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Cytotoxicity of the dental composite component TEGDMA and selected metabolic by-products in human pulmonary cells

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ABSTRACT

Objectives. The comonomer triethyleneglycoldimethacrylate (TEGDMA) is a commonly used constituent of resin-based dental materials. Upon placement, light-cured dental polymers may release a wide spectrum of residual compounds due to incomplete monomer-conversion during polymerization. Apart from liberating unreacted monomers, additional compound release might occur due to mechanical wear and enzymatic degradation on the salivary surface of resin fillings. Following delivery into the local bio phase, leached compounds may encounter a variety of different enzymes, which might be present in their oral or systemic environment. Metabolic by-products formerly associated with TEGDMA-degradation include triethylene glycol (TEG), methacrylic acid (MA), 2,3-epoxymethacrylic acid (2,3-EMA), and formaldehyde.

Methods. Cytotoxicitiy of TEGDMA-derived intermediates was measured as mitochondrial dehydrogenase activity assessed by colorimetric measurement of formazan formation as a cleavage-product from the tetrazolium salt XTT by metabolically active A549 cells. EC_{50}-values were calculated by using curve fitting software (GraphPad Prism).

Results. The following EC_{50}-values (mmol/L) (95% confidence interval) were obtained: 2,3-EMA 1.65 (1.28–2.13), TEGDMA 1.83 (1.46–2.30), MA 4.91 (4.22–5.71), and paraformaldehyde (PFA) 5.48 (4.56–6.58). For TEG no cytotoxic effects up to a concentration of 10 mM could be found.

Significance. The epoxy compound 2,3-EMA induced comparable toxic effects as the raw comonomer TEGDMA. It is therefore concluded that the formation of toxic intermediates might significantly contribute to TEGDMA-induced cytotoxicity in human pulmonary cells.

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1. Introduction

Persistent concerns regarding the safety of dental amalgams [1] along with a preference for aesthetically advanced tooth-colored restorations among patients have prompted a widespread use of composite resins in restorative dentistry [2]. Despite their common employment in dental applications, information concerning the biochemical stability and biocompatibility of resin-based materials as compared to silver mercury fillings is still rather scarce [3]. With initial research having primarily focused on an improvement of the physical structure and mechanical function of resin materials, in order to optimize handling of composite resins, further questions regarding their biological safety had only been raised later on. The first biocompatibility studies mainly had focused on the release of unpolymerized residual monomers [15,23], whereas recently gathered knowledge on the biodegradation of resin-based dental materials has instigated additional concerns on the toxicity of monomer-related metabolic by-products, e.g. formaldehyde [4,24,25]. However, initial investigations concerning the issue of biocompatibility soon made clear that composites can liberate a wide spectrum of residual compounds due to deficiencies in monomer-conversion during polymerization [5]. In addition to the release of unpolymerized compounds into the oral cavity [6], also a rapid systemic distribution of unreacted monomers by diffusion through dentine and pulp might occur [7]. Apart from the elution of residual monomers immediately after placement, diverse chemical (e.g. solvolysis, hydrolysis, and alcoholysis) and physical (e.g. wear and erosion) reactions at the salivary surface of fillings furthermore promote a constant disintegration and dissolution of resin polymers [4]. Moreover, recent findings indicate that dental polymers are also quite extensively subjected to enzymatic hydrolysis [8], thus presumably leading to a generation of a variety of different metabolic by-products. A metabolic pathway for the degradation of monomers has formerly been proposed [9]. In this context, it has been hypothesized that monomers can be degraded via two different catabolic pathways, one of them leading to the formation of toxic epoxides from the metabolic by-product methacrylic acid (Fig. 1). The generation of 2,3 epoxymethacrylic acid (2,3-EMA) as an intermediate in the metabolism of dental materials in human liver microsomes could recently be confirmed [10]. Furthermore, a selectivity of human saliva-derived enzyme activities (HSDEA) for certain monomer types and the preferential scission of specific sites could be shown [11,12].

Although an increasing number of studies which have investigated the cytotoxic potentials of different comonomers in various in vitro systems, only limited data are available concerning the interactions between composite resin degradation by-products and their biological environment [13]. The comonomer triethyleneglycoldimethacrylate (TEGDMA) is a commonly used diluent of many resin-based dental composites [14], which belongs to the main compounds released [15,16]. Due to its lipophilic nature, TEGDMA can easily penetrate the cytosol and membrane lipid compartments of mammalian cells [17].

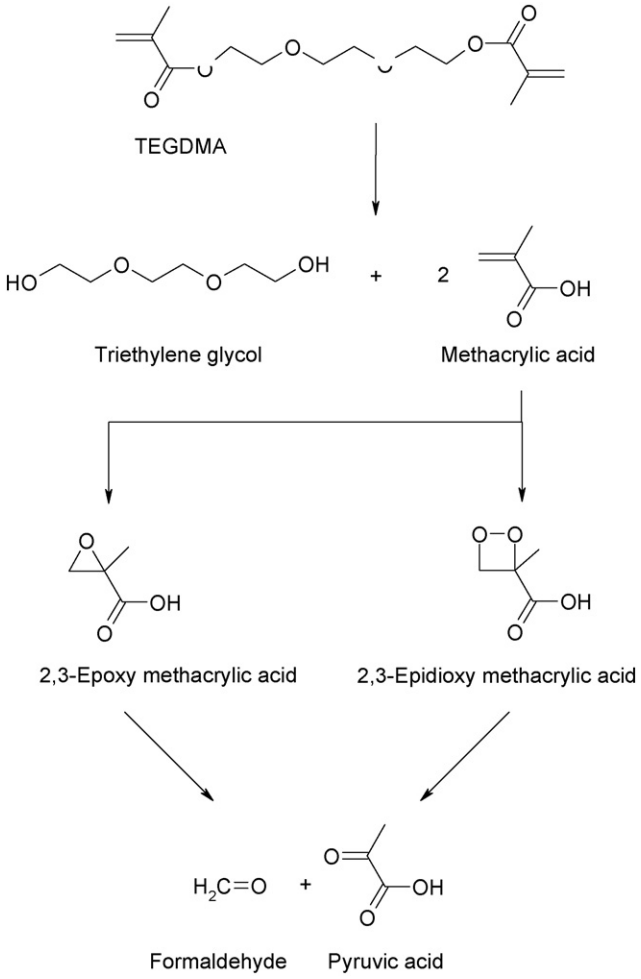

Fig. 1 – Proposed metabolites of methacrylic acid during the degradation of triethyleneglycoldimethacrylate (TEGDMA) according to Reichl et al. [9].

Scarce information is available in the literature on the effects of TEGDMA-derived degradation products on host cell metabolism and function. Therefore, the present study was designed to assess the relevant cytotoxic concentrations of selected TEGDMA-associated metabolites in human pulmonary A549 cells, which have been used previously for testing cytotoxicity of various dental materials [32,35].

2. Materials and methods

2.1. Chemicals

TEGDMA was obtained from ESPE Dental AG (Seefeld, Germany). Methacrylic acid, triethylene glycol and paraformaldehyde were purchased from Sigma–Aldrich Chemicals (Taufkirchen, Germany). 2,3-Epoxymethacrylic acid was synthesized from methacrylic acid according to the procedure described by Yao and Richardson [18]. For the determination of cytotoxic effects the cell proliferation kit II from Roche Diagnostics (Mannheim, Germany) was used.

2.2. Cell culture

Human bronchoalveolar carcinoma-derived A549 cells (ATCC, Rockville, MD, USA) were cultured in a humidified atmosphere at 37 °C with 5% CO_2 (v/v), using RPMI 1640 medium (Gibco BRL, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom, Munich, Germany).

Cells were maintained in logarithmic growth phase by routine passage every 3–4 days. Experiments were performed with confluent cell monolayers at a density of 80–90%.

2.3. Cytotoxicity assay

Cell proliferation and viability was determined by means of a colorimetric XTT-based assay, as it has previously been described [19]. A549 cells were seeded in 96-well tissue culture plates at an initial density of 5×10^3 cells per well and were cultured for 24 h prior to assay performance. Medium was then removed and the proliferating cell monolayers were exposed to the following reagents/reagent combinations in HBSS: TEGDMA (0.003–10 mM), TEG + MA (equimolar TEGDMA) (0.03–10 mM), TEG (0.03–10 mM), MA (0.03–30 mM), 2,3-EMA (0.001–30 mM), and PFA (0.03–10 mM). After an incubation period of 20 h according to previous studies [32,35], a XTT reagent mix was added according to the supplier's instructions. 4 h later formazan formation was quantified spectrophotometrically at 450 nm (reference wavelength 670 nm) using a microplate reader (Victor 3, PerkinElmer LAS, Rodgau-Jügesheim, Germany). Each experiment was performed in triplicate and was repeated twice. Data are expressed as mean ± S.D.

2.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.03 for Windows (GraphPad Software, San Diego, CA, USA). Half-maximum-effect-concentrations (EC_{50}-values) were calculated by fitting the obtained data to a sigmoidal curve with variable slope ($n = 6$). Statistical significance between experimental groups was determined by comparing best-fit log EC_{50}-values using the *F*-test and was assumed for $p < 0.05$.

3. Results

The cytotoxic potential of TEGDMA-related metabolic by-products was determined by assessing metabolic activity of pulmonary cells after compound exposure. Within 24 h, all tested metabolites (exception TEG) induced a dose-dependent loss of viability in exposed A549 cells (Fig. 2). For the diol compound TEG, however, no cytotoxic effects up to the concentration of 10 mM were observed. Correspondingly, toxic effects after exposure to MA did not significantly differ from reduction of metabolic activity following exposure to TEG + MA applied in equimolar masses to TEGDMA-derived degradation by-products. According to calculated half-maximum-effect-concentrations (EC_{50}), only the epoxy compound 2,3-EMA induced comparable cytotoxic effects as the raw dental material TEGDMA.

According to the measured mitochondrial dehydrogenase activity in exposed cells the following order of toxicity (EC_{50}-values) was obtained (mean (95% confidence interval in brackets) [mmol/L]): 2,3-EMA 1.65 (1.28–2.13), TEGDMA 1.83 (1.46–2.30), TEG + MA 4.68 (4.32–5.08), MA 4.91 (4.22–5.71), and PFA 5.48 (4.56–6.58). The relative cytotoxic potentials of the tested metabolites versus TEGDMA-associated cytotoxic effects are summarized in Table 1.

4. Discussion

The determination of cellular viabilities by tetrazolium-based colorimetric assays (e.g. XTT-assay) has been described to provide a useful in vitro screening tool to compare the cytotoxicity of dental materials [20].

In the present study, the toxicity of the comonomer TEGDMA, a widely used constituent of dentin-bonding systems and major component of dental restorative biomaterials [14], was compared to the toxic potentials of formerly identified metabolic by-products in human pulmonary cells. It is a drawback, that isolated cell systems only partially reflect clinical reality. However, the biological fate of TEGDMA is at first a biochemical question. Enzymes, e.g. cytochrome P450 (CYP450), might catalyze the previously proposed TEGDMA metabolism, which is present in many cell lines and mainly in the liver and lung of human beings. Thus, metabolic com-

Table 1 – Log EC_{50} (mol/L), EC_{50} (mmol/L) (mean values and 95% confidence interval) and relative toxicities of TEGDMA-related metabolites in A459 cells

Substance	Log EC_{50} (mol/L) (95% C.I.)	EC_{50} (mmol/L) (95% C.I.)	Relative toxicity vs. TEGDMA
TEGDMA	−2.737 (−2.835 to −2.639)	1.83 (1.46 to 2.30)	1
TEG + MA equimolar TEGDMA	−2,329* (−2.365 to −2.294)	4.68 (4.32 to 5.08)	0.39
TEG	–	>10	<0,18
MA	−2,309* (−2.375 to −2.243)	4.91 (4.22 to 5.71)	0,37
2,3-EMA	−2,784* (−2.894 to −2.674)	1.65 (1.28 to 2.13)	1.10
PFA	−2,261* (−2.341 to −2.181)	5.48 (4.57 to 6.58)	0.33

EC_{50}-values were calculated from fitted curves ($n = 6$ from repeated experiments). Cell-viability was assessed 24 h after exposure to the specified compounds by XTT-based assay.

* Log EC_{50} significantly different to TEGDMA ($p < 0.05$).

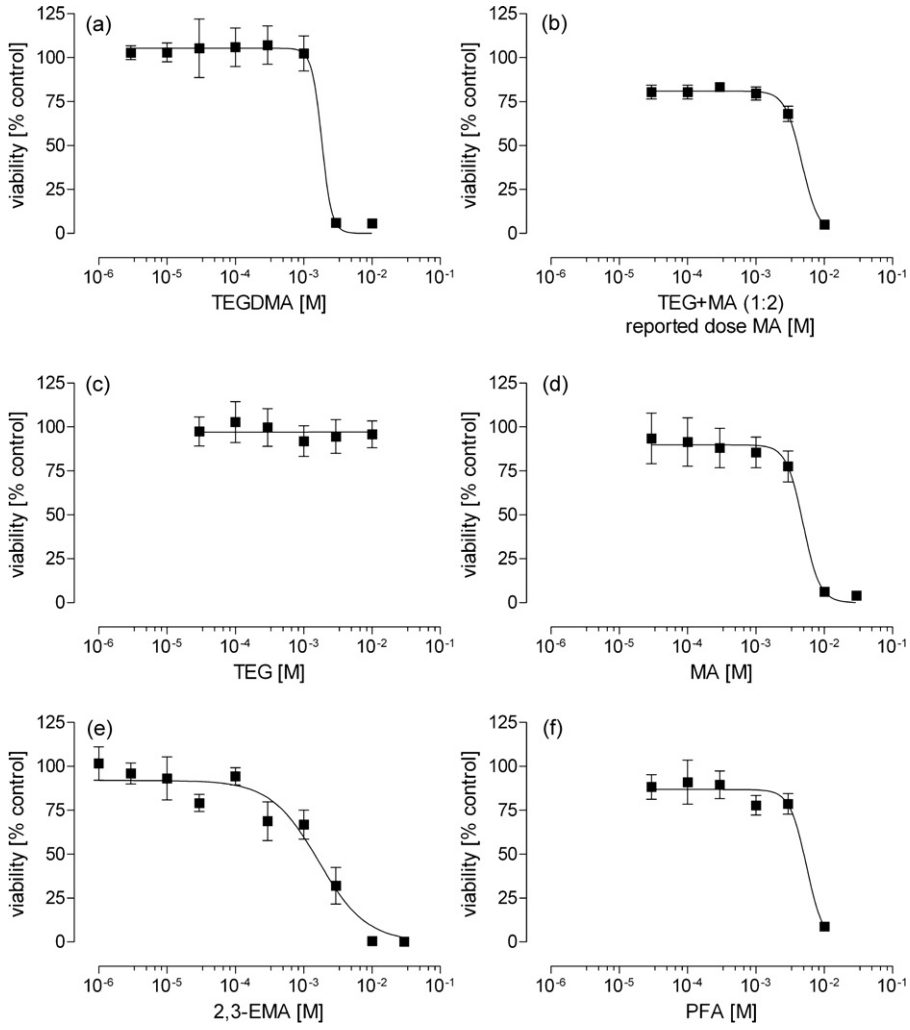

Fig. 2 – Effects of TEGDMA (a), TEG + MA (equimolar TEGDMA) (b), TEG (c), MA (d), 2,3-EMA (e), and PFA (f) on the viability of A549 cells. Cell viabilities were assessed by XTT-based assay. Curves are drawn from parameters obtained by fitting a sigmoid function to the data points. Data points (■) represent mean values ± S.D. from repeated experiments ($n = 6$).

petent lung cell cultures are advantageous to investigate the cytotoxicity of dental materials, because it is possible to investigate the effect of dental materials on target cells, where metabolic activation might occur.

In our experimental setup, A549 cells were chosen as an in vitro model of acute lung injury. Previously, a constant release of resinous compounds from dental composites followed by systemic distribution and the exertion of toxic systemic effects had been assumed [2]. Moreover, studies had also documented adverse health effects in dental personnel with increased incidence of respiratory symptoms due to inhalation of respirable composite compounds [21]. Apart from displaying characteristics of type II pneumocytes, the adenocarcinoma-derived cell line A549 has also been identified as a superior candidate for metabolism-related toxicity studies, as its xenobiotic-metabolizing capability exceeds that of other cellular models and expression of P450 is not only limited to the CYP1-enzyme family but also includes other biotransformation enzymes [22].

First studies addressing the problem of composite biodegradation demonstrated that unspecific esterases and saliva-derived enzymes are capable of softening the surface of dimethacrylate polymers by hydrolyzing methacrylate ester bonds [24]. The concomitantly observed liberation of methacrylic acid and the corresponding alcohol was assumed to originate from a degradation of dimethacrylates only attached in the matrix by one end of the molecule [25]. Recent studies confirmed, that matrix composition seems to be a key parameter for the sorption and solubility behavior of composite resin materials [26] and interactions between commercial composite resins with human saliva-derived esterases have repeatedly been described [27]. Concerning the relation of dental composite formulations to extent of degradation and release of hydrolyzed polymeric-resin-derived products further studies revealed that inflammatory and salivary enzymes preferentially cause a breakdown of components in dental resin materials, e.g. bisphenol-A diglycidyl dimethacrylate (BisGMA) and TEGDMA [4]. In this context, a higher specificity

of salivary enzymes towards TEGDMA, compared to BisGMA, could be shown. It therefore has been concluded that the amount and type of degradation products depends on esterase make-up and content of an individual's saliva in combination with the specific formulation of monomer components used [8].

Apparently being the main target of enzymatic hydrolysis, a generation of various TEGDMA-associated toxic by-products such as methacrylic acid (MA), formaldehyde [28] and 2,3-EMA [9] has been proposed.

According to obtained EC_{50}-values, in our study none of the tested metabolites (exception 2,3-EMA) caused an equivalent reduction in metabolic activity in exposed A549 cells. The cytotoxic potential of 2,3-EMA exceeded the cytotoxic effects of the raw dental material TEGDMA. When incubating cells with a combination of TEG + MA in equimolar masses to TEGDMA-derived degradation products, it could furthermore be observed that cytotoxic effects seem to be mainly originated from MA, since after TEG-treatment no significant reduction of cellular metabolic activity could be seen (Fig. 2). Lower toxicity of the metabolite MA corresponds to observations of former investigations describing a decline in hemolytic and cytotoxic activity of MA compared to TEGDMA in different cell lines [29,30]. However, assuming that the hydrolytic degradation of each TEGDMA molecule can provide two molecules of MA (Fig. 1), these underlying molar proportions cannot sufficiently explain a higher than 2-fold increase in toxicity of TEGDMA compared to MA. In this context, former in vivo studies on the metabolism of TEGDMA [31] had proposed that a bioactivation of MA to highly toxic intermediates, like the additionally tested compound 2,3-EMA, might occur.

Metabolic biotransformation reactions, e.g. P450-mediated Phase I oxidations, are known to frequently result in the generation of more reactive intermediates (i.e. electrophils such as epoxides), thus eliciting toxic injury to the lung [22]. Since it has been shown that intracellular MA could serve as a precursor molecule for epoxidation [10] our results support the hypothesis that after membrane permeation of the lipophilic comonomer TEGDMA toxic intermediates like 2,3-epoxymethacrylic acid might be generated, which then could contribute to TEGDMA-related cytotoxicity in vitro.

For the comonomer TEGDMA, cytotoxic effects [32] and the induction of apoptotic processes [33] have formerly been described. Likewise, TEGDMA-induced GSH-depletion in various cell models could previously be shown [34,35]. It also has been suggested that components of resin materials may lead to damage of DNA as prior findings provide evidence for the induction of micronuclei, gene mutations and DNA sequence deletions by TEGDMA [36]. It has been well established that TEGDMA-intermediates, like the detected epoxide 2,3-EMA [10], are highly reactive and unstable and therefore tend to exert toxicity in situ rather than in distant tissues. An involvement of P450 enzymes in the mechanisms of toxicity of a wide variety of lung toxicants has been well documented [22]. Due to the high metabolizing capacity of A549 cells, a bioactivation of the tested monomer with the formation of highly toxic intermediates, which might then react with different cell biomolecules, seems plausible. A high reactivity of epoxides towards biological nucleophils, like GSH and the DNA, has earlier been described [37]. However, it has to be assumed that due to subsequent detoxification processes of potentially cytotoxic and mutagenic epoxides by soluble and microsomal epoxide hydrolases [38] a possibly occurring intermediate-associated damage is strictly limited to the cellular level.

5. Conclusion

The results of this study indicate, that from all investigated TEGDMA-intermediates, 2,3-EMA was identified to have the highest cytotoxicity in pulmonary A549 cells. In vivo, TEGDMA-intermediates are excreted via the lungs, but do not reach cytotoxic levels [9]. However, possible mutagenic effects of 2,3-EMA will need further investigations.

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