponds to a calculated maximum elutable concentration of about 57 μM Asc or 61 μM NAC. However, elution mechanism depends on the molecular weight, hydrophobicity, filler content, investigated material and the final network characteristics of the resin-matrix [41,42,49–51]. Consequently, in the present study concentrations of released Asc or NAC were always lower than the calculated corresponding maximal elutable Asc or NAC concentration.

In the present study, elution of Asc or NAC at 5 min, 1 day and 7 day was determined to estimate the maximum available concentration of released antioxidant in eluates. However, degradations of Asc and NAC in solutions were reported in other studies [52,53]. This is in accordance with our data because a degradation of eluted Asc and NAC in methanol and water was observed. Nevertheless, an increased release of antioxidants can also be found for Venus® -1 wt% Asc (methanol) and Filtek™ Supreme XTE-1 wt% NAC (water) with increasing elution time.

As described above, concentrations higher than 50 μM Asc or NAC can significantly reduce DNA-DSBs [14]. In the present study, NAC or Asc concentrations over this value were found in methanol or water eluates of Venus® -1 wt% Asc, Grandio® -1 wt% Asc, Filtek™ Supreme XTE-1 wt% Asc and Filtek™ Supreme XTE-1 wt% NAC. Hence, it can be concluded that these composites containing 1 wt% Asc or 1 wt% NAC might offer sufficient amounts of eluted antioxidants to reduce genotoxicity induced by dental composite (co)monomers, their metabolization intermediates and epoxy metabolites in human oral cells.

4.4. *Risk assessment*

Among all investigated composite-antioxidant mixtures, only Venus®, Grandio® and Filtek™ Supreme XTE (each 1 wt% Asc) and Filtek™ Supreme XTE (1 wt% NAC) can offer sufficient amounts of eluted antioxidants to reduce DNA-DSBs [14]. However, the incorporation of 1 wt% Asc into Venus®, Grandio® and Filtek™ Supreme XTE showed a significant decreased DC and a significant increased elution of TEGDMA. The increase of composite component elution (e.g. TEGDMA) can lead to adverse effects [13,35]. Only Filtek™ Supreme XTE (1 wt% NAC) showed no significant change as well on DC as on composite component elution and supplies sufficient amount

of antioxidant to reduce toxicity. Therefore, Filtek™ Supreme XTE-1 wt% NAC represents a beneficial mixture.

If we assume the methanol elution as a worst-case scenario, in the present study for Filtek™ Supreme XTE-1 wt% NAC (1 day methanol elution), the total concentration of eluted TEGDMA from 10 filling (100 mg each) would reach about 360 μM, which can induce DNA-DSBs [13]. Similarly, the maximum concentration of released NAC from one filling was 43 μM (Filtek™ Supreme XTE-1 wt% NAC, 1 day, methanol). For 10 fillings this would result in 430 μM total released NAC. This is 9-fold higher than 50 μM NAC, which can reduce DNA-DSBs [14]. Therefore, the incorporation of 1 wt% NAC into Filtek™ Supreme XTE would be a useful step to reduce dental (co)monomer induced DNA-DSBs. However, it is emphasized that these data may not be easily transformed into the clinical situation, because in the physiological situation the continuous formation of saliva may lead to a dilution of released composite components.

5. Conclusion

Incorporation of NAC (1 wt%), as a novel composite component, into Filtek™ Supreme XTE, had no effect on DC and composite component elution and supplies sufficient amount of antioxidant which may reduce toxicity. Therefore, it represents a beneficial mixture.

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3. Diskussion

3.1. Freisetzung von Inhaltsstoffen aus dentalen Kompositen

Zur Bestimmung der Freisetzung von (Co)Monomeren und Additiven aus dentalen Kompositen werden polymerisierte Kompositprüfkörper in die Extraktionsmedia Methanol oder Wasser eluiert. Zur Herstellung dieser Prüfkörper werden die unpolymerisierten Komposite mit einer LED Lampe gemäß den Anweisungen des Herstellers lichtgehärtet/polymerisiert. Mittels GC/MS lassen sich anschließend freigesetzte Inhaltsstoffe in den Eluaten qualifizieren und quantifizieren. Die Verwendung von Wasser als Elutionsmittel lässt einen Vergleich mit den physiologischen intraoralen Konditionen zu [50, 51]. Methanol ermöglicht eine gute Löslichkeit organischer Matrixkomponenten und eine tiefere Matrixpenetration [7]. Ein (unbeabsichtigter) Abbau von freigesetzten (Co)Monomeren durch Methanol (und Wasser) konnte in unserer Studie über eine 180d Elution ausgeschlossen werden [51] und ist somit viel geringer als bei anderen organischen Lösungsmitteln wie z.B. Aceton [52, 53]. Daher ermöglicht Methanol die Elution eines maximalen Anteils an unpolymerisierten Substanzen und somit eine Abschätzung im Rahmen einer „Worst-Case“-Situation.

3.1.1. Einfluss der Schichtdicke auf die Freisetzung von Inhaltsstoffen aus dentalen Bulk-Fill Kompositen [44]

Um die Polymerisationsschrumpfung zu reduzieren und eine ausreichende Durchhärtungstiefe zu erreichen sowie die Freisetzung von Kompositinhaltsstoffen aus konventionellen Kompositmaterialen zu reduzieren wird bisher für Schichtdicken > 2 mm die sogenannte Inkrementschichttechnik bei posterioren Kompositrestaurationen mit einer maximalen Schichtdicke von 2 mm [54]. Die Entwicklung von Bulk-Fill Kompositen versprechen eine Beschleunigung des Restaurationsprozesses, durch die Möglichkeit der Aushärtung einer mindestens 4 mm dicken Schicht in einem Schritt [55] und somit in vielen Fällen eine schnelle Ein-Inkrementschichttechnik zum Füllen einer kompletten Kavität [56]. Dies ist auf eine erhöhte Transluzenz durch einen reduzierten Füllungsmaterialanteil oft bei gleichzeitig erhöhter Füllungspartikelgröße oder/und ein effektiveres Initiator System zurückzuführen [57]. Die mechanischen Eigenschaften von Bulk-Fill Kompositen wurden bereits in mehreren Studien untersucht [56, 58-62]. So wurden für Bulk-Fill Komposite beispielsweise eine geringere Höckerablenkung [62], höhere Randdichtigkeit der Füllung [60, 61] und eine höhere Durchhärtungstiefe [61] im Vergleich zu konventionellen Kompositen in Inkrementschichttechnik festgestellt. Allerdings zeigten sich im Vergleich zu konventionellen Kompositen auch nachteilige Ergebnisse, wie z.B. beim Polymerisationsgrad (DC) von Bulk-

Fill Kompositen [63]. Der DC von > 55 % für Bulk-Fill Komposite liegt noch im klinisch akzeptablen Bereich, ist aber geringer als bei konventionellen Kompositen [63]. Je niedriger die Umwandlungsrate eines Komposits ist, desto mehr Restmonomere können eluiert werden [64]. Diese eluierbaren Kompositinhaltsstoffe können zu allergischen Reaktionen [65] wie Asthma, allergischer Rhinokonjunktivitis oder Kontaktdermatitis [20] führen. In früheren Studien zeigten einige Kompositinhaltsstoffe konzentrationsabhängige zytotoxische Effekte, wie erhöhten Zelltod, Schädigung der Plasmamembran oder einen erhöhten Gehalt an freigesetzter Laktatdehydrogenase [1, 66, 67]. Allerdings konnte gezeigt werden, dass die Elution von Bulk-Fill Kompositen trotz der erhöhten Schichtdicke mit konventionellen Materialien vergleichbar ist [68] und die Menge an eluierten (Co)Monomeren mit der Elutionszeit zunimmt [47, 69].
Vor der Durchführung unserer Studie „Einfluss der Schichtdicke auf die Freisetzung von Inhaltsstoffen aus dentalen Bulk-Fill Kompositen“ [44] lagen in der wissenschaftlichen Literatur jedoch keine Daten darüber vor, inwieweit sich eine Schichtdicke von bis zu 6 mm (entgegen der Herstellerempfehlung) im Vergleich zu einer Schichtdicke von 2 und 4 mm (gemäß Herstellerangaben) auf die Menge an eluierbaren Inhaltsstoffen aus Bulk-Fill Kompositen auswirkt. In der vorliegenden Studie [44] wurde daher der Einfluss der Schichtdicke auf die Elution von Inhaltsstoffen aus den Bulk-Fill Kompositen SDR Bulkfill, Venus Bulkfill und ELS Bulkfill untersucht.

Laut den Herstellerangaben werden die untersuchten fließfähigen Bulk-Fill Komposite als Liner und Base verwendet und müssen mit einem konventionellen Komposit überschichtet werden. Daher kann die Exposition mit freigesetzten Kompositinhaltsstoffen gegenüber der oralen Umgebung von untergeordneter Bedeutung sein. Dennoch können freigesetzte Inhaltsstoffe durch die Dentintubuli in die Pulpa eindringen [50, 70] und dort die Vitalität und Regenerationsfähigkeit der Pulpa beeinträchtigen [71]. Darüber hinaus wurden die Proben in der vorliegenden Studie [44] sowohl in Methanol als auch in Wasser eluiert, da Wasser den größtmöglichen physiologischen Vergleich mit Dentinflüssigkeit und menschlichem Speichel erlaubt [45, 51].
In der vorliegenden Studie [44] wurden die Eluate mittels GC/MS analysiert. Langkettige Methacrylate wie UDMA und ethoxyliertes Bisphenol-A-Dimethacrylat (Bis-EMA) können im Injektor von GC thermisch diskriminieren [10, 72, 73]. UDMA kann beispielsweise zu geringen Mengen HEMA diskriminieren [10, 72]. In den Eluaten von SDR Bulkfill und Venus Bulkfill der vorliegenden Studie konnte HEMA nachgewiesen werden. Da einerseits Eluate der Komposite untersucht werden und andererseits auch Verunreinigungen der Ausgangsmaterialien (z.B. UDMA, Bis-EMA) des Komposits durch HEMA möglich sind, ist die Quelle von HEMA unklar. Daher wurden die Abbauprodukte von UDMA und Bis-EMA in der vorliegenden Studie nicht

quantifiziert. Denn nach der Diskriminierung von UDMA sind nur geringe Mengen an HEMA nachweisbar [10, 72]. In der vorliegenden Studie wurden in den Eluaten von SDR Bulkfill und Venus Bulkfill relativ hohe HEMA-Mengen gefunden, obwohl HEMA nach Herstellerangaben nicht Bestandteil der Zusammensetzung beider Komposite ist. Die Quelle von HEMA ist somit nicht eindeutig zurückzuführen. Hingegen wurden in den Eluaten von ELS Bulkfill keine kleineren (Co)Monomere wie HEMA (oder TEGDMA) nachgewiesen. Beispielsweise würden bei der Diskriminierung von UDMA geringe Mengen HEMA entstehen [10, 72]. Daher ist davon auszugehen, dass nur geringe Mengen an langkettigen Methacrylaten, wie z.B. Bis-EMA und/oder UDMA aus dem Komposit ELS Bulkfill freigesetzt werden.
Das Ausmaß und die Geschwindigkeit der Elution von Inhaltsstoffen aus Kompositen hängt von mehreren Faktoren ab: dem DC von (Co)Monomeren, der Zusammensetzung und Löslichkeitseigenschaften des Extraktionslösungsmittels sowie der Größe und chemischen Eigenschaften der eluierbaren Spezies [53]. In der vorliegenden Studie [44] wurde die Elution bis zu einer Schichtdicke von 6 mm untersucht. Dabei entspricht eine Schichtdicke von bis zu 4 mm den Vorgaben der verwendeten Hersteller und eine Schichtdicke von 6 mm einer „Worst-Case“-Situation entgegen der Herstellerangaben. In einer früheren Studie wurde der Zusammenhang von Aushärtetiefe und DC für ein konventionelles Komposit untersucht [74]. Basierend auf der Lampenenergie der vorliegenden Studie (22-24 J/cm^2) kann eine Umwandlungsrate von etwa > 55% bei einer Aushärtetiefe von 6 mm für konventionelles Komposit der oben zitierten Studie [74] geschätzt werden. Unter Berücksichtigung einer erhöhten Transluzenz durch reduzierten Füllungskörperanteil bei gleichzeitig erhöhter Füllungspartikelgröße für Bulk-Fill-Komposite [57] sollten die in der vorliegenden Studie untersuchten Bulk-Fill Proben mit einer Schichtdicke von 6 mm ausreichend ausgehärtet sein. Dennoch zeigten andere Studien über Bulk-Fill Komposite bei einer Schichtdicke von 4 mm einen signifikant niedrigeren DC an der unteren Oberfläche im Vergleich zur oberen Oberfläche [75, 76]. Jedoch wurde weder an der oberen noch an der unteren Oberfläche eine Korrelation zwischen DC und (Co)monomerfreisetzung gefunden [75]. Bisher gibt es keine Hinweise auf eine Korrelation zwischen DC und (Co)monomer-Freisetzung, da der Elutionsmechanismus unter anderem vom Molekulargewicht und der Hydrophobie von (Co)Monomeren sowie dem Füllstoffgehalt abhängt [76] und somit vom untersuchten Material sowie der endgültigen Netzwerkcharakteristik der Harzmatrix abhängt [68, 75, 77].

TEGDMA ist aufgrund seiner geringen Viskosität und der Fähigkeit, die organische Harzmatrix von Kompositen mit einem Maximum an anorganischen Füllstoffpartikeln anzureichern, ein häufig in Kompositen eingesetztes (Co)monomer [78]. TEGDMA wurde in den Eluaten von SDR Bulkfill und Venus Bulkfill nachgewiesen. Die höchste eluierte TEGDMA-Konzentration in der vorliegenden Studie beträgt 146 µg/mL für SDR Bulkfill bei einer Schichtdicke von 6 mm

nach 7 Tagen Elution in Wasser. Diese Konzentration ist signifikant höher als die Konzentration im Eluat bei einer Schichtdicke von 2 mm (83 µg/mL). Die zytotoxische Konzentration für TEGDMA beträgt 1058 µg/ml für HGFs [79]. Obwohl die Elution von TEGDMA aus SDR Bulkfill bei einer Schichtdicke von 6 mm gegenüber einer Schichtdicke von 2 mm signifikant erhöht ist, liegt dieser Wert fast 10 Mal unter der angegebenen zytotoxischen Konzentration.

In den Eluaten von SDR Bulkfill und Venus Bulkfill konnte HEMA nachgewiesen werden. Die Schichtdicke beeinflusste die Menge an eluiertem HEMA nicht signifikant. HEMA wird aufgrund seiner hydrophilen Eigenschaft in dentalen Kompositen als (Co)monomer der organischen Harzmatrix verwendet. HEMA kann zytotoxische und genotoxische Wirkungen auslösen [80]. Die höchste gemessene HEMA-Konzentration mit 108 µg/mL wurde im Methanoleluat von Venus Bulkfill nach 7 d bei einer Schichtdicke von 6 mm nachgewiesen. In früheren Studien wurden zytotoxische Konzentrationen für HEMA von 312 µg/ml in HPFs [81] und 1548 µg/ml in HGFs [79] bestimmt. Die in unserer Studie nachgewiesenen HEMA-Konzentrationen liegen daher weit unter diesen zytotoxischen Konzentrationen.

Der Photoinitiator Campherquinon (CQ) ist ein aus allen untersuchten Bulkfill Kompositen freigesetzter Inhaltsstoff. CQ gilt als starkes Allergen [82], das auch oxidativen Stress und DNA-Schäden verursachen kann [83]. Nach 7 d wurde die höchste CQ-Konzentration in den Methanoleluaten von ELS Bulkfill mit 22 µg/mL gemessen. Diese Konzentration war signifikant höher als die Konzentration der Eluate bei einer Schichtdicke von 2 mm (6 µg/mL). Lediglich bei den Methanoleluaten konnte im Gegensatz zu den Wassereluaten ein Einfluss der Schichtdicke auf die Menge an eluierbaren Kompositinhaltsstoffen beobachtet werden. Frühere Studien zeigten einen signifikanten konzentrationsabhängigen Anstieg von intrazellulären reaktiven Sauerstoffspezies (ROS) und DNA-Schäden bei HGFs ab einer Konzentration von 8,3 µg/ml [83]. Die in der vorliegenden Studie nachgewiesene Konzentration ist fast dreimal höher als dieser Wert. Eine frühere Studie unserer Gruppe zeigte, dass CQ im nativen menschlichen Speichel nicht nachweisbar ist [45]. Aufgrund dieser Ergebnisse sind toxische Wirkungen in der humanphysiologischen Situation nicht zu erwarten.

Für das Komposit SDR Bulkfill führte nach 24h und 7d in Methanol eine Schichtdicke von 6 mm zu einer signifikant höheren Freisetzung von 4-N,N-dimethylaminobenzoesäurebutylethoxyester (DMABEE) im Vergleich zu einer Schichtdicke von 4 mm bzw. 2 mm. In den Wassereluaten beeinflusste die Schichtdicke die Freisetzung von DMABEE nicht signifikant. DMABEE ist ein Coinitiator, der in Kompositen verwendet wird, um den Abbau von Initiatoren in Radikale und damit die Polymerisation zu beschleunigen [84]. Der höchste Wert für DMABEE (78 µg/mL) wurde in der vorliegenden Studie in den Methanoleluaten von SDR Bulkfill nach 7d bei einer Schichtdicke von 6 mm gemessen. Dies

ist fast dreimal weniger als die für HGFs beschriebene zytotoxische Konzentration von 237 µg/ml [81].

2-Hydroxy-4-methoxybenzophenon (HMBP) wird dentalen Kompositen als UV-absorbierende Verbindung zugesetzt, um die Verfärbung des Kompositharzes zu reduzieren [85]. Nach 7d wurde die höchste HMBP-Konzentration im Methanoleluat von SDR Bulkfill bei einer Schichtdicke von 6 mm (246 µg/mL) nachgewiesen. Eine Schichtdicke von 6 mm führte zu einer signifikant höheren HMBP-Elution im Vergleich zu einer Schichtdicke von 4 mm (188 µg/mL) bzw. einer Schichtdicke von 2 mm (163 µg/mL). HMBP zeigte östrogene Wirkungen in humanen embryonalen Nierenfibroblasten. HMBP zeigte mit 228 µg/ml eine höhere relative Luciferase-Aktivität im Vergleich zu den Negativkontrollen [86]. Die Konzentration von HMBP in den Methanoleluaten war fast 30-mal höher als die Konzentration der entsprechenden Wassereluate. Aufgrund dieses Ergebnisses sind toxische Wirkungen in der humanphysiologischen Situation nicht zu erwarten.

Interessanterweise führt bei unseren Untersuchungen eine doppelte Schichtdicke nicht zu doppelt so hohen Elutionswerten, jedoch nehmen die Elutionswerte der Methacrylate mit steigender Oberfläche zu. Dies steht im Einklang mit vielen früheren Studien [87-91].

Schlussfolgerung

Die Ergebnisse der vorliegenden Studie [44] zeigen, dass die Herstellerangaben strikt befolgt werden sollten, da in vielen Fällen eine laut Hersteller nicht freigegebene Schichtdicke von 6 mm („Worst-Case“-Situation) zu einer höheren Menge an eluierten Bulk-Fill Kompositinhaltsstoffen führt. Dies kann zu einer höheren Exposition bei Patienten führen.

3.1.2. Freisetzung und Proteinbindung von Inhaltsstoffen aus dentalen Kompositen in nativem Speichel und anderen Extraktionsmedien [45]

Die Menge an eluierten (Co)Monomeren und deren Wiederfindungsrate ist von dem zur Extraktion verwendeten Medium abhängt. So ist z.B. die Wiederfindungsrate von TEGDMA geringer, wenn ein Zellkulturmedium mit fötalem Kälberserum, welches einen hohen Anteil an Proteinen enthält verwendet wird [92]. Für die Beurteilung der Toxizität und Biokompatibilität von Dentalkompositen ist es nicht nur wichtig zu wissen, welche (Co)Monomere und Additive in welcher Menge freigesetzt werden, sondern ob diese Stoffe auch an Proteine binden können. Diese Proteinbindung könnte in vivo zu einer reduzierten Bioverfügbarkeit führen [93]. Nur der freie, nicht proteingebundene Anteil eines Wirkstoffs kann pharmakologische oder toxikologische Wirkungen entfalten, Zellmembranen durchdringen und eliminiert werden [93].

Proteine sind im Speichel in einer Konzentration von 2,5 g/L vorhanden und haben einen entscheidenden Einfluss auf die Eigenschaften des Speichels [94]. Insgesamt enthält Speichel mehr als 1000 Proteine mit einem Molekulargewicht von 40 bis 1000 kDa, die fast ausschließlich Glykoproteine sind [95]. Quantitativ dominieren die Muzine, die in zwei Grundformen im Speichel vorkommen. Mucine werden im Allgemeinen in Proteine mit hohem Molekulargewicht (MUC5B, früher MG1) und Proteine mit niedrigem Molekulargewicht (MUC7, früher MG2) eingeteilt [95]. Biologisch aktive und antibakterielle Speichelproteine sind: Agglutinin, Lactoferrin, Lysozym, Peroxidasen, histidinreiche Proteine (Histatine), Defensine und Cystatine [96, 97].

In der vorliegenden Studie „Freisetzung und Proteinbindung von Inhaltsstoffen aus dentalen Kompositen in nativem Speichel und anderen Extraktionsmedien“ [45] wurde die Bindung von freisetzbaren Inhaltsstoffen aus den Kompositen Admira flow, Venus Diamond flow, Filtek Supreme XTE flow, Tetric EvoCeram und Tetric EvoFlow an die oben genannten Proteine untersucht. Außerdem wurde nativer Speichel (mit Proteinen) neben proteinfreiem Speichel, Wasser und Ethylacetat als Extraktionsmedium für die Elutionsversuche verwendet. Die Konzentration von (Co)Monomeren und Additiven, die in nativem Speichel freigesetzt wurden, wurde mit der Konzentration von (Co)Monomeren und Additiven, die in proteinfreiem Speichel, Wasser bzw. Ethylacetat freigesetzt wurden, verglichen. (Co)Monomere und Additive können verschluckt werden und durch Aufnahme über den Darmtrakt ins Blut und schließlich in den Organismus gelangen [13]. Neben der Kenntnis der Bindung von (Co)Monomeren an Speichelproteine ist auch die Bindung an Plasmaproteine wichtig, um die Toxizität von Dentalmaterialien zu beurteilen. Daher wurde auch die Bindung einiger Methacrylate und Additive an die Plasmaproteine human serum albumin (HSA) und human α_1-acid glycoprotein (AGP) untersucht.

Verschiedene Faktoren können die Freisetzung von (Co)Monomeren aus Kompositen beeinflussen. In früheren Studien konnte gezeigt werden, dass die freigesetzte Menge neben dem Polymerisationsgrad (DC) des Komposits und der Porosität des Materials wesentlich vom verwendeten Extraktionsmedium abhängt [98, 99]. Der Einfluss des Extraktionsmediums lässt sich mit dem Nernst-Verteilungskoeffizienten erklären, der von Durner et al. für zahlreiche freisetzbare Inhaltsstoffe aus Kompositen bestimmt wurde [100]. Wasser- und proteinfreier Speichel eluierte nach dem Nernst-Verteilungskoeffizienten in unserer Studie fast die gleiche Menge an (Co)Monomeren und Additiven, stark lipophile Verbindungen wie 1,10-Decandiol dimethacrylat (DDDMA) wurden meist durch Ethylacetat eluiert. Dieses Ergebnis steht im Einklang mit einer früheren Studie [7], die eine sehr ähnliche Eigenschaft von künstlichem Speichel und Wasser zeigte, (Co)Monomere und Additive zu eluieren. Weder Wasser noch künstlicher Speichel spiegeln jedoch die reale physiologische Situation in der Mundhöhle wieder. In der vorliegenden Studie [45] wurde daher der Einfluss von nativem Speichel auf die

Elution von (Co)Monomeren und Additiven evaluiert. Um Erkenntnisse über den Einfluss des Extraktionsmediums zu gewinnen, wurde nativer Speichel (mit Proteinen), proteinfreier Speichel, Wasser bzw. Ethylacetat verwendet. Die vorliegende Elutionsstudie zeigt, dass die Menge an nachweisbaren (Co)Monomeren und Additiven stark vom verwendeten Extraktionsmedium abhängt. Jedes der fünf getesteten Komposite zeigte einen großen Unterschied zwischen nativem Speichel, in dem nur sehr geringe Mengen an (Co)Monomeren und Additiven nachgewiesen werden konnten, und den drei anderen Extraktionsmedien (proteinfreier Speichel, Wasser und Ethylacetat).

Beispielsweise wurde für Admira flow beobachtet, dass die in der physiologischen Situation (in nativem Speichel) gemessene maximale TEGDMA-Konzentration mit 0,08 mmol/L signifikant – etwa um den Faktor fünf – niedriger war als die TEGDMA-Konzentration im Wasser Eluat (0,39 mmol/L) bzw. im proteinfreien Speichel-Eluat (0,34 mmol/L). Diese Beobachtung der signifikant unterschiedlichen TEGDMA Konzentrationen in nativem Speichel und proteinfreiem Speichel bzw. Wasser traf auch für Venus Diamond flow und Filtek supreme XTE flow zu. Des Weiteren wurde festgestellt, dass CQ und HEMA in nativem Speichel nicht nachweisbar waren, im Gegensatz zu proteinfreiem Speichel und Wasser.

Der Grund für die geringe Wiederfindungsrate von (Co)momomeren könnte in der Zusammensetzung des nativen Speichels liegen. Nativer Speichel enthält bis zu 3% verschiedene Salze und Glykoproteine (hauptsächlich Mucine) [95], die (Co)Monomere und Additive binden können. Es gibt einige Studien zu (Co)Monomeren und Additiven im menschlichen Speichel [101-103], jedoch keine Studie, die die Extraktionsmedien Wasser, proteinfreier Speichel und nativer Speichel hinsichtlich der Bindung von (Co)Monomeren und Additiven an Speichelproteinen vergleichen. Nur für TEGDMA wurde in einer früheren Studie gezeigt, dass die Wiederfindungsrate in einem serumhaltigen Zellkulturmedium viel geringer im Vergleich zur Wiederfindungsrate von TEGDMA in einem serumfreien Zellkulturmedium, destilliertem Wasser, Kochsalzlösung oder künstlichem Speichel war [92]. Folglich wurde eine Wechselwirkung von TEGDMA mit den Inhaltsstoffen des serumhaltigen Zellkulturmediums postuliert. Die in dieser Studie beobachtete geringe Wiederfindungsrate von (Co)Monomeren und Additiven in nativem Speichel im Vergleich zu der höheren Wiederfindungsrate von (Co)Monomeren und Additiven in proteinfreiem Speichel und Wasser unterstreicht die Bedeutung das richtige Extraktionsmedium für die Elution zu wählen. Sowohl für die Bewertung der Toxizität als auch für eine sinnvolle Risikobewertung der (Co)Monomere und Additive ist es daher wichtig zu wissen, in welchen Mengen diese Stoffe aus dem Komposit freigesetzt werden und in welchen Konzentrationen sie in nativem menschlichen Speichel vorliegen. In früheren Studien wiesen (Co)Monomere und Additive auf konzentrationsabhängige zytotoxische Effekte, wie erhöhten Zelltod, Schädigung der

Plasmamembran oder einen erhöhten Gehalt an freigesetzter Laktatdehydrogenase hin [1, 66, 67].

TEGDMA wurde in der vorliegenden Studie in den Eluaten der Komposite Filtek supreme XTE flow, Venus Diamond flow und Admira flow nachgewiesen. Die höchste TEGDMA-Konzentration wurde im Wassereluat von Filtek supreme XTE flow nach 72 h (0,43 mmol/L) gefunden und war fast zehnmal niedriger als die bekannte zytotoxische Konzentration für HGFs (3,7 mmol/L) [79].

TEGDMA und HEMA sind die am häufigsten verwendeten (Co)Monomere in dentalen Kompositen. In den Eluaten von Tetric EvoFlow und Tetric EvoCeram konnte HEMA nachgewiesen werden. Es ist bekannt, dass HEMA zytotoxische und genotoxische Wirkungen haben kann [80]. Außerdem kann HEMA bei 3 mmol/L eine höhere Konzentration an reaktiven Sauerstoffspezies (ROS) induzieren [104, 105]. Die höchste gemessene HEMA-Konzentration von 0,18 mmol/L wurde im Ethylacetat-Eluat von Tetric EvoFlow nach 24 h nachgewiesen. In früheren Studien wurden zytotoxische Konzentrationen für HEMA von 2,4 mmol/L in HPFs [81] und 11,9 mmol/L in HGFs [79] gefunden. Die in unserer Studie in allen Extraktionsmedien nachgewiesenen HEMA-Konzentrationen liegen daher weit unter den zytotoxischen Konzentrationen, die von Geurtsen et al. [81] und Reichl et al. [79] beschrieben wurden.

Der Photoinitiator CQ ist Bestandteil aller untersuchten Komposite. In der vorliegenden Studie wurde CQ in den Eluaten mit proteinfreiem Speichel, Wasser und Ethylacetat gefunden. In den nativen Speichel-Eluaten blieb CQ unterhalb der Nachweisgrenze. CQ gilt als starkes Allergen [82], das auch oxidativen Stress und DNA-Schäden verursachen kann [83]. Die höchste CQ-Konzentration wurde in den Wassereluaten von Tetric EvoFlow nach 72h mit 0,05 mmol/L gemessen. Frühere Studien zeigten einen signifikanten konzentrationsabhängigen Anstieg von intrazellulären ROS und DNA-Schäden in HGFs ab einer Konzentration von 0,05 mmol/L [83]. Da die Konzentrationen von CQ in allen Eluaten mit nativem Speichel unterhalb der Nachweisgrenze (0,0006 mmol/L) lagen, dürfte die physiologische Konzentration in der Mundhöhle daher – etwa um den Faktor 80 – unter der genotoxischen Konzentration liegen.

Um den DC eines Komposits zu verbessern, werden üblicherweise Co-Initiatoren wie DMABEE zugesetzt. DMABEE kann Apoptose in humanen monoblastoiden Zellen und Nekrose bei 10 µM induzieren [106]. Die höchste DMABEE-Konzentration (0,23 mmol/L) wurde im Ethylacetat-Eluat von Tetric EvoFlow nach 72 h nachgewiesen. Diese ist fünfmal niedriger als die zytotoxische Konzentration für HGFs (1,23 mmol/L) [81] und 20-mal höher als die minimale apoptotische bzw. nekrotische Konzentration [106]. Diese Erkenntnis sollte jedoch nicht besorgniserregend sein, da die Konzentration in der physiologischen Situation (nativer Speichel: 0,009 mmol/L) nicht die apoptotische oder nekrotische Konzentration erreicht.

Plasmaproteinbindung von Methacrylaten und Additiven an HSA und AGP

(Co)Monomere können aus Dentalmaterialien in den Speichel freigesetzt werden und durch Aufnahme über den Darmtrakt in den systemischen Kreislauf gelangen [13]. Darüber hinaus können (Co)Monomere aus Kompositen und Adhäsiven durch das Dentin in die Pulpa diffundieren und für den Stoffwechsel bioverfügbar werden [11]. In Lebermikrosomen kann TEGDMA in vivo zu 2,3-Epoxymethacrylsäure (EMPA) metabolisiert werden [41]. Die Bildung der toxischen EMPA als Zwischenprodukt im Stoffwechsel von Dentalmaterialien konnte nicht nur in Lebermikrosomen, sondern auch in HPFs und HGFs beobachtet werden [42, 43]. Epoxide gelten als zytotoxische und mutagene Substanzen [107-109]. Nur der freie, nicht proteingebundene Anteil der Wirkstoffe kann pharmakologische oder toxikologische Wirkungen entfalten, Zellmembranen durchdringen und eliminiert werden [93]. Betrachtet man die Ergebnisse des vorliegenden Plasmaproteinbindungsassays, so stünden aufgrund der Bindung an HSA bzw. AGP nur etwa 18 % TEGDMA für den Metabolismus zur Bildung von Epoxymetaboliten zur Verfügung (Die Bindung der getesteten Methacrylate an HSA und AGP betrug 82 – 85%). Die ermittelten Werte für die Negativkontrolle Coffein und die Positivkontrolle Propranolol entsprachen den bekannten Werten aus der Literatur (Coffein: 41 %; Propranolol: 94 %) [110, 111].

Die Bindung von Wirkstoffen bzw. Schadstoffen an Proteine (Plasmaproteine, Transportproteine) ist in der Regel reversibel [112]. Aufgrund der hohen Reaktivität von Methacrylaten könnte auch die Bildung kovalenter Bindungen (z.B. mit Thiolgruppen von Proteinen) möglich sein [113]. Methacrylate stellen aufgrund ihrer alpha-beta-ungesättigten Carbonylsäureeinheit Michael-Akzeptoren dar, die leicht mit Bionukleophilen reagieren können. Dies wurde bereits durch die Reaktion von Michael-Akzeptoren mit Glutathion beschrieben [114]. Die Proteinbindung von Methacrylaten könnte zu einer geringeren Exposition gegenüber Epoxiden beitragen, da die Ausgangsverbindungen möglicherweise nur in geringeren Mengen für die Epoxidierung in Leber- oder Zahnfleischzellen zur Verfügung stehen oder sogar noch langsamer in das entsprechende Epoxid umgewandelt werden könnten. Epoxidhydrolasen und Glutathion-S-Transferasen sind in allen Körpergeweben allgegenwärtig. Sie wandeln Epoxide in die entsprechenden ungiftigen Dihydrodiole und Glutathion-Konjugate um [115]. Daher könnten sie potenziell zu niedrigeren Epoxidkonzentrationen führen und somit zu einer geringeren Exposition dieser toxischen Zwischenprodukte beitragen. Die Ergebnisse der vorliegenden Studie [45] zeigten, dass (Co)Monomere und Additive sowohl in der Mundhöhle als auch im Plasma an Proteine gebunden werden können.

Schlussfolgerung

Unsere Studie über die Freisetzung und Protein Bindung von Kompositinhaltsstoffen in nativen Saliva, proteinfreien Saliva (künstlicher Speichel) und Wasser zeigte, dass künstlicher Speichel bzw. Wasser als Extraktionsmedium nicht die tatsächliche physiologische Situation im Körper widerspiegeln [45]. Die Konzentration an (Co)Monomeren und Additiven, die in nativem Speichel freigesetzt werden, ist deutlich niedriger als die Konzentration, die in proteinfreiem Speichel bzw. Wasser freigesetzt wird. Speichel- und Plasmaproteine können (Co)Monomere und Additive binden und dadurch zu einer geringeren Bioverfügbarkeit von freigesetzten Inhaltsstoffen in vivo als bisher angenommen, führen. Allerdings ist die Bindung von Wirkstoffen und wohl auch von freigesetzten Inhaltsstoffen aus Kompositen an Proteine in der Regel reversibel [45, 112].

3.1.3. Einfluss von Bleachinggelen auf die Freisetzung von Inhaltsstoffen aus konventionellen dentalen Kompositen und Bulk-Fill Kompositen [46, 47]

Neben Veneers und zahnfarbenen Kronen ist zum Beispiel Bleaching das häufigste Verfahren, um die Zähne ästhetisch zu verschönern [116]. Es gibt verschiedene Möglichkeiten der Zahnaufhellung, so dass die persönlichen ästhetischen Erwartungen des Patienten erfüllt werden können [117]. Hierfür gibt es drei verschiedene Standard-Bleichmethoden, die auf Carbamid- oder Wasserstoffperoxid-Gel basieren. 1. In-Office-Bleaching-Methoden (35% Peroxide) bzw. Chairside-Bleaching-Methoden (38% Peroxide) [118], 2. Home-Bleaching-Methoden (15% Peroxide) [119] und 3. rezeptfreie Produkte (Over-the-counter-Produkte) (maximal 10% Peroxide). Bleichschienen zum Auftragen des Bleichgels werden nur beim Home-Bleaching und beim In-Office-Bleaching verwendet [120]. Carbamidperoxid zerfällt während des Bleichvorgangs bei Kontakt mit dem Speichel in Wasserstoffperoxid und Harnstoff, der wiederum in Ammoniak und Kohlendioxid zerfällt. Wasserstoffperoxid spaltet sich in Sauerstoff und Wasser auf [121, 122]. Sowohl bei der Chairside- als auch bei der In-Office-Bleaching-Methode sind jedoch durch die Verwendung von hochprozentigen (38% oder 35%) Carbamid- bzw. Wasserstoffperoxid-Gelen, Überempfindlichkeiten an den Zähnen [123] und Schädigungen der Pulpa-Zellen [124] zu beobachten. Durch eine Reduzierung des prozentualen Anteils des Bleichgels, wie bei den Home-Bleaching-Methoden (15% Peroxide), kann eine Verringerung der Hypersensibilitäten erreicht werden [125].

Werden jedoch bei einem Patienten vor einem Bleichvorgang insuffiziente Restaurationen festgestellt, müssen diese in jedem Fall vor Beginn des Bleichvorgangs ausgetauscht werden, da es beim Kontakt der Bleichgele mit der Pulpa zu einer Schädigung der Pulpazellen kommen kann [126]. Zu diesem Zweck wurden verschiedene Kompositmaterialien wie Bulk-Fill oder

andere konventionelle Kompositmaterialien in Betracht gezogen. Letztere werden in der inkrementellen Technik eingesetzt, d.h. in maximal 2 mm dicke Schichten integriert [61], um laut Hersteller die vollständige Polymerisation des Komposits zu gewährleisten. Bulk-Fill Komposite hingegen garantieren eine ausreichende Polymerisationstiefe bei Inkrementen bis zu 4 mm Stärke [55], was auf eine erhöhte Transluzenz durch reduzierten Füllstoffgehalt bei gleichzeitig erhöhter Füllkörpergröße zurückzuführen ist [57]. Die genannten Eigenschaften erlauben bei Bulk-Fill Kompositen in vielen Fällen eine schnelle Ein-Inkrement-Technik zum Füllen einer kompletten Kavität [56].

In der Vergangenheit wurden bereits einige dentale Komposite im Hinblick auf ihre Reaktion auf Bleichmittel untersucht. So wurden zum Beispiel Veränderungen der Oberflächenstruktur [122, 127-131] und der Mikrohärte [128, 132-134] getestet. Ebenso wurde in früheren Studien der Einfluss von Bleaching-Behandlungen auf die Farbe von dentalen Kompositen untersucht [135-137].

Die Polymerisation von dentalen Kompositen ist jedoch unvollständig. Freisetzbare Inhaltsstoffe können allergische Reaktionen [65] wie Asthma, Rhinoconjunctivtis allergica oder Kontaktdermatitis [20] auslösen. Unsere früheren Studien zeigten, dass Bleichbehandlungen die Freisetzung von (Co)Monomeren und anderen Substanzen aus dentalen Kompositen beeinflussen können [138-140]. Zur Monomerelution aus dentalen Kompositen wurden bisher zahlreiche Studien durchgeführt [92, 141, 142].

Vor der Durchführung der beiden vorliegenden Studien „Einfluss von Opalescence®-Bleichgelen auf die Elution von dentalen Kompositinhaltsstoffen“ [46] und „Einfluss von Opalescence® Bleichgelen auf die Elution von Bulk-Fill Kompositinhaltstoffen“ [47] lagen in der wissenschaftlichen Literatur relativ wenig Daten zum Einfluss auf eluierbare Substanzen aus dentalen Kompositen und keine Daten für Bulk-Fill Komposite nach vorangegangenem Bleaching vor. In den vorliegenden Studien [46, 47] wurden daher der Einfluss von Bleaching-Behandlungen mit den Opalescence-Bleichgelen PF 15% (PF 15%) (Home-Bleaching) und PF 35% (PF 35%) (In-Office-Bleaching) auf die Freisetzung von Kompositinhaltsstoffen aus den konventionellen Kompositen Tetric EvoCeram, CLEARFIL AP-X, Tetric EvoFlow, Filtek Supreme XT, Ceram X mono+, Admira und Filtek Silorane, und den Bulk-Fill Kompositen Tetric EvoCeram Bulk Fill, QuiXFil und X-tra Fill untersucht.

Fließfähige Bulk-Fill Komposite wurden für die vorliegende Studie nicht ausgewählt, da diese mit einer Schicht aus konventionellem Komposit [58] überdeckt werden müssen und somit nicht in direktem Kontakt mit dem Bleichgel stehen würden. Zur Elution wurden die Proben sowohl in Methanol als auch in Wasser überführt, da Wasser den größtmöglichen physiologischen Vergleich zum menschlichen Speichel erlaubt [51]. Darüber hinaus ist Wasser der Dentinflüssigkeit sehr ähnlich, so dass freigesetzte Bestandteile durch die

Dentinkanälchen in die Pulpa eindringen [50, 70] und dort die Vitalität und Regenerationsfähigkeit der Pulpa beeinflussen können [71].

Reduzierte Elution durch Bleichbehandlungen im Vergleich zu den Kontrollen

Beim konventionellen Komposit Tetric EvoCeram führte die PF 15%-Behandlung nach 24h in Wasser zu einer 1/3 geringeren Elution von UDMA im Vergleich zu den Kontrollen. Hersteller verwenden UDMA häufig als Basismonomer in Kompositen, um eine bessere Haltbarkeit, Biokompatibilität und weniger Schrumpfung des Komposits zu gewährleisten [78]. Die zytotoxische Konzentration von UDMA für HGFs beträgt 0,27 mmol/l [79]. Der höchste Elutionswert bei den in der vorliegenden Studie [46] untersuchten konventionellen Kompositen für UDMA (0,01 mmol/l) (Tetric EvoCeram, Wasser, 24h, Kontrollen) liegt etwa 30-mal unter der angegebenen zytotoxischen Konzentration.

Beim Komposit Tetric EvoFlow nach 24h in Methanol führte die PF 15%-Behandlung zu einer 3/5 geringeren Elution von HEMA im Vergleich zu den Kontrollen. Aufgrund seiner hydrophilen Eigenschaften wird HEMA als Comonomer der organischen Harzmatrix in dentalen Kompositen verwendet. Frühere Studien berichten von oxidativem Stress und Apoptoseeffekten, die durch HEMA verursacht werden [80, 143, 144]. In der vorliegenden Studie [46] wurde für konventionelle Komposite die höchste HEMA-Konzentration (0,74 mmol/l) in den Methanoleluaten des Komposites Tetric EvoFlow (PF 35%; 7d) gemessen. HEMA wurde nur in den Methanoleluaten nachgewiesen. Da die HEMA-Konzentration in den Wassereluaten unter der Nachweisgrenze (0,14µmol/l) lag, bleibt diese Konzentration etwa um den Faktor 21000 unter der Konzentration für oxidativen Stress in HGFs (3mmol/l) [143] und etwa um den Faktor 71000 unter der Konzentration, die Apoptose in HPFs (10mmol/l) auslöst [144].

Die PF 15%-Behandlung beim Komposit CLEARFIL AP-X führte nach 7d in Wasser zu einer 1/5 geringeren Freisetzung von TEGDMA im Vergleich zu den Kontrollen. Nach 7d in Methanol zeigten sowohl die PF 15%- als auch die PF 35%-Behandlungen etwa 1/4 der TEGDMA-Freisetzung im Vergleich zu den Kontrollen. TEGDMA ist ein Comonomer, das aufgrund seiner geringen Viskosität und seiner Fähigkeit, die organische Harzmatrix von Kompositen mit einem Maximum an anorganischen Füllstoffpartikeln anzureichern, in Kompositen verwendet wird [78]. Laut Literatur [79] beträgt die zytotoxische Konzentration für TEGDMA in HGFs 3,7 mmol/l. In der vorliegenden Studie [46] wurde für konventionelle Komposite die höchste TEGDMA-Menge bei den Kontrollen von CLEARFIL AP-X nach 7d in Wasser (0,28 mmol/l) gefunden. Dieser Wert ist 13-mal niedriger als die angegebene zytotoxische Konzentration. Bei den untersuchten Bulk-Fill Kompositen [47] zeigt das Komposit QuiXFill mit PF 35%-Behandlung nach 24h in Wasser eine um etwa die Hälfte geringere TEGMA-Freisetzung im Vergleich zu den Kontrollen. Unter den untersuchten Bulk-Fill Kompositen hatte die PF 35%-

Behandlung nur bei X-tra Fill keinen Einfluss auf die Freisetzung der Komponenten im Vergleich zur Kontrolle.
Der Mechanismus des Wasserstoffperoxid- und Carbamidperoxid-Bleichens ist noch nicht vollständig geklärt, da unspezifische Oxidationsprozesse auftreten [145]. Insbesondere langkettige Moleküle wie UDMA und Bis-GMA können durch Wechselwirkung mit Peroxiden abgebaut werden [138]. Dies könnte sowohl die reduzierte Freisetzung von UDMA, TEGDMA und HEMA durch Bleichbehandlungen im Vergleich zu den Kontrollen bei den oben genannten konventionellen Komposite Tetric EvoCeram, Tetric EvoFlow und CLEARFIL AP-X, als auch die reduzierte Freisetzung von DMABEE und TEGDMA im Bleichprozess mit PF 35% im Vergleich zu den Kontrollen der untersuchten Bulk-Fill Kompositen Tetric EvoCeram Bulk Fill und QuiXFill erklären.

Reduzierte Elution durch PF 15%-Behandlung im Vergleich zur PF 35%-Behandlung

Eine signifikant reduzierte Elution durch PF 15%-Behandlung im Vergleich zur PF 35%-Behandlung konnte nur bei den untersuchten konventionellen Kompositen [46] und nicht bei den untersuchten Bulk-Fill Kompositen [47] festgestellt werden.
Beim konventionellen Komposit Tetric EvoCeram nach 7d in Methanol führte die PF 15%-Behandlung zu einer 1/3 geringeren Elution von UDMA im Vergleich zur PF 35%-Behandlung. Das Komposit Tetric EvoFlow zeigte nach 7d in Methanol und PF 15%-Behandlung auch eine 1/3 geringere Elution von HEMA im Vergleich zur PF 35%-Behandlung. Die Oxidation von Peroxiden innerhalb des Bleichprozesses ist unspezifisch [145] und daher bisher nicht im Detail geklärt. Dennoch konnte gezeigt werden, dass Peroxide die Mikrohärte der Kompositoberfläche modifizieren können [146, 147]. Darüber hinaus haben Marson et al. berichtet, dass das Eindringen von Wasserstoffperoxid in die Zahnstruktur zeitabhängig ist [148]. Ausgehend von der Anwendung von Bleaching-Gelen gemäß den Herstellerangaben lassen diese Erkenntnisse und die oben genannten Ergebnisse der konventionellen Komposite Tetric EvoCeram und Tetric EvoFlow folgende Annahme zu: Längere Einwirkungszeit (5h) von PF 15% kann zum Abbau bzw. zur Oxidation eluierbarer Substanzen führen, wenn Peroxide durch die Kompositoberfläche in tiefere Schichten eindringen. Bei Verwendung von PF 35% mit einer kürzeren Einwirkzeit von 30 Minuten würde dieser Effekt nicht in gleichem Maße auftreten. Offensichtlich ist nicht die Peroxidkonzentration des Bleichgels ausschlaggebend, sondern die unterschiedliche Einwirkzeit beider Bleichgele, die die reduzierte Elution (7d, Methanol) von UDMA und HEMA bei Behandlung mit PF 15% im Vergleich zu PF 35% bestimmt.

Erhöhte Elution durch PF 15%-Behandlung im Vergleich zur PF 35%-Behandlung

Beim Komposit Ceram X mono+ nach 24h in Methanol führte die PF 15%-Behandlung zu einer 2-fach höheren Elution von UDMA im Vergleich zur PF 35%-Behandlung. Gleiches gilt für das Komposit Admira nach 24h in Wasser, das nach PF 15%-Behandlung 1/3 mehr TEGDMA freisetzt als nach PF 35%-Behandlung.

Für die mit PF 15% untersuchten Bulk-Fill Komposite Tetric EvoCeram Bulk Fill (24h und 7d) und QuiXFil (7d) weisen alle in Methanol eluierten Substanzen im Vergleich zur PF 35%-Behandlung deutlich höhere Konzentrationen auf. So konnten z.B. für Tetric EvoCeram Bulk Fill nach 7d in Methanol 2,5 mal höhere Werte für alle eluierten Substanzen durch PF 15%-Behandlung im Vergleich zur PF 35%-Behandlung ermittelt werden. Das Komposit X-tra Fill zeigte nach 7d in Wasser (PF 15%) nur für TEGDMA eine 1,5-fach höhere Konzentration im Vergleich zur PF 35%-Behandlung.

Diese Ergebnisse deuten darauf hin, dass eine längere Einwirkungszeit (PF 15%: 5 h, PF 35%: 30 min), anders als unterschiedliche Konzentrationen von Peroxiden in den Bleichgelen, eine erhöhte Freisetzung von Inhaltsstoffen für die oben genannten Komposite Ceram X mono+, Admira, Tetric EvoCeram Bulk Fill und QuiXFill nach der Bleichbehandlung verursacht. Auch hier scheint der Einfluss von Peroxiden auf die Mikrohärte der Kompositoberfläche [146, 147] und das zeitabhängige Eindringen von Wasserstoffperoxid in die Zahnstruktur [148] ausschlaggebend zu sein.

Erhöhte Elution durch Bleichbehandlungen im Vergleich zu den Kontrollen

Beim konventionellen Komposit Filtek Supreme XT führte die PF 15%-Behandlung nach 24h in Methanol zu einer 2-fach höheren Elution von UDMA im Vergleich zu den Kontrollen. Beim Komposit Ceram X mono+ ergab die PF 15%- und die PF 35%-Behandlung nach 7d in Wasser eine etwa 3-mal höhere DMABEE Konzentration im Vergleich zu den Kontrollen. DMABEE ist ein Coinitiator, der in Kompositen verwendet wird, um den Abbau von Initiatoren in Radikale und damit die Polymerisation zu beschleunigen [84]. Die höchste DMABEE-Konzentration wurde in den Wassereluaten (0,10 mmol/l) für das konventionelle Komposit Ceram X mono+ nach 7d Behandlung mit PF 35% nachgewiesen. Dieser Wert ist 12-mal niedriger als die zytotoxische Konzentration in HGFs (1,23 mmol/l) [81].

Für das Bulk-Fill Komposit Tetric EvoCeram Bulk Fill wurde nach 24h in Methanol mit PF 15%-Behandlung fast doppelt so viel HEMA nachgewiesen wie in den Kontrollen. Der höchste Wert für HEMA in der vorliegenden Studie [47] mit Bulk-Fill Kompositen wurde für Tetric EvoCeram Bulk Fill (PF 15%; 7d) in Methanol mit 0,22 mmol/l gemessen. HEMA konnte nur im Methanol-Eluat nachgewiesen werden. Da die HEMA-Konzentration in den Wassereluaten unter der Nachweisgrenze (0,14 µmol/l) lag, bleibt diese Konzentration etwa um den Faktor 21000 unter

der Konzentration für oxidativen Stress in HGFs (3 mmol/l) [143] und etwa um den Faktor 71000 unter der Konzentration, die Apoptose in HPFs (10 mmol/l) auslöst [144].
Nach 24h in Methanol weist das Komposit QuiXFil mit PF 15%-Behandlung eine fast doppelt so hohe DMABEE-Freisetzung auf wie die Kontrollen. Der höchste in der vorliegenden Studie [47] mit Bulk-Fill Kompositen gemessene Wert für DMABEE in Wasser beträgt 0,05 mmol/l für QuiXFill (7d; PF 35%). Dies ist fast 25 Mal weniger als die zytotoxische Konzentration von 1,23 mmol/l, die für Konzentration in HGFs beschrieben wurde [81].
Bei dem Komposit X-tra Fill konnte nach 7d in Wasser und einer PF 15%-Behandlung 1/3 mehr TEGDMA nachgewiesen werden als bei den Kontrollen. Die höchste eluierte TEGDMA-Konzentration in der vorliegenden Studie [47] mit Bulk-Fill Kompositen beträgt 0,3 mmol/l für X-tra Fill nach 7d in Wasser und PF 15%-Behandlung. Dies ist also etwa 12 Mal weniger als die zytotoxische Konzentration von 3,7 mmol/l in HGFs [79].
In unserer früherer Studie konnte gezeigt werden, dass Bleichprozesse zu einer erhöhten Freisetzung von Inhaltsstoffen, wie z.B. TEGDMA oder DMABEE aus dem konventionellen Komposit Filtek Supreme XT im Vergleich zu den Kontrollen führen können [139]. Die Oxidation durch Peroxide im Bleichprozess ist unspezifisch [145] und daher nicht im Detail geklärt. Es konnten jedoch Veränderungen der Mikrohärte der Kompositoberfläche durch Peroxide festgestellt werden [146, 147]. So ist es möglich, dass das Polymernetzwerk der Kompositoberfläche durch das im Bleichgel enthaltene Carbamid-/Wasserstoffperoxid geschädigt wird und somit eine verstärkte Freisetzung der Inhaltsstoffe nachgewiesen werden kann [139]. Dies könnte auch die erhöhte Freisetzung der Inhaltsstoffe HEMA, DMABEE, UDMA und TEGDMA aus den oben beschriebenen konventionellen Komposite Filtek Supreme XT und Ceram X mono+ sowie den Bulk-Fill Kompositen Tetric EvoCeram Bulk Fill, QuiXFill und X-tra Fill nach Bleichbehandlung erklären.

Polydorou et al. [122] konnten bei einigen konventionellen Kompositmaterialien signifikante Veränderungen der Oberflächentextur (Verlust von Harzanteilen und Oberflächenrissen) nach dem Bleichprozess feststellen. Es wurde daher angenommen, dass diese Effekte durch die Auflösung der adhäsiven Bindung zwischen den Füllstoffen und der organischen Matrix verursacht werden [122]. Des Weiteren postulierten Wattanapayungkul et al. [147], dass die Aufrauhung der Oberfläche des Komposits von der Bleichbehandlung abhängt; dieser Effekt wird nicht nur durch Unterschiede in den Harzmatrixkomponenten, sondern auch durch die unterschiedlichen Füllstoffgrößen innerhalb der Komposit-Subtypen verursacht. Im Vergleich zu konventionellen Kompositen haben Bulk-Fill Komposite einen geringeren Füllstoffgehalt bei gleichzeitig erhöhter Füllpartikelgröße, was zu einer erhöhten Transluzenz führt [57]. In der vorliegenden Studie [46] wurde die Auswirkung von PF 15% und PF 35% auf die Elution von konventionellen Kompositen untersucht. Zum Beispiel zeigten die Elutionswerte von HEMA in

Tertic Evo Ceram (Ivoclar Vivadent AG) keine signifikanten Unterschiede nach der Behandlung mit PF 15% im Vergleich zur Kontrolle (24h und 7d; Methanol) [46]. In der vorliegenden Studie [47] mit Bulk-Fill Kompositen wurde das Bulk-Fill Komposit Tetric Evo Ceram Bulk Fill desselben Herstellers und mit denselben Inhaltsstoffen (laut Hersteller) untersucht: HEMA wurde bei der PF 15%-Behandlung im Vergleich zur Kontrolle (24h, Methanol) in einer etwa doppelt so hohen Konzentration nachgewiesen. Offensichtlich scheint die erhöhte Füllkörpergröße bei Bulk-Fill Kompositen die Freisetzung von Inhaltsstoffen nach dem Bleichverfahren verglichen mit konventionellen Kompositen mit geringerer Füllkörpergröße zu beeinflussen.

In der vorliegenden Studie [46] wurde für konventionelle Komposite gezeigt, dass die Elution von Inhaltsstoffen nach dem Bleichprozess von der Zusammensetzung jedes einzelnen Komposites abhängig ist. Dies wird auch in der vorliegenden Studie [47] für die untersuchten Bulk-Fill Komposite bestätigt. Den stärksten Effekt durch einen Bleichprozess zeigt demnach Tetric EvoCeram Bulk Fill, gefolgt von QuiXFill und X-tra Fill (schwächster Effekt) für PF 15% und auch für PF 35%. Folglich variieren die Ergebnisse innerhalb der untersuchten Bulk-Fill Komposite in Abhängigkeit von der Zusammensetzung des jeweiligen Materials.

Von allen untersuchten Kompositen konnten ausschließlich in den Methanoleluaten von Filtek Silorane, Ceram X mono+ und Tetric EvoCeram Bulk Fill Methacrylate und somit keine Methacrylate in der physiologischen Situation des Wassereluats nachgewiesen werden. Daher sollten bei einem Patienten mit Methacrylatallergie Filtek Silorane, Ceram X mono+ und Tetric EvoCeram Bulk Fill den anderen untersuchten Kompositen vorgezogen werden, wenn eine Kompositrestauration erforderlich ist.

Schlussfolgerung

Bleaching-Behandlungen können je nach Kompositmaterial zu einer reduzierten und/oder erhöhten Elution von Inhaltstoffen aus konventionellen und Bulk-Fill Kompositen führen.

3.1.4. Elutionsverhalten von 3D-gedruckten, gefrästen und konventionellen Aufbissschienen auf Methacrylatharzbasis [4]

Die Herstellung von Kronen, Zahnersatz und Aufbissschienen sind nur einige der vielen Anwendungen des 3D-Drucks in der Zahnmedizin. Die zunehmende Digitalisierung ermöglicht konventionelle Techniken, bei denen üblicherweise Polymethylmethacrylat (PMMA), ein Material auf Methacrylbasis bzw. Harzbasis verwendet wird zu ersetzen. PMMA bietet Vorteile wie eine hohe Ästhetik und gute chemische und physikalische Eigenschaften und ist gleichzeitig relativ kostengünstig. Das computergestützte Design und die computergestützte

Fertigung (CAD/CAM) basieren entweder auf subtraktiven (Fräsen oder Schleifen von vorgefertigten Materialblöcken) oder additiven (3D-Druck) Methoden. Die subtraktive Fertigung ist in der Zahnmedizin nicht neu und erreichte die Dentalindustrie in den späten 1980er Jahren. Daher wurden Fräsmaterialien ausgiebig untersucht und es hat sich gezeigt, dass sie den konventionell verarbeiteten Materialien hinsichtlich ihrer physikalischen Eigenschaften überlegen sind [149-152]. Der 3D-Druck ist eine neuere Technologie, bei der im Gegensatz zu industriell vorgefertigten Fräsblöcken meist niedrigvisköse Materialien verwendet werden. Da diese neuartigen, für den 3D-Druck entwickelten Materialien je nach Verfahren eine bestimmte Zusammensetzung erfordern, gibt es bisher nur wenige Erkenntnisse über ihre Biokompatibilität und andere Eigenschaften [153].

Eine nachteilige Eigenschaft aller Materialien auf Methacrylatbasis ist die unvollständige Umwandlung von (Co)Monomeren in Polymere, selbst unter idealen Polymerisationsbedingungen. Der Polymerisationsgrad (DC) wird hauptsächlich von Parametern des Herstellungsverfahrens wie dem Verhältnis von Monomer zu Polymer, der Polymerisationszeit und -temperatur und, im Falle der Fotopolymerisation, der Lichtdichte und der Aushärtungszeit beeinflusst [154, 155]. Die unvollständige Umsetzung von (Co)Monomeren birgt ein potenzielles Risiko für die Freisetzung von ungebundenen Rest(co)monomeren, Additiven und Reaktionsprodukten [156-159]. Darüber hinaus können Teile der nicht umgesetzten Komponenten in dem dreidimensionalen Polymernetzwerk eingeschlossen bleiben und können im Laufe der Zeit freigesetzt werden [160]. Abbauprozesse durch mechanische Einwirkung [161], chemische Wechselwirkungen in der oralen Umgebung wie Speichel [162] und bakterielle Enzyme [163] sowie Hydrolyse [164] können zu einer Elution von Inhaltsstoffen führen.

In vitro Studien ergaben, dass (Co)Monomere wie TEGDMA, HEMA, MMA und Additive negative Auswirkungen wie Mutagenität, Teratogenität, Genotoxizität, Zytotoxizität und östrogene Aktivität in Zellen der Mundhöhle hervorrufen können [25-29].

Während einige Studien Elutionsdaten für konventionelle und vorgefertigte Fräsmaterialien erheben [165, 166], finden sich nur sehr wenige Untersuchungen für additiv verarbeitete Materialien [3]. Ziel der vorliegenden in vitro Studie [4] ist, die Elution/Freisetzung von Inhaltsstoffen und das zytotoxische Potenzial von PMMA-basierten Aufbissschienenmaterialien unter Berücksichtigung physiologischer Schienengrößen zu untersuchen. Eine hohe Biokompatibilität und damit gesundheitliche Unbedenklichkeit von Dentalmaterialien ist sowohl für Patienten als auch für Behandler von entscheidender Bedeutung. In der vorliegenden Studie [4] wurden drei verschiedene PMMA-Materialien auf Methacrylharzbasis für die Herstellung von Zahnschienen in additiven (3D-Druck), subtraktiven (Fräsen) und konventionellen Herstellungsverfahren (Pulver und Flüssigkeit) untersucht.

SHERAprint-ortho plus, das für die relativ neue Technik der additiven Fertigung entwickelt wurde, ist als Medizinprodukt der Klasse IIa gemäß der europäischen Medizinprodukterichtlinie (Richtlinie 93/42/EWG) für eine kontinuierliche oder wiederholte intraorale Anwendung von bis zu 30 Tagen zugelassen und wurde daher in dieser Studie als Testmaterial ausgewählt. Für die etablierten Verfahren der subtraktiven und konventionellen Herstellung wurden SHERAeco-disc PM20 (subtraktiv) und das konventionelle Kaltpolymerisationsmaterial SHERAORTHOMER.
In Anlehnung an unsere früheren Studien mit Materialien auf Methacrylharzbasis [45, 53] wurden in der vorliegenden Studie ebenfalls Probenkörper in Form eines Zylinders mit einem Durchmesser von 6 mm und einer Höhe von 2 mm verwendet. Alle untersuchten Probekörper wurden der gleichen Oberflächenbehandlung unterzogen. In Anlehnung an die Herstellung von Zahnschienen, bei denen die Innenseite wegen eines sonst drohenden Passungsverlustes nicht nachbearbeitet wird, blieb eine Oberfläche unpoliert. Dies ist relevant, da gezeigt werden konnte, dass Polieren die Restmonomerelution verringert [167, 168].
Die Anzahl und Menge der eluierten Verbindungen hängen in hohem Maße vom Lösungsmittel ab; daher sollten die Lösungsmittel entsprechend den Anforderungen ausgewählt werden [46, 92]. In der vorliegenden Studie [4] wurden Wasser und Methanol als Lösungsmittel gewählt. Wasser wird als dem menschlichen Speichel und der Dentinflüssigkeit am ähnlichsten angesehen und daher als relevantes Lösungsmittel bezeichnet, um einen physiologischen Vergleich mit intraoralen Bedingungen zu ermöglichen [50, 51]. Organische Lösungsmittel wie Methanol haben eine wesentlich bessere Löslichkeit der organischen Matrixbestandteile und eine tiefere Matrixpenetration [7]. Der (unbeabsichtigte) Abbau von (Co)Monomeren durch Methanol ist viel geringer als bei anderen organischen Lösungsmitteln wie z.B. Aceton [53, 169]. Daher ermöglicht Methanol die Elution eines maximalen Anteils an unpolymerisierten Substanzen und spiegelt somit die „Worst-Case“-Situation der freigesetzten Verbindungen wider.

Da keine Daten über das Volumen oder die Oberfläche von Aufbissschienen vorlagen, haben wir eine Michigan-Schiene für Ober- und Unterkiefer entworfen und vermessen. Dies ermöglichte die Hochskalierung unserer Ergebnisse auf die durchschnittliche Schienengröße für ein „Worst-Case“-Szenario in Schienengröße. Die Elutionseigenschaften und der Mechanismus von Materialien auf Methacrylharzbasis hängen jedoch vom Molekulargewicht, der Hydrophobie, dem Füllstoffgehalt, dem untersuchten Material und den endgültigen Netzwerkeigenschaften der Harzmatrix ab [68, 75-77, 139]. Für die Berechnung einer „Worst-Case“-Situation wurde ein Splint/Proben-Faktor von 55,7 (Mittelwert aus Volumen- und Oberflächenfaktor im Vergleich zum untersuchten Probenkörper) verwendet, um die maximal mögliche Elution in physiologischer Schienengröße zu berechnen. Da ein Großteil der Mundhöhle mit HGFs ausgekleidet ist, kommen diese teilweise in direkten Kontakt mit dem

Schienenmaterial. Daher wurde im Folgenden ihre EC_{50}-Werte verwendet, um das zytotoxische Potenzial der berechneten Elutionsdaten in Schienengröße zu bewerten.

MMA als Hauptbestandteil von PMMA wurde in dem Lösungsmittel Methanol aller drei getesteten Materialien gefunden. In einer früheren Studie wurde der EC_{50}-Wert für MMA in HGFs mit 70 mmol/L bestimmt [170]. In den Methanol-Eluaten wurde die höchste MMA-Konzentration in SHERAORTHOMER mit 8768 µmol/L nach 72h gefunden. Hochgerechnet auf Schienendimension könnte MMA im ungünstigsten Fall nach 72h in Methanol eine Konzentration von 488378 µmol/L erreichen, was den oben genannten EC_{50}-Wert in HGFs um das Siebenfache übersteigen würde. Die zweithöchste MMA-Konzentration wurde in der SHERAeco-disc PM20 mit 3514 µmol/L nach 72h gefunden. Extrapoliert auf die Schienendimensionen könnte MMA in Konzentrationen von bis zu 195729 µmol/L innerhalb von 72h in Methanol eluieren, was die oben genannte EC_{50} in HGFs um einen Faktor von etwa 3 übersteigt. Die geringste MMA-Konzentration wurde in SHERAprint-ortho plus mit 70 µmol/L nach 72 h gefunden. Bezogen auf die Schienendimensionen in „Worst-Case“-Situation könnte die MMA-Konzentration in Methanol nach 72h 3899 µmol/L erreichen und läge damit etwa um den Faktor 18 unter zytotoxischen Konzentrationen. In den Wassereluaten aller drei untersuchten Materialien konnte jedoch kein MMA nachgewiesen werden. Da Wassereluate eine Annäherung an die Dentinflüssigkeit und den menschlichen Speichel darstellen [45, 51], sind keine zytotoxischen Effekte von MMA in humanphysiologischer Situation zu erwarten.
Tetrahydrofurfurylmethacrylat (THFMA) ist nur in den Herstellerangaben von SHERAprint-ortho plus aufgeführt und wurde auch nur in dessen Eluaten nachgewiesen. THFMA kann z.B. als Verdünner verwendet werden, um die Viskosität des Materials zu verringern [171]. In der vorliegenden Studie [4] wurde die Zytotoxizität von THFMA in HGFs mittels XTT-Viabilitätsassay mit einer EC_{50} von 3006 µmol/L bestimmt. Die höchste THFMA-Konzentration in der vorliegenden Studie [4] wurde in den Methanol-Eluaten von SHERAprint-ortho plus nach 72h mit einem Wert von 132 µmol/L gefunden. Extrapoliert auf Schienengröße würde die THFMA-Konzentration in Methanol innerhalb von 72h 7352 µmol/L betragen und damit die EC_{50} in HGFs um einen Faktor von etwa 2 übersteigen. Die höchste THFMA-Konzentration in den Wassereluaten von SHERAprint-ortho plus wurde jedoch nach 72h mit einem Wert von 7 µmol/L gemessen. Hochgerechnet auf die Schienendimensionen wäre die THFMA-Konzentration (416 µmol/L) etwa 7mal niedriger als die ermittelte zytotoxische Konzentration und 18 mal niedriger als die Konzentration der entsprechenden Methanol-Eluate. Aufgrund dieser Ergebnisse sind zytotoxische Wirkungen von eluiertem THFMA in humanphysiologischer Situation nicht zu erwarten.
HEMA wurde nur in den Methanol-Eluaten von SHERAprint-ortho plus gefunden. HEMA ist hydrophil, reduziert die Oberflächenspannung und wird deshalb oft als Primer-Komponente in Dentin-Bonding-Systemen eingesetzt [172]. HEMA wurde von den Herstellern nicht als

Bestandteil der untersuchten Materialien aufgeführt. Es wurde beschrieben, dass das langkettige UDMA bei der GC/MS-Analyse im Injektor teilweise zu HEMA diskriminiert [72, 173]. Darüber hinaus wird HEMA als eine mögliche Verunreinigung von Materialkomponenten auf Methacrylharzbasis beschrieben [44]. Daher ist die Quelle von HEMA in den Eluaten unbekannt. Ab einer Konzentration von 1 mmol/L induziert HEMA entzündliche Wirkungen und eine Störung des zellulären Redox-Gleichgewichts durch übermäßige Bildung reaktiver Sauerstoffspezies (ROS) [174]. Nach Urcan et al. [175] beträgt die EC_{50} von HEMA in HGFs 11 mmol/L. Die höchste HEMA-Konzentration, die in SHERAprint-ortho plus gefunden wurde, betrug 379 µmol/L nach 72h in Methanol. In einem „Worst-Case"-Szenario in Splintgröße könnte die HEMA-Elution 21110 µmol/L betragen, was den EC_{50}-Wert von Urcan et al. um etwa den Faktor 2 übertrifft. In den Wassereluaten konnte kein HEMA nachgewiesen werden, so dass in der humanphysiologischen Situation keine zytotoxischen Effekte zu erwarten sind.

1,4-Butylenglycoldimethacrylate (BDDMA) konnte nur im SHERAORTHOMER mit dem Lösungsmittel Methanol nachgewiesen werden. Nocca et al. [176] stellten fest, dass 0,4 mmol/L BDDMA die Zellproliferation um 80% verringert und die Zelltodrate in einer humanen Leukämie-Zelllinie HL-60 signifikant erhöht. In der vorliegenden Studie [4] ergab der XTT-basierte Viability-assay für BDDMA in HGFs eine EC_{50} von 2570 µmol/L. Die höchste BDDMA-Konzentration, die in SHERAORTHOMER gefunden wurde, betrug 161 µmol/L nach 72h. Hochgerechnet auf Splintgröße führt Methanol zu einer möglichen Elution von 8968 µmol/L innerhalb von 72h. Dies wäre etwa dreimal so hoch wie die bestimmte EC_{50} in HGFs. Da in allen Wasserproben keine Elution von BDDMA detektiert wurde, sind toxische Effekte in der humanphysiologischen Situation nicht zu erwarten.

Erstaunlicherweise konnte Tripropylenglycoldiacrylat (TPGDA), ein multifunktionelles Acrylat, das als Verdünnungsmittel und Vernetzungsmittel in ultraviolett härtenden Harzen fungiert und vom Hersteller als Bestandteil von SHERAprint-ortho plus aufgeführt wird nicht nachgewiesen werden. Dies könnte auf seine Vernetzungseigenschaften und eine hohe Umwandlungsrate zurückzuführen sein. Es wird von Björker als moderater Sensibilisator beschrieben [177]. Die EC_{50} von TPGDA in HGFs beträgt 597 µmol/l, was eine relative Toxizität von 5 gegenüber THFMA bedeutet. TPGDA ist also fünfmal so zytotoxisch wie THFMA.

Zusammenfassend eluierte das Lösungsmittel Methanol in Annäherung an mögliche zytotoxische Wirkungen in einem „Worst-Case"-Szenario in Splintgröße Verbindungen in Konzentrationen, die teilweise zytotoxische Konzentrationen in HGFs übersteigen. Diese Ergebnisse sollten nicht als besorgniserregend interpretiert werden, da Methanol nicht mit Speichel vergleichbar ist. Die Wassereluate, die einen guten Vergleich mit Speichel erlauben, zeigten für SHERAORTHOMER und SHERAeco-disc PM20 keine Elution der untersuchten Kompositinhaltsstoffen. Allerdings eluierte SHERAprint-ortho plus das Methacrylat THFMA in

Konzentrationen, die etwa 7-mal unter der bestimmten EC_{50} in HGFs lagen. Es kann daher davon ausgegangen werden, dass unter normalen physiologischen intraoralen Bedingungen von keinem der getesteten Schienenmaterialien unerwünschte zytotoxische Effekte zu erwarten sind. Rothmund et al. ermittelten eine signifikant geringere (Co)monomerfreisetzung in nativem Speichel im Vergleich zu proteinfreiem Speichel und Wasser [45]. Eine Bindung von (Co)Monomeren und Additiven an Speichelproteine könnte eine geringere Bioverfügbarkeit und damit ein geringeres zytotoxisches Potential in vivo bewirken [45]. Darüber hinaus wurde gezeigt, dass große Unterschiede in der MMA-Konzentration zwischen dem gesamten Speichel (40µg/ml) und dem Speichelfilm auf Gaumenapparaturen (180µg/ml) auftreten können [178]. Folglich könnten sich die Substanzen in der physiologischen Situation vor allem an unpolierten inneren Passflächen anreichern, wo die Speichelflussrate gering ist. Daher könnte die Konzentration an den spezifischen Ort gebunden sein, was zu einem höheren zytotoxischen Potenzial führt.

Außergewöhnlich ist auch die deutlich höhere Anzahl verschiedener Methacrylat-Typen, die in den Eluaten von SHERAprint-ortho plus (fünf) im Vergleich zu SHERAORTHOMER (zwei) und SHERAeco-disc PM20 (einer) nachgewiesen werden konnten. Die Materialzusammensetzung des Additivmaterials SHERAprint-ortho plus könnte aufgrund höherer Materialanforderungen in der Verarbeitungskette hinsichtlich der Druckbarkeit oder der Tatsache, dass es in vorgemischter Form bereitgestellt wird, komplexer sein. Es ist wichtig zu beachten, dass sich die Zytotoxizität einer einzelnen Substanz bei einer Mehrkomponenten-Exposition nicht unbedingt aufsummiert. Komplexe Wechselwirkungen und antagonistische Effekte zwischen verschiedenen Verbindungen können manchmal sogar zu einer geringeren Zytotoxizität bei Mehrkomponenten-Exposition führen [26, 179, 180]. Außerdem könnten orale Bakterien einen Einfluss auf die Zytotoxizität von (Co)Monomeren haben. Bei der Co-Kultivierung von HGFs mit Streptococcus mitis-Stämmen und HEMA wurde ein Kreuzschutz mit einem positiven Effekt, nämlich dem Rückgang des Zelltods von HGFs beobachtet [181]. Dennoch sind zahlreiche (Meth)acrylate und Additive starke Sensibilisatoren und können unerwünschte Wirkungen wie die Auslösung und Bildung von Allergien und Kreuzreaktionen verursachen [182, 183]. Ist eine Allergie bereits bekannt und durch einen Patch-Test bestätigt, sollte besonders auf die Verwendung geeigneter Materialien geachtet werden.

Schlussfolgerung

Derzeit sind industriell vorgefertigte Polymerrohlinge für die subtraktive Fertigung (SHERAeco-disc PM20) in Bezug auf die Biokompatibilität überlegen. Neu entwickelte Werkstoffe für die additive Fertigung scheinen eine komplexere Zusammensetzung aufzuweisen und damit das Potenzial für eine Elution einer größeren Anzahl von (Co)Monomeren und Additiven zu haben. Die freigesetzten Inhaltsstoffe im Elutionsmittel Methanol aus den untersuchten

Schienenmaterialien überstiegen die zytotoxischen Konzentrationen in HGFs, die für ein „Worst-Case“-Szenario in der physiologischen Schienengröße berechnet wurden. In den Wassereluaten konnte dagegen nur das Methacrylat THFMA aus SHERAprint-ortho plus in Konzentrationen unterhalb zytotoxischer Konzentration in HGFs bestimmt werden. Daher ist das Gesundheitsrisiko in der physiologischen (Wasser/Speichel) Situation von geringer Bedeutung.

3.2. Toxikologie der freigesetzten Inhaltsstoffe aus dentalen Kompositen

3.2.1. Einfluss von Antioxidantien auf Comonomer-Epoxy-Metaboliten-induzierte DNA-Doppelstrangbrüche in humanen Gingivafibroblasten [48]

Die unpolymerisierten (Co)Monomere TEGDMA und HEMA können aus den unvollständig polymerisierten Kompositmaterialien freigesetzt werden [1] und dadurch die Aktivität von Pulpa-Zellen beeinträchtigen oder durch Verschlucken in den Darm gelangen und anschließend den Blutkreislauf und die Organe erreichen [1, 15]. In unseren früheren Studien wurde die Aufnahme, Verteilung und Ausscheidung von radiomarkiertem ^{14}C-TEGDMA und ^{14}C-HEMA bei Meerschweinchen untersucht [13, 39]. Daraufhin wurde der Metabolismus von TEGDMA und HEMA, und die Bildung von Methacrylsäure (MA), einem Metabolisierungszwischenprodukt von TEGDMA und HEMA, beschrieben [13, 39-41]. MA kann über zwei verschiedene Wege metabolisiert werden [40]. So zeigten frühere Studien mit ^{14}C-TEGDMA und ^{14}C-HEMA in A549-Zellen, dass diese hauptsächlich über den sogenannten Epoxyweg metabolisiert werden [41]. In diesem Metabolismus (Epoxyweg) kann die C-C-Doppelbindung von MA oxidiert werden, wodurch der Epoxy-Metabolit 2,3-Epoxy-2-methylpropionsäure (EMPA) gebildet werden kann, der sodann in humanen oralen Zellen (z.B. HGFs und HPFs) nachgewiesen werden konnte [29, 42, 43]. An diesem Prozess ist einerseits Wasserstoffperoxid als chemischer Katalysator beteiligt [184] und andererseits spielt das Cytochrom P450 2E1 (CYP2E1) eine wichtige Rolle [42]. Außerdem ist sehr wahrscheinlich, dass in vivo 2,3-Epoxy-2-methyl-propionsäuremethylester (EMPME) gebildet werden kann [29].

In einer früheren Studie wurde die Toxikologie von EMPME und EMPA mit Hilfe eines modifizierten Fluoreszenz-Stammzelltests untersucht; als Ergebnis wurde für EMPA eine teratogene Wirkung und für EMPME eine embryotoxische Wirkung auf die embryonalen Stammzellen von Mäusen festgestellt [29]. Eine ähnliche Genotoxizität von Epoxiden wurde auch bei Glycidamid festgestellt, dem Epoxy-Metaboliten von Acrylamid, der häufig in frittierten Lebensmitteln vorkommt [185]. Glycidamid kann durch die Bildung kovalenter Addukte an der N7-Position von Guanin, der N3-Position von Adenin und der N1-Position von Desoxyadenosin sehr reaktiv gegenüber der DNA sein [186]. Da das Glycidamid eine ähnliche Epoxidstruktur wie EMPME und EMPA aufweist, ist es wahrscheinlich, dass diese zu einer ähnlichen Gentoxizität führen könnten. DNA-Schäden können zu karzinogenen und mutagenen Wirkungen führen [187]. Epoxide gelten als hochreaktive Moleküle und toxische Stoffe [43]. Werden DNA-Schäden nicht repariert, können sie zum Zelltod führen; werden sie falsch repariert, kann es zu chromosomalen Translokationen und genomischer Instabilität kommen [188].

Viele Studien haben sich mit der Toxikologie von (Co)Monomeren (z.B. TEGDMA und HEMA), die DNA-Doppelstrangbrüchen (DNA-DSBs) induzieren können befasst [175, 189]. Schweikl et al. wiesen nach, dass HEMA-induzierte Apoptose eine Reaktion auf DNA-Schäden ist [190]. Des Weiteren konnten Studien zeigen, dass die Zugabe von Antioxidantien wie Asc bzw. NAC die zytotoxische Wirkung und DNA-DSBs von Kompositinhaltsstoffen ((Co)Monomeren) reduzieren kann [36-38]. Ob die Comonomer-Epoxy-Metaboliten im Vergleich zu ihren metabolischen Vorläufern TEGDMA, HEMA und dem Zwischenprodukt MA mehr DNA-DSBs induzieren können, und ob Antioxidantien zur Verringerung von DNA-DSBs in Gegenwart von Comonomer-Epoxy-Metaboliten führen können, war vor der Durchführung der vorliegenden Studie „Einfluss von Antioxidantien auf Comonomer-Epoxy-Metaboliten-induzierte DNA-Doppelstrangbrüche in humanen Gingivafibroblasten“ [48] jedoch noch unbekannt. In der vorliegenden Studie [48] wurde daher der Einfluss der Comonomer-Epoxy-Metaboliten EMPME und EMPA auf DNA-DSBs in humanen Gingivafibroblasten und der Einfluss der Antioxidantien Asc und NAC auf die epoxidinduzierten DNA-DSBs untersucht.

In der vorliegenden Studie [48] wurde eine 9-mal höhere relative Toxizität von EMPA gegenüber MA, und eine 6-mal höhere von EMPME gegenüber MA gemessen. Zusätzlich zur Bestimmung der Zytotoxizität wurde, um die Genotoxizität von MA, EMPME und EMPA zu untersuchen ein γ-H2AX-Test durchgeführt. Die Epoxidverbindungen EMPME und EMPA verursachten im Vergleich zu MA eine höhere Anzahl an DNA-DSBs. Eine Erklärung dafür könnte sein, dass die Epoxy-Metabolite EMPME und EMPA hochreaktive und instabile Moleküle sind und daher eine höhere Toxizität aufweisen. Es wurde gezeigt, dass MA eine Schlüsselrolle im DNA-Zellbindungsassay spielen kann [191], und daher ist es sehr wahrscheinlich, dass die Epoxy-Metaboliten von MA stammend in der Lage sind, im Vergleich zu MA selbst höhere toxische Wirkungen, wie DNA-DSBs, zu induzieren. Im Gegensatz zu EMPME wurden in der vorliegenden Studie für EMPA eine höhere Zytotoxizität und eine höhere Anzahl an DNA-DSBs beobachtet. Es wurde beschrieben, dass Säuren den pH-Wert des Zellmediums senken können, was zu einer erhöhten Zytotoxizität und DNA-Schäden in vielen Zelllinien führt [192, 193]. Daher könnte in der vorliegenden Studie EMPA (Carbonsäure) ebenfalls den pH-Wert senken, was zu einer höheren Zytotoxizität und einer stärkeren Induktion von DSBs führt.
In einer früheren Studie wurden die durch TEGDMA (EC_{50}=3,6 mM, 1/3EC50=1,2 mM) und HEMA (EC_{50}= 11,2mM, 1/3EC_{50}=3,7 mM) induzierten DNA-DSBs untersucht [175]. Im Vergleich zu diesen früheren Ergebnissen wurden in der vorliegenden Studie höhere Raten von DSBs-Foci für EMPME bei 2,58 mM (EC_{50}), und EMPA bei 1,72 mM (EC_{50}) und 0,57 mM (1/3EC_{50}) gefunden. Diese Daten deuten darauf hin, dass Epoxy-Metabolite selbst bei niedrigeren Konzentrationen schwerwiegendere DNA-Schäden verursachen können als ihre

metabolischen Vorläufer TEGDMA und HEMA. In diesem Zusammenhang stellt sich folgende interessante Frage: Wird die DNA-Schädigung wirklich durch die Comonomere TEGDMA und HEMA verursacht oder wird sie tatsächlich durch ihre Epoxy-Metabolite EMPME und EMPA ausgelöst. Es wurde nachgewiesen, dass die durch Acrylamid (z.B. in frittierten Lebensmitteln) [185] induzierten DNA-Schäden durch dessen Epoxy-Metabolit Glycidamid [194] ausgelöst werden können. Aufgrund der strukturellen Ähnlichkeit von EMPME und EMPA mit Glycidamid ist es sehr wahrscheinlich, dass bei der Inkubation von HGFs mit den Comonomeren TEGDMA und HEMA die daraus gebildeten Epoxy-Metabolite EMPME und EMPA an der Induktion von DNA-DSBs beteiligt sind.

Ferner wurde in der vorliegenden Studie [48] der Einfluss der Antioxidantien Asc bzw. NAC auf die durch MA und dessen Epoxy-Metabolite induzierten DNA-DSBs in HGFs untersucht. Die vorliegenden Daten zeigten, dass die Zugabe von Asc bzw. NAC bei der Exposition von HGFs mit MA (15,64 und 5,21 mM), EMPA (1,72 und 0,57 mM) und EMPME (2,58 mM) die Anzahl der DSBs-Foci/Zelle signifikant verringern konnte. Diese Ergebnisse stehen im Einklang mit früheren Studien, die nach Zugabe von Asc bzw. NAC eine Reduktion der zytotoxischen Wirkung von Comonomeren und der durch Comonomere induzierten DNA-DSBs feststellten [36-38, 195]. Antioxidantien gelten als Radikalfänger, können also Radikale binden und somit die Toxizität verringern [196]. Die Zugabe von Asc bzw. NAC zum Zellkulturmedium kann die Zytotoxizität [37, 38, 197] verringern und NAC kann die DNA-Deletionen in ATM-defizienten Mäusen reduzieren [198]. Schließlich können oral verabreichte Antioxidantien, einschließlich Asc und NAC, durch ionisierende Strahlung induzierte DSBs vermindern [199].

In der vorliegenden Studie [48] wurden ebenso die Genotoxizität in HGFs der verwendeten Antioxidation Asc (50; 100; 200; 500 µM) und NAC (50; 100; 200; 500 µM) untersucht. Die höchste getestete Asc-Konzentration (500 µM) induzierte im Vergleich zur Negativkontrolle signifikant mehr DSBs-Foci. Eine Erklärung für dieses Phänomen könnte mit der Fähigkeit von Asc bei höheren Konzentrationen verbunden sein reaktive Sauerstoffspezies (ROS) und oxidativen Stress zu induzieren [200]. Wie von Chen et al. beschrieben [201], ist in der vorliegenden Studie auch eine durch Asc (500 µM) induzierte Bildung von H_2O_2 möglich, welche zu einem Anstieg der DNA-DSBs in HGFs geführt haben könnte. Umgekehrt wurde die antigenotoxische Rolle von Asc nachgewiesen [38, 202] und gezeigt, dass die Anwesenheit von Asc die Bildung von DNA-Addukten verhindern kann [203]. Dies stimmt mit der vorliegenden Studie [48] für die Asc Konzentrationen 50-200 µM überein, die eine schützende Wirkung auf HGFs durch Reduktion der DNA-DSBs zeigten. Der schützende Mechanismus, der durch Asc in einer niedrigeren Konzentration reguliert wird, ist daher dominanter als die durch das Xenobiotikum und Asc selbst verursachte Toxizität.

Im Gegensatz zu Asc zeigte NAC im Vergleich zur Negativkontrolle keine signifikante Induktion von DNA-DSBs, selbst bei der höchsten Konzentration von 500 µM. NAC ist als thiol-haltiges Antioxidans bekannt und schützt zelluläre Komponenten, indem es durch ROS verursachte Schäden verringert [198]. Frühere Studien haben gezeigt, dass NAC die Zytotoxizität und Genotoxizität von dentalen (Co)Monomeren auf Methacrylatbasis reduzieren kann [36, 38]. Obwohl der Mechanismus der Senkung der Genotoxizität von Epoxiden durch NAC noch nicht bekannt ist, wird davon ausgegangen, dass ein Schutzmechanismus im Zusammenhang mit der Glutathion (GSH)-Synthese eine Schlüsselrolle bei diesem Prozess spielt. So wurde berichtet, dass eine Verringerung des interzellulären GSH-Spiegels mit einer erhöhten ROS-Bildung einhergeht, wenn Zellen (Co)Monomeren ausgesetzt waren [104]. Außerdem erhöhte die Zugabe von NAC zu Glycidamid den GSH-Level in Hepatozyten [196]. In der vorliegenden Studie [48], in der HGFs EMPME (2,58 mM) und EMPA (1,72 und 0,57 mM) in Gegenwart von NAC (50-500 µM) ausgesetzt wurden, war die Anzahl der DSBs-Foci/Zelle signifikant reduziert. Diese Schutzwirkung von NAC auf Epoxid-induzierte DSBs stimmt mit einer früheren Studie überein, die zeigte, dass NAC eine schützende Rolle gegenüber Acrylamid-induzierten DNA-Schäden spielen kann, was möglicherweise auf die Verringerung der Genotoxizität dessen Epoxy-Metaboliten Glycidamid zurückzuführen ist [204]. Epoxid-induzierte DNA-Addukte wurden bereits nachgewiesen [186], wobei die Zugabe von NAC die Bildung von DNA-Addukten reduzieren kann [198]; die Ursache für die Bildung von EMPME- und EMPA-induzierten Addukten ist jedoch noch unklar, was in weiteren Studien untersucht werden sollte.

Die Ergebnisse der vorliegenden Studie [48] zeigen, dass NAC (50-500 µM) im Vergleich zu Asc (50-200 µM) zu einer stärkeren Verringerung der DSBs-Foci führt. Dies könnte durch die Bildung endogener ROS erklärt werden, die durch Asc ausgelöst werden und zu einer Verringerung des GSH-Spiegels führen [205], während der gegenteilige Effekt, der durch NAC verursacht wird, den GSH-Spiegel erhöht, was die DNA vor oxidativen Schäden und der Bildung von DNA-Addukten schützt [186, 198]. NAC sollte daher als ein dem Asc vorzuziehendes Antioxidans in Erwägung gezogen werden, wenn es um DNA-Schäden geht, die durch dentale (Co)Monomere sowie durch deren Metaboliten verursacht werden.

Des Weiteren wurde berichtet, dass Epoxide Mikronuklei (MN) induzieren können, die eng mit DNA-DSBs oder nicht reparierten DNA-Brüchen in Verbindung stehen [206, 207]. Demnach konnten auch in der vorliegenden Studie [48] MN bei 2,58 mM EMPME beobachtet werden; Diese MN deuten auf eine Genmutation hin, die nach Exposition der HGFs mit EMPME stattgefunden haben könnte.

Die in der vorliegenden Studie [48] aufgetretene Genotoxizität der Epoxide macht eine grobe Risikobewertung erforderlich. Ausgehend von einem „Worst-Case“-Szenario, bei dem 32 Zähne mit einem TEGDMA-haltigem Komposit gefüllt werden und gemäß der durchschnittlichen Volumenschätzung einiger typischer Restaurationen [88] ist von maximal

0,2 g Komposit pro Zahn auszugehen. Der durchschnittliche Anteil von TEGDMA in einem Komposit beträgt etwa 10%, und maximal 10% des TEGDMA werden tatsächlich in einem Methanol-Wasser-Gemisch innerhalb von 96h freigesetzt [99]. Theoretisch kann also nach der von Seiss et al. [43] beschriebenen Berechnungsmethode eine Menge von 38,4 mg MA gebildet werden. Geht man von einer täglichen Speichelproduktion von ca. 1L aus [208], und einer Umwandlungsrate von MA in EMPA von ca. 5% [43], so ergäbe sich eine Konzentration von 60 µM EMPA. Dieser Wert liegt weit unter der niedrigsten Konzentration ($1/3EC_{50}$) von EMPA, die in der vorliegenden Studie eine signifikant höhere Anzahl an DNA-DSBs auslöste. Diese Daten, die aus einem Elutionsexperiment in einer menschlichen „Worst-Case"-Situation berechnet wurden, sollten jedoch nicht beunruhigen, da Speichel bei der Elution von unpolymerisiertem (Co)monomer weniger effektiv ist als eine Methanollösung [45]. Darüber hinaus ist es klinisch unrealistisch, 32 Zähne gleichzeitig mit je 0,2 g zu füllen; daher kann das eluierte TEGDMA keine so hohe Konzentration erreichen, so dass die Menge der in biologischen Systemen gebildeten Epoxide viel niedriger ist als im „Worst-Case"-Szenario berechnet wurde.

Schlussfolgerung

Die Comonomer-Epoxy-Metaboliten EMPME und EMPA weisen im Vergleich zu ihrem metabolischen Vorläufer, MA eine höhere Zytotoxizität auf und induzieren mehr DNA-DSBs. Die Antioxidantien Asc bzw. NAC können die Anzahl der durch EMPME und EMPA induzierten DNA-DSBs-Foci in HGFs verringern. NAC weist im Vergleich zu Asc eine bessere Schutzwirkung auf.

3.2.2. Zytotoxizität und Induktion von DNA-Doppelstrangbrüche in humanen Gingivafibroblasten bei Exposition mit Eluaten aus dentalen Kompositen [26]

Geurtsen et al. bestimmten die Zytotoxizitäten von insgesamt 35 Kompositmonomeren und -additiven in humanen Fibroblasten [81]. Des Weiteren wurde von einer durch freigesetzte (Co)Monomere induzierten Mutagenität, Embryotoxizität und Teratogenität berichtet [29]. TEGDMA und HEMA können zu der Epoxyverbindung 2,3-Epoxy-2-methylpropionsäure (EMPA) metabolisiert werden [43]. Ebenso wurde die Bildung eines weiteren Epoxids, 2,3-Epoxy-2-methyl-propionsäuremethylester (EMPME) postuliert [29]. Die Bildung dieser Epoxide konnte in humanen oralen Zellen (z.B. HGFs und HPFs) nachgewiesen werden [42]. EMPME und EMPA zeigen nicht nur Zytotoxizität-Effekte, sondern induzieren auch höhere Raten von DNA-Doppelstrangbrüchen (DNA-DSBs) in HGFs im Vergleich zu ihren

metabolischen Vorläufern TEGDMA und HEMA [48, 175]. DNA-DSBs gelten als die toxischste Form von DNA-Läsionen [187].

Bisherige Studien über die durch dentale Kompositmaterialien induzierte Zytotoxizität und DNA-DSBs befassten sich seither ausschließlich mit den Auswirkungen einzelner Kompositinhaltsstoffe [36, 175, 187]. Vor der Durchführung der vorliegenden Studie „Zytotoxizität und Induktion von DNA-Doppelstrangbrüchen in humanen Gingivafibroblasten bei Exposition mit Eluaten aus dentalen Kompositen“ [26] lagen für Komposite-Eluate, die aus mehreren Inhaltsstoffen bestehen, jedoch wenige Daten zur Zytotoxizität und keine Daten zur Induktion von DSBs vor. Experimente mit Komposit-Eluaten, deren Inhaltsstoffe durch vorherige qualitative und quantitative Analyse ermittelt werden, spiegeln möglicherweise eine Situation wider, die der Physiologie näher kommt als Versuche mit einzelnen Kompositkomponenten/-inhaltsstoffen. In der vorliegenden Studie [26] wurde daher die durch dentale Komposit-Eluate resultierende Zytotoxizität und Induktion von DNA-DSBs in HGFs untersucht. Die aus den untersuchten Kompositen freigesetzten Inhaltsstoffe wurden mittels GC/MS qualifiziert und quantifiziert.

Zur Bestimmung der Zytotoxizität und Gentoxizität der Eluate aus den Kompositmaterialien EsthetX HD, Venus, X-tra fil, CLEARFIL AP-X, Admira Fusion und QuiXfil wurden XTT- und γ-H2AX-Tests durchgeführt. Normalerweise werden Wasser, Speichel, Ethanol, Methanol usw. für die Elution von dentalen Kompositen verwendet [45, 53, 209, 210]. Studien zeigten, dass vor allem Dulbecco’s modified Eagle’s medium (DMEM) ein mit Speichel vergleichbares Elutionsmedium und repräsentativ für die orale Umgebung ist [53, 211]. DMEM als Elutionsmedium in Verbindung mit der Qualifizierung und Quantifizierung der Zusammensetzung der Komposit-Eluate könnte daher eine physiologischere Situation abbilden als bei Experimenten mit einzelnen Kompositinhaltsstoffen. In der vorliegenden Studie wurden die freigesetzten Kompositinhaltsstoffe im Zellmedium DMEM qualifiziert und quantifiziert, um deren Relevanz als multiple Zusammensetzung aus den freigesetzten Kompositinhaltsstoffen im Eluat für die HGF-Inkubation im XTT- und γ-H2AX-Assays bestmöglich bewerten zu können.

Komposit-Additive wurden in allen Eluaten der untersuchten Komposite nachgewiesen. In einer früheren Studie wurde BHT (EC_{50}: 170 µM) als das zytotoxischste Additiv unter den getesteten Initiatoren, Koinitiatoren, Inhibitoren und Photostabilisatoren ermittelt [81]. In der vorliegenden Studie [26] wurde die höchste BHT-Konzentration für QuiXfil gefunden (1 µM), was mehr als 100-mal niedriger ist als die oben genannte zytotoxische Konzentration von BHT [81]. Der Photoinitiator CQ wird als Allergen angesehen [82] und wurde in allen untersuchten Eluaten nachgewiesen. Es wurde gezeigt, dass CQ bei Konzentrationen >50 µM in HGFs DNA-Schäden induziert und intrazelluläre ROS erhöht [83]. In der vorliegenden Studie wurde

die höchste CQ-Konzentration (9,7 µM) im Eluat von Venus gefunden. Dieser Wert ist fünfmal niedriger als die oben genannte toxische Konzentration von CQ [83]. DMABEE kann die Apoptose und Nekrose von Zellen auslösen [106]. Die höchste DMABEE-Konzentration (55 µM) in der vorliegenden Studie wurde bei QuiXfil gemessen. Dieser Wert ist 22-mal niedriger als die zytotoxische Konzentration von 1,2 mM, die in HGFs beschrieben wurde [81]. Zusammenfassend waren also die Konzentrationen der oben genannten Komposit-Additive in Bezug auf ihre Toxizität als Einzelkomponenten stets niedriger als die entsprechenden toxischen Konzentrationen aus früheren Studien [81, 83]. Bezüglich der Toxizität der untersuchten Eluate in multipler Zusammensetzung aus freigesetzten Kompositinhaltsstoffen zeigten alle Eluate in der vorliegenden Studie [26] keine Zytotoxizität. Daher gibt es keine Hinweise darauf, dass Komposit-Additive die Zytotoxizität in Komposit-Eluaten, die mehrere Inhaltsstoffe enthalten erhöhen.

HEMA wurde in den Eluaten von Esthet.X HD, X-tra fil und QuiXfil nachgewiesen. HEMA ist jedoch in den Herstellerangaben nicht aufgeführt. HEMA wurde im GC/MS-Analyseverfahren als Diskriminierungsprodukt von Urethandimethacrylat (UDMA) beschrieben [72], aber auch Verunreinigungen von Einzelkompositkomponenten (z.B. UDMA) sind möglich [44]. Daher ist der Ursprung von HEMA unbekannt. Es wurde gezeigt, dass die HEMA-induzierte Apoptose eine Reaktion auf DNA-Schäden ist [190]. In der vorliegenden Studie [26] wurde die höchste HEMA-Konzentration für QuiXfil mit 110 µM gemessen. Frühere Studien zeigten eine zytotoxische Konzentration von 2,4 mM [81] und eine genotoxische Konzentration von 1,1 mM für HEMA in HGFs [175]. Zusammenfassend lässt sich sagen, dass die in der vorliegenden Studie festgestellten Konzentrationen von HEMA hinsichtlich der Toxizität als Einzelkomponenten weit unter den zitierten zytotoxischen und genotoxischen Konzentrationen lagen [81, 175]. Hinsichtlich der Toxizität von Eluaten in multipler Zusammensetzung aus freigesetzten Kompositinhaltsstoffen steht dies im Einklang mit den Zytotoxizitätsergebnissen der untersuchten HEMA-haltigen Eluate (Esthet.X HD, X-tra fil und QuiXfil). Von den untersuchten HEMA-haltigen Eluaten induzierte jedoch nur Esthet.X HD mit der niedrigsten HEMA-Konzentration (2,5 µM) ein signifikant höhere Anzahl von DSBs im Vergleich zur Kontrolle, während QuiXfil mit einer 44-fach höheren gemessenen HEMA-Konzentration keine signifikante Induktion von DSBs-Foci zeigte. Es gibt also keine Hinweise darauf, dass HEMA die Zytotoxizität und die DNA-DSBs in multipel-zusammengesetzten Eluaten erhöht.

In der vorliegenden Studie [26] wurde die höchste Konzentration von TEGDMA in den Eluaten von Venus und Esthet.X HD gefunden (1080 µM und 1019 µM). Dies stimmt mit einer früheren Studie überein, in der eine Konzentration von 1448 µM TEGDMA für Esthet.X nach 24-stündiger Elution in DMEM nachgewiesen wurde [211]. Unsere frühere Studie zeigte, dass eine Exposition mit TEGDMA als Einzelkomponente bei Konzentrationen von 1200 µM (1/3 EC_{50}) und 360 µM (1/10 EC_{50}) eine 7- bzw. 4-fach höhere Anzahl von DSBs-Foci im Vergleich

zur Kontrolle induziert [175]. In der vorliegenden Studie [26] induzierten die in den Eluaten gemessenen TEGDMA-Konzentrationen von Venus und Esthet.X HD (entsprechen in etwa 1/3 EC_{50} der vorherigen Studie [175]) jedoch nur eine 2-fach höhere Anzahl von DSBs-Foci im Vergleich zur Kontrolle. Außerdem wurde bei der Exposition mit den Eluaten von X-tra fil und CLEARFIL AP-X keine signifikante DNA-DSBs-Induktion beobachtet, obwohl die in den Eluaten gemessenen Konzentrationen von TEGDMA (494 µM und 479 µM) höher sind als 360 µM (1/10 EC_{50}), die in der Einzelkomponenten Inkubation eine signifikant höhere Anzahl von DSBs-Foci induzierte [175]. Zusammenfassend spielen offensichtlich die Konzentrationen von TEGDMA eine dominante Rolle bei der Induktion von DNA-DSBs in den untersuchten Komposit-Eluaten, wobei die multipel-zusammengesetzten Eluate insgesamt geringere Raten von DSBs im Vergleich zur Einzelkomponentenexposition mit TEGDMA induzierten [175]. Die geringeren DSB-Raten könnten auf die Zugabe von 10 % Fetalem Kälberserum (FKS) zu DMEM während der XTT- und γ-H2AX-Tests zurückzuführen sein, da FKS (Co)Monomeren und Additive durch Proteinbindung abfangen kann [45, 211] und so weniger (Co)Monomere und Additive zur Induktion von DNA-DSBs zur Verfügung stehen.

Darüber hinaus können auch interaktive Effekte zwischen den freigesetzten Inhaltsstoffen in den Eluaten die Toxizität verringern. So wurde beschrieben, dass ein interaktiver Effekt zwischen Kompositinhaltsstoffen in bestimmten Konzentrationen und unter bestimmten Zeitbedingungen auftritt [180]. Ratanasathien et al. wiesen nach, dass ein antagonistischer Effekt nach 24h eine dominante Rolle spielt, wenn Maus Fibroblasten einer Mischung aus zwei verschiedenen dentalen (Co)Monomeren ausgesetzt sind [179]. Daher kann davon ausgegangen werden, dass bei der Exposition von HGFs gegenüber den Eluaten von Esthet.X HD, Venus, X-tra fil und CLEARFIL AP-X, ein antagonistischer Effekt zwischen den freigesetzten Kompositinhaltsstoffen auftritt und folglich sich die Rate der DNA-DSBs im Vergleich zu einer Einzelkomponenten Exposition mit TEGDMA reduzieren.

Abschließend ist anzumerken, dass die signifikant höhere Anzahl von DNA-DSBs, die durch die Eluate von Esthet.X HD und Venus induziert werden, jedoch nicht beunruhigen sollte. In der vorliegenden Studie [26] wurde ein „Worst-Case“-Szenario für die maximale Freisetzung von Inhaltsstoffen mit einer Probenoberfläche von 220 mm^2 und einer Sauerstoffinhibitionsschicht, basierend auf früheren Studien [87, 212, 213] erstellt. Die Oberfläche der untersuchten Proben ist viermal größer als die von typischen Restaurationen (52 mm^2) [212]. Eine größere Oberfläche der Probe kann die Freisetzung von Inhaltsstoffen erhöhen [87]. Außerdem trägt das Vorhandensein einer Sauerstoffinhibitionsschicht ebenfalls zu einer erhöhten Freisetzung von Komponenten bei [87]. In der klinischen Praxis ist die exponierte Oberfläche jedoch begrenzt und die Sauerstoffinhibitionsschicht wird durch Schleifen und Polieren entfernt [214]. Außerdem muss berücksichtigt werden, dass in der physiologischen Situation die freigesetzten Inhaltsstoffe auch durch Speichelproteinbindung

reduziert werden können [45, 211]. Darüber hinaus können interaktive Effekte zwischen den freigesetzten Kompositinhaltsstoffen die Toxizität beeinflussen. Dies ist vor allem im Hinblick auf die Sicherheit und die potenziellen Gefahren nach der Restauration mit Zahnkunststoffen von großer Bedeutung.

Schlussfolgerung

Die Exposition von HGFs mit den Eluaten von Esthet.X HD und Venus zeigten eine signifikante Induktion von DNA-DSBs. In allen untersuchten Eluaten wurde keine signifikante Zytotoxizität festgestellt. Interaktive Effekte zwischen den freigesetzten (Co)Monomeren und Additiven können die Zytotoxizität und die Induktion von DNA-DSBs im Vergleich zur Exposition mit Einzelkomponenten positiv (abschwächend) beeinflussen.

3.2.3. Einfluss von Antioxidantien, als neue Komponente in dentalen Kompositen auf die Freisetzung von Inhaltsstoffen und den Polymerisationsgrad [49]

Die Polymerisation von lichtgehärteten Kompositmaterialien ist unvollständig und (Co)Monomere und Additive können freigesetzt werden [210]. Frühere Studien haben gezeigt, dass die (Co)Monomere TEGDMA und HEMA zu dem Zwischenprodukt Methacrylsäure (MA) und weiter zu den Epoxy-Metaboliten 2,3-Epoxy-2-methyl-propionsäure-methylester (EMPME) und 2,3-Epoxy-2-methylpropionsäure (EMPA) metabolisiert werden können [13, 39, 42, 43]. Diese Epoxyverbindungen gelten als hochreaktive Moleküle und werden als mutagene und karzinogene Stoffe angesehen [15, 29, 43, 109, 215]. Dementsprechend wurden für EMPA und EMPME teratogene/embryotoxische Wirkungen in den embryonalen Stammzellen von Mäusen beobachtet [29]. Darüber hinaus wurde berichtet, dass EMPME und EMPA bei HGFs eine höhere Zytotoxizität und höhere Raten von DNA-Doppelstrangbrüchen (DNA-DSBs) als ihre metabolischen Vorläufer TEGDMA oder HEMA hervorrufen können [48, 175]. DNA-DSBs können zu karzinogenen und mutagenen Wirkungen führen [187].

Die Antioxidantien Asc und NAC gelten als Radikalfänger [196] und können die durch TEGDMA und HEMA induzierte Zytotoxizität verringern [38, 216]. Des Weiteren kann die Zugabe von Asc bzw. NAC in vitro die Genotoxizität dentaler (Co)Monomere und ihrer Epoxymetaboliten EMPA und EMPME verringern [36, 48, 217]. Oral verabreichte Mischungen von Antioxidantien, einschließlich Asc und NAC, können durch ionisierende Strahlung induzierte DSBs reduzieren [199]. Eine Studie über die Einmischung von NAC in ein autopolymerisierendes Poly-Methyl-Methacrylat (PMMA)-basierendes Komposit führte zu einer Verringerung des Polymerisationsgrades (DC) im Vergleich zum unbehandelten PMMA [218].

Vor der Durchführung der vorliegenden Studie „Einfluss von Antioxidantien, als neue Komponente in dentalen Kompositen auf die Freisetzung von Inhaltsstoffen und den Polymerisationsgrad“ [49] lagen keine Daten über die Auswirkungen auf den DC und die Elution von Kompositinhaltsstoffen nach der experimentellen Einmischung von Asc bzw. NAC in lichthärtende Methacrylat-basierende Komposite vor. Daher wurden in der vorliegenden Studie [49] die Antioxidantien Asc bzw. NAC (1, 0,1 und 0,01 Gew.-%) als neue/zusätzliche Kompositkomponente in den drei lichthärtende Kompositmaterialien Venus, Grandio und Filtek supreme XTE hinsichtlich ihrer Auswirkungen auf den DC und die Elution von Kompositinhaltsstoffen mittels GC/MS untersucht. Zusätzlich wurde die Freisetzung von Asc bzw. NAC mittels „high-performance liquid chromatography/diode array detection“ (HPLC/DAD) und „high-performance liquid chromatography/fluorescence detection“ (HPLC/FLD) in diesen neuen Mischungen bestimmt.

Die untersuchten Kompositmaterialien wurden ausgewählt, weil unsere früheren Studien verschiedene Zusammensetzungen und hohe Freisetzungen von Methacrylaten und Additiven gezeigt haben [26, 46, 51]. Von allen untersuchten Kompositen konnten nur die Venus-Proben nach der experimentellen Einmischung von 1 Gew.-% NAC nicht untersucht werden, da die Zugabe von 1 Gew.-% NAC zu einer instabilen Kompositmischung führte, die in der Lage war, ohne Photoinitiation zu polymerisieren. Grundsätzlich handelt es sich bei allen Proben um experimentelle Einmischungen der Antioxidantien. Deshalb geben die vorliegenden Untersuchungen nur Anhaltspunkte über mögliche Auswirkungen auf den DC und die Elution von Kompositinhaltsstoffen. In weiteren Studien sind daher Lagerstabilität und weitere Faktoren, wie z.B. physikalische Eigenschaften zu prüfen.

Einfluss von Asc/NAC auf den DC

Die photoinitiierte Polymerisation erfolgt durch eine Kettenreaktion zwischen den vom photoinitiierenden System gebildeten freien Radikalen und den Monomeren [219]. CQ, ein Photoinitiator, wird häufig für lichthärtende Kompositmaterialien verwendet [219]. Die Photopolymerisation wird durch das CQ/Amin-Photoinitiatorsystem eingeleitet. In diesem Prozess wurde ein Aminoalkylradikal, ein Schlüsselradikal, das die Polymerisation initiiert, durch photoinduzierten Elektronentransfer erzeugt [219]. Darüber hinaus kann auch eine direkte Abstraktion von Wasserstoffatomen aus dem Triplett-Zustand von CQ am Monomer reaktive Radikale bilden [219]. Daher könnte der signifikant reduzierte DC nach dem Einmischen von Asc bzw. NAC in die Kompositmaterialien der vorliegenden Studie [49] auf diese Antioxidantien zurückzuführen sein, die initiierende Radikale abfangen können [220] und folglich die Initiierung der Kettenpolymerisation unterdrücken. Darüber hinaus hängt die Effizienz der Photopolymerisation auch von der sterischen Struktur der von Aminen abgeleiteten Radikale ab, die sich der reaktiven ungesättigten Bindung in einem Monomer

nähern müssen [221]. Daher könnte die Einmischung von Asc bzw. NAC die Diffusion von Elektronen stören, was die Bildung von Exziplexen [222] oder die weitere Polymerisation behindert.

Bei allen untersuchten Kompositen, die 1, 0,1 und 0,01 Gew.-% Asc enthielten (mit Ausnahme von Venus-0,01 Gew.-% Asc), wurde eine signifikant niedrigere DC im Vergleich zur Kontrolle festgestellt. Bei der NAC-Einmischung wurde für Venus und Grandio mit 0,1 und 0,01 Gew.-% NAC sowie für Filtek Supreme XTE mit 1 Gew.-% NAC keine signifikante Änderung des DC festgestellt. Dies zeigt, dass Asc einen stärkeren Einfluss auf den DC hat als NAC. Neben einer stärkeren Fähigkeit Radikale zu fangen und die Diffusion von Elektronen zu stören, könnte eine weitere Erklärung darin liegen, dass Asc kettenunterbrechende Eigenschaften besitzt, indem es durch freie Radikale vermittelte Kettenreaktionen hemmt [223].

Elution von Kompositinhaltsstoffen und Einfluss von Asc/NAC

Für die GC/MS-Analyse wurden Methanol und Wasser als Elutionsmedia für 1d- und 7d-Elutionsversuche gemäß unseren früheren Studien [44, 46] verwendet.

TEGDMA ist ein (Co)monomer, das häufig in Kompositen verwendet wird, um die organische Harzmatrix des Komposits mit einem Maximum an anorganischen Füllstoffpartikeln anzureichern [78]. Ein signifikanter Anstieg der eluierten Kompositinhaltsstoffe (z.B. TEGDMA) im Vergleich zur Kontrolle kann zu schädlichen Wirkungen führen [70, 175]. Unsere frühere Studie zeigte, dass eine Exposition mit einer TEGDMA-Konzentration von 360 µM eine 4-fach höhere Anzahl von DNA-DSBs/Foci im Vergleich zur Kontrolle in HGFs induziert [175]. TEGDMA wurde in den Eluaten aller untersuchten Komposite nachgewiesen. Die höchste TEGDMA-Konzentration in den Methanol-Eluaten betrug 1148 µM (Venus-1 Gew.-% Asc, 7 d). Dies ist etwa dreimal so hoch wie die angegebene genotoxische Konzentration. Allerdings stellen Methanol-Eluate die maximale eluierbare Konzentration von (Co)Monomeren und Additiven dar [224]. Wassereluate hingegen erlauben einen physiologischen Vergleich mit Dentinflüssigkeit und menschlichem Speichel [45, 51]. Im Vergleich dazu betrug in dieser Studie die höchste Konzentration von TEGDMA in Wasser 260 µM (Venus-1 Gew.-% Asc, 7 d). Dies ist 1,4 Mal niedriger als die zitierte genotoxische Konzentration.

HEMA wird aufgrund seiner hydrophilen Anwendung in Dentalkompositen als (Co)Monomer der organischen Harzmatrix verwendet. In früheren Studien wurde für HEMA eine genotoxische Konzentration von 1100 µM in HGFs gefunden [175]. HEMA wurde nur in den Eluaten von Grandio nachgewiesen. Die höchste HEMA-Konzentration wurde mit 28 µM im Methanol-Eluat von Grandio-0,1 Gew.-% NAC nach 7 d nachgewiesen. Dies ist 39-mal niedriger als die zitierte genotoxische Konzentration.

Die Einmischung von Asc in Venus (1, 0,1 und 0,01 Gew.-%, 1 d / 7 d) und Grandio (1 Gew.-%, 1 d / 7 d) sowie die Einmischung von NAC in Venus (0,1 Gew.-%, 1 d) führte im Vergleich zur Kontrolle zu einem signifikanten Anstieg von TEGDMA in Methanol- und Wassereluaten. Diese Ergebnisse deuten darauf hin, dass die Einmischung von Asc bzw. NAC bei einigen Kompositen die Freisetzung von TEGDMA steigern und dementsprechend unerwünschte (Neben-)Wirkungen hervorrufen kann (z.B. Methacrylat-Allergie, zytotoxische / genotoxische Wirkungen [70, 175]). Die Daten der vorliegenden Studie [49] zeigten jedoch auch, dass die Einmischung von Asc bzw. NAC in bestimmten Gewichtsprozenten keinen Einfluss auf die TEGDMA-Freisetzung hatte (z.B. Filtek Supreme XTE-1, 0,1 und 0,01 Gewichtsprozent Asc bzw. NAC, 1 und 7 Tage in Methanol). In Grandio konnte eine signifikant verringerte Freisetzung von TEGDMA nach Einmischung von 1 Gew.-% NAC (7 d, Methanol) und eine signifikant verringerte Freisetzung von HEMA nach Einmischung von Asc (1, 0,1, 0,01 Gew.-%, Methanol / Wasser, 1 d / 7d) festgestellt werden. Diese Ergebnisse könnten auf die Wechselwirkung von Asc bzw. NAC mit den Inhaltsstoffen des Komposits zurückzuführen sein, so dass weniger HEMA bzw. TEGDMA freigesetzt wird. Dies stellt einen positiven Effekt dar. Unsere frühere Studie zeigte, dass ein geringerer DC mit einer höheren Menge an eluierten Kompositinhaltsstoffen einhergeht [225], was einen negativen Effekt darstellt. Allerdings spielen auch das Molekulargewicht und die Hydrophobizität der (Co)Monomere sowie der Füllstoffgehalt eine Rolle für den Elutionsmechanismus. Die Korrelation zwischen DC und der Elution der Komponenten ist daher nicht bekannt [68, 76]. Die zusätzlichen Einmischungen der Antioxidantien Asc bzw. NAC (wie zuvor beschrieben, als Radikalfänger) können die Polymerisation beeinflussen und zeigten sowohl einen positiven als auch negativen bzw. keinen Effekt auf die Elution von Inhaltsstoffen der untersuchten Komposite. Insgesamt konnte in der vorliegenden Studie [49] kein Zusammenhang zwischen DC und der Elution der Kompositinhaltsstoffe nach der Einmischung von Asc bzw. NAC festgestellt werden.

Freisetzung von Asc bzw. NAC aus den Komposit-Antioxidans Mischungen

Asc kann DNA-Läsionen vermindern, indem es reaktive Spezies direkt abfängt und ihre Bildung reduziert oder Proteine schützt, die DNA Schäden reparieren [226]. NAC kann freie Radikale sowohl direkt über die Thiol-Seitenkette als auch durch gleichzeitige Erhöhung des intrazellulären Glutathion (GSH)-Gehalts abfangen [104]. Darüber hinaus gibt es Hinweise darauf, dass NAC die Verfügbarkeit von freien Kompositmonomeren reduziert, indem es mit der Methacrylgruppe durch Michael-Addition reagiert [30, 227]. Sowohl von Asc als auch von NAC wurde berichtet, dass diese der Bildung von DNA-Addukten entgegenwirken [198, 203].

In unseren früheren Studien wurden die Zytotoxizität und Gentoxizität von dentalen (Co)Monomeren (z.B. TEGDMA) und ihren Epoxy-Metaboliten (z.B. EMPA) nachgewiesen [48, 175]. Darüber hinaus berichteten wir, dass Zellkulturmedium-Eluate von dentalen

Kompositen, die alle eluierbaren Inhaltsstoffe enthalten, ebenfalls DNA-DSBs induzieren [26]. Außerdem haben wir gezeigt, dass die Anwesenheit von Asc bzw. NAC die durch dentale (Co)Monomere und ihre Epoxy-Metaboliten induzierte Zytotoxizität und Genotoxizität deutlich reduziert [36, 38, 48, 217]. Konzentrationen größer als 50 µM Asc bzw. NAC bewirkten eine signifikante Verringerung der durch dentale (Co)monomer-Zwischenprodukte und deren Epoxy-Metaboliten induzierten DNA-DSBs im Vergleich zur Kontrolle in HGFs [48]. Daher spielen diese Konzentrationen eine Schlüsselrolle bei der Verringerung der Zelltoxizität.

Auf der Grundlage dieser 50 µM Asc / NAC [48] wurde die vorliegende Studie [49] für die Einmischung von NAC bzw. Asc ab 0,01 Gew.-% konzipiert, was einer berechneten maximalen eluierbaren Konzentration von etwa 57 µM Asc bzw. 61 µM NAC entspricht. Der Elutionsmechanismus hängt jedoch vom Molekulargewicht, der Hydrophobie, dem Füllstoffgehalt, dem untersuchten Material und den endgültigen Netzwerkeigenschaften der Harzmatrix ab [68, 75-77, 139]. Folglich waren in der vorliegenden Studie [49] die Konzentrationen von freigesetztem Asc bzw. NAC immer niedriger als die berechnete maximale eluierbare Asc- bzw. NAC-Konzentration.

In der vorliegenden Studie [49] wurde die Elution von Asc bzw. NAC nach 5 min, 1d und 7d bestimmt, um die maximal verfügbare Konzentration des freigesetzten Antioxidans in den Eluaten abzuschätzen. In anderen Studien wurde jedoch über einen Abbau von Asc bzw. NAC in Lösung berichtet [228, 229]. Dies steht im Einklang mit den Ergebnissen der vorliegenden Studie [49], da ein Abbau von eluiertem Asc bzw. NAC in Methanol und Wasser beobachtet wurde. Dennoch konnte auch für Venus-1% Gew.-% Asc (Methanol) und Filtek Supreme XTE-1 Gew.-% NAC (Wasser) eine erhöhte Freisetzung von Antioxidantien mit zunehmender Elutionszeit festgestellt werden.

Wie oben ausgeführt, können Konzentrationen mit mehr als 50 µM Asc bzw. NAC DNA-DSBs signifikant reduzieren [48]. In der vorliegenden Studie [49] wurden NAC- bzw. Asc-Konzentrationen über diesem Wert in den Methanol- oder Wassereluaten von Venus-1 Gew.-% Asc, Grandio-1 Gew.-% Asc, Filtek Supreme XTE-1 Gew.-% Asc und Filtek Supreme XTE-1 Gew.-% NAC gefunden. Dementsprechend lässt sich schließen, dass aus den vorstehenden Kompositen mit 1 Gew.-% Asc bzw. 1 Gew.-% NAC ausreichende Mengen an eluierten Antioxidantien zur Verfügung stünden, um die durch dentale Komposit-(Co)Monomere, ihre Zwischenprodukte und Epoxy-Metaboliten induzierte Gentoxizität in humanen oralen Zellen zu reduzieren.

Risikobewertung

Von allen untersuchten Komposit-Antioxidans Mischungen konnten nur Venus, Grandio und Filtek Supreme XTE (jeweils 1 Gew.-% Asc) und Filtek Supreme XTE-1 Gew.-% NAC, ausreichende Mengen an eluierten Antioxidantien freisetzen, um DNA-DSBs zu reduzieren

[48]. Die Beimischung von 1 Gew.-% Asc in Venus, Grandio und Filtek Supreme XTE führte jedoch zu einer signifikanten Verringerung des DC und einer signifikant erhöhten Elution von TEGDMA. Der Anstieg der Elution von Kompositinhaltsstoffen (z.B. TEGDMA) kann zu negativen Auswirkungen führen [70, 175]. Im Rahmen der vorliegenden Studie zeigte nur Filtek Supreme XTE-1 Gew.-% NAC sowohl beim DC als auch bei der Elution von Kompositinhaltsstoffen keine signifikante Veränderung und setzte eine ausreichende Menge an Antioxidans frei, um die Toxizität zu reduzieren. Daher stellt Filtek Supreme XTE-1 Gew.-% NAC eine vorteilhafte experimentelle Mischung dar.

Die Methanol-Elution stellt ein „Worst-Case"-Szenario und würde in der vorliegenden Studie [49] für Filtek Supreme XTE-1 Gew.-% NAC (1d Methanol-Elution) eine Gesamtkonzentration des eluierten TEGDMA aus 10 Füllungen (je 100 mg) von etwa 360 µM erreichen, die bereits DNA-DSBs induzieren kann [175]. In ähnlicher Weise betrug die maximale Konzentration an freigesetztem NAC aus einer Füllung 43 µM (Filtek Supreme XTE-1 Gew.-% NAC, 1d, Methanol). Bei 10 Füllungen würde dies eine Konzentration von 430 µM freigesetztem NAC ergeben. Dieser Wert ist 9-mal höher als 50 µM NAC, welche DNA-DSBs reduzieren kann [48]. Daher wäre eine Einmischung von 1 Gew.-% NAC in Filtek Supreme XTE ein nützlicher Schritt zur Verringerung der durch dentale (Co)Monomere induzierten DNA-DSBs. Es ist jedoch hervorzuheben, dass diese Daten nicht ohne weiteres auf die klinische Situation übertragen werden können, da in der physiologischen Situation die kontinuierliche Speichelbildung zu einer Verdünnung der freigesetzten Kompositinhaltsstoffe führen kann.

Schlussfolgerung

Beide in die untersuchten Komposite experimentell eingemischten Antioxidantien (Asc bzw. NAC) werden nach der Polymerisation freigesetzt und erreichen entsprechende effektive Konzentrationen zur möglichen Reduktion der Toxizität von (Co)Monomeren und deren Metaboliten. NAC hatte im Vergleich zu Asc einen geringen Einfluss auf den DC. Dennoch führten beide Antioxidantien teilweise zu einer erhöhten Freisetzung von Inhaltsstoffen der untersuchten Komposite im Vergleich zu Kompositen ohne Antioxidans-Einmischung, wodurch dementsprechend nachteilige toxische Effekte entstehen können. Die experimentelle Einmischung von 1 Gew.-% NAC als neue Kompositkomponente in Filtek Supreme XTE hatte keine Auswirkungen auf den DC, die Elution von Kompositinhaltsstoffen und setzte eine ausreichende Menge an Antioxidans frei, die die Toxizität von (Co)Monomeren und deren Metaboliten verringern könnte. Daher stellt Filtek Supreme XTE-1 Gew.-% NAC eine vorteilhafte Mischung dar.

4. Zusammenfassung

Aufgrund ihrer ästhetischen und physikalischen Eigenschaften sind dentale Komposite das meist verwendete Zahnfüllungsmaterial. Dentale Komposite auf Methacrylatbasis bestehen aus verschiedenen (Co)Monomeren (Methacrylaten) und Additiven [1, 7]. Wegen der unvollständigen (Co)Monomer-Polymer-Umwandlung wird eine Freisetzung von unpolymerisierten (Co)Monomeren und Inhaltstoffen aus den dentalen Kompositen beschrieben [210, 230]. Diese können über die Dentintubuli mit der Pulpa in Kontakt kommen, die Aktivität der Zahnpulpa-Zellen beeinträchtigen oder durch Verschlucken in den Darm gelangen, wo sie in den Blutkreislauf und die Organe geraten können [8-10]. Außerdem wird über allergische Reaktionen wie Asthma und Kontaktdermatitis berichtet, die durch Methacrylate verursacht werden [20]. Es gibt zahlreiche In-vitro-Studien zur Toxizität und Biokompatibilität, die gezeigt haben, dass einige der eluierten (Co)Monomere und Additive östrogene, mutagene, teratogene und genotoxische Wirkungen haben [29, 81, 231, 232].
Zur Bestimmung der Freisetzung von (Co)Monomeren und Additiven aus dentalen Kompositen werden polymerisierte Kompositprüfkörper in Extraktionsmedia Methanol oder Wasser eluiert. Mittels GC/MS lassen sich anschließend die freigesetzten Inhaltsstoffe qualifizieren und quantifizieren. Trotz der Einhaltung der vom Hersteller empfohlenen Polymerisationszeiten ist der relativ niedrige Polymerisationsgrad (DC) von dentalen Kompositen ein Grund für die Freisetzung von Inhaltsstoffen aus Kompositen. Dieser liegt nur etwa bei 55-65% [64].
Um die Polymerisationsschrumpfung zu reduzieren und eine ausreichende Durchhärtungstiefe zu erreichen sowie die Freisetzung von Kompositinhaltsstoffen aus konventionellen Kompositmaterialen zu reduzieren wird bisher für Schichtdicken > 2 mm die sogenannte Inkrementschichttechnik bei posterioren Kompositrestaurationen mit einer maximalen Schichtdicke von 2 mm oder weniger angewendet [54]. Die Entwicklung von Bulk-Fill Kompositen verspricht eine Beschleunigung des Restaurationsprozesses, durch die Möglichkeit der Aushärtung einer bis zu 4 mm dicken Schicht in einem Schritt [55] und somit in vielen Fällen die Füllung einer kompletten Kavität [56, 57]. Inwieweit sich eine Schichtdicke von bis zu 6 mm (entgegen der Herstellerempfehlung) im Vergleich zu einer Schichtdicke von 2 und 4 mm (gemäß Herstellerangaben) auf die Menge an eluierbaren Inhaltsstoffen aus Bulk-Fill Kompositen auswirkt, wurde in der vorliegenden Studie [44] mit den Kompositen SDR Bulkfill, Venus Bulkfill und ELS Bulkfill untersucht. Die Ergebnisse der vorliegenden Studie [44] zeigen, dass die Herstellerangaben strikt befolgt werden sollten, da in vielen Fällen eine laut Hersteller nicht freigegebene Schichtdicke von 6 mm zu einer höheren Menge an eluierten Bulk-Fill Kompositinhaltsstoffen führt. Dies kann zu einer höheren Exposition bei Patienten führen.

Die Menge an eluierten (Co)Monomeren und deren Wiederfindungsrate ist von dem zur Extraktion verwendeten Medium abhängt. So ist z.B. die Wiederfindungsrate im Zellkulturmedium mit fötalem Kälberserum aufgrund des hohen Anteil an Proteinen geringer [92]. Für die Beurteilung der Toxizität und Biokompatibilität von Dentalkompositen ist es nicht nur wichtig zu wissen, welche (Co)Monomere und Additive in welcher Menge freigesetzt werden, sondern ob diese Stoffe auch an Proteine binden können. Diese Proteinbindung könnte in vivo zu einer reduzierten Bioverfügbarkeit führen [93]. In der vorliegenden Studie [45] wurde die Bindung von freisetzbaren Inhaltsstoffen aus den Kompositen Admira flow, Venus Diamond flow, Filtek Supreme XTE flow, Tetric EvoCeram und Tetric EvoFlow an Speicherproteinen untersucht. Als Extraktionsmedien wurden nativer Speichel (mit Proteinen), proteinfreier Speichel, Wasser und Ethylacetat für die Elutionsversuche verwendet. Neben der Kenntnis der Bindung von (Co)Monomeren an Speichelproteine ist auch die Bindung an Plasmaproteine wichtig, um die Toxizität von Dentalmaterialien zu beurteilen. Daher wurde auch die Bindung einiger Methacrylate und Additive an die Plasmaproteine human serum albumin (HSA) und human α_1-acid glycoprotein (AGP) untersucht. So konnte gezeigt werden, dass künstlicher Speichel bzw. Wasser als Extraktionsmedium nicht die tatsächliche physiologische Situation im Körper widerspiegeln [45]. Die Konzentration an (Co)Monomeren und Additiven, die in nativem Speichel freigesetzt werden, ist deutlich niedriger als die Konzentration, die in proteinfreiem Speichel bzw. Wasser freigesetzt wird. Speichel- und Plasmaproteine können (Co)Monomere und Additive binden und dadurch zu einer geringeren Bioverfügbarkeit von freigesetzten Inhaltsstoffen in vivo als bisher angenommen führen. Allerdings ist die Bindung von Wirkstoffen und wohl auch von freigesetzten Inhaltsstoffen aus Kompositen an Proteine in der Regel reversibel [45, 112].

Neben Veneers und zahnfarbenen Kronen ist zum Beispiel Bleaching das häufigste Verfahren, um die Zähne ästhetisch zu verschönern [116]. Hierfür gibt es drei verschiedene Standard-Bleichmethoden, die auf Carbamid- oder Wasserstoffperoxid-Gel basieren. 1. In-Office-Bleaching-Methoden (35% Peroxide) bzw. Chairside-Bleaching-Methoden (38% Peroxide) [118], 2. Home-Bleaching-Methoden (15% Peroxide) [119] und 3. rezeptfreie Produkte (Over-the-counter-Produkte) (maximal 10% Peroxide). Bleichschienen zum Auftragen des Bleichgels werden nur beim Home-Bleaching und beim In-Office-Bleaching verwendet [120]. Werden jedoch bei einem Patienten vor einem Bleichvorgang insuffiziente Restaurationen festgestellt, müssen diese in jedem Fall vor Beginn des Bleichvorgangs ausgetauscht werden, da es beim Kontakt der Bleichgele mit der Pulpa zu einer Schädigung der Pulpazellen kommen kann [126]. In den vorliegenden Studien [46, 47] wurden daher der Einfluss von Bleaching-Behandlungen mit den Opalescence-Bleichgelen PF 15% (PF 15%) (Home-Bleaching) und PF 35% (PF 35%) (In-Office-Bleaching) auf die Freisetzung von Kompositinhaltsstoffen aus den konventionellen Kompositen Tetric EvoCeram, CLEARFIL AP-X, Tetric EvoFlow, Filtek Supreme XT, Ceram X

mono+, Admira und Filtek Silorane, und den Bulk-Fill Kompositen Tetric EvoCeram Bulk Fill, QuiXFil und X-tra fil untersucht. Für die untersuchten konventionellen und Bulk-Fill Komposite konnte gezeigt werden, dass die Elution von Inhaltsstoffen nach dem Bleichprozess von der Zusammensetzung des jeweiligen Komposits abhängig ist, also Bleichbehandlungen sowohl zu einer verringerten als auch zu einer erhöhten Elution von Inhaltsstoffen im Vergleich zur unbehandelten Kontrolle führen können [46, 47].

Der 3D-Druck (additive Methode) ist eine neuere Technologie, bei der im Gegensatz zu industriell vorgefertigten Fräsblöcken flüssige bzw. niedrigvisköse Materialien verwendet werden. Da diese neuartigen, für den 3D-Druck entwickelten Materialien je nach Verfahren eine bestimmte Zusammensetzung erfordern, gibt es bisher nur wenige Erkenntnisse über ihre Biokompatibilität und andere Eigenschaften [153]. Während einige Studien Elutionsdaten für konventionelle und vorgefertigte Fräsmaterialien erheben [165, 166], finden sich nur sehr wenige Untersuchungen für additiv verarbeitete Materialien [3]. Ziel der vorliegenden In-vitro-Studie [4] war daher, die Elution/Freisetzung von Inhaltsstoffen aus drei verschiedenen methacrylharzbasierenden PMMA-Materialien, die für die Herstellung von Zahnschienen im additiven (3D-Druck, SHERAprint-ortho plus), subtraktiven (Fräsen, SHERAeco-disc PM20) und konventionellen Verfahren (Pulver und Flüssigkeit, SHERAORTHOMER) verwendet werden, sowie deren zytotoxisches Potenzial unter Berücksichtigung physiologischer Schienengrößen zu untersuchen. Die Ergebnisse der vorliegenden in vitro Studie [4] zeigten, dass derzeit industriell vorgefertigte Polymerrohlinge für die subtraktive Fertigung (SHERAeco-disc PM20) in Bezug auf die Biokompatibilität überlegen sind. Neu entwickelte Werkstoffe für die additive Fertigung scheinen eine komplexere Zusammensetzung aufzuweisen und damit das Potenzial für eine Elution einer größeren Anzahl von (Co)Monomeren und Additiven zu haben. Die freigesetzten Inhaltsstoffe im Elutionsmittel Methanol aus den untersuchten Schienenmaterialien überstiegen die zytotoxischen Konzentrationen in HGFs, die für ein „Worst-Case“-Szenario in der physiologischen Schienengröße berechnet wurden. In den Wassereluaten konnte dagegen nur das Methacrylat Tetrahydrofurfuryl methacrylat (THFMA) aus SHERAprint-ortho plus in Konzentrationen unterhalb zytotoxischer Werte in HGFs bestimmt werden. Daher ist das Gesundheitsrisiko in der physiologischen (Wasser/Speichel) Situation von geringer Bedeutung.

Die (Co)Monomere Triethylenglycoldimethacrylat (TEGDMA), 2-Hydroxyethylmethacrylat (HEMA) können aus den unvollständig polymerisierten Kompositmaterialien freigesetzt werden [1] und dadurch die Aktivität von Pulpa-Zellen beeinträchtigen oder durch Verschlucken in den Darm gelangen und anschließend den Blutkreislauf und die Organe erreichen [1, 15]. Die Aufnahme, Verteilung und Ausscheidung von radiomarkiertem ^{14}C-TEGDMA und ^{14}C-HEMA bei Meerschweinchen wurde in unseren früheren Studien untersucht [13, 39]. TEGDMA und HEMA werden zu Methacrylsäure (MA) und darauffolgend

hauptsächlich über den sogenannten Epoxyweg metabolisiert [13, 39-41]. In diesem Metabolismus (Epoxyweg) kann die C-C-Doppelbindung von MA oxidiert werden, wodurch der Epoxy-Metabolit 2,3-Epoxy-2-methylpropionsäure (EMPA) gebildet werden kann [29, 42, 43]. Ebenfalls ist sehr wahrscheinlich, dass in vivo der 2,3-Epoxy-2-methyl-propionsäuremethylester (EMPME) gebildet werden kann [29]. Epoxide gelten als hochreaktive Moleküle und mutagene/carcinogene Substanzen [43]. Bisher wurden Studien zur Toxizität und Induktion von DNA-Doppelstrangbrüchen (DNA-DSBs) von den (Co)Monomeren (u.a. TEGDMA und HEMA) durchgeführt [175, 189, 190]. Des Weiteren zeigten Studien, dass die Zugabe von Antioxidantien wie Ascorbinsäure (Asc) bzw. N-Acetylcystein (NAC) die zytotoxische Wirkung und DNA-DSBs von Kompositinhaltsstoffen ((Co)Monomeren) reduzieren können [36-38]. Ob die Comonomer-Epoxy-Metaboliten EMPME und EMPA im Vergleich zu ihren metabolischen Vorläufern TEGDMA, HEMA und dem Zwischenprodukt MA mehr DNA-DSBs in HGFs induzieren können sowie, ob Antioxidantien zur Verringerung von DNA-DSBs in Gegenwart von Comonomer-Epoxy-Metaboliten führen, wurde in der vorliegenden Studie [48] untersucht. Schließlich zeigten die Comonomer-Epoxy-Metaboliten EMPME und EMPA, im Vergleich zu ihrem metabolischen Vorläufer, MA eine höhere Zytotoxizität und induzierten mehr DNA-DSBs. Die Antioxidantien Asc bzw. NAC können die Anzahl der durch EMPME und EMPA induzierten DNA-DSBs in HGFs verringern. NAC wies im Vergleich zu Asc eine bessere Schutzwirkung auf.

Bisherige Studien über die durch dentale Kompositmaterialien induzierte Zytotoxizität und DNA-DSBs befassten sich ausschließlich mit den Auswirkungen einzelner Kompositinhaltsstoffe [36, 175, 187]. Versuche mit Komposit-Eluaten, deren Inhaltsstoffen durch vorherige qualitative und quantitative Analyse ermittelt werden, spiegeln möglicherweise eine Situation wider, die der Physiologie näher kommt als Versuche mit einzelnen Kompositkomponenten/-inhaltsstoffen. In der vorliegenden Studie [26] wurde daher die durch dentale Komposit-Eluate resultierende Zytotoxizität und Induktion von DNA-DSBs in HGFs untersucht. Zur Bestimmung der Zytotoxizität und Gentoxizität der Eluate aus den untersuchten Kompositmaterialien EsthetX HD, Venus, X-tra fil, CLEARFIL AP-X, Admira Fusion und QuiXfil wurden XTT- und γ-H2AX-Tests durchgeführt. Die freigesetzten Kompositinhaltsstoffen im Zellmedium Dulbecco's modified Eagle's medium (DMEM) wurden mittels GC/MS qualifiziert und quantifiziert, um deren Relevanz als multiple Zusammensetzung aus den freigesetzten Kompositinhaltsstoffen bestmöglich abschätzen zu können. So zeigte die Exposition von HGFs mit den Eluaten von Esthet X HD und Venus eine signifikante Induktion von DNA-DSBs. In allen untersuchten Eluaten wurde keine signifikante Zytotoxizität festgestellt. Interaktive Effekte zwischen den freigesetzten (Co)Monomeren und Additiven können also die Zytotoxizität und die Induktion von DNA-DSBs im Vergleich zur Exposition mit Einzelkomponenten beeinflussen.

Die Antioxidantien Asc und NAC gelten als Radikalfänger [196] und können in vitro durch TEGDMA und HEMA induzierte Zytotoxizität sowie die Genotoxizität von dentalen (Co)Monomeren und ihren Epoxymetaboliten EMPA und EMPME verringern [36, 38, 48, 216, 217]. Oral verabreichte Mischungen von Antioxidantien, einschließlich Asc und NAC, können durch ionisierende Strahlung induzierte DSBs reduzieren [199]. In der vorliegenden Studie [49] wurden die Antioxidantien Asc bzw. NAC (1, 0,1 und 0,01 Gew.-%) als neue/zusätzliche Kompositkomponente in den drei lichthärtenden Kompositmaterialien Venus, Grandio und Filtek supreme XTE hinsichtlich ihrer Auswirkungen auf den Polymerisationsgrad (DC) und die Elution von Kompositinhaltsstoffen untersucht. Zusätzlich wurde die Freisetzung von Asc bzw. NAC mittels „high-performance liquid chromatography/diode array detection“ (HPLC/DAD) und „high-performance liquid chromatography/fluorescence detection“ (HPLC/FLD) in diesen neuen Mischungen bestimmt. Beide experimentell eingemischten Antioxidation werden nach der Polymerisation freigesetzt und erreichen entsprechende effektive Konzentrationen zur möglichen Reduktion der Toxizität von (Co)Monomeren und deren Metaboliten. Vor allem NAC hatte im Vergleich zu Asc einen geringen Einfluss auf den DC. Dennoch führten beide Antioxidantien teilweise zu einer erhöhten Freisetzung von Inhaltsstoffen der untersuchten Komposite im Vergleich zu Kompositen ohne Antioxidans-Einmischung, wodurch dementsprechend nachteilige toxische Effekte entstehen könnten. Die experimentelle Einmischung von 1 Gew.-% NAC als neue Kompositkomponente in Filtek Supreme XTE hatte keine Auswirkungen auf den DC, die Elution von Kompositinhaltsstoffen und setzte eine effektive Konzentration an Antioxidans frei. Daher stellt Filtek Supreme XTE-1 Gew.-% NAC eine vorteilhafte Mischung dar.

Insgesamt erreichen (Co)Monomere und Additive im Speichel des Menschen nach der Elution aus Kompositen maximal nur mikromolare Konzentrationen. Toxische Wirkungen dieser Stoffe treten jedoch meist erst im höheren millimolaren Konzentrationensbereich auf. Für eine exakte Risikoabschätzung eines Schadstoffes sind wissenschaftlich fundierte epidemiologische Untersuchungen unumgänglich. Daher zeigen aus toxikologischer Sicht dentale Komposite, vor allem wenn die Herstellerangaben strikt befolgt werden, eine gute Verträglichkeit. Da die Zusammensetzung von dentalen Materialien stetig verbessert werden, kann es aber trotzdem immer wieder zu Verunreinigungen der Rohprodukte kommen. Deshalb ist eine ständige Kontrolle der eluierbaren Inhaltsstoffe obligatorisch. Ein Teil möglicher unbekannter Inhaltsstoffe ist bis heute noch nicht nachgewiesen und nicht toxikologisch bewertet worden. Für ein tieferes Verständnis des Metabolismus von Abbauprodukten, die aus freigesetzten Inhaltsstoffen gebildet werden und toxische Wirkungen (z.B. Kanzerogenität/Mutagenität) verursachen können, ist ebenfalls die weitere Aufklärung biochemischer

Stoffwechselvorgänge, wie z.B. das Alkylierungsverhalten auf DNA-Ebene und eventuelle Möglichkeiten zur Reparatur unumgänglich.

Neben den möglichen toxischen Effekten von Inhaltsstoffen aus Zahnmaterialien, stellen allergische Reaktionen/Nebenwirkungen (z.B. Lichen planus, Gingivitis, Ulzerationen, Ekzeme, Erytheme) und Atemwegserkrankungen, für Patienten und zahnärztliches Personal gegenüber Kompositinhaltsstoffen ein Risiko dar. Grundsätzlich ist die Konzentration eines Allergens nicht maßgebend. D.h. bereits geringste Konzentrationen können allergische oder andere Nebenwirkungen hervorrufen. Daher ist es wichtig, alle eluierbaren Bestandteile von Dentalmaterialien zu identifizieren und zu charakterisieren. Darüber hinaus können freigesetzte Kompositinhaltsstoffe zur Bildung von Entzündungsfaktoren führen und/oder das Bakterienwachstum (Parodontitis) beeinflussen [104]. Deshalb ist der Nachweis solcher Substanzen und die Aufklärung der daraus resultierenden Stoffwechselvorgänge von aktueller Bedeutung.

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Diese kumulative Habilitationsschrift beruht auf folgenden Aufsätzen:

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