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Effects of dental composite resin monomers on dental pulp cells

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Methacrylate monomers found in many dental materials cause toxicity to dental pulp cells but the mechanism of the toxicity is poorly understood. We used cultured human dental pulp cells to test the effects of three commonly used monomers; bisphenol-A-glycidyl methacrylate (Bis-GMA), urethane dimethacrylate (UDMA), and triethyleneglycol dimethacrylate (TEGDMA). The order of toxicity was Bis-GMA>UDMA>TEGDMA. The toxicity correlated inversely with cystine uptake, with TEGDMA stimulating uptake and Bis-GMA and UDMA inhibiting uptake. Bis-GMA and UDMA induced oxidative stress, while TEGDMA did not. Toxicity correlated poorly with glutathione levels, as all compounds decreased cellular glutathione. TEGDMA is less toxic than Bis-GMA and UDMA likely because it stimulates cystine uptake and does not induce oxidative stress, the enhanced uptake of cystine appears to compensate for TEGDMA’s direct interaction with glutathione. Bis-GMA and UDMA both deplete glutathione and inhibit cystine uptake leading to oxidative stress and cell death.

Keywords: Bis-GMA, Dental pulp, TEGDMA, Toxicity, UDMA

INTRODUCTION

Resin containing compounds are used for a wide variety of dental applications including restorations, sealants, liners, bonding agents, and pulp capping. Among the most commonly used compounds in dental resins are bisphenol-A-glycidyl methacrylate (Bis-GMA), urethane dimethacrylate (UDMA), and triethyleneglycol dimethacrylate (TEGDMA). The degree of polymerization appears to vary depending on the viscosity of the monomers², the concentration of the photoinitiator used³, and the conditions to which the material is exposed⁴,⁵). The essential question is whether enough methacrylate monomers leach out of dental materials to cause toxicity to dental pulp cells. An important aspect of the potential toxicity is whether once the monomers leach out of the dental material does the polymerization process occur in the oral environment. Interestingly, methacrylate monomers have been shown to be able to diffuse through the dentin layer to potentially interact with pulp cells even when the dental material is not in direct contact with the pulp cells⁶,⁷). The toxicity of a number of dental materials to pulp cells involves oxidative stress, and specifically, depletion of cellular glutathione (GSH)⁸). The methacrylates found in resins used in dentistry are known to cause toxicity that involves GSH depletion and this toxicity has been shown to be attenuated by the promoter of GSH production, N-acetylcysteine⁹). Also, dentin bonding agents with the only common component being Bis-GMA cause toxicity to dental pulp cells that is enhanced by the GSH synthesis inhibitor buthionine sulfoximine and attenuated by the GSH booster 2-octothiazolidine-4-carboxylic acid¹⁰).

The cystine/glutamate antipporter (system x⁰) is a Na⁺-independent amino acid transport system that mediates the uptake of cystine. Levels of intracellular cysteine are the rate limiting factor in the production of the main intracellular free radical scavenger GSH. Most cysteine is generated from the cellular reduction of cystine, which is mainly supplied by system x⁰. We have shown previously that about 90% of the cystine uptake into dental pulp cells is through system x⁰. Also, the composite restoration materials Flow line and Durafill VS, both of which contain methacrylates, decrease GSH levels in dental pulp cells, and upregulation of system x⁰ attenuates that decrease¹³). Therefore, regulation of system x⁰ can alter cell death in conditions that involve GSH depletion.

Because of the common role of oxidative stress in the toxicity of dental materials, upregulation of system x⁰, with subsequent enhancement of GSH production, system x⁰ may have general protective effects against that toxicity. In the current study, we tested the effects of the commonly used methacrylate monomers Bis-GMA, UDMA, and TEGDMA on toxicity, regulation of system x⁰, induction of oxidative stress, and GSH levels.

MATERIALS AND METHODS

Materials

Serum was obtained from Atlanta Biologicals (Atlanta, GA, USA). 14-C cystine was from PerkinElmer (Waltham, MA, USA). 5-(and –6)-2'7'-dichlorodihydrofluorescein diacetate (DCF-DA) was from Fisher Scientific.
(Waltham, MA, USA). Bis-GMA, UDMA, TEGDMA and all other chemicals were obtained from Sigma (St. Louis, MO, USA). Bis-GMA, UDMA, and TEGDMA were diluted in dimethyl sulfoxide (DMSO) with a final DMSO concentration of 1%, control cultures were also exposed to 1% DMSO.

Subjects and human dental pulp cell cultures
Normal human impacted third molars were collected from adults at the Marquette University School of Dentistry Surgical Services Department. Tooth surfaces were cleaned and cut around the cementum-enamel junction using sterilized diamond stones to access the pulp chamber. The pulp tissue was separated from the tooth and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 h at 37°C. The cells were plated onto flasks and grown to confluence. The cells were then removed and plated on 24-well plates coated with poly-D-lysine and laminin in Eagle’s medium supplemented with 20% fetal calf serum, 100 μm L-ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and then incubated at 37°C with 5% CO2. Experiments were performed on cultures 7–9 days in vitro on cells that were passed from 1–5 times, at this time point the cells form a confluent layer.

Cell death assay
Cell death was assessed in mixed cultures by the measurement of lactate dehydrogenase (LDH) released from damaged or destroyed cells, in the extracellular fluid 48 h after the beginning of the insult. Control LDH levels were subtracted from insult LDH values and results normalized to 100% cell death caused by exposure to 20 µm of the calcium ionophore A23187. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed14,15. Advantages of the LDH release assay for the current studies is that it can be performed at multiple time points in the same experiment and is a measure of true cell death. The commonly used 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) metabolism assay can only be performed at one timepoint and is a measure of cell activity which does not always correlate to cell survival, particularly when using dividing cells16.

14C-cystine uptake
For 14C-cystine uptake experiments following methacrylate monomer exposure for 2 or 48 h, cultures were washed into HEPES buffered saline solution and immediately exposed to 14C-cystine (0.025 µCi/mL) for 20 min16. Following the exposure to 14C-cystine, the cultures were washed 3 times HEPES buffered saline solution and dissolved in 1% SDS (250 µL). An aliquot (200 µL) was removed and added to scintillation fluid for counting. Values were normalized to control 14C-cystine uptake (20-min exposure to 14C-cystine without growth factor or dental material treatment).

MCB Assay
Cellular GSH levels were measured by monochlorobimane (MCB) fluorescence. MCB forms a fluorescent compound when it reacts with GSH through a reaction catalyzed by glutathione-S-transferase13. MCB (10 µm) was added to the media following a 2 or 48 h treatment with methacrylate monomers. After 30 min the cells were washed three times with cold HEPES buffered saline and dissolved with 250 µL of 100% DMSO. A 200 µL sample was placed into a 96-well plate. Samples were read at 355/460 with a Fluoroskan Ascent Microplate Fluorometer (Thermo LabSystems). Background (no MCB added) was subtracted and values normalized to control (MCB but no methacrylate monomer treatment).

DCF Assay
Oxidative stress was measured with 5-(and -6)-2′7′-dichlorodihydrofluorescein diacetate (DCF-DA) using a fluorescent plate reader following a modification of a previous method17,18. The non-fluorescent, lipophilic compound DCF-DA (10 µm) was added to the cultures for 30 min where it is de-esterified and may be oxidized to fluorescent DCF (2′7′-dichlorofluorescein). Fluorescence was read using a Fluoroskan Ascent plate reader (Thermo LabSystems) with excitation and emission filters set to 485 and 538 nm, respectively. Background fluorescence (no DCF added) was subtracted and the results normalized to control conditions.

Statistical analysis
n Values for all experiments were n=8–16. Each value represents 1 well in a 24 well plate, with each experiment representing from 2–4 separate studies (different 24 well plates). Statistical calculations were performed using one-way ANOVA followed by the Bonferroni t-test. p Values<0.05 were considered to indicate significant differences.

RESULTS
Dental pulp cultures were exposed to varying concentration of each of the monomers for 48 h, after which time the amount of cell death was assayed by the LDH release assay. The concentrations of the compounds were chosen to provide a range of toxicity for the compounds that induced cell death. We found that both Bis-GMA and UDMA were highly toxic (Figs. 1A and B), with Bis-GMA causing toxicity beginning at a concentration of 30 µm and UDMA beginning at 100 µm. TEGDMA caused no toxicity at any of the concentrations tested (Fig. 1C).

We have shown previously that changes in cystine uptake can alter cellular glutathione levels and toxicity13. Therefore, we tested the effects of the monomers on cystine uptake (Table 1). We measured 14C-cystine uptake at both an early timepoint before any toxicity occurs (2 h) and at the 48 h timepoint. At 2 h, 100 and 300 µm concentrations of Bis-GMA and UDMA caused a decrease in cystine uptake, while those concentrations of
Toxicity of 48-h exposure of dental pulp cultures to Bis-GMA, UDMA, and TEGDMA was determined by measuring LDH release. Significant toxicity was induced by Bis-GMA (A) and UDMA (B), but not TEGDMA (C). Bars show % cell death quantified by release of the cytosolic enzyme LDH normalized to 100% cell death caused by exposure to 20 μm of the calcium ionophore A23187 (mean±SEM, n=8–16). * indicates significant difference from control (p<0.05).

Table 1 14C-cystine uptake (20 min) after 2 or 48 h exposure to monomers

| Assay          | Hours | Monomer | Control | Concentration (µm) |
|----------------|-------|---------|---------|--------------------|
|                |       |         |         | 10        | 30      | 100     | 300     |
| 14C-cystine uptake (14C-cystine uptake (% Control)) | 2     | Bis-GMA | 100±3   | 108±5    | 98±6   | 34±6*   | 27±2*   |
|                |       | UDMA    | 100±4   | 87±6    | 80±7   | 73±6*   | 37±6*   |
|                |       | TEGDMA  | 100±3   | 122±10  | 122±8   | 129±7*   | 167±7*   |
|                | 48    | Bis-GMA | 100±4   | 100±5   | 21±4*   | 9±1*   | 8±1*   |
|                |       | UDMA    | 100±11  | 180±14* | 200±16* | 26±5* | 9±1*   |
|                |       | TEGDMA  | 100±5   | 129±7   | 141±9*   | 202±11*   | 191±15*   |

* indicates significant difference from control (p<0.05).

Table 2 Oxidative stress measured by DCF fluorescence after 2 or 48 h exposure to monomers

| Assay         | Hours | Monomer | Control | Concentration (µm) |
|---------------|-------|---------|---------|--------------------|
|                |       |         |         | 10        | 30      | 100     | 300     |
| DCF Fluor. (% Control) | 2     | Bis-GMA | 100±6   | 107±17  | 149±11* | 170±14* | 160±8* |
|                |       | UDMA    | 100±6   | 99±9    | 131±16 | 137±16 | 163±5* |
|                |       | TEGDMA  | 100±13  | 92±21   | 101±19  | 102±17  | 119±17 |
|                | 48    | Bis-GMA | 100±7   | 173±29  | 172±23  | 170±23  | 234±26* |
|                |       | UDMA    | 100±12  | 136±23  | 157±27  | 255±44* | 236±18* |
|                |       | TEGDMA  | 100±10  | 121±19  | 116±6   | 120±18  | 154±17 |

* indicates significant difference from control (p<0.05).

TEGDMA caused an increase in uptake. At 48 h, as would be expected, the toxic concentrations of Bis-GMA and UDMA caused decreased cystine uptake. Interestingly, non-toxic concentrations of UDMA caused increased uptake. Also, at 48 h, TEGDMA at concentrations of 30, 100 and 300 µm caused increased uptake (Table 1).

Since cystine uptake is required for the production of GSH, we expected GSH levels to track the cystine uptake. However, this was not always observed. Cellular GSH levels were assayed using the fluorescent dye MCB. At the 2 h timepoint there was a significant decrease in cellular GSH with each of the monomers at all concentrations (Table 2). At the 48 h timepoint, the 10 µm concentrations of Bis-GMA and UDMA did not cause further decrease or recovery, but at all higher concentrations there was a dramatic further decrease in GSH. After 48 h of TEGDMA treatment, GSH levels showed significant recovery from the levels at 2 h at all of the concentrations tested, and at the 100 µm concentration there was actually an increase in GSH levels above control (Table 2).

With lowered GSH levels there would be expected
Table 3  Glutathione measured by MCB fluorescence after 2 or 48 h exposure to monomers

| Assay  | Hours | Monomer   | Control | Concentration (µm) |
|--------|-------|-----------|---------|-------------------|
|        |       |           |         | 10     | 30     | 100    | 300    |
|        | 2     | Bis-GMA   | 100±2   | 65±2*  | 86±4  | 58±7*  | 61±6*  |
|        |       | UDMA      | 100±1   | 64±2*  | 43±3*  | 42±3*  | 44±3*  |
|        |       | TEGDMA    | 100±5   | 58±4*  | 64±6*  | 51±3*  | 36±2*  |
| 48     | Bis-GMA| 100±12    | 108±8   | 4±2*   | 6±4*  | 2±1*   |
|        | UDMA  | 100±8     | 60±11   | 18±6*  | 7±4*  | 3±1*   |
|        | TEGDMA| 100±2     | 93±3    | 95±3   | 118±3*| 56±8*  |

*indicates significant difference from control (p<0.05).

Fig. 2  Bis-GMA, UDMA, and TEGDMA all decrease glutathione (GSH) in a cell free system.

GSH standards (100 µm) were exposed to Bis-GMA, UDMA, or TEGDMA (300 µm) for 6 h at which time GSH levels were measured by MCB fluorescence. Bars show % MCB fluorescence normalized to control fluorescence (GSH standard with no dental monomers added) (mean±SEM, n=8). * indicates significant difference from control (p<0.05).

discussion

The results of the current study are consistent with previous studies in finding that the toxicity of dental monomers follows the pattern of Bis-GMA>UDMA>TEGDMA19). The additional information provided in the current study is the complex effects the compounds have on antioxidant systems. The simple explanation that depletion of cellular GSH causes the toxicity is clearly not the full story. Each of the compounds depleted cellular GSH, but Bis-GMA and UDMA were toxic, while TEGDMA was not. Also, the mechanism by which they deplete GSH is the same for each compound in that they each had a direct effect on GSH in a cell free system, a result that has been observed previously20).

Also, while Bis-GMA and UDMA caused increased oxidative stress, as measured by DCF fluorescence, TEGDMA did not, and yet TEGDMA caused decreased cellular GSH levels. What can account for this diverse set of results? The most striking difference between the toxic and non-toxic monomers is their effects on cystine uptake. Bis-GMA and UDMA caused dramatic decreases in cystine uptake, while TEGDMA causes an increase in cystine uptake. System xc$^{-}$ is responsible for transport of cystine into dental pulp cells where it is converted into cysteine which is required for GSH production13). The most likely explanation for the results is that the reason TEGDMA does not cause toxicity is that it upregulates cystine uptake leading to enhanced GSH production.

A complexity of the interpretation of these studies is that since Bis-GMA and UDMA are toxic, biochemical changes measured after toxicity has occurred may simply reflect cell death and may not be causal to the death. For that reason, we also performed the biochemical measurements after 2-h exposure, a time point before any cell death occurred. An interesting result from this strategy was that while TEGDMA did cause a decrease in GSH levels at 2 h of exposure that was similar to the decrease caused by Bis-GMA and UDMA, after 48 h, the GSH levels following TEGDMA treatment recovered, while those following Bis-GMA
and UDMA treatment decreased further. This finding is consistent with TEGDMA stimulating cystine uptake leading to increased GSH production. The increased GSH production appears to be adequate to attenuate the direct effects of TEGDMA on GSH and to prevent the increased oxidative stress observed with both Bis-GMA and UDMA.

It is well established that compounds such as Bis-GMA, UDMA, and TEGDMA leach out of resin containing dental materials\(^6\). However, the concentrations of those compounds reached in the restricted space on the interior of teeth were the dental pulp cells are found is unknown, but considering the limited volume of that space, it seems possible that levels of released monomers may become quite high.

We have previously shown that upregulating cystine uptake in dental pulp cells with the growth factors IGF-1 or TGF-β prevents oxidative stress mediated cell death induced by exposure to composite restoration materials or depletion of cellular GSH by buthionine sulfoximine\(^13\). That result raised the possibility of using growth factors to increase cystine uptake as a means of preventing dental pulp cell death when composite restoration materials are used. The results of the current study suggest that composite materials that contain TEGDMA may provide the same type of benefit that the addition of growth factors could provide.

### CONCLUSION

The current study addresses the issue of determining the mechanism by which methacrylate monomers that may be leached from composite materials cause death of pulp, and specifically asking whether effects on cystine uptake play a role in the toxicity. The fact that there is a strong relationship between the up or down regulation of cystine uptake and the toxicity of the materials suggest that levels of cystine uptake are an important factor in determining the death of dental pulp cells.

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