*Dental Materials Journal 2013; 32(5): 709–717*

# The effects of restorative composite resins on the cytotoxicity of dentine bonding agents

Kyunghwan KIM, Kyung Mi SON, Ji Hyun KWON, Bum-Soon LIM and Hyeong-Cheol YANG

*Department of Dental Biomaterials Science and Dental Research Institute, School of Dentistry, Seoul National University, 28 Yeonkun-dong, Chongro-ku, Seoul 110-749, Korea*
*Corresponding author, Hyeong-Cheol YANG; E-mail: yanghc@snu.ac.kr*

During restoration of damaged teeth in dental clinics, dentin bonding agents are usually overlaid with restorative resin composites. The purpose of this study was to investigate the effects of restorative resin composites on cytotoxicity of dentin bonding agents. Dentin bonding agents were placed on glass discs, pre-cured and uncured resin composite discs. Bonding agents on the glass discs and composite resins discs were light cured and used for agar overlay cytotoxicity testing. Dentin bonding agents on composite resin discs exhibited far less cytotoxicity than that on glass discs. The polymerization of resin composite increased the surface hardness and decreased the cytotoxicity of bonding agents. In conclusion, composite resins in dental restorations are expected to enhance the polymerization of dentin bonding agents and reduce the elution of resin monomers, resulting in the decrease of cytotoxicity.

*Keywords*: Dentin bonding agents, Cytotoxicity, Resin composites, Oxygen inhibition, Surface hardness

## INTRODUCTION

Dentine bonding agents are being used to generate a tight bond between dentine and restorative resin composites and to minimize microleakage, a cause of restoration failure. In bonding processes, the polymerization of resin monomers is important from the view point of biocompatibility as well as bonding properties, because unreacted resin monomers can damage pulpal tissues by diffusion through dentinal tubules. Previous studies demonstrated severe cytotoxicity from various bonding agents, suggesting a possibility of pulpal damage with the application of the agents in dental clinics[1-4]. It is evident that the adhesives are able to cause an inflammatory reaction when applied for direct pulp capping[5,6]. However, dentine bonding agents currently being used do not exhibit any practical adverse effects on teeth in dental clinics, suggesting that the conventional *in vitro* methods to evaluate cytotoxicity overestimate the hazard of the bonding agents to pulpal tissues. To reflect the clinical situation, a dentine barrier system was applied to assess the cytotoxicity of the bonding agents[7-9]. The dentine barrier system included a dentine disc interposed between target cells and a bonding agent in order to mimic the environment of pulpal tissues. Under this condition, it was evident that the cytotoxicity of the dentine bonding agent was greatly attenuated by dentine discs, suggesting that dentine provided protective effects by interfering with the free diffusion of hazardous chemicals to the target cells. Therefore, it is expected that pulp tissue is minimally damaged, if at all, by bonding agents in clinical situations due to the presence of adequate amounts of dentine[10,11]. However, there are several bonding materials that still exert cytotoxic effects on pulp cells even in the presence of a dentine barrier[12]. Because clinical evidence relating to pulp damage by these materials has yet to be reported, it is likely that the cytotoxicity of bonding agents is still overestimated even in dentine barrier tests. In this context, we focused on another clinical condition, the presence of resin composite. In restorative practice, dentine bonding systems are applied on the surface of dentine, followed by layering of restorative composite resins over the bonding agents. Since bonding agents and restorative composite resins share an identical mechanism for the polymerization of resins, the polymerization process of resin composite is expected to result in the secondary polymerization of unreacted resin monomers in bonding agents. Furthermore, the overlaid composite resins are expected to remove or neutralize the oxygen inhibition layer which is generated on the surface of bonding agents and contains an amount of unreacted resin monomers[13]. In a previous study, bonding agents combined with composite resins were found to not affect the cytotoxicity of resin composites, although the effects of composite resins on the cytotoxicity of bonding agents were not directly demonstrated[14].

The purpose of this study is to investigate the effects of restorative composite resins on the cytotoxicity of dentine bonding agents. The bonding agents or bonding systems were placed on composite resin discs, and the cytotoxicity was compared with that on inert glass discs. Our hypothesis was that the cytotoxicity of bonding agents was reduced by the polymerization of restorative composite resins. The surface hardness of the bonding agents was also measured on resin composite discs which were pre-polymerized or polymerized after coating with bonding agents in order to demonstrate that restorative composite resins affect the polymerization of bonding agents.

Received Jan 2, 2013: Accepted Jun 12, 2013
doi:10.4012/dmj.2012-335 JOI JST.JSTAGE/dmj/2012-335

## MATERIALS AND METHODS

*Dentine bonding agents, a restorative resin composite, and chemicals*

In dental clinics, are currently being used various types of dentine bonding agents which are classified into generations. In this study, we selected bonding agents arbitrarily from fourth to seventh generations. The dentine bonding agents used in this study were as follows: Adper Scotchbond Multipurpose Plus (3M ESPE, St. Paul, MN, USA), Adper Single Bone 2 (3M ESPE), Prime & Bond NT (Dentsply De Trey, Konstanz, Germany), Clearfil SE Bond (Kuraray Medical Inc. Osaka, Japan), One-Up Bond F Plus (Tokuyama Corporation, Tokyo, Japan), and iBond (Heraeus Kulzer, Hanau, Germany). Ceram X (Dentsply De Trey) was used as a restorative resin composite. The components of each bonding agent are listed in Table 1. Unless otherwise noted, all chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

*Cell culture*

For the cytotoxicity test, L929 mice fibroblasts (ATCC, Rockville, MD, USA) were used according to ISO 10993-5 (Tests for cytotoxicity, *in vitro* method). L929 cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotic solution (100 U $mL^{-1}$ of penicillin-G and 100 μg $mL^{-1}$ streptomycin) at 37°C in a humidified atmosphere (5% $CO_2$/95% air). MEM and FBS (Lot No. 0000185451) were purchased from GIBCO (Carlsbad, CA, USA) and Lonza (Basel, Switzerland), respectively.

*Cytotoxicity test*

The cytotoxicity of the dentine bonding agents were evaluated by serial dilution and agar overlay methods. For the serial dilution method, the L929 cells were incubated in 96-well plates until confluence, and the culture media was replaced with fresh media containing serial dilutes of bonding agent extracts. Cell viability was measured using a Cell Count Kit-8 (WST-8; Dojindo Laboratories, Kumamoto, Japan). After 24 h of treatment, the cells were further incubated for 1 h with WST-8. The optical density at 450 nm was then measured using a plate reader (Sunrise; TECAN, Salzburg, Austria). For the agar overlay method, the L929 cells were incubated in a 100 mm culture dish until confluence, and the culture medium was replaced with 3 mL MEM containing 1.6% agar. After the agar overlay was solidified, sample discs were placed on the agar medium and incubated for 24 h at the culture condition described above. The sample discs were then removed, and the culture was stained with phosphate-buffered

Table 1 Dentin bonding agents used in this study

| Product | Composition | Manufacturer |
|---|---|---|
| Adper Scotchbond Multipurpose Plus | bisphenol-A-diglycidyl methacrylate (Bis-GMA), 2-hydroxyethyl methacrylate (HEMA), glycerol 1,3-dimethacrylate, copolymer of acrylic and itagonic acids, diurethane dimethacrylate, water, ethanol | 3M ESPE, St. Paul, MN, USA |
| Adper Single Bone 2 | Bis-GMA, HEMA, dimethacrylate, fluoride, amines, silica, water, ethanol | 3M ESPE, St. Paul, MN, USA |
| Prime & Bond NT | Dipentaerythritol penta acrylate (PENTA), urethane dimethacrylate (UDMA), cetylamine hydrofluoride, silica, acetone | Dentsply De Trey, Konstanz, Germany |
| Clearfil SE Bond | <Primer> 10-methacryloyloxydecyl dihydrogen phosphate (MDP), (HEMA), DL-camphorquinone, N,N-diethanol-p-touidine, water <Bond> MDP, Bis-GMA, hydrophobic dimethacrylate, HEMA, di-camphorquinone, N,N-diethanol-p-touidine, silica | Kuraray Medical Inc. Osaka, Japan |
| One-Up Bond F Plus | <Bonding Agent A> methacryloylalkyl acid phosphate, multi-functional methacrylic monomers, photo-initiator <Bonding Agent B> methyl methacrylate (MMA), HEMA, fluoro-aluminosilicate glass, photo-initiator, dye-sensitizer, borate derivate, water | Tokuyama Corporation, Tokyo, Japan |
| iBond | UDMA, 4-methacryloxyethyl trimellitate anhydride (4-META), glutaraldehyde, photo-initiator, stabilizer, acetone, water | Heraeus Kulzer, Hanau, Germany |

saline (PBS, pH 7.3) containing 0.02% neutral red for 4 h. Finally, the stained cells were observed microscopically, and the size of the clear zone was measured.

*Preparation of the sample discs and extraction of dentine bonding agents*

Dentine bonding agents (5 μL) were dropped on the upper side of the glass discs (5 mm diameter ×2 mm depth) and cured with a curing light device (Spectrum 800, Dentsply De Trey). Light intensity was set to 300 mW/cm$^2$, and the distance between bonding agents and a light bulb was less than 1 mm. When the primer and adhesive were supplied in separate bottles, 2.5 μL aliquots of both components were dropped in order and cured. In the cytotoxicity test using the serial dilution method, the glass discs covered with bonding agents were immersed in culture media (60 mm$^2$ mL$^{-1}$) for 24 h at 37°C, and the extracts were filtered through 0.22 μm sterile filters and serially diluted before use. To investigate the effects of resin composite on the cytotoxicity of the dentine bonding agents, the agar overlay tests were modified as shown in Fig. 1 and Table 2. In condition A, 5 μL of the dentine bonding agents were applied onto the glass discs, and the glass discs were placed on agar medium with the bonding agents facing the agar. In condition B, dentine bonding agents were smeared inside a circle (5 mm in diameter) on a polypropylene (PP) film (1 mm in width), cured, overlaid by resin composite prepared in a Teflon mold (5 mm in diameter ×2 mm in depth), and then light cured. Ceram X was used as a resin composite since cured Ceram X proved to be non-cytotoxic in the agar overlay test (data not shown). After curing the resin composite by irradiation for 20 s, the PP film was removed. The resin composite discs were then withdrawn from the mold and placed on agar medium with bonding agents facing the agar and then used for cytotoxicity tests. The transfer of bonding agents from the PP film to the resin composite was confirmed by field emission scanning electron microscopy (JSM-6500F, JEOL, Japan). Conditions C, D, and E were devised to investigate the effects of polymerization of the composite resins and oxygen inhibition layer on the cytotoxicity of the bonding agents. In condition C, the bonding agents were directly applied onto the surface of the uncured resin composite discs, and both bound materials were cured. Unlike condition B in which the bonding agents were allowed to contact air only through PP film during polymerization, the air was freely accessible to the surface of the bonding agents in condition C. As a result, it was believed that the oxygen inhibition layer was readily formed in condition C due to sufficient oxygen in the environment. In condition D, the bonding agents were dropped on PP films, and the uncured resin composite discs were laid over the bonding agents. Bonding agents and composite resins were then cured as in condition C, and the PP films were removed, leaving the bonding agents on the resin composites. In condition E, the bonding agents were placed between the PP films and pre-cured bonding agents. The bonding agents were then cured, and the PP films were removed.

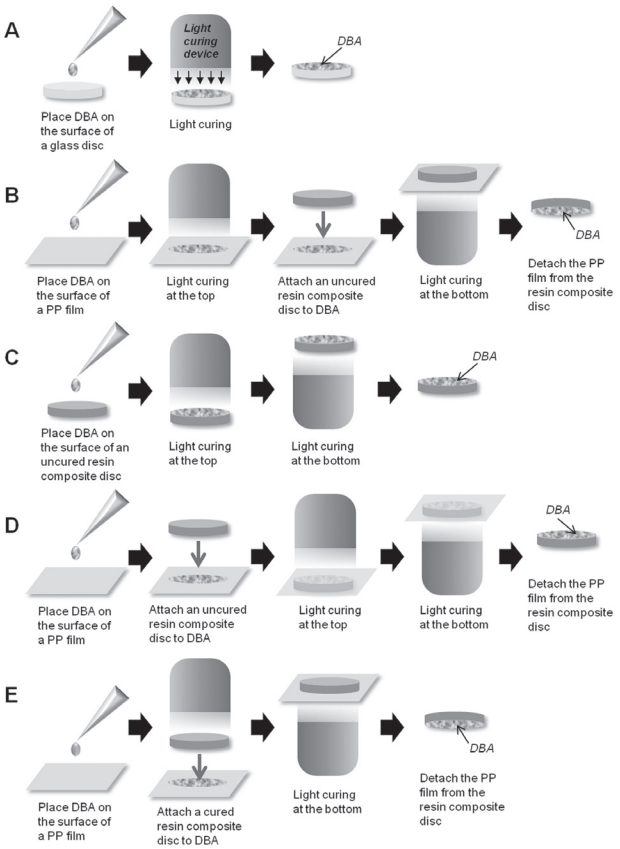



Fig. 1 Schematic diagram of the preparation of test samples for agar overlay cytotoxicity tests. DBA, dentin bonding agent.

*Vickers micro-hardness tests of the dentine bonding agents*

The dentine bonding agents were placed on PP films as described above, cured for 10 s, and adhered to the pre-cured (irradiation for 20 s) or uncured Ceram X resin composite discs. The resin composite discs were then cured by light irradiation to the opposite side for 20 s. The pre-cured and uncured resin composites were irradiated for a total of 40 and 20 s, respectively. After removing the PP film, the surface hardnesses of the dentine bonding agents were measured by a Vickers Micro-Hardness Tester (HMV-2, Shimadzu, Kyoto, Japan) with a load of 98.07 mN 10$^{-1}$ s. At this load, the resin composite discs cured for 20 s and 40 s were equal, with a Vickers hardness number of 100. Thus, the difference in the surface hardness of the bonding agents did not originate from the hardness of the composite resins beneath the bonding agents.

*Statistical analysis*

In Fig. 3, the cytotoxicity of the bonding agents on the glass discs (A) was compared with that obtained under a condition mimicking clinical tooth restoration (B)

Table 2 Conditions for the agar diffusion cytotoxicity test of dentine bonding agents

| Test conditions | Application of a DBA[1] | Location of a DBA in the curing step | RRC[2] curing step |
|---|---|---|---|
| A: Conventional | Onto a glass disc | On a glass disc | — |
| B: Similar to clinical application | Onto a PP[3] film | On a PP film (before attachment to an RRC disc) | After attachment of a DAB to an RRC disc (DAB located between a PP film and an RRC disc at the RRC curing step) |
| C: Affected by polymerization of RRC, sufficient oxygen environment | Onto an uncured RRC disc | On an uncured RRC disc (in an open-air environment) | After curing of a DAB on an RRC disc |
| D: Affected by polymerization of RRC, limited oxygen environment | Onto a PP film | Between a PP film and an uncured RRC disc (after attachment to an uncured RRC disc) | After curing of a DAB (DAB located between a PP film and an RRC disc at the RRC curing step) |
| E: Unaffected by polymerization of RRC, limited oxygen environment | Onto a PP film | Between a PP film and a pre-cured RRC disc (after attachment to a pre-cured RRC disc) | Before attachment of a DAB to an RRC disc |

[1]DBA: Dentine bonding agent
[2]RRC: Restorative resin composite
[3]PP: Polypropylene

using a paired *t*-test. Condition D was compared with conditions C and E to investigate the effects of resin composite polymerization and the oxygen inhibition layer on the cytotoxicity of the bonding agents. The surface hardnesses of the bonding agents on the pre-cured composite resins were compared to those of the post-cured composite resins in Fig. 5. The values with $p<0.05$ were considered statistically significant.

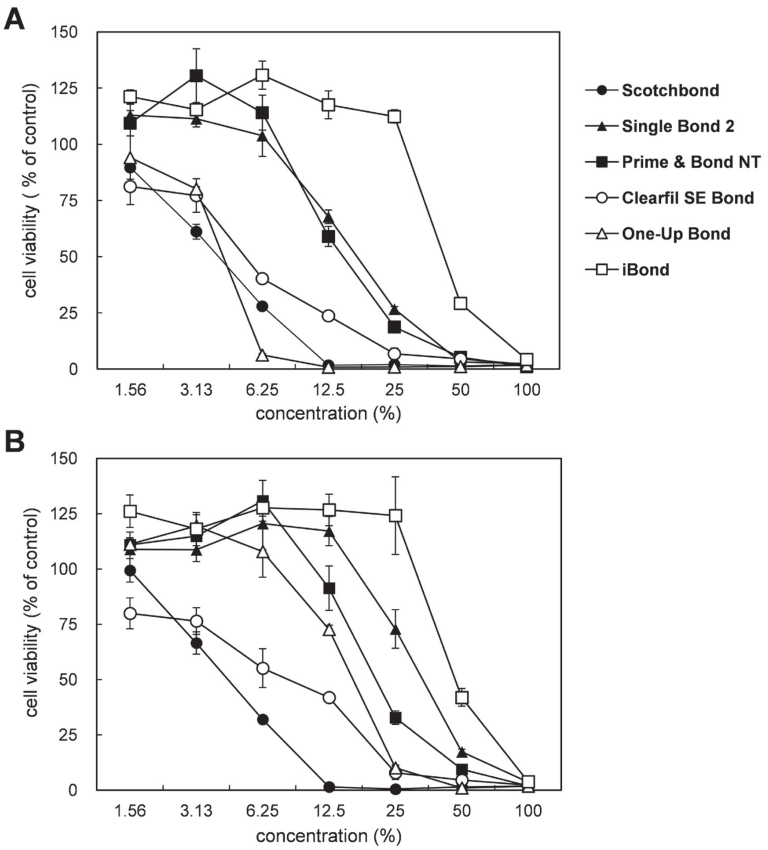
## RESULTS

*Cytotoxicity of the extracts of the dentine bonding agents*

Cytotoxicity of the various dentine bonding agents were evaluated by a serial dilution method. Glass discs covered with bonding agents were left in the air to evaporate the solvent and then cured with an LED light or were cured without evaporation of the solvent in order to investigate the effects of solvent on cytotoxicity. Cytotoxicity values of the extracts obtained from the unevaporated and evaporated bonding agents are shown in Fig. 2. Cell viability was completely abolished by treatment with the undiluted extracts of all the bonding agents regardless of evaporation. As shown in Fig. 2A, the bonding agents not included in the evaporation process were divided into three groups according to cytotoxicity: highly cytotoxic (Adper Scotchbond Multipurpose Plus (Scotchbond), One-Up Bond F Plus (One-Up Bond), and Clearfil SE Bond), moderately cytotoxic (Prime & Bond NT and Adper Single Bone 2 (Single Bond 2)), and weakly cytotoxic (iBond). After evaporation of the solvent, cytotoxicity was attenuated in all the bonding agents, especially in One-Up Bond and Single Bond 2 (Fig. 2B). The $LC_{50}$ increased 3.7- and 1.9-fold in One-Up Bond and Single Bond 2, respectively, while the cytotoxicity values of Scotchbond and iBond were less affected by the evaporation process. After evaporation and light-curing, the ranking of cytotoxicity based on $LC_{50}$ was Scotchbond > Clearfil SE Bond > One-Up Bond > Prime & Bond NT > Single Bond 2 > iBond (Fig. 2C).

*Effects of restorative composite resins on the cytotoxicity of the dentine bonding agents*

As described in Table 2, agar overlay tests were performed in five different conditions in order to observe the effects of restorative composite resins on the cytotoxicity of dentine bonding agents (Fig. 3). The width of the clear zone represents the cytotoxicity of the intact bonding agents in condition A. It was found that Scotchbond was the most cytotoxic agent, followed by One-Up Bond, Prime & Bond NT, Clearfil SE Bond, iBond, and Single Bond 2. Although the rankings of cytotoxicity were not identical between the serial dilution and agar overlay methods, Scotchbond was the most toxic bonding agent, and iBond was the least toxic in all conditions. In condition B that mimicked the clinical restoration process, the width of the clear zone was considerably reduced to less than 2 mm in highly and moderately toxic agents including Scotchbond, One-Up Bond, Prime & Bond NT, and Clearfil SE Bond. Statistically significant differences between the results of condition A and B were shown in all bonding agents except for Single Bond 2. This result suggests that




C

| Dentin bonding agent | $LC_{50}$ (%) | |
|---|---|---|
| | unevaporated | evaporated |
| Scotchbond | 4.1 | 5.2 |
| Single Bond 2 | 18.3 | 35.6 |
| Prime & Bond NT | 15.4 | 21.1 |
| Clearfil SE Bond | 5.8 | 9.4 |
| One-Up Bond | 4.8 | 17.6 |
| iBond | 44.7 | 47.0 |

Fig. 2 Cytotoxicity of the dentine bonding agent extracts.
(A) The dentine bonding agents were placed on glass discs, cured by irradiation, and immersed in culture media to prepare extracts. Cells were treated with serial dilution of the extracts for 24 h, and the cell viability was measured with WST-8. Each value and error bar represents the mean±SD of triplicate experiments.
(B) Solvents of the bonding agents on glass discs in (A) were evaporated before light curing, and the extracts were prepared for the evaluation of cytotoxicity.
(C) $LC_{50}$ obtained from (A) and (B).

polymerization of adjacent resin composite attenuated cytotoxicity, or that the cytotoxic oxygen inhibition layer was not formed in this condition due to the PP film functioning as a barrier between the air and bonding agents. To determine which factor contributed to the reduction of cytotoxicity, conditions C, D, and E were employed. Reduction of cytotoxicity in condition C showed that polymerization of composite resins affected the toxicity. On the other hand, a decrease in cytotoxicity in condition E demonstrated the importance of the cytotoxic oxygen inhibition layer. In condition D, for either or both factors, the polymerization of the composite resins and removal of the oxygen inhibition layer contributed to the reduction of cytotoxicity. Interestingly, the determining factor for cytotoxicity was not the same for the different bonding agents. The

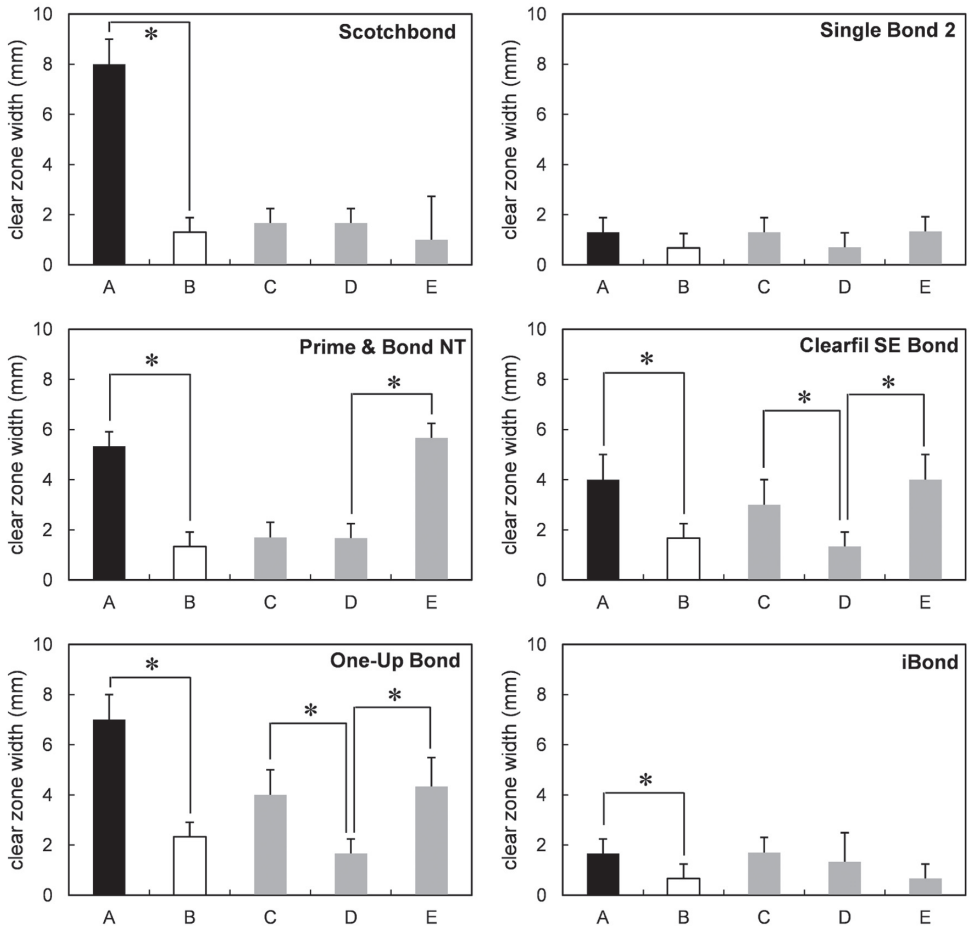



Fig. 3 Effects of composite resins on the cytotoxicity of dentine bonding agents.
Preparation of the test samples and light curing were performed according to the test conditions in Table 1 and 'Materials and Methods' section. Cytotoxicity of the test samples was assessed by the agar overlay method. Each value and error bar represents the mean±SD of triplicate experiments. *Significant difference at $p<0.05$.

clear zone width of Prime & Bond NT was reduced in condition C. There was a significant difference between D and E. In Clearfil and One-Up Bond bonding agents, only condition D was able to reduce the size of the clear zone to the level of condition B. As for Scotchbond, cytotoxicity in conditions C and E was reduced to the level of condition D. The complete reduction of the clear zone in condition C indicated that the oxygen inhibition layer was not involved in the cytotoxicity of Scotchbond. Pre-cured resin composite was also effective in down-regulating the toxicity of the agent, as shown in condition E. Therefore, it is likely that light-curing polymerization of Scotchbond is readily accomplished even in the pre-cured composite resins regardless of air condition.

In conditions B, D, and E, shown in Fig. 3, the tests were performed on a premise that dentine bonding agents on the PP films were transferred to the surface of the resin composite discs after removal of the PP films. To confirm the transfer of the bonding agents, the surface of the PP film and composite resins was observed with a scanning electron microscope (Fig. 4). The surface of an untreated PP film was seen as flat and smooth at 1,000× magnification (Fig. 4A), while the composite resins exhibited an uneven and rough surface due to the presence of silicate fillers (Fig. 4B). The samples C1, D1, E1, F1, G1, and H1 represent bonding agents on PP films after light curing. Scotchbond, Single Bond 2, Prime & Bond NT, and iBond exhibited a smooth and undulated surface. The undulation seemed to occur due to alteration with electron beam microscopy. Clearfil SE Bond exhibited a wrinkled surface, and One-Up Bond was fuzzy with no undulations. Therefore, the surfaces of all the bonding agents were clearly distinguishable from that of the PP film and resin composite. After the PP films were removed from the resin composites, the PP films exhibited flat and smooth surfaces which were identical to the intact PP film, and the rough surfaces disappeared from the resin composites. These changes in

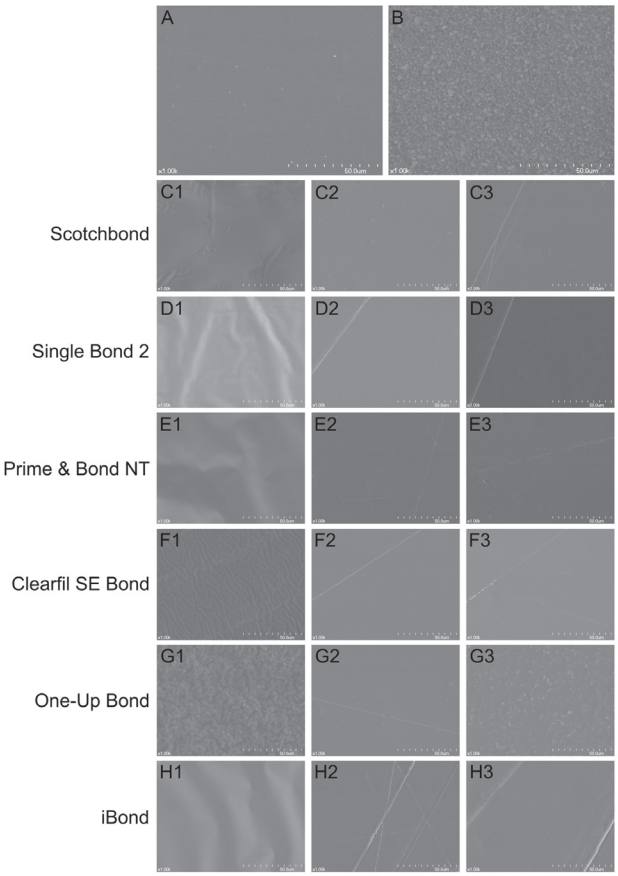



Fig. 4 SEM images of the bonding agents on PP films and resin composites.
A clean PP film (A) was covered with bonding agents, air-dried, cured, and analyzed with SEM (C1–H1). The PP films were laid on composite resins to position the bonding agents between the PP films and resin composites. Opposite sides of the composite resins were irradiated for light curing. After removing the PP films, the surface of the detached PP films (C2–H2) and composite resins (C3–H3) were observed. Panel B is a surface of cured composite resins without bonding agents.

surface morphology indicate that all the bonding agents were transferred from PP films to the resin composites.

*Vickers micro-hardness of the dentine bonding agents*

As shown in Fig. 3, the cytotoxicity of the dentine bonding agents was attenuated by the polymerization of the resin composites. This suggests that the composite resins promoted the polymerization of the bonding agents, reducing the release of cytotoxic components. To verify the effects of polymerization of composite resins on the physical properties, we measured the surface hardness of the bonding agents on resin composite discs. The Vickers hardness numbers of the bonding agents on pre-cured composite resins were compared

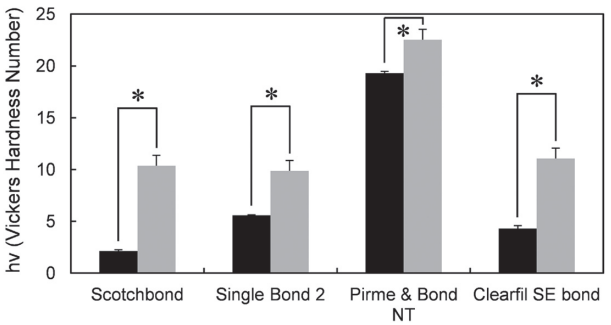



Fig. 5 Vickers hardness of the dentine bonding agents on pre-cured and post-cured resin composites. Bonding agents on the PP films were transferred to pre-cured and uncured resin composites, and the uncured composite resins were irradiated on opposite sides. After removing the PP films, the surface hardnesses of the bonding agents were measured with a Vickers hardness tester. Each value and error bar represents the mean±SD of triplicate experiments. *Significant difference at $p<0.05$.

to those obtained on post-cured resin composites. When the bonding agents on the PP films were cured and transferred onto pre-cured resin composites, the Vickers hardness numbers of Prime & Bond NT, Single Bond 2, Clearfil SE Bond, and Scotchbond were 19.3, 5.6, 4.3, and 2.1, respectively (Fig. 5). The hardness number of One-Up Bond and iBond were not constant, indicating instability of surface properties among the detection areas. When the composite resins were cured after binding to the bonding agents, the Vickers hardness number of all the bonding agents increased significantly compared to values obtained with the pre-cured resin composites. This result suggests that the polymerization of composite resins strengthened the surface hardness of the attached bonding agents through the augmentation of resinous polymerization.

## DISCUSSION

Resin monomers such as triethyleneglycol dimethacrylate and 2-hydroxyethyl methacrylate have been found to deplete glutathione and induce cellular oxidative stress, resulting in cytotoxicity[15,16]. Resin-based dentine bonding agents were also found to reduce intracellular glutathione[17]. Their cytotoxicity effects were prevented with antioxidants[18,19], suggesting that resin monomers play a role in the cytotoxicity of bonding agents. Eluting resin monomers from resin-based materials generally depends on the degree of polymerization. Therefore, the cytotoxicity effects of the bonding agents shown in Fig. 2 are likely due to the incomplete polymerization of the resin matrix, which allows for the release of a large amount of resin monomers. In Fig. 2, the bonding agents were cured

according to the recommendation of the manufacturers. However, it is possible that the bonding agents are cured further with the curing of overlaid composite resins during tooth restorations because both materials are used in combination in dental clinics. To investigate whether composite resins affect the cytotoxicity of bonding agents, we applied the various conditions shown in Table 2. As shown in Fig. 3, it was evident that composite resins reduced the cytotoxicity of the bonding agents except for the slightly cytotoxic Single Bond 2. This suggests that the polymerization of the bonding agents was enhanced by application of the resin composites. The polymerization reaction initiated from composite resins is thought to cross the boundary of two different materials because the pre-cured composite resins were less effective in reducing the cytotoxicity of the Prime & Bond NT, Clearfil SE Bond, and One-Up Bond bonding agents. However, the cytotoxicity of Scotchbond was reduced by curing after contact even with pre-cured resin composites. This result suggests that the resin matrix facilitates the polymerization of Scotchbond even though the bonding agents do not make contact with the pre-cured composite resins in clinical practice.

In addition to the curing effect of resin composites, we also considered the oxygen inhibition layer in assessing the toxicity of bonding agents. In a conventional evaluation method of cytotoxicity, the bonding agents were irradiated after being exposed to air before extraction or before making direct contact with cells or agar. On the surface of the bonding agents, it is expected that the resin monomers in the oxygen inhibition layer remain unpolymerized[20]. The resin monomers in the oxygen inhibition layer are known to affect the biological and physical properties of resin biomaterials. In tooth restorations, dentine bonding agents were applied on dentine, cured, and overlaid with resin composites. As a result, the oxygen inhibition layer would not form on dentine but between the bonding agents and resin composites. Considering the effects of composite resins on the polymerization of bonding agents, the oxygen inhibition layer is expected to disappear after curing of the overlaid resin composites. Therefore, the oxygen inhibition layer is not expected to remain after the restoration is complete. In condition B of Table 2 where clinical application was mimicked, PP films were used instead of dentine. The limited penetration of oxygen through the PP films was expected to inhibit the generation of the oxygen inhibition layer and reduce cytotoxicity. As shown in Fig. 3, the cytotoxicity of only the Clearfil SE Bond and One-Up Bond bonding agents was likely affected by the presence of the oxygen inhibition layer, which was demonstrated by the significant difference of cytotoxicity between conditions C and D. In the Prime & Bond NT bonding agent, it is possible that the PP films could not prevent the access of oxygen to the surface. However, it is certain that the polymerization of composite resins alone is sufficient to prevent the cytotoxicity of bonding agents.

Generally, cytotoxic resin monomers released from resin-based dental materials that is incompletely polymerized is thought to be a major reason for cytotoxicity of the materials, suggesting that the less polymerized resin-based materials release the more cytotoxic resin monomers. In this study, the cytotoxicity of dentin bonding agents was decreased by resin composites, leading us to speculate that less amount of resin monomers are released from bonding agents due to an increase in the degree of polymerization *via* curing of adjacent composite resins. To investigate whether the curing of composite resins enhanced the polymerization of bonding agents, we measured the surface hardness of bonding agents instead of using chemical analysis such as Fourier Transform Infrared (FTIR) spectrometry because the composite resins and bonding agents could not be physically separated in the assay system of this study. As shown in Fig. 5, the four bonding agents exhibited different Vickers hardness numbers on pre-cured resin composite. Prime & Bond NT exhibited the highest value, followed by Single Bond 2, Clearfil SE bond, and Scotchbond. Among those materials, Single Bond 2 showed the weakest cytotoxicity (Figs. 2, 3). Therefore, the degree of cytotoxicity of bonding agents cannot be predicted by the surface hardness. However, an increase in surface hardness by curing of attached composite resins resulted in a decrease in cytotoxicity. Therefore, the polymerization reaction of composite resins likely propagated to the bonding agents over the boundary of those materials, resulting in the reduction of resin monomer elution and the cytotoxicity of the bonding agents.

The cytotoxicity of dentine bonding agents has been reported in previous studies in which the toxicity was evaluated without considering the clinical conditions. In the study using a combination of dentine bonding agents and composite resins[13], the cytotoxicity of the bonding agents could not be evaluated independently from that of resin composites. By employing a non-toxic resin composite and agar overlay method in this study, the cytotoxicity of the bonding agents was successfully evaluated without resin composite intervention. The combination of restorative composite resins and bonding agents significantly reduced the cytotoxicity of bonding agents, possibly due to the reinforced polymerization of the resin components.

The current result may explain the insignificant pulp reactions to bonding agents in animal models and dental clinics. Furthermore, the evaluation method devised in this study is expected to provide a more precise prediction of biocompatibility to more closely resemble *in vivo* or clinical situations. Although this study showed a significant reduction of cytotoxicity of bonding agents by resin composites, dentine undoubtedly plays a role as a barrier preventing the free diffusion of toxic components from the bonding agents. Inflammatory reactions by the direct application of dentine bonding agents to pulpal tissue in human teeth, even with composite resin overlays, indicate the importance of dentine barriers for the reduction of biological side

effects[21]. Therefore, a combination of dentine barrier systems and resin composite overlays are expected to closely mimic tooth restorations in the clinic and to provide precise information relating to the biological events of dentine bonding agents in the oral cavity.

## CONCLUSION

This study showed that restorative resin composites dramatically reduced the cytotoxicity of dentine bonding agents. The increased surface hardness of bonding agents by light-curing of overlaid resin composites suggests that curing of resin composites enhanced the resin polymerization of bonding agents and reduced the release of cytotoxic resin components. These results explain the reason why the severely cytotoxic dentine bonding agents are successfully applied to tooth restoration in clinics.

## ACKNOWLEDGMENTS

This research was supported by a grant (11172KFDA501) from Korea Food & Drug Administration in 2011.

## REFERENCES

1) Chen RS, Liu CC, Tseng WY, Jeng JH, Lin CP. Cytotoxicity of three dentin bonding agents on human dental pulp cells. J Dent 2003; 31: 223-229.
2) Hashieh IA, Cosset A, Franquin JC, Camps J. *In vitro* cytotoxicity of one-step dentin bonding systems. J Endod 1999; 25: 88-92.
3) Karapınar-Kazandağ M, Bayrak OF, Yalvaç ME, Ersev H, Tanalp J, Sahin F, Bayırlı G. Cytotoxicity of 5 endodontic sealers on L929 cell line and human dental pulp cells. Int Endod J 2011; 44: 626-634.
4) Yasuda Y, Inuyama H, Maeda H, Akamine A, Nör JE, Saito T. Cytoxicity of one-step dentin-bonding agents toward dental pulp and odontoblast-like cells. J Oral Rehabil 2008; 35: 940-946.
5) Costa CAS, Teixeira HM, Nascimento ABL, Hebling J. Biocompatibility of two current adhesive resins. J Endod 2000; 26: 512-516.
6) Cui C, Zhou X, Chen X, Fan M, Bian Z, Chen Z. The adverse effect of self-etching adhesive systems on dental pulp after direct pulp capping. Quintessence Int 2009; 40: 26-34.
7) Meryon SD, Brook AM. *In vitro* comparison of the cytotoxicity of twelve endotontic materials using a new technique. Int Endod J 1990; 23: 203-210.
8) Ulker HE, Sengun A. Cytotoxicity evaluation of self adhesive composite resin cements by dentin barrier test on 3D pulp cells. Eur J Dent 2009; 3: 120-126.
9) Vajrabhaya LO, Pasasuk A, Harnirattisai C. Cytotoxicity evaluation of single components dentin bonding agents. Oper Dent 2003; 28: 440-444.
10) Bouillaguet S, Virtiliggo M, Wataha J, Ciucchi B. The influence of dentine permeability on cytotoxicity of four dentine bonding systems *in vitro*. J Oral Rehabil 1998; 25: 45-51.
11) Kusdemir M, Gunal S, Ozer F, Imazato S, Izutani N, Ebisu S, Blatz MB. Evaluation of cytotoxic effects of six self-etching adhesives with direct and indirect contact tests. Dent Mater J 2011; 30: 799-805.
12) Vajrabhaya L, Korsuwannawong S, Bosl C, Schmalz G. The cytotoxicity of self-etching primer bonding agents *in vitro*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009; 107: 86-90.
13) Koga K, Tsujimoto A, Ishii R, Iino M, Kotaku M, Takamizawa T, Tsubota K, Miyazaki M. Influence of oxygen inhibiton on the surface free-energy and dentin bonding strength of self-etch adhesives. Eur J Oral Sci 2011; 119: 395-400.
14) Franz A, König F, Luca T, Watts DC, Schedle A. Cytotoxic effects of dental bonding substance as a function of degree of conversion. Dent Mater 2009; 25: 232-239.
15) Chang HH, Guo MK, Kasten FH, Chang MC, Huang GF, Wang YL, Wang RS, Jeng JH. Stimulation of glutathione depletion, ROS production and cell cycle arrest of dental pulp cells and gingival epithelial cells by HEMA. Biomaterials 2005; 26: 745-753.
16) Englemann J, Leyhausen G, Leibfritz D, Geurtsen W. Effect of TEGDMA on the intracellular glutathione concentration of human gingival fibroblasts. J Biomed Mater Res 2002; 63: 746-751.
17) Huang FM, Li YC, Lee SS, Chang YC. Cytotoxicity of dentine bonding agents on human pulp cells is related to intracellular glutathione levels. Int Endod J 2010; 43: 1091-1097.
18) Demirci M, Hiller KA, Bosl C, Galler K, Schmalz G, Schweikl H. The induction of oxidative stress, cytotoxicity and genotoxicity by dental adhesives. Dent Mater 2008; 24: 362-371.
19) Kim NR, Park HC, Kim I, Lim BS, Yang HC. *In vitro* cytocompatibility of N-acetylcysteine-supplemented dentin bonding agents. J Endod 2010; 36: 1844-1850.
20) Gauthier MA, Stangel I, Ellis TH, Zhu XX. Oxygen inhibition layer in dental resins. J Dent Res 2005; 84: 725-729.
21) Subay RK, Demirci M. Pulp tissue reactions to a dentin bonding agents as a direct capping agent. J Endod 2005; 31: 201-204.