Cytotoxicity of Dental Resin Monomers in the Presence of S9 mix Enzymes

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The purpose of this study was to evaluate the cytotoxicity of dental resin monomers in the presence of a rat liver S9 mix containing cytochrome P 450 enzymes. JTC-12 cells derived from a monkey kidney were seeded on a 96-well multi-well-plate at 9×10^3 cells per well. After cultivation, the S9 mix was added to the wells as an S9 mix group (+S9), and PBS⁻ was added to the other wells as a none-S9 mix group (–S9), then 7 different concentrations of various monomers were added to each well. All the specimens were cultured for another 24 hrs. The cell survival ratios (CSR) were calculated by using a neutral red cytotoxicity assay.

CSR for 50 μ g/mL of Bis-GMA/S9 mix was 92.6% while for none-S9 mix it was 6.6%. The values of CSR for UDMA, Bis-MPEPP, EGDMA, TEGDMA, DMAEM, 4-META and HEMA exhibited a reduction in cytotoxicity in the presence of the S9 mix. There were significant differences between +S9 and –S9 for respective monomers ($p < 0.05$). However, there were no significant differences between +S9 and –S9 for MMA ($p < 0.05$).

Key words : S9 mix enzyme, Dental resin monomer, Biotransformation

INTRODUCTION

In dental materials cytotoxicity research, many *in vitro* tests have been employed to examine the *in vitro* toxicity of materials before metabolization¹⁾. In that respect, we feel that it is equally necessary to evaluate the cytotoxicity of resin based biomaterials after metabolism, especially as some resin based biomaterials are actually metabolized, and absorbed into the body, and excreted from the body^{2–4)}.

Cytochrome P 450 species (P 450) in S9 mix are oxidative enzymes which are present in the liver, kidney, lung and skin. The overall purpose of metabolism of xenobiotics is to increase their water solubility, and thus facilitate their excretion from the body. In some cases, however, the reactions to which xenobiotics are subjected actually increase their biological activity and consequently their toxicity.

It has been discussed by some research laboratories that certain dental restorative resin based materials may be metabolized in the body, because the dental resin monomers are absorbed through the dentine and the dental pulp tissue^{5,6)}. Borenfreund *et al.*⁷⁾ reported that the neutral red assay was suitable for the rapid screening

of a broad spectrum of substances, including pharmaceuticals, carcinogens, and anti-neoplastic agents.

The purposes of this study were 1) to evaluate the cytotoxicity of various dental resin monomers in the presence of a rat liver S9 mix containing cytochrome P 450 enzymes and 2) to establish a systematic cytotoxicity screening test for dental resin monomers in the presence of the S9 mix using cultured cells.

MATERIALS AND METHODS

Dental resin monomers

The dental resin monomers used in the present study are shown in Table 1.

All of the monomers except for HEMA were dissolved with dimethylsulfoxide (DMSO, Dojin, Kumamoto, Japan) and then diluted with the cell culture medium. HEMA was directly dissolved and diluted with the medium, because it is a water soluble monomer.

Cyclophosphamide (Lot #08928TZ, Aldrich Chemicals, Milwaukee, USA) and Benzo(α)pyrene (Lot #DSL6942, Wako Pure Chemical, Tokyo, Japan) were used as positive controls. The negative control was the cell culture medium including 0.5

Table 1 Monomers used

Monomer	Molecular weight	Lot No.
Bis-GMA ¹	512.65	0314H
UDMA ²	498.64	150412
Bis-MPEPP ³	478	0867B
EGDMA ⁴	198	0124K
TEGDMA ⁵	286.36	0302U
HEMA ⁶	130.14	PTE5111
DMAEM ⁷	157.21	SAL4470
4-META ⁸	304	T1002
MMA ⁹	100.12	ECJ6564

¹ 2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)phenyl]propane, Shin-Nakamura Chemical, Wakayama, Japan

² di-2-methacryloyloxyethyl trimethylhexamethylenedicarbamate, Negami Industrial, Ishikawa, Japan

³ 2,2-bis-4(methacryloyloxypropoxy)phenyl propane, Shin-Nakamura Chemical, Wakayama, Japan

⁴ ethylenglycol dimethacrylate, Shin-Nakamura Chemical, Wakayama, Japan

⁵ triethylenglycol dimethacrylate, Shin-Nakamura Chemical, Wakayama, Japan

⁶ hydroxyethylmethacrylate, Wako Pure Chemical, Tokyo, Japan

⁷ 2-(dimethylamino)ethyl methacrylate, Wako Pure Chemical, Tokyo, Japan

⁸ 4-methacryloyloxyethoxycarbonylphthalic anhydride, Sun Medical, Moriyama, Japan

⁹ methyl methacrylate, Wako Pure Chemical, Tokyo, Japan

vol% DMSO.

Cell and cell culture medium

The cells used for the cytotoxicity test were JTC-12⁸⁾, an established cell line of epithelial cells derived from the cynomolgous monkey kidney. The cell culture medium used for this experiment was DM160AU medium (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) supplemented with 10% fetal bovine serum (ICN Flow, Costa Mesa, CA), 2 mM L-glutamine (Nissui Pharmaceutical, Tokyo, Japan), 10 mM HEPES (N-2-hydroxyethylpiperadine-N'-2-ethane sulfonic acid, Research Organics Inc., Cleveland, USA), 20 mM sodium bicarbonate (Wako Pure Chemical, Tokyo, Japan), and 60 mg/L kanamycin (Meiji Seika Kaisha, Tokyo, Japan).

In addition, HeLa-S3 cells derived from human ovary cancer tissue were used for the cytotoxicity of S9 mix itself to compare with JTC-12 cells. The medium for HeLa cells was Eagles MEM medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% calf bovine serum (ICN Flow, Costa Mesa, CA).

Preparation of S9 mix

An S9 (Lot #92121113, Oriental Yeast Co., Tokyo, Japan) was prepared from rat liver induced with phenobarbital and 5,6-benzoflavone (Table 2). The cofactor (Lot #999202, Oriental Yeast Co., Tokyo, Japan, Table 2) of one vial (268.6mg) was dissolved in 9 mL distilled water, and the solution of the cofactor was filtered through a membrane filter (Millipore Co., Bedford, USA) with a pore size of 0.45 μ m. The 0.5 mL rat liver S9 was added to 4.5 mL of the cofactor solution. This solution was then used as the S9 mix. The S9 mix was added at 5 vol% level in the medium.

Table 2 Component of phenobarbital & 5,6-benzoflavone-induced S9 and cofactor

1) S9

Rat Liver S9 (Phenobarbital & 5,6-Benzoflavone-Induced)	
Protein content	22.78 mg/mL S9 12.69 mg/mL Microsome
Cytochrome P-450 content	2.15 nmol/mg protein

2) Cofactor (in one vial)

Component	(mg)
MgCl ₂ ·6H ₂ O	16.3
KCl	24.6
G-6-P	17.0
NADPH	36.2
NADH	30.2
Na ₂ HPO ₄	119.6
NaH ₂ PO ₄	24.7

Neutral red bioassay and method of the evaluation

The confluent JTC-12 cells were dispersed with 0.1% trypsin (Flow Laboratories, Irvine, Scotland) containing 0.04% ethylenediaminetetraacetate (EDTA, Flow Laboratories, Irvine, Scotland) and suspended 10^5 cells/mL of culture medium. The cells were seeded into a 96-well multiwell-plate (Falcon, Becton Dickinson, Franklin Lakes, USA) at 9×10^3 cells/ $90 \mu\text{L}$ per well, and then cultured in a humidified 5% CO_2 –95% air atmosphere at 37°C for 24 hours. After cultivation, a $10 \mu\text{L}$ of the S9 mix was added to half of the wells as the S9 mix group (+S9), and $10 \mu\text{L}$ of Ca^{++} and Mg^{++} free phosphate buffered saline (PBS^- , Nissui Pharmaceutical, Tokyo, Japan) was added to the other half as a non-S9 mix group (–S9), then a $100 \mu\text{L}$ of 7 different concentrations of various monomers were added to each well. All the specimens were cultured for another 24 hours. Specimens cultured without monomer or the S9 mix were used as the control.

After 24 hours' cultivation, the medium of each well was changed to neutral red (NR, Merck, Darmstadt, Germany) solution. The neutral red solution was prepared by diluting a stock solution of 5 mg/mL NR with the medium at a volume ratio of 1 : 100. The neutral red solution was removed 3 hours later. In addition, cells were fixed with 40% formaldehyde solution containing 10% CaCl_2 . Neutral red was extracted from cells with 1% citric acid–50% ethyl alcohol solution at room temperature for 20 minutes, and the solution was stirred by a Plate mixer (PMX-01, Fuji-Rebio Inc., Tokyo, Japan) for 2 minutes. The absorbance of neutral red was measured at a wave length of 540 nm with a Micro Plate Reader (model-450, Bio-Rad, Richmond, CA). The cell survival ratios (CSR, % of control) were calculated by $100 \times E_t/C_t$ (E_t : the absorbance of experimental group, C_t : the absorbance of negative control). In addition, the IC_{50} values (the drug concentration required to inhibit viability by 50%) were determined by dose-response curves. The data were statistically analyzed by ANOVA. The cytotoxicity of dental resin monomers metabolized by S9 mix enzymes was then evaluated from the data.

Four wells were used for one concentration. This experiment was repeated two times.

HeLa cells were also examined, using the same method, to evaluate the cytotoxicity of S9 mix itself.

RESULTS

Cytotoxicity of S9 mix

The cytotoxicity of the S9 mix is shown in Fig. 1. The cytotoxicity appeared over 10 vol% concentration after 24 hours' cultivation in JTC-12 cells. It was found that the S9 mix had a minimal toxic effect on JTC-12 cells at concentrations of less than 5 vol% after cultivation for 24 hours.

The cell survival ratios (CSR) of the 5% S9 mix in JTC-12 cells and HeLa cells after 24 hours were $97.5 \pm 5.3\%$ and $74.0 \pm 6.4\%$, respectively.

Fig. 1 Cytotoxicity of S9 mix after 24 hour cultivation in JTC-12 cells. The different concentrations of S9 mix are indicated on the logarithmic concentration axis. Each point is the mean of 8 cultures.

Fig. 2 Cytotoxicity of cyclophosphamide (CP) with or without S9 mix after 24 hour cultivation in JTC-12 cells. The different concentrations of cyclophosphamide are indicated on the logarithmic concentration axis. Each point is the mean of 8 cultures. ●: CP alone, ○: CP+S9 mix

Cytotoxicity of cyclophosphamide and benzo(α)pyrene

Cyclophosphamide and benzo(α)pyrene without the S9 mix had almost no cytotoxicity in JTC-12 cells. However, cytotoxicity of cyclophosphamide with the 5 vol% S9 mix appeared at a concentrations greater than 6.2 μg/mL (Fig. 2). Cyclophosphamide, the anti-neoplastic agent, was highly toxic when incubated in the

presence of the S9 mix. Benzo(α)pyrene, the carcinogen, was also highly toxic in the presence of the S9 mix.

Effects of S9, cofactor and S9 mix on the cytotoxicity of Bis-GMA monomer

Cytotoxicity of Bis-GMA with a cofactor was the same as that of Bis-GMA alone. At 25 and 50 $\mu\text{g/mL}$, CSR for Bis-GMA with the S9 was higher than that of Bis-GMA alone. In addition, the cytotoxicity of Bis-GMA with the S9 mix was significantly reduced compared with Bis-GMA with the S9 (Fig. 3).

Fig. 3 Cytotoxicity of Bis-GMA with cofactor, S9 or S9 mix after 24 hour cultivation in JTC-12 cells.

The different concentrations of Bis-GMA are indicated on the logarithmic concentration axis. Each point is the mean of 4 cultures. ●: Bis-GMA alone, △: Bis-GMA+cofactor, □: Bis-GMA+S9, ○: Bis-GMA+S9 mix

Table 3 IC50 of dental resin monomers with or without S9 mix in JTC-12 cells

Monomers	IC50 (without S9 mix)		IC50 (with S9 mix)	
	$\mu\text{g/mL}$	mM	$\mu\text{g/mL}$	mM
Bis-GMA	39	0.681	820	4.141
UDMA	28	0.096	106	0.260
Bis-MPEPP	32	0.054	100<	0.195<
EGDMA	135	0.068	200<	0.425<
DMAEM	180	1.146	710	4.516
TEGDMA	400	1.398	1000<	3.496<
HEMA	220	1.692	500	3.842
4-META	1040	3.421	1060	3.486
MMA	1070	10.70	1080	10.80
Cyclophosphamide*	200<	0.765<	34	0.130
Benzo(α)pyrene**	100<	0.396<	10	0.039

*teratogen, **carcinogen

Fig. 4 Cytotoxicity of dental resin monomers with or without S9 mix after 24 hour cultivation in JTC-12 cells.

The different concentrations of dental resin monomers are indicated on the logarithmic concentration axis. Each point is the mean of 4 cultures. ●: Bis-GMA alone, ○: Bis-GMA+S9 mix, ▲: UDMA alone, △: UDMA+S9 mix, ■: Bis-MPEPP alone, □: Bis-MPEPP+S9 mix

Fig. 5 Cytotoxicity of dental resin monomers without S9 mix after 24 hour cultivation in JTC-12 cells.

The different concentrations of dental resin monomers are indicated on the logarithmic concentration axis. Each point is the mean of 4 cultures. ●: EGDMA, ▲: TEGDMA, ■: DMAEM, ◆: HEMA, ▼: MMA

Fig. 6 Cytotoxicity of dental resin monomers with S9 mix after 24 hour cultivation in JTC-12 cells.

The different concentrations of dental resin monomers are indicated on the logarithmic concentration axis. Each point is the mean of 4 cultures. ○: EGDMA+S9 mix, △: TEGDMA+S9 mix, □: DMAEM+S9 mix, ◇: HEMA+S9 mix, ▽: MMA+S9 mix

Cytotoxicity of the resin monomers

Most resin monomers for this experiment reduced cytotoxic effects by P 450 mediated biotransformation in the S9 mix (Table 3). CSR for 50 $\mu\text{g/mL}$ of Bis-GMA with the S9 mix was $92.6 \pm 0.5\%$, while that for the non-S9 mix was $6.6 \pm 0.3\%$ (Fig. 4). The values of CSR for UDMA, Bis-MPEPP, EGDMA, TEGDMA, DMAEM, 4-META and HEMA exhibited a reduction in cytotoxicity in the presence of the S9 mix (Table 3 and Fig. 4-6). There were significant differences between +S9 and -S9 for the respective monomers ($p < 0.05$). However, there were no significant differences between +S9 and -S9 for MMA (Table 3 and Fig. 5-6) ($p < 0.05$).

DISCUSSION

The results obtained in this study demonstrated that some dental resin monomers show reduced cytotoxicity by P 450 mediated biotransformation.

The S9 mix itself has toxic effect on cultured cells. The cytotoxicity of the S9 mix appeared at concentration above 10 vol% after 24 hour cultivation in JTC-12 (Fig. 1). However, on addition of the 5 vol% S9 mix to the culture medium, the CSR for S9 mix in JTC-12 cells was $97.5 \pm 5.3\%$ of the negative control and that in HeLa cells was $74.0 \pm 6.4\%$ of the negative control. This CSR in JTC-12 cells was not significantly different from the negative control, but that in HeLa cells was significantly different from the negative control ($p < 0.05$). Therefore, in comparison with HeLa

cells, the 5 vol% S9 mix did not have a pronounced effect on the CSR after 24 hour cultivation in JTC-12 cells. However, cyclophosphamide and benzo(α)pyrene as the positive control were highly toxic with the 5 vol% S9 mix after 24 hours (Table 3). Cyclophosphamide is metabolized by P 450 which is a major mono-oxygenase⁹⁻¹², and phosphoramidate mustard and acrolein are finally formed¹³. These metabolites are highly cytotoxic and teratogenic. Benzo(α)pyrene is also activated by P 450, and epoxides are finally formed. Epoxides are highly reactive and mutagenic and/or carcinogenic.

In Figure 3, the cytotoxicity of Bis-GMA with the S9 mix is seen to be reduced more than that of Bis-GMA with the S9. This suggests that the reduction of the cytotoxicity of Bis-GMA was not due merely to the binding of a protein, but to a response with enzymes of P 450 in the S9 mix, because the response with P 450 proceeded in the presence of a cofactor, especially NADPH.

DeMarini *et al.*¹⁴ reported that S9 was able to render the mixtures less toxic to the cells by metabolic detoxification and/or nonspecific binding of some of the cytotoxins. Our results show that Bis-GMA and the other monomers in the presence of the S9 mix, except for MMA, reduced cytotoxicity in JTC-12 cells (Fig. 4-6). This reduction of cytotoxicity suggests that the monomers might be metabolized to lower toxic metabolites by the S9 mix.

Fujisawa *et al.*¹⁵ reported that Bis-GMA and UDMA showed strong migration into the lipid bilayer, and that TEGDMA and MMA were weaker in migration into the lipid bilayer than Bis-GMA and UDMA. The metabolites of the dental resin monomers in the presence of the S9 mix, except for MMA, may be weaker in migration into the lipid bilayer.

Ruyter *et al.*¹⁶ reported the release of formaldehyde from denture base polymers at 37°C *in vitro*, and the release of formaldehyde was also found in dental composites¹⁷. They discussed the possible mechanisms of formaldehyde formation in dental resin monomers. Formaldehyde and methyl pyruvate are formed by the primary oxidation of unreacted monomers in methacrylate groups. Even if the formaldehyde was formed in the medium, in this study, it would not reach a sufficiently high the concentration to have a strong toxic effect because the *in vitro* cytotoxicity of formaldehyde is dependent on the concentration in the cell culture medium.

On the other hand, metabolites of MMA may have toxic effects on cells. The metabolite of MMA by the S9 mix was not identified. However, we recognized that it was not methacrylic acid by analysis of gas chromatography.

Corkill *et al.*² reported that a major metabolic pathway of MMA in human blood was by hydrolysis to metacrylic acid. The metabolic pathway of MMA in the presence of the S9 mix may be different from that in human blood. It should be pointed out that MMA is split by an unspecified esterase which occurs ubiquitously in the body, and is formed when oxidized to CO₂⁴.

In this present study, most of the investigated dental resin monomers did not increase their level of cytotoxicity after biotransformation in the presence of the S9 mix, in contrast to cyclophosphamide and benzo(α)pyrene. Moreover, it was found

through this present study that JTC-12 cells can be useful in the evaluation of cytotoxicity after biotransformation in a short term assay using the S9 mix.

CONCLUSIONS

From the results of this study, it can be concluded that:

1) The values of cell survival ratio for Bis-GMA, UDMA, Bis-MPEPP, EGDMA, TEGDMA, DMAEM, 4-META and HEMA demonstrated reduced in cytotoxicity by cytochrome P 450 mediated biotransformation.

2) Cytotoxicity of MMA in the presence of the S9 mix was the same as that of MMA alone.

3) JTC-12 cells can be used as a cell line to evaluate the cytotoxicity of chemicals after *in vitro* biotransformation.

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