Doctoral (Ph.D.) Thesis

Investigations into the pulpal responses to TEGDMA monomer exposure

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List of abbreviations

AIF	apoptosis-inducing factor
Bis-GMA	bisphenol a-glycidyl methacrylate
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
HEMA	Hydroxyethyl methacrylate
HRP	horseradish-peroxidase
JNK	c-Jun N-terminal kinase
kDa	kilodalton
MMP	matrix metalloproteinase
OPF	oxidative protein folding
PVDF	polyvinylidine fluoride
RBC	resin-based composites
TEGDMA	triethylene glycol dimethacrylate
TIMP	tissue inhibitor of matrix metalloproteinases
TNF-α	tumour necrosis factor- α
WST-1	water-soluble tetrazolium salt-1

Introduction

Composite resin filling materials have been used for over 50 years for the restoration of destructed teeth and represent one of the great successes of biomaterials as they adequately replace dental hard tissue as well as aesthetics. They not only possess comparable mechanical and longevity properties but they successfully overcome many drawbacks of their amalgam precursors such as aesthetics, handling, and preservation of tooth structure. Composites are used for a variety of dental applications not limited to restorations, cores, build-ups, onlays, crowns, cavity liners, cements, and orthodontic appliances. Due to this versatility their popularity is likely to continue to rise.

As a result of the inherent chemistry of the material formulations, complete polymerisation of resin-based composites is currently not possible which gives rise to unreacted monomers. In light of their wide application the toxicity of various leachable components of the material mixture has been a popular topic among researchers. Resin monomers including Bis-GMA, HEMA, and TEGDMA - all commonly used in composites- have already been confirmed to be cytotoxic on various cell types such as human gingival fibroblasts, dental pulp cells, and immortalised keratinocytes. Although resin chemistry has developed significantly over the last decade, leading to overall safer composite filling materials, well designed investigations into monomer toxicity have again and again uncovered potentially deleterious cellular consequences many details of which are thus far unclear.

Aims of the investigations

Based on the above:

1. The overarching aim of both of our investigations was to delineate further the specific pulp cell responses to TEGDMA monomer exposure. Although many details of monomer-induced cell death have been uncovered, there are still areas of great ambiguity. Clinically relevant concentrations of TEGDMA have predominantly been shown to lead to apoptosis in pulp cells. Studies have attempted to uncover which of the two principle apoptotic pathways -intrinsic or extrinsic- is chiefly activated, with conflicting results. To the best of the author's knowledge, no study has so far investigated the possible role of ER stress or the activation of caspase-independent apoptotic pathways -such as AIF- in TEGDMA-induced pulp cell death. In light of the above the aim of this in vitro study was three-fold; to confirm the dose- and time-dependent cytotoxicity of TEGDMA monomers, to confirm the predominant apoptotic pathway activated, as well as to determine whether ER stress and/or caspase-independent pathways could also be involved in mediating cell death in TEGDMA-exposed pulp cells.

2. Aim of the second study was to investigate the effect of TEGDMA monomers on protease synthesis and activity in pulp cells. Clinical and histological observations have shown that the placement of resin-based composite restorations may lead to temporary pulp inflammation which is thought to at least in part be related to the leaching of unreacted monomers from the set composite fillings. The role of matrix metalloproteinases (MMPs) in various dental pathologies such as caries, pulpitis, and periapical inflammation is well documented. Many studies have recently demonstrated the role of *in situ* dentine MMPs, thought to

be activated by leaching unreacted monomers, in degrading the bond at the tooth/restoration interface. Information however, on the possible effects of leachable monomers on *intrapulpal* MMP production and activity is lacking. A rise in intrapulpal MMP production and activity would potentially suggest a role for MMPs in post restoration pulp inflammation, residual caries progression and bond weakening. Thus, the aim of the second study was to establish the effect of low concentrations of TEGDMA monomers on MMP-2, -8, and -9 production and total collagenase/gelatinase activity in pulpal cells. Secondary objective was to correlate changes in the activation of ERK 1 and 2, p38, and JNK signalling molecules with possible changes in MMP production and activity.

Materials and Methods

Investigation into the cytotoxicity of TEGDMA monomers and the mechanisms of monomer-induced pulp cell death

Pulp tissue was isolated from five healthy third molar teeth extracted for orthodontic reasons. Informed consent was obtained as per a protocol approved by the University of Pecs. Following extraction, pulp tissue was isolated according to a technique previously described in the literature and cultured through an explant method in minimum essential medium eagle-alpha modification (Alpha MEM) containing ultraglutamine 1, ribonucleosides, and deoxyribonucleosides (Lonza, Basel, Switzerland) with the addition of 10% fetal bovine serum (FBS, Euroclone, Milan, Italy), and antibiotics. Following three passages, cells were seeded at an arbitrary density of 2×10^4 cells/cm^2 based on previous experience with similar populations.

Pulp cells were initially exposed to 0.75, 1.5, and 3 mM TEGDMA for a period of 5 days based on relevant literature data. Two cell-counting methods, and a water-soluble tetrazolium salt-1 (WST-1) colorimetric assay was subsequently used to assess cytotoxicity.

WST-1 colorimetric assay is frequently used as an indicator of mitochondrial metabolism, hence viability. If the cell is viable WST-1 is reduced to a yellow-coloured formazan dye by cellular dehydrogenases which can be measured with a spectrophotometer. Thus, colour change is directly proportional to viability. As per manufacturer's instructions, following TEGDMA exposure the medium was removed and 200 µl of WST-1 reagent (Hoffmann-La Roche, Basel, Switzerland) in a 1:9 WST to 2 % Alpha MEM medium ratio (180 µl of medium and 20 µl of WST dye) was added. Cells were subsequently stored at 37 °C for 4 hours and then transferred to a 96-

well plate. Absorbance in 100 µl samples was measured with a FluoStar Optima plate reader (BMG Labtech, USA) at 440 nm.

We assessed cell death also through cell counting in marked viewing fields as well as with the application of a Hemocytometer. For the latter, after monomer exposure cells were collected with trypsin followed by their suspension in 10% FBS containing Alpha MEM medium. One drop of the cell suspension was subsequently transferred into the counting chamber of a hemocytometer and viable cells were counted under a phase contrast microscope. For cell counting in marked fields of view, three areas were labeled on the bottom of each well of the culture plates. Similarly, a phase contrast microscope was used to count the number of viable cells in the same designated areas on days 1, 2, and 5 of the monomer-exposure.

Due to the kinetics of cell death observed in the 5-day study, for the investigations into the activation of various apoptotic pathways, cells were exposed to 0.1, 0.2, 0.75, 1.5, and 3 mM TEGDMA concentrations, however this time for only 24 hours. Western blotting was subsequently employed to assess changes in the concentrations of cleaved caspase-3, caspase-8, caspase-9, caspase-12 and apoptosis inducing factor (AIF)- all specific to various apoptotic pathways.

Briefly, after TEGDMA treatment, cells were harvested and lysed as detailed in published studies. Pulp cells were first collected in cold lysis buffer (50 mM Tris-base, pH 7.4, 10% glycerol, 150 mM NaCl, 1 mM EGTA, 1 mM Na-orthovanadate, 100 mM NaF, 5 µM $ZnCl_2$, 10 µg/mL aprotinin, 1 µg/mL leupeptin, 1 mM PMSF, 1% Triton X-100), homogenized for 20 s, and then centrifuged for 30 min at 4 °C (RCE=40,000x *g*). The protein concentrations of the supernatants were determined with Lowry's method and then the samples were diluted to contain an equal amount of 30 µg of protein. Following addition of the Laemmli buffer and denaturation, proteins were separated based on molecular size in a 10% SDS-containing polyacrylamide

gel and then blotted to polyvinylidine fluoride (PVDF) membranes. Nonspecific binding sites on the membrane were blocked with nonfat dry milk (3%). For the labelling of proteins rabbit polyclonal primary antibodies specific to cleaved caspase-9, caspase-3, caspase-8, caspase-12, AIF and horseradish-peroxidase (HRP)-conjugated polyclonal goat anti-rabbit secondary antibodies were used. The enhanced chemiluminescent signal was detected using a G:box gel documentation system (Syngene International Ltd., Bangalore, India). Densitometry analysis was performed using the ImageJ software (National Institutes of Health, Bethesda, USA). Results were gathered from a series of four independent experiments. Statistical analysis was undertaken with the Graphpad Prism software. The normality distribution of the data was tested with the Kolmogorov–Smirnov test, while a one-way analysis of variance (ANOVA) test, supplemented with a Tukey's post hoc test for multiple samples were used to highlight the significances in the differences.

Investigation into the influence of TEGDMA monomer on MMP-2, -8, -9 production and total collagenase activity in pulp cells.

Pulp tissue was isolated and cultured as described above. For preliminary viability testing a 1-day exposure to concentrations of 0.1, 0.2, 0.75, 1.5, and 3 mM was carried out in order to find the exposure conditions not yet causing significant cell death but allow for the monitoring of intracellular stress responses. Based on the results of our pilot studies, cells were subsequently exposed to TEGDMA concentrations of 0.1, 0.2, and 0.75 mM for 24 hours. Viability changes were assessed with a WST-1 colorimetric assay as described above. Western blotting and a specific EnzCheck Gelatinolytic/ Collagenolytic activity assay kit were employed to detect changes in MMP-

2, -8, -9 and total collagenase/gelatinase activity, respectively. Changes in MMP production and localization were also analyzed and visualized with immunofluorescence microscopy.

The EnzCheck Gelatinolytic/ Collagenolytic activity assay kit contains fluorescein-bound gelatin conjugated to such a high degree that fluorescence is quenched. The specific gelatin substrate is digested by most, if not all gelatinases and collagenases liberating fluorescent peptides. Since MMPs represent most of such enzymes, fluorescence can be considered to be directly proportional to total MMP activity and can be monitored with a special spectrophotometer. The assay uses Clostridium histolyticum collagenase as reference.

Following the manufacturer's instructions, the highly labelled fluorescent gelatin substrate was mixed with reaction buffer in a final volume of 200 µL. This was subsequently added to the cell cultures or dilutions of mechanically homogenized cell extracts suspended in lysis buffer. The samples were finally collected into 96-well plates. Proteolysis was determined with a Promega Glo Max plate reader (Madison, Wisconsin, USA), operated at a fluorescent excitation maximum of 495 nm and emission maximum of 515 nm.

The method for the western blot analysis was the same as described above. For the labelling of proteins rabbit polyclonal primary antibodies specific to MMP-8, MMP-9, ERK1/2, p-p38, and p-JNK, rabbit monoclonal antibody specific to MMP-2 and horseradish-peroxidase (HRP)-conjugated polyclonal goat anti-rabbit secondary antibodies were used. As per above, samples were analyzed using a G:box gel documentation system. Densitometry analysis was performed using the ImageJ software (National Institutes of Health, USA).

Changes in the production and localization of MMPs was also visualized with immunofluorescence microscopy. Briefly, a quick rinse in 37 °C PBS was followed by a 4% paraformaldehyde fixation. The fixative was removed,

followed by permeabilization with 0.1% Triton X-100 in TBS at 4°C. Nonspecific binding sites were blocked with 5% nonfat dry milk. This was subsequently followed first by incubation with the primary antibodies specific to MMP-2, MMP-8, and MMP-9 and then with Cy3-conjugated polyclonal donkey-anti-rabbit secondary antibodies. Prior to analysis nuclei were also counterstained with Hoechst 33342 (Calbiochem, La Jolla, CA, USA). Images were obtained using an Olympus FV-1000 laser scanning confocal system (Olympus Europa, Hamburg, Germany). Results presented are representative of a series of four independent experiments with similar results. Likewise for this investigation, Graphpad Prism software was used for the statistical analysis, while the determination of raw integrated density values was carried out using the ImageJ software (National Institutes of Health, USA).

Results

Investigation into the cytotoxicity of TEGDMA monomers and the mechanisms of monomer-induced pulp cell death

Cell counting undertaken by two distinctive methods as well as the WST-1 staining showed TEGDMA toxicity to be concentration- and time-dependent on pulp cells. While significant cell death was detected at 24 h after exposure to 1.5 and 3 mM TEGDMA, 0.75 mM TEGDMA did not cause a significant increase in the number of dead cells. Second- and fifth-day results showed the continued destruction of cells with near-complete cell death evident on the fifth day especially with exposure to 3 mM TEGDMA.

One-day exposure to TEGDMA led to an increase in the cleaved variants of all investigated caspases as well as to an induction in AIF production. Significant elevations in cleaved caspases were apparent after exposure to TEGDMA concentrations of 1.5 mM and 3 mM for caspase-3, to

concentrations of 0.2 mM and above for caspase-8, and after exposure to all tested concentrations in the case of caspase-9. The increase in caspase-12 was determined to be significant with treatments above a concentration of 0.75 mM, while significant rise in AIF production at 24 h occurred after exposure to 0.2 mM, 0.75 mM, and 1.5 mM TEGDMA.

Investigation into the influence of TEGDMA monomer on MMP-2, -8, -9 production and total collagenase activity in pulp cells.

The 5-day pilot experiment, carried out to identify the ideal exposure conditions for the subsequent phases of the investigation, confirmed also in this independent experiment the concentration and time-dependent toxicity of TEGDMA monomers on pulp cells. Results show, after 24 hours, a significant decrease in viability upon exposure to 1.5 and 3 mM TEGDMA, while 0.1, 0.2, and 0.75 mM did not affect viability in a significant manner. 2nd and 5th day results proved to be erratic, and extermination of cells was apparent at 3 mM by the 2nd day and at 1.5 mM by the 5th day. Therefore, the highest concentration and longest treatment time applicable for the subsequent experiments was decided to be 0.75 mM and one day, which are conditions not yet causing substantial cell death.
Results obtained with the EnzCheck Gelatinolytic/ Collagenolytic activity assay kit showed after one day, a mild increase in total collagenase/gelatinase activity upon exposure to 0.1 and 0.2 mM TEGDMA (also from the cell culture media upon exposure to 0.75 mM TEGDMA).
TEGDMA exposure also led to increased levels of MMP-2, -8, and -9 in pulp cells, as detected by the Western blot analysis. The lowest TEGDMA concentration of 0.1 mM caused an increase in MMP-2 production only. A strong increase in all tested MMPs was seen after exposure to 0.2 mM TEGDMA. 0.75 mM TEGDMA increased MMP-2 and MMP-8 levels

marginally, without influencing MMP-9 production. As per the images obtained with the immunofluorescence microscopy, while untreated cells labelled weakly or not at all for the MMPs, exposure to 0.1, 0.2, and 0.75 mM TEGDMA resulted in increased MMP-2, -8, and -9 immunostaining in pulp cells. In the cases of MMP-2 and MMP-8 the antigens showed a cytoplasmic distribution, with a grainier appearance for MMP-8. MMP-9 produced a grainy, as well as a filamentous cytoplasmic signal, the latter often in the vicinity of the cell membrane.

With regards to the results pertaining to signal transduction, western blot images showed an increase in the levels of both 44 and 42 kDa variants of phosphorylated (activated) ERK as well as p-JNK, for all tested TEGDMA concentrations after one day. A considerable p38 activation could only be seen after exposure to 0.1 mM TEGDMA.

Discussion

Although slightly tailored to their specific applications, resin-based composites (RBC) in general contain three distinct phases; a polymerizable oligomer resin-based matrix, inorganic fillers and the resin-filler interface, each of which influence material properties in different ways. Polymerisation is initiated with the exposure to external energy (blue light of a specific wavelength) and proceeds through the generation of free radicals eventually forming a highly crosslinked matrix. Additional constituents such as an initiator, coupling agent and various colorants are added to optimise curing behaviour, setting reaction and aesthetics.

Clinical observations suggest that the placement of monomer containing adhesives/ RBCs may lead to pulp inflammation which lasts longer than what could only be attributed to the mechanical irritation of cavity preparation. Investigations have now shown that any component of a set composite restoration may leach out and interact with host tissues. Literature concerning the biocompatibility of various composite components has mainly focused on the possible toxicity of the resin monomer constituents. Even more so since current composite formulations allow for a maximum conversion of approximately 60-70% giving rise to unreacted monomers. Moreover, certain salivary enzymes as well as the mechanical irritation of chewing have shown to cause a slow but prolonged breakdown of the set polymer liberating additional monomers. The cytotoxicity of various monomers has been confirmed on both continues cell lines and cells isolated from target tissues in contact with the filling, however since the observation that composites may cause a low degree of temporary pulp inflammation more studies have employed pulp cells as subjects. Investigations have now confirmed that RBC components can indeed access and interact with the pulp tissue via the dentine tubules. Leaching has been described to be biphasic, with an acute elution of

unreacted monomers over the first day followed by a slower, more prolonged release as fluid penetrates the resin, opens the spaces between the polymer chains further facilitating the leaching of unreacted monomers. Considering that the full cure of modern composites is reached only after 24 hours, the presence of unreacted monomers may be even greater during the initial periods post-placement. Elution studies have consistently found TEGDMA to be leaching in the greatest amount of all the monomers commonly employed in modern resin-based composites and therefore has been a popular target for cytotoxicity studies. This may partially be explained by its chemical structure. In contrast to Bis-GMA, the other main resin constituent, this monomer has a linear backbone which negates the need for intramolecular hydrogen bonding. This imparts greater flexibility, lower molecular weight which leads to better mobility and easier elution into dentine tubules. Their hydrophilicity, surfactant, detergent-like properties render them capable of easily passing the cell membrane. Theoretical calculations based on the molar concentration of TEGDMA used in commercially available composites and the supposed dilution effect of the remaining dentine suggest that intrapulpal concentrations of TEGDMA may reach 4 mM. Although many details of monomer-induced cell death have been uncovered, there are still areas of great ambiguity, therefore the overarching aim of our investigations was to further delineate the details of the specific intracellular responses to TEGDMA exposure in pulp cells.

Our first investigation showed a concentration- and time-dependent cytotoxicity for TEGDMA monomers on pulp cells over a period of 5 days. The number of viable cells decreased exponentially at all time-points upon exposures to 1.5 and 3 mM TEGDMA, with statistically significant deaths occurring already at 24 h compared to the untreated population. 0.75 mM TEGDMA failed to cause a significant rise in the number of dead cells. Aside from confirming the cytotoxic potential of TEGDMA monomers this allowed

us to set sublethal experimental conditions ideal for our subsequent investigations into the specific stress responses. The kinetics of cell death is thought to be dependent on the applied concentration of monomers, exposure time, cell culture, and the sensitivity of the detection method. Considering all, the toxic concentration threshold of 0.75 mM of TEGDMA at 24 hours on pulp cells observed in the present results can be considered to be in line with the vast majority of the literature.

Several studies have causally related TEGDMA monomer-induced cell death to the generation of oxidative stress which is a result of a direct increase in reactive oxygen species (ROS) and cytokine production, and concurrent reduction in antioxidant glutathione. This may cause cell death via the induction of oxidative DNA damage, DNA fragmentation, and micronuclei formation. Underlining the importance of redox stress, TEGDMA monomer exposure was also shown to lead to an increased expression of antioxidant enzymes catalase and heme oxygenase involved in the detoxification of H_2O_2 and heme, respectively.

The second finding of the present study was the confirmation of apoptosis as the mechanism of cell death as observed by the increase in all investigated apoptosis-specific caspases. The significance of findings pertaining to the pattern of pulp cell death lies in the lack of inflammation accompanying apoptosis as opposed to necrosis. Few studies have attempted to distinguish whether apoptosis or necrosis is the primary mechanism of cell death in cells exposed to TEGDMA, and only a handful of those investigated pulp cells. Some have suggested a concentration-dependent shift from apoptosis to necrosis. Two studies attempted to delineate which classical pathway is in principle responsible for mediating apoptosis with conflicting results. One study found TEGDMA to increase the release of mitochondrial cytochrome c and activate caspase-9 thereby activating predominantly the intrinsic pathway, while another detected increased levels of Bid, cytochrome c,

caspase-8, and caspase-3, thus suggested the activation of both pathways. In accordance with the latter the current study has also found both pathways to be activated. According to our results, TEGDMA exposure led to a significant rise in cleaved caspase-8 as well as cleaved caspase-9 and caspase-3 levels. The previously described increase in ROS production has been shown to be capable of activating both the intrinsic and extrinsic caspase-dependent apoptotic pathways. In addition, TEGDMA exposure has also been demonstrated to lead to a contemporaneous rise in TNF-α expression and decline in the levels of the anti-apoptotic protein BCL-xL, which could provide further mechanisms for the activations of both apoptotic pathways.

To the best of the authors' knowledge alternative apoptotic pathways have not yet been investigated in connection with TEGDMA exposure. Additional to the aforementioned findings, the present study demonstrated a rise in AIF and cleaved caspase-12 levels in pulp cells upon exposure to TEGDMA monomers. A recent development has been the identification of endoplasmic reticulum (ER) stress as a further possible initiator of apoptosis through the release of caspase-12. The ER is principally responsible for the post-translational modification of proteins and oxidative protein folding (OPF). These processes rely on a tightly regulated intraluminal redox homeostasis ensured, among other things, by a very specific intraluminal ratio of GSH to reduced glutathione (GSSG). Although the oxidative environment favours OPF, excessive ROS production and GSH depletion, both of which have also been shown to be a consequence of TEGDMA exposure in pulp cells, can lead to the destruction of this redox balance. As a primary site of tertiary and quaternary folding of proteins, this leads to the formation of unfolded protein aggregates. Failure of clearance of the unfolded proteins may in turn activate, through diverse signalling mechanisms, membrane-bound caspase-12. Translocation of caspase-12 from the ER membrane to the cytosol/nucleus

connects ER stress to apoptosis through the direct activation of procaspase-9 and convergence with other pathways on caspase-3.
AIF is a mitochondrial protein residing in the intermembranous space with both resident housekeeping and possible apoptosis effector functions. Redox energy crisis may lead to the loss of mitochondrial membrane potential and permeabilization. Upon release, AIF preferentially associates with Cyclophilin A and translocates to the nucleus. This protein complex then induces large-scale DNA fragmentation to 20 kb and 50 kb fragments and subsequent chromatin condensation in a caspase-independent way, thereby leading to cell death. The present findings of increased intracellular levels of AIF and caspase-12 in pulp cells exposed to TEGDMA have furthered our understanding of monomer toxicity.
In our second investigation pulp cells were exposed to 0.1, 0.2, and 0.75 mM TEGDMA solutions for 24 hours. Exposure time and concentrations were chosen based on the substantial cell death seen in our pilot studies for populations exposed to TEGDMA concentrations above 0.75 mM and for over 48 hours. Regarding the employed concentration and study time-frame, similar exposure conditions were used also in other studies to detect gene expression changes and production of specific proteins, all of which yielded representative findings. Results of this investigation suggest that cells exposed to sublethal TEGDMA concentrations for 24 hours show a mild increase in total collagenase/gelatinase activity, as well as a rise in MMP-2, -8, and -9 production. Few studies, if any so far have explicitly aimed at investigating the possible changes in the quantities of intrapulpal MMPs in the context of TEGDMA exposure. No studies could be found which examined the direct effect of TEGDMA on dentally relevant MMP-2, -8, and -9 production. Currently, the mechanisms for TEGDMA-induced MMP production can only be speculated. A possible explanation could be signaling alterations taking place in the background. TEGDMA seems to influence

intracellular signal transduction through direct alterations in mitogen-activated protein kinase (MAPK) cascades. ERK, JNK, p38, all MAPKs, have been shown to play a role in changes in MMP-1 and -13 levels in pulp cells. The possible relationship between these signaling molecules and the presently investigated MMPs has not been clarified yet. Our results have demonstrated an increased activation of MAPK members ERK1/2, JNK, and p38 with the concurrent increase in MMP production. However, given the lack of specific inhibitors used in the experimental set-up, a causative relationship based on these results cannot be concluded. Considering the roles of the above molecules in general cellular processes, the observed concurrent rise may only be part of a global stress response. Further studies, possibly with the use of signaling inhibitors, would be required to confirm the causative role of these molecules in TEGDMA-induced MMP rise.

The second finding of this study was the increase in total collagenase/gelatinase activity seen in pulp cells upon TEGDMA treatment. Since MMPs represent most of such enzymes, the measured rise in activity can be considered to be representative of total MMP activity. MMP activation is regulated at multiple levels including transcription, synthesis, and activation of pro-enzymes. Few theories exist about the specific activation mechanism of MMPs but the so-called "cysteine conformational switch" is thought to be an important step. MMPs have also been shown to be activated in an acidic medium, which led to the speculation, that the mildly acidic monomers may be responsible for dental adhesive-induced increase in MMP activity. A further factor may be TEGDMA-induced changes in the concentrations of proteins known to regulate MMP activity such as tissue inhibitors of metalloproteinases (TIMPs) and cathepsins. No studies have been found which investigated the possible changes in the production of these proteins in the setting of monomer exposure. The hereby detected concurrent rise in activated JNK, p38, ERK with the rise in total collagenase/gelatinase

activity may also suggest a role for signaling in enzyme activation. Again, additional studies would be required to further clarify a possible relationship. Interestingly, contrary to our results, evidence also exists suggesting an inhibitory role to such monomers on MMP-2 and -9 activity in pulp cells. According to the authors of this investigation, this may occur due to a complexation between the ether and carbonyl groups of TEGDMA and the bivalent zink ion and nucleophilic centers of the MMPs thereby physically restricting the catalytic domain.

Within the limitations of the above results, it is important to consider that in a healthy tooth there are several processes which may offset the above-mentioned toxic effects *in vivo* such as the pulp circulation as well as the outward dentinal fluid flow which limit the access of the toxic metabolites to the pulp. Moreover, in the present study, pulp cells were obtained from healthy teeth extracted for orthodontic reasons. Composite restorations are placed in destructed teeth. Stressed pulp cells may respond slightly differently to monomer exposure. And finally, in the present study cells were exposed only to TEGDMA. As commercially available composite mixtures contain various other monomers, combinatorial studies would be useful to elicit possible synergistic effects that would apply better to the *in vivo* situation and are among the future plans for the research group.

Theses of the dissertation

1. *Investigation into the cytotoxicity of TEGDMA monomers and the mechanisms of monomer-induced pulp cell death*

The objective of the first investigation was to reveal further details about the cytotoxicity and apoptotic mechanisms leading to pulp cell death in the setting of exposure to clinically relevant concentrations of TEGDMA.
Centred on the results of this study the following conclusions are drawn:

- TEGDMA displays a concentration and time-dependent cytotoxicity on human pulp fibroblasts.
- Cell death, as a result of exposure to clinically relevant monomer concentrations predominantly occurs via apoptosis and is mediated by both extrinsic and intrinsic caspase-dependent pathways.
- ER stress and AIF may be novel, previously unreported, caspase-independent mediators of monomer-induced pulp cell death.

2. *Investigation into the influence of TEGDMA monomer on MMP-2, -8, -9 production and total collagenase activity in pulp cells.*

The second study aimed to establish whether exposure to TEGDMA could influence dentally relevant MMP-2, -8, -9 production and total collagenase/gelatinase activity in pulp cells, thereby suggesting a possible role in post-restoration pulp inflammation, bond degradation, and progression of residual caries. Secondary aim was to correlate possible collagenase/gelatinase changes with the activation of certain MAPK cascades.
Within the limitations of the investigation the results demonstrated the following:

- Low concentrations of TEGDMA monomers (0.1 and 0.2 mM) cause a mild elevation in total collagenase/gelatinase activity, suggestive of MMP activation, in pulp cells.
- Monomer presence induces dentally relevant MMP-2, -8, and -9 production.
- Exposure to low concentrations of TEGDMA also led to the activation of ERK1/2, p38, and JNK. Specific roles of these signaling molecules in the stress response and/or MMP induction are yet to be determined.

List of publications

Publications serving as the basis for the dissertation:

1. Lovász BV, Berta G, Lempel E, Sétáló G Jr, Vecsernyés M, Szalma J (2021) TEGDMA (triethylene-glycol-dimethacrylate) induces both caspase-dependent and caspase-independent apoptotic pathways in pulp cells. *Polymers.* 13(5): 699. doi: 10.3390/polym13050699 **IF$_{2019}$ 3.426 Q1**
2. Lovász BV, Lempel E, Szalma J,Sétáló Jr. G, Vecsernyés M, Berta G (2021) Influence of TEGDMA monomer on MMP-2, -8, and -9 production and collagenase activity in pulp cells. *Clin Oral Investig.* 25(4):2269-2279 doi: 10.1007/s00784-020-03545-5. **IF$_{2019}$ 2.812 D1**

Publications within the topic of the dissertation:

1. Lempel E, Lovász BV, Meszarics R, Jeges S, Tóth Á, Szalma J (2017) Direct resin composite restorations for fractured maxillary teeth and diastema closure: A 7 years retrospective evaluation of survival and influencing factors. *Dent Mater.* 33(4): 467-476. doi: 10.1016/j.dental.2017.02.001 **IF 4.039 D1**
2. Lempel E, Lovász BV, Bihari E, Krajczár K, Jeges S, Tóth Á, Szalma J (2019) Long-term clinical evaluation of direct resin composite

restorations in vital vs. endodontically treated posterior teeth – Retrospective study up to 13 years. *Dent Mater.* 35(9): 1308-1318. doi: 10.1016/j.dental.2019.06.002 **IF 4.495 D1**

3. Lempel E, Németh KD, Lovász BV, Szalma J (2021) Adhesive management of anterior tooth wear in combination with the Dahl concept. Observational case-series. *Oper Dent.* **IF$_{2019}$ 2.213 D1**
4. Lempel E, Őri Zs, Kincses D, Lovász BV, Kunsági-Máté S, Szalma J (2021) Degree of conversion and in vitro temperature rise of pulp chamber during polymerization of flowable and sculptable conventional, bulk-fill and short-fibre reinforced resin composites. *Dent Mater.* S0109-5641(21)00075-0
doi: 10.1016/j.dental.2021.02.013 **IF$_{2019}$ 4.495 D1**
5. Lempel E, Õri Zs, Szalma J, Lovász BV, Kiss A, Tóth Á, Kunsági-Sándor M (2019) Effect of extended exposure time and pre-heating on the conversion degree of conventional, bulk-fill, fiber reinforced and polyacid-modified resin composites in 8 mm deep cavities. *Dent Mater.* 35(2): 217-228. doi: 10.1016/j.dental.2018.11.017 **IF 4.495 D1**

Other publications:

1. Szalma J, Vajta L, Lovász BV, Kiss C, Soós B, Lempel E (2020) Identification of specific panoramic high-risk signs in impacted third molar cases where cone beam computed tomography changes the treatment decision. *J Oral Maxillofac Surg.* 78(7): 1061-1070. doi: 10.1016/j.joms.2020.03.012 **IF$_{2019}$ 1.642 Q2**
2. Szalma J, Lovász BV, Vajta L, Soós B, Lempel E, Möhlhenrich SC (2019) The influence of the chosen in vitro bone simulation model on intraosseous temperatures and drilling times. *Sci Rep.* 9(1): 11871. doi: 10.1038/s41598-019-48416-6 **IF 3.998 D1**
3. Szalma J, Lovász BV, Lempel E, Maróti P (2019) Three-dimensionally printed individual drill sleeve for depth-controlled sections in third molar surgery. *J Oral Maxillofac Surg.* 77(4): 704.e1-704.e7. doi: 10.1016/j.joms.2018.11.028 **IF 1.642 Q1**
4. Szalma J, Klein O, Lovász BV, Lempel E, Jeges S, Olasz L (2018) Recommended drilling parameters of tungsten carbide round drills for the most optimal bone removals in oral surgery. *BioMed Res Int.* 2018:3108581. doi: 10.1155/2018/3108581 **IF 2.197 Q2**

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