Doctoral (Ph.D.) Dissertation

Investigations into the pulpal responses to TEGDMA monomer exposure

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List of Abbreviations

AFCT	addition-fragmentation chain transfer
AIF	Apoptosis inducible factor
ALP	Alkaline phosphatase
Alpha MEM	minimum essential medium eagle-alpha modification
BHT	butylated hydroxytoluene
Bis-EMA	Bisphenol-A ethoxylated dimethacrylate
Bis-GMA	bisphenol a-glycidyl methacrylate
BPA	Bisphenol-A
COX	Cyclooxygenase
CQ	camphorquinone
DC	degree of conversion
DEJ	dentino-enamel junction
DMP	dentine matrix protein
DMPT	N,N-dimethyl-p-toluidine
DSB	double strand break
ECM	extracellular matrix
EDMAB	ethyl-4-dimethylamino-benzoate
EDTA	ethylene-diamine-tetraacetic acid
EGMPM	glycol-3-morpholine-propionate methacrylate
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FGF	Fibroblast growth factor
GF	growth factor
GSH	glutathione
HEMA	hydroxyethyl methacrylate
HL	hybrid layer
HMOX1	heme oxygenase-1
HRP	horseradish-peroxidase
IL	interleukin
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LED	Light-emitting diode
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP-1	monocyte chemotactic protein-1
MMP	matrix metalloproteinase
mM	millimole
MN	micronuclei
MPC	2-methacryloyloxyethyl phosphorylcholine
NAC	N-acetyl cysteine
NfκB	nuclear factor kappa b

NOX4	NADPH-oxidase 4
Nrf2	nuclear factor like-2
OPF	oxidative protein folding
PBS	phosphate-buffered saline
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PI3K	phosphatidylinositol 3-kinase
PPD	1-phenyl-1,2-propanedione
PPRF	pre-polymerised resin fillers
PSC	pulp stem cells
PVDF	polyvinylidine fluoride
QAM/C	quaternary ammonium (di)methacrylates/compounds
RBC	resin-based composite
RDT	residual dentine thickness
ROS	reactive oxygen species
SBC	silorane-based composite
SD	standard deviations
SOD	superoxide dismutase
TEGDMA	Triethylene glycol dimethacrylate
TGF-β	transformation growth factor-β
TNF-α	tumour necrosis factor α
UDMA	urethane dimethacrylate
VEGF	vascular endothelial growth factor
WST-1	water-soluble tetrazolium-1
3-MPS	γ-methacryloyloxypropyl trimethoxysilane

I. Introduction (literature review)

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 since they adequately replace dental hard tissue and aesthetics. They 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 use is likely to continue to rise [12]. 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 [1].

Due to the inherent chemistry of the material formulations, complete polymerisation is currently not possible which gives rise to unreacted monomers. In light of the wide application of composite restorative materials the cytotoxicity of various leachable components of the material mixture has been a popular topic among researchers. Monomers including Bis-GMA, HEMA, and TEGDMA have confirmed to be cytotoxic on various cell types such as human gingival fibroblasts, dental pulp cells, or immortalised keratinocytes [2, 3]. Research into the biocompatibility of resin monomers has also raised concerns over the potential genotoxic and carcinogenic effects.

Although resin chemistry has developed significantly over the last decade, leading to overall safer materials, well designed investigations have again and again uncovered potentially deleterious cellular consequences. It is this relentless desire to test and investigate various constituents of composites that is the driving force behind the development of material formulations. Detailed knowledge of the cellular responses and mechanisms elicited by various components of dental materials are vital in the quest for developing safer, more biocompatible materials.

In view of the above, the aim of the present thesis is to provide an overview of the material chemistry and polymerisation kinetics of composite resin restorative materials with a specific focus on the cytotoxic mechanisms of resin monomers confirmed in the literature so far.

Additional aim is to describe and discuss the investigations which have been undertaken within the framework of this doctoral study to delineate further cellular responses with the aim of improving our current understanding of monomer toxicity.

I.1. Composition of dental composites

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 [4]. 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 *(figure 1)*. Additional constituents such as an initiator, coupling agent and various colorants are added to optimise curing behaviour, setting reaction and aesthetics [5].
Composites may be classified from numerous angles such as filler particle size, modes of curing, composition, and clinical application, just to mention a few. *Luts and Phillips* classified composites in 1983 onto a continuum from larger to smaller filler sizes into macro-, micro-filled, hybrids, modern hybrids, and nanofill composites [6]. Depending on the mechanism of initiation there are the historically significant self-, or chemical cured composites and their currently almost exclusively used light-cured counterparts.
From the aspect of clinical application, packable composites are used for conventional posterior restorations, flowable composites are applied in low stress-bearing areas, novel self-adhesive composites eliminate the need for a separate adhesive system, infiltration resins are used mainly in interproximal lesions, and bulk-fill composites allow the application of larger increments thereby reducing operative time [5].

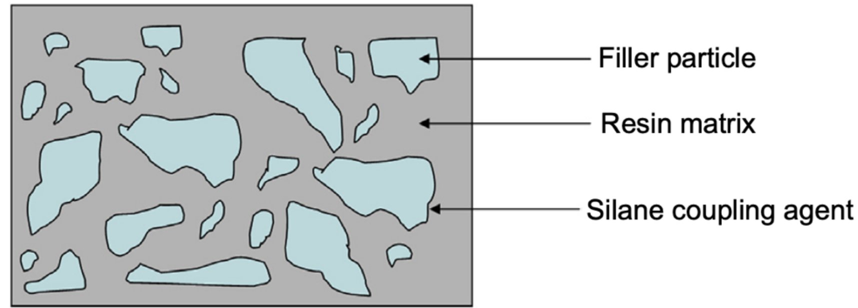


Figure 1: Schematic illustration of resin-based composites showing the dispersion of filler particles in the resin matrix. Chemical linkage is established via the silane coupling agent lining the surfaces of each particle [185].

I.1.1. Organic resin matrix

The resin phase is composed of polymerizable monomers which upon exposure to an appropriate wavelength of light convert from a predominantly liquid phase to a highly crosslinked solid polymer. Resin mixtures contain relative proportions of the base monomers and diluents below with various degrees of trade-offs in viscosity, mechanical properties, and monomer conversion [4].

By content bisphenol a-glycidyl methacrylate (Bis-GMA) is the major constituent of the mixture. It is chemically characterised by extremely high viscosity and low mobility owing to its stiff central aromatic phenyl ring and two diametrically-positioned pendant hydroxyl groups *(figure 2)* [7]. The high viscosity not only hinders ease of handling but also conversion of the monomers as evidenced by data showing a degree of conversion of only 39% for the homopolymer, Bis-GMA. Its large size however imparts a high elastic modulus, impact strength, relatively low polymerization shrinkage, low volatility and diffusivity thereby reducing the potential for cytotoxicity [8, 10]. Despite the desirable properties an appropriate diluent, TEGDMA, must be added to the mixture to negate the high viscosity and to allow for the incorporation of high filler loading.

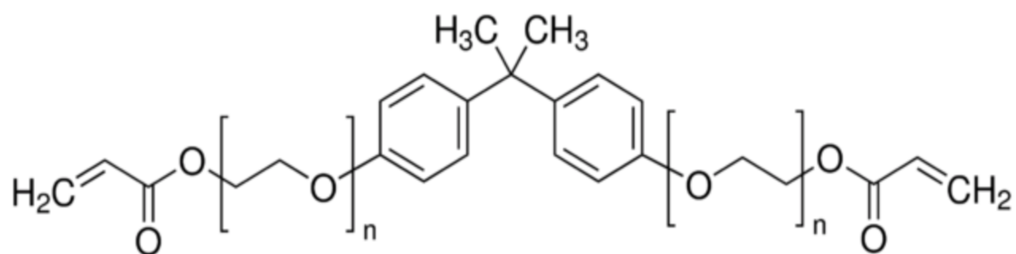


Figure 2: Structure of Bis-GMA

Ethoxylation of bisphenol a-glycidyl methacrylate produces an analogous dimethacrylate also frequently used in dental resin monomers whereby the only difference is an ethoxy species replacing the pendant hydroxy groups. Bisphenol-A ethoxylated dimethacrylate (Bis-EMA) is a frequently used additive or alternative in resin mixtures due to its lower viscosity, water sorption and polymerization shrinkage *(figure 3)* [8]. Despite its seemingly favourable structure, the process of polymerization is limited by steric hindrance and thereby results in low degree of conversion [11].

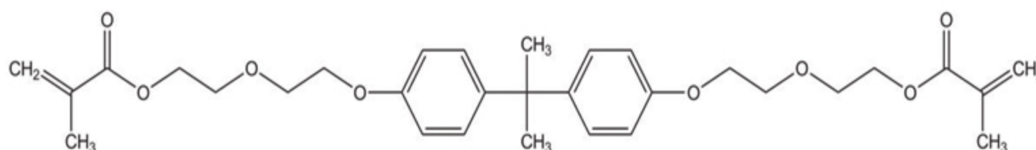


Figure 3: Structure of Bis-EMA

Triethylene glycol dimethacrylate (TEGDMA) is a highly flexible, low-molecular-weight monomer formed as a product of the reaction between triethylene glycol and two molecules of methacrylic acid [7]. It generally constitutes 20-50% *by weight* of the resin mixture [8]. The most important difference in the chemical structure of TEGDMA as compared to Bis-GMA is the linear rather than aromatic link between the two methacrylate functional groups *(figure 4)* which has multiple implications. Firstly, weaker polar (hydrogen bond) interactions between the chains which imparts greater flexibility [9]. This plasticising effect has been shown to lead to a synergistic increase in initial polymerization reactivity and hence rate, ultimately resulting in an increase in maximum double bond conversion [8]. Secondly, the relatively small size of TEGDMA and numerous double bonds bring radical polymerization sites closer together which also helps conversion [31]. Thirdly, the reduction in viscosity helps increase filler loading, reduces stiffness and thus improves handling ability. Offsetting

the beneficial properties however, TEGDMA also increases water sorption, curing shrinkage, hence the generation of undue stress, decreases colour stability and mechanical properties [7, 32].

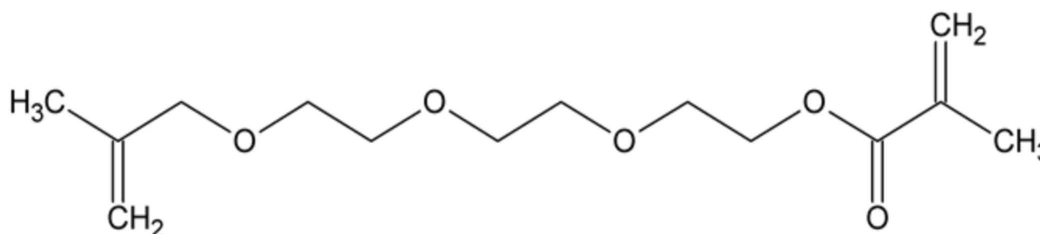


Figure 4: Structure of TEGDMA

Modern RBCs may also contain two further monomers; the low molecular weight hydrophilic monomer, hydroxyethyl methacrylate (HEMA) -mainly used in adhesives- and the rather new dimethacrylate; urethane dimethacrylate (UDMA). In the latter, the lack of phenol ring lends to higher flexibility and greater conversion in comparison to Bis-GMA *(figure 5)* [7, 8]. Strong hydrogen bonding allows for a very strong set polymer, however also increases viscosity above that of TEGDMA and thus necessitates the addition of a diluent. UDMA is the only commercially available alternative to Bisphenol-A containing dental dimethacrylates. The only significant downside of employing UDMA in place of Bis-GMA is the comparatively lower molecular weight leading to a slightly greater polymerization shrinkage [10]. Considering all, one can't help but think that with the increasing scrutiny surrounding the potential health effects of Bis-GMA degradation product Bisphenol-A (BPA), UDMA may be an appropriate BPA-free formulation to turn to.

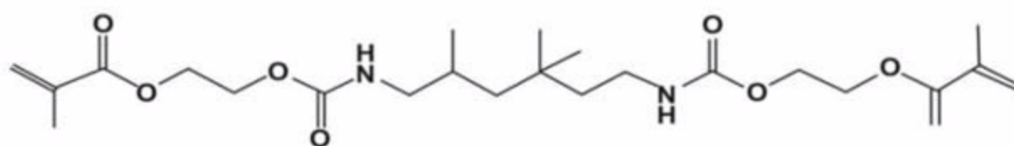


Figure 5: Structure of UDMA

Developments in resin technology

Although current material mixtures have adequate mechanical properties, undergo fast conversion with relatively low polymerization shrinkage there is still dramatic potential for improvement. Researchers have worked relentlessly over the past decade to address some of the inherent issues with current composites.

It has been shown that up to 25% of composite replacements are due to some form of fracture [35, 36, 167, 168]. Current conviction is that repeated thermal stresses and mastication cause microcrack formation and propagation. Of this end, *Wu et al,* in a recent study, showed the effective application of poly (urea-formaldehyde) microcapsules containing TEGDMA liquid in order to generate 'self-healing' composites [34]. Microcapsules containing the monomer were shown to rupture upon crack initiation followed by a flow of TEGDMA to the site of damage where it would polymerise with the help of a tertiary amine catalyst. Mechanical properties were shown to be comparable to conventional formulations.

Secondary caries is another main reason for restoration failure [36, 167, 168]. Resin mixtures with the incorporation of silver nanoparticles and quaternary ammonium dimethacrylates (QAMs) have been shown to give composites high antibacterial efficiency without compromising mechanical properties. QAM is believed to lead to bacterial redox destabilisation due to the interaction between the positively charged amine and the negatively charged bacterial cell membrane [37]. Silver inactivates vital bacterial enzymes. For any material to be effective on the long run sufficient activity needs to be present both at the surface and in the bulk of the biofilm. Since QAM efficiency depends on physical contact, appropriate levels in the bulk shown may only be achieved by leaching which then gives rise to questions regarding biocompatibility [38]. Moreover, the formation of acquired pellicle may physically impede contact between the QAM and bacteria. 2-methacryloyloxyethyl phosphorylcholine (MPC) has been shown to be a successful additive in preventing protein adsorption to the acquired pellicle [39].

Attempts have also been undertaken to try to eliminate polymerization stress generated during curing. This is caused by the conversion of van der Waals connections to tighter covalent bonds, which may ultimately lead to adhesion inadequacy, sensitivity, and

secondary caries [40]. Novel strategies include the utilisation of the mechanism of addition-fragmentation chain transfer (AFCT). This is enabled by the incorporation of an allyl sulfide functional group. Reduction of shrinkage stress in AFCT is the result of the rearrangement of network connectivity through recurring scission and recombination without altering crosslink density. There is a delay to gel point conversion as a result of limitation in particle diffusion which reduces stress [41]. This method has been shown to reduce stress by 90% while increasing the degree of conversion however was accompanied also by a reduction of mechanical properties. Further evolution of this technique resulted in the incorporation of multimethacrylates; thio-urethane oligomers. While the thiol/vinyl groups enable polymerization via chain transfer, the oligomers and strong urethane bonds give rise to a more homogenous and stronger polymer structure [42].

Latest and probably most promising approach to relieve stress has been the development of silorane-based composites (SBC). Contrary to the linear structure of methacrylates, silorane is composed of a siloxane core with four oxygen-containing rings attached. Unlike in conventional resins, polymerization is not linear but achieved through a cationic ring-opening mechanism. Shrinkage has been reported to be less than 1% in comparison to 2.6-7.9% for Bis-GMA-based products. Mixed reports exist pertaining to the impact on mechanical properties with some studies claiming comparable strength, while some found them to be inferior [43]. Water sorption and solubility has been found to be significantly lower due to the hydrophobic backbone which translates to more stability in biological fluids [44].

Finally, with the aim of eliminating the most technique-sensitive step of composite placement, the application of the adhesive system, self-etching composites were developed. Various hydrophilic monomers such as glycerolphosphate dimethacrylate, dihydrogen phosphate or trimetallitic acid were added to resin mixtures all capable of simultaneously etching dentine, interacting with hydroxyapatite, and polymerising to the resin matrix. Studies have found bond strength to be inferior in comparison to conventional composites. This was attributed to the water absorbing property of these novel hydrophilic monomers leading to the weakening of the polymer matrix [53].

I.1.2. Fillers

Over the course of the past several decades continuous material research and development among manufacturers have led to the use of various filler technologies and designs. Fillers, usually mixtures of glass/silica, increase modulus, surface hardness while decreasing volumetric shrinkage. Owing to the evolution of manufacturing processes filler sizes have decreased from tens of microns to submicron dimensions. Based on the filler-size distribution, composites may grossly be classified into; macrofill, containing particle sizes exceeding 50 μm, microfill, composed of much smaller fillers, and nanofill, with glass particle sizes truly in the nm range [12]. It has been a general academic and industry objective to maximise filler loading. With uniform particle sizes, gaps smaller than the particles would be inevitable with increasing filler content leaving the mixture underfilled [21]. Therefore, most commercial composites are hybrids containing a mixture of various sizes of filler particles and are further subdivided into micro and nanohybrids (<1um) based on the dimensions of the largest micron-sized particle. Reduction in particle sizes came with alterations in physical properties. Micrometric particles conveyed excellent strength at the expense of polishability and smoothness. With the incorporation of smaller particle sizes came improved aesthetics but generally lower filler content compromising strength. Nano-sized particles represent an additional asset in being smaller than the wavelength of light thereby reducing scatter and refraction leading to a greater depth of cure [13, 19]. Thus, current formulations composed of a mixture of larger and smaller glass particles represent a compromise between adequate strength and desirable aesthetics [6].

The driver behind research into filler technology has been the desire to maximise mechanical properties. Increasing volume % has been shown to increase modulus until an optimal percentage (approx. 80%) [6]. Regarding filler morphology, studies have found irregular filler shapes to impart increased wear resistance in comparison to their spherical counterpart [14, 19]. Fairly recently an investigation demonstrated the beneficial effect of the incorporation of nano-sized silanised glass fibers on elastic modulus, flexural strength and fracture propagation [20]. Building on this idea, the addition of pre-polymerised resin fillers (PPRF) has been shown to allow for a further increase in filler content leading to increased strength. The study also found an

improvement in aesthetics and polish retention in the investigated PPRF-reinforced hybrid composites [15].
Compared to amalgam, RBCs are more likely to be colonised by bacteria. Interesting novel attempts have been made to make RBCs more antimicrobial through the incorporation of specific fillers. Of the tested chemicals, the addition of quaternary ammonium compounds (QAC) at 1 wt. % to a TEGDMA/bis-GMA mixture imparted antibacterial effects against S. Mutans [22]. An alternative approach involved the surface treatment of inorganic fillers -in a way similar to silanisation- with QAC. The cured product exhibited antimicrobial effectivity against S. Mutans, S. Aureus, E. Coli, Pseudomonas [23].
Prompted by the ongoing concern about biofilm formation on polymeric surfaces, research groups have proposed the technology of 'remineralising-RBCs' reinforced with amorphous calcium phosphate. Results showed a successful release of calcium and phosphate ions in a pH <4 followed by recharge in pH 7, thereby preventing demineralisation and facilitating remineralisation. Mechanical properties were comparable to conventional composites [24].

I.1.3. Coupling agent

Bifunctional silanes serve the dual purpose of providing a functional interface enabling a covalent bond between the polymer matrix and the inorganic filler and improving wettability and dispersion of fillers thereby leading to improved physical and mechanical properties [16]. Without silane, nanoparticles may agglomerate leading to a heterogenous distribution within the set polymer.
Silanes are organic silicides (X_3SiY) where X may be chloro, alkoxy, acetoxy and Y may be vinyl, epoxy, amino, or mercapto groups. Most widespread silane used is γ-methacryloyloxypropyl trimethoxysilane (3-MPS). The methoxy group at one end binds covalently to the hydroxy group of the filler and other silane molecules while the methacryloxy end copolymerises with the methacrylic resin polymer matrix *(figure 6)* [17]. Many attempts have been made to change the functional groups at each end of the silane molecule with a view to improving hydrolytic stability and/or mechanical properties with varying success. Of note *Craig et al* investigated the effect of various hydrophobic functional groups on chemical stability and found vinyltriethoxysilane to hydrolytically stabilise composites [18]. Another interesting preposition was the

utilisation of dual silanes. Filler treatment with conventional 3-MPS and either styryl functionalized silane or non-functional methoxy silane was shown to increase modulus and improve handling, respectively [4].

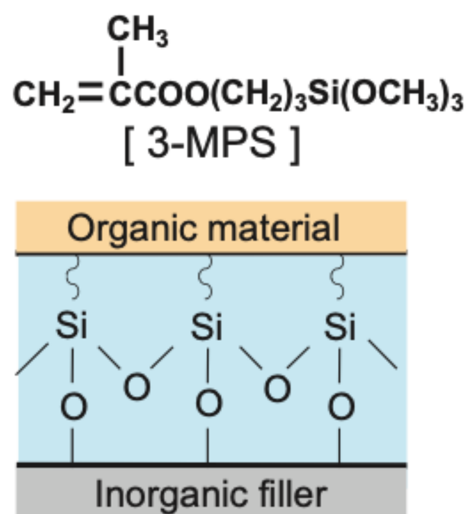


Figure 6. Schematic illustration of the mechanism of silane coupling. The methacryloxy group radically polymerises to the double bonds of the organic resin matrix, while the methoxy group binds covalently to the hydroxy group of the filler particles [17].

I.1.4. Photoinitiator

Photoinitiated polymerization has enormous value as it controls setting kinetics and allows the appropriate placement and manipulation of the restoration prior to curing. Clinically used visible light has reduced the energy of the individual photons, subsequently necessitating the application of multicomponent initiators. Current material designs use camphorquinone (CQ) as the main initiator and various amines as co-initiators/electron donors [4]. On exposure to visible light CQ temporarily reaches an excited-state and complexes with the amine to liberate the active α-amino-alkyl initiating radical which then drives the conversion process *(figure 7)*. CQ remains to be the most widely used photoinitiator mainly due to convenience as it has a wide absorption spectrum from 360-510 nm with peak absorption at 468 nm. On dispersion in TEGDMA, CQ undergoes a bathochromic shift moving the absorption peak to 474 nm, ideal for blue LEDs [7]. The obvious benefit of practicability is offset by the potential to disrupt membrane integrity and the generation of yellow by-products during curing due to the presence of chromophore groups [25, 26].

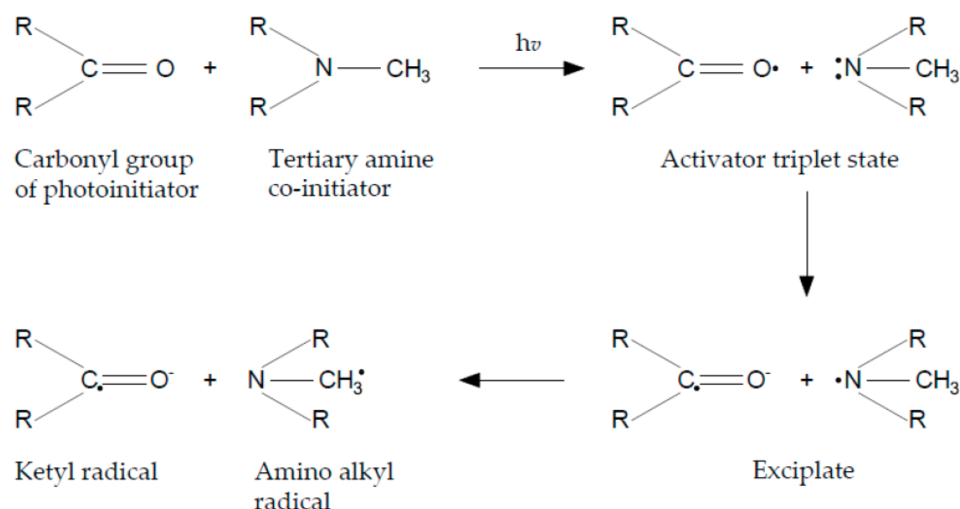


Figure 7. Mechanism of photoinitiation by the camphorquinone (CQ)/tertiary amine system. On exposure to visible light, electron exchange between the initiator and coinitiator yields a ketyl and amino-alkyl radical, respectively, capable of initiating the polymerisation process [7].

There is concern about the possible biocompatibility of the co-initiator too. Widely used amines, N,N-dimethyl-p-toluidine (DMPT) and ethyl-4-dimethylamino-benzoate (EDMAB), are aromatic in nature. They have relatively low molecular weight and have been shown to increase the production of reactive oxygen species and glutathione leading to potential DNA damage [27]. In a quest to increase curing efficiency, reduce toxicity, and the production of by-products researchers have been busy investigating alternative systems. From the numerous biochemicals targeted the most notable may be 1-phenyl-1,2-propanedione (PPD). This alpha-diketone photosensitiser generates radicals through cleavage, which is markedly different from CQ. It is not only less yellow but also very compatible with the resin matrix. Tests have indicated similar conversion values when used alone. However, when added in conjunction with CQ or tertiary amine a synergistic effect was apparent on the degree of conversion and mechanical properties [28, 29]. This was most likely due to the complimentary absorption spectra (410 nm) when compared with CQ (470 nm). This effect together with the potential benefit to the aesthetics makes PPD a seemingly appropriate CQ replacement or additive.

Promising results were obtained also with the application of polymerizable monomers such as ethylene glycol-3-morpholine-propionate methacrylate (EGMPM) as co-initiators instead of tertiary amines. They are bifunctional monomers presenting an amine at one end and a methacrylate at the other. On curing, total conversion and polymerization rates were found to be higher than when CQ was used alone, however in comparison with the CQ/amine system the overall degree of conversion was slightly lower at 60%. Considering this together with the reported diluent effect such monomers may be great targets for future investigations [30].

I.1.5. Additional components

Composites may also contain additional ingredients such as inhibitors and pigments. Internal stresses generated during free radical polymerization can either be transferred to the tooth resin interface and lead to marginal failure/secondary caries or may be transmitted to other parts of the restoration/tooth resulting in microcrack formation and ultimately fracture. One strategy employed to eliminate premature polymerisation, thus reduce stress generated, is the incorporation of specific inhibitors such as butylated hydroxytoluene (BHT) [51].

Pigments optimise the shade of the final restoration in order to achieve a better match with the native tooth. In standardised curing conditions a reduced polymerisation degree has been observed in the case of darker commercially-available composites which may be due to the attenuating effect of the darker pigments selectively filtering out certain wavelengths of light [52].

I.1.6. Mechanism of polymerisation

Liquified monomer mixtures of RBCs convert to hardened, highly crosslinked polymers through chain growth polymerisation. Current material technology allows for a maximum conversion of approximately 60-70% [47, 56]. Phases of the process are initiation, propagation, and termination. During initiation on exposure to light the initiator/co-initiator complex decays into ketyl and amino alkyl radicals which then drive the breakage of the C = C bonds initiating conversion [7]. Propagation involves further radical addition to double bonds ultimately resulting in chain growth. Termination is characterised by chain growth arrest *(figure 8)* [46]. Polymerisation is

a complex process, the kinetics of which is controlled in different ways during various phases of the network formation. From the onset there is a dramatic increase in the rate of reaction due to a process known as *autoacceleration* [45]. Even at low conversions, radicals become hindered due to reduction in mobility and cannot terminate by segmental movement (diffusion) but must propagate via double bonds. This results in the accumulation of radicals and hence acceleration of conversion. As the rigidity of the polymer increases radicals become trapped and effectively terminated [46]. The nature of the propagation phase is such that it doesn't become diffusion controlled until significantly higher conversions; when the composite becomes glassy i.e., vitrifies. This is termed *autodeceleration* and is important as it gives rise to unreacted monomers [4]. The final polymer network is extremely heterogenous characterised by both areas of high and low crosslink densities. This is due to the formation of microgels which are areas with high crosslink density at the independent sites of initiation. As the polymerisation process progresses these areas trap radicals leading to also areas of low crosslink density with a relatively high amount of unreacted monomers [46].

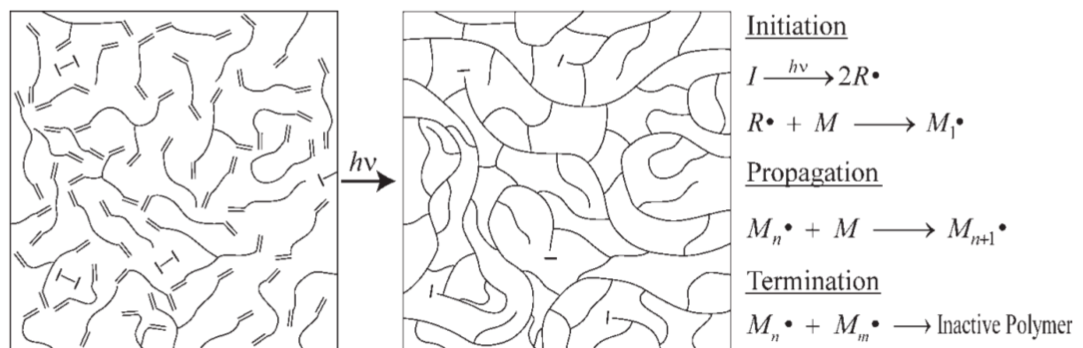


Figure 8. Monomer to polymer conversion is achieved via chain growth polymerisation involving three distinct chemical steps driven by free radicals; initiation, propagation and termination [46].

Polymerisation inherently results in shrinkage and generation of stress, as described above, which alternative polymerisation technologies have attempted to alleviate. Accordingly, the mechanism of AFCT relieves some of the stress by alternating propagation and chain transfer in a step-growth pattern. This results in alleviation of shrinkage as flow rather than stress [41]. Another promising approach has been the

ring-opening silorane system which leads to markedly reduced shrinkage due to a lower molar shrinkage coefficient [43].

Factors influencing polymerisation

Polymerisation may be influenced by many factors such as the composition of the RBC, increment size, light curing unit, irradiation time [47, 169]. Increasing filler-matrix ratio leads to reduction in the degree of conversion (DC) as inorganic glass particles hinder chain growth [7]. Monomer composition, concentration of inhibitor, accelerator, initiator have also been shown to affect depth of cure [49]. Energy density and spectral flux of the light source influence conversion too. Energy density is the product of the light intensity and the duration of exposure. Lowering the energy density significantly decreases conversion degree [48]. On the contrary increasing the communicated energy gives rise to a higher degree of conversion up to an optimal energy density level after which no further increase was seen [47, 169]. An explanation for this may be a saturated photo-initiator system in which the radical content is too high leading to mutual annihilation [50].

The importance of increment thickness has also been widely investigated in recent literature. In standardised curing conditions a significantly better DC was found when increment size was less then 2 mm in comparison to when it was applied thicker. As mentioned earlier modern composites are heterogenous in structure characterised by increased light attenuation via scatter, absorption, and reflection with increasing thickness. This leads to insufficient photo-inititator activation at depths greater then 2 mm [47, 50].

I.1.7. Monomer elution

Biocompatibility is the most important prerequisite for biomaterials to be placed inside living organisms. It may be defined as an ability of the material to exist in harmony with the surrounding tissues without eliciting toxic, immunologic, or any other deleterious effects. Although currently there is no evidence that modern composites may hold serious health hazards a lot of research effort has been directed at the potential biotoxic effects of various RBC constituents. Even though every component of the mixture has been shown to elute into a solvent, unbound monomers,

present due to the incomplete conversion process, in particular have been the subjects of focus of many toxicity studies. Eluted monomers may then contact mucosa through the salivary fluids, or may penetrate to the pulp through the dentine tubules [54, 184]. Elution studies have historically analysed elution over a short period of time (24h), however more and more recent studies have investigated elution over a longer period (up to 1 year) which seems to be more relevant considering the lifetime of a restoration [56, 57].

Ferrecane suggested that elution is inversely proportional to DC along with all factors influencing polymerisation degree (detailed above), and also dependent on composite composition and extraction solution [54, 47]. Significantly more elution is seen in organic (typically alcohol containing) than water-based solvents. More acidic solutions, for instance containing HCl, have also led to the elution of more degradation products as a result of hydrolysis of the ester bonds in the polymer matrix [56]. More recently, salivary hydrolytic processes have been shown to result in chain scission and release of polymeric breakdown products [55]. Aging and wear of composites leading to micro porosities, water sorption and subsequent mechanical swelling has also been implicated in the release of unbound monomers.

Both short and long-term studies have shown a biphasic release of monomers characterised by an initial acute phase followed by a substantially slower release lasting in certain solvents for up to 1 year. Acute phase has been suggested to be due to the faster surface/subsurface degradation whilst the subsequent deceleration was explained by the limited access to methacrylates in the core of the restoration in addition to the inherent in-vitro limitations [58]. Of the most widely employed RBC constituents HEMA and TEGDMA monomer elution has been detected in the largest amount [54, 56]. TEGDMA in particular has a lower molecular weight and more flexible backbone allowing for more mobility and easier elution. Both HEMA and TEGDMA are relatively hydrophilic when compared with other monomer components which also helps elution into water-based solvents [57]. On the contrary, Bis-GMA was consistently found to leach the least from all commercial composites, which is thought to be due to its aromatic structure imparting a very large molecular weight and viscosity. These characteristics significantly reduce mobility and thereby elution [56].

Results from in-vitro studies should be appreciated with caution. Most studies employed test set-ups in which a block of composite was immersed in a volume of

solvent. As the release of monomers is a chemical reaction, it must be assumed that elution arrests when equilibrium is reached. This is a major limitation of *in-vitro* studies and has been regarded as a possible explanation for the biphasic monomer release mentioned earlier [56]. Few studies have attempted to mimic intraoral condition with a periodic change of medium, however this can hardly be considered an adequate recreation of the continuous salivary flow and intrapulpal circulation [56, 57]. Based on this logic, monomer elution should be suspected to continue for longer periods then the initial 24 hours and may ultimately result in substantially more monomer exposure then quoted by the above studies.

I.2. Biological Safety

I.2.1. Biocompatibility of resin-based composites

Although the introduction of RBCs has opened up a new avenue for tooth restoration their success also depends on biological safety. The claim that various components of the dental resin mixture are completely inert is nowadays untannable. Huge effort has gone into elucidating the possible toxic effects of various resin constituents.

Allergic reactions have long been associated with the application of dental resins both in the patient as well as in clinical staff. Allergy to dental base resins has been documented to lead to mouth soreness and burning sensation on the palate, tongue, and oral mucosa. This is more prominent in undercured prosthesis and thus has been attributed to the leaching of the residual unreacted monomers [59].

Moreover, cured composite disks were found to possess estrogenic activities [60]. Bisphenol-A (BPA) has long been labelled as a possible endocrinological disruptor. It may be present in modern mixtures as a direct constituent; however, this is nowadays rare. What is more common is their presence as a degradation product of various other components such as Bis-GMA. It may also be present as an impurity in poorly controlled synthetic reactions [60, 62]. Studies have shown leached composite constituents to decrease fertility and implantation rate in mice as well as be directly toxic to ovaries. It was speculated that BPA may disrupt endocrine functions by altering signalling along the hypothalamo-pituitary axis leading to the inhibition of follicle stimulating hormone (FSH) and subsequent alterations in follicular

development [61]. Bisphenol-A was also found to lead to cell proliferation and progesterone receptor expression in MCF-7 breast cancer cells prompting groups of scientists to call for the re-evaluation of resin composites by the FDA [62]. Additionally, BPA is claimed to be associated with diseases such as peripheral arterial disease, diabetes mellitus, and infertility [140, 141].

In spite of the previous findings, claims have largely been controversial, studies have demonstrated contradicting results. The general position is that more relevant experiments need to be undertaken in order to draw more definitive conclusions regarding the potential impact on human health [60, 62].

I.2.2. Pulp response to resin-based restorations

On comparing pulp response to amalgam and composite restorations, *Chadwani et al* found moderate to severe pulp inflammation in the form of necrosis and abscesses to last longer (>7days) in the case of RBC restorations than amalgam (<7 days) [63]. It's generally accepted that inflammation seen after 7 days can no longer be attributed to the trauma of the cavity preparation and thus is more likely due to chemical irritation. This claim was supported by several additional studies. *Murray et al* observed a continued pulpal inflammatory response beyond 28-days post-placement and has correlated this with the chemical nature of the restorative material as well as bacterial microleakage [65]. Moderate degree but persistent pulp inflammation with tissue disorganisation, macrophage infiltration, and delayed dentine bridge formation was seen histologically in multiple investigations after the application of resinous adhesives/composites. Changes were directly proportional to the length of resin tags and monomer content imparting a potential role to leachable resin monomers [64, 66, 142, 143].

Uncured monomers are widely thought to be able to penetrate dentine tubules and reach the pulp [67, 68, 69, 184]. Although they are thought to leach from polymerised samples, the moments prior to curing may also be a significant source of uncured monomers especially in deep carious lesions, close to the pulp, where the density and size of dentine tubules are the greatest [184]. Dentine permeability was inferred from the cytotoxicity detected in a pulp cell culture placed under a block of dentine with a resin-based restoration polymerised on the surface [69]. In further support of this hypothesis fluid analysis from the pulps of composite-restored teeth has confirmed

that monomers, HEMA and TEGDMA, can indeed diffuse through various thicknesses of residual dentine in order to access the pulp [67]. An inverse relationship exists between residual dentine thickness (RDT) and post-procedure pulp inflammation with 0.5mm being the critical thickness [142, 172, 65]. Remarkably, monomers have been reported to be able to diffuse through even 2mm RDT [143]. Theoretical calculations based on the molar concentration of monomers used in commercially available composites and the supposed dilution effect of the remaining dentine indicate that intrapulpal concentrations of TEGDMA may reach 4 mM [58, 68, 69, 67]. This monomer has consistently been found 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 [54, 57, 58]. As described above, this is most likely a result of their relatively low molecular weight and flexible backbone imparting lower viscosity and easier diffusion through the dentine tubules [58].

I.2.3. TEGDMA and mineralisation

The understanding of how the dentin-pulp complex responds to exogenous irritation by reparative dentine production has advanced significantly over the last decade. Integral to this process is the activity of uninjured odontoblasts as well as the replenishment of those injured by pulp stem cells (PSC). The mechanism of reparative dentinogenesis is proposed to involve PSC proliferation, migration to the injured site followed by differentiation into protein-secreting odontoblast-like cells [81, 82].
Pulp capping with monomer-based materials has been shown histologically to not only exacerbate pulp inflammation, as detailed above, but also result in a lack of mineralised dentine formation [142, 143, 66, 63, 64, 150]. This was also attributed to the pulpal displacement of leachable monomers since small globules of various sizes -thought to be storing monomers- were repeatedly identified in the dentine tubules. Additionally, other studies found a significant reduction in the expression of markers typical of odontogenic differentiation in pulp cells exposed to TEGDMA [130, 82, 83, 124]. Crystal nucleation and growth of tertiary/reparative dentine is controlled by non-collagenous proteins such as osteopontin, osteocalcin, and bone sialoprotein produced by resident pulp cells [83]. Several of these genes have been subjects of recent investigations. The expression of collagen α1, bone sialoprotein, osteocalcin, Runx2,

dentine sialophosphoprotein have all been shown to decrease in a time- and concentration-dependent manner in pulp cells after a relatively short exposure to TEGDMA [82, 124, 130, 131].

Furthermore, during the process of mineralisation alkaline phosphatase (ALP) cleaves pyrophosphate to inorganic phosphate. A decrease in both ALP transcription and activity was also demonstrated in pulp cells upon TEGDMA exposure [81, 124]. All above observations were counteracted by the addition of radical scavenger, N-acetyl cysteine (NAC), thus highlighting the potential involvement of oxidative stress in the down-regulation of differentiation-related genes and reduction in ALP activity [81].

It's important however to remember that the above experiments were largely carried out on healthy teeth/pulps. Concurrent presence of bacterial toxins and resinous materials may lead to more dramatic pulp responses [66, 149]. Moreover, they were undertaken in normoxic conditions (21% O_2). Recent studies have suggested that due to the limitations in sources of oxygen, the oxygen tension in the pulp may in fact only be 3%. Accordingly, investigations carried out in hypoxic conditions showed TEGDMA exposure to lead to an even greater decrease in the expression of mineralisation specific genes [151].

I.2.4. TEGDMA and cytokine production

The temporary increase in pulp inflammation seen secondary to the placement of resin-based restorations may partly be due to TEGDMA-induced changes in intrapulpal cytokine production. Prostaglandins have been suggested to induce leukocyte recruitment and modulate vascular permeability in inflamed rat pulps [84]. Cyclooxygenase (COX) is the eicosanoid-converting enzyme responsible for prostanoid synthesis. While COX-1 is a ubiquitous enzyme, COX-2 is inducible and only present in inflamed tissues. Prostaglandin E_2 (PGE2), regarded as a proinflammatory cytokine, is the most widely produced prostanoid. Levels in pulp and periapical exudates have been correlated with failure of radiologic and clinical outcome. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is principally vasoactive, may decrease ALP expression and upregulate IL-8 production in pulp cells [85]. Studies have attempted to elucidate whether TEGDMA may influence COX-2 or prostaglandin $E_2/F_{2\alpha}$ levels in pulp tissue. Results show an induction of COX-2, PGE_2, and PGF_2 in pulp cells and macrophages exposed to TEGDMA [86, 152]. PGE_2 is thought to affect the

proliferation, apoptosis of lymphocytes and influence cytokine production in T cells thus may play a particularly important role in the intensification of pulp inflammation following an operative procedure [153].

Changes in various interleukins (ILs) in response to TEGDMA exposure have also been studied. IL-6 and -8 are thought to be crucial cytokines for immune response leading to the induction of acute phase proteins and recruitment of inflammatory cells such as lymphocytes, neutrophils to the site of inflammation, respectively. Contrastingly IL-10 is important for limiting the response as it inactivates nuclear factor kappa b (NfκB) leading to the reduction in pulpal IL-6 and -8 levels [154]. TEGDMA was found to have an immune-modulating effect by decreasing IL-6 and -10 and inducing IL-8 production in pulp cells [131, 116]. Similarly, TEGDMA also impedes lipopolysaccharide (LPS)-induced production of IL-6, -10, and tumour necrosis factor α (TNF-α) in macrophages [74]. Considering the above observations, TEGDMA may temporarily worsen pulp inflammation by impeding an appropriate immune response and bacterial clearance.

Furthermore, TEGDMA has also been shown to induce the production of monocyte chemotactic protein-1 (MCP-1) with the subsequent induction of hydrolase activity in pulp cells. While the function of MCP-1 is to recruit white blood cells to the site of inflammation, hydrolases target ester bonds susceptible to hydrolysis in the dental polymer. It is believed that, the increased generation of breakdown products may further contribute to intrapulpal inflammation and restoration failure [87].

I.2.5. Cytotoxicity of TEGDMA

Studies have confirmed the cytotoxicity of TEGDMA on various continuous cell lines as well as target cell isolates including human dental pulp cells [144, 70, 145]. *Walther et al* employed a lactate dehydrogenase (LDH) test to confirm the cytotoxicity of low concentrations of TEGDMA (0.1 mM to 3mM) on L2 and A549 Lung cells [70]. In this case the purpose of employing a continuous cell line was to assess the effect of the monomers on the production of reactive oxygen species rather than to confirm the toxicity on a specific target organ. A549 Lung cells contain especially high levels of glutathione as compared with normal cell isolates allowing the demonstration of changes in redox status. Immortal cell lines are frequently chosen in place of primary cells due to their ease of use, low cost, pure population

characteristics and reproducible results. Findings, however, have to be appreciated with caution as cells are genetically manipulated which may alter their phenotype and responsiveness to stimuli in comparison to target cell isolates [147].

Monomers from composite restorations may not only diffuse through dentine but can also leach out towards the oral cavity and have been shown to exert deleterious effects on mucosal/gingival cells [2, 72, 79, 87, 135]. This is believed to be accelerated by dental erosion and bacterial, salivary enzymatic degradation [71]. TEGDMA has been shown to decrease viability in human gingival fibroblasts in a dose-dependent way, which is speculated to occasionally lead to mucosal reactions [72, 79]. In spite of this, levels in the saliva are still thought to be way below the toxic range.

Macrophages have also been targets of various cytotoxicity studies [68, 73, 74, 96, 125, 136, 145]. The rationale for testing this cell population is that macrophages are key effectors of the innate immune system. They provide non-specific first-line defence against inflammation due to microorganisms or foreign bodies and subsequently drive tissue repair. TEGDMA concentrations above 1.5 mM have been found to reduce viability in macrophage cultures at 24h via the mechanisms of both apoptosis and necrosis [73].

Studies have occasionally used lymphocytes as subjects of investigations as they form a group of standardised cells ideal for chromosomal tests and identifying genotoxic effects [75, 146]. TEGDMA concentrations of 0.1 mM have been found to lead to significant toxicity at 24h and increase the number of apoptotic/necrotic lymphocytes [146]. Although the above cell types show slightly different sensitivities and hence toxic threshold to TEGDMA, in general all cell types show complete destruction below 5 mM TEGDMA.

Cell cultures are widely used in vitro to assess toxicity on tissues presumed to be in physical contact with leachable monomers. While continuous cell lines are advantageous due to their homogenous morphology and behaviour, their metabolic potential is hardly that of the recipient cells'. Thus, primary cells derived from the target tissue provide better inferable data [76, 147].

Since the observation that RBC restorations and resin-based pulp capping biomaterials may lead to various degrees of pulp inflammation, pulp cells have enjoyed increasing popularity as subjects of cytotoxicity studies [67, 125, 137, 143]. Multiple effects of TEGDMA have been uncovered on pulp cell metabolism however there remains to be a need for further understanding of the molecular details.

I.2.6. TEGDMA and intracellular redox balance

Monomer-induced apoptosis/necrosis has been casually related to the generation of oxidative stress. Significant reduction in cell death was observed in the presence of well-known experimental antioxidants such as Trolox (vitamin E), N-acetylcysteine, or ascorbic acid [70, 78, 127].

Several studies have shown TEGDMA to lead to an increase in reactive oxygen species (ROS) production exceeding the capacities of intracellular antioxidant systems [70, 77, 78, 79, 96, 123, 127, 137, 148]. ROS are present physiologically, formed from the incomplete reduction of oxygen and include superoxide anions, hydrogen peroxide, and hydroxyl radicals. Their levels are tightly regulated by complex antioxidative responses under the control of transcription factor, Nrf2 [136, 138]. They function as signalling molecules in normal cellular process and can also lead to covalent protein modifications. If not restored, redox disbalance leads to oxidative DNA damage, membrane lipid peroxidation, and apoptosis *(figure 9).*

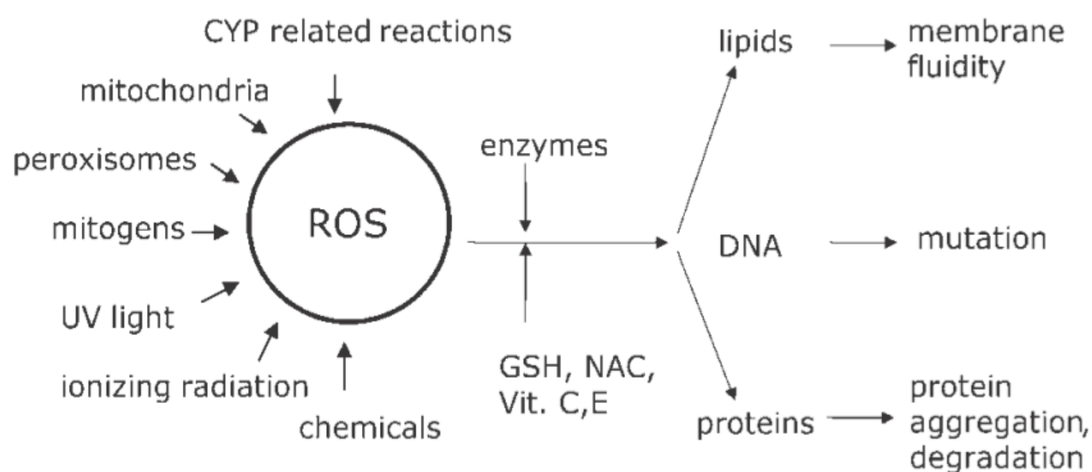


Figure 9. Various external and internal signals may lead to the generation of oxidative stress. Reactive oxygen species (ROS) disrupt cellular homeostasis by targeting primarily lipids, DNA and various intracellular proteins [97].

Accordingly, a recent study detected a rise in the production of oxidative membrane degradation products after exposure to mM range TEGDMA [127]. Reactive oxygen

species are thought to interact with the methylene groups of the membrane to liberate lipid peroxyl radicals which then react further forming degradation products. Membrane degradation products impair DNA repair and intracellular antioxidant functions [128].

ROS may be generated via multiple mechanisms upon exposure to TEGDMA. Balanced intracellular redox homeostasis is tightly controlled by various antioxidant enzymes of which, most notably, TEGDMA has been associated with a depletion in intracellular glutathione (GSH) level [77, 78, 79, 137, 148]. The mechanism is suggested to be partly adduct formation. TEGDMA monomers are esters of unsaturated carboxylic acid which function as bifunctional molecules for addition reactions via the mechanism of Michael addition. The carbonyl groups next to the C=C are electron withdrawing leading to the formation of a positively charged beta carbon, which can then react with nucleophiles such as amino and thiol groups of GSH or DNA [77, 79]. This physically impedes the activity of GSH allowing unrestricted ROS production. It also explains why a reduction in GSH in pulp cells exposed to TEGDMA is not accompanied by a rise in oxidised GSH (GSSG) [77].

TEGDMA has also been shown to directly lead to the loss of mitochondrial membrane potential by allowing the entry of calcium ions through pore complexes. This results in the uncoupling of electron transfer and subsequent perpetuation of ROS production [127]. Moreover, resin metabolites such as methacrylic acid may also impede the activities of ROS-neutralising enzymes such as superoxide dismutase [80, 78]. In human cells TEGDMA is preferentially metabolised through an epoxy pathway leading to the generation of especially toxic metabolites such as 2,3-epoxymethacrylic acid [129].

The role of NADPH-oxidase 4 (NOX4) has recently been identified as a ROS-producing enzyme in the setting of TEGDMA exposure. *Yeh et al* found not only NOX4 mRNA, protein levels to increase in pulp cells but NOX4 knockout mice showed significantly reduced ROS production in the presence of TEGDMA. Considering that this enzyme has the sole function of ROS generation, this may be the most important source of radicals in TEGDMA-exposed pulp cells [78].

I.2.7. TEGDMA and DNA damage

Dental resin monomers, specifically TEGDMA, has been shown to increase the frequency of gene mutations by up to 25-fold resulting in micronuclei (MN) formation and large DNA deletions in various cells *in vitro* [98, 133, 155]. With the help of comet assays and novel investigative methods targeting the detection of phosphorylated double strand break (DSB)-associated protein H2AX and DSB damage sensor protein 53BP1 researchers have also confirmed the generation of DNA breaks in standardized lymphocytic cell populations [134, 146]. Surprisingly, a recent study showed structural chromosomal aberrations to occur already at TEGDMA concentrations 100-1000-fold lower than employed in cytotoxicity studies [75]. As mentioned before, TEGDMA exposure leads to MN formation [133, 98, 75, 73]. This represents the endpoint of DNA damage when broken chromosome fragments aggregate and form extranuclear bodies following cell division [133].

Central to the likely mechanism of DNA damage is thought to be the production of reactive oxygen species *(figure 10)* [77, 97]. Lengthy exposure to TEGDMA has led to elevated levels of 8-oxoguanine, a common oxidative DNA product, as well as to the induction of 8-hydroxyguanine DNA glycosylase 1, the principal enzyme tasked with the repair of such lesions [96]. Expression of the oxidative-stress-response gene, DNA damage inducible transcript 4, has also been found to be induced by TEGDMA. NAC addition successfully prevented micronuclei and DSB formation further supporting the potential key role of ROS in TEGDMA-induced DNA damage [97, 133, 134]. ROS may interact with chromatin proteins as well as purine and pyrimidine bases. If base repair processes fail, single/double strand breaks and DNA crosslinks ensue with subsequent stalling of replication fork and potentially initiation of apoptosis [97, 125].

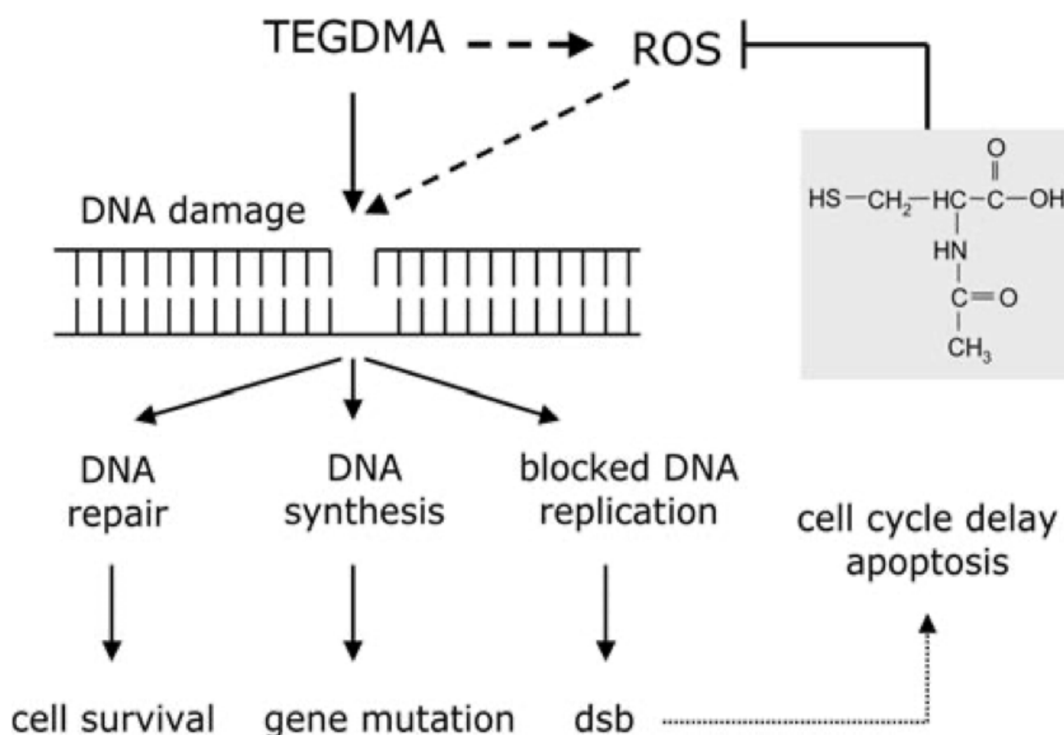


Figure 10: TEGDMA may cause DNA damage directly by adduct formation or indirectly via the generation of reactive oxygen species. If subsequent DNA repair fails, cell cycle arrest and ultimately induction of apoptosis may follow [97].

Although the exact signalling following monomer-induced DNA damage has not been completely mapped yet, cell cycle arrest in G1/G2/M or S phases have consistently been detected as an early response [86, 88, 97, 133, 73, 156]. The transcription of critical genes involved in the progression past cell cycle checkpoints have been found to decrease in several studies, while others have detected a slight increase in the expression of checkpoint-controlling p53 in the nuclear fraction of human pulp-derived cells [86, 88]. Cell cycle arrest allows for DNA repair or alternatively for the induction of programmed cell death [133].

Aside from ROS production, in a mechanism similar to adduct formation with GSH, positively charged carbonyl groups of methacrylates may also react *directly* with the nucleophilic groups of DNA via a Michael addition resulting in the formation of intrastrand crosslinks [77, 79, 97]. Double strand breaks found in connection with the previously mentioned metabolite of TEGDMA, 2,3-epoxymethacrylic acid, could yet implicate epoxides as another mechanism for indirect DNA damage [135].

I.2.8. TEGDMA and apoptosis

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. *Yeh et al* found apoptosis to be the primary mechanism of pulp cell death upon TEGDMA exposure [78]. Others have shown a concentration-dependent shift from apoptosis to necrosis [86, 3, 77, 88]. While apoptosis is a controlled active cell death initiated by various exogenous and endogenous signals, necrosis is a result of massive cell damage leading to passive loss of protein function, cell rupture and leakage of organelles. Clinical importance lies in the lack of inflammation during apoptosis [89]. Pulp cells were found to undergo primarily apoptosis upon exposure to 1 mM TEGDMA, however there was a clear shift to necrotic cell death with concentrations above 2 mM [3, 86]. The expression of critical cell cycle regulating genes were shown to be decreased contemporaneously suggesting an arrest, which may be the initiating mechanism of apoptosis [86]. Antioxidants such as NAC, vitamin E, ascorbate seem to protect cells from monomer-induced apoptosis implying a role for ROS in the initiation of apoptosis [70, 88].

To date, research has shown that there are caspase-dependent and -independent apoptotic pathways. The former relies on the sequential activation of various proteolytic enzymes, caspases, and can be further subclassified based on the instigating signal. The intrinsic pathway involves mitochondrial damage with a subsequent release of cytochrome c and activation of procaspase-9. Conversely the extrinsic pathway relies on signals originating from a death receptor activated by ligands such as Fas ligand or TNF-α, involves the activation of procaspase-8 and subsequent convergence with the intrinsic pathway on caspase-3. Effector caspases target DNA and bring about cell death via *'death by a thousand cuts*.' Both processes lead to controlled cell death characterized by shrinkage, nuclear, cytoskeletal protein fragmentation and formation of apoptotic bodies [89].

Aside from mitochondrial dysfunction another key organelle change implicated in apoptosis may be endoplasmic reticulum (ER) stress leading to caspase-12 translocation from the ER with subsequent activation of procaspase-9 and -3 [90]. ER is the primary site for tertiary and quaternary protein folding. Excess demand for protein synthesis such as that encountered in redox disbalance, abnormalities of Ca^{2+} homeostasis causes ER stress manifested as an unfolded protein response. This is

followed by cell cycle arrest, attenuation of further protein synthesis, and disposal of unfolded proteins through ER-associated degradation [91]. If the mitigation of ER stress fails, apoptosis may follow. Several studies have highlighted the role of caspase-12, found exclusively in the endoplasmic reticulum, in linking ER stress to apoptosis in a manner independent of the mitochondria and death receptors [92]. Procaspase-9 and -3, in particular, have been found to be targets of caspase-12 [90-92].

Recent findings corroborate the existence of a caspase-independent apoptotic pathway also. Central to this mechanism is a mitochondrial polypeptide -apoptosis inducible factor (AIF)- causing chromatin condensation and DNA degradation [94]. Physiologically, this 57 kDa protein is tethered to the inner membrane of the mitochondria. Cellular stress frequently leads to an intracellular energy crisis abolishing mitochondrial membrane potential. Subsequent permeabilization of the mitochondria and cleavage by proteases such as calpain and cathepsins translocate AIF to the cytosol [93]. Differential interaction with Cyclophilin A and heat-shock-protein 70 determine AIF's nuclear or cytosolic localisation, respectively [95]. During apoptosis AIF preferentially translocates to the nucleus and promotes DNA degradation to 20 kb and 50 kb fragments by modulating the activity of non-specific endonucleases [94].

Eliciting which apoptotic pathway leads to cell death would add valuable data to the understanding of monomer-induced pulpal toxicity. Two studies attempted to delineate which pathway is in principle responsible for mediating apoptosis with conflicting results. *Yeh et al* found TEGDMA to increase the release of mitochondrial cytochrome c and activate caspase-9 thereby activating the intrinsic pathway, while *Batarseh* et al detected increased levels of Bid, cytochrome c, caspase-8, and caspase-3, thus suggested the activation of both pathways [78, 99].

I.2.9. Cellular defence mechanisms against TEGDMA

Since redox disbalance leads to the most deleterious cellular effects of TEGDMA exposure, unsurprisingly, so far identified cell responses aim overwhelmingly at trying to restore redox homeostasis. Many investigative methods have been employed to elucidate which antioxidant defence responses could be activated upon TEGDMA exposure. Most fruitful of them have been gene expression studies. Nuclear factor like-2 (Nrf2) regulates the transcription of detoxifying and

cytoprotective enzymes and is believed to be the key controller of intracellular antioxidant mechanisms secondary to TEGDMA exposure [127, 136, 138]. Although the conversion of transcriptome to actual proteins involves very complex signals, identifying which genes are targeted in the first instance gives a good indication of cell response priority. *Baldion et al* found the transcription of antioxidant enzyme, catalase, to be strongly upregulated upon TEGDMA exposure implying that H_2O_2 may be the predominant radical species produced by monomers [127]. Proteomic analysis identified Heme oxygenase (HMOX1) to be a key additional cytoprotectant [116, 136, 132]. During oxidative stress, this enzyme mediates the metabolism of toxic heme to Fe(II), carbon dioxide and biliverdin. The latter in turn is converted to antioxidant bilirubin which similarly to glutathione protects cellular components from oxidative stress *(figure 11).* The same study detected also an increase in the production of auxiliary enzymes such as 6-phosphogluconate dehydrogenase, NADPH dehydrogenase, and sulfiredoxin 1, all of which are thought to strengthen the reductive power of cells in the setting of TEGDMA exposure [136].

Glutathione is the most abundant intracellular ROS scavenger. TEGDMA exposure has been associated with an early reduction in GSH levels. Hardly surprisingly, cysteine uptake, phosphoserine aminotransferase-1, and cysteine ligase regulatory subunit levels -all essential for glutathione regeneration- have been shown to be upregulated in TEGDMA-exposed pulp cells [136, 137].

An interesting recent investigation was carried out to see whether secreted growth factors from pulp cells exposed to TEGDMA could provide a positive feedback through an autocrine mechanism on viability, migration, and mineralisation potential. Results suggest that the secretome not only counteracts TEGDMA cytotoxicity but also increases stem cell migration and mineralisation potential which could provide yet another mechanism for pulp recovery [139].

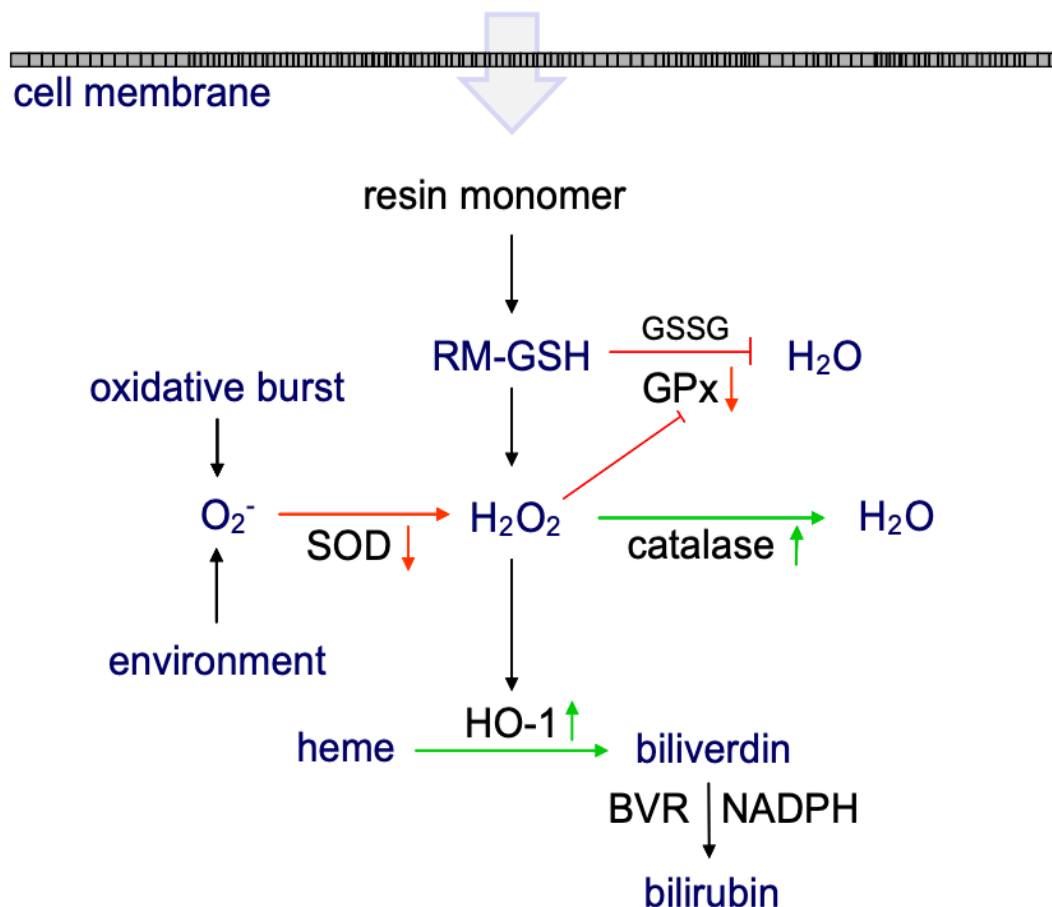


Figure 11: Redox response in cells exposed to dental resin monomers. Current literature suggests that TEGDMA, as a resin monomer representative, causes oxidative stress by decreasing glutathione levels. The subsequent rise in H_2O_2 induces the antioxidant enzyme catalase and inhibits the expression of superoxide dismutase (SOD). Redox disbalance enhances the expression of Heme oxygenase (HO-1), which catalyses the formation of biliverdin. A reduction carried out by biliverdin reductase (BVR) results ultimately in the generation of antioxidant bilirubin [77].

I.3. Matrix Metalloproteinases (MMPs)

I.3.1. Role of MMPs in caries

Matrix metalloproteinases are zinc- and calcium-dependent endoproteinases with a wide range of overlapping substrates, that are synthesised as inactive zymogens. They influence essential cellular functions such as cell migration, proliferation, angiogenesis, and bone remodelling. They have recently been increasingly implicated in various pathological processes such as cancer, vascular diseases and various

inflammatory conditions [100]. MMPs have been shown to be present in intact pulp, odontoblasts, and predentine suggesting a physiological function [104]. Gelatinases such as MMP-2 and -9 have a wide range of extracellular matrix (ECM) targets and play physiologically crucial roles in remodelling and tertiary dentine formation [101]. Collagenases such as MMP-8 cut type 1 collagen into ¾ and ¼ fragments and are thought to influence dentine organisation prior to mineralisation during tooth development. More recently, their role in caries progression has been investigated [101].

Caries involves demineralisation of inorganic particles by bacterial acids followed by degradation of the organic matrix. Bacterial proteases were thought to be responsible for the latter, however their intolerance of acidic pH and inability to degrade collagen, shown in vitro, suggests limited contribution to the process [102, 160]. Increased levels of MMPs-2, -8, -9, -13, -20 have all been shown to be present in carious dentine and are suspected to be involved in the degradation of the organic, collagenous matrix as well as liberation of inflammatory cytokines [104, 112, 116, 117, 118]. Increased presence at the dentino-enamel junction (DEJ) could indicate a role in the extension of lesions along the border of the two tissues [104, 119, 158]. During matrix degradation MMPs cleave C-telopeptides from collagen fibrils, disrupting the fibrillar structure, and leading to a loss of remineralisation ability [102, 158, 118]. By cleaving large insoluble components, MMPs may also liberate bioactive peptides/cytokines and thereby influence differentiation, proliferation, angiogenesis [157]. Gelatinase (MMP-2) has been found to be able to release transformation growth factor-β (TGF-β) and dentine matrix protein (DMP) 1 thereby driving the formation of a dense reparative dentine bridge [119].

In situ dentine MMPs may initially be secreted by odontoblasts and subsequently get trapped during the mineralisation phase of tooth development. The acidic environment characteristic of carious lesions facilitates their activation [112]. Evidence also suggests that MMPs can be actively secreted by stressed odontoblasts. Higher levels of MMP-2, -9 have been detected in caries affected dentine tubules and in partially demineralised dentine [103, 159, 118]. An additional matrix-degrading contribution has been attributed to salivary MMPs also. In support of this, recent studies detected increased MMP levels in the outer layers of carious lesions [104, 112, 119]. MMP-8, -9 are perhaps the most abundant salivary MMPs implicated in caries [117].

I.3.2. Role of MMPs in pulp/periapical inflammation

As caries disrupts the integrity of the hard tissues, pulp is exposed to inflammation. Tissue destruction is believed to be dependent on the nature of the immune response [105].

Similarly to caries, in pulpitis and periapical lesions, MMPs are suspected to be involved in ECM proteolysis, remodelling and release of inflammatory cytokines, thereby regulating the immune response. ECM degradation is considered to be crucial in the initiation and progression of pulpitis. MMP-1, -2, -3, -8, -9, -13 have all been shown to be significantly increased in pulp inflammation, abscess, and necrosis [101, 102, 106, 107]. Both inflammatory as well as resident pulp cells may be sources of MMPs [106, 107]. Immunohistochemistry has shown MMP-9 to be present in specific intracellular areas of pulp cells, which may mean they are pre-synthesized and stored in vesicles [105]. The induction of MMP synthesis can happen directly by bacterial antigens, LPS and indirectly through the production of inflammatory cytokines such as IL-1, TNF-α generated by resident pulp cells [86, 161]. No information is available as to whether leachable composite monomers can induce the production or activation of MMPs and thereby contribute to pulpal inflammation and/or caries progression.

Periapical disease represents the progression of the bacterial infection through the apical foramen resulting in a localised inflammatory response and bone resorption [162]. Immunohistochemistry has shown MMP-2, -8, -9, -13 to be abundantly present in both acute and chronic periapical lesions likely originating from various inflammatory cells. Their principal roles are defined to matrix degradation, tissue disorganisation, as well as repair [102, 106, 107, 120]. MMP-9 in particular serves an important function for osteoclast activity by removing collagen from the bone surfaces before the start of demineralisation [163]. Subsequent decline in MMP levels after treatment of apical periodontitis further confirms their possible role in the pathogenesis of apical disease [106, 107].

I.3.3. MMPs and hybrid layer integrity

As mentioned earlier, one of the main reasons for restoration failure is secondary caries [167, 168, 169]. The hybrid layer (HL), established at the tooth-resin interface through the adhesive procedure, has been shown to be susceptible to

hydrolytic and enzymatic degradation in vitro. Durability of the HL ultimately depends on the relative resistance of the components - collagen and resin polymer- to degradation [170]. MMPs are synthesised during tooth development by odontoblasts and get trapped in inorganic tissue as it mineralises. Acidic resinous adhesives or bacterial products may activate in-situ MMPs directly as well as induce further production of various MMPs in the pulp [108, 109, 111, 177]. Low pH environments may activate MMPs indirectly through the induction of cathepsins and alteration of TIMP levels too [110, 121]. Demineralised dentine is the scaffold for adhesive infiltration. Demineralised but not infiltrated dentine lacks the protection of the resin and thus may subsequently become substrates of activated MMPs resulting in the failure of the hybrid layer, nanoleakage and secondary caries [102, 121, 122]. Especially, levels and activity of MMP-2 and -9 were shown to be high in the hybrid layer of adhesive treated dentine [108, 113, 121, 171]. Multiple additives have been investigated with the aim of improving HL durability by inhibiting MMPs with varying success [113, 171]. Although the effect of various dental adhesives on MMP production and activity has been investigated, their correlation with exposure to specific leachable components is lacking. TEGDMA has been shown to inhibit MMP-2 activity in mouse gingival tissue, while HEMA inhibited MMP-2 and -9 activity and production in pulp cells [114, 115]. The gene expression of various other MMPs such as MMP-1, -3, -10, -12 increased in pulp cells exposed to TEGDMA [116]. However there seems to be a lack of studies examining the direct effect of TEGDMA on dentally relevant MMP-2, -8, and -9 production. Eliciting how leachable components may influence intrapulpal MMP levels and activity would allow us to understand better the impact of a restoration on pulp tissue, as well as the possible effect of leaching monomers on long term HL integrity and caries progression.

I.3.4. MMP/TEGDMA and Signalling

Cellular responses to endogenous and exogenous stressors are directed by signals. Mitogen activated protein kinase (MAPK) cascades are among the most essential for cell survival and mediate growth factor (GF) and mitogen signals from the cytoplasm to the nucleus. Each MAPK, specifically extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 are serine-threonine kinase modules regulating the response to various stimuli including ROS. ERK leads to cell

survival and relies on the activation of receptor tyrosine kinases RAF/MEK1/2. While p38 and JNK are usually activated by exogenous signals such as cytokines, UV light and regulate stress-related apoptosis [123, 125, 96]. Although TEGDMA-induced activation of MAPKs has been shown in various cell lines, their direct causative relationship with apoptosis has not been unequivocally proven yet. *Kwon et al* suggested TEGDMA to inhibit odontogenic differentiation by decreasing p38 and ERK phosphorylation while activating JNK in pulp cells [124]. *Krifka et al* demonstrated the onset of apoptosis and necrosis in macrophages and pulp cells to be paralleled with simultaneous ERK, p38 and JNK activation [125]. The use of specific signalling inhibitors however failed to prove the direct causation of ERK, p38, or JNK in TEGDMA-induced apoptosis [126]. THP-1 monocytes showed reduced TEGDMA-induced activation of p38 and ERK 1/2 in the presence of ROS scavenger NAC suggesting a role for redox balance in MAPK activation [96].

Literature exists describing the possible role of ERK, JNK, p38 in the production of MMP-1 and -13 in pulp cells [164]. One study found MAPK cascades not to play a role in MMP-2 production in pulp cells [165]. On the contrary, *Shin et al* demonstrated reduced MMP-2 and -9 production in pulp cells with the use of specific p38, JNK, and ERK inhibitors in the setting of TNF-α/LPS exposure [166].

The possible role of the above-mentioned signalling molecules in the induction of MMP activity and expression by TEGDMA has not been investigated yet. Detailed knowledge of the mechanisms leading to MMP induction would enable the development of specific counter-strategies. The first step of implicating signalling molecules in MMP rise would be to correlate their levels with changes in MMP production.

II. Aims of the Investigations

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

Although many details of monomer-induced cell death have been uncovered, there are still areas of great ambiguity. Investigations pertaining to the pattern of cell death are significant with respect to the biocompatibility of resin monomers. While apoptosis is a controlled form of cell death, necrosis is an uncontrolled process characterised by inflammation. The latter may contribute to post-restoration pulp sensitivity and pulpitis. Clinically relevant concentrations of TEGDMA have predominantly led 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. Uncovering the precise details of cytotoxicity would improve our understanding of the pulpal repercussions of restoration placement as well as allow the development of strategies to protect the tissues of the oral cavity.

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 are also involved in mediating cell death in TEGDMA-exposed pulp cells.

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

The role of matrix metalloproteinases in various dental pathologies such as caries, pulpitis, and periapical inflammation is well documented. In inflammation they have typically been described to serve dual functions; activation of innate/adaptive immunity as well as degradation of the extracellular matrix. In caries MMPs may aid the progression of demineralisation. Based on their localisation MMPs may originate from the saliva, be present as in-situ MMPs in the dentine, or may in fact be actively produced by pulp cells and reach the restoration/caries via the dentine tubules.

Commonest reason for the failure of resin composite restorations is secondary caries [167, 168, 169]. Most susceptible area for microbial infiltration is the tooth-resin interface, therefore the integrity of the hybrid layer is paramount for the longevity of restorations.
Many studies have recently demonstrated the role of *in situ* MMPs, thought to be activated by leaching unreacted monomers, in hybrid layer degradation. Information however, on the possible effects of monomers, such as TEGDMA, on *intrapulpal* MMP production and activity is lacking. A rise in intrapulpal MMP production and activity would not only suggest an additional source of MMPs for HL degradation but would also further our understanding of the consequences of tooth restorations on pulp/periapical inflammation and residual caries progression.

TEGDMA exposure, a form of exogenous stress, has been shown to lead to a differential activation of MAPK cascades. Several studies have correlated this with the occurrence of TEGDMA-induced apoptosis however no causative relationship has been established. To the best of the author’s knowledge no literature has so far investigated the signalling background of monomer-induced MMP changes.

In light of the above the aim of this *in vitro* study was to establish the effect of low TEGDMA monomer concentrations on MMP-2, -8, and -9 production and total collagenase/gelatinase activity in pulpal cells with the help of western blotting, immunocytochemistry, and a specific activity assay. Secondary objective was to

correlate changes in ERK 1 and 2, p38, and JNK activation with possible changes in MMP production, activity, and cytotoxicity thereby providing a foundation for further investigations into the signalling background of the above changes.

III. Materials and Methods

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

III.1.1. Reagents

All chemicals used were obtained from Sigma-Aldrich (now Merck KGaA, Darmstadt, Germany) unless stated otherwise.

III.1.2. Pulp Cell Culture

Pulp tissue was isolated from five healthy third molar teeth extracted for orthodontic reasons. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki or comparable standards. All data were anonymized in line with patient confidentiality guidelines. Informed consent was obtained as per the protocol approved by the University of Pecs (Pecs, Hungary, under license No. PTE3026/2007).

Following extraction, pulp tissue was isolated according to a technique described by Sun et al. [115] 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 (100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B). Culturing took place in a humidified atmosphere containing 5% CO_2 at 37 °C. At 90% confluence, the passage to additional Petri dishes was undertaken. Cell cultures were first washed with phosphate-buffered saline (PBS, 1.37 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4 \cdot 7H_2O$, 0.14 mM KH_2PO_4, pH 7.4) followed by trypsin (0.25% trypsin + 0.02% ethylene-diamine-tetraacetic acid (EDTA); Gibco, Grand Island, NY, USA) digestion for 10 min in a controlled, 37 °C, environment. Following two or three passages, cells were seeded at an arbitrary density of 2×10^4 cells/cm^2 based on previous experience with similar populations. Forty-eight hours prior to the start of the TEGDMA exposure, the medium was changed from 10% to 2% fetal bovine serum

(FBS)-containing medium (without antibiotics) in order to decrease the potential signaling interference.

III.1.3. Monomer exposure

In order to assess cytotoxicity, pulp cells were exposed to 0.75, 1.5, and 3 mM TEGDMA for a period of 5 days based on relevant literature data. 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.

III.1.4. Cell counting

Following monomer exposure in 6-well plates, trypsin digestion was applied to collect the cells, followed by their suspension in Alpha MEM medium containing 10% FBS. One drop of the cell suspension was subsequently transferred into a counting chamber of a hemocytometer *(figure 12, 13)*. Cells were counted using a phase contrast microscope, cell numbers were calculated for each well.

For cell counting in marked fields of view, three areas were labeled on each well on the bottom of each culture plate. A phase contrast microscope was used to count the number of viable cells in the areas on days 1, 2, and 5 of the monomer exposure.

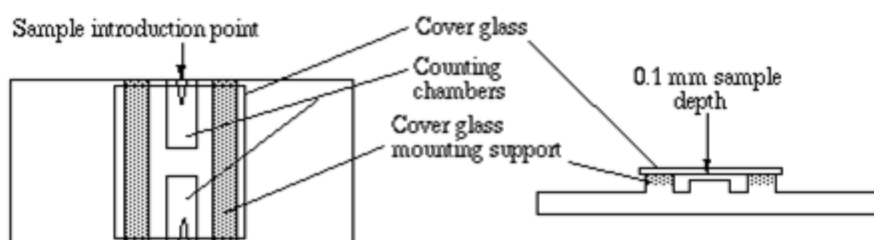
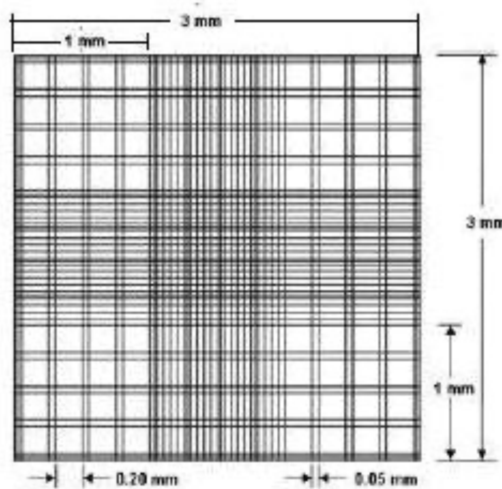


Figure 12. Schematic representation of a Hemocytometer which was used to determine the number of cells per unit volume. Sample of the suspension is introduced into one of the V-shaped wells with a pipet. The charged counting chamber is then evaluated with a microscope at low power [186].

a)



b)

$$\text{Measured cell density} = \frac{\text{Average cells per small square} \cdot \text{Dilution factor}}{\text{Volume of a small square } (mL)}$$

Figure 13: Bürker counting grid as seen in the microscope (a) and calculation method (b) used to estimate cell number [186].

III.1.5. Fluorescence Microscopy

For illustrative purposes, nuclei were counterstained with Hoechst 33,342 (Calbiochem, La Jolla, CA, USA) to obtain representative images. Briefly, cells were rinsed with PBS at 37 °C, which was followed by 4% paraformaldehyde fixation at pH 7.4 and 4 °C for 4 h. Fixative was subsequently removed by further TBS washes (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and nuclei were counterstained with Hoechst 33,342 (Calbiochem, La Jolla, CA, USA). An Olympus FV-1000 laser scanning confocal system (Olympus Europa, Hamburg, Germany) with a 20× phase contrast dry objective was used to obtain the representative single optical slice images, which were merged with the pictures taken of the same viewing fields by the same phase contrast mode of the microscope *(figure 14)*.

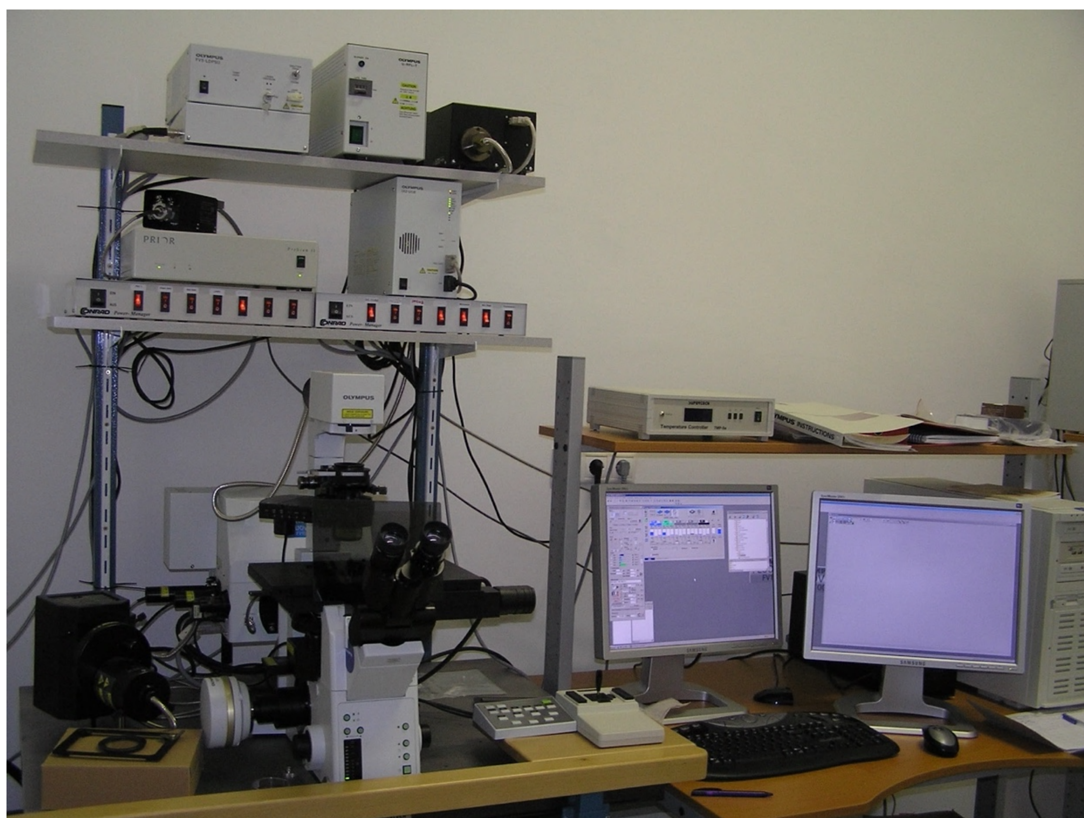


Figure 14. Olympus FV-1000 laser scanning confocal system used to obtain fluorescent microscopic images (Olympus Europa, Hamburg, Germany) [personal image]

III.1.6. WST-1 (water- soluble tetrazolium salts) colorimetric viability assay

A WST-1 colorimetric assay, as an indicator of mitochondrial metabolism, was employed to demonstrate changes in viability *(figure 15).* 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 was measured in 100 µl samples by a FluoStar Optima plate reader (BMG Labtech, USA) at 440 nm.

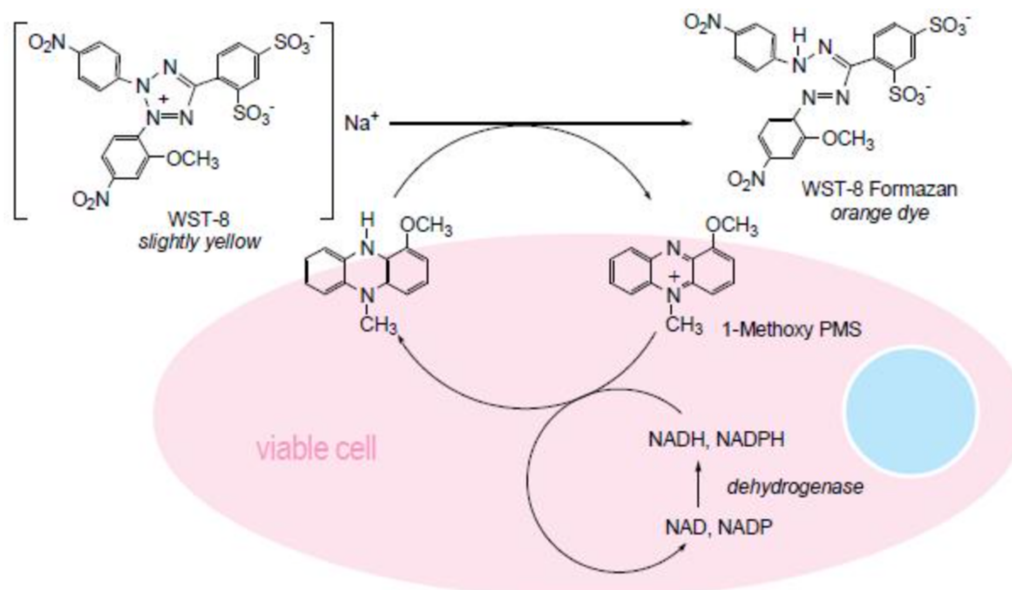


Figure 15. Biochemical basis of Water Soluble Tetrazolium-1 (WST) colorimetric viability assay. If the cell is viable WST-1 is reduced to a yellow-colour formazan dye by cellular dehydrogenases. Colour change, measured by a plate reader, is directly proportional to viability [187].

III.1.7. Western Blotting

After TEGDMA treatment, cells were harvested and lysed as detailed in published studies [12]. 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 and at 40,000× *g*. Protein concentrations of the supernatants were measured (Lowry's method, Detergent Compatible Protein Assay Kit, Bio-Rad, Hercules, CA, USA) and then diluted to contain an equal amount of 30 µg of protein. Following the addition of Laemmli buffer (prepared from 25 mL 1M Tris-HCl, pH 6.8, 40 mL glycerol, 8 g SDS, 10 mL 100 mM EGTA, 10 mL 100 mM EDTA, 1 mL 1% bromophenol blue; and distilled water to a total volume of 100 mL), samples were boiled for denaturation. Proteins were separated based on molecular size in a 10% SDS-containing polyacrylamide gel and then blotted to polyvinylidine fluoride (PVDF) membranes (Hybond-P, GE Healthcare, Little Chalfont, United Kingdom) by the Trans-Blot Turbo system (Bio-Rad, Hercules, CA, USA). Nonfat dry milk (3%) in TBS-Tween

(10 mM Tris-base, 150 mM NaCl, 0.2% Tween-20, pH 8.0) was used to block nonspecific binding on the membrane. Rabbit polyclonal primary antibodies were added, specific to cleaved caspase-9, cleaved caspase-3, caspase-8, AIF (Cell Signaling Technology, Beverly, MA, USA), and caspase-12 (MBL International Corporation, Woburn, MA, USA), diluted to 1:1000 in the blocking solution, and then incubated overnight. Five washes with TBS-Tween were undertaken to remove excess antibodies. Incubation with a horseradish-peroxidase (HRP)-conjugated polyclonal goat anti-rabbit secondary antibody (Pierce, Thermo Fischer Scientific, Rockford, IL, USA) diluted to 1:10,000 in blocking solution followed. The enhanced chemiluminescent signal (Immobilon Western, Millipore Corporation, Billerica, MA, USA) was detected using a G:box gel documentation system (Syngene International Ltd., Bangalore, India). Membranes were then chemically stripped of antibodies (0.2M glycin-HCl, 0.2% Tween-20, 0.05%, pH 2.5) and reprobed using β-actin or GAPDH (Cell Signaling Technology, Beverly, MA, USA) rabbit polyclonal primary antisera as mentioned above to control the disparity in protein concentration among samples. Densitometry analysis was performed using the ImageJ software (National Institutes of Health, Bethesda, USA).

III.1.8. Plotting of experimental data and statistical analysis

Data presented in the diagrams were gathered in a series of four independent experiments. Values shown are the means and standard deviations (± S.D.). The Kolmogorov–Smirnov test was used to test the normality of the distribution of the data. A one-way analysis of variance (ANOVA) test, supplemented with a Tukey's post hoc test for multiple samples, was used to highlight the significance of differences. P values < 0.05 were considered to be significant. Relevant significant differences are marked in the graphs and their corresponding P values are indicated in the figure legend.

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

III.2.1. Reagents

All chemicals used were obtained from Sigma-Aldrich (now Merck KGaA, Darmstadt, Germany) unless stated otherwise.

III.2.2. Pulp cell culture

As described in section *III.1.2*. above.

III.2.3. Monomer exposure

Cells were exposed to TEGDMA concentrations of 0.1, 0.2, and 0.75 mM for 24 hours as suggested by our pilot viability studies. 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 concentration range not yet causing significant cell death.

III.2.4. WST-1 (water- soluble tetrazolium salts) colorimetric viability assay

As described in section *III.1.6.* above.

III.2.5. EnzCheck Gelatinolytic/ Collagenolytic activity assay

EnzCheck Gelatinolytic/ Collagenolytic activity assay kit (Molecular Probes; Eugene, OR, USA) was used to investigate the possible effect of various concentrations of TEGDMA monomers on enzyme activity in pulp cell lysates and media derived from cells. Following the manufacturer's instructions, the highly labelled fluorescent gelatin substrate was mixed with reaction buffer in a final volume of 200 μL. Cell culture media or dilutions of mechanically homogenized cell extracts suspended in lysis buffer were collected into 96-well plates. As per the information from the manufacturer, increase in fluorescence is proportional to enzymatic activity. Proteolysis was determined by a Promega Glo Max plate reader (Madison, Wisconsin,

USA), operated at a fluorescent excitation maximum of 495 nm and emission maximum of 515 nm. Absolute enzyme activity values were plotted and analyzed [188].

III.2.6. Western Blotting

After TEGDMA treatment, cells were harvested and lysed as described in previous studies [12]. Briefly, the pulp cells were collected in cold lysis buffer (50mM Tris-base, pH 7.4, 10% glycerol, 150mM NaCl, 1mM EGTA, 1mM Na-orthovanadate, 100mM NaF, 5μM $ZnCl_2$, 10μg/ml aprotinin, 1μg/ml leupeptin, 1mM PMSF, 1% Triton X-100) and homogenized for 20 seconds. The homogenate was centrifuged at 4°C and at 40,000 x g for 30 minutes. Protein concentrations of the supernatants were measured (Lowry's method, Detergent Compatible Protein Assay Kit, Bio-Rad, Hercules, CA, USA). Protein concentrates were then diluted to contain equal 30 μgs of protein. Laemmli buffer (prepared from 25 ml 1M Tris-HCl, pH 6.8, 40 ml glycerol, 8 g SDS, 10 ml 100mM EGTA, 10 ml 100mM EDTA, 1 ml 1% bromophenol blue; and distilled water to a total volume of 100 ml) was added followed by boiling for denaturation. Subsequently, 10% SDS-containing polyacrylamide gels were used to separate proteins based on molecular size. Proteins were then transferred to PVDF membranes (Hybond-P, GE Healthcare, United Kingdom) by the Trans-Blot Turbo system (Bio-Rad, Hercules, CA, USA). Nonspecific binding on the membrane was blocked by 3% nonfat dry milk in TBS-Tween (10mM Tris-base, 150mM NaCl, 0.2% Tween-20, pH 8.0). Rabbit polyclonal primary antibodies were added specific to MMP-8 (Thermo Fisher Scientific, Waltham, MA, USA); MMP-9 (Abcam, Cambridge, UK); p-(phospho-) ERK1/2 (Thr202/Tyr204), p-p38 (Thr180/Tyr182) and p-JNK (Thr183/Tyr185) (Cell Signaling Technology, Beverly, MA, USA), and rabbit monoclonal antibodies specific to MMP-2 (D4M2N, Cell Signaling Technology, Beverly, MA, USA) diluted 1:1000 in the blocking solution and then incubated overnight. Excess antibody was removed by five washes of TBS-Tween. Incubation with a horseradish-peroxidase (HRP)-conjugated polyclonal goat anti-rabbit secondary antibody (Pierce, Thermo Fischer Scientific, Rockford, IL, USA) diluted 1:10,000 in blocking solution followed. Enhanced chemiluminescent signal (Immobilon Western, Millipore Corporation, Billerica, MA, USA) was detected using a G:box gel documentation system (Syngene International Ltd, Bangalore, India).

Membranes were then chemically stripped of antibodies (0.2M glycin-HCl, 0.2% Tween-20, 0.05%, pH 2.5) and reprobed using β-actin, ERK1/2, p38, or JNK (Cell Signaling Technology, Beverly, MA, USA) rabbit polyclonal primary antisera as mentioned above to prove the absence of disparity in protein concentration among samples. Densitometry analysis was performed using the ImageJ software (National Institutes of Health, USA).

III.2.7. Immunofluorescence Microscopy

Pulp cells seated onto glass coverslips were exposed to the conditioned or control media, and processed for immunocytochemistry [47]. Briefly, a quick rinse in 37 °C PBS was followed by a 4% paraformaldehyde fixation in PBS at pH 7.4, 4°C for 12 hours. The fixative was removed by three changes of TBS (50mM Tris-HCl, pH 7.4, 150mM NaCl). Permeabilization was achieved by a 30-minute wash with 0.1% Triton X-100 in TBS at 4°C. Nonspecific binding sites were blocked by incubation in 5% nonfat dry milk in TBS at 4 ˚C for 1 hour. The primary antibodies MMP-2 (D4M2N, Cell Signaling Technology, Beverly, MA, USA), MMP-8 (Thermo Fisher Scientific, Waltham, MA, USA), and MMP-9 (Abcam, Cambridge, UK) were diluted 1:100 in the blocking solution and incubated with the cells overnight at 4 ˚C. After five washes in TBS, Cy3-conjugated polyclonal donkey-anti-rabbit antibodies (Jackson Immuno Research, Cambridgeshire, UK) were diluted 1:200 in the blocking solution and added to the cells for overnight incubation at 4 ˚C. For each antigen respectively, control samples prepared by the omission of the primary antibodies produced no visible fluorescence signal using the same microscope. During the final five TBS washes nuclei were counterstained with Hoechst 33342 (Calbiochem, La Jolla, CA, USA). Fluorescence microscopy images were obtained using an Olympus FV-1000 laser scanning confocal system (Olympus Europa, Hamburg, Germany). The presented single optical slice pictures were generated using a 40X dry objective and are representative of series of four to five independent experiments with similar results. Determination of raw integrated density values was carried out using the ImageJ software (National Institutes of Health, USA).

III.2.8. Plotting of experimental data and statistical analysis

WST-1, collagenolytic activity assay, Western Blot and immunocytochemistry density data presented in diagrams were gathered in a series of four independent experiments, values shown are the means and standard deviations (± S.D.). Significance of differences was determined using one-way analysis of variance (ANOVA) test with the application of Tukey post hoc tests for multiple samples. P values < 0.05 were considered to be significant. Significant differences considered as relevant to major findings were marked in the graphs and their corresponding P values were indicated in the figure legend.

IV. Results

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

IV. 1.1. Cell Counting

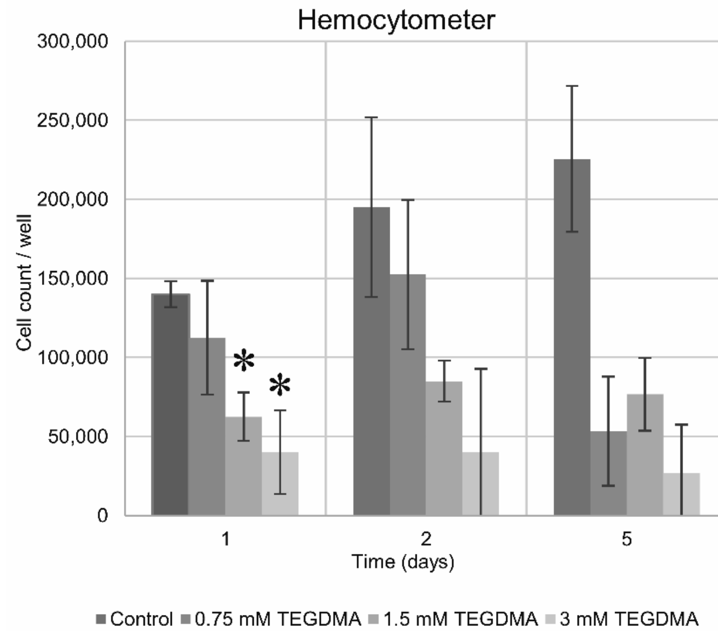
Results of cell counting undertaken by two distinctive methods show a concentration- and time-dependent TEGDMA monomer toxicity on pulp cells. While significant cell death was detected at 24 h after exposure to 1.5 and 3 mM TEGDMA, 0.75 mM TEGDMA did not cause a significant increase in the number of dead cells *(figures 16-19).* Second- and fifth-day results showed the continued destruction of cells with near-complete cell death evident on the fifth day with exposure to 3 mM TEGDMA. Microscope images have been included to illustrate the changes in cell number over the course of the investigation.

	Hemocytometer (average cell count/well, SD)		
	Day 1	Day 2	Day 5
Control	140,000 (8,164.9)	195,000 (56,862.4)	225,555.6 (46,127.8)
0.75 mM	112,500 (35,939.7)	152,500 (47,169.9)	53,333.3 (34,641)
1.5 mM	62,500 (15,275.2)	85,000 (12,909.9)	76,666.6 (22,912.8)
3 mM	40,000 (26,457.5)	40,000 (52,915)	26,666.6 (30,822)

Figure 16. Number of viable pulp cells at various time-points of the 5-day exposure to 0.75, 1.5, and 3 mM TEGDMA, as measured by a hemocytometer (sample number: n = 2).

	Marked viewing field (average cell count/viewing field (fold), SD)		
	Day 1	Day 2	Day 5
Control	1.309 (0.192)	2.328 (0.462)	4.477 (1.058)
0.75 mM	0.969 (0.109)	0.891 (0.084)	0.578 (0.074)
1.5 mM	0.924 (0.192)	0.753 (0.183)	0.007 (0.006)
3 mM	0.624 (0.168)	0 (0)	0 (0)

Figure 17. Number of viable pulp cells at various time-points of the 5-day exposure to 0.75, 1.5, and 3 mM TEGDMA, as counted in marked fields of view (sample number: n = 2).



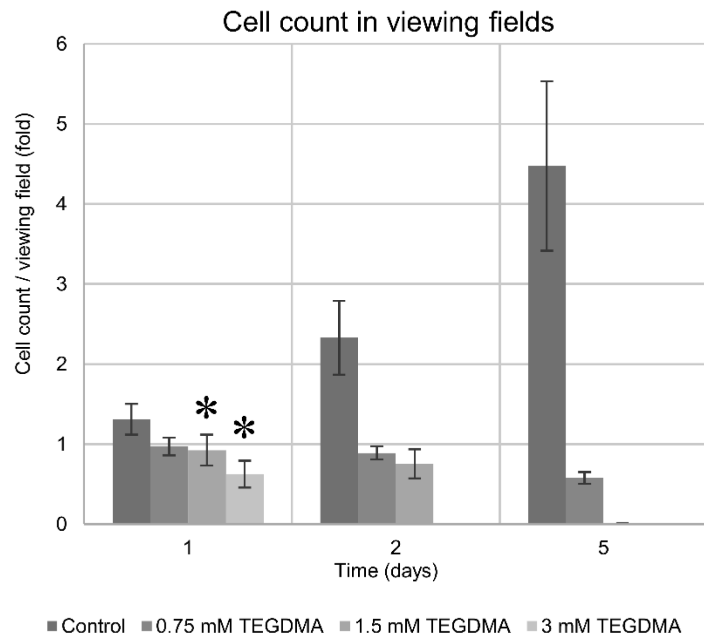


Figure 18. Number of viable pulp cells at various time-points of the 5-day exposure to 0.75, 1.5, and 3 mM TEGDMA, as measured by a hemocytometer and counted in marked fields of view. The hemocytometer graph presents the absolute number of viable cells, while the marked fields of view graph plots the decrease in the number of viable cells as a ratio relative to the control cell number at the start of the experiment. * = significantly different from the untreated control of the first day (for the hemocytometer, P = 0.0018, P = 0.0002 at 1.5 mM and 3 mM TEGDMA concentrations, and P = 0.0293, P = 0.0004 at 1.5 mM and 3 mM TEGDMA concentrations for the viewing field data, respectively, sample number: n = 2)

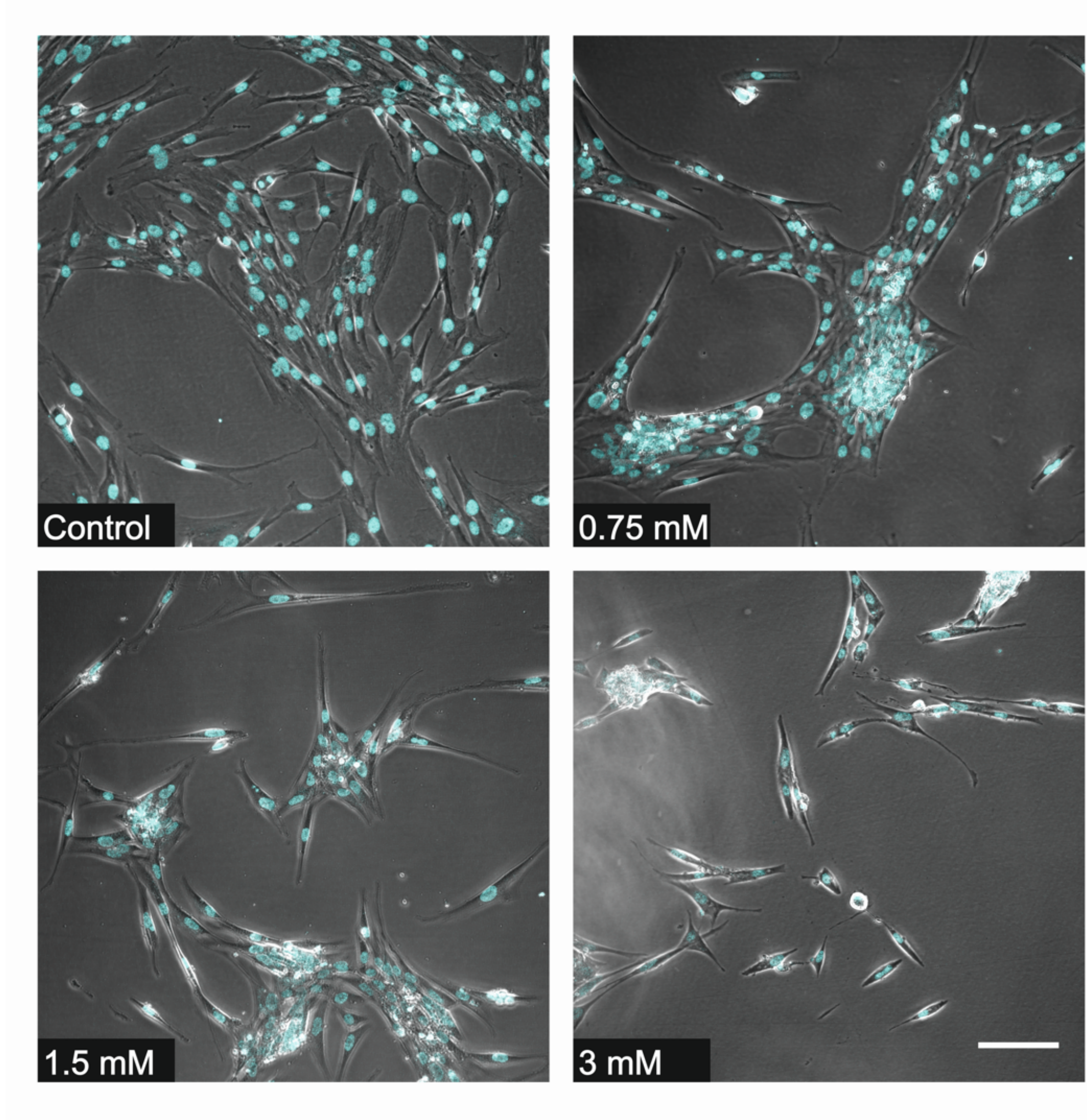


Figure 19. Illustrative microscope images showing the decrease in viable cell number with increasing concentration of TEGDMA (sample number: n = 2, 20X dry objective, scale bar represents 100 µm, blue: Hoechst DNA staining).

IV.1.2. WST-1 Colorimetric Viability Assay

Similar to the results of the above cell counting, WST-1 staining showed a significant reduction in cell viability at 24 h upon exposure to 1.5 and 3 mM TEGDMA. A concentration of 0.75 mM failed to decrease viability significantly at 24 h *(figures 20 and 21)*. Second- and fifth-day readings confirmed the findings established by the above cell counting with minimal viability readings after 5 days of exposure to 1.5 and 3 mM TEGDMA. Based on the kinetics of cell death seen in the above results, it was decided that the treatment time applicable for the subsequent investigations would be 24 h.

	WST-1 (average fold of day 0 untreated control, SD)		
	Day 1	Day 2	Day 5
Control	1.237 (0.120)	1.961 (0.676)	2.259 (0.115)
0.75 mM	0.970 (0.193)	1.290 (1.041)	0.893 (0.243)
1.5 mM	0.814 (0.228)	0.472 (0.586)	0.105 (0.135)
3 mM	0.518 (0.084)	0.056 (0.065)	0.089 (0.116)

Figure 20. Viability changes in pulp cells over the course of the 5-day exposure to 0.75, 1.5, and 3 mM TEGDMA as detected by the Water-Soluble Tetrazolium-1 assay (sample number: n = 3).

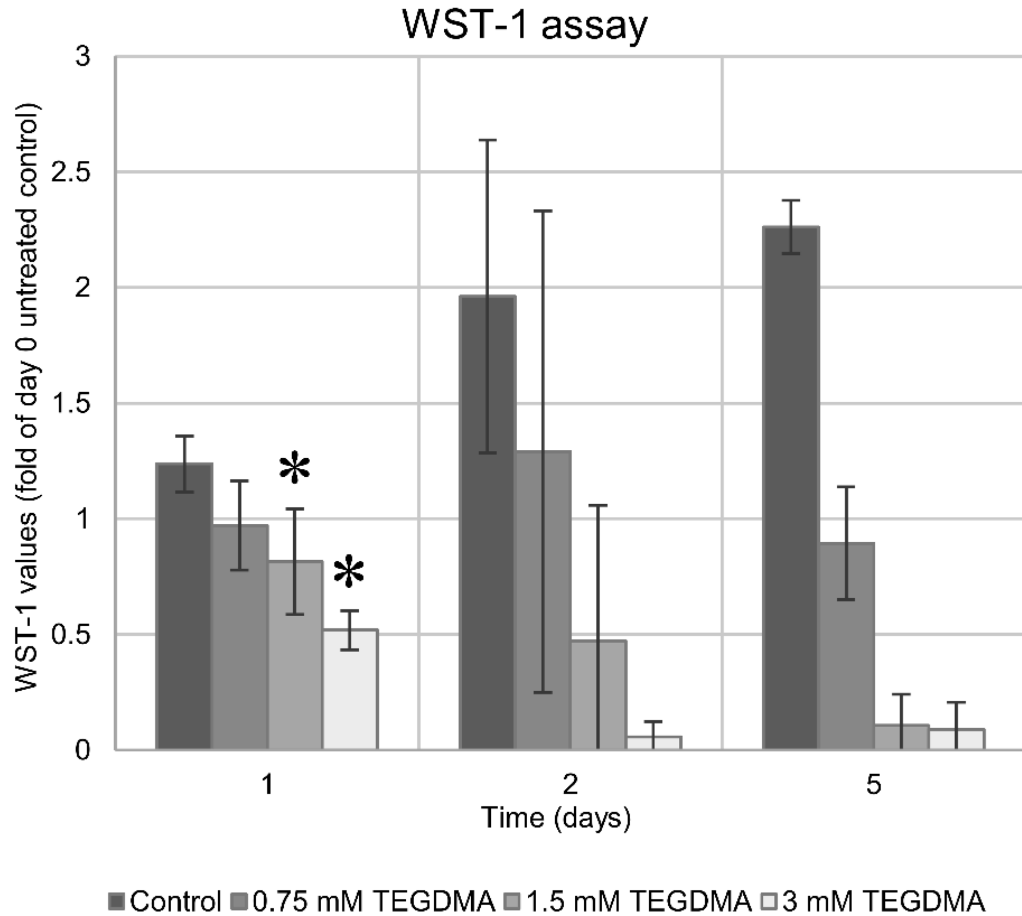


Figure 21. Viability changes in pulp cells over the course of the 5-day exposure to 0.75, 1.5, and 3 mM TEGDMA as detected by the Water-Soluble Tetrazolium-1 assay. The graph depicts viability values as a ratio relative to the untreated cells at the start of the experiment. * = significantly different from the 1st day untreated control (P = 0.0293, 0.0004 for 1.5 mM and 3 mM, respectively, sample number: n = 3).

IV.1.3. Western Blotting

One-day exposure to TEGDMA led to an increase in the cleaved variants of all investigated caspases as well as to an induction of AIF production. Significant elevations in cleaved caspase-3, -8, and -9 were apparent after exposure to concentrations of 1.5 mM and 3 mM for caspase-3 (*figure 22*), 0.2 mM and above for caspase-8, and for all tested concentrations in the case of caspase-9. The increase in caspase-12 *(figure 23)* was determined to be significant above the concentration of 0.75 mM, while significant AIF production at 24 h occurred after exposure to 0.2 mM, 0.75 mM, and 1.5 mM TEGDMA *(figure 24).*

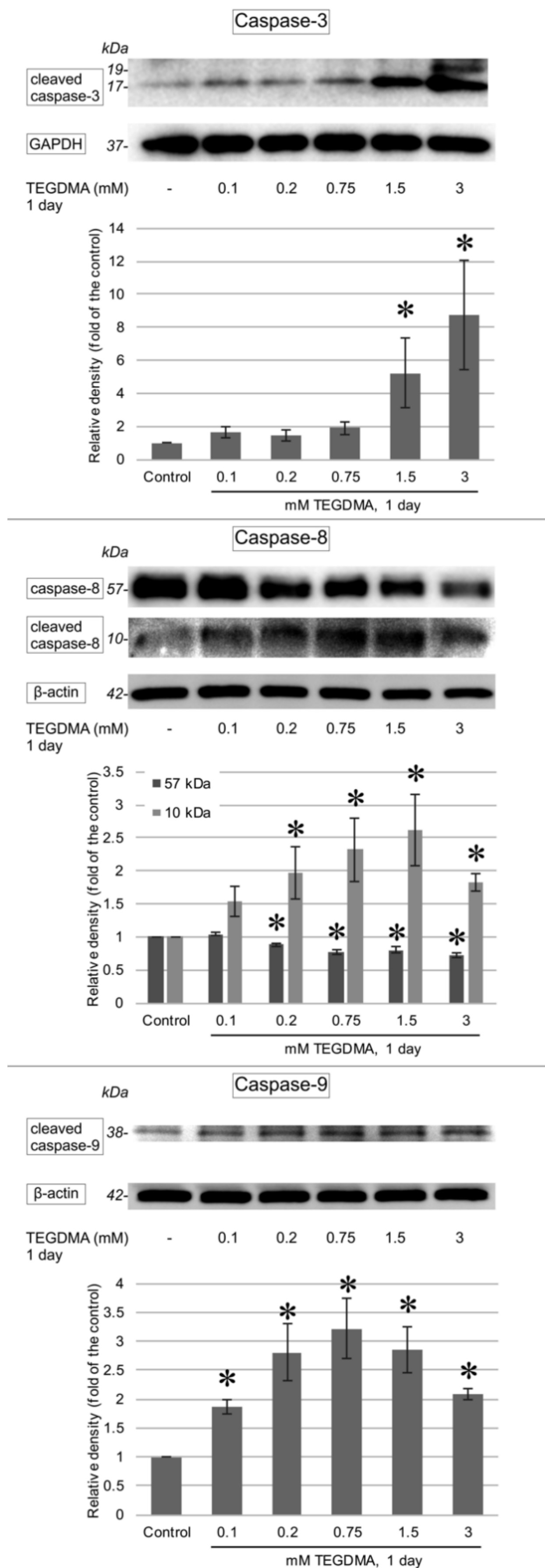


Figure 22. Immunoblots showing the changes in the levels of cleaved caspase-3, -8, and -9 in pulp cells after a 1-day exposure to 0.1, 0.2, 0.75, 1.5, and 3 mM TEGDMA. GAPDH or β-actin bands, obtained by reprobing the Western blot membrane, served as a loading control each time. Results of the quantitative analysis of densitometry data are illustrated below each blot (gathered by ImageJ). * = significantly different from the untreated control (in the case of the cleaved caspase-3 $P = 0.0174$ and $P < 0.0001$ at 1.5 mM and 3 mM, respectively; for the uncleaved caspase-8 $P = 0.0002$, $P < 0.0001$, $P < 0.0001$, and $P < 0.0001$ at 0.2 mM, 0.75 mM, 1.5 mM, and 3 mM, respectively; for the cleaved caspase-8 $P = 0.0002$, $P < 0.0001$, $P < 0.0001$, and $P < 0.0001$ at 0.2 mM, 0.75 mM, 1.5 mM, and 3 mM, respectively; in the case of the cleaved caspase-9 $P = 0.0208$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, and $P = 0.0035$ at 0.1 mM, 0.2 mM, 0.75 mM, 1.5 mM, and 3 mM, respectively, sample number: n = 3).

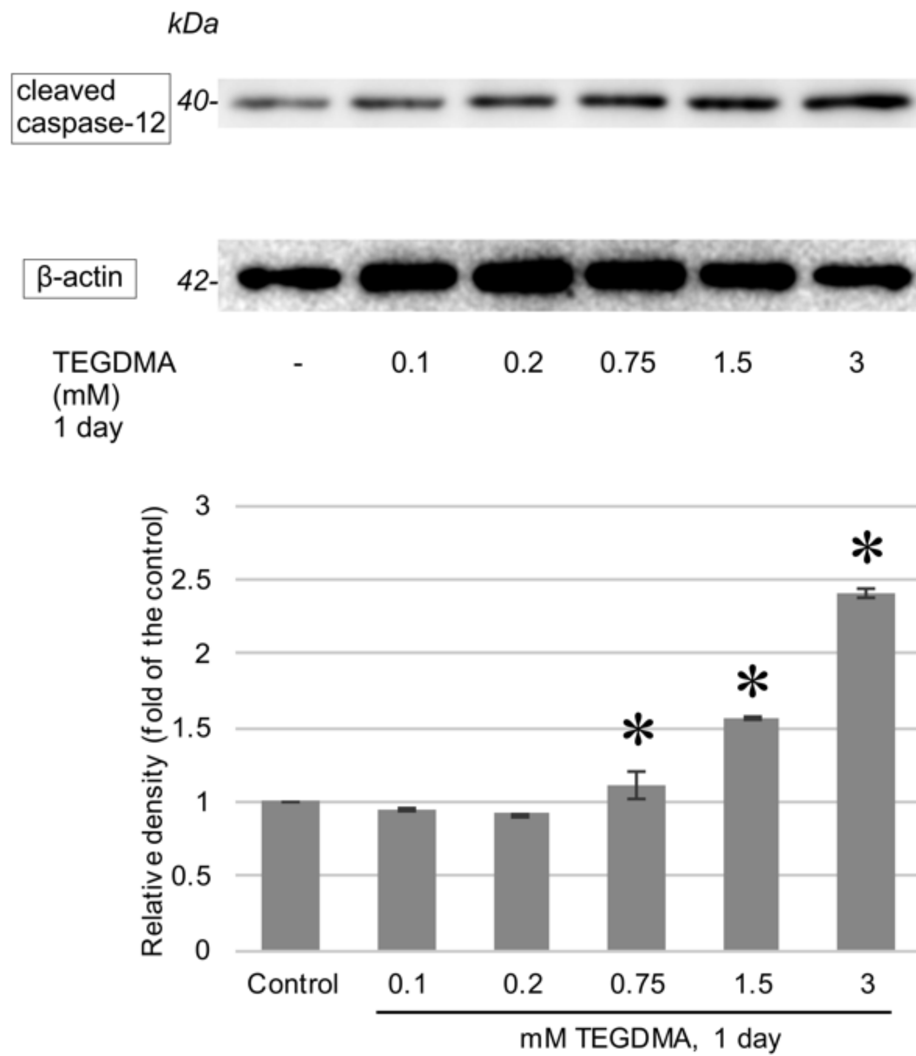


Figure 23. Immunoblots showing the changes in the levels of cleaved caspase-12 after a 1-day exposure to 0.1, 0.2, 0.75, 1.5, and 3 mM TEGDMA. β-actin bands, obtained by reprobing the Western blot membrane, served as a loading control each time. Results of the quantitative analysis of densitometry data are illustrated below the blot. * = significantly different from the untreated control ($P = 0.0074$, $P < 0.0001$, and $P < 0.0001$ at 0.75 mM, 1.5 mM, and 3 mM, respectively, sample number: n = 3).

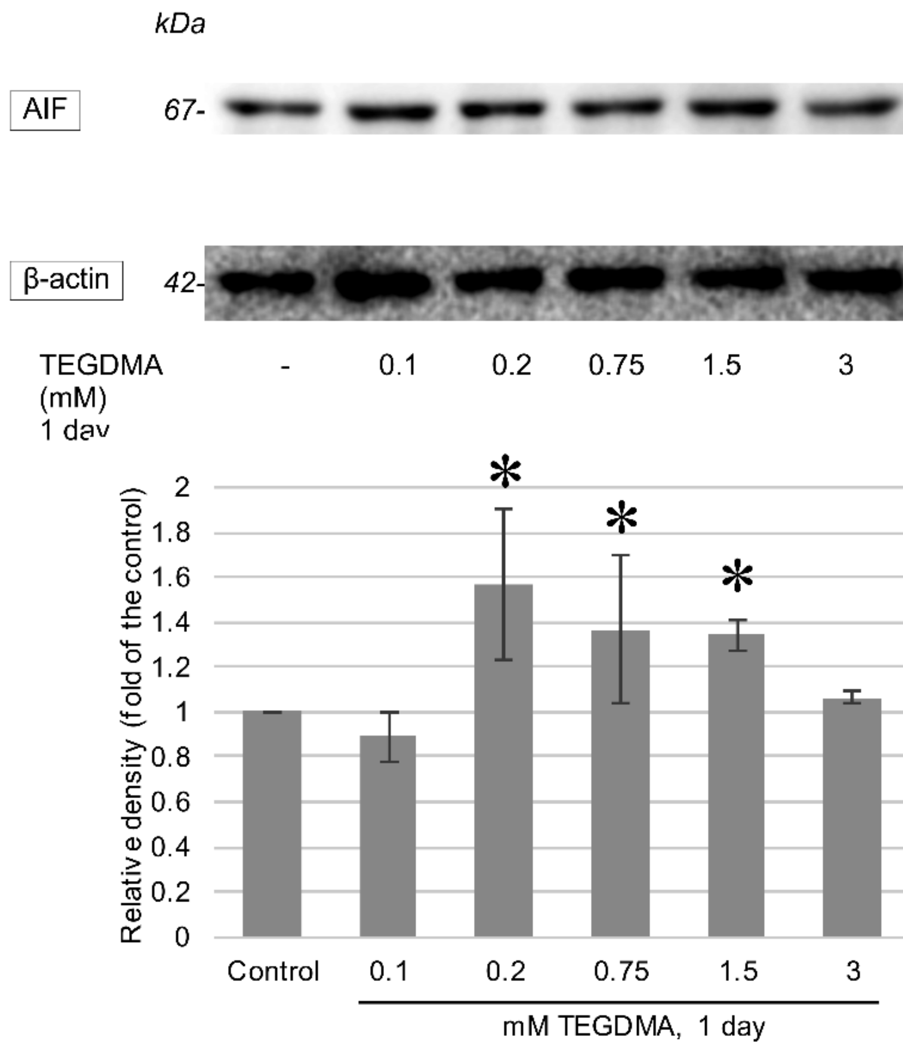


Figure 24. Western blots representing the alterations in apoptosis-inducing factor (AIF) concentrations after a 1-day exposure to 0.1, 0.2, 0.75, 1.5, and 3 mM TEGDMA. β-actin is presented as a means of loading control. Results of the quantitative analysis of densitometry data are illustrated below the blot. * = significantly different from the untreated control ($P < 0.0001$, $P = 0.0074$, and $P = 0.0158$ at 0.2 mM, 0.75 mM, and 1.5 mM respectively, sample number: n = 3).

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

IV.2.1. WST-1 colorimetric viability assay

The time- and dose dependent effect of TEGDMA on cell viability was investigated by a WST-1 assay *(figure 25)*. 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 (data not presented). Therefore, the highest concentration and longest treatment time applicable for further experiments was decided to be 0.75 mM and one day, which are conditions not yet causing substantial cell death.

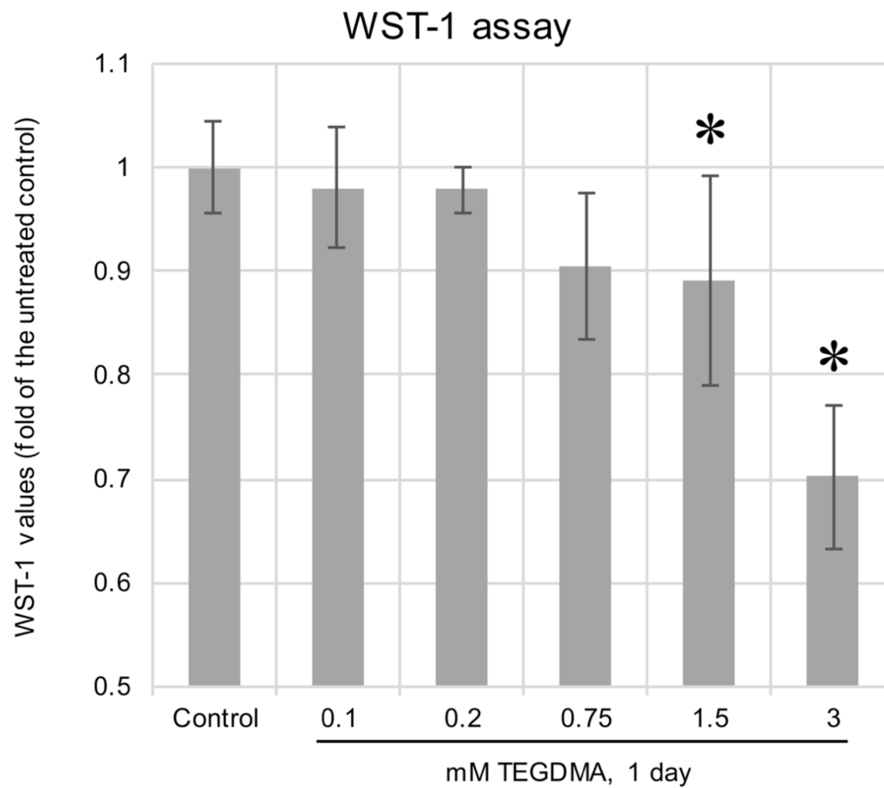
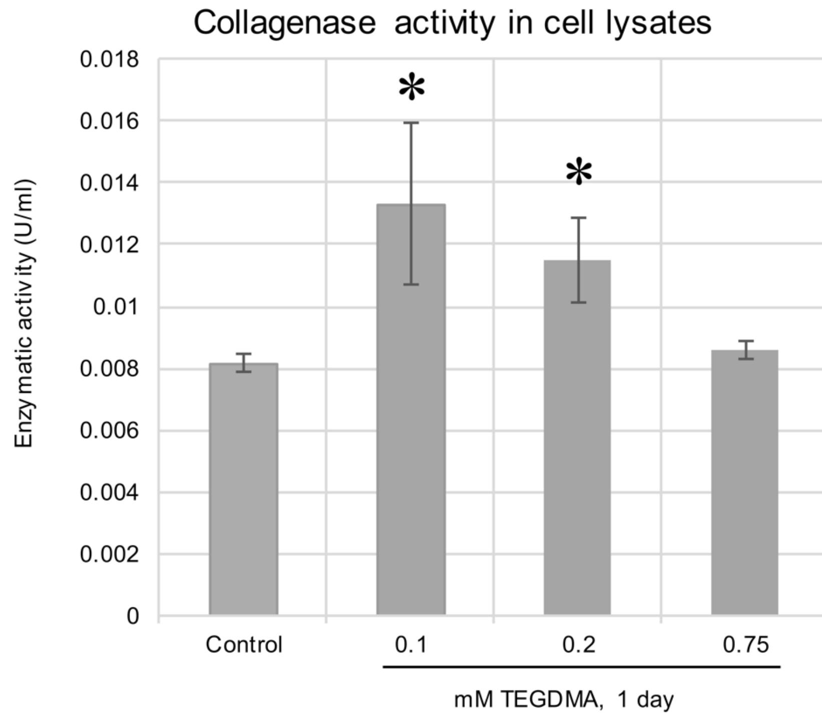


Figure 25. Viability changes in pulp cells after a 1-day exposure to 0.1, 0.2, 0.75, 1.5, and 3 mM TEGDMA as detected by a Water-Soluble Tetrazolium-1 assay. The graph represents the WST-1 values as a ratio compared to the average value of the day 1 control. *= significantly different from the untreated control of day 1 (P=0.0251 and P<0,0001 at 0.75 mM and 3 mM TEGDMA concentrations respectively)

IV.2.2. Gelatinolytic/ Collagenolytic activity assay

Compared to the control, pulp cell lysates showed after one day, a mild increase in total collagenase/gelatinase activity upon exposure to 0.1 and 0.2 mM TEGDMA *(figure 26),* (also from media upon 0.75 mM TEGDMA exposure). Longer exposures resulted in a stagnation and decrease in collagenase/gelatinase activity both from the lysates and medium for all groups including the control (results not presented).



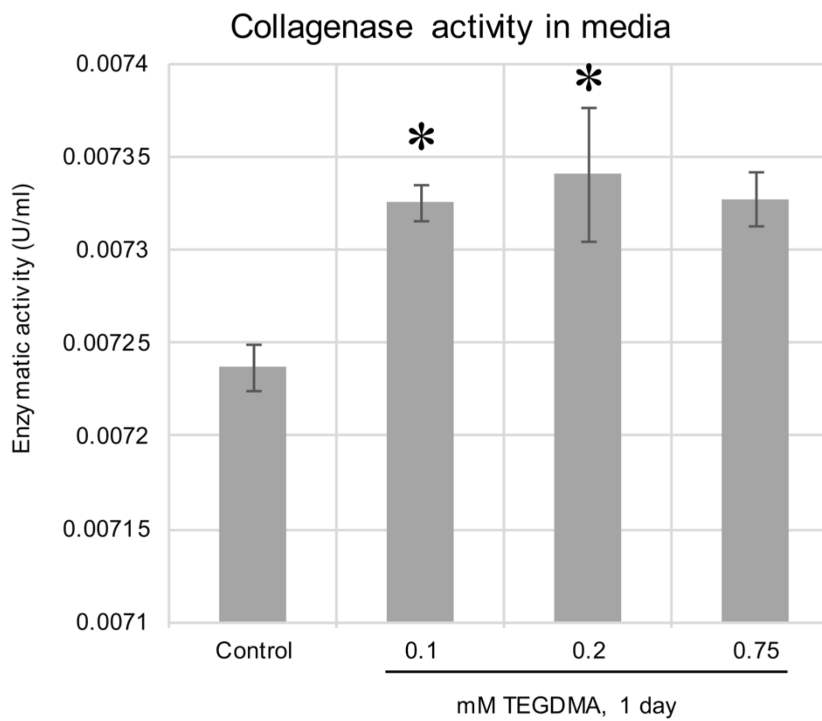


Figure 26. Total collagenase/gelatinase activity after exposure to 0.1, 0.2, and 0.75 mM TEGDMA. The diagram represents enzyme activity values detected by a specific kit from lysates of pulp cells and the media removed from cultures. *= significantly different from the day 1 untreated control in cell lysate sample (P=0.002 and P=0.0381 for 0.1 mM and 0.2 mM respectively), from 1st day untreated control in medium sample (P=0,0003; P<0,0001 and P=0.0003 for 0.1 mM; 0.2 mM and 0.75 mM respectively)

IV.2.3. Western Blotting

TEGDMA exposure also led to increased levels of MMP-2, -8, and -9 in pulp cells, as detected by the Western blot analysis *(figure 27).* 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. The presented photos are representative of series of four to five independent experiments with similar results.

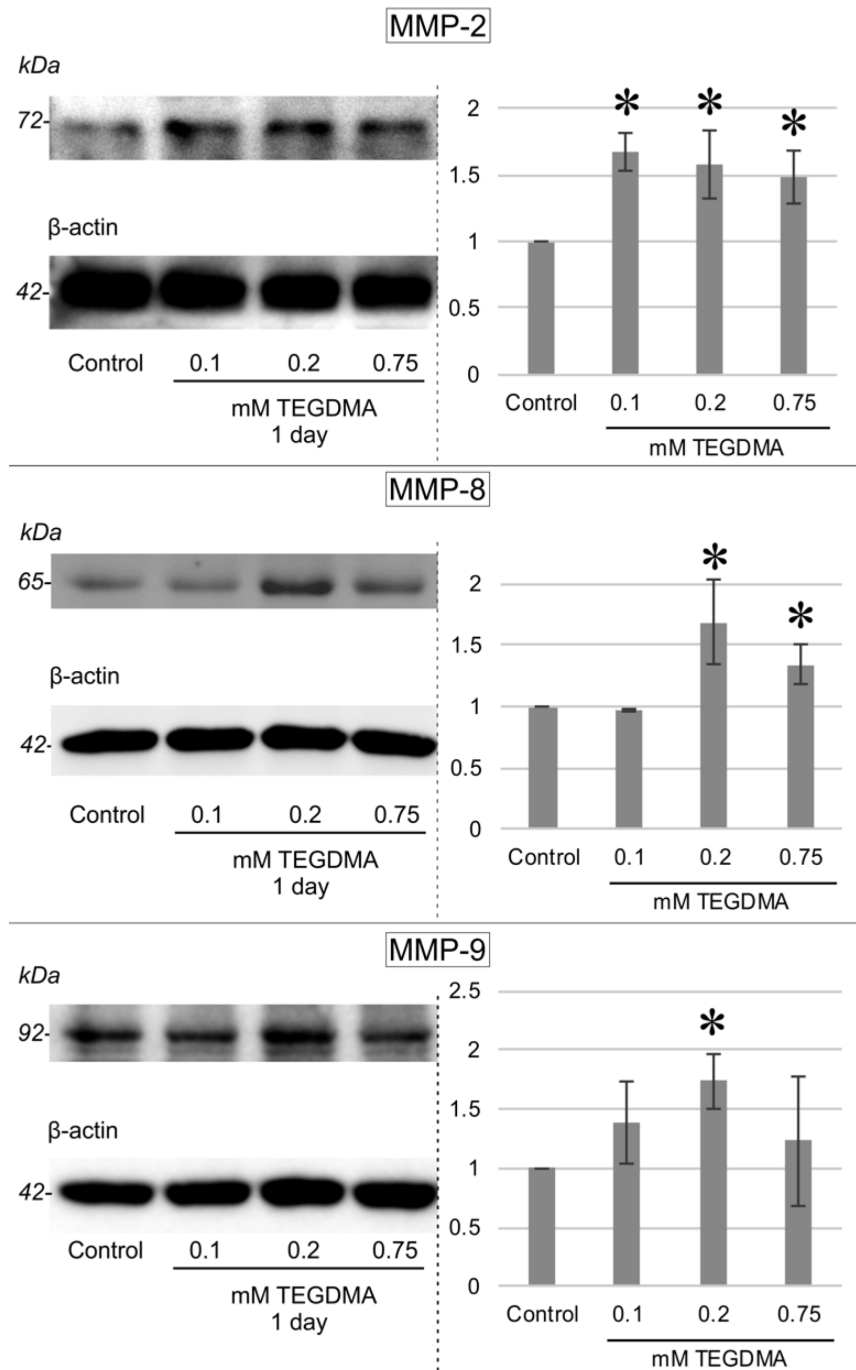


Figure 27. Immunoblots illustrating the changes in MMP-2, -8, and -9 concentrations in pulp cells upon a 1-day exposure to TEGDMA concentrations of 0.1, 0.2, and 0, 75 mM. β-actin bands are results of Western blot membrane reprobing which served as loading control each time. Results of the quantitative analysis of densitometry data gathered by ImageJ are presented on the right side. *= significantly different from the 1 day untreated control, in the case of MMP-2 $P= 0.0026$; 0.0079 and 0.0249 at 0.1 mM; 0.2 mM and 0.75 mM respectively. For MMP-8 $P< 0.0001$; $P= 0.0410$ at 0.2 mM and 0.75 mM respectively. For MMP-9 $P= 0.0021$ at 0.2 mM.

IV.2.4. Immunofluorescence Microscopy

Immunocytochemistry was also performed to further confirm the findings acquired by Western blot, and to investigate possible changes in antigen distribution. 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 *(figures 28, 29* and *30)* with the exception of the 0.75 mM treatment and MMP-9 levels. In 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, cytoplasmic as well as a filamentous signal, the latter often in the vicinity of the cell membrane. There was no detectable difference in antigen localization between the control and treated pulp cells.

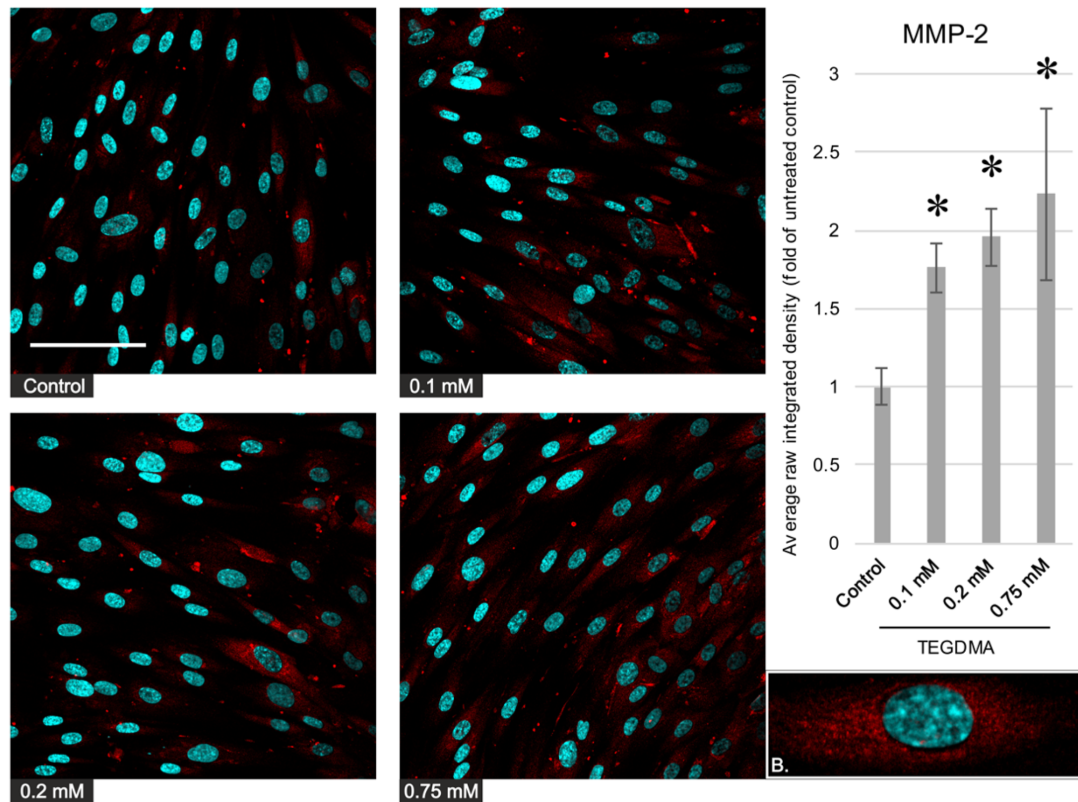


Figure 28. Left panel: Immunocytochemistry images depicting the level and distribution of MMP-2 (red) in pulp cells after a 1-day exposure to 0.1, 0.2, and 0.75 mM TEGDMA, nuclei were counterstained with Hoechst 33342 (blue). The scalebar represents the length of 100µm. Right panel: average fluorescence intensity values of images gathered by analysis using the ImageJ software, compared to the untreated control. *= significantly different from the 1-day untreated control, P= 0.0191; 0.0039 and 0.0005 at 0.1 mM; 0.2 mM and 0.75 mM respectively. Part B.: a representative cutout magnified image showing the intracellular distribution of the antigen (from the control picture).

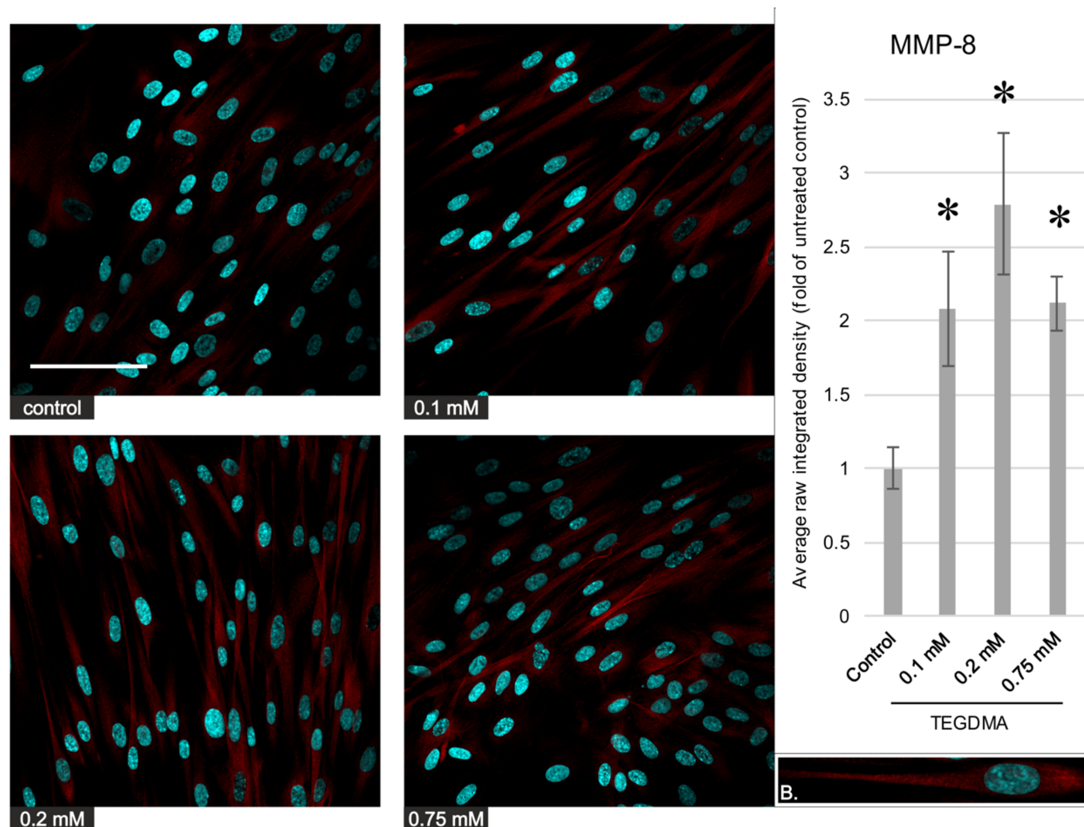


Figure 29. Left panel: Immunocytochemistry images depicting the level and distribution of MMP-8 (red) in pulp cells after a 1-day exposure to 0.1, 0.2, and 0.75 mM TEGDMA, nuclei were counterstained with Hoechst 33342 (blue). The scalebar represents the length of 100µm. Right panel: average fluorescence intensity values of images gathered by analysis using the ImageJ software, compared to the untreated control. *= significantly different from the 1-day untreated control, P= 0,0028; 0,0001 and 0,0021 at 0.1 mM; 0.2 mM and 0.75 mM respectively. Part B.: a representative cutout magnified image showing the intracellular distribution of the antigen (from the 0.2 mM picture).

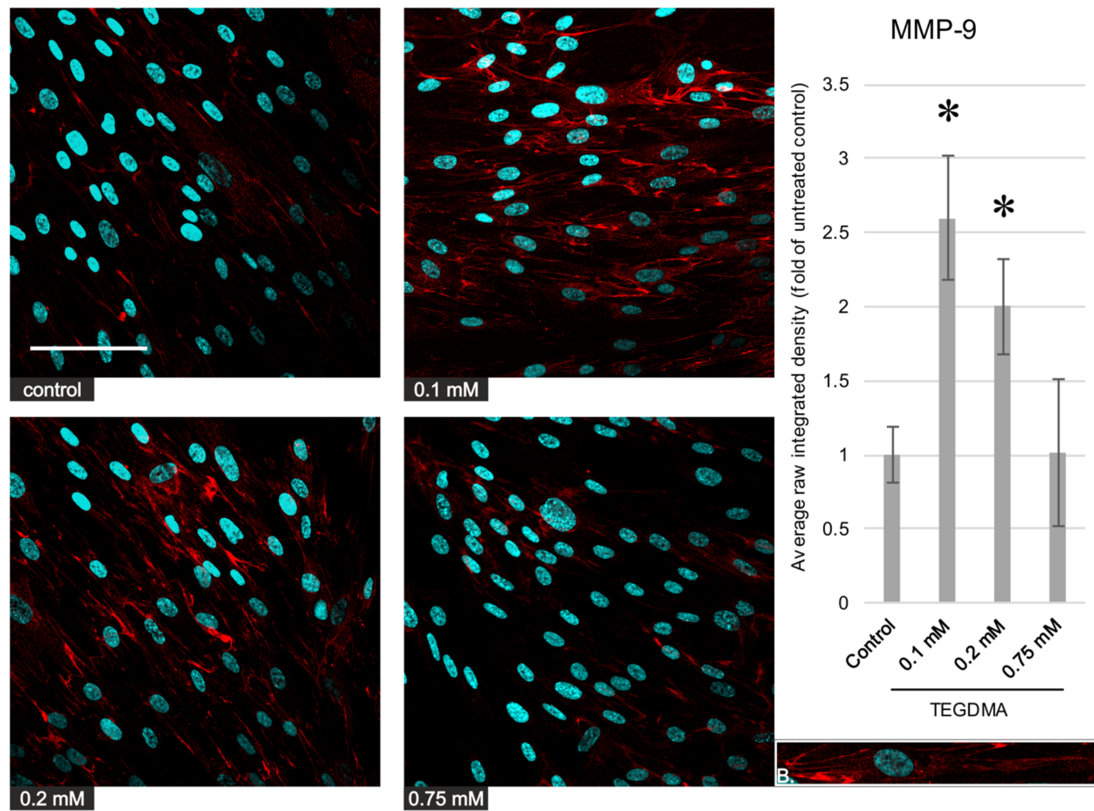


Figure 30. Left panel: Immunocytochemistry images depicting the level and distribution of MMP-9 (red) in pulp cells after a 1-day exposure to 0.1, 0.2, and 0.75 mM TEGDMA, nuclei were counterstained with Hoechst 33342 (blue). The scalebar represents the length of 100µm. Right panel: average fluorescence intensity values of images gathered by analysis using the ImageJ software, compared to the untreated control. *= significantly different from the 1-day untreated control, P= 0.0003 and 0.0125 at 0.1 mM and 0.2 mM respectively. Part B.: a representative cutout magnified image showing the intracellular distribution of the antigen (from the 0.1 mM picture).

IV.2.5. ERK, p38 and JNK activation

The identification of concurrent signaling pathway activation was also attempted. Western blotting (*figure 31*) showed after one day, increased levels of both 44 and 42 kDa variants of phosphorylated (activated) ERK as well as p-JNK, for all tested TEGDMA concentrations. A considerable p38 activation could only be seen after exposure to 0.1 mM TEGDMA. After the removal of antibodies from the blots, ERK1/2, p38, and JNK proteins were detected on the corresponding membranes using the proper antisera.

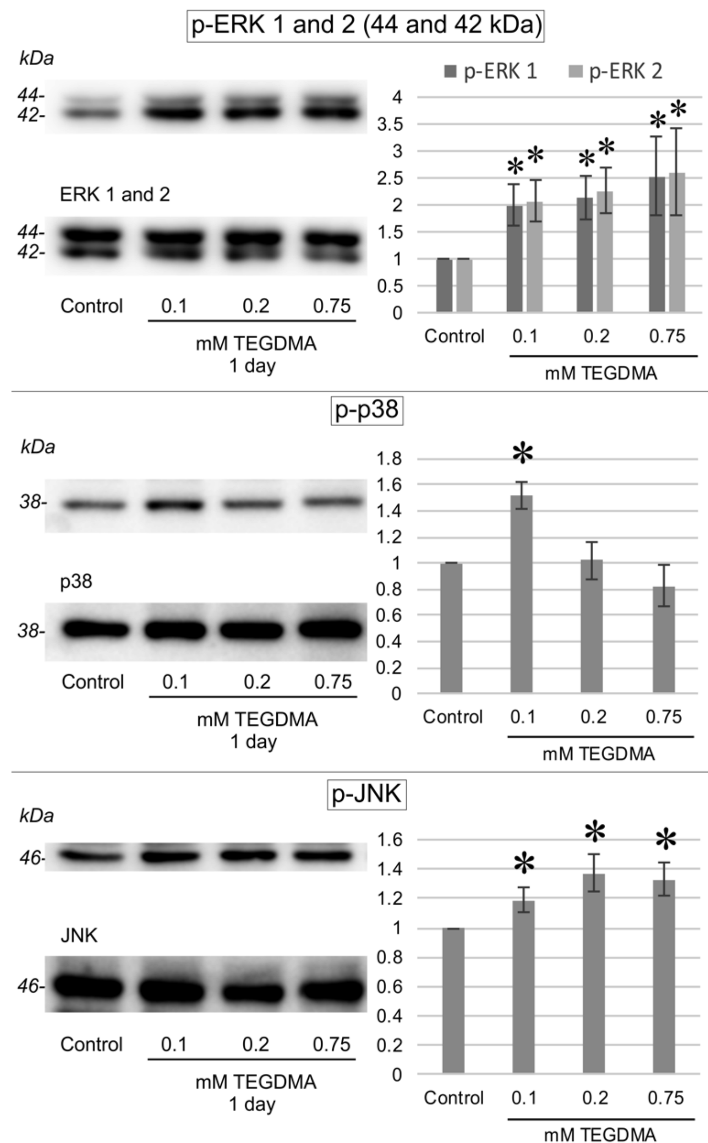


Figure 31. Immunoblot of changes in the concentrations of 42/44 kDa versions of phosphorylated (activated) ERK, p38, and JNK in pulp cells after a 1-day exposure to 0.1, 0.2, and 0.75 mM TEGDMA. The membranes were reprobed using anti-ERK1/2, anti-p38 and anti-JNK antibodies for the purpose of loading controls. The equal level of proteins throughout control and treated pulp cells in cases of JNK, p38, ERK respectively shows that the increased p-ERK, p-JNK and p-p38 concentration upon TEGDMA treatment is the result of the activation (phosphorylation) of these signaling proteins and not a change in their amount. Results of the quantitative analysis of densitometry data gathered by ImageJ are presented on the right side. *= significantly different from the 1-day untreated control, in the case of p-ERK 1 $P= 0.0345$; 0.0149 and 0.0013 at 0.1 mM; 0.2 mM and 0.75 mM respectively. For p-ERK 2 $P= 0.0131$; 0.033 and 0.0003 at 0.1 mM, 0.2 mM and 0.75 mM respectively. For p-p38 $P< 0.0001$ at 0.1 mM. For p-JNK $P= 0.025$; $P<0.0001$; and $P= 0.0001$ at 0.1 mM; 0.2 mM and 0.75 mM respectively.

V. Discussion

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

Since the observation that monomers from resin-based restorative materials may reach the pulp in the millimolar range and may be a cause of chronic pulp inflammation, an increasing number of studies have employed pulp cells in their toxicity investigations [63, 64, 65, 67]. Although resin-based composites typically contain a mixture of monomers, the subject of this current study was chosen to be TEGDMA. In contrast to Bis-GMA, this monomer has a linear backbone which negates the need for intramolecular hydrogen bonding [9]. This imparts greater flexibility, lower molecular weight which leads to better mobility and easier elution into dentine tubules [57]. Unsurprisingly, TEGDMA and HEMA have consistently been detected to be leaching in the greatest amount in elution studies [54, 56] Their hydrophilicity, surfactant, detergent-like properties render them capable of easily passing the cell membrane. These chemical properties coupled with its relatively high proportion in modern composite formulations, 20–50%, makes TEGDMA a relevant target for RBC toxicity studies [57]. Pure TEGDMA has a concentration of 3.8 mol/L. Considering its relative content in RBCs and the postulated 500x dilution effect of 0.5 mm of dentine, the concentration applied herein was in the millimolar range, which is comparable to all recent cytotoxicity studies [58, 172].

Results of the current investigation show 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. The kinetics of cell death is dependent on the applied concentration of monomers, exposure time, cell culture, and detection method. Continuous cell lines display slightly different sensitivities from target cell isolates [70], while macrophages, gingival fibroblasts, and lymphocytes all show significant cytotoxicity at different monomer concentrations [2, 72, 73, 125].

The findings of the present study can be considered to be in line with the findings of *Galler et al.*, who found 3 mM TEGDMA to reduce viability in pulp cells to 20% by

48 h while 0.3 and 1 mM TEGDMA only influenced viability minimally by the 96-h end-point of the study [82]. The viability assay employed within the framework of the current study confirmed the toxic concentration threshold to be somewhere between 0.75 and 1.5 mM. Similar threshold-values have been observed in earlier studies too on pulp cells, which found significant cell death to occur at 24 h above a concentration of 1 mM TEGDMA, which corresponded to a viability decrease of circa 20–30% [3, 78, 86]. However, toxic thresholds outside this range have also been reported. Two millimolar (2 mM) TEGDMA was the lowest concentration causing significant cell death in a study conducted by *Paschalidis et al.* [139]. Conversely, *Batarseh et al.* found significant cell death to take place already with exposure to 0.5 mM TEGDMA [99]. The former exclusively examined pulp stem cells, which are a subset of pulp cells. As mentioned earlier, it is well documented that various cell populations have different sensitivities to TEGDMA [2, 145]. As for the study conducted by *Batarseh et al*., although the principle of the lactate dehydrogenase (LDH) viability assay is the same as the WST-1 and MTT assays used in the current and aforementioned studies, respectively, the LDH test relies on the detection of different molecules and hence could have a slightly different sensitivity.

Mechanisms of TEGDMA toxicity may include an increase in reactive oxygen species (ROS) and cytokine production as well as the induction of oxidative DNA damage, DNA fragmentation, and micronuclei formation [75, 78, 127]. Recent studies have also focused on mapping out possible pulpal/cellular recovery mechanisms following exposure to sub-toxic concentrations of TEGDMA. Owing to the central role ROS plays in TEGDMA-mediated toxicity widespread activation of antioxidant mechanisms, thought to be directed by Nrf2, have been detected by recent studies [136, 138]. Monomer exposure was shown to lead to an increased expression of catalase and heme oxygenase involved in the detoxification of H_2O_2 and heme, respectively [116, 127, 136]. Moreover, *Schneider et al*. demonstrated an increase in cysteine uptake with a subsequent rise in intracellular glutathione formation as pulp cells were exposed to 0.3 mM TEGDMA [137]. Monomers such as TEGDMA have also been shown to induce the intrapulpal production of the anti-inflammatory molecule TGFβ-1 and modify the expression of various other growth factors such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), all of which play important roles in tissue repair and may contribute to pulp recovery [139, 173].

The second finding of the present study was the confirmation of apoptosis as the mechanism of cell death as observed by the increase in 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. Apoptosis has been demonstrated in a number of prior studies and among other things was found to correlate with the inhibition of phosphatidylinositol 3-kinase (PI3K) signaling [3]. *Chang et al.* detected a decrease in the expression of cdc2, cyclin B1, and cdc25C in pulp cells upon exposure to low toxic TEGDMA concentrations, leading to S phase arrest and a concurrent rise in the number of apoptotic cells [86]. Implicating NADPH oxidase 4 as a possible inducer of apoptosis in pulp cells, *Yeh et al.* found that silencing the expression of the above enzyme resulted in a steep decline in ROS production and almost completely abolished TEGDMA-induced apoptosis [78].

In a bid to specify which apoptotic pathway is employed, *Batarseh et al.* used an antibody array to detect changes in the levels of various apoptosis-specific proteins. The levels of Bid, Bim, cytochrome c, caspase-8, and caspase-3 were found to increase in pulp cells upon exposure to low concentrations of TEGDMA, implying that both intrinsic and extrinsic pathways had been activated [99]. Contrastingly, *Yeh et al.* observed a rise only in caspase-9 cleavage, thus suggesting the intrinsic pathway to mediate TEGDMA-induced cell death [78]. In accordance with the first author, the current study has also found both pathways to be activated. TEGDMA exposure led to a significant rise in cleaved caspase-8 as well as cleaved caspase-9 and caspase-3 levels. Multiple studies have confirmed the induction of ROS production to be one of the main mechanisms of monomer toxicity [78, 96, 137]. ROS in turn has been shown to play a role in the activation of both intrinsic and extrinsic caspase-dependent apoptotic pathways [89]. In addition, TEGDMA exposure has also been demonstrated to lead to a significant rise in TNF-α expression in pulp cells, which could provide yet another mechanism for the initiation of the extrinsic pathway [99]. A decline in anti-apoptotic protein BCL-xL leading to mitochondrial depolarization and cytochrome release, as observed in pulp cells in connection with TEGDMA exposure, may activate the intrinsic pathway [89].

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 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 been shown to be a consequence of TEGDMA exposure, can lead to the destruction of this redox balance [174]. 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 [174]. Translocation of caspase-12 from the ER membrane to the cytosol/nucleus connects ER stress to apoptosis by the direct activation of procaspase-9 and convergence with other pathways on caspase-3 [90, 91, 92].

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 [127]. Upon release, AIF preferentially associates with Cyclophilin A and translocates to the nucleus. It 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 [94]. 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.

Limitations of the current study may include the *in vitro* nature of the investigation. The present study demonstrated significant cytotoxicity in controlled conditions. Many additional factors may influence intrapulpal monomer concentration and toxicity in vivo, such as circulation, pressure, outward dentinal fluid flow, as well as chemical interactions with dentine. 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. Additionally, in the present study cells were exposed to TEGDMA only. 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.

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

Polymerization degree of resin-based composite restorative materials correlates well with the quantity of leached monomers [47, 175]. Current composite formulations allow for a maximum conversion of approximately 60-70% [47, 54, 56]. 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 facilitating the leaching of unreacted monomers. Considering that 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 [56, 176]. Monomers have been shown to reach the pulp via the dentine tubules *in vitro* [67, 68, 69]. Theoretical calculations suggest a worst case intrapulpal concentration of 4 mM [58]. Clinical observations suggest that monomer containing adhesives/ RBCs may lead to pulp inflammation [63-66] and MMP induction [108, 177]. Pulp derived MMPs have been suggested to play a role in caries [101], hybrid layer degradation [102] as well as pulpal inflammation [105]. With a lack of studies examining the effect of specific adhesive/ RBC components on MMP activity, the aim of this study was to explore how TEGDMA might affect MMP expression, collagenase/gelatinase activity in pulp cells, and what signaling molecules may play a role.

In this experimental design pulp cells were exposed to 0.1, 0.2, and 0.75 mM TEGDMA solution for 24 hours. Exposure time and concentrations were chosen based on the substantial cell death seen for all treated populations after 48 hours and above concentrations of 0.75 mM in our pilot studies. 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 [124, 136].

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 possible intrapulpal MMP quantity changes upon TEGDMA exposure. Some however, have documented changes in these proteins

as findings in gene expression studies. *Torun et al.*, in their study on pulp cells showed, that 1 mM TEGDMA caused a 10-fold increase in MMP-10 and -12 expression, as measured by DNA microarray and real-time PCR [116]. Similarly, although only a fraction of RNA transcripts translates to proteins, *Cho et al.* detected elevated levels of MMP-1 and MMP-3 mRNA in human dental pulp cells after a 48-hour exposure to low-toxic, 1.3 mM, TEGDMA [132]. The current study has identified MMP-2, -8, and -9 production to increase in a non-linear way with applied TEGDMA concentrations. Currently, the mechanisms for TEGDMA-caused 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 MAPK cascades [123, 124]. ERK, JNK, p38, all MAPKs, have been shown to play a role in changes in MMP-1 and -13 levels in pulp cells [164]. Conflicting results exist on the possible regulation of MMP-2, -9 production by such cascades with some studies finding no correlation and some confirming a direct inducive relationship in pulp cells [165, 166]. ERK1/2 has been found to induce MMP-2 expression in deciduous pulp cells, triggered by TNF-α [178]. The present study also demonstrated an increased activation of MAPK members ERK1/2, JNK, and p38 with the concurrent increase in MMPs. p38 and JNK, are activated by oxidative stress and proinflammatory cytokines, while ERK -downstream of Raf- mostly mediates growth factor stimulus leading to cell differentiation, survival, and migration [96, 125]. No literature has been found confirming the function of the above-mentioned signaling molecules in TEGDMA-induced MMP production. The concurrent activation of signaling molecules and induction of MMP expression found in the present experimental set-up cannot confirm the role of p38, JNK or ERK1/2 in MMP increase. Considering the above-mentioned roles of these signaling molecules, they may only be part of a broader stress response. TEGDMA has in fact been found to increase the activation of p38, ERK, and JNK in pulp cells concurrently with the induction of apoptosis, however the use of specific inhibitors failed to establish a causative relationship. Further studies, possibly with the use of signaling inhibitors, would be required to confirm the possible causative role of these molecules in TEGDMA-mediated MMP rise in pulp cells.

Furthermore, consideration needs to be given also to the trapped growth factors liberated as a consequence of dentine demineralization by adhesive treatment.

Specifically, TGF-β and TNF-α are believed to reach pulp cells via the dentine tubules and induce MMP production [112, 178].

Findings of the present study are in line with those of *Lehmann et al*, who observed an elevation in MMP-2 and proMMP-9 expression in the pulp upon self-etching adhesive treatment of tooth slices and speculated this to be caused by either the acidity or the monomer content [111]. Adhesive treatment was found to lead to a rise in MMP-2 expression in pulp cells by a later study also. Comparatively, due to the lack of acid pre-treatment in the latter study, this author attributed the observed effect to the monomer content [177]. Interestingly, the current findings are in contradiction with those of *Sun et al*, who found HEMA, another leachable monomer, to inhibit MMP-2 and -9 expression in pulp cells, thereby suggesting a potential hybrid layer protective role to this constituent [115].

The second finding of this study is the increase in total collagenase/gelatinase activity seen in pulp cells upon TEGDMA treatment. The employed EnzCheck assay uses Clostridium histolyticum collagenase as reference. Bacterial cleavage sites could be different from human enzymes', however prior studies have shown it to be a useful model in dental *in vitro* inhibition investigations [112, 180]. Moreover, the specific gelatin substrate utilized within this assay is digested by most, if not all, gelatinases and collagenases [179]. Since MMPs represent most of such enzymes, fluorescence can be considered to be directly proportional to total MMP activity. Measurements at later time points were disregarded due to the concurrent drop in viability detected in all groups by the supplementary WST-1 stain.

MMP activation is regulated at multiple levels including transcription, synthesis, and activation of pro-enzymes. Few theories exist pertaining to the mechanism of induction secondary to adhesive exposure. MMP activity in the pro-peptide form is limited by a cysteine sulfhydryl group which interacts with a zinc ion in the catalytic domain of the enzyme [100]. Scission by another protease or removal of this interaction, by a conformational 'cysteine' switch, is a key step in enzyme induction. MMPs have been shown to be activated in acidic pH [112], which led to the speculation, that the mildly acidic monomers may be responsible for adhesive-induced increase in MMP activity [108, 177]. Tissue inhibitors of metalloproteinases (TIMPs) are also believed to physically regulate MMPs. They contain functional groups at the N-terminal capable of chelating the active site catalytic zinc ion. The expression of all four members of the TIMP family are tightly regulated during development and

remodeling thereby controlling MMP activity and ECM degradation [181]. No studies have been found investigating the possible changes in this protein in the setting of monomer/adhesive exposure.

Cathepsins have recently been proposed as a potential regulator of MMP activity [110]. *Tersariol et al* demonstrated both MMP and cathepsin presence in the dentine as well as in the pulp. Although the interplay between cathepsins and dentally relevant MMPs (MMP-2, -8, -9) is yet unknown, studies have shown cathepsin B to activate MMP-1 in human gingival fibroblasts [183]. Cathepsins have been suggested to control matrix degradation in caries via regulating the activity of various MMPs [113, 118]. The mechanism for activity induction can only be speculated at this stage, but the concurrent detection of JNK, p38, ERK activation with the rise of total collagenase/gelatinase activity in the current study may also suggest a role for signaling in enzyme activation. Further studies, ideally with the use of specific inhibitors, would be required to elucidate the possible TEGDMA influence on the previously mentioned proteins in pulp cells.

Although TEGDMA induction of collagenase/gelatinase activity has hardly been demonstrated so far, the findings of the present study can be considered to agree with studies that found MMP activity to increase upon exposure to various adhesives [102, 108, 109]. These materials in turn have been shown to leach significant amounts of monomers capable of reaching the pulp via the dentine tubules [184]. Interestingly, contrary to the findings of the current study, evidence also exists suggesting an inhibitory role to such monomers on MMP-2 and -9 activity in pulp cells. According to the authors, this may happen via multiple mechanisms. Ether bonds in TEGDMA may complex with the bivalent zinc ion in the catalytic domain [114, 182]. Alternatively, such a complexation may happen through the two carbonyl groups of TEGDMA in a Lewis acid-base reaction or TEGDMA may directly react with the nucleophilic centers of the MMP outside the catalytic domain [155].

Regarding the limitations of the present study, demonstration of MMP changes with the application of ERK, p38, JNK inhibitors would have provided additional data. A lack of MMP elevation in the presence of ERK, p38, JNK inhibitors would have provided evidence for the direct involvement of these signaling molecules in triggering MMP increase in the setting of TEGDMA exposure. Therefore, based on the current experimental set-up the role of these signaling molecules could only be suggestive and requires further investigation. Moreover, combinatorial studies

involving 2 or more resin monomers, as present in commercially available RBCs, in combination and individually would have provided information not just about another constituent but also any potential synergistic effect which would apply better to the *in vivo* situation.

VI. Summary of the novel findings

VI.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.

VI.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, HL 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 that:

- 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.

Closing remarks

Persistent concerns about the safety of dental amalgams as well as a desire for more life-like dental restorations led to the introduction of resin-based composites. Despite their wide-spread employment many studies have raised questions about their biocompatibility. Investigations soon uncovered the inherent limitation in the conversion degree of modern composites giving rise to residual, leachable unreacted monomers. For this reason, RBCs may be considered a lasting source of bioactive compounds both in the short and long-term.

A lot has been mapped pertaining to the possible cellular consequences of monomer exposure, however there remain to be areas of great obscurity. A detailed understanding of the toxic and adaptive mechanisms is vital as it allows the development of new materials with intrinsic counter-strategies thereby limiting the irritation on the bordering oral tissues.

Although there is minimal concern regarding the overall safety of composites, the investigations presented here have confirmed that leachable monomers do in fact interfere with normal pulp function and may indirectly influence processes such as caries, pulp inflammation, and HL degradation. Most importantly, the extensive literature review as well as the additional contributions of the described studies highlight the importance of following the manufacturer's instructions during restoration placement in order to maximise conversion and thus minimise the presence of unreacted monomers. This will not only improve physical properties and longevity of the repair but will also reduce unwanted tissue side-effects.

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VIII. 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:

3. 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**
4. 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**
5. 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**
6. 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**
7. 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:

8. 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**

9. 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**
10. 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**
11. 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**

Presentations:

- **The 33rd National Conference of Student Research Societies**,
  Medical and Health Sciences Section
  University of Pécs, Hungary
  18/04/2017 – 21/04/2017

  Presentation title: *Examination of the toxic effects of triethylene-glycol (TEGDMA) resin.*

- **Perspectives in Perio-implantology and Comprehensive Dentistry Conference**
  Oral Biology Section
  Szeged, Hungary
  6/05/2016 – 07/05/2016

  Presentation title: *Examination of the toxic effects of triethylene-glycol (TEGDMA) resin.*

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