TEGDMA INDUCTION OF APOPTOTIC PROTEINS

IN PULP FIBROBLASTS

by

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DEDICATION

This thesis is dedicated to my wonderful family, who believed in me and supported me through the completion of this Master of Science in Dentistry degree.

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INTRODUCTION

Resin composites (RC) are widely used due to increases in esthetic demand and increased concerns about mercury toxicity. Dentists choose RC because of the conservative approach of this preparation when compared with amalgam restorations. Mechanical properties of these restorations are improving continuously through ongoing research in this area. Unfortunately, much research is still needed to test the biocompatibility of these materials and their biological behavior. Cytotoxicity tests done *in vitro* and *in vivo* have shown that there are monomers that leach from the RC and that can cause cell death and damage to the surrounding tissues, specifically pulp tissue. In a study by Qvest et al. in 1989, they showed that teeth restored with RC using different reducing-leakage methods cause increases in pulp reactions and inflammation/necrosis.[1] Studies that compare amalgam to resin-based composite restorations show that composites do have higher failure rates than that of amalgam.[2] In a primary care setting, pulp breakdown and endodontic complications were four times more likely with composite restorations than with amalgam restorations.[2] One of the components of RC that was found to leach was triethylene glycol dimethacrylate (TEGDMA). These monomers were found to leach either due to incomplete polymerization of the resin or mechanical or chemical degradation of the restoration.[3]

TEGDMA is a small hydrophilic monomer (Figure 1). It forms approximately 30 percent to 50 percent of almost all the resin-based composites. TEGDMA is added to improve viscosity and to make the RC more manageable clinically. These monomers can reach the pulp and can cause cell death either by necrosis or apoptosis. Apoptosis is a

programmed, energy dependent cell death which causes cells to shrink with no loss of the membrane integrity. In contrast, necrosis is an uncontrolled, pathological process that does not require energy and the cells swell and lose their membrane integrity. The main difference between necrosis and apoptosis is that there are minimal inflammatory responses initiated in apoptosis.

Different dental materials can affect pulp tissue and can even result in apoptosis and necrosis. Although TEGDMA-induced apoptosis in primary human pulp has been reported,[4] the exact molecular mechanisms and the signal transduction pathways through which it occurs are not clear. Therefore, the aim of this study was to determine which apoptotic proteins are involved in TEGDMA-induced apoptosis.

PURPOSE OF THE STUDY

The purpose of this study was to determine which apoptotic proteins (anti-apoptotic proteins and pro-apoptotic proteins) are involved in TEGDMA-induced apoptosis in human pulp fibroblasts.

HYPOTHESIS

TEGDMA will increase the concentrations of pro-apoptotic proteins in the extrinsic pathway in human pulp fibroblasts.

REVIEW OF LITERATURE

Resin composites (RC) were introduced in the mid-sixties by R. L. Bowen.[5] He prepared the monomer 2,2-bis[4-(2-hydroxy-3 methacryloyloxypropoxy) phenyl]propane (Bis GMA) from combining bisphenol A with glycidyl methacrylate and after that added diglycidyl ether of bisphenol. Bis-GMA, molecular weight (MW) 512, is considered to be the backbone of RC.[5]

To have a better understanding of RC, one should define RC in general and in dental terms. RC is defined as "three-dimensional combination of at least two chemically different materials with a distinct interface separating the components."[5] Fundamentally, dental RC are formed from three chemically-distinct materials or phases: 1) the organic phase or the matrix, 2) the disperse phase or the inorganic filler, and 3) the coupling phase with an organosilane agent that connects the disperse phase with the matrix.[6]

The matrix of the RC is composed primarily of mono-, di- or tri-monomers. Manufacturers still focus on the traditional molecule Bis-GMA alone or in combination with urethan dimethacrylate (UDMA). These molecules represent around 20 percent (v/v) of the constituents of the RC. These molecules are highly viscous substances. The viscosity of water is approximately 1 mPa-s (23°C), while the viscosity of BisGMA is approximately 1,000,000 mPa-s (23°C). However to enhance the properties of RC and make them clinically manageable, diluents with lower viscosity are added such as TEGDMA. TEGDMA is a small (MW 286) hydrophilic molecule.[7] TEGDMA's viscosity is 10 mPa-s (23˚C), which makes TEGDMA one of the major diluents added to the Bis-GMA. TEGDMA actually composes around 30 percent to 50 percent of the RC.[6] While

its addition enhances the clinical properties of the RC, other challenges are faced in regard to the biocompatibility of these RC. Due to incomplete polymerization and continuous degradation attributed either to mechanical or chemical wear through the masticatory forces and salivary esterases, these monomers were found to leach into the surrounding tissues.[7]

Unpolymerized TEGDMA is responsible for some of the cytotoxic effects of RC and dental adhesives on pulp and gingival fibroblasts.[8] Walther et al.[9] found that a key element in TEGDMA-induced apoptosis was the production of reactive oxygen species (ROS).[10] This ROS production was found to be associated with reduced amounts of the natural ROS scavenger glutathion (GSH).[11] It was found that TEGDMA plays a role in decreasing GSH levels, for example to 30 percent to 50 percent.[12, 13] GSH is the most abundant non-protein thiol in eukaryotic cells.[14] GSH plays an important role in protecting the cells from the damage caused by ROS.

Despite the efforts to decrease the amounts of unpolymerized monomers remaining in the RC after polymerization, a percentage (1.5 percent to 5 percent) of the monomers remain unreacted and may leach.[12] There are two factors that hinder the complete polymerization, especially when direct pulp capping is involved. These issues are oxygen and humidity, which are excessively present especially in the previously mentioned scenario (direct pulp capping).[15] However, though this percentage is small, it is still enough to induce apoptosis in pulp fibroblasts.[8]

According to a study conducted by Janke et al.[16] in gingival fibroblasts, TEGDMA caused apoptotic cell death rather than necrotic cell death. Other dental materials that may also induce pulp apoptosis are used as pulp capping agents such as:

calcium hydroxide, zinc oxide eugenol cement, or 4-Methyl methacrylate–tri-*n*-butylborane (4MMT).[17] Necrosis can be a pulp cell reaction that occurs in response to different dental materials. Necrosis is defined as a passive irreversible catastrophic cell death that results when ATP is depleted to an incompatible level for cell survival. The depletion of ATP levels lead to malfunction of the ATP-dependent ion pump. This malfunction of the ATP-dependent ion pump leads to the activation of the so -called cytoplasmic death channel. Opening of the death channel causes cationic ions to leak into the cells, resulting in disruption in the colloidal osmotic pressure of the cell. This cationic entry and the increase in the colloidal osmotic forces cause the cell to swell and then rupture.[18] Therefore, a main criterion of necrosis is the loss of cellular membrane integrity and induction of inflammation in the surrounding tissues. Inflammation results from the release of the cellular content and pro-inflammatory products of the dying cells.[19] Pulp necrosis and inflammation extends through the apical foramen to the surrounding bone and periodontium resulting in periapical inflammation.[20] In contrast, apoptosis is defined as a programmed suicidal death that requires energy for execution. The main differences between apoptotic cell death and necrosis is that in necrosis the cells are unable to maintain their electrochemical potentials, which results in the rupture of cell membrane. When a cellular membrane ruptures, the cell contents are released to the surrounding tissue and cause the second important difference, which is the initiation of inflammatory response. In apoptosis, cells modify their surface proteins so they can be phagocytized by macrophages.[21] Based on that, apoptosis causes a lower level of inflammatory responses than does necrosis.[22] This could be important in evaluating the biocompatibility of a material.[22] It is important to realize that apoptosis is induced at

lower concentrations, while higher concentrations cause necrosis.[18] TEGDMA was shown to cause an increase of necrotic cells at concentrations higher than 3 mM, while at concentrations lower than that, apoptotic cells were dominant.[4] Since the concentration of TEGDMA used for this study is less than 3mM, the focus is going to be on apoptosis rather than necrosis. The main differences between apoptosis and necrosis are noted in Table I.

There are two major pathways for apoptosis, extrinsic and intrinsic pathways. The extrinsic pathway occurs via binding of cell surface death receptors to their specific ligands. These transmembrane receptors belong to the tumor necrosis factor receptor (TNFR) superfamily and include Fas, TRAIL (TNF-related apoptosis inducing ligand) receptor, and TNFR. Upon activation of these transmembrane receptors by their corresponding ligands, caspase 8 is activated. Caspase 8 activates caspase 3, which mediates apoptosis (Figure 2).[23] The intrinsic pathway occurs from activating signals from within the cells due to intense cellular stresses such as DNA damage, growth factor, severe deprivation, and oxidative stress (ROS production) as shown in Figure 2.[24] The intrinsic pathway depends on the balance between pro-apototic and anti-apoptotic proteins of the BCL-2 superfamily (Table II). The balance between the pro-apoptotic and anti-apoptotic determines if the cell lives or die.[25] Any shift in balance changes mitochondrial permeabilty.[26, 27] Mitochondrial permeability increases when the balance shifts toward the pro-apoptotic proteins and allows SMAC (second mitochondria-derived activator of caspases), HtrA (high temperature requirement protein A) and then cytochrome c to be translocated from the mitochondria to the cytosol. SMAC activates apoptosis through binding directly to inhibitory apoptotic family (IAP) family members.

The release of cytochrome c plays a critical role in mammalian apoptosis.[26] Cytochrome c binds to apoptotic protease activating factor 1 (APAF-1) to form a multiprotein structure called apoptosome. The primary function of the apoptosome is the activation of caspase 9.[28, 29] Caspase 3 is then activated by caspase 9, which causes oligonucleosomal DNA fragmentation to occur (i.e., apoptosis) (Figure 2).[30]

Both pathways result in the activation of the caspases. Caspases play a crucial role in the induction of apoptosis, and their inhibition can cause failure in apoptosis even if the mitochondrial cytochrome c was released.[31] Caspases are intracellular proteases that execute the apoptotic processes through the destruction of cellular proteins that are vital for the cells, which ultimately results in cell death. Caspases are usually kept in an inactivated form by IAP (inhibitors of apoptosis) proteins such as IAP 1 and IAP2, as well as by Survivin. These inhibitory effects can either be achieved directly by binding or indirectly through suppression of the caspase initiators.[32] Some caspases can be initiators (caspase 2, caspase 8, and caspase 9) that are activated through signaling pathways, which in turn activate the effector caspases (caspase 3, caspase 6, and caspase 7) that carry out the apoptotic processes.[32] Figure 3 illustrates the inhibitory roles of the IAPs on the caspases (adapted from Ashkenazi, 2002).

Caspase 8 plays a major role in initiating the extrinsic pathway. Procaspase 8 engages the death effecter domains (DED) to form the death inducer signaling complexes (DISC). This complex is then ligated to one of the tissue necrotizing factor receptors (TNFR). These interactions cause several molecules of procaspase 8 to be in close proximity to other molecules of procaspase 8, and this proximity leads to their activation through a mechanism called auto-proteolysis as shown in Figure 4. In most cell types, the

activation of caspase 8 through the activation of the TRAIL receptors is not enough to fully induce apoptosis. In these cells, caspase 8 activates the Bcl-2 pro-apoptotic protein Bid, which in turn activates the intrinsic pathway.[30] Bid is an intermediary proapoptotic protein that connects the extrinsic and the intrinsic pathways (Figure 2).[33, 34]

A study conducted by Spanguolo et al.[4] showed TEGDMA-induced apoptosis rather than necrosis in pulp fibroblasts. This TEGDMA- induced apoptosis was time- and concentration-dependent.[4] Noda et al.[35] were able to calculate the amount of TEGDMA leaching from RC to the pulp. They noted that the concentration of TEGDMA in many composites is approximately 30 percent to 50 percent. Pure TEGDMA has a concentration of 3.8 mol/L. Therefore, the molar concentration in composites is slightly less than 2 mol/L.[36] The dilution factor of TEGDMA across 0.5 mM of dentin was determined to be 500.[36] Based on that, the amount of TEGDMA that reaches pulp fibroblasts is around 4 mM/L.[35] Small concentrations of TEGDMA in the lower millimolar range are clinically significant[35] and may cause pulp tissue injury.[37] Pulp is connective tissue that responds to TEGDMA leaching. The most common response of the pulp is the formation of tertiary dentin in an attempt to preserve the integrity and structure of the tissue.[38] Another response is the decrease in the volume of the pulp chamber upon the induction of apoptosis.[39]

One way that TEGDMA exposure leads to apoptosis could be through oxidative stress. TEGDMA causes a drastic depletion of glutathione (GSH) resulting in oxidative stress conditions.[40] GSH is the most abundant non-protein thiol in eukaryotic cells.[14] GSH plays an important role in antioxidant defense.[14]The depletion of GSH leads to an excessive production of reactive oxygen species (ROS) in the cells. ROS formation

results in the release of cytochrome c from the mitochondria. Cytochrome c then activates the caspases, which leads to apoptosis.

Although TEGDMA-induced apoptosis in primary human pulp has been reported,[4] the exact molecular mechanisms and the signal transduction pathways through which apoptosis occurs are not clear. Therefore, the aim of this study was to determine which apoptotic proteins are altered in TEGDMA-induced apoptosis.

MATERIALS AND METHODS

CELL CULTURE

Human pulp tissues were obtained from healthy impacted wisdom teeth. The use of the teeth was approved by Indiana University-Purdue University of Indianapolis Review Board. The pulp tissues were removed from the pulp cavities using tweezers after cutting the teeth in half using a high-speed hand piece with a 330 fissure bur and water spray. The fissure bur was changed regularly to eliminate the friction heat that is produced after it becomes dull to avoid heat damage to the pulp tissues. The pulp tissues were then minced with a blade into several fragments approximately (1 mm x 1mm x 2 mm in size). These fragments were then placed in 100-mm culture dishes and air dried, and then Dulbecco's Modified Essential Media (DMEM) was added, supplemented with 10-percent fetal bovine serum, 4 mM L-glutamine, 2.5 g/mL fungizone, 100 unit/mL penicillin, and 50 g/mL gentamicin.[41] The tissues were maintained at 37˚C in a humidified atmosphere of 5-percent CO_2. The pulp cells that grew out from the tissue fragments were then allowed to reach confluence (Figure 5). Confluent cells were detached with 0.25-percent trypsin and 0.05-percent ethylenediaminetetraacetic acid, and subcultured as needed. Cells were used at passages 3 through 8.

MEASUREMENT OF TEGDMA CYTOTOXICITY ON HUMAN PULP FIBROBLASTS (HPFs) BY LACTATE DEHYDROGENASE (LDH) ASSAYS

Cellular membrane integrity was monitored using the permeability assay based on the determination of the release of LDH from cells into the media. The Cytotoxicity

Detection Kit^{PLUS} (Roche Applied Science, Mannheim, Germany), which measures the conversion of tetrazolium salt into a red formazan product, was used as described previously.[42] Cells were treated with 0.125 mM, 0.25 mM, 0.50 mM, 0.75 mM, and 1.00 mM of TEGDMA in 100-mm incubation dish with serum-free DMEM for 24 hrs. The high control (total cell death) was generated by adding 1.9 ml of serum-free DMEM and 100-µl lysis solution to the control cells as described by the manufacturer after 24 hours, which gave the maximum release of LDH. The low control consisted of serum-free DMEM from the untreated control cells after 24 hours and gave the minimal release of LDH. Serum-free DMEM without HPFs was utilized as the background level of the assay. After 24 hours, media from each of the wells were transferred to a 96-well plate and 100 µl of reconstituted mix as per the manufacturer (Roche) was added to each well and the plates incubated for 15 minutes at room temperature. Absorbance was recorded at 490 nm in a microplate reader (Titertek, Multiskan MCC, Flow Laboratories, McLean, VA). The experiments were repeated five times and the mean value calculated. The percentage release of LDH from the treated cells was calculated by comparing it to the maximum release of LDH. To determine the cytotoxicity, the absorbance values of the background were subtracted from those of the experimented samples, and the cytotoxicity was calculated by the following equation:

Cytotoxicity (%) = (experiment value-low control)/ (high control–low control) $\times$100%

CELL TREATMENT WITH TEGDMA

Sub-confluent layers of HPFs at passages 3 through 8 were utilized for the RayBio Antibody Apoptosis kit (Norcross, GA). HPFs (2 x 10^5 cells/100 mm dish) were

incubated with or without 0.25 mM TEGDMA for 6 and 24 hours.

PREPARATION OF CELL LYSATES

Cell lysates were prepared as per the manufacturer at (www.raybiotech.com).

Briefly, cells were rinsed twice with cold phosphate-buffered saline (PBS) and the remaining PBS removed before adding the lysis buffer. The cells were then solubilized at $2x10^7$cells/ml in lysis buffer containing protease inhibitor cocktail as per the manufacturer. The cells were then pipetted up and down, and the lysate rocked gently at 4 °C for 30 minutes. The extracts were transferred to microfuge tubes and centrifuged at 14,000 x g for 10 minutes and then the supernatant was collected (cell lysate).

The protein concentrations of the cell lysates were determined using Bio-Rad Protein Assay kit (Hercules,CA). All the lysates were diluted at least five-fold with blocking buffer to the same protein concentration of 200 µg/ ml per the manufacturer.

THE RAY BIOTECH APOPTOSIS ARRAY

A Human Apoptosis Antibody Array kit was purchased from Ray Biotech (Norcross, GA). The relative level of 43 apoptosis-related proteins in the cell lysates were detected with the RayBio® Human Apoptosis Antibody Array kit according to manufacturer's instructions; the experiment was repeated four times. The arrays were analyzed with a Gel-Doc XR imaging system (Bio-Rad, Hercules, CA). Quantity one analysis software (Bio-Rad, Hercules, CA) was used to analyze the images obtained. Measurements of the protein images were repeated three times.

Briefly, the treated or untreated cell lysates were added to the antibody array membranes. After extensive washing, the membranes were incubated with a cocktail of

biotin-conjugated anti-apoptotic protein antibodies. After incubation with horseradish peroxidase (HRP)-streptavidin, the signals were visualized by chemiluminescence.

STATISTICAL ANALYSES

Data were reported as mean± SD (standard deviation). The sample size was three samples for each group (0.25 mM and the control). The density of the proteins was measured in three different arrays. Since each sample was measured multiple times, the density of each protein expression was modeled using a repeated measures analysis of variance (ANOVA) model. The ANOVA model had a fixed effect for each group, which allowed for estimation of mean density; a random run effect, which allowed for correlation of measurements from the same run; and a random sample effect, which allowed for the correlation of measurements from the same sample. Pair-wise comparisons between the 6 treatment combinations were performed using Tukey's method to control the overall significance level of the comparisons at 5 percent.

RESULTS

CYTOTOXICITY RESULTS (LDH)

The LDH assay was repeated five times and the averages with standard deviation (SD) were determined. The p-values were then calculated; significance was established where $p < 0.05$. The highest non-toxic concentration of TEGDMA was determined based on the LDH assays. This concentration was the highest concentration that was not significantly higher than that of the control in regard to cytotoxicity. TEGDMA at 0.50, 0.75, and 1.00 mM were statistically higher than that of the control (Table III). The concentration that was chosen for the human apoptosis antibody arrays was 0.25 mM TEGDMA, which was not significantly different than the control (p=.806)

RAYBIO APOPTOSIS ARRAY RESULTS

The relative expression of apoptotic proteins using the RayBio human apoptosis antibody array kit that were significantly higher at 6 hours were: B cell lymphoma-w (Bcl-w) (p=.010); BH3-interacting domain death agonist (BID) (p= .001); (Bim) (p=.009); heat shock protein 27 (HSP 27) (p= .022); HSP 60 (p= .007); HSP 70 (p= .010); heat shock-inducible protein (HTRA) (p=.001); Insulin like growth factor binding protein-1 (IGFBP-1) (p= .004); IGFBP-2 (p= .011); P21(p= .016); (P27) (p= .015); second mitochondria-derived activator of caspases (SMAC) (p= .018) (Figure 7). All of these were pro-apoptotic proteins.

The only anti-apoptotic proteins that were significantly increased compared with the control at 6 hours were Survivin (p=.015); IGFBP-5 (p= .012); and Livin (p= .006). The pro-apoptotic proteins that significantly decreased compared with the control at 6

hours were tumor necrosis factor ligand superfamily member 6 (FASL) (p=.010); TNF-β (p= .046); and TRAIL-r2 (p=.003).

However at 24 hours, more pro-apoptotic proteins had significant increases compared to the control than at 6 hours. These were Bad (p= 0.021); Bax (p= 0.027); Bcl-2 (p= 0.005); Bcl-w (p= 0.017); Bid (p= 0.005); Bim (p= 0.031); Caspase 3 (p= 0.028); Caspase 8 (p= .028); CD40L (p= 0.006); Cytochrome c (Cyto c) (p=.001); IGFBP-5 (p= 0.020); IGFBP-6 (p= 0.005); cyclin-dependent kinase inhibitor 1B (p27, kip1) (p= 0.009); P27 (p= 0.006); serum tumor necrosis factor receptor 1 (sTNF-R1) (p= 0.001); sTNF-r2 (p= 0.004); Tissue necrotizing factor –α (TNF-α) (p= 0.001); TNF-β (p= 0.003); TNF-related apoptosis-inducing ligand receptor 1 (TRAILR 1) (p= 0.016); TRAILR 2 (p= 0.028); TRAILR 3 (p= 0.039), and TRAILR 4 (p= 0.010). The anti-apoptotic proteins that had greater relative expression significant from the control were (Bcl-2) (p= 0.017); Livin (p= 0.001); Survivin (p= 0.001); IGF-II (p= 0.001).

The only anti-apoptotic protein that significantly decreased was HSP 70 (p= 0.034). There were no pro-apoptotic proteins that were significantly decreased in their relative expression at 24 hours compared to the controls.

TABLES AND FIGURES

TABLE I

Apoptosis (programmed cell death) and necrosis (pathologic cell death)

Apoptosis	**Necrosis**
Controlled process, physiological stimuli	Uncontrolled process, pathological stimuli
Energy dependent (ATP required)	No energy required (passive)
Cells shrink (apoptotic bodies)	Cells swell (leading to cell lysis)
No loss of membrane integrity	Membrane integrity lost
Non-inflammatory	Inflammatory
Nuclear fragmentation	Nuclear dissolution
Individual or small cell groups	Large cell groups (organ segments)

TABLE II

BCL-2 superfamily[43]

BCL-2 superfamily		
Pro-apoptotic		Anti-apoptotic
BH3-only proteins	Multidomain (BH)	BCL-2
BIK	BAX	$BCL\text{-}X_L$
BAD	BAK	BCLW
BIM	BOK	MCL1
HRK	BOO	BCLB
BCLG	BCLG	+ viral homologs
HR	BCLB	
NOXA	BCL-RAMBO	
PUMA		
+ others		

TABLE III

LDH results and p-values

Concentration of TEGDMA	Cytotoxicity±SD	P-value
NC (negative control, 0 mM)	0±0.00	
0.13 Mm	-0.74±0.02	1.00
0.25 mM	1.46±0.01	.806
0.50 mM	4.96±0.02	.004*
0.75 mM	10.7±0.02	.000*
1.00 Mm	20.6±0.01	.000*

* Denotes statistical significance (p-value < 0.05).

TABLE IV

RayBio human apoptosis antibody array (www.**raybio**tech.com).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Pos	Pos	Neg	Neg	Blank	Blank	Bad	Bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
2	Pos	Pos	Neg	Neg	Blank	Blank	Bad	Bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
3	CD40	CD40L	cIAP-2	CytoC	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
4	CD40	CD40L	cIAP-2	CytoC	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
5	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	Blank	livin	p21	p27	p53	SMAC	Survivin
6	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	Blank	livin	p21	p27	p53	SMAC	Survivin
7	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAIL R-2	TRAILR-3	TRAILR-4	XIAP	Blank	Neg	Neg	Neg	Pos
8	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAIL R-2	TRAILR-3	TRAILR-4	XIAP	Blank	Neg	Neg	Neg	Pos

BAD: Bcl2 antagonist of cell death; BAX: Bcl-2–associated X protein; BCL-2: B-cell lymphoma 2; BID: BH3-interacting domain death agonist; BIM: BCL2 interacting protein BIM; Bcl-w: Apoptosis regulator BclW; IAP: inhibitor of apoptotic protein; Cyto c: Cytochrome c; DR: Death receptor; FAS: Fatty acid synthetase; FAS L: Fatty acid synthetase ligand; HSP: Heat shock protein; HTRA2: High temperature requirement protein A2; IGF: Insulin like growth factor; IGFBP: Insulin like growth factor binding protein; SMAC: second mitochondria- derived activator of caspases; caspase 8: Cysteinyl aspartic acid-protease 8; TNF: Tissue necrotizing factor; TRAIL-R: TNF-related apoptosis-inducing ligand receptor; and XIAP: X-linked inhibitor of apoptosis protein.

TABLE V

Relative expressions of apoptotic proteins and p-values

Apoptotic protein	6hrs ± SD	P value	24 hrs ± SD	P value
Bad	1.65 ± 0.71	.116	1.57 ± 0.37	.021*
Bax	1.29 ± 0.38	.184	1.20 ± 0.17	.027*
Bcl-2	2.24 ± 0.75	.046	1.59 ± 0.34	.005*
Bcl-w	2.11 ± 0.41	.010*	1.20 ± 0.12	.017*
Bid	2.08 ± 0.53	.001*	1.17 ± 0.12	.005*
Bim	1.63 ± 0.54	.009*	1.21 ± 0.18	.031*
Caspase3	1.41 ± 0.48	.344	1.54 ± 0.28	.028*
Caspase8	1.52 ± 0.54	.557	1.59 ± 0.30	.028*
CD40	1.19 ± 0.46	.510	0.83 ± 0.45	.545
CD40L	0.74 ± 0.38	.124	1.15 ± 0.07	.006*
cIAP-2	1.23 ± 0.17	.905	1.15 ± 0.29	.335
Cyto c	0.88 ± 0.20	.174	1.21 ± 0.05	.001*
DR6	0.89 ± 0.24	.303	1.02 ± 0.10	.724
Fas	1.02 ± 0.21	.792	1.12 ± 0.13	.108
Fasl	0.71 ± 0.22	.010*	1.12 ± 0.11	.072
HSP27	1.91 ± 0.35	.022*	1.00 ±0.08	.966
HSP60	1.20 ± 0.10	.007*	0.95 ± 0.13	.355
HSP70	1.06 ± 0.05	.010*	0.87 ± 0.13	.034*
HTRA	1.24 ± 0.23	.020*	1.16 ± 0.13	.101
IGF-I	13.22 ± 5.74	.021*	0.92 ± 0.06	.147
IGF-II	0.82 ± 0.72	.681	2.91 ± 0.41	.001*
IGFBP-1	1.61 ± 0.47	.004*	1.47 ± 0.49	.104
IGFBP-2	1.53 ± 0.29	.011*	1.13 ± 0.20	.100
IGFBP-3	1.63 ± 0.54	.113	1.11 ± 0.19	.288
IGFBP-4	1.89 ± 1.14	.247	1.01 ± 0.25	.963
IGFBP-5	1.33 ± 0.19	.012*	1.23 ± 0.14	.020*
IGFBP-6	1.60 ± 0.68	.200	1.41 ± 0.02	.005*
IGF-1sr	0.97 ± 0.03	.214	1.55± 0.41	.036
Livin	2.74± 0.83	.006*	1.61 ± 0.20	.001*
P21	1.14± 0.12	.016*	1.15 ± 0.08	.009*
P27	2.01± 0.43	.015*	1.27 ± 0.17	.006*
P53	0.97 ± 0.18	.671	1.31 ± 0.15	.070
SMAC	1.32 ± 0.20	.018*	1.06 ± 0.12	.258
Survivin	1.29 ± 0.12	.015*	1.21 ± 0.10	.001*
sTNF-r1	0.55 ± 0.48	.183	1.62 ± 0.02	.001*
sTNF-r2	1.57 ± 0.39	.063	2.24 ± 0.37	.004*
TNF-alpha	1.01 ± 0.08	.242	2.25 ± 0.01	.001*
TNF-beta	0.57 ± 0.20	.046*	1.53 ± 0.18	.003*
TRAIL- R1	1.28 ± 0.37	.257	1.42 ± 0.06	.016*
TRAIL- R2	0.78 ± 0.14	.003*	1.20 ± 0.07	.028*
TRAIL- R3	1.00 ± 0.20	.944	1.64 ± 0.36	.039*
TRAIL- R4	1.39 ± 0.35	.068	1.31 ± 0.13	.010*
XIAP	1.15 ± 0.24	.259	1.15 ±0.14	.074

* Denotes statistical significance ($p < 0.05$).

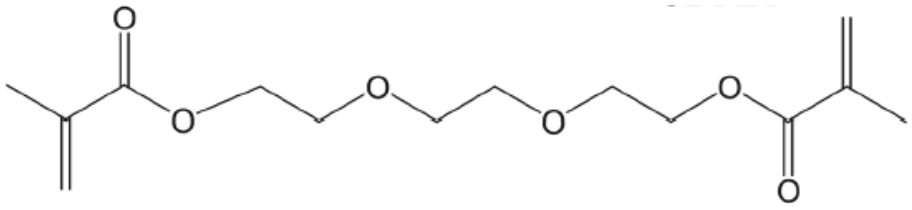


FIGURE 1. Triethylene glycol dimethacrylate (TEGDMA).

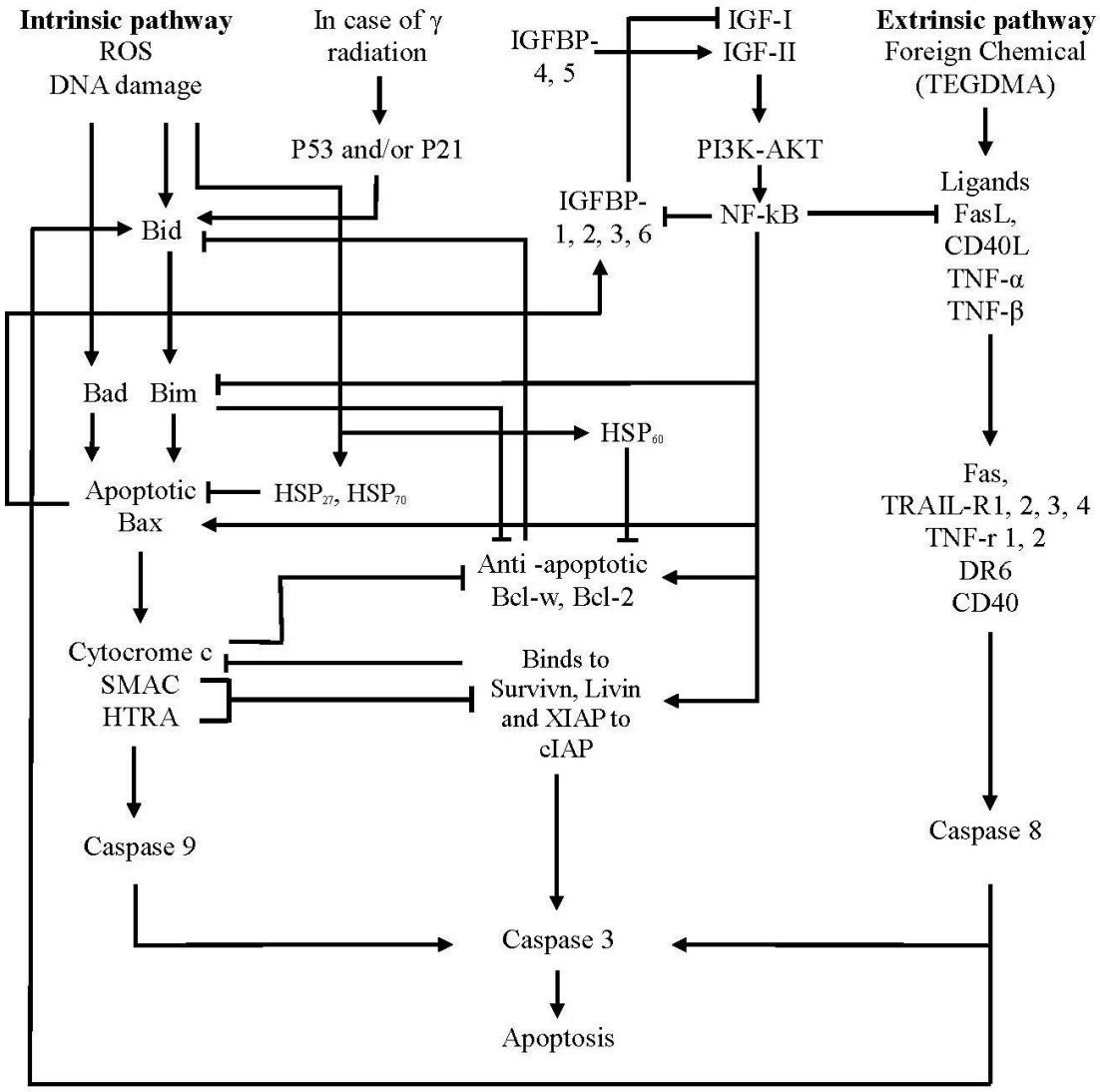
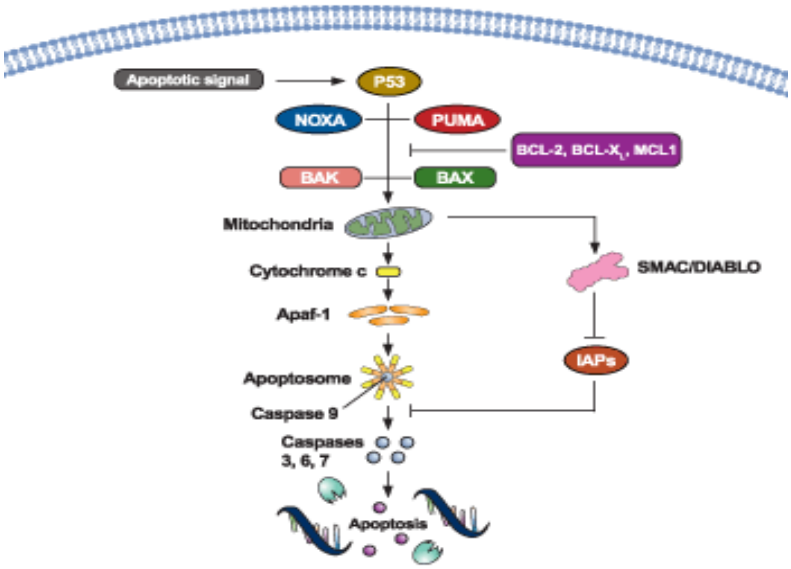


FIGURE 2. Extrinsic and intrinsic pathway.



http://www.biooncology.com/research/apoptosis/pathways/caspase-cascade/index.html

FIGURE 3. IAPs and caspase inhibition.

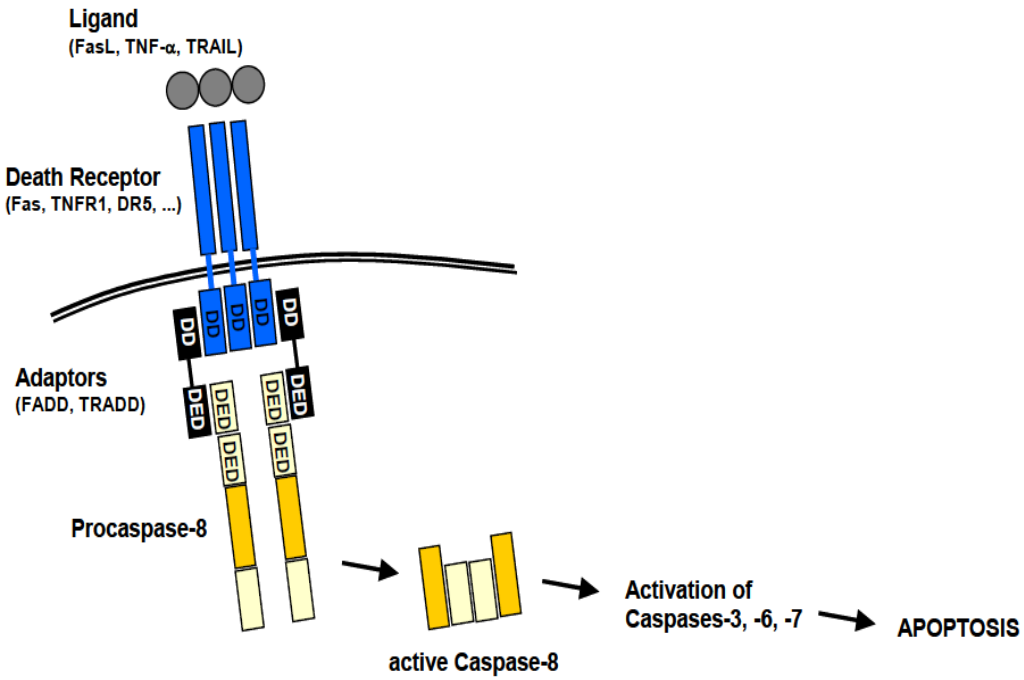


FIGURE 4. Extrinsic pathway.[44]

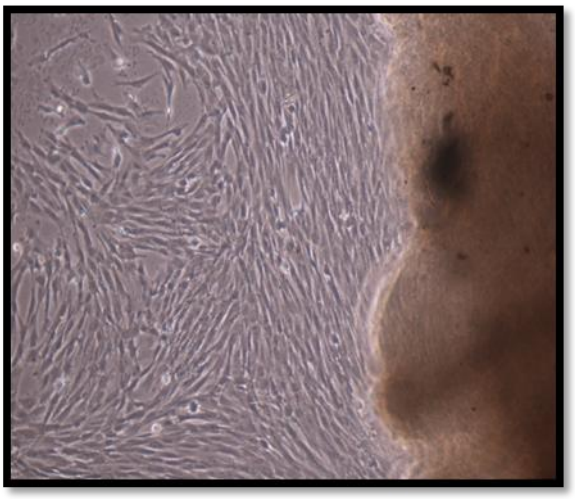


FIGURE 5. Human pulp fibroblasts growing out of pulp tissue.

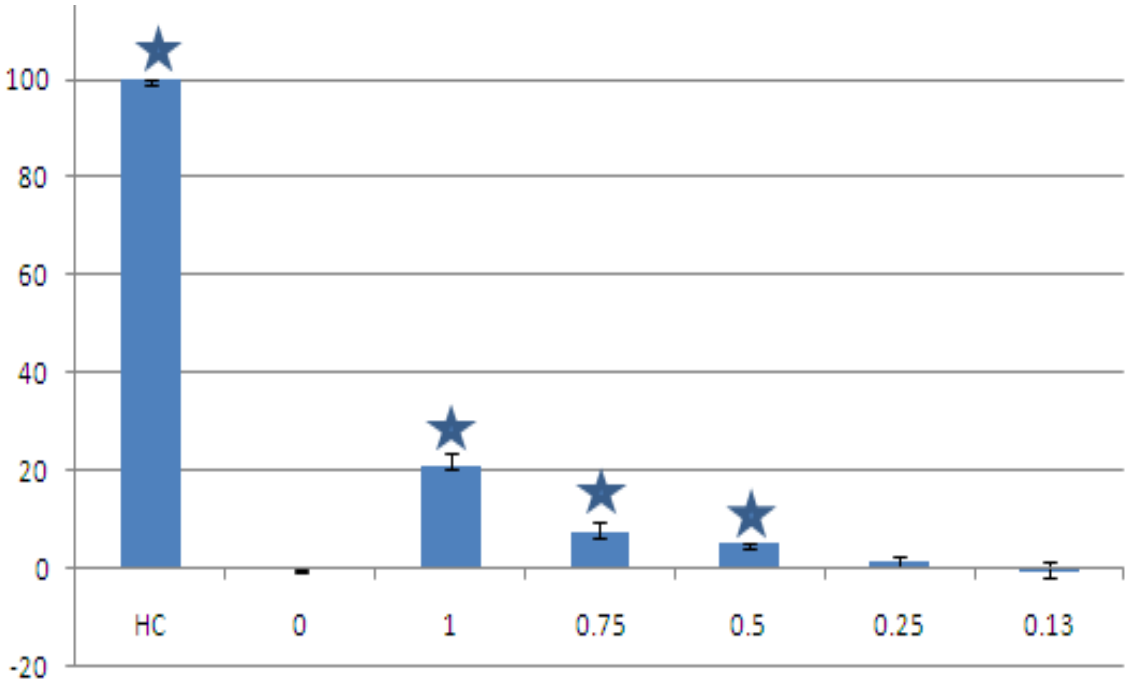


FIGURE 6. Concentration of TEGDMA versus percent of cytotoxicity after 24-hour exposure at various concentrations. *Denotes statistical significance (p-value < 0.05). HC denotes high control (maximum-released LDH/total cell death).

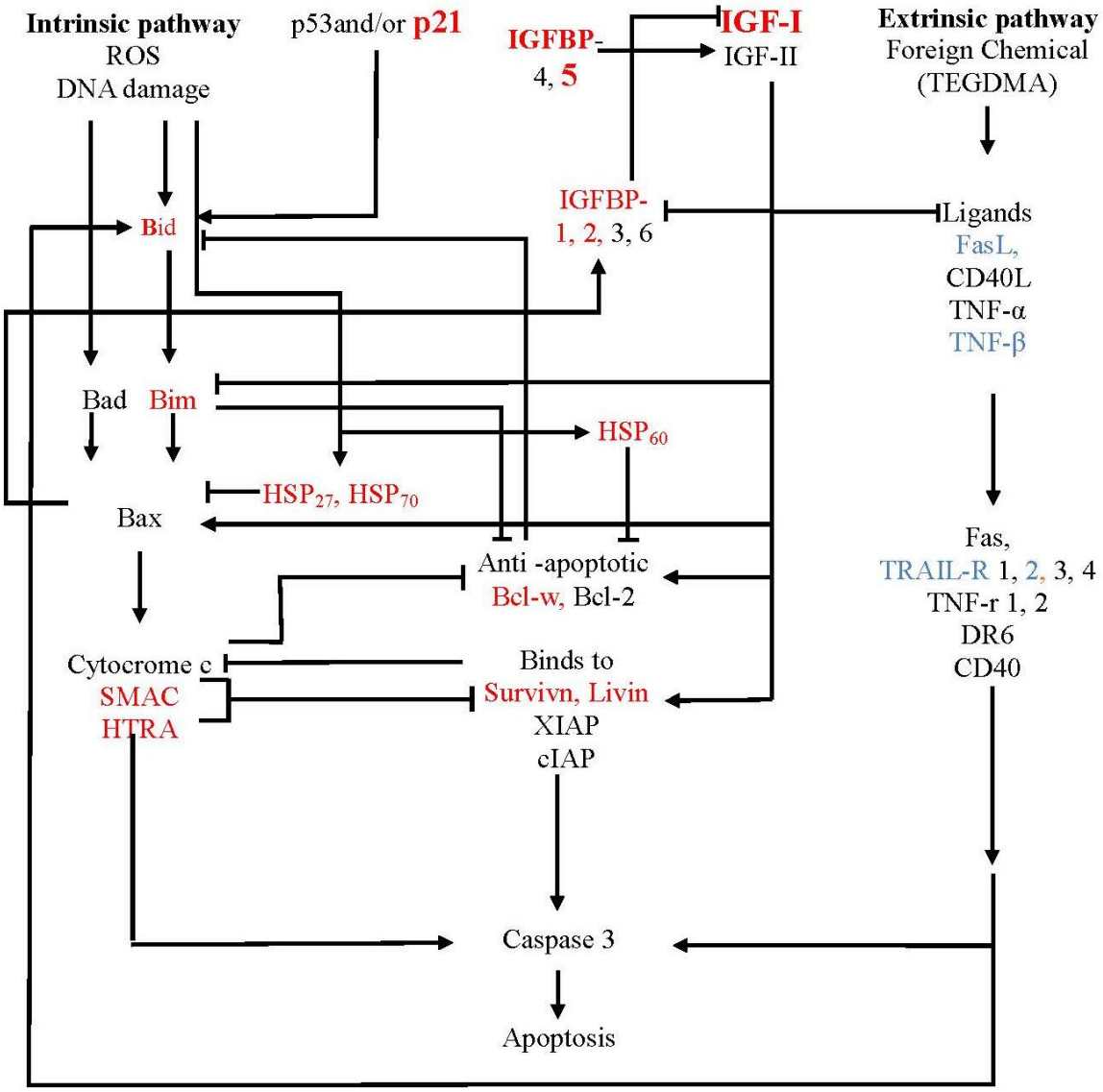


FIGURE 7. Induced apoptotic proteins at 6 hours.

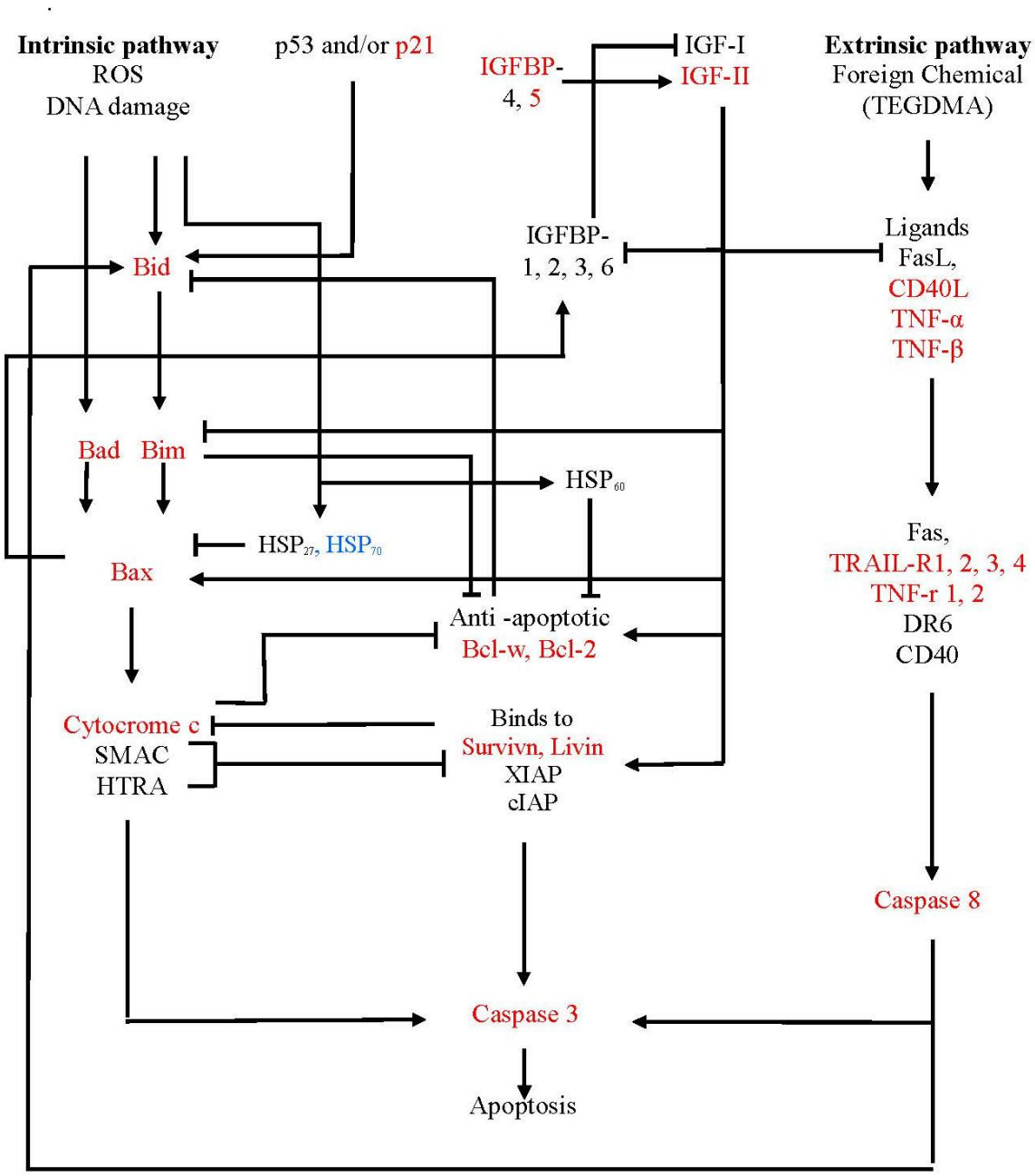


FIGURE 8. Induced apoptotic at 24 hours.

DISCUSSION

The biological effects of TEGDMA on apoptosis of pulp fibroblasts were examined. LDH assay was used to measure cell cytotoxicity. The rationale for using the LDH assays was to verify the concentration at which necrosis starts and to hopefully ensure the presence of apoptotic cells rather than necrotic. The results of this current study showed that TEGDMA had statistically significant cytotoxic effects at 0.5 mM and above. Therefore, 0.25 mM of TEGDMA was used in this study and is clinically relevant as discussed previously.[35]

The results of this study demonstrated that TEGDMA increases the expression of the multiple pro-apoptotic proteins. There were statistically significant increases in the expression of these proteins after 6 h and 24 h when compared with the control. The extrinsic apoptotic pathway is activated through the activation of the transmembrane receptors of the TNF family. Members of the TNF family include TNFR 1 and 2, Fas, death receptor (DR) 6, CD40, and TRAIL-R 1-4, which bind to their corresponding ligands (i.e., TNF-α, TNF-β, fasL, CD40L, and TRAIL, respectively). This study showed that there were statistically significant increases in TNF-α and TNF-β after 24 h (Figure 8). TRAIL-R 1 and 2 contain death domains, which mean they are able to induce apoptosis by activating caspase 8, whereas TRAIL-R 2-4 are considered to be antagonistic decoys and do not induce death signals.[45] This study showed statistically significant increases in all TRAIL receptors after 24 hours (Figure 8). Fas increased slightly when compared with the control; at 24 h, it was not statistically significant. Fas L

at 24 h showed a slight increase that was not statistically significant; however, it showed a statistically significant decrease at 6 hours (Figure 7). This decrease in the expression of Fas L could be explained by the presence of anti-apoptotic proteins that could down-regulate it.[46]

Iwama K et al.[47] reported that excessive ROS production due to arsenic trioxide (ATO) toxicity resulted in changing membrane permeability. ATO rapidly induced TRAIL and then activates caspase 8, which resulted in the phosphorylation of Bid.[47]TEGDMA treatment leads to the production of ROS is documented in the literature.[48] This study showed significant increases of all TRAIL receptors at 24 hours (Figure 8), which is in line with the previous study.[47] Activation of the extrinsic pathway will result in caspase activation, specifically caspase 8. The process then is induced by two parallel pathways. The first pathway is through the cleavage and activation of caspase 3 and the second is created when the TRAIL receptors activates the extracellular pathway. In that case the intrinsic pathway (mitochondrial pathway) is activated after the activation of the pro-apoptotic protein Bid by caspase 8 (Figure 2).[27, 29] In this study, the caspase pathway was activated, because there were statistically significant increases in both caspase 8 and 3 after 24 h, which indicated the extrinsic pathway was activated (Figure 7). Bid had increased and was statistically significant after 24 h, which also indicated that the mitochondrial pathway had been activated. Upon Bid activation, Bcl-2 family proteins are activated. The Bcl-2 family includes both pro- and anti-apoptotic proteins. Some pro-apoptotic proteins that were induced were Bim, Bax, and Bad. When Bad and Bim are phosphorelated, they are sequestered and enter into the mitochondria to cause the release of cytochrome c, SMAC and HtrA. Bad also binds to anti-apoptotic

proteins (Bcl-2 and Bcl-w) and prevents their inhibitory effects on apoptosis.[27, 49] The interactions between the pro- and anti-apoptotic proteins results in no inhibition of apoptosis and thus causes depolarization of the mitochondria. Depolarization of the mitochondria results in increases in the permeability of their membranes, thus releasing more pro-apoptotic factors like SMAC, HtrA and cytochrome c. This study showed significant increases in some of the Bcl-2 family members that are involved in the mitochondrial pathway. Bid and Bim (pro-apoptotic proteins of the Bcl-2 family) were both significantly increased at 6 h (Figure 8) and 24 h (Figure 7). Bcl-w (anti-apoptotic protein) was also significantly increased at 6 h and 24 h. SMAC and HtrA are two pro-apoptotic proteins that are released upon the activation of the intrinsic pathway. These two pro-apoptotic proteins will promote apoptosis through binding to the cIAP anti-apoptotic protein preventing its inhibitory effects on apoptosis.[50] SMAC and HtrA were increased significantly at 6 h (Figure 8) and increased slightly at 24 h, but was not significant.

Another important pro-apoptotic protein that is released from the mitochondria upon the activation of the intrinsic pathway is cytochrome c. Cytochrome c activates caspase 9 and then caspase 9 activates caspase 3. Cytochrome c was statistically significantly increased compared with the control at 24 h (Figure 7).[44]

Members of the heat shock protein (HSP) family are over-expressed under biological stress such as heat or when treated with toxic materials. Their function in general is to prevent cellular protein aggregation and to increase levels of reduced glutathione to protect the cell from reactive oxygen species (ROS). [51] HSP 27 and 70 are anti-apoptotic proteins, while HSP 60 is a pro-apoptotic protein. The current study agrees

with Noda et al.[35] They showed that TEGDMA inhibits the phosphorylation of HSPs, thereby decreasing their levels.[35, 51]This study showed there was activation of HSP 70 and its increase was statistically significant at 6 h (Figure 8), but its level at 24 h was decreased significantly (Figure 7), which indicated that the anti-apoptotic effects of HSP 70 was counteracted. As for HSP 60, there was a slight decrease at 24 h, yet it was not significant statistically. HSP 27 was unaltered.

IGF-1 plays an important role in cell survival pathway and inhibiting apoptosis. IGF-1 activation causes induction of two major signaling pathways: the phosphatidylinositol-triphosphate kinase/AKTransforming (PI3K/AKT) pathway and the mitogen-activated protein kinase (MAPK) pathway.[52] This leads to lower concentrations of pro-apoptotic proteins like Bax and Bad, but increases the expression of anti-apoptotic proteins like Bcl-w. These pathways tend to inhibit caspases specifically caspase-3.[52] Spagnuolo et al.[4] showed that AKT is a main target in TEGDMA-induced apoptosis. This study showed significantly higher levels of IGF-1, especially after 6 h. These results are in an agreement with Spagnuolo et al., since IGF-1 activates the PI3K/AKT pathway.[4] However, IGF-1 expression at 24 h slightly decreased. One of the signs of apoptosis is decrease in IGF levels.

Insulin-like growth factor-binding proteins (IGFBP) had been described to have both pro- and anti-apoptotic effects. The effects of IGFBP-5 are variable depending on the tissue and cell type.[53, 54] IGFBP 5 was shown to be anti-apoptotic in gingival fibroblasts. However, in the current study, the increase of IGFBP-5 at 24 hours may have led to activation of caspase 3. The increase of caspase 3 is an important sign of apoptosis activation.[30] This study also showed elevated levels of IGFBP 6. IGFBP-6 is associated

with the apoptotic pathway c-jun N-terminal kinase (JNK) activation and the inhibition of nuclear factor kappa B (NFkappaB). Both these pathways were shown by Samuelsen et al.[48] to be involved in the TEGDMA-induced apoptosis. This study showed significant increases in the expression of IGFBP-6 in comparison with the control at 24 h.

There are several reports about TEGDMA causing genotoxicity and cell-cycle delay.[55] The p21 and p27 are cell cycle regulators. The tumor suppressor protein p53 is the main regulator of p21. Krifta et al.[56] showed that there was a slight increase in p53 expression, while there was a noticeable increase in the expression of p21. This study agrees with their findings in that significant increases in p21 occurred at 6 h and 24 h. While p53 showed slightly elevated levels at 24 h only and was not significant.

IAP proteins are caspase inhibitor proteins. Survivin, Livin, XIAP, and cIAP are members of this family. It has been shown that XIAP has high affinity for caspase 3 and tries to inhibit apoptosis once it started.[46] SMAC and HtrA (mitochondrial pro-apoptotic proteins) are known to bind to these inhibitory apoptotic proteins and inhibit their functions.[46] Survivin and Livin significantly increased at 6 h and 24 h. However, these increases were not enough to inhibit apoptosis. Caspase 8 and caspase 3 were both activated at 24 h, indicating the apoptotic process was still continuing.[46]

This current study showed that TEGDMA-activated apoptosis within 24 h. The extrinsic pathway at 24 h is clearly activated and was activated after the activation of members of the TNFR family. The activation of the intrinsic pathway started at 6 h, but it was amplified at 24 h. Bax and cytochrome c, are essential pro-apoptotic proteins for the intrinsic pathway. Bax and cytochrome c were not activated until the extrinsic pathway

was also activated. Both pathways play a role in inducing apoptosis in TEGDMA-treated HPFs.

SUMMARY AND CONCLUSION

The results of this study showed statistically significant increases of multiple examined pro-apoptotic proteins. The anti-apoptotic proteins were also altered. Most pro-apoptotic proteins involved in the intrinsic (mitochondrial) pathway were significantly increased after 6 h and 24 h. Numerous pro-apoptotic proteins of the extrinsic pathway were activated at 24 h. The activation of these pro-apoptotic proteins in the extrinsic pathway amplified the intrinsic pro-apoptotic proteins at 24 h. More pro-apoptotic proteins in the intrinsic pathway were activated at 24 h than at 6 h. TEGDMA has effects on both the extrinsic and intrinsic apoptotic pathways. The results of this study showed involvement of some proteins involved in the TEGDMA- induced apoptosis that coincides with the aim of this study. More research is still needed to elucidate the net effects of this apoptotic process on the pulp tissue and to find more clinically relevant ways to stop or even reverse this process if possible.

REFERENCES

1. Qvist V, Stoltze K, Qvist J. Human pulp reactions to resin restorations performed with different acid-etch restorative procedures. Acta Odontol Scand 1989;47(5):253-63.

2. Whitworth JM, Smith J, Walls, AW, McCabe JF. Endodontic complications after plastic restorations in general practice. Int Endodontic J 2005;38:409–16.

3. Geurtsen W. Biocompatibility of resin-modified filling materials. Crit Rev Oral Biol Med 2000;11(3):333-55.

4. Spagnuolo G, Galler K, Schmalz G, et al. Inhibition of phosphatidylinositol 3-kinase amplifies TEGDMA-induced apoptosis in primary human pulp cells. J Dent Res 2004;83(9):703-7.

5. Peutzfeldt A. Resin composites in dentistry: the monomer systems. Eur J Oral Sci 1997;105(2):97-116.

6. Hervas-Garcia A, Martinez-Lozano MA, Cabanes-Vila J, Barjau-Escribano A, Fos-Galve P. Composite resins. A review of the materials and clinical indications. Med Oral Patol Oral Cir Bucal 2006;11(2):E215-20.

7. Geurtsen W, Leyhausen G. Chemical-Biological Interactions of the resin monomer triethyleneglycol-dimethacrylate (TEGDMA). J Dent Res 2001;80(12):2046-50.

8. Szep S, Kunkel A, Ronge K, Heidemann D. Cytotoxicity of modern dentin adhesives--in vitro testing on gingival fibroblasts. J Biomed Mater Res 2002;63(1):53-60.

9. Walther UI, Siagian, II, Walther SC, Reichl FX, Hickel R. Antioxidative vitamins decrease cytotoxicity of HEMA and TEGDMA in cultured cell lines. Arch Oral Biol 2004;49(2):125-31.

10. Krifka S, Seidenader C, Hiller KA, Schmalz G, Schweikl H. Oxidative stress and cytotoxicity generated by dental composites in human pulp cells. Clin Oral Investig. 2011/01/18 ed; 2011.

11. Eckhardt A, Gerstmayr N, Hiller KA, et al. TEGDMA-induced oxidative DNA damage and activation of ATM and MAP kinases. Biomaterials 2009;30(11):2006-14.

12. Goldberg M. In vitro and in vivo studies on the toxicity of dental resin components: a review. Clin Oral Investig 2008;12(1):1-8.

13. Engelmann J, Leyhausen G, Leibfritz D, Geurtsen W. Effect of TEGDMA on the intracellular glutathione concentration of human gingival fibroblasts. J Biomed Mater Res 2002;63(6):746-51.

14. Dickinson DA, Moellering DR, Iles KE, et al. Cytoprotection against oxidative stress and the regulation of glutathione synthesis. Biol Chem 2003;384(4):527-37.

15. Mantellini MG, Botero TM, Yaman P, et al. Adhesive resin induces apoptosis and cell-cycle arrest of pulp cells. J Dent Res 2003;82(8):592-6.

16. Janke V, von Neuhoff N, Schlegelberger B, Leyhausen G, Geurtsen W. TEGDMA causes apoptosis in primary human gingival fibroblasts. J Dent Res 2003;82(10):814-8.

17. Kitamura C, Ogawa Y, Morotomi T, Terashita M. Differential induction of apoptosis by capping agents during pulp wound healing. J Endod 2003;29(1):41-3.

18. Zong WX, Thompson CB. Necrotic death as a cell fate. Genes Dev 2006;20(1):1-15.

19. Edinger AL, Thompson CB. Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 2004;16(6):663-9.

20. Torabinejad M, Walton RE, editors. Endodontics: principles and practice 4th ed. St. Louis, MO: Sanunders/Elsevier; 2009.

21. Arends MJ, Morris RG, Wyllie AH. Apoptosis. The role of the endonuclease. Am J Pathol 1990;136(3):593-608.

22. Becher R, Kopperud HM, Al RH, et al. Pattern of cell death after in vitro exposure to GDMA, TEGDMA, HEMA and two compomer extracts. Dent Mater 2006;22(7):630-40.

23. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 2006;25(34):4798-811.

24. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer 2002;2(6):420-30.

25. Yang H, Zhu YT, Cheng R, et al. Lipopolysaccharide-induced dental pulp cell apoptosis and the expression of Bax and Bcl-2 in vitro. Braz J Med Biol Res;43(11):1027-33.

26. Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? Genes Cells 1998;3(11):697-707.

27. Coultas L, Strasser A. The role of the Bcl-2 protein family in cancer. Semin Cancer Biol 2003;13(2):115-23.

28. Ashkenazi A. Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. Nat Rev Drug Discov 2008;7(12):1001-12.

29. Srinivasula SM, Datta P, Fan XJ, et al. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. J Biol Chem 2000;275(46):36152-7.

30. Kim R, Emi M, Tanabe K. Role of mitochondria as the gardens of cell death. Cancer Chemother Pharmacol 2006;57(5):545-53.

31. Kurokawa M, Kornbluth S. Caspases and kinases in a death grip. Cell 2009;138(5):838-54.

32. Lavrik IN, Golks A, Krammer PH. Caspases: pharmacological manipulation of cell death. J Clin Invest 2005;115(10):2665-72.

33. Broaddus VC, Dansen TB, Abayasiriwardana KS, et al. Bid mediates apoptotic synergy between tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and DNA damage. J Biol Chem 2005;280(13):12486-93.

34. Liu W, Bodle E, Chen JY, et al. Tumor necrosis factor-related apoptosis-inducing ligand and chemotherapy cooperate to induce apoptosis in mesothelioma cell lines. Am J Respir Cell Mol Biol 2001;25(1):111-8.

35. Noda M, Wataha JC, Kaga M, et al. Components of dentinal adhesives modulate heat shock protein 72 expression in heat-stressed THP-1 human monocytes at sublethal concentrations. J Dent Res 2002;81(4):265-9.

36. Gregson KS, O'Neill JT, Platt JA, Windsor, LJ. In vitro induction of hydrolytic activity in human gingival and pulp fibroblasts by triethylene glycol dimethacrylate and monocyte chemotatic protein-1. Dent Mater 2008;24(11):1461-7.

37. Costa CA, Teixeira HM, do Nascimento AB, Hebling J. Biocompatibility of an adhesive system and 2-hydroxyethylmethacrylate. ASDC J Dent Child 1999;66(5):337-42, 294.

38. Smith AJ, Murray PE, Sloan AJ, Matthews JB, Zhao S. Trans-dentinal stimulation of tertiary dentinogenesis. Adv Dent Res 2001;15:51-4.

39. Goldberg M, Smith AJ. Cells and extracellular matrices of dentin and pulp: a biological basis for repair and tissue engineering. Crit Rev Oral Biol Med 2004;15(1):13-27.

40. Stanislawski L, Lefeuvre M, Bourd K, et al. TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. J Biomed Mater Res A 2003;66(3):476-82.

41. Sawa Y, Horie Y, Yamaoka Y, et al. Production of colony-stimulating factor in human dental pulp fibroblasts. J Dent Res 2003;82(2):96-100.

42. Issa Y, Watts DC, Brunton PA, Waters CM, Duxbury AJ. Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. Dent Mater 2004;20(1):409–16.12-20.

43. Mayer B, Oberbauer R. Mitochondrial regulation of apoptosis. News Physiol Sci 2003;18:89-94.

44. Gewies A. Introduction to Apoptosis; 2003. "http:// www.celldeath.de/encyclo/aporev/aporev.htm". Accessed February 10, 2011.

45. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ 2003;10(1):66-75.

46. Schimmer AD. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res 2004;64(20):7183-90.

47. Iwama K, Nakajo S, Aiuchi T, Nakaya K. Apoptosis induced by arsenic trioxide in leukemia U937 cells is dependent on activation of p38, inactivation of ERK and the Ca2+-dependent production of superoxide. Int J Cancer 2001;92(4):518-26.

48. Samuelsen JT, Dahl JE, Karlsson S, Morisbak E, Becher R. Apoptosis induced by the monomers HEMA and TEGDMA involves formation of ROS and differential activation of the MAP-kinases p38, JNK and ERK. Dent Mater 2007;23(1):34-9.

49. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 1996;87(4):619-28.

50. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007;35(4):495-516.

51. Csermely P, Yahara I. Heat shock proteins. In: Keri G, Toth I, eds. Molecular pathomechanisms and new trends in drug research. London: Taylor & Francis; 2002.

52. Novosyadlyy R, Kurshan N, Lann D, et al. Insulin-like growth factor-I protects cells from ER stress-induced apoptosis via enhancement of the adaptive capacity of endoplasmic reticulum. Cell Death Differ 2008;15(8):1304-17.

53. Han X, Amar S. Role of insulin-like growth factor-1 signaling in dental fibroblast apoptosis. J Periodontol 2003;74(8):1176-82.

54. Perks CM, Bowen S, Gill ZP, Newcomb PV, Holly JM. Differential IGF-independent effects of insulin-like growth factor binding proteins (1-6) on apoptosis of breast epithelial cells. J Cell Biochem 1999;75(4):652-64.

55. Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. J Dent Res 2006;85(10):870-7.

56. Krifka S, Petzel C, Bolay C, et al. Activation of stress-regulated transcription factors by triethylene glycol dimethacrylate monomer. Biomaterials;32(7):1787-95.

ABSTRACT

TEGDMA INDUCTION OF APOPTOTIC PROTEINS IN PULP FIBROBLASTS

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Monomers like triethylene glycol dimethacrylate (TEGDMA) leach from dental composites and adhesives due to incomplete polymerization or polymer degradation. The release of these monomers causes a variety of reactions that can lead to cell death. This death can be either necrotic, which is characterized mainly by inflammation and injury to the surrounding tissues, or apoptotic, which elicits little inflammatory responses, if any at all. TEGDMA-induced apoptosis in human pulp has been reported recently. However, the molecular mechanisms and the apoptotic (pro and anti) proteins involved in this process remain unclear.

The objective of this study was to determine the apoptotic proteins expressed or

suppressed during TEGDMA-induced apoptosis. Human pulp fibroblasts (HPFs) were incubated for 24 hours with different TEGDMA concentrations (0.125-1.0 mM). Cytotoxicity was determined using the cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Mannheim, Germany). TEGDMA was shown to cause cell cytotoxicity at concentrations of 0.50 mM and up. The highest concentration with no significant cytotoxicity was used. Cells were incubated with or without 0.25 mM TEGDMA for 6 h and 24 h. Cell lysates were then prepared and the protein concentrations determined using the Bradford protein assay. A Human Apoptosis Array kit (Bio-Rad Hercules, CA) was utilized to detect the relative levels of 43 apoptotic proteins. The results of this study showed statistically significant increases of multiple examined pro-apoptotic proteins. The anti-apoptotic proteins were also altered. Pro-apoptotic proteins involved in the intrinsic and extrinsic apoptotic pathways were increased significantly. The results indicated that TEGDMA has effects on both the extrinsic and intrinsic apoptotic pathways.

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