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TEGDMA Reduces Mineralization in Dental Pulp Cells

ABSTRACT

Direct application of dentin bonding agents onto the exposed pulp has been advocated, but *in vivo* studies indicate a lack of reparative dentin formation. Our objective was to investigate the role of triethylene glycol dimethacrylate (TEGDMA), a commonly used compound in dentin bonding agents, as a potential inhibitor of mineralization. Human pulp cells were exposed to different concentrations of TEGDMA, and expression of the mineralization-related genes collagen I, alkaline phosphatase, bone sialoprotein, osteocalcin, Runx2, and dentin sialophosphoprotein was analyzed. Gene expression studies by real-time polymerase chain-reaction revealed a concentration- and time-dependent decrease of mineralization markers. A subtoxic TEGDMA concentration (0.3 mM) reduced expression levels by 5 to 20% after 4 hrs and by 50% after 12 hrs. Furthermore, alkaline phosphatase activity and calcium deposition were significantly lower in dental pulp cells treated with TEGDMA over 14 days. These findings indicate that even low TEGDMA concentrations might inhibit mineralization induced by dental pulp cells, thus impairing reparative dentin formation after pulp capping with dentin bonding agents.

KEY WORDS: triethylene glycol dimethacrylate (TEGDMA), dental pulp cells, mineralization.

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INTRODUCTION

Materials for direct pulp capping have been studied extensively, and several of the different procedures resulted in hard tissue formation. The application of calcium-hydroxide-based compounds is widely accepted due to their favorable effects on regeneration. Successful treatment is indicated by maintained pulp vitality and formation of reparative dentin, which separates the soft tissue from the site of injury.

With the widespread use of dentin bonding agents in combination with resin composites, the application of dental adhesives for pulp capping has been proposed as an alternative to $Ca(OH)_2$-containing agents. Although experiments in monkeys showed little inflammation and formation of a dentin bridge (Hafez *et al*., 2002; Kitasako *et al*., 2002), manifold studies in other animals and in humans have documented a failure of hard tissue formation after pulp capping with different bonding agents. However, dentin bridging could be observed in $Ca(OH)_2$-treated controls (Hörsted-Bindslev *et al*., 2003; Silva *et al*., 2006; Tziafas *et al*., 2007). Inflammatory responses of various degrees were reported for both treatment methods, but pulp vitality and odontoblast function remained intact. These results suggest that adhesive systems are not acceptable as capping agents, since they interfere with reparative dentin formation.

Little is known about the underlying mechanisms, though biocompatibility of dentin bonding agents has been investigated in numerous studies, because their organic matrix contains cytotoxic compounds, namely, acrylic monomers such as triethylene glycol dimethacrylate (TEGDMA) or 2-hydroxyethyl methacrylate (HEMA). Even after polymerization, sufficient amounts of residual monomers are released from resin-based materials to damage the subjacent cell population (Hume and Gerzina, 1996). Although a remaining dentin layer reduces adverse biological effects, effective monomer concentrations have been estimated to reach 0.2-3.6 mM in dental pulp, even in the presence of an intact dentin barrier (Bouillaguet *et al*., 1996).

TEGDMA is a cytotoxic monomer, and even sublethal amounts might impair cellular function and alter pathways regulating homeostasis, tissue repair, and mineralization (Schweikl *et al*., 2006). Results from clinical studies and recent findings on stress response in human pulp cells indicate an interference of bonding agents with reparative dentin formation. In the present study, primary human dental pulp cells were used to test whether low TEGDMA concentrations decrease expression levels of mineralization-related genes, and lead to reduced mineral deposition.

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MATERIALS & METHODS

Cell Cultures and TEGDMA Exposure

Primary human pulp cell cultures were established from freshly extracted human third molars as described previously (Galler *et al*., 2006), following an informed consent protocol approved by an appropriate Institutional Review Board (University of Regensburg). Cells from 4 wisdom teeth were pooled, and cultured in αMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Invitrogen Corporation, Karsruhe, Germany). Cells of passage 3 were used to evaluate cell viability and proliferation, gene expression levels, alkaline phosphatase (ALP) activity, and calcium deposition after TEGDMA exposure. To test whether the observed effects were cell-specific, we further evaluated ALP activity and calcium deposition in dental pulp stem cells (DPSC), which possess a high mineralization activity and have been shown to form mineralized nodules *in vitro* (Gronthos *et al*., 2000; Galler *et al*., 2008). DPSC were cultured in αMEM supplemented with 15% fetal bovine serum, 50 μg/mL L-ascorbic acid 2-phosphate, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Invitrogen Corporation, Karsruhe, Germany).

TEGDMA concentrations were chosen based on a previous study with primary human pulp cells (Spagnuolo *et al*., 2004). After preparation of a TEGDMA stock solution in dimethyl sulfoxide (DMSO) at 1 M (chemicals from Sigma Aldrich, Taufkirchen, Germany), the solution was diluted in cell culture medium to final concentrations of 0.3, 1.0, and 3.0 mM.

For real-time PCR and visualization of calcium deposition by alizarin red stain, 5 x 10^4 cells/well were seeded in 6-well plates. For cell viability and proliferation, alkaline phosphatase assays were conducted, and for quantification of calcium deposition, cells were seeded at 2 x 10^3 cells/well in 96-well plates.

Cell Viability and Proliferation

Cell viability and proliferation were determined after exposure to different TEGDMA concentrations for up to 96 hrs and compared with untreated controls. For viability assays, a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared in PBS. Cells were incubated with 100 μL MTT for 2 hrs at 37°C, the solution was removed and subjected to lysis in DMSO, and absorbance was measured on a spectrophotometer at 570 nm (Infinite 200, TECAN, Crailsheim, Germany).

Cell numbers were determined by quantification of DNA content with the CyQuant cell proliferation assay kit (Invitrogen, Molecular Probes, Carlsbad, CA, USA) on a fluorescent plate reader (Infinite 200). Cell numbers were calculated based on standard curves created from known cell densities.

Real-time PCR

Real-time PCR was performed at time-points and TEGDMA concentrations matching those for cell viability assays. RNA was extracted by means of an RNeasy mini kit (QIAGEN, Hilden, Germany). Reverse transcription was performed according to standard protocols for cDNA synthesis (Omniscript RT-Kit, QIAGEN, Hilden, Germany). Human-specific primers were designed with primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and synthesized as follows: collagen α(1)I (sense 5′-AAAAGGAAGCTTGGTCCACT-3′; antisense 5′-GTGTGGAGAAAGGAGCAGAA-3′; GenBank NM_000088), alkaline phosphatase (sense 5′-CCACGTCTTCACATTTGGTG-3′; antisense 5′-AGACTGCGCCTGGTAGTTGT-3′; GenBank NM_000478), bone sialoprotein (sense 5′-GTGGATGAAAACGAACAAGG-3′; antisense 5′-CCCCTTCTTCTCCATTGTCT-3′; GenBank NM_000582), osteocalcin (sense 5′-ACTGTGACGAGTTGGCTGAC-3′; antisense 5′-CAAGGGCAAGAGGAAAGAAG-3′; GenBank X_53698), Runx2 (sense 5′-GAACTGGGCCCTTTTTCAGA-3′; antisense 5′-GCGGAAGCATTCTGGAAGGA-3′; GenBank NM_004348), and dentin sialophosphoprotein (sense 5′-TTAAATGCCAGTGGAACCAT-3′, antisense 5′-ATTCCCTTCTCCCTTGTGAC-3′; GenBank NM_014203). Glyceraldehyde-3-phosphate dehydrogenase (sense 5′-GAGTCAACGGATTTGGTCGT-3′; antisense 5′-GACAAGCTTCCCGTTCTGAG-3′, GenBank M_33197) and beta-actin (sense 5′-AAACTGGAACGGTGAAGGTG-3′; antisense 5′-TTTTTAGGAGGGCAAGGGACT-3′; GenBank BC0 92424) were used as internal controls. Conditions for real-time PCR were as follows: denaturation at 95°C (15 min); 50 cycles at 95°C (15 sec), 58°C (30 sec) (annealing), and 72°C (30 sec), followed by a final dissociation step. Reactions were measured 3 times (ABI Prism 7900), and gene expression was quantified with SYBR green (QuantiTect SYBR green PCR kit, Qiagen Inc., Valencia, CA, USA).

Alkaline Phosphatase Activity and Calcium Deposition

ALP activity was quantified prior to the application of 0.3 mM TEGDMA (D0), and subsequently at D1, 3, 5, 7, 10, and 14 of exposure. Cells were rinsed with PBS, then frozen (-20°C) and thawed (37°C) 3 times to disrupt cell membranes. ALP activity was determined by hydrolysis of p-nitrophenol phosphate (SIGMA-Aldrich, St. Louis, MO, USA) in 2-amino-2-methyl-propanole (pH 10.3) (Sigma-Aldrich) at 37°C for 1 hr. Liberated p-nitrophenol was measured spectrophotometrically (405 nm), and samples were compared with a p-nitrophenol standard (Sigma-Aldrich). ALP activity was related to the cell number obtained from proliferation assays at matching time-points, and expressed as nanomoles of p-nitrophenol liberated *per* 1000 cells after 1 hr.

For visualization and quantification of calcium deposition, cells were fixed in cold methanol and stained with 2% alizarin red for 5 min. Pictures were taken of samples in 6-well plates. For quantification, cells in 96-well plates were washed 5 times in PBS, and crystals were allowed to dissolve in 100 μL of a 10% cetylpyridinium chloride monohydrate solution for 30 min at room temperature. Absorbance was determined at 540 nm.

Data Analysis

Gene expression was quantified as described based on GAPDH in corresponding samples (Pfaffl, 2001). Expression in treated samples is presented as a fold-change compared with controls, which were set to 1. For statistical analysis, 3 samples from 3

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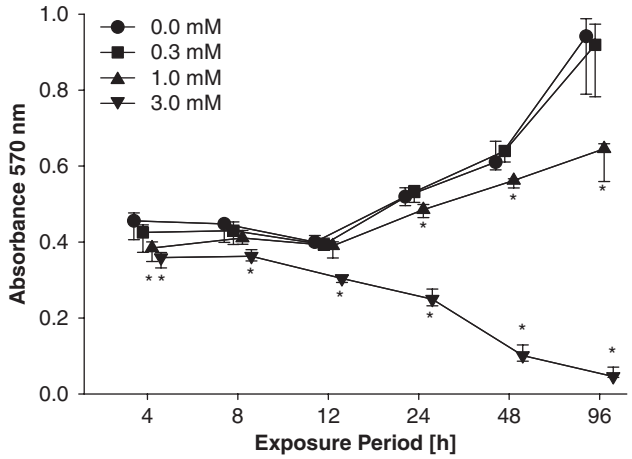

Figure 1. Viability of primary human pulp cells after exposure to different concentrations of TEGMDA as determined by MTT assay. Symbols and bars represent medians (25th and 75th percentiles) calculated from 10 individual values (n = 10) resulting from 2 independent experiments. Asterisks indicate significant differences in cell viability of TEGDMA-treated cultures compared with untreated controls (0.0 mM). Viability of cells treated with 0.3 mM TEGDMA and untreated control cells showed a nearly identical trend, whereas viability decreased with increasing TEGDMA concentration.

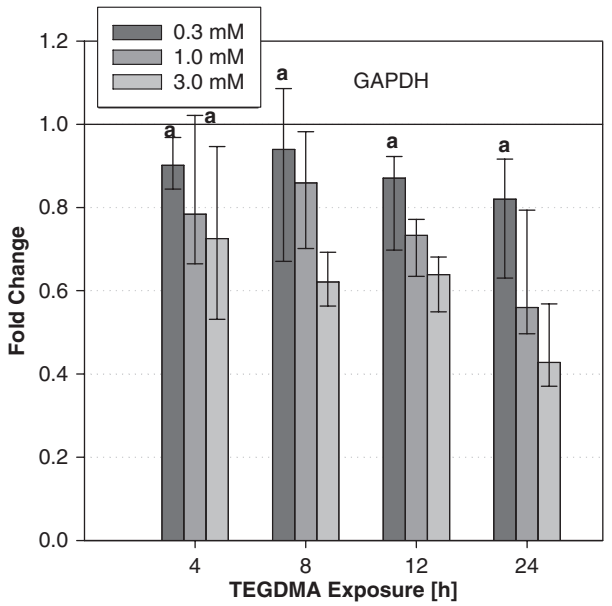

Figure 2. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in primary human pulp cells exposed to 0.3 mM, 1 mM, and 3 mM TEGDMA after 4, 8, 12, and 24 hrs. Bars represent medians (25th and 75th percentiles) calculated from triplicates in 3 independent experiments (n = 9). Untreated controls were set to 1, and results are presented as a fold-change relative to untreated controls. GAPDH expression is slightly reduced at 0.3 mM; differences become significant at 1 mM and 3 mM. Lower-case letters indicate non-significant differences.

independent experiments were combined (n = 9). For MTT and DNA assays, quantification of ALP, and calcium deposition, 10 samples for each concentration and exposure period were obtained from 2 independent experiments. For all experiments, medians and 25th and 75th percentiles were calculated, and statistical analysis was performed with the Mann-Whitney U-test ($\alpha = 0.05$). Tables containing means and standard deviations are available in the Appendix.

RESULTS

Cell Viability and Proliferation

Cell viability and proliferation were not affected at 0.3 mM TEGDMA ((Figs. 1, 4A). However, significant differences could be observed at 1 mM and 3 mM. After 48 hrs at 3 mM TEGDMA, cell numbers and viability were reduced to 20%.

Real-time PCR

GAPDH expression decreased slightly in cell cultures exposed to 0.3 mM TEGDMA compared with untreated controls. However, a statistically significant reduction of GAPDH expression was observed only at higher concentrations of 1 and 3 mM TEGDMA (Fig. 2). Expression levels of beta-actin followed a similar pattern (see Appendix), where reduction of expression at 0.3 mM TEGDMA remained non-significant.

Changes in gene expression normalized to GAPDH for increasing TEGDMA concentrations and exposure periods are shown in Fig. 3. Our analysis showed a time- and concentration-dependent decrease of expression levels of mineralization-related genes in relation to GAPDH expression. Changes in gene expression were significant in all genes except COL I after 4 hrs at 3 mM TEGDMA and DSPP after 8 hrs at 3 mM. At the lowest TEGDMA concentration, the range in expression levels decreased from 5-20% (COL I, Runx2) after 4 hrs to about 50% after 12 hrs. Higher concentrations impaired mainly COL I and Runx2 expression; OC and DSPP were the least affected over the course of the experiment. Data from measurements at 48 and 96 hrs are incomplete because of missing samples due to cytotoxicity (not shown).

Alkaline Phosphatase Activity and Mineral Formation

ALP activity in primary human pulp cells was significantly reduced at 0.3 mM TEGDMA, whereas cell numbers in corresponding samples showed little difference compared with untreated controls (Fig. 4A). After 14 days in culture, ALP appeared five-fold higher in untreated cells. The difference in color intensity between treated cells and controls after alizarin red stain indicates reduced mineral formation after TEGDMA exposure (Fig. 4B). Quantification of calcium deposition showed three-fold higher absorbance in untreated controls (Fig. 4C). Reduced ALP activity and calcium deposition could be confirmed in DPSC, where ALP activity in untreated cells was six-fold increased, and the difference in color intensity between treated cultures and controls indicated a lack of mineral formation after TEGDMA exposure (see Appendix).

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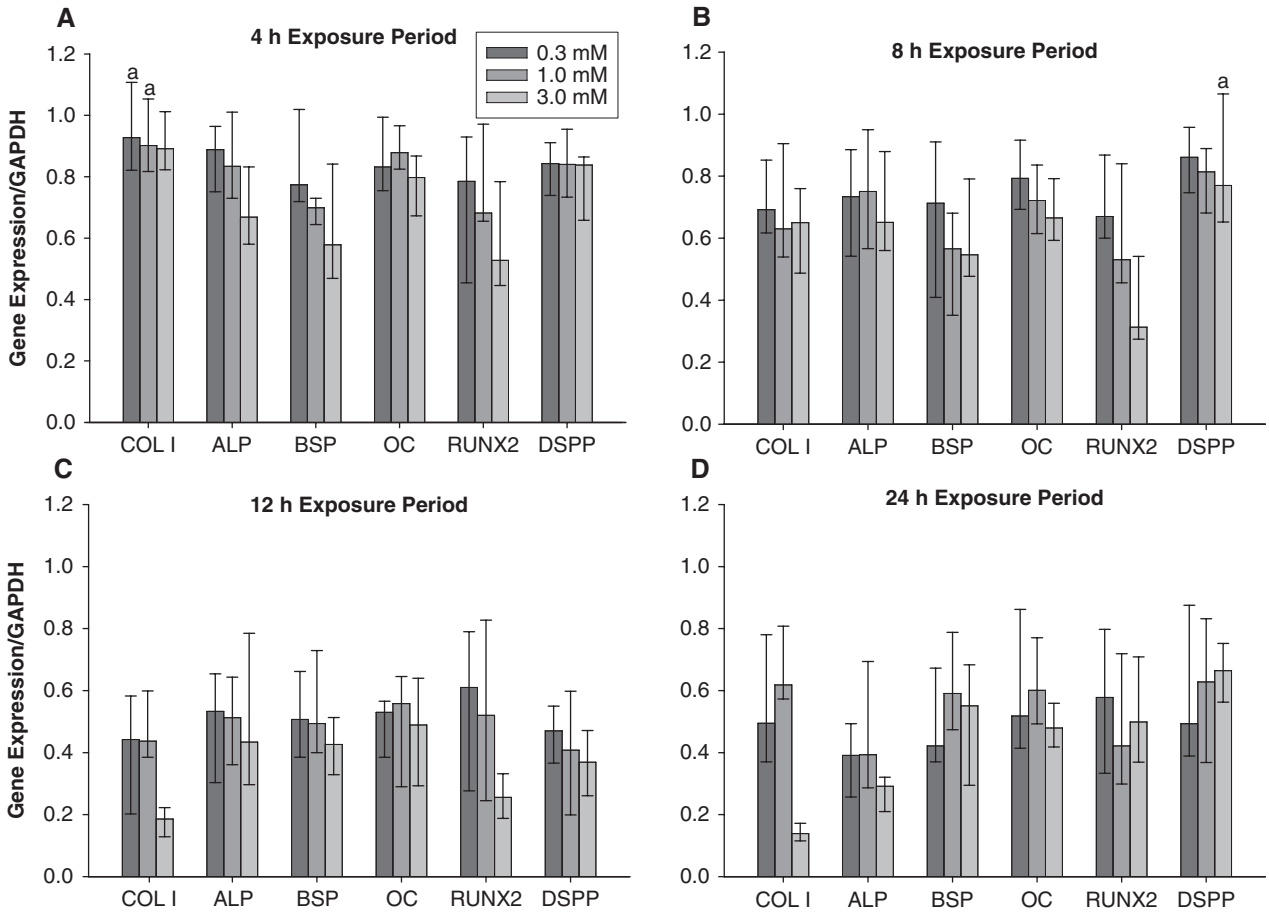

Figure 3. Dose-dependent decrease of expression levels of 6 genes critical for mineralization determined by quantitative real-time PCR analysis in primary human pulp cells. RNA was extracted after 4, 8, 12, and 24 hrs **(A-D)** after exposure to 0.3 mM, 1 mM, and 3 mM TEGDMA. Gene expression was normalized to GAPDH in corresponding samples. Untreated controls were set to 1, as indicated by the dotted line, and results are presented as a fold-change relative to untreated controls. Bars represent medians (25th and 75th percentiles) calculated from triplicates in 3 independent experiments (n = 9). Expression of all genes in TEGDMA-treated cell cultures is significantly different from gene expression in untreated controls, except for those cases indicated by the lower-case letter (a). Col I, collagen αI(I); ALP, alkaline phosphatase; BSP, bone sialoprotein; OC, osteocalcin; Runx2, runt-related transcription factor 2; DSPP, dentin sialophosphoprotein.

DISCUSSION

The production of dentin is a complex process by which differentiated odontoblasts secrete a collagenous matrix that serves as a template for mineral deposition. Non-collagenous proteins control crystal nucleation and growth. After loss of dentin and disruption of the odontoblast layer, progenitor cells residing in the dental pulp can differentiate and form reparative dentin as an active defense mechanism to create a barrier at the site of injury (Mitsiadis and Rahiotis, 2004). Reduced mineralization might be attributed to a down-regulation of dentin-specific proteins. In this study, the resin monomer TEGDMA inhibited the expression of parameters of the odontoblastic phenotype of dental pulp cells. Levels of the marker genes collagen α(1)I, ALP, bone sialoprotein (BSP), osteocalcin (OC), Runx2, and dentin sialophosphoprotein (DSPP) decreased in a time- and concentration-dependent fashion. These findings suggest a TEGDMA-induced down-regulation of genes necessary for matrix deposition and crystal formation after relatively short exposure. In similar studies, the expression of collagen I and DSPP genes was inhibited in rat pulp cells after exposure to a poly-methylmethacrylate (PMMA)-based dental resin, where inhibition was specific since expression levels of the housekeeping gene GAPDH remained the same (Kojima *et al*., 2008; Yamada *et al*., 2008). Moreover, concentrations as low as 10 μM of the monomers HEMA and TEGDMA reduced the protein levels of collagen I, osteonectin, and DSP in human pulp cells after 4 wks. Signs of acute cytotoxicity were not detected (About *et al*., 2002).

Here, the levels of the internal standards GAPDH and beta-actin slightly decreased at low TEGDMA concentrations after short exposure, and cell viability and proliferation remained stable up to 14 days. Thus, we suppose that the inhibition of the expression of the mineralization-related genes analyzed here was not primarily caused by acute cytotoxic effects. Additional evidence exists of a specific influence of physiologically relevant amounts of TEGDMA on the cellular response at the transcriptional level. The monomer changed the expression of genes related to the regulation of redox-homeostasis, cellular

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proliferation, and cell death. Collagen I was among the most down-regulated genes (Schweikl *et al*., 2008).

As expression of mineralization-related genes decreases, less protein product is available for their physiological functions. ALP cleaves extracellular pyrophosphate to inorganic phosphate, which is needed for matrix calcification (Hessle *et al*., 2002). In our case, in parallel to a down-regulation of ALP at the transcriptional level, ALP protein function was decreased in cells treated with a low TEGDMA concentration. Whereas both untreated primary human pulp cell cultures and DPSC cultures formed calcified nodules after 14 days, mineralization was reduced or nearly absent after TEGDMA exposure. Likewise, other studies reported decreased ALP activity in the presence of resin monomers (About *et al*., 2002). ALP-positive stain or Von Kossa-positive areas indicating mineralizing capability could not be detected in pulp cells cultured on a PMMA-based dental resin for up to 20 days (Kojima *et al*., 2008; Yamada *et al*., 2008).

From recent investigations, we are beginning to understand how monomers impair the cells' basic function. Concentrations well below those inducing acute cytotoxic effects alter various cellular endpoints, modify signal transduction pathways, and disrupt the cell's redox balance (Schweikl *et al*., 2006). Oxidative damage occurs after generation of reactive oxygen species (ROS), and a simultaneous reduction of anti-oxidants like glutathione. Ample experimental evidence exists that the coordinated induction of genes coding for oxidative stress response and anti-oxidant proteins is a critical mechanism to protect cells against adverse effects of monomers (Schweikl *et al*., 2008). It appears that the generation of oxidative stress induces down-regulation of genes that control cell differentiation and mineralization processes. Reduced bone mineral density involved a decrease in anti-oxidant defense and impairment of osteoblast maturation and mineralization (Mody *et al*., 2001; Maggio *et al*., 2003). Exposure of osteoblastic cells to hydrogen peroxide decreased mineralization and expression of Runx2, COL I, and ALP (Bai *et al*., 2004; Arai *et al*., 2007). Furthermore, N-acetylcysteine (NAC), a scavenger of ROS, protected pulp cells from PMMA-induced inhibition of ALP activity (Kojima *et al*., 2008; Yamada *et al*., 2008). The application of NAC prior to tooth restoration with a resin material even restored ALP activity in pulp cells in an *in vivo* model (Paranjpe *et al*., 2008). Elevated ROS levels have been demonstrated to activate NF-κB, which counteracted HEMA-induced apoptosis in human pulp cells (Spagnuolo *et al*., 2006). Interestingly, the anti-oxidant-responsive proteins AP-1 and NF-κB were also identified as regulators of bone metabolism (Wagner and Eferl, 2005).

In a clinical situation, monomers like HEMA and TEGDMA are constantly released from resin materials. These compounds can diffuse across dentin in relevant amounts to impair cellular functions. It has been suggested that the responses of pulp cells will depend on their differentiation status and the level of exposure. Heavy exposure to dental resins in the case of direct pulp capping or application of materials in cavities with thin remaining dentin barriers may cause cell necrosis, but lower exposure may result in the activation of physiological pathways including protective mechanisms (Paranjpe *et al*., 2007). Until recently, the cell's response to stressors was thought to involve primarily unspecific reactions characteristic of all cells, but accumulating evidence is contradictory. Activation of signaling pathways by oxidative stress seems cell-type-specific, resulting in regulated modulation of cytodifferentiation and controlled changes in gene expression patterns. Our results suggest a negative effect of TEGDMA on expression levels of mineralization-related genes, resulting in reduced mineralization activity and calcium deposition, which might explain the failure of reparative dentin formation after direct pulp capping with bonding agents.

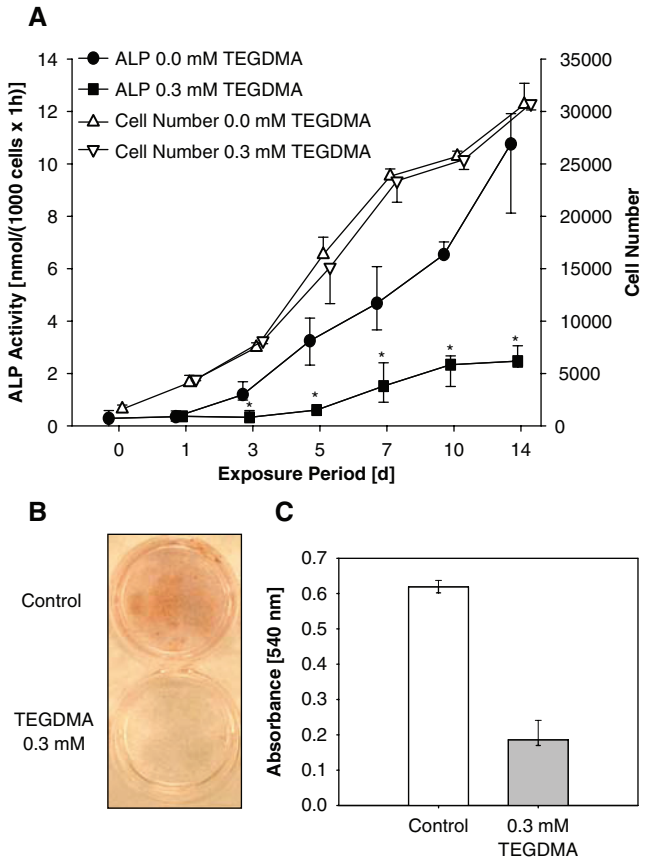

Figure 4. Proliferation and mineralization activity of primary human pulp cells in controls and TEGDMA-treated cultures. **(A)** Alkaline phosphatase activity (Y1-axis) and cell numbers (Y2-axis) of corresponding samples are shown for control cells and cells treated with 0.3 mM TEGDMA for 14 days. Symbols and bars represent medians (25th and 75th percentiles) calculated from 10 individual values (n = 10) resulting from 2 independent experiments. ALP activity is normalized to cell numbers in corresponding samples and expressed in nmol/1000 cells/1 hr. Whereas cell numbers in treated samples and controls were nearly identical, ALP activity was significantly reduced in TEGDMA-treated cells after 3 days. Asterisks indicate significant differences between ALP activities found in untreated cell cultures (medium) and cultures treated with 0.3 mM TEGDMA. **(B)** Alizarin Red stain for calcium deposition after 14 days. Top row shows control cells, bottom row shows cells exposed to 0.3 mM TEGDMA, which visibly reduced mineral deposition, as indicated by the difference in intensity of alizarin red. Representative results from 3 experiments (n = 9) are shown. **(C)** Relative quantification of calcium deposition after 14 days in culture in TEGDMA-treated cells and controls. Columns and bars represent median values with corresponding 25th and 75th percentiles (n = 10).

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