Biological effects of filling materials widely used in dentistry

Experimental study of mitochondrial toxicity of TEGDMA

Doctoral thesis

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INTRODUCTION

Due to the continuous growth of human life and the aging of the population, long-lasting biocompatible materials are needed in dentistry. Unfortunately, materials used to replace the teeth and the missing tooth tissues still do not meet all expectations. Mechanical properties, biocompatibility and adhesion of direct restorative materials to dental tissues are generally not ideal and need further development. In 2013, the Uited Nations Environment Program emphasized the harmful effects of mercury on the human body and the surrounding environment. In May 2017, the European Union adopted the so called Mercury Regulation based on the United Nations Minamata Convention, which regulates the phase-down of dental amalgam. In this thesis I discuss the advantages and disadvantages of possible amalgam alternatives; of glass ionomer cements and resin-based aesthetic composites.

Composites are resin-based restorative materials and in dentistry they play an essential role when it comes to functional and aesthetic restoration of dental hard tissues. Most resin-based composites contain anorganic substances (e.g., quartz, ceramic, lithium-aluminum silicate particles, etc.), and organic resin monomers (e.g., A-glycidyl dimethacrylate (Bis-GMA), hydroxyethyl methacrylate (HEMA), urethane dimethacrylate (UDMA), triethylene glycol dimethacrylate (TEGDMA)) and a silane phase that provides bonding effect between the two major components. Dental resin monomer mixture (Bis-GMA, TEGDMA, HEMA, UDMA) widely used in dentistry provides good mechanical properties, fast polymerization, ease of handling and fastening to enamel, for example in aesthetic filling materials, bonding systems, and fixing cements. To reduce viscosity, low molecular weight co-monomer of lower viscosity, TEGDMA, is added to the aesthetic filling materials, which contribute to the increase of filler concentration. The prevalence of TEGDMA in resin-based composites, glass ionomer cements and bonding systems is high at 25-50%. Increased quantities of TEGDMA leading to undesirable properties are increased polymerization shrinkage, resulting in reduced life of restorations. During the polymerization process a cross-linked polymer is formed, which results in a reduction in the distance between the molecules and leads to polymerization shrinkage. Consequently, internal tension and stress may develop in the material during adhesive technique. This can cause deformation, fracture cracking and micro cracks in restorations. Polymerization shrinkage may be the main cause of postoperative pain, microcracks, cusps fracture and the micro gap between the surface of the tooth and restoration, resulting in secondary caries. Incomplete resin-based composite polymerization may lead to undesired leaching of further monomers can be released by biodegradation and

erosion throughout the lifetime of the filling. The non-reacted monomers in the composite fillings may reduce the clinical durability of the materials, and may increase the discoloration of the fillings by oxidation and hydrolytic degradation.

The biocompatibility of dental materials has become a challenging issue in the last decades. Despite the significant development of composites in recent time, their biocompatibility is questionable, due to their long-term chemical and biological degradation in addition to insufficient polymerization. Cholesterolesterase and pseudo-cholinesterase can break down the monomer components of composites, which can release methacrylic molecules. BisGMA and TEGDMA released from composites may alter the proliferation and metabolism of S. mutans, thereby facilitating the formation of biofilm. Polymerization shrinkage, dissolving methacrylate monomers (TEGDMA, UDMA, HEMA, Bis-GMA, etc.) and degradation of the composites (chemical, mechanical, enzymatic, light and heat) are responsible for the main clinical disadvantages.

The research activity on biocompatibility of dental materials is increasing, the most common are in vitro experiments on cytotoxicity and genotoxicity. The topic is highly relevant; not only researchers but also dentists show increasing interest in literary data.

Due to incomplete polymerization, residual monomers (TEGDMA, HEMA) are released in millimolar amounts in the pulp and saliva, which can contact the oral tissue and enter the gastrointestinal system. Monomers may be present for a long time in the oral cavity even with the appropriate polymerization time. Monomers are able to cross the dentin, enter the pulp and may indeed affect cellular functions. The role of allergy in the adverse effects of monomers and many components of composites is also emerging. However, the presence of free monomers in the human body is negligible, probably no systemic adverse effects can be detected apart from allergies. Most studies have investigated the effect of resin components on the essential functions of cells: e.g. cell proliferation, inhibition of enzyme activites, cell membrane integrity, cellular metabolism (DNA, RNA and protein synthesis), cell viability, genotoxicity and estrogen-like effects, etc.

The TEGDMA is of amphiphilic character and the most common co-monomer released from polimerized composites. Depending on the thickness of the dentin and the quality of the polymerization, TEGDMA in the pulp was shown to reach levels up to 4 mM. Apart from clinically manifested contact allergies, the systemic blood concentration of the monomers are orders of magnitude lower, therefore the probability of the development of TEGDMA-

induced adverse systemic effects is negligible. TEGDMA and HEMA were reported to affect various cellular functions and the viability of the cells in the oral cavity. It was reported earlier that TEGDMA could initiate cellular stress responses, induce reactive oxygen species (ROS) generation, and cause glutathione (GSH) depletion in a wide variety of eukaryotic cells in vitro. The genotoxic and mutagenic effects of TEGDMA are likely consequences of ROS-triggered DNA damages; the toxic effects could apparently be eliminated applying ROS scavengers. It was demonstrated that TEGDMA could induce, apoptotic or necrotic cell death depending on the concentration. Many studies have shown that monomer-induced apoptosis is associated with oxidative stress with increased ROS formation.

All these results assume the central role of ROS in the cytotoxicity and genotoxicity of TEGDMA and in inducing pathways leading to cell death. These results in the literature raise the possibility of a common mechanism and perhaps molecular target which would be responsible for the cellular effects described. Given that mitochondria play a very important role in apoptosis, cell viability determination, ROS formation and elimination, it seems to be interesting to look for mitochondrial targets in the toxicity of TEGDMA. Considering that mitochondrial damage may be primary or secondary, but such differentiation cannot always definately be made in cellular systems, isolated mitochondria have been selected as the focus of our studies. According to our hypothesis, most of the toxic effects of TEGDMA could potentially be explained by the development of bioenergetic insufficiency and increased ROS generation. The hypothesis that mitochondrial dysfunctions could be responsible for the onset of either necrotic or apoptotic cell death does not demand any sophisticated verification. It is likely that mitochondrial dysfunctions are responsible for necrotic or apoptotic cell death.

In the present study, low millimolar concentrations of TEGDMA were used to challenge isolated brain mitochondria and the most important bioenergetic functions were evaluated in parallel with the measurement of mitochondrial H_2O_2 homeostasis. We propose that inhibition of the mitochondrial respiratory CI may be responsible for most of the cytotoxic effects of TEGDMA.

AIMS AND OBJECTIVES

The phase-down of amalgam is a main issue nowadays. At the beginning of my dissertation, I tried to find answers to possible filling materials alternatives. In Hungary resin based composites are widely used. Due to the biodegradation of the composites, the mechanical properties and biocompatibility of the material are still imperfect today and need further improvement. The composites contain a number of components which make it very difficult to specify the mechanisms behind their toxicity, that in literature mostly have been studied in vitro in gingival fibroblasts.

The aim of my work is to investigate the background of the cytotoxic and genotoxic effects of TEGDMA resin monomer which is the most soluble resin component in composites. In the international literature, there is still limited data available for understanding the cytotoxic mechanism of TEGDMA. The adverse effects described in the literature, the increased ROS production, the DNA damage caused by the suspected ROS and the apoptosis were assumed to be common targets. Regarding that mitochondria play a significant role in apoptosis, cell viability determination, ROS generation and elimination, it seemed logical to look for mitochondrial targets in the toxicity of TEGDMA. Most of the toxic effects of TEGDMA are assumed to be due to adverse bioenergetic parameters and increased ROS production. Therefore, we investigated the bioenergetic effects of TEGDMA on isolated brain mitochondria and parallel ROS homeostasis. We examined the effect of TEGDMA on mitochondrial O_2 consumption and membrane potential in the presence of NADH- and $FADH_2$-dependent respiratory substrates. In parallel, we investigated the effect of TEGDMA on ROS production and elimination. The effects of TEGDMA on ATP synthesis and GSH were also investigated in our study. In our experiments, we wanted to answer the following questions:

I. How does TEGDMA affect mitochondrial O_2 consumption in CI and CII-dependent (NADH-dependent and $FADH_2$-dependent substrates) respiratory substrates energized isolated brain mitochondria (in vitro)?

II. In parallel with the studies of O_2 consumption, how does TEGDMA affect mitochondrial membrane potential ($\Delta\psi m$)?

III-IV.

How does TEGDMA change the mitochondrial H_2O_2 generation and NAD(P)H level in the presence of CI or CII-dependent respiratory substrates?

Is there any relationship between TEGDMA and reduced glutathione level?

V. What is the impact of TEGDMA on ATP synthesis?

VI. Does direct measurement support that CI is a primary target of TEGDMA?

MATERIALS AND METHODS

Mitochondria were isolated from guinea pig brain cortex using a Percoll gradient, by differential centrifugation. Animal experiments were performed in accordance with the relevant guidelines of the National Institute of Health (USA) and the Semmelweis University.

I. Measurement of mitochondrial oxygen consumption

Mitochondrial respiration was monitored using the highresolution respirometry system Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). Oxygen sensors were calibrated at air-saturation and in oxygen-depleted media. During the measurements 0.1, 1.0, 2.0 and 5 mM TEGDMA were investigated and dimethylsulfoxide (DMSO) was added as control compound.

II. Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial $\Delta\psi_m$ was detected using safranine O fluorescent dye (2M). This lipophilic cationic dye gets distributed among mitochondria and the surrounding medium; the value of the partition coefficient is the function of the actual mitochondrial ($\Delta\psi_m$). Measurements were performed using a spectrophotometer at 495 and 585nm excitation and emission wavelengths and at 37 C°.

III-IV. Parallel measurements for H_2O_2 and NAD(P)H

H_2O_2 formation by isolated mitochondria was detected using the Amplex Ultra Red fluorescence assay. In this assay, Amplex Ultra Red (3μM) is converted to the highly fluorescent compound resorufin by H_2O_2 and horseradish peroxidase. Fluorescence was detected using spectrophotometer; for excitation and emission, the 550 and 585nm wavelengths were applied, respectively. Each measurement was calibrated by 100 pmol H_2O_2 at the end of the experiment. H_2O_2 production were detected in glutamate *plus* malate (GM) (5-5 mM) or succinate (Succ) (5 mM) energized mitochondria in the presence and absence of TEGDMA.

In a parallel experiment, using the double-excitation/double-emission mode of the spectrofluorimeter, NAD(P)H fluorescence was also measured at 340 and 466 nm excitation and emission wavelengths, respectively.

Measurement of mitochondrial H_2O_2 elimination

Mitochondrial elimination of H_2O_2 was measured as previously described. Briefly: glutamate plus malate supported mitochondria (0.1mg/mL) were preincubated in either the presence or absence of TEGDMA (5mM) or DMSO for 5min. At 5min H_2O_2 (10 µM) was given, then 50 µl samples were taken at 30 s intervals for 2min, and the residual H_2O_2 was determined by Amplex Ultra Red fluorimetry.

Measurement of reduced glutathione (GSH) consumption

The measurement of reduced glutathione (GSH) is based on the reaction of the thiol SH group with 5,5'–dithiobis (2- nitrobenzoic acid) (DTNB). Freshly diluted GSH (2mM) was incubated in the reaction medium at 37 ◦C for 15 minute intervals in the presence of TEGDMA or DMSO. Aliquots were taken and the generated TNB was measured via spectrofotometry at 412 nm.

V. Measurement of ATP production

ATP production by isolated mitochondria was detected using a coupled enzyme assay that applies hexokinase and glucose6-phosphate dehydrogenase. In the medium, ATP phosphorylated glucose via hexokinase and the produced glucose-6-phosphate got further converted to 6-phosphogluconate via glucose-6-phosphate dehydrogenase; with the concomitant reduction of NADP+ to NADPH. NADPH formation is proportional to ATP synthesis in a 1: 1 stoichiometric ratio after calibration. Light absorbance of NADPH was recorded at 37 ◦C and 340nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) using a spectrophotometer.

VI. Detection of mitochondrial respiratory Complex I activity

The assay measures the rate of the conversion of NADH to NAD^+ while the electron acceptor is Coenzyme Q1 (CoQ1). The initial rate ofthe change in absorbance at 340 nm was detected at 37 ◦C using a spectrophotometer.

VII. Statistics

Data was evaluated using SigmaPlot. Pairwise comparisons were evaluated by Student's t-test. Statistical differences for multiple comparisons were evaluated using the one-way

ANOVA method, or for data not following normal distribution ANOVA on ranks Kruskar Wallis test was applied. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

I. The effect of TEGDMA on mitochondrial respiration

Mitochondrial respiration is a sensitive indicator of mitochondrial functions. Mitochondria were energized with either alpha-ketoglutarate (α-KG), or glutamate plus malate (GM) or succinate (Succ) as respiratory substrate.
α-KG or GM entering the mitochondria are metabolized in the citrate cycle, which results in NADH gets oxidized on CI. Since the produced NADH is oxidized by CI, α-KG and GM are both considered to be Complex I substrates; whereas $FADH_2$ related to oxidation of succinate is predominantly associated with Complex II. In the presence of respiratory substrates, oxygen consumption was detected, followed by ADP (2mM) treatment initiated oxidative phosphorylation. Afterwards, mitochondria were challenged with TEGDMA. Experiments were terminated with carboxyatractylate (CAT) (2µM) which inhibits ADP-ATP exchange.

- **Oxidation of Complex I substrates**

In the presence of ADP oxygen consumption was significantly increased both in α–KG- and GM-supported mitochondria. The high respiratory control ratio (RCR: oxidation rate in the presence of ADP vs oxidation rate in the absence of ADP) indicated the high quality of mitochondrial preparations used in this study (RCR > 7). A subsequent administration of TEGDMA (5mM) resulted in 69% decrease in oxygen consumption in α–KG supported mitochondria. Challenging mitochondria with an ANT inhibitor decreased the α–KG -supported respiration both in the absence and presence of TEGDMA.

Similarly to the effect observed with α–KG, addition of TEGDMA (5mM) decreased the rate of the ADP-stimulated respiration by 88% in GM supported mitochondria. Significant effect of TEGDMA on ADP-stimulated O_2 consumption was detected from 2mM concentration. At lower TEGDMA concentrations (0.5, 1.0 mM) there was no significant effect. CAT lowered the rate of oxygen consumption both in TEGDMA-treated and non-treated mitochondria. The DMSO solvent used in our studies had no toxic effect on mitochondria. In the experiments with buffer and DMSO, the same effects were detected in our measurements.

- **Oxidation supported by a Complex II substrate**

Unlike with the NADH-linked substrates, in Succ supported mitochondria TEGDMA given after ADP did not influence significantly the rate of oxygen consumption. These results unequivocally imply that TEGDMA does not inhibit Complex II function in the respiratory chain.

In the present study, the reduced rates of mitochondrial respiration observed in the presence of CI substrates can potentially be explained by: (i) a decreased ATPase activity, (ii) the inhibition of ANT, (iii) a reduced rate of transport for metabolites and/or inorganic phosphate, or (iv) the compromised activities of CI and/or selected dehydrogenase enzymes. However, the normal rate of respiration which was detected in the presence of succinate excluded the reasons listed under i, ii, and partially iii. In order to elucidate the mechanism that lies behind the depression of respiration the effects of TEGDMA on mitochondrial Δψm were assessed.

II. Effect of TEGDMA on the mitochondrial membrane potential (Δψm)

The (Δψm) is an important parameter of mitochondrial bioenergetic competence, whose building up is a prerequisite for efficient oxidative phosphorylation-dependent ATP synthesis. Δψm was measured in isolated mitochondria supported by either α-KG or GM or Succ. In the presence of respiratory substrates, the inner membrane became hyperpolarized, indicated by a decrease of safranin fluorescence. Δψm was higher in GM-supported mitochondria than in mitochondria respiring on α-KG, reflecting the higher rate of oxidation of GM. Subsequent addition of ADP caused depolarization with both substrates. Both in α-KG and in GM supported mitochondria, TEGDMA induced further depolarization. Addition of CAT to TEGDMA treated mitochondria resulted in a complete depolarization, as opposed to control mitochondria where CAT induced membrane hyperpolarization. The total depolarization of mitochondria was achieved using uncoupler carbonylcyanidep-trifluoromethoxy-phenylhydrazone (FCCP).

Contrary to that observed with CI substrates in succinate supported mitochondria TEGDMA did not depolarize mitochondria in the presence of ADP and subsequent addition of CAT resulted in an elevation of Δψm both in TEGDMA-treated mitochondria and in control condition. Consequently Δψm measurements also support the notion that Complex I activity is selectively inhibited by TEGDMA.

These results again demonstrate that TEGDMA does not inhibit ANT or ATP-ase; if selective inhibitors of these molecules are used, Δψm would become hyperpolarized, regardless of the respiratory substrate used. The results so far are consistent with the assumption that TEGDMA selectively inhibits CI activity.

III. The effect of TEGDMA on H_2O_2 generation and elimination

As we discussed earlier, there is a general agreement that exposition of cells to TEGDMA provokes oxidative stress. However, the sources and mechanisms of the redox imbalance have not been elucidated, yet. Our hypothesis that mitochondrial targets are also involved in the cellular toxicity of TEGDMA would be substantiated if administration of TEGDMA would be able to stimulate the mitochondrial ROS production. We have examined this hypothesis in the following. In the following experiments, mitochondrial H_2O_2 production and elimination were both measured in the presence of CI substrates and H_2O_2 production was also measured with non-CI substrates like Succ and alpha-glycerophosphate (α-GP).

➢ H_2O_2 formation in mitochondria supported by NADH-linked substrates

GM and α-KG both initiated H_2O_2 production in mitochondria. Both α-KG and GM stimulated the H_2O_2 production, but to different extent in the absence of ADP. The higher rate of H_2O_2 production with GM can be ascribed to the higher rate of GM-induced respiration and to the consequently higher $\Delta\psi_{m.}$ There is a direct correlation between the membrane potential and H_2O_2 production (Starkov et al). Addition of ADP differently inhibited H_2O_2 formation on α-KG and GM respiring mitochondria. The higher was the membrane potential before the ADP addition, the greater was the decrease of H_2O_2 production. TEGDMA augmented the rate of H_2O_2 production both in α- KG- and in GM-supported mitochondria after ADP treatment. Elevated rates of H_2O_2 production in the presence of CI inhibitors previously described by several authors were observed in our study. CI possesses several sites that are capable of producing ROS. In the electron transport chain, those electron carrier complexes that are upstream to the binding site of a respiratory chain inhibitor are in highly reduced states. Reduction of a ROS-forming site increases the likelihood for an electron leakage. Inhibition of CI in CI substrate (glutamate plus malate) supported mitochondria stimulated H_2O_2 formation in either the presence or absence of ADP. Enhanced H_2O_2 production therefore can deplete endogenous antioxidants such as glutathione and inhibit glutathione-related antioxidant systems.

Effect of pretreatment with TEGDMA on ROS production

Continuous monitoring of mitochondrial H_2O_2 production with Amplex Ultrared plus horseradish perosidase (HRP) not only detects, but also detoxifies the generated H_2O_2 mitigating the oxidative damage. To eliminate this, mitochondria were pretreated with

TEGDMA for 10 minutes in the presence of GM and in the absence of Amplex + HRP. In GM-energized mitochondria, in the absence of Amplex UltraRed, higher H_2O_2 formation was detected than in the presence of Amplex UltraRed. After the 10 min preincubation in the presence of TEGDMA (5mM) and ADP, 95% stimulation of H_2O_2 formation was detected compared to the absence of TEGDMA.

H_2O_2 elimination in mitochondria supported by NADH-linked substrates

Considering that mitochondria play roles in both ROS formation and ROS elimination, the effect of TEGDMA on the elimination of exogenous H_2O_2 was also investigated. Mitochondria were challenged with exogenous H_2O_2 in the presence or absence of TEGDMA (5mM) and the disappearance of H_2O_2 was tested. In the presence of TEGDMA a significant decrease in H_2O_2 elimination was observed in GM supported mitichondria. Our results indicate slower elimination in the presence of TEGDMA, therefore these results further confirm the hypothesis drawn from the preincubation experiment that TEGDMA can impair the antioxidant system. This phenomenon can also be attributed to the inhibition of CI, which results in a decrease in the mitochondrial membrane potential. Due to CI inhibition, NADH accumulates, increases the NADH / NAD^+ ratio, which inhibits the NADH-dependent dehydrogenases of the citrate cycle, thereby retarding the slowing of the citrate cycle. $\Delta\psi m$ is a driving force for the energy-dependent transhydrogenase enzyme that can transfer electrons from NADH to $NADP^+$, thus can maintain the NADPH level, which is necessary for the regeneration of GSH. If $\Delta\psi m$ decreases, NADPH will not be sufficient for GSH regeneration.

The interaction of TEGDMA with GSH

However, it is still obscure whether TEGDMA can directly interfere with the antioxidant system. We reinvestigated this question.
2 mM GSH was incubated with 5 mM TEGDMA for 15 min in mitochondrion-free medium. At 15 min of incubation time significant (but slight change) 3.7% decrease in GSH concentration was detected in the presence of TEGDMA (5mM). There are two possible ways of interaction between TEGDMA and glutathione:

a). direct oxidation of GSH by TEGDMA,

b). direct chemical coordination of TEGDMA and GSH.

The chemical structure of TEGDMA does not justify increased GSH oxidation. In contrast, the chemical coordination of TEGDMA and GSH cannot be excluded by the chemical structures of TEGDMA and GSH. This coordination can potentially reduce the available GSH concentration. For this reduction, it is not necessary that the SH group in the GSH be in direct contact with any of the atoms of TEGDMA, because then the thiol group will not be available in the adduct. In our opinion, this contributes to a small extent to oxidative stress, independently of mitochondria.

- **H_2O_2 formation in the courses of oxidation of non-Complex I substrates**

From $FADH_2$ linked respiratory substrates most of the electrons are transported to oxygen as the final electron acceptor via the Complexes III and IV. In this case, most of the electrons go forward from succinate dehidrogenase (SDH) (forward; forward electron transport - FET) to O_2 and contribute to protone motive force generation. It is well documented however, that with succinate in the absence of ADP a high $\Delta\psi_m$ is generated that drives a certain flux of electrons from coenzyme Q (CoQ) to flow towards the CI, in a direction that is inverse to normal. This phenomenon is referred to as the reverse electron transport (RET) mechanism that results in the conversion of NAD^+ to $NADH+H^+$ at the CI. RET is the predominant ROS-generator when no ADP is present and the mitochondria are supported by either succinate or α-GP. In our experiments, RET was used as a tool to demonstrate the effect of TEGDMA on CI. In such circumstances, the mitochondrial membrane potential is high enough for the electrons to flow from CII to CI, which is reflected in major increase in ROS production. There are no RET for CI-dependent respiratory substrates, hence we detected lower levels of H_2O_2 generation.

H_2O_2 generation in mitochondria supported by succinate

TEGDMA or DMSO were applied to mitochondria energized with succinate. Significant decrease of H_2O_2 formation has been observed already at 0.5mM of TEGDMA. Addition of ADP further decreased H_2O_2 production that was observed in the presence of TEGDMA (5mM) and in DMSO-treated mitochondria. Inhibition of ANT by CAT stimulated again the ROS production. H_2O_2 generation in TEGDMA-treated group was significantly lower than that of control group owing to the CI inhibition. It is important to note that when inhibiting CI (for example rotenone), at high levels of NADH / NAD^+ ratio, citrate dehydrogenases may also be involved in ROS production.

H_2O_2 formation in mitochondria supported by α-GP

In brain mitochondria alpha-glycerophosphate dehydrogenase (α-GPDH) is highly active. This enzyme (such as succinate dehydrogenase) provides the respiratory chain with electrons and can also generate RET. In α-GP-energized mitochondria TEGDMA mediated a 47% suppression of H_2O_2 production. Addition of ADP lowered the rate of H_2O_2 generation in both groups (DMSO or TEGDMA) because ADP stimulation eliminates RET conditions. Under control conditions CAT restored the rate of H_2O_2 production to the level detected in the ADP-free state because CAT hyperpolarized $\Delta\psi_m$ and therefore restored the RET in the DMSO-treated control group. In TEGDMA-treated mitochondria there was also a CAT-mediated stimulation of H_2O_2, but the effect was much smaller than the one measured in solvent-treated mitochondria. This phenomenon can also be explained by the inhibition of CI.

IV. Effects of TEGDMA on the NAD(P)H level

The application of both NADH-linked (α-KG, GM) and $FADH_2$- linked substrates (Succ and α-GP) elevated the mitochondrial NAD(P)H level. Complex I substrates (α-KG, GM) led to NADH formation are oxidized by CI. In the absence of ADP NADH / NAD^+ ratio is high and in the presence of ADP most of the NADH became oxidized. Addition of ADP to energized mitochondria decreased the steady state level of NADH for all the tested respiratory substrates because (i.) ADP stimulates the respiration, (ii.) a higher rate of the electron flow promotes NADH oxidation, and (iii.) an ADP-induced decrease in the membrane potential abolishes the reverse electron flow. In mitochondria respiring on both α-KG and GM TEGDMA induced elevation of NAD(P)H level. With CI substrates TEGDMA-mediated inhibition of the Complex I withheld electrons from reaching the distal sequences of respiratory chain; consequently, NAD^+ mostly got reduced to NADH. This observation is crucial for the mechanism, since it means that TEGDMA inhibits CI but does not affect the dehydrogenases involved in the reaction, because if it were affected, the NADH level would not increase by TEGDMA.

With CII substrates, in the case of high mitochondrial membrane potential, RET is provided, leading to increased NADH formation by CI. In the presence of succinate or α-GP the addition of TEGDMA preceded the administration of ADP to mitochondria because we wanted to know how TEGDMA affects RET. If TEGDMA was given after ADP, we would not have been able to observe the effect of TEGDMA on RET due to ADP depolarization. In

succinate-supported mitochondria addition of ADP was unable to drop NADH concentration to the control level, because CI was inhibited, thus the accumulated NADH was unable to be oxidized. In the presence of CAT there was no difference in the NAD(P)H steady state between control and TEGDMA challenged mitochondria.

V. ATP production in TEGDMA-challenged mitochondria

For a better characterization of the relevant mitochondrial bioenergetics, in addition to oxygen consumption and $\Delta\psi_m$ the rate of ATP production was also assessed. After administering ADP, in the absence of respiratory substrates, a compromised rate of the adenylate kinase (AK) coupled ATP synthesis could still be detected as 200 μM P^1,P^5-di(adenosine-5') pentaphosphate (AP5) was apparently not sufficient to fully inhibit the activity of AK.

Oxidative phosphorylation was initiated with the addition of respiratory substrates in the presence of ADP. In GM-supported TEGDMA-treated mitochondria the rate of ATP production was lowered by 86% as compared with controls. This observation suggests that TEGDMA, which was inhibiting the CI, caused depolarization and the remaining proton gradient was not sufficient to support a high rate of oxidative phosphorylation; compromised ATP synthesis leads to cellular bioenergetic insufficiency and eventually necrotic cell death. However, addition of TEGDMA (5mM) to succinate-energized mitochondria resulted in a small drop in the rate of ATP synthesis.

This is consistent with our results so far; since TEGDMA inhibits CI, electrons from SDH to CII can flow freely to the CIV and the electrochemical potential can be translated into ATP production.

VI. The effect of TEGDMA on CI activity

In order to prove our hypothesis that TEGDMA was an inhibitor of CI, the CI activity was directly measured. The CI activity was significantly reduced in the presence of 5 and 2mM TEGDMA. The direct measurement of CI activity proved that TEGDMA can indeed inhibit the CI. The immediate effect of TEGDMA suggests that CI can in fact be a primary target of this resin monomer.

CONCLUSIONS

The phase-down of amalgam is an essential factor for reduce environmental mercury pollution, regulated by the EU Directive 2017/852. Along with the complete phase out, the additional amalgam alternatives (glass ionomer cements and resin-based composites) require further material development to develop ideal restorative materials. The aim of the dissertation was to identify a direct molecular target in cytotoxic, genotoxic effects and oxidative stress induced by TEGDMA, which is the most common co-monomer released from resin based composites due to their inadequate polymerization and biodegradation.

In the present thesis, the effects of TEGDMA have been studied on the bioenergetic functions and ROS homeostasis of mitochondria isolated from brain, because mitochondria play an important role in ROS generation and elimination, apoptosis and cell viability. Our new results are as follows:

- In CI-dependent respiratory substrates supported isolated brain mitochondria the O_2 consumption was inhibited by TEGDMA resin monomer. Contrary to that respiration was not influenced by CII substrates.
- The administration of TEGDMA induced the depolarization of the mitochondrial membrane *via* CI substrates, resulting in decreased ATP production *via* oxidative phosphorylation.
- TEGDMA significantly stimulated the rate of H_2O_2 (ROS) generation in CI-dependent respiratory substrates supported mitochondria and also limited the H_2O_2 elimination. However, direct interaction between TEGDMA and reduced glutathione has not been verified in our studies, therefore the decreased GSH level is attributed to the mitochondrial effects of TEGDMA.
- Our study investigated on isolated mitochondria has shown that TEGDMA impairs mitochondrial functions and inhibits CI in the mitochondrial respiration chain. Inhibition of CI explains the cellular toxic effects of TEGDMA described in the literature.

In our study, we have shown that the cytotoxic effects of -a highly active methacrylate molecule - TEGDMA resin monomer are greatly explained by CI inhibition. Consequences of CI inhibition include decreased O_2 consumption, increased H_2O_2 production, and decrease of mitochondrial H_2O_2 elimination, resulting in oxidative stress and apoptosis. Decreased ATP

production at high TEGDMA concentrations can cause necrotic cell death. A number of publications in the literature discuss the positive effects of antioxidants, which can eliminate the harmful effects of TEGDMA, and research results on the development of new monomers are also encouraging. In our opinion, finding a probable molecular target for the TEGDMA resin monomer may contribute to further successful material development in dentistry.

ABBREVIATIONS

α-GP: alpha-glycerophosphate

α-KG: alpha-ketoglutarate

α-KGDH: alpha-ketoglutarate dehidrogenase

AK: adenilate kinase

ANT: adenin-nukleotid translocase, ADP/ATP transporter

AP5: P^1,P^5-di(adenosine-5′) - pentaphosphate

CI: mitochondrial complex I

CII: mitochondrial complex II, succinate dehidrogenase

CIII: mitochondrial complex III

CIV: mitochondrial complex IV, citochrome c oxidase

CAT: carboxyatractylate

DMSO: dimethyl-sulfoxide

ΔpH: transmembrane pH gradient

$\Delta\Psi_m$: mitochondrial membrane potential

FET: forward electron flow

FCCP: carbonylcyanide-p-trifluoromethoxy-phenylhydrazone

GM: glutamate *plus* malate

GSH: glutathione

H_2O_2: hidrogen peroxide

KoA: koenzim-A

pmf: proton motive force

RET: reverse electron transfer

ROS: reactive oxygen species

Succ: succinate

LIST OF PUBLICATIONS

Publications of the author in the scope of the present work

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