Dental Materials (2006) **22**, 338–345

dental materials

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In vitro cytotoxicity of a remineralizing resin-based calcium phosphate cement

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Received 6 January 2005; accepted 12 May 2005

KEYWORDS
Cytotoxicity;
Dental material;
Dimethacrylate resin;
$Ca\text{–}PO_4$ cement;
Dental cell sensitivity

Summary Recently, a resin-based calcium phosphate cement (RCPC) has been reported as a remineralizing pulp-capping or lining cement. RCPC consists mainly of tetracalcium and dicalcium phosphates, ethoxylated bisphenol A dimethacrylate and pyromellitic glycerol dimethacrylate monomers and photo- and chemical initiators.
Objectives. Here, the cytotoxic effects of RCPC were evaluated. The hypothesis was that RCPC induced only minor cytotoxic response in immortalized murine odontoblast and pulp cells, comparable to that produced by similar dimethacrylates due to unpolymerized dimethacrylate monomer present after curing.
Methods. Cytotoxicity was determined following the changes in cell succinate dehydrogenase activity after 24 h exposure to the cement components and after a 24 h recovery period. A fourfold range of concentrations was tested of the monomers, the eluate of cured RCPC leached in Dulbecco's modified Eagle's medium, and crushed cured cement in dimethyl sulfoxide.
Results. The monomers themselves had cytotoxicities similar to those reported for other dimethacrylates, although they are significantly less toxic than Bis-GMA. Differential cell sensitivity was demonstrated, with the pulp cells having greater sensitivity to the unpolymerized monomer than the odontoblast-like cells. The leached components have cytotoxicity similar to that of the free monomers. The crushed material demonstrated no apparent cytotoxicity at the dilutions tested.
Significance. These data demonstrate that RCPC has an in vitro cytotoxicity that is comparable to other materials containing dimethacrylate monomers and suggest that the material may be suitable for use in dental restorations. The data also

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doi:10.1016/j.dental.2005.05.004

indicate that the pulp cells appear more sensitive to dimethacrylates than the odontoblasts.

Introduction

Hydroxyapatite based materials are commonly used in dentistry and orthopedics due to their high biocompatibility and osteoconductivity [1]. The hydroxyapatite ceramics that are currently employed, however, often lack plasticity and thus cannot be molded during dental and surgical applications [2]. Recently, novel resin based calcium phosphate cements (RCPC) consisting of tetracalcium and dicalcium phosphates (TTCP, DCPA) combined with dimethacrylate monomers have been reported as potential pulp capping or dental lining cements [3]. Combining calcium phosphates with monomers, polymerization initiators and water in the form of two pastes that are mixed at use, produces a dough-like material. After curing, the set material is comprised of calcium phosphate microcrystals surrounded by a polymer matrix. The presence of the polymer inhibits the complete conversion of the calcium phosphate components to hydroxyapatite observed with water-based TTCP/DCPA cements [4]. This results in a material capable of providing more soluble calcium phosphates for the diffusion of Ca^{2+} and PO_4^{3-} ions for the remineralization of mineral-deficient tissue. The ion release and in vitro remineralization of artificial dentin lesions has been demonstrated in previous experiments [3].

Residual monomer resulting from incomplete polymerization during curing can leach out soon after application and has the potential to cause irritation, inflammation and an allergic response [5]. Dimethacrylates, in particular, have been shown to have inherent biotoxicity when used clinically [6,7] and as such, require risk assessment to determine the potential for inducing toxic responses [8].

The risk that novel materials can pose to pulpal tissue in vivo can be partly estimated by assessing the cytotoxicity of these materials in vitro and current thinking suggests that in vitro assessment of cytotoxicity should be the first step in the determination of the bio-utility of novel compounds [9]. Most current cytotoxicity tests determine the level of cell survival following exposure to a compound versus the same amount of unexposed control cells [10]. A variety of tests are available for analysis of cellular response to biomaterials including mitochondrial function tests [MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; WST-1: tetrazolium compounds (Boehringer Mannheim, Indianapolis, IN)] neutral red uptake, lactate dehydrogenase release and labeled nucleotide incorporation [10]. Generally, comparable results are produced by the various tests and all have been demonstrated to have validity and reliability. The cytotoxicity of RCPC has been assessed by analyzing the response of immortalized dental cell lines using the WST-1 mitochondrial activity assay for determination of cell survival. It was hypothesized that the resin-based cement will induce a minor cytotoxic response due to residual unpolymerized dimethacrylate monomer present after curing, which is comparable to that produced by dimethacrylates of similar chemical structure.

Materials and methods[1]

Sample preparation. The preparation of resin samples was done as described previously [3,11]. Briefly, the resin mixture used to formulate the cements consisted of the two monomers, ethoxylated bisphenol A dimethacrylate (EBPADMA, Sartomer Co., West Chester, PA) and the acidic monomer pyromellitic glycerol dimethacrylate (PMGDM). The resin mixture was activated with camphorquinone and the aromatic tertiary amine, *N*,*N*,-dimethylaminophenethanol. Benzoyl peroxide was added to ensure a thorough through-cure of the rather opaque cement. The solid phase consisted of $TTCP_{2.05}$ and DCPA. To maintain a higher pH in the final cement mixture, a calcium-enriched TTCP, termed $TTCP_{2.05}$, was prepared. The subscript 2.05 denotes that the TTCP preparation had a Ca to P ratio of 2.05, as it contained a small amount of calcium oxide. $TTCP_{2.05}$ was ground to a median particle diameter of 16 μm and DCPA to 1.1 μm. Two pastes were prepared, which were then mixed

[1] Certain commercial materials and equipment identified in this paper are for adequate definition of the experimental procedure. In no instance does such identification imply recommendation or endorsement by the National Institute of Standards and Technology or the ADA Foundation or that the material or equipment identified is necessarily the best available for the purpose.

to produce the final cement. In Paste 1, the ingredients PMGDM, water, DCPA, sodium hexafluorosilicate, camphorquinone and benzoyl peroxide were hand mixed. For Paste 2, EBPADMA, $TTCP_{2.05}$ and *N*,*N*-dimethylaminophenethanol were added to methylene chloride, tumbled for 30 min and the solvent was removed by vacuum suction. Immediately before sample preparation, the pastes were mixed together for about 30 s at a ratio of one part of Paste 1 to two parts of Paste 2. The final mixed cement then contained mass fractions of 8% PMGDM, 2.9% water, 19.3% DCPA, 1.6% sodium hexafluorosilicate, 0.1% camphorquinone, 0.4% benzoyl peroxide, 8% EBPADMA, 59.5% $TTCP_{2.05}$ and 0.1% *N*,*N*-dimethylaminophenethanol.[2]

To evaluate the cytotoxicity of the monomers, PMGDM and EBPADMA were dissolved in sterile dimethyl sulfoxide (DMSO) to a concentration of 0.5 mol/L. Stock solutions were created by a 1:1000 dilution of the sample in medium [Alpha Minimum Essential Medium, αMEM; 1% fetal bovine serum, FBS, 1% PSF (penicillin, streptomycin, fungizone)] producing a volume fraction of 0.1% DMSO solution in the highest concentration of monomer tested (5×10^{-4} mol/L). At this concentration DMSO has been demonstrated to produce no significant cytotoxicity.

To test the leached material, three cement specimens, 15 mm in diameter and 1 mm thick were made, light cured for 1 min on each side, and incubated in 5 mL Dulbecco's modified Eagle's medium (DMEM) for 24 h at 37 °C. The disks were removed and the DMEM solution was analyzed.

For the crushed material, three cement specimens, 6 mm in diameter and 3 mm thick, were prepared, light cured for 1 min on each side, and crushed with mortar and pestle. The crushed sample, about 0.08 g, was put into 1 mL DMSO.

Cytotoxicity assays

Cell response was assessed in vitro using the tetrazolium compound WST-1 (Boehringer Mannheim, Indianapolis, IN), for mitochondrial succinate dehydrogenase [9,12,13] activity as outlined by the ISO 10993 specification and similar studies. Immortalized MO6-G3 odontoblast – like cells [14] and MD10-H1 cells (pulp-like cell) were cultured using αMEM augmented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 μg/mL) and ascorbic acid until used. For the assay, cells were harvested by trypsinization (Trypsin/ethylenediaminetetraacetic acid) and plated, 10,000 cells/well in a 96 well format at 100 μL volume, and allowed to attach overnight. The next day, the used medium was replaced with fresh αMEM containing 1% FBS to induce quiescence in the cells. After 24 h, the medium was removed and replaced with an aliquot of 1% FBS medium containing the appropriate concentration or dilution of the test sample ranging from 5×10^{-4} to 5×10^{-7} mol/L for the monomer. The dilutions of the leached sample ranged from undiluted to 1:1000. The dilution of the DMSO containing the crushed sample was 0.1 mass fraction%, which was further diluted to 1:10, 1:100 and 1:1000. These solutions were allowed to remain in contact with the cells for 24 h. The treatment media were then removed and the cells were either immediately tested for mitochondrial activity (see below) or the treatment medium was removed and replaced by 10% FBS medium and the cells returned to the incubator for a 24 h recovery period, after which they were subjected to mitochondrial activity assessment.

For the mitochondrial activity assay, the medium was removed by aspiration. The cells were washed three times with Hank's balanced salt solution and 100 μL of a 1:10 dilution of WST-1 reagent was added according to the manufacturer's instructions. The cells were incubated for 2 h at 37 °C and the absorbance read at 450 nm for each well. At each concentration eight wells were run for PMGDM, EBPADMA, and for each leached or crushed specimen. The recovery experiments were performed measuring all eight wells of the monomer concentrations and Specimen 1 of the leached or crushed cement experiments. A mean and SD as a measure of uncertainty was calculated for each sample regime and the means compared using a multiple comparison analysis with the Statgraphics program (Manugistics, Inc., Rockville, MD). Statistical significance was given to values with a $P<0.05$. Means from treatment regimes were normalized against the mean from the negative control to produce a percent survival versus control (PSVC) number as a measure of the relative cytotoxicity of the compound. The cytotoxic dose at which mitochondrial activity was reduced by 50% (CD_{50}) was determined in the following manner. The means and SD for the mitochondrial activity of each treatment concentration were plotted on a semilog graph using the Statgraphics 5 Plus software (Manugistic, Inc., Rockville, MD) and a curve was best-fit to the data. The CD_{50} value was determined by evaluating the curve at the 50% inhibition level. The 2 h absorbance readings from each treatment level were converted to a mean $\pm$ SD and expressed as percentage control (where control is the

[2] All percentages are given as mass fraction percent unless said differently.

absorbance of untreated wells). Multiple sample comparison analysis (ANOVA), and Fisher's LSD test for post-hoc analysis (Statgraphics) was used to determine if the effects of the test substances are significant compared to the untreated wells at the $P<0.05$ level.

The negative control comprised eight wells in which the cells were plated and treated as above except that the treatment media did not contain a test compound. The positive control involved treatment of the cells with the compound 2,2′-bis-[4-(methacryloxypropoxy)-phenyl]-propane (Bis-GMA) at 10^{-4} mol/L. Bis-GMA is a dimethacrylate monomer found in composite resins currently in use (e.g. Z100, 3M company) and has been shown to have a cytotoxic dose of 3×10^{-5} mol/L at which the cell activity decreases by 50% [15]. Treatment of the cells with Bis-GMA at a concentration of 10^{-4} mol/L is well above the cytotoxic dose for this compound and should produce a 0% PSVC value in assays that are working correctly. As most dimethacrylates have a CD_{50} of between 10^{-3} and 10^{-5} mol/L, the Bis-GMA concentration is seen as a compromise to allow for a comparison of the relative cytotoxicity of a resin monomer.

Results

The effect of the monomers PMGDM and EBPADMA on the mitochondrial activity of odontoblast-like cells (MO6-G3) and pulp-like cells (MD10-H1) is shown in Fig. 1. For both odontoblasts and pulp cells, the monomers PMGDM and EBPADMA were significantly more cytotoxic at the highest test dosage (5×10^{-4} mol/L) than the negative control. At lower concentrations, only EBPADMA showed cytotoxicity that was significantly different from the control. The pulp cells appeared more sensitive to the EBPADMA monomer because dosage levels, which had no effect on the odontoblasts, had a significant effect on the pulp cells. Complete recovery of cells from the cytotoxic insult of the two monomers was not observed for either cell type at the highest concentration, with cell levels remaining significantly below that of the control wells. This indicated that the highest concentration of the monomers produced cell death and not merely an inhibition of mitochondrial activity. At the highest concentration PMGDM seemed to produce a long-term effect on odontoblast-like cells, as the level of survival at the 24 h recovery mark was significantly below that of the 24 h treatment level.

At the highest concentration, the eluate showed a significant decrease in MD10-H1 cell activity when

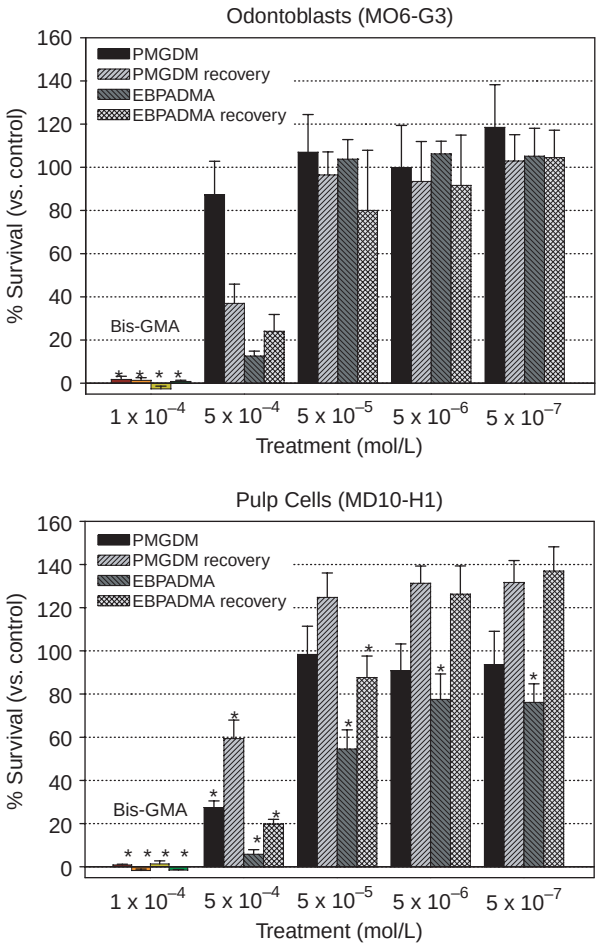

Figure 1 Survival of MO6-G3 cells and MD10-H1 cells following 24 h exposure to Bis-GMA, PMGDM and EBPADMA monomer and after 24 h recovery. For Bis-GMA, the two left bars show cell survival after Bis-GMA exposure and recovery as controls for the PMGDM experiments, and the two right bars show cell survival after Bis-GMA exposure and recovery for the EBPADMA experiments. For all treatment levels, $n=8$; *The asterisk designates significance at $P<0.05$. The error bars represent one SD as a measure of the standard uncertainty.

compared to untreated cells (Fig. 2). This decrease is attributable to cell death as no recovery occurred following removal of the cytotoxic entity. Cured resin-cement samples crushed in 100% DMSO did not produce a significant level of cytotoxicity (Fig. 3). The calculated CD_{50} values for MO6-G3 and MD10-H1 cells are presented in Table 1.

Consistency of plating was evaluated by determining the coefficient of variation (c.v.) for the measured optical densities (o.d.) of the samples using the control wells. The average c.v. was 7% with a SD of 2.5%. All SD reported in this paper express the estimated standard uncertainty. The data for each individual treatment is given in Table 2.

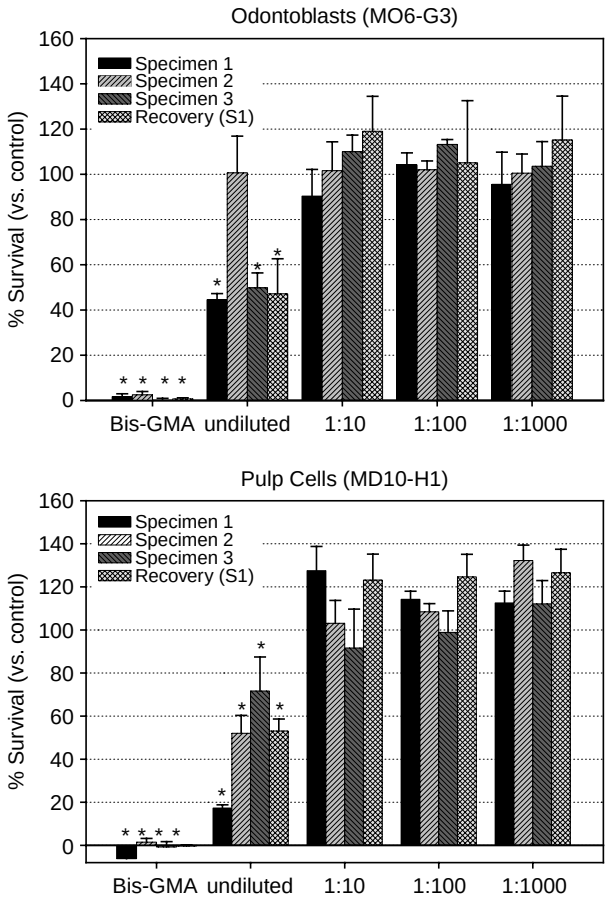

Figure 2 Survival of MO6-G3 cells and MD10-H1 cells after exposure to serial dilutions of eluates produced by leaching polymerized cement into media. For all treatments a total of eight wells were run ($n=8$). For Specimen 1, the 24 h recovery of cells is also shown. For each specimen, Bis-GMA was run as a control. *The asterisk designates significance at $P<0.05$. The error bars represent one SD as a measure of the standard uncertainty.

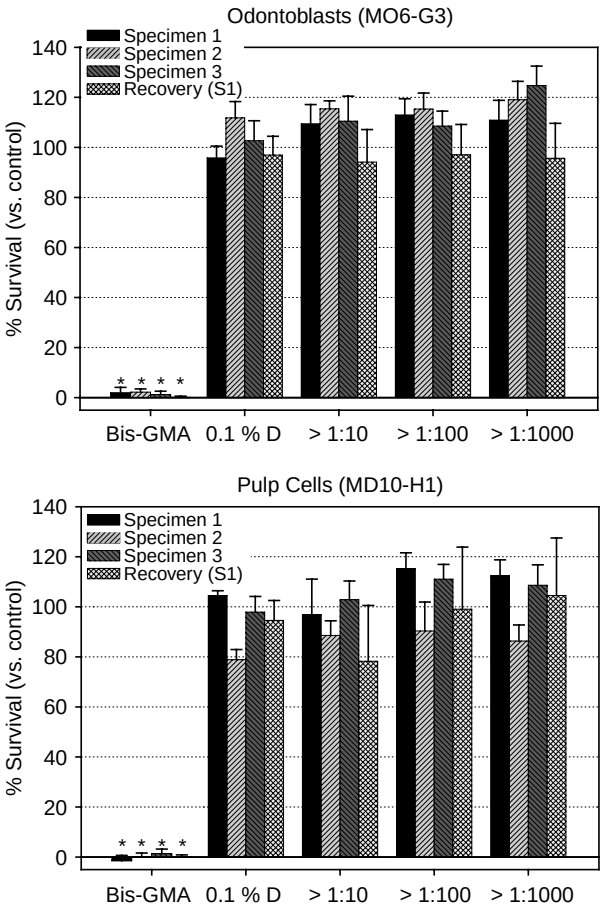

Figure 3 Survival of MO6-G3 and MD10-H1 dental cells after exposure to crushed cement samples and after 24 h recovery. The crushed cement was dissolved in 100% dimethyl sulfoxide. The final concentration of dimethyl sulfoxide (D) in test media was 0.1 mass fraction%, which has been demonstrated to be non-cytotoxic under the conditions of this assay. For all treatments a total of eight wells were run ($n=8$). For Specimen 1, the 24 h recovery of cells is also shown. For each specimen, Bis-GMA was run as a control. *The asterisk designates significance at $P<0.05$. The error bars represent one SD as a measure of the standard uncertainty.

Discussion

In vitro cytotoxicity assays can be used as a first level risk assessment when working with new biomaterials [7]. A variety of assays exist [10] and in general these assays produce approximately equivalent results when used to determine the apparent cytotoxicity of novel biomaterials [16]. Using these assays, the relative cytotoxicity of acrylates and methacrylate monomers has been established [17]. Generally, acrylate-based resins have a greater biotoxicity than analogous methacrylate resins [15,18]. Further, acute toxicity is correlated with increasing molecular mass of alkyl chain acrylates and methacrylates and water solubility of the monomers. The lower the water solubility the more toxic the monomer appeared leading to the hypothesis that these compounds induce a cytotoxic response through a disruption of normal membrane integrity or functionality [15,19]. In contrast, hydroxylated acrylates and methacrylates are more toxic than their non-hydroxylated counterparts [15]. The cytotoxicity of acrylate and methacrylate resin based materials

Table 1 CD_{50} values in mol/L.

	MO6-G3	MD10-H1
PMGDM	$\gg 5\times10^{-4}$	1.9×10^{-4}
EBPADMA	1.9×10^{-4}	3.5×10^{-5}

Table 2 Plating consistency based on control optical density (o.d.) readings.

$n \gg$	8	3×8	3×8
MO6-G3	Monomer	Leach	Crushed
Mean (o.d.)	1.19	1.1	1.31
SD	0.07	0.05	0.08
c.v. (%)	5	4	6
MD10-H1			
Mean (o.d.)	0.69	0.67	1.03
SD	0.04	0.08	0.13
c.v. (%)	5	12	11

in dental applications has been verified, although the mechanism is undetermined [7,20].

Dimethacrylate resins have been shown to have a cytotoxic impact in vitro [6,8,15,20]. This has been shown to be related to the relative molecular mass of the compound as well as the hydrophilicity (more hydrophobic monomers having higher toxicity) [21]. The data for dimethacrylate resin cytotoxicity presented here is in agreement with previous studies [15,18]. The PMGDM and EBPADMA resins are only cytotoxic at the highest resin concentration tested (5×10^{-4} mol/L). These monomers have a cytotoxicity comparable with that reported for other dimethacrylates, but significantly less than that of Bis-GMA, which has been reported of having the highest measured cytotoxicity ($IC_{50}=3\times10^{-5}$ mol/L) of a number of acrylates and methacrylates [15].

In the clinic, complete polymerization of the polymer is unlikely given the nature of multimethacrylate polymerization kinetics, the oral temperature and the variability in the technique of the clinicians. It is assumed that unpolymerized monomer will leach out of the material into the surrounding cell and fluid layers. In vitro data suggest that the majority of monomer leaching is accomplished within 24 h [22] and subsequent monomer release is reduced and primarily due to polymer hydrolysis. Data from the leached samples (Fig. 2) suggest that polymerization of the resin component of the cement is incomplete and that the released compounds are capable of producing a cytotoxic response. Significant cytotoxicity after 24 h exposure and after a 24 h recovery period was observed with both cell types at the highest concentration. As expected, dilution of the eluate ameliorated the cytotoxic response.

Previous (unpublished) leaching experiments of the cured cement and analyses of the leached components by nuclear magnetic resonance spectroscopy showed that approximately 1.8% of EBPADMA originally used for the mixture leached out into water (SH Dickens, unpublished data). No PMGDM was detected. Based on this, the concentration of the undiluted leached monomer (Fig. 2) was approximately 4×10^{-4} mol/L. However, this concentration is much higher than that experienced by pulpal tissue. Assuming that approximately 20 mg of RCPC is deposited in a pulp capping procedure and given the amount of EBPADMA that leached into water in the aqueous extraction, there will be approximately 50 μg (0.09 μmol) of EBPADMA that could leach into the 15 mL of blood flowing through the pulp in 24 h. The pulpal blood flow was estimated from Kim et al. and Matthews and Andrew [23,24], for 20 mg pulp tissue to be about 15 mL in 24 h. This would amount to a concentration of about 6×10^{-6} mol/L EBPADMA. At this concentration EBPADMA did not have a significant cytotoxic effect on MO6-G3 cells, while MD10-H1 cells were affected, but showed full recovery.

None of the crushed samples demonstrated cytotoxic potential in these assays. One would not expect, a priori, a lower level of residual monomer in the crushed samples. Crushing the material should have increased the contact surface area and decreased diffusion distance between the cement and solvent thereby increasing the efficiency of monomer extraction. This would have resulted in significant amounts of resin monomer in the solvent and a cytotoxic response similar to that of the leached samples. This did not occur. The lack of a cytotoxic response could be the result of diminished cytotoxicity due to some interaction of monomer with the filler components. However, it is also possible that the dilution required to eliminate the solvent's (DMSO) intrinsic effect [25] placed the level of leached monomer below the cytotoxic threshold.

Several workers have proposed that toxicity tests in vitro will be more convincing when performed on cells that are homologous with the tissue with which they will be in contact in vivo [26–28]. MO6-G3 cells are odontoblast like cells while MD10-H1 cells are pulp like cells, each expressing characteristic markers of the cells from which they derive [14]. Previous work in our laboratory has suggested that these cells demonstrate a differential response to cytotoxic insult and may represent a more relevant cytotoxic assay for use with dental materials [25]. In most experiments in which a cytotoxic response was elicited, the pulp-like cells (MD1010-H1) demonstrated a higher sensitivity than did those of the odontoblast-like cells (MO6-G3). This suggests that the pulpal cells are more sensitive to cytotoxic agents than the more highly differentiated odontoblasts. The in vivo relevance of these findings is debatable. Given the anatomy of

the pulp cavity, cytotoxic agents applied to the outside of the dentin would interact at a higher concentration with the odontoblast before interaction with the underlying pulp cell. This may be a factor in the higher tolerance of the odontoblast to cytotoxic materials as these cells may have mechanisms which allow for survival as they are normally insulted first and at higher intensity.

In conclusion, these data clearly demonstrate that the recently developed RCPC has an inherent cytotoxicity most likely due to the dimethacrylate component of the cement. This cytotoxicity is comparable to levels measured for this family of compounds, but falls well below that of the commonly used dimethacrylate compound Bis-GMA. In combination with an in vivo (animal) pulp capping study [29], where long-term exposure to RCPC consistently induced secondary dentin with low inflammatory response, the here presented data suggest that the new resin-based calcium phosphate cement may be suitable for clinical use.

Acknowledgements

The support by the American Dental Association Foundation, the National Institute of Standards and Technology, and the National Institute of Dental and Craniofacial Research, Grant No. DE13298 is gratefully acknowledged. We also wish to express our thanks to the Esstech Co. for supplying the EBPADMA monomer used in this study, to Ms Helen Hoffer for cell maintenance, and to Mr Glenn Flaim and Ms Cynthia Floyd for sample preparations.

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