Cytotoxicity of one-step dentin-bonding agents toward dental pulp and odontoblast-like cells

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SUMMARY The purpose of this study was to compare the cytotoxicity of five one-step dentin-bonding agents on human dental pulp and odontoblast-like cells (MDPC-23). Photopolymerized and unpolymerized samples of these dentin-bonding agents were prepared and incubated with dental pulp or MDPC-23 cells. After 24 or 72 h of incubation, the number of unstained cells with trypan blue was counted. The staining of cells with trypan blue stands for a cytotoxicity. The pulp cell and MDPC-23 cytotoxicity of polymerized sample treatment increased in the order of AQ Bond Plus (AQ)<Clearfil Tri-S Bond (TS)=G-bond (GB)<Absolute (AB)<Adper Prompt (AP) for 24 and 72 h. The pulp cell cytotoxicity of unpolymerized sample treatment for 24 h increased in the order of AQ<GB = AB<TS<AP. The MDPC-23 cytotoxicity of unpolymerized sample treatment for 24 h increased in the order of AQ<GB<TS = AB<AP. Whether polymerized or unpolymerized, AQ was the least cytotoxic agent, while AP was the strongest. All polymerized dentin-bonding agents exhibited lower cytotoxicity by 2–65% than their unpolymerized counterparts. The appearance of the cytotoxicity of dentin-bonding agents was time-dependent, and cell viability was lower at 72 h by 2–46% than at 24 h. The cytotoxicity to MDPC-23 cells was about 5–24% higher than that to pulp cells. These results indicate that one-step dentin-bonding agents differ markedly in their cytotoxicity. Differential cytotoxic effects of one-step dentin-bonding agents should be considered during clinical application of operative restoration.

KEYWORDS: one-step dentin-bonding agents, dental pulp cells, odontoblasts, trypan blue staining, cell viability, photopolymerization

Introduction

Minimal intervention (MI) has become the preferred treatment for caries with recent advances in dentin-bonding agents (1, 2). If their bonding strength is weak, then microleakage can occur, thereby affecting the survival of the teeth (3, 4). Bonding agent-induced dental pulp inflammation has been demonstrated histopathologically (5). This pulp reaction is considered to be caused by the diffusion from dental tubules of unpolymerized monomers remaining after photopolymerization (6, 7). Unpolymerized monomers may come in direct contact with odontoblasts and pulp cells. Furthermore, the monomers have been recognized as antigen and induced inflammatory reactions in the pulp tissue (8).

In the past, many studies have investigated the influence of dental restorative materials on dental pulp cells (9, 10). In particular, methods of screening for cytotoxicity in cultured cells are widely used

because of their inexpensiveness and high reproducibility (11–15). Their cytotoxicity has been evaluated in terms of changes in cell number, membrane permeability, intracellular metabolism and cell morphology. Cytotoxicity was detected in most of the previously investigated bonding agents. Clearfil SE bond was less cytotoxic than Prime & Bond and Single Bond on human pulp cells (16), and Scotchbond 1 was reported to be more toxic than Syntac Sprint and Prime & Bond (17). Chen *et al.* reported that Syntac Sprint was more toxic than Prime & Bond and Single Bond (18). Based on these findings, Scotchbond 1 is the most toxic and Clearfil SE bond is the least toxic. Furthermore, various monomers comprising dentin-bonding agents have been compared regarding cytotoxicity in cultured cell systems. Ratanasathien *et al.* reported that 2,2-bis[4′-(x-hydroxy-3′-methacryloyoxy)phenyl] propane (bis-GMA) showed the highest toxicity against mouse fibroblasts, followed by urethane dimethacrylate (UDMA), triethylene-glycoldimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) in order of decreasing toxicity (19). In a comparison of 35 resin components concerning cytotoxicity, Geurtsen *et al.* found that UDMA was more cytotoxic than TEGDMA on human gingival fibroblasts and periodontal ligament cells, but less toxic on human dental pulp cells (20). The biological effects of bonding agents have also been clarified. Recently, unpolymerized monomers have been reported to influence the immune system by inhibiting the secretion of cytokines from macrophages (21). Furthermore, dentin-bonding agents induce apoptosis and cell cycle arrest was noted in most cells that escaped from apoptosis (22).

One-step bonding agents have been developed and shown to exhibit good bonding strength despite their easier handling properties compared with two-step bonding agents (23, 24). However, little is known about the cytotoxicity of one-step bonding agents, compared with two-step bonding agents. Moreover, no comparison has been made between their cytotoxicity before and after polymerization. We hypothesized that the cytotoxicity varies among one-step dentin-bonding agents because each component exhibits different cytotoxicity. To confirm our hypothesis, we selected five one-step dentin-bonding agents consisting of different solvents and component monomers. The purpose of this study was to compare the cytotoxicity of five one-step dentin-bonding agents on human dental pulp and MDPC-23 cells.

Materials and methods

Dentin-bonding agents

Five one-step dentin-bonding agents consisting of different solvents and component monomers were selected. Absolute (AB),* Adper Prompt (AP),[†] AQ Bond Plus (AQ),[‡] G-bond (GB)[§] and Clearfil Tri-S Bond (TS).[¶] Their components are listed in Table 1.

Cells and cell culture conditions

Human dental pulp cells were obtained from the healthy third molars which were extracted during normal treatment (19 to 21-year-old patients). The protocol for these experiments was reviewed and approved by the Health Sciences University of Hokkaido Research Ethics Committee, and informed consent was obtained from the tissue donors. To avoid the contamination of experimental materials with gingival tissues and the periodontal ligament attached to the tooth, any residues of these were carefully removed before further processing. The teeth were placed into calcium-free and magnesium-free phosphate-buffered saline (PBS),** and scored sagittaly with a sterile diamond bur flushed with PBS and open. The pulp cells were prepared by pooling pulps collected from three patients. The extruded pulp tissues were rinsed once in PBS and cut into 2-mm^3 cubes using a dental surgical knife. Next, the fragmented pulp tissues were digested with 2mg mL^{-1} collagenase and 0·25% tripsin at 37 °C for 30 min. The resultant suspension was then centrifuged to collect the released cells. These cells were subsequently cultured in Dulbecco's Modified Eagle's Medium (DMEM),[††] supplemented with 10% fetal bovine serum,[††] 10 000 U mL^{-1} penicillin[‡‡] and 10 mg mL^{-1}streptomycin[‡‡] at 37 °C in a humidified atmosphere of 5% CO$_2$. All experiments were per-

Table 1. The components of the five one-step dentin-bonding agents tested

One-step dentin-bonding agent	Components	Lot number
Absolute (AB)	4-MET, methacryloxyethyl pyrophosphate-fluoridated filler, SiO_2, initiator, acetone	409-029
Adper Prompt (AP)	Methacrylated phosphoric acid esters, bis-GMA, HEMA, water	133495
AQ Bond Plus (AQ)	Base: 4-META, methacrylate, initiator, acetone, water Sponge: aromatic amine, Sodium *p*-toluensulfinate	LW3
G-Bond (GB)	4-MET, phosphate ester, di-methacrylate, water, acetone, initiator	0403021
Clearfil Tri-S Bond (TS)	MDP, bis-GMA, HEMA, microfiller, initiator, ethanol, water	011174

4-MET, 4-methacryloxyethyl trimellitic acid; 4-META, 4-methacryloxyethyl trimellitate anhydride; MDP, 10-methacryloxydecyl dihydrogen phosphate; HEMA, 2-hydroxyethyl methacrylate; bis-GMA, 2,2-bis[4'-(x-hydroxy-3'-methacryloyoxy)phenyl] propane.

formed on human dental pulp cells between passages 5 and 8.

MDPC-23 is a cell line established from dental papilla cells of 18 to 19 foetal day CD-1 mouse mandibular first molars by Hanks *et al.* (25). The cells were cultured by the standard method for pulp cells using DMEM. The cells have properties of odontoblast precursor cells, being characterized by high alkaline phosphatase (ALP) activity. We confirmed the retention of this property every passage.

Sample preparation

Two microliters of each dentin-bonding agent were placed with a micropipette in the centre of each well of a 6-well plate and irradiated with a halogen irradiator. Regarding the irradiation conditions, AQ was exposed for 5 s, and the other materials for 10 s, following the manufacturer's instructions. Each well was seeded with a 2 mL suspension of cells at a concentration of 2×10^5 cells mL^{-1}. The same cell suspension was dispensed into wells without a dentin-bonding agent to serve as controls. In another group of similar wells, dentin-bonding agents were not irradiated before placing the cells. Plates were incubated at 37 °C for 24 and 72 h. The pH of media containing polymerized or unpolymerized agents was measured by pH meter.§§

Cell viability assay

After culture, the medium was transferred to a tube and floating cells were collected. The adherent cells were removed by 0·25% trypsin treatment for 5 min and

§§Horiba Instruments Ltd, Kyoto, Japan.

transferred to the same tube. The tube was centrifuged, the supernatant was removed, and all cells were suspended in PBS. The absence of residual cells in the well or supernatant was confirmed under a microscope. The cells were then stained with trypan blue (Sigma), and the unstained cells were counted with haemocytometer chamber under a light microscope. The cell viability was calculated as a percentage using the following formula:

$$\text{Percentage of viable cells} = (A/B) \times 100$$

where A is the number of viable cells in the experimental well and B is that in the control well.

Statistical analysis

A statistical analysis was performed with the data obtained from three independent experiments. The data are expressed as the mean ± s.d. and analyzed using One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests, and a value of $P < 0.05$ was considered to be significant.

Results

Table 2 shows the cytotoxicity of polymerized and unpolymerized one-step dentin-bonding agents toward human dental pulp cells. The cell count of untreated human pulp cells was $2·49 \times 10^5$ after 24 h and $3·90 \times 10^5$ after 72 h. The pulp cell viability after treatment with polymerized AB, AP, AQ, GB and TS for 24 h were 50·6 ± 7·2%, 12·4 ± 8·8%, 88·8 ± 9·2%, 63·5 ± 17·7% and 67·5 ± 10·4%, respectively, and those after 72 h were 7·9 ± 4·9%, 0%, 60·3 ± 3·1%, 26·2 ± 3·8% and 21·0 ± 2·1%, respectively. The pulp cell cytotoxicity of polymerized sample treatment

Table 2. The cytotoxicity of five one-step dentin-bonding agents toward human dental pulp cells

Materials	24 h		72 h	
	Polymerized	Unpolymerized	Polymerized	Unpolymerized
Absolute	1·26 (0·18)	0·69 (0·21)	0·31 (0·19)	0
	50·6% (7·2%)[c]	27·7% (8·4%)[b]	7·9% (4·9%)[c]	0%
Adper Prompt	0·31 (0·22)	0	0	0
	12·4% (8·8%)[d]	0%	0%	0%
AQ Bond Plus	2·21 (0·23)	1·28 (0·19)	2·35 (0·12)	0·22 (0·12)
	88·8% (9·2%)[a]	51·4% (7·6%)[a]	60·3% (3·1%)[a]	5·6% (3·1%)
G-bond	1·58 (0·44)	0·60 (0·15)	1·02 (0·15)	0
	63·5% (17·7%)[b]	24·1% (6·0%)[b]	26·2% (3·8%)[b]	0%
Clearfil Tri-S	1·68 (0·26)	0·07 (0·05)	0·82 (0·08)	0
Bond	67·5% (10·4%)[b]	2·8% (2·0%)[c]	21·0% (2·1%)[b]	0%

Data are shown as the number of viable cells (upper; $\times 10^5$ cells) and percentage of viable cells compared with the control (lower) at 24 and 72 h in photopolymerized and unpolymerized groups ($n = 3$). Data are expressed as the mean (s.d.) and were analyzed using One-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison tests. $P < 0.05$ was considered to be significant.
[a,b,c,d]Different letters indicate significant differences between groups.

increased in the order of AQ<TS=GB<AB<AP for 24 and 72 h. The cytotoxicity of unpolymerized sample treatment for 24 h increased in the order of AQ<AB=GB<TS<AP, and no viable cell after treatment for 72 h excluding AQ treatment was found. The viable rates of human pulp cells treated with the unpolymerized AQ for 24 h ($P < 0.01$), AQ for 72 h ($P < 0.001$), GB for 24 h ($P < 0.01$) and TS for 24 h ($P < 0.001$) were significantly lower than those treated with the polymerized samples by 37–65%.

Next, the cytotoxicity of one-step bonding agents was evaluated in the MDPC-23 odontoblast-like cells

(Table 3). The cell count of untreated MDPC-23 was $2·61 \times 10^5$ after 24 h and $4·10 \times 10^5$ after 72 h. The MDPC-23 cell viability after treatment with polymerized AB, AP, AQ, GB and TS for 24 h were $26·1 \pm 4·6\%$, 0%, $82·0 \pm 12·6\%$, $42·5 \pm 13·4\%$ and $44·8 \pm 5·4\%$, respectively, and those after 72 h were $1·7 \pm 2·0\%$, 0%, $38·0 \pm 4·1\%$, $6·3 \pm 3·2\%$ and $12·0 \pm 2·7\%$, respectively. The MDPC-23 cytotoxicity of polymerized sample treatment for 24 and 72 h increased in the order of AQ<TS = GB<AB<AP. The cytotoxicity of unpolymerized sample treatment for 24 h increased in the order of AQ<GB<TS = AB<AP,

Table 3. The cytotoxicity of five one-step dentin-bonding agents toward MDPC-23 odontoblast-like cells

Materials	24 h		72 h	
	Polymerized	Unpolymerized	Polymerized	Unpolymerized
Absolute	0·68 (0·12)	0·12 (0·04)	0·07 (0·08)	0
	26·1% (4·6%)[c]	4·6% (1·5%)[c]	1·7% (2·0%)[c]	0%
Adper Prompt	0	0	0	0
	0%	0%	0%	0%
AQ Bond Plus	2·14 (0·33)	1·12 (0·16)	1·56 (0·17)	0·16 (0·11)
	82·0% (12·6%)[a]	43·0% (6·1%)[a]	38·0% (4·1%)[a]	3·9% (2·7%)
G-bond	1·11 (0·35)	0·44 (0·07)	0·26 (0·13)	0
	42·5% (13·4%)[b]	16·9% (2·7%)[b]	6·3% (3·2%)[b]	0%
Clearfil Tri-S	1·17 (0·14)	0·15 (0·05)	0·49 (0·11)	0
Bond	44·8% (5·4%)[b]	5·7% (1·9%)[c]	12·0% (2·7%)[b]	0%

Data are shown as the number of viable cells (upper; $\times 10^5$ cells) and percentage of viable cells compared with the control (lower) at 24 and 72 h in photopolymerized and unpolymerized groups ($n = 3$). Data are expressed as the mean (s.d.) and were analyzed using One-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison tests. $P < 0.05$ was considered to be significant.
[a,b,c,d]Different letters indicate significant differences between groups.

and no viable cell after treatment for 72 h excluding AQ treatment was found. The viable rates of MDPC cells treated with the unpolymerized AB for 24 h ($P < 0.01$), AQ for 24 h ($P < 0.001$), AQ for 72 h ($P < 0.001$), GB for 24 h ($P < 0.01$) and TS for 24 h ($P < 0.001$) were significantly lower than those treated with the polymerized samples by 21–40%.

Whether polymerized or unpolymerized, AQ exhibited the lowest cytotoxicity, while AP exhibited the highest. The appearance of the cytotoxicity of dentin-bonding agents was time-dependent, and cell viability was lower at 72 h by 2–46% than at 24 h. The viable rates of MDPC cells treated with polymerized AB for 24 h ($P < 0.01$), GB for 72 h ($P < 0.05$) and TS for 24 h ($P < 0.01$), and that treated with unpolymerized AB for 24 h ($P < 0.01$) were significantly lower than those of pulp cells by 20–24%. No significant differences were noted in cells treated with other samples.

The pH of media after 24 h of treatment with polymerized samples, AB, AP, AQ, GB and TS was 6.47, 6.23, 7.07, 6.73 and 6.95, respectively. The pH of media added unpolymerized samples, AB, AP, AQ, GB and TS was 6.21, 5.84, 6.86, 6.51 and 6.60, respectively. The pH was 0.2–0.4 lower in the media the unpolymerized samples.

Discussion

To date, the mouse fibroblast cell line L929 (11), human fibroblasts (12, 13), human dental pulp cells (16, 18) and the mouse odontoblast-like cell line MDPC-23 (14, 15) have been used to test the cytotoxicity of dentin-bonding agents. In this study, to evaluate their cytotoxicity on dental pulp tissue, we used the dental pulp and MDPC-23 cells because these cells mainly constitute pulp tissue. As it was difficult to isolate odontoblasts from crowns, an odontoblast-like cell line was used. The toxicity to MDPC-23 cells was about 5–24% higher than that to pulp cells, but the reason for it: whether it was because of cell differentiation, cultured cell line or species difference could not clarified in this study.

MDPC-23 is known to have mineralization ability (25). These cells are very useful for analyzing the mechanism of calcification by odontoblasts (26). In control cells to which no extracts from dentin-bonding agents were added, the ALP activity peaked on day 4 and decreased thereafter. Although we examined the influence of dentin-bonding agents on cell differentia-

tion, even polymerized samples were so cytotoxic that we were not able to culture cells for a long period of time. It was difficult to ascribe the inhibition of ALP activity to the inhibition of cell differentiation or cell damage.

The viability of human dental pulp and MDPC-23 cells incubated with a polymerized AQ for 24 h were the highest at 88% and 82%, respectively, among the dentin-bonding agents tested. AQ is characterized by the lower acidity of phosphate ester monomers and the thinner hybrid layer compared with other dentin-bonding agents (27). Acid removal from the medium reduced the cytotoxicity in a previous report (15). Furthermore, we found in this study that on measurement of the culture medium pH, the toxicity increased as the acidity increased. These results suggest that the cytotoxicity may be because of a shift of medium pH toward acidic. The differences in the component monomer type and concentration may have affected the cytotoxicity. Unpolymerized AP and TS exhibited relatively high cytotoxicity, suggesting that bis-GMA may have strong toxicity among the monomers, as previously reported (19). In an experimental system in which unpolymerized bonding agents were directly added to cell cultures, all bonding agent samples showed a higher cytotoxicity than equal volumes of their polymerized counterparts. This is in agreement with the previous report (28) that polymerized resins released fewer polymers and were less stimulatory and completely polymerized resins were non-stimulatory.

We analyzed the cell viability using the trypan blue staining method. This dye strongly binds to protein. It passes through injured cell membranes, and specifically stains the dead cells. The viable cell rate can be calculated by counting the unstained cells using a haemocytometer. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method measuring succinic dehydrogenase (SDH) activity is generally used as a simple method for investigating cytotoxicity. However, we stained the cells with trypan blue according to previous reports (29) as the residual monomers may affect the medium colour. Cellular SDH activity measurement may also be necessary.

Regarding the irradiation conditions, a halogen irradiator was used. AQ was exposed for 5 s and the other materials for 10 s, following the manufacturer's instructions. For comparison with other studies, the ISO guidelines should be followed, but we followed the

instructions for each material because the protocol specified in the instructions may maximize the specific properties of the material. Furthermore, inhibition of polymerization by oxygen was also considered (30), but we did not employ irradiation in the presence of nitrogen or coverage by glycerol. Therefore, it cannot be ruled that the polymerized samples may exhibit stronger toxicity.

Recently, dentin-bonding agents have been suggested as replacement for calcium hydroxide for pulpal protection (31). The dentin-bonding agents were observed to seal the exposed dental pulp and induce the formation of repaired dentin (32). On the other hand, studies reported that dentin-bonding agents induced persistent inflammation in pulpal tissue and completely inhibited the repair process (33). Therefore, it is desirable that dentin-bonding agents not only exhibit strong bonding strength, but also are harmless to dental pulp, and do not inhibit the differentiation of dental pulp cells into odontoblasts to promote the formation of repaired dentin in the dental pulp.

In conclusion, we found that AQ was the least cytotoxic while AP was most toxic among the five one-step dentin-bonding agents examined. A sufficient thickness of the hybrid layer may be obtained as the monomer acidity increases, strengthening adhesive force (34). Therefore, consideration is necessary for clinical application such as application of AP for Class IV cavities requiring strong adhesive force and less toxic AQ for deep cavities.

Acknowledgments

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