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Triethylene Glycol Dimethacrylate Induction of Apoptotic Proteins in Pulp Fibroblasts

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Clinical Relevance

Pulp exposed to triethylene glycol dimethacrylate (TEGDMA) containing resin composite may be at higher risk of endodontic treatment.

SUMMARY

Objective: Monomers such as triethylene glycol dimethacrylate (TEGDMA) can leach from dental composites. TEGDMA-induced apoptosis in human pulp has been reported. However, the apoptotic (pro or anti) proteins involved in this process remain unclear. Therefore, the purpose of this study was to determine which apoptotic proteins are enhanced or suppressed during TEGDMA-induced apoptosis.

Materials and Methods: Human pulp fibroblasts (HPFs) were incubated with different TEGDMA concentrations (0.125-1.0 mM) and cytotoxicity was determined. TEGDMA was shown to be cell cytotoxic at concentrations of 0.50 mM and higher. The highest concentration with no significant cytotoxicity was then incubated (0.25 mM TEGDMA) with the HPFs. Cell lysates were then prepared and the protein concentrations determined. Human Apoptosis Array kits were utilized to detect the relative levels of 43 apoptotic proteins.

Results: HPFs exposed to TEGDMA showed significant increases in multiple pro-apoptotic proteins such as Bid, Bim, Caspase 3, Caspase 8, and Cytochrome c at 24 hours. Some anti-apoptotic proteins were also altered.

Conclusions: The results indicated that TEGDMA activates both the extrinsic and intrinsic apoptotic pathways.

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INTRODUCTION

Resin composites (RC) are widely used as a result of increases in esthetic demands and increased concerns related to mercury toxicity. Dentists choose RC because of the conservative approach of this preparation when compared to amalgam restorations. The

mechanical properties of these restorations are improving continuously as a result of the ongoing research in this area. More research is still needed to test the biocompatibility of these materials. Cytotoxicity tests done *in vitro* and *in vivo* have shown that there are monomers that leach from the RC that can cause cell death and damage to the surrounding tissues, specifically pulp tissue. A study by Qvist and others in 1989[1] showed that teeth restored with RC using different leakage-reducing methods experienced increases in pulp inflammation as well as necrosis. Studies that compared amalgam to resin-based composite restorations showed that composites do have higher failure rates than does 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). TEGDMA comprises small hydrophilic monomers. They form approximately 30-50% of almost all of the resin-based composites. TEGDMA is added to improve viscosity and to make the RC more manageable clinically. These monomers were found to leach as a result of either incomplete polymerization of the resin or mechanical/chemical degradation of the restoration.[3]

Unpolymerized TEGDMA is responsible for some of the cytotoxic effects of RC and dental adhesives on pulp and gingival fibroblasts.[4] According to a study conducted by Janke and others[5] on gingival fibroblasts, TEGDMA caused apoptotic cell death rather than necrotic cell death. A study conducted by Spagnuolo and others[6] showed that TEGDMA induced apoptosis rather than necrosis in pulp fibroblasts. Apoptosis is a programmed, energy-dependent cell death that 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.

TEGDMA-induced apoptosis was time and concentration dependent.[6] Noda and others[7] 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 30-50%. Pure TEGDMA has a concentration of 3.8 mol/L (3.8 M). Therefore, the molar concentration in composites is slightly less than 2 mol/L (2 M).[8] The dilution factor of TEGDMA across 0.5 mm of dentin was determined to be 500.[8] Based on that, the amount of TEGDMA that reaches pulp fibroblasts is around 4 mM.[7] Small concentrations of unpolymerized TEGDMA in the lower millimolar range are clinically significant[7] and may cause pulp tissue injury.[9]

Although TEGDMA-induced apoptosis in primary human pulp has been reported,[6] the exact molecular mechanisms and the signal transduction pathways through which apoptosis occurs are not clear. The purpose of this study was to determine which anti-apoptotic and pro-apoptotic proteins are involved in TEGDMA-induced apoptosis in human pulp fibroblasts (HPFs). The hypothesis of this study was that TEGDMA will increase the concentrations of pro-apoptotic proteins in the apoptosis extrinsic pathway in HPFs.

MATERIALS AND METHODS

Primary Cell Culture

Human pulp tissues were obtained from extracted healthy impacted wisdom teeth. The use of the teeth was approved by the Indiana University-Purdue University Indianapolis Institutional 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 avoid heat damage to the pulp tissues. The pulp tissues were then minced with a blade into several fragments measuring approximately 1 mm × 1 mm × 2 mm in size. These fragments were then placed in 100-mm^2 culture dishes and air-dried, and then Dulbecco Modified Essential Media (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 2.5 g/mL fungizone, 100 unit/mL penicillin, and 50 g/mL gentamicin was added.[10] The tissues were maintained at 37°C in a humidified atmosphere of 5% CO_2. The pulp cells that grew out from the tissue fragments were then allowed to reach confluence. Confluent cells were detached with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid and subcultured as needed. Cells were used at passages 3-8.

Cytotoxicity 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.[11] Cells were treated with 0.125, 0.25, 0.50, 0.75, and 1.00 mM of TEGDMA in 100-mm^2 culture dishes with serum-free DMEM for 24 hours. The positive control (total cell death) was generated by adding 1.9 mL of serum-free DMEM and 100 μL of lysis solution to the control cells, as described by the manufacturer, after 24 hours, which resulted in the maximum release of LDH. The negative 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 cells was utilized as the background level of the assay. After 24 hours, media from each of the wells was transferred to a 96-well plate and 100 μL of reconstituted mix (per the manufacturer, Roche) was added to each well and the plates were incubated for 15 minutes at room temperature. Absorbance was recorded at 490 nm in a microplate reader (Titertek, Multiskan MCC, Flow Laboratories, McLean, VA, USA). The experiments were repeated five times and the mean value was 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:

$$\text{Cytotoxicity}(\%) = (\text{experiment value} - \text{low control})/(\text{high control} - \text{low control}) \times 100\%.$$

Cell Treatment with TEGDMA and Preparation of Cell Lysates

HPFs at passages 3-8 were utilized for the Ray Bio Antibody Apoptosis kit (Norcross, GA, USA). HPFs (2×10^5 cells/100-mm dish) were incubated with or without 0.25 mM TEGDMA for six and 24 hours. Cell lysates were prepared per the manufacturer. Briefly, the cells were rinsed twice with cold phosphate-buffered saline. The cells were then solubilized in lysis buffer containing a protease inhibitor cocktail, as per the manufacturer. The cells were then pipetted up and down, and the lysate was rocked gently at 4°C for 30 minutes. The extracts were transferred to tubes and centrifuged at 14,000*g* for 10 minutes and then the supernatant was collected (cell lysate). The protein concentrations of the cell lysates were determined using a Bio-Rad Protein Assay kit (Hercules, CA, USA). All of the lysates were diluted at least fivefold with blocking buffer to the same protein concentration of 200 μg/mL, per the manufacturer.

RayBio Apoptosis Array

A Human Apoptosis Antibody Array kit (Ray Biotech) was used to detect the relative levels of 43 apoptosis-related proteins in the cell lysates according to the manufacturer's instructions. The experiment was repeated four times. The arrays were analyzed with a Gel-Doc XR imaging system (Bio-Rad). Quantity one analysis software (Bio-Rad) was used to analyze the images obtained. Measurements were repeated three times.

Briefly, for the arrays, the treated or untreated cell lysates were added to the antibody array membranes and incubated overnight with rocking at 4°C. After extensive washing, the membranes were incubated with a cocktail of biotin-conjugated anti-antibodies to apoptotic proteins at room temperature for two hours, as per the manufacturer's recommendations. After incubation with horseradish peroxidase–streptavidin at room temperature for an hour and a half, the signals were visualized by chemiluminescence.

Statistical Analyses

Data were reported as mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance followed by Tukey test. Level of significance was $p < 0.05$.

RESULTS

Cytotoxicity Results (LDH)

The LDH assays were performed and the averages with SD determined. TEGDMA at 0.50 mM (p=0.004), 0.75 mM (p=0.000), and 1.00 mM (p=0.000) was significantly higher than the control (Table 1). The highest nontoxic concentration of TEGDMA was 0.25 mM TEGDMA (p=0.806), which then was used for the human apoptosis antibody arrays.

RayBio Apoptosis Array Results

The relative expression of apoptotic proteins (Table 2) that were significantly higher at six hours were B-cell lymphoma-w (Bcl-w, p=0.010), BH3-interacting domain death agonist (BID, p=0.001), Bim (p=0.009), heat shock protein 27 (HSP 27, p=0.022), HSP 60 (p=0.007), HSP 70 (p=0.010), heat shock-inducible protein (HTRA, p=0.001), in-

Table 1: *Lactate Dehydrogenase (LDH) Assays*

Concentration of TEGDMA, mM NC (negative control, 0 mM)	Cytotoxicity ± SD 0 ± 0.00	*p*-Value
0.13	−0.74 ± 0.02	1.00
0.25	1.46 ± 0.01	0.806
0.50	4.96 ± 0.02	0.004*
0.75	10.7 ± 0.02	0.000*
1.00	20.6 ± 0.01	0.000*

Abbreviations: SD, standard deviation; TEGDMA, triethylene glycol dimethacrylate.
** Denotes statistically significant (p<0.05) differences compared to the negative control.*

sulin-like growth factor-1 (IGF-1, p=0.021), insulin-like growth factor binding protein-1 (IGFBP-1, p=0.004), IGFBP-2 (p=0.011), P21 (p=0.016), P27 (p=0.015), and second mitochondria-derived activator of Caspases (SMAC, p=0.018) (Figure 1). All of these were pro-apoptotic proteins. The only anti-apoptotic proteins that were significantly increased compared to the control at six hours were Survivin (p=0.015), IGFBP-5 (p=0.012), and Livin (p=0.006). The pro-apoptotic proteins that significantly decreased compared to the control at six hours were tumor necrosis factor (TNF) ligand superfamily member 6 (FasL, p=0.010), TNF-"β (p=0.046), and TNF-related apoptosis-inducing ligand receptor 2 (TRAILR 2, p=0.003).

At 24 hours, more pro-apoptotic proteins were significantly increased compared to the control than at six hours. These were Bad (p=0.021), Bax (p=0.027), Bcl-w (p=0.017), Bid (p=0.005), Bim (p=0.031), Caspase 3 (p=0.028), Caspase 8 (p=0.028), CD40L (p=0.006), Cytochrome c (Cyto c, p=0.001), IGFBP-5 (p=0.020), IGFBP-6 (p=0.005), cyclin-dependent kinase inhibitor 1 (p21, kip1, p=0.009), P27 (p=0.006), serum TNF receptor 1 (sTNF-R1, p=0.001), sTNF-r2 (p=0.004), tumor necrotizing factor–α (TNF-α, p=0.001), TNF-"β (p=0.003), TRAILR 1 (p=0.016), and TRAILR 2 (p=0.028). The anti-apoptotic proteins that increased significantly compared to the control at 24 hours were Bcl-2 (p=0.005), Livin (p=0.001), Survivin (p=0.001), TRAILR 3 (p=0.039), TRAILR 4 (p=0.010), and IGF-2 (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.

DISCUSSION

The effects of TEGDMA on apoptosis of pulp fibroblasts were examined. First, LDH assays were used to measure cell cytotoxicity. The rationale for using the LDH assays was to verify the concentration at which necrosis starts and to hopefully insure the presence of apoptotic cells rather than necrotic cells. The results 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.[7] It was verified by a ss DNA Elisa apoptosis kit (EMD Millipore Corporation, Billerica, MA, USA) that these cells were going through apoptosis at 0.25 mM of TEGDMA (data not shown). 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 some pro-apoptotic proteins after six and 24 hrs when compared to the control.

The extrinsic apoptotic pathway is activated through the activation of the transmembrane receptors of the TNF family. Members of the TNF family

Table 2: *RayBio Human Apoptosis Antibody Array*

	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	Caspase 3	Caspase 8
2	Pos	Pos	Neg	Neg	Blank	Blank	BAD	BAX	Bcl-2	Bcl-w	BID	BIM	Caspase 3	Caspase 8
3	CD40	CD40L	cIAP-2	Cyto C	DR6	Fas	FasL	Blank	HSP 27	HSP 60	HSP 70	HTRA	IGF-I	IGF-II
4	CD40	CD40L	cIAP-2	Cyto C	DR6	Fas	FasL	Blank	HSP 27	HSP 60	HSP 70	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-α	TNF-"β	TRAIL-R1	TRAIL-R2	TRAIL-R3	TRAIL-R4	XIAP	Blank	Neg	Neg	Neg	Pos
8	sTNF-R1	sTNF-R2	TNF-α	TNF-"β	TRAIL-R1	TRAIL-R2	TRAIL-R3	TRAIL-R4	XIAP	Blank	Neg	Neg	Neg	Pos

Abbreviations: BAD, Bcl-2 antagonist of cell death; BAX, Bcl-2–associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-w, apoptosis regulator Bcl-W; BID, BH3-interacting domain death agonist; BIM, Bcl2 interacting protein BIM; Caspase 8, cysteinyl aspartic acid-protease 8; Cyto c, Cytochrome c; DR, death receptor; Fas, fatty acid synthetase; FasL, fatty acid synthetase ligand; HSP, heat shock protein; HTRA2, High-temperature requirement protein A2; IAP, inhibitor of apoptotic protein; IGF, insulin-like growth factor; IGFBP, insulinlike growth factor binding protein; neg, negative; pos, positive; SMAC, second mitochondria-derived activator of Caspases; TNF, tissue necrotizing factor; TRAIL-R, TNF-related apoptosis-inducing ligand receptor; XIAP, X-linked inhibitor of apoptosis protein.

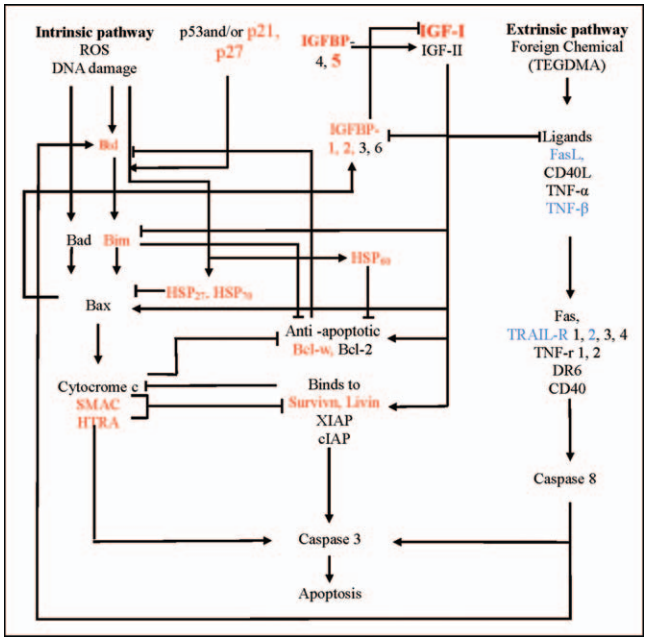

Figure 1. *Induced apoptotic proteins at six hours. Red indicates increases in relative expression, blue indicates decreases in relative expression, and black indicates no change in the relative expression.*

include TNFR 1 and 2, Fas, death receptor (DR) 6, CD40, and TRAIL-R 1-4, which bind to their corresponding ligands (TNF-α, TNF-"β, FasL, CD40L, and TRAIL, respectively). There were statistically significant increases in TNF-α and TNF-"β after 24 hours (Figure 2). TRAIL-R 1 and 2 contain death domains, which mean they are able to induce apoptosis by activating Caspase 8. However, TRAIL-R 3 and 4 are considered to be antagonistic decoys and do not induce death signals.[12] There were statistically significant increases in all of the TRAIL receptors after 24 hours (Table 3). Fas increased slightly when compared to the control at 24 hours, but this increase was not statistically significant. FasL showed a slight increase at 24 hours, but this increase was not statistically significant. However, it showed a statistically significant decrease at six hours (Table 3). This decrease in the expression of FasL could be explained by the presence of anti-apoptotic proteins, which could downregulate FasL.[13]

Iwama and others[14] reported that excessive reactive oxygen species (ROS) production due to arsenic trioxide toxicity resulted in changes in membrane permeability. Arsenic trioxide rapidly induced TRAIL and then activated Caspase 8, which resulted in the phosphorylation of Bid.[14] TEGDMA treatment leading to the production of ROS has been documented in the literature.[15] The current study

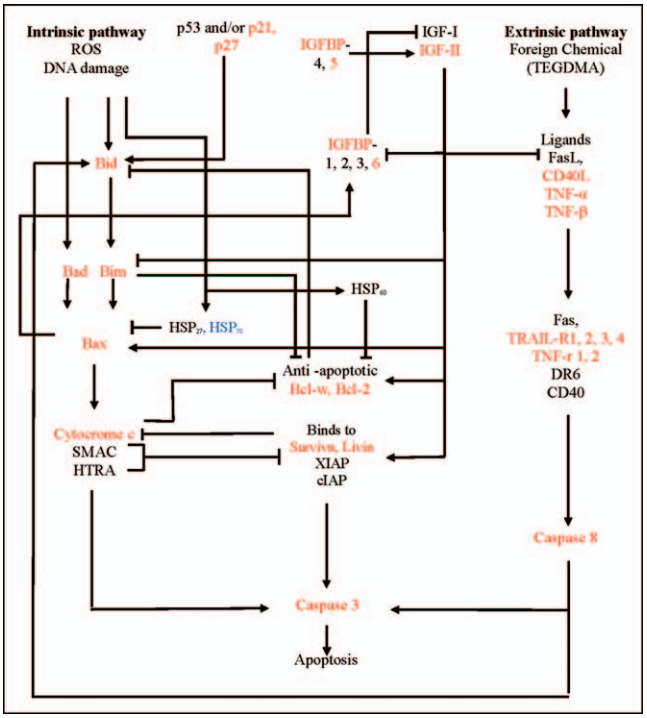

Figure 2. *Induced apoptotic proteins at 24 hours. Red indicates increases in relative expression, blue indicates decreases in relative expression, and black indicates no change in the relative expression.*

showed significant increases in all of the TRAIL receptors at 24 hours (Table 3), which is in agreement with the findings of a previous study.[14] Activation of the extrinsic pathway results in Caspase activation, specifically Caspase 8. The process is induced by two parallel pathways. The first pathway is through the cleavage and activation of Caspase 3, and the second one gets activated when the TRAIL receptors activate the extrinsic pathway. The intrinsic pathway (mitochondrial pathway) gets activated through the activation of the pro-apoptotic protein Bid by Caspase 8.[16,17] In the current study, the Caspase pathway was activated, as is evident by the statistically significant increases in both Caspase 8 and 3 after 24 hours, thus indicating that the extrinsic pathway had been activated (Figure 2). Bid increased significantly after 24 hours and demonstrated that the intrinsic pathway had been activated. Upon Bid activation, Bcl-2 family proteins get activated. The Bcl-2 family includes both pro- and anti-apoptotic proteins. Some of the pro-apoptotic proteins that were increased were Bim, Bax, and Bad. When Bad and Bim are phosphorylated, they become sequestered and enter into the mitochondria, causing the release of Cyto c, SMAC, and HtrA. Bad also binds to anti-apoptotic proteins (Bcl-2 and Bcl-w) and prevents their inhibition of apoptosis.[16,18]

Table 3: *Relative Expression of Apoptotic Proteins and* p-*Values*

Apoptotic Protein[1]	Mean (at 6 h) ± SD	*p*-Value	Mean (at 24 h) ± SD	*p*-Value
BAD	1.65 ± 0.71	0.116	1.57 ± 0.37	0.021*
BAX	1.29 ± 0.38	0.184	1.20 ± 0.17	0.027*
Bcl-2	2.24 ± 0.75	0.046	1.59 ± 0.34	0.005*
Bcl-w	2.11 ± 0.41	0.010*	1.20 ± 0.12	0.017*
BID	2.08 ± 0.53	0.001*	1.17 ± 0.12	0.005*
BIM	1.63 ± 0.54	0.009*	1.21 ± 0.18	0.031*
Caspase 3	1.41 ± 0.48	0.344	1.54 ± 0.28	0.028*
Caspase 8	1.52 ± 0.54	0.557	1.59 ± 0.30	0.028*
CD40	1.19 ± 0.46	0.510	0.83 ± 0.45	0.545
CD40L	0.74 ± 0.38	0.124	1.15 ± 0.07	0.006*
cIAP-2	1.23 ± 0.17	0.905	1.15 ± 0.29	0.335
Cyto c	0.88 ± 0.20	0.174	1.21 ± 0.05	0.001*
DR6	0.89 ± 0.24	0.303	1.02 ± 0.10	0.724
Fas	1.02 ± 0.21	0.792	1.12 ± 0.13	0.108
FasL	0.71 ± 0.22	0.010*	1.12 ± 0.11	0.072
HSP 27	1.91 ± 0.35	0.022*	1.00 ±0.08	0.966
HSP 60	1.20 ± 0.10	0.007*	0.95 ± 0.13	0.355
HSP 70	1.06 ± 0.05	0.010*	0.87 ± 0.13	0.034*
HTRA	1.24 ± 0.23	0.020*	1.16 ± 0.13	0.101
IGF-1	13.22 ± 5.74	0.021*	0.92 ± 0.06	0.147
IGF-2	0.82 ± 0.72	0.681	2.91 ± 0.41	0.001*
IGFBP-1	1.61 ± 0.47	0.004*	1.47 ± 0.49	0.104
IGFBP-2	1.53 ± 0.29	0.011*	1.13 ± 0.20	0.100
IGFBP-3	1.63 ± 0.54	0.113	1.11 ± 0.19	0.288
IGFBP-4	1.89 ± 1.14	0.247	1.01 ± 0.25	0.963
IGFBP-5	1.33 ± 0.19	0.012*	1.23 ± 0.14	0.020*
IGFBP-6	1.60 ± 0.68	0.200	1.41 ± 0.02	0.005*
IGF-1sr	0.97 ± 0.03	0.214	1.55± 0.41	0.036
Livin	2.74± 0.83	0.006*	1.61 ± 0.20	0.001*
p21	1.14± 0.12	0.016*	1.15 ± 0.08	0.009*
p27	2.01± 0.43	0.015*	1.27 ± 0.17	0.006*
p53	0.97 ± 0.18	0.671	1.31 ± 0.15	0.070
SMAC	1.32 ± 0.20	0.018*	1.06 ± 0.12	0.258
Survivin	1.29 ± 0.12	0.015*	1.21 ± 0.10	0.001*
sTNF-r1	0.55 ± 0.48	0.183	1.62 ± 0.02	0.001*
sTNF-r2	1.57 ± 0.39	0.063	2.24 ± 0.37	0.004*
TNF-α	1.01 ± 0.08	0.242	2.25 ± 0.01	0.001*
TNF-"β	0.57 ± 0.20	0.046*	1.53 ± 0.18	0.003*
TRAIL-R1	1.28 ± 0.37	0.257	1.42 ± 0.06	0.016*
TRAIL- R2	0.78 ± 0.14	0.003*	1.20 ± 0.07	0.028*
TRAIL-R3	1.00 ± 0.20	0.944	1.64 ± 0.36	0.039*
TRAIL-R4	1.39 ± 0.35	0.068	1.31 ± 0.13	0.010*
XIAP	1.15 ± 0.24	0.259	1.15 ±0.14	0.074

Abbreviation: SD, standard deviation.
[1] Reference abbreviation footnote for Table 2 for definition of terms.
** Denotes statistically significant (p<0.05) differences compared to the control.*

The interactions between the pro- and anti-apoptotic proteins result in no inhibition of apoptosis and thus cause depolarization of the mitochondria. Depolarization of the mitochondria results in increases in the permeability of their membranes, thus releasing more pro-apoptotic factors, such as SMAC, HtrA, and Cyto c. This study showed significant increases in some of the Bcl-2 family members that are involved in the intrinsic pathway. Bid and Bim (pro-apoptotic proteins of the Bcl-2 family) were both significantly increased at six (Table 3) and 24 hours (Table 3). Bcl-w (anti-apoptotic protein) was also significantly increased at six and 24 hours. SMAC and HtrA are two pro-apoptotic proteins that are released upon activation of the intrinsic pathway. These two pro-apoptotic proteins promote apoptosis through binding to the cIAP anti-apoptotic protein and prevent its attribution to apoptosis.[19] SMAC and HtrA were increased significantly at six hours (Table 3) and increased slightly at 24 hours, but the increases were not significant.

Another important pro-apoptotic protein that is released from the mitochondria upon the activation of the intrinsic pathway is Cyto c. Cyto c activates Caspase 9 and then Caspase 9 activates Caspase 3. Cyto c was statistically significantly (p=0.001) increased compared to the control at 24 hours (Table 3).

Members of the HSP family are overexpressed under biological stress such as heat or when treated with toxic materials. Their general function is to prevent cellular protein aggregation and to increase levels of reduced glutathione to protect the cell from ROS.[20] HSP 27 and 70 are anti-apoptotic proteins, while HSP 60 is a pro-apoptotic protein. The current study agrees with that of Noda and others,[7] which showed that TEGDMA inhibits the phosphorylation of HSPs, thereby decreasing their levels. The current study showed that there was activation of HSP 70 and that its increase was statistically significant at six hours (Table 3), but its level at 24 hours was decreased significantly (Table 3), which indicated that the anti-apoptotic effects of HSP 70 were counteracted. As for HSP 60, there was a slight decrease at 24 hours, yet it was not significant statistically. HSP 27 was unaltered.

IGF-1 plays an important role in the cell survival pathway and inhibits apoptosis. IGF-1 activation causes induction of two major signaling pathways: the phosphatidylinositol-triphosphate kinase/AK-Transforming (PI3K/AKT) pathway and the mitogen-activated protein kinase (MAPK) pathway.[21] 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, especially Caspase-3.[21] Spagnuolo and others[6] showed that AKT is a main target in TEGDMA-induced apoptosis. The current study showed significantly higher levels of IGF-1, especially after six hours. These results are in an agreement with those of Spagnuolo and others,[6] since IGF-1 activates the PI3K/AKT pathway. However, IGF-1 expression at 24 hours slightly decreased. One of the signs of apoptosis is a decrease in IGF levels.

IGFBPs have been described to have both pro- and anti-apoptotic effects. The effects of IGFBP-5 are variable depending on the tissue and cell type.[22,23] IGFBP-5 was shown to be anti-apoptotic in gingival fibroblasts.[22] 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.[24] This study also showed increased 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 of these pathways were shown by Samuelsen and others[15] to be involved in TEGDMA-induced apoptosis. The current study showed a significant increase ($p=0.005$) in the expression of IGFBP-6 in comparison to the control at 24 hours.

There are several reports about TEGDMA causing genotoxicity and cell-cycle delay.[25] The p21 and p27 proteins are cell cycle regulators. The tumor suppressor protein p53 is the main regulator of p21. Krifka and others[26] 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 six hours and 24 hours. However, p53 showed a slight increase at 24 hours only and was not significant.

IAP proteins are Caspase inhibitor proteins. Survivn, Livin, XIAP, and cIAP are members of this family. It has been shown[13] that XIAP has a high affinity for Caspase 3 and tries to inhibit apoptosis once it is started. SMAC and HtrA (mitochondrial pro-apoptotic proteins) are known to bind to these IAP inhibitory apoptotic proteins and inhibit their functions.[13] Survivin and Livin significantly increased at six ($p=0.015$ and $p=0.006$, respectively) and 24 hours ($p=0.001$ and $p=0.001$, respectively). However, these increases were not enough to inhibit apoptosis. Caspase 8 and Caspase 3 were both activated at 24 hours, indicating that the apoptotic process was still continuing.[13]

This current study showed that TEGDMA activated apoptosis within 24 hours. The extrinsic pathway at 24 hours was clearly activated and appears to be activated through the activation of members of the TNFR family. The activation of the intrinsic pathway started at six hours, but it seems to be further amplified at 24 hours. Bax and Cyto c are essential pro-apoptotic proteins for the intrinsic pathway. However, Bax and Cyto c were not activated until the extrinsic pathway was also activated. Both the extrinsic and intrinsic pathways play a role in inducing apoptosis in TEGDMA-treated HPFs.

CONCLUSIONS

The results of this study showed significant increases in multiple examined pro-apoptotic proteins, and several anti-apoptotic proteins were also altered. Pro-apoptotic proteins involved in the intrinsic (mitochondrial) pathway were significantly increased after six and 24 hours. Numerous pro-apoptotic proteins of the extrinsic pathway were activated at 24 hours. The activation of these pro-apoptotic proteins in the extrinsic pathway seemed to amplify some of the intrinsic pro-apoptotic proteins at 24 hours. More pro-apoptotic proteins in the intrinsic pathway were activated at 24 hours than at six hours. TEGDMA had effects on both the extrinsic and intrinsic apoptotic pathways. Additional research is needed to elucidate the net effects of this apoptotic process on the pulp tissue and to find ways to prevent or even reverse this process, if possible.

Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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