J Med Dent Sci 2006; 53: 127−133

Original Article

Effects of exposure to 4-META/MMA-TBB resin on pulp cell viability

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Adhesive restorative systems have expanded the range of possibilities for direct pulp-capping technique, with evidences of clinical success in vital pulp therapy. However, quite few studies have described the direct responses of pulp cells following the application of resinous materials to pulp exposure. To address this issue, effects of exposure to an adhesive resin, 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butyl borane (4-META/MMA-TBB) resin on cellular activity were investigated in an established rat dental pulp cell line (RPC-C2A). RPC-C2A cells were cultured on normal plastic plates or the disks prepared from 4-META/MMA-TBB resin (Super Bond® C&B) in a-MEM containing 10% FBS. After 3, 7 and 14 days, DNA content and alkaline phosphatase (ALP) activity were measured. Total RNA in each group was extracted and RT-PCR analysis was performed. Moreover, the live cell ratio was also evaluated by cytotoxicity assay after treatment with various concentrations of 4-META/MMA-TBB. At day 3, 7 and 14, amount of DNA and ALP activity of the cells on normal plastic plates and the one on the 4-META/MMA-TBB were comparable. Cells of both groups expressed mRNA of type I collagen (CoII), ALP, osteopontin (OPN), osteocalcin (OC), and bone morphogenetic protein (BMP-2). Furthermore, 4-META/MMA-TBB (10^{-1}% or less) did not influence dead cell ratio in the confluent state. According to the results of these *in vitro* studies, exposure to this resinous material would not induce cytotoxic response in the pulp cells.

Key words: 4-META/MMA-TBB, pulp cell, cytotoxicity, cell differentiation

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Received January 27; Accepted March 17, 2006

Introduction

Development of adhesive resin materials has expanded the range of possibilities for restoration, direct pulp-capping technique,[1] reinforcement of fractured teeth[2-5] and root canal sealing.[6,7] Recently, dentin adhesive systems have been proposed as an alternative to $Ca(OH)_2$-based materials in direct pulp capping treatment. However, side effects by effluents of the adhesive resin on dental pulp tissue are of great concern. Although a number of studies have investigated the effects of adhesive resin materials on pulp tissue, there are still conflicts in their views. [8-19]

In previous studies effects of resin-based restorative materials on pulp tissue or pulp cells have been

reported and the results of these studies are summarized in Table 1. A histopathological study reported the changes in the condition of vital teeth after direct pulp capping with adhesive resins in nonhuman primates.[20] Another article described that resin-based materials inhibited the growth of human pulp cells *in vitro*.[21] In contrast, adhesive resins induced only a slight initial reaction and secondary dentin was observed in other studies.[22,23] Thus, diverse effects of the resin materials have been previously reported. Moreover, few *in vitro* studies have described the direct pulp cell responses following application of the resinous materials.[24,25] In the present study, we evaluated effects of exposure to an adhesive resin, 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate -tri-n-butyl borane (4-META/MMA-TBB, Super Bond® C&B, Table 2) on cellular activity in an established rat dental pulp cell line (RPC-C2A).

Materials and Methods

Preparation of 4-META/MMA-TBB Plates

4-META/MMA-TBB was polymerized on Teflon-coated 12-well and 6-well culture plates at room temperature for 24 h. Normal plastic plates (SUMITOMO

Table 1. The previous reports concerning effects of resin-based materials on pulp tissue and/or pulp cells.

Authors	Materials	*In vivo*	*In vitro*	Toxic	Not toxic
Pameijer CH et al.[20]	ProBond™ (Nickel-chelating resin)	O		O	
	AllBond 2® (Na-N-tolylglycine glycidylmethacrylate, Biphenyl dimethacrylate)	O		O	
	Permagen A&B® (2-Hydroxyethyl methacrylate)	O		O	
Huang FM et al.[21]	Fuji IX® (Polyacrylic acid)		O	O	
	Superfil® (Urethane modified Bis-GMA dimethacrylate)		O	O	
Tsuneda Y et al.[22]	Super Bond® C&B (4-META/MMA-TBB)	O		O	
Inoue T et al.[23]	HEMA	O		O	
	MMA	O			O
	4-META/MMA	O			O
Mishima K et al.[24]	Super Bond® D Liner DUAL (4-META/MMA-TBB)		O		O
Costa CA et al.[25]	Single Bond™ (Bisphenol a diglycidyl ether dimethacrylate, HEMA)		O	O	
	Prime & Bond2.1® (Urethane dimethacrylate)		O	O	
	Syntac®Sprint (HEMA, Methacrylate modified polyacrylic acid)		O	O	

Table 2. Composition of 4-META/MMA-TBB resin

Product trade name	Ingradients	Percent
Super Bond® C&B monomer	MMA 4-META	Balance Approx. 5 %
Super Bond® C&B catalyst	TBB, partial oxidized (TBB-O) Hydrocarbon	80 % Balance
Super Bond® C&B polymer	PMMA Metal oxides	Balance 0~50 %

BAKELITE CO, LTD, Japan) for cell culture were used as positive controls. Both plates were sterilized by UV irradiation for 30 min before inoculation.

Cell Culture

The rat clonal dental pulp cell line RPC-C2A was used in the present study. RPC-C2A cells were maintained in a-minimal essential medium (a-MEM; GIBCO, Gland Island, NY, USA) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Inc, Street Lenexa, KS, USA) and 1% (vol/vol) penicillin-streptomycin solution (Sigma Chemical, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Assessment of Cell Viability

The cells were inoculated into 96-well plates at a cell density of 2×10^5 cells/cm^2. At confluence, the cells were treated with various concentrations (0% to 10%) of 4-META/MMA-TBB monomer for 24 h. Zero % 4-META/MMA-TBB group was used as control. Then, cell viability was assessed using a LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Molecular Probes, USA) which provided a fluorescence cell viability assay with calcein AM. The fluorescence was read at 485 nm excitation and 535 nm emission using a micro-plate reader, Wallac 1420 ARVOsx (Parkin Elmer Co, Ltd, USA). The percentage of live cells was calculated from the fluorescence readings defined below as: % of Live Cells = (Fsam -Fmin)/(Fmax -Fmin) ×100%, Fsam; Fluorescence in the experimental cell sample, labeled with calcein AM, Fmin; Fluorescence in a control sample where nearly all cells are alive without dye added, Fmax; Fluorescence in a control sample where nearly all cells are alive, labeled with calcein AM, F0; Fluorescence in the cell-free sample without dye added. Background fluorescence readings (F0) were subtracted from all values of Fsam, Fmin, Fmax prior to calculation of results.

Quantitative analysis of DNA

The cells were inoculated into 12-well normal plastic plates or the one prepared from 4-META/MMA-TBB at a density of $2x10^5$ cells/cm^2. After 3, 7, and 14 days of culture period, cells were washed with phosphate-buffered saline (PBS, GIBCO, Gland Island, NY, USA), scraped and centrifuged for 5 min at 8000 x g. Samples were transferred into 0.1 % Triton X-100® (ICN Biomedicals Inc, Aurora, OH, USA) and sonicated on ice for 30 min. Fifty ml of sample were used for DNA measurement. One thousand five hundred μl of Hoechst 33342 solution was added to each sample. The fluorescence value was read at 356 nm excitation and 458 nm emission using a micro-plate reader, Wallac 1420 ARVOsx.

Measurement of Alkaline Phosphatase (ALP) Activity

ALP activity assay was performed by an Alkaline Phospha-B Test Wako Kit® (Wako Pure Chemical Industries, Ltd, Japan) accordingtothemanufacturer'sinstructions. One hundred ml of measurement buffer was added to 50 μl of each sample. After heating at 37 °C for 15 min, the absorbance was measured with a spectrophotometer (Model 450 MICROPLATE READER, Bio-Rad Laboratories, USA).

Analyses of mRNA Expression

RPC-C2A cells were inoculated into 6-well normal plastic plates or the one prepared from 4-META/MMA-TBB at a density of 2 x 10^5 cells/cm^2. After 14 days of culture period, the cells were rinsed with PBS and digested in 1 ml of ISOGEN (NIPPON GENE, Co, Ltd, Japan) based on the acid guanidinium-phenol-chloroform (AGPC) method. Total RNA was extracted and purified according to the manufacturer's instructions. Single-strand cDNA for PCR templates was synthesized from 1.5 μg of total RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™ life technologies, USA). An aliquot (2 ml) of cDNA was amplified employing puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, UK). Each amplification step was performed as follows: denaturation for 45 s at 94°C, annealing for 30 s at 60°C and extension for 1 min at 72°C, for 23 cycles. The nucleotide sequences of primers for PCR are shown in Table 3. The PCR products were electrophoresed in a 2% agarose gel, stained with 10 μg / ml ethidium bromide and photographed on the ultraviolet transluminator.

Statistical Analyses

We repeated the set of experiments five times and presented the representative data. Numerical data was presented as mean + 1 standard deviation. Statistical analyses were performed by Mann Whitney U test (JSTAT).

Table 3. Primers used for RT-PCR

Gene		Primer sequence	Fragment size (bp)
ALP	sense	5'GGAAGGAGGCAGGATTGACCAC3'	338
	antisense	5'GGGCCTGGTAGTTGTTGTGAGC3'	
ColI	sense	5'TTGACCCTAACCAAGGATGC3'	197
	antisense	5'CACCCCTTCTGCGTTGTATT3'	
OPN	sense	5'TTTCCCTGTTTCTGATGAACAGTAT3'	228
	antisense	5'CTCTGCTTATACTCCTTGGACTGCT3'	
OC	sense	5'AGCTCAACCCCAATTGTGAC3'	190
	antisense	5'AGCTGTGCCGTCCATACTTT3'	
BMP2	sense	5'AAGGCACCCTTTGTATGTGG3'	189
	antisense	5'CATGCCTTAGGGATTTTGGA3'	
GAPDH	sense	5'AACTCCCATTCCTCCACCTT3'	200
	antisense	5'GAGGGCCTCTCTCTTGCTCT3'	

Results

Assessment of Cell Viability

The cell viability test was performed after treatment with 0, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1 and 10 % of 4-META/MMA-TBB monomer (Fig. 1). In the 0.1 % and 1 % group, the rate of live cells was significantly lower than control group. No significant difference was found in the 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} % groups compared with the control group. It was impossible to perform fluorescence measurement in the 10% group, because each sample was dried and coagulated within 24 h after treatment with the monomer.

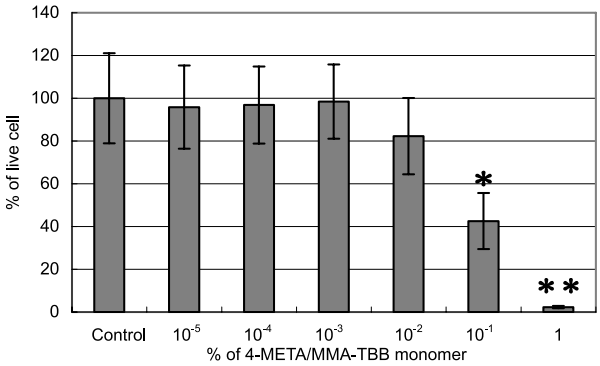

Fig. 1. Toxic effect of 4-META/MMA-TBB monomer in RPC-C2A cells. Live cell ratio in the pulp cell cultures was evaluated with cytotoxicity assay kit after treatment with various concentrations of 4META/MMA-TBB monomer. The bar represents mean + 1 SD (n=17). Statistical analysis was performed using the Mann Whitney U test to compare each sample and control. The probability level (* $p < 0.05$, ** $p < 0.01$) was considered to be statistically significant.

Cellular Proliferation

The proliferation of RPC-C2A on 4-META/MMA-TBB plates was determined at 3, 7 and 14 days of culture (Fig. 2). The DNA content of both experimental and control groups gradually increased through 14 days of culture. The presence of 4-META/MMA-TBB did not affect the rate of cellular proliferation.

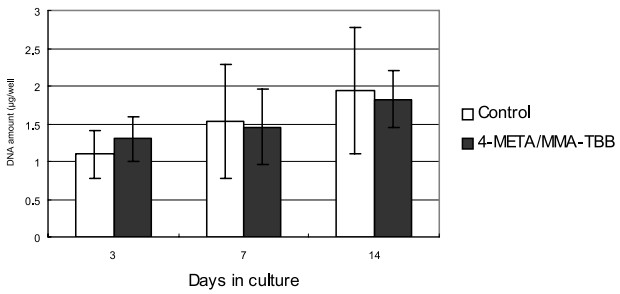

Fig. 2. Cellular proliferation on 4-META/MMA-TBB plates. RPC-C2A cells were cultured on normal plastic plates or the one prepared from 4-META/MMA-TBB in a-MEM containing 10 % FBS. At 3, 7 and 14 days of culture period, pulp cells were collected and DNA contents were measured (n=6).

ALP Activity

ALP activity was determined in the cells on 4-META/MMA-TBB plates and on control plates up to day 14 of culture period (Fig. 3). ALP activity of each group gradually increased, and their values on 4-META/MMA-TBB were not significantly different from control at day 3, day 7 and day 14.

mRNA Expression on 4-META/MMA-TBB Plates

After cells were differentiated on each plate, mRNA expression related to mineralization was evaluated. At day 14, gene expression for ALP, type I collagen (ColI), osteopontin (OPN), osteocalcin (OC) and bone morphogenetic protein-2 (BMP2) in RPC-C2A cells was detected by RT-PCR analyses. All of the mRNA expression, which examined in the present study, was detected in both control and 4-META/MMA-TBB groups, although we did not employ quantitative PCR analyses (Fig. 4).

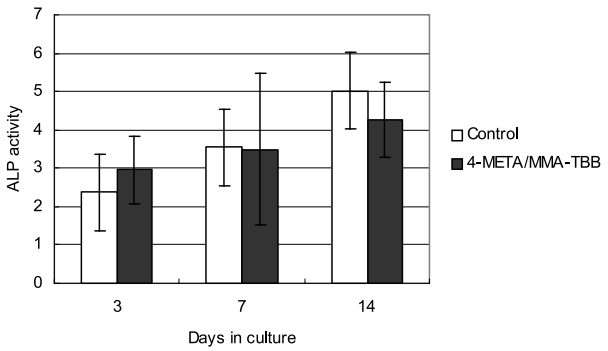

Fig. 3. ALP activity on 4-META/MMA-TBB plates. RPC-C2A cells were cultured on normal plastic plates or the one prepared with 4-META/MMA-TBB in a-MEM containing 10 % FBS. At 3, 7 and 14 days of culture period, pulp cells were collected and ALP activity was examined (n=6).

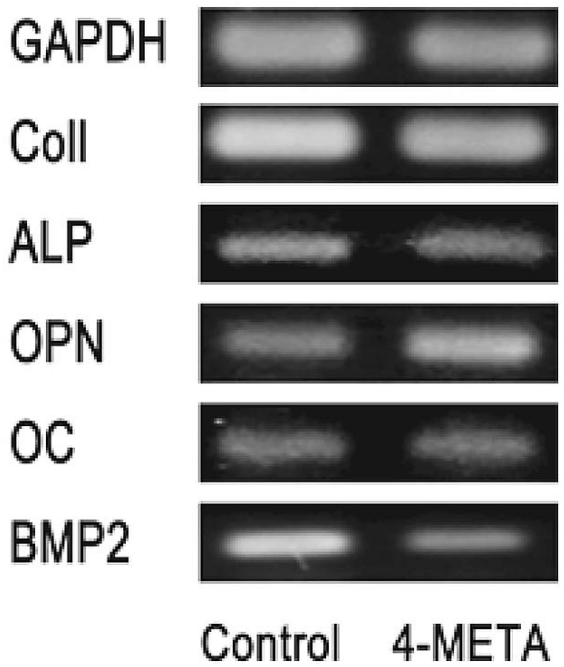

Fig. 4. Messenger RNA expression of ALP, Coll, OPN, OC and BMP2 on 4-META/MMA-TBB plates. RPC-C2A cells were cultured on normal plastic plates (Control) or the one prepared with 4-META/MMA-TBB (4META) in a-MEM containing 10 % FBS. At 14 days of culture period, total RNA in each group was extracted and RT-PCR analyses were performed.

Discussion

In this study, cellular viability after exposure to 4-META/MMA-TBB was examined employing an *in vitro* culture system, and the results of our experiments demonstrated several aspects of the direct pulp cell response to the resinous material. It was previously reported that the cytotoxic effects induced by 4-META/MMA-TBB resin may be associated with TBB.[26] Yamamoto *et al*. have also reported that sealing the perforation using 4-META/MMA-TBB resin with activated liquid induces more serious inflammation than sealing without activated liquid.[27] The components of 4-META/MMA-TBB resin may cause degeneration of the pulp and periodontal tissues, but does not interfere with wound healing.[28,29] Another study revealed that macrophages appeared during dentin bridge formation after application of an adhesive resin to human dental pulp.[30] Thus, a number of studies have shown the effects of exposure to the resin materials on the pulp tissue, reproducing the environment in which direct pulp capping technique was employed. According to the results of previous studies described above, it might be necessary to assume that chronic cytotoxicities as well as acute cytotoxicities against the cell functions were observed when cells were exposed to polymerizing or polymerized resin for a long time. Moreover, the direct response of individual cells, especially effects on cellular proliferation and cellular differentiation, to adhesive resin materials has not been studied in detail yet.

The results of the cell viability test, in which various concentrations of 4-META/MMA-TBB monomer were examined, indicated that 4-META/MMA-TBB monomer caused weak cytotoxic damage to the cells. This monomer suppressed cell proliferation in higher concentration, whereas it did not influence live cell ratio in lower concentration. These results suggested that the toxic effects of 4-META/MMA-TBB monomer on the cells were concentration dependent and lower concentration of the monomer, released during its polymerizing process, may not be cytotoxic. Moreover, polymerized resin material contains little monomer and that should have less negative effects.

Several studies have described the influences of 4-META/MMA-TBB resin on exposed pulp. Nakamura *et al*. previously reported that wound healing and dentin bridge formation occurred after the application of 4-META/MMA-TBB resin.[31-33] It has also been reported that 4-META/MMA-TBB adhesive resin components may not inhibit the calcification of pulp tissue.[34] In contrast, another study have shown that 4-META/MMA-TBB resin inhibited osteogenic activity *in vivo*[35] and it is still ambiguous whether 4-META/MMA-TBB resin chronically affects cellular proliferation, differentiation and consequent dentin bridge formation. To examine the chronic effects of 4-META/MMA-TBB on pulp cells, RPC-C2A pulp cell line was employed and cultured on the polymerized resin. In this culture system,

DNA content and ALP activity of both experimental and control groups gradually increased and those values of 4-META/MMA-TBB group were similar to the control group at day 3, 7 and 14. These results suggested that 4-META/MMA-TBB which was completely polymerized did not exert any negative regulatory effects on pulp cell culture. On the other hand, there is a contradicting report that polymerized 4-META/MMA-TBB suppressed cell proliferation for 4 days of the culture period.[30] However, fibroblastic cells were employed in that study and the characteristics of fibroblasts may be quite different from that of pulp cells. Furthermore, the culture period in the present study was much longer than that of the previous study and there were several differences in the experimental conditions.

In a histopathological study of direct pulp capping with adhesive resins, 4-META/MMA-TBB induced only a slight initial inflammatory reaction, and formation of secondary dentin was observed.[22] RPC-C2A cells are able to synthesize ColI and OPN, and have great ALP activity.[36-39] In the present study, RPC clonal pulp cells could grow normally, expressing mRNA of ColI, ALP, OPN, OC and BMP2 on 4-META/MMA-TBB plates. As previously reported, the mature odontoblasts synthesize extracellular matrix, mainly ColI.[40-42] Also, ALP is used as a histochemical marker for mineralizing tissue,[43,44] and OPN is a major phosphoprotein found in bone and dentin. [45-47] OC is produced by both odontoblasts and osteoblasts and is expressed at the later stages of cytodifferentiation especially in teeth.[48,49] In addition, BMP2 expressed in the pulp coordinates odontoblast differentiation and dentin formation.[50] The results of RT-PCR analyses suggested that the dental pulp cells on the 4-META/MMA-TBB plate might possibly be mineralized, preserving the original phenotype and pluripotent stem cells in dental pulp might differentiate into odontoblasts to form dentin bridges adjacent to application of 4-META/MMA-TBB.

Therefore, this resinous material could maintain pulp cell activity rather than exert inhibitory effects on cellular proliferation and differentiation. In conclusion, 4-META/MMA-TBB has little cytotoxic effect on the pulp cells and would not disturb cellular proliferation and differentiation, suggesting that this resinous material could be applied for direct pulp-capping procedures.

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