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Effect of light curing type on cytotoxicity of dentine-bonding agents

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Abstract

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Aim To compare the cytotoxic effects of dentine-bonding agents (DBAs) polymerized with two different curing units at 24 h and 72 h on L-929 cells.

Methodology Disc-shaped test samples of light-activated DBAs were prepared according to manufacturers' instructions and cured with either conventional quartz tungsten halogen or light-emitting diode light curing units (LCUs). After curing, the samples were transferred into a culture medium for 24 h. Eluates were obtained and pipetted onto L-929 mouse fibroblast cultures (3×10^4 cells per well), incubated for evaluation after 24 and 72 h. After both incubation periods, measurements were performed by an dimethylthiazol diphenyltetrazolium assay. The degree of cytotoxicity for each sample was determined according to the reference value represented by the cells with a control (culture without sample). Statistical significance was determined by a three-way analysis of variance followed by the Mann–Whitney *U*-test.

Results No significant three-factor interaction occurred amongst LCUs, DBAs and time factors ($P = 0.955$). LCUs and DBAs had a significant two-factor interaction ($P < 0.001$). In general, the test materials cured with the light-emitting diode LCU demonstrated higher cell survival rates when compared with the those cured with the quartz tungsten halogen.

Conclusions Differential toxic effects of the DBAs cured with the quartz tungsten halogen or the light-emitting diode on the fibroblast cells may prove to be very important when suitable DBAs or LCUs are used for operative restorations.

Keywords: cytotoxicity, dentin bonding agents, light curing units, quartz tungsten halogen.

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Introduction

Various dentine-bonding agents (DBAs) have been developed for bonding restorative resin to dentine (Chen *et al.* 2001). The major goals of using DBAs are to enhance the bonding between resin and the tooth structure to increase the retention of restorations, to reduce microleakage across the dentine–resin interface and to dissipate the occlusal stress (Chen *et al.* 2003). The recent developments in DBAs have greatly changed the way restorative dentistry is accomplished (Szep *et al.* 2002).

Different types of light curing units (LCUs) have been proposed for the photopolymerization of light-activated restorative materials including conventional quartz tungsten halogen lights and new photoactivation techniques, such as intermittent light (Tarle *et al.* 1998, Obici *et al.* 2002), plasma arc curing (PAC) (Peutzfeldt *et al.* 2000) and, more recently, a new technology employing light-emitting diode (Jandt *et al.* 2000, Kurachi *et al.* 2001) or laser (Price *et al.* 2003). The differences amongst the LCUs reflect the differences amongst materials in terms of depth and degree of polymerization. The degree of light induced

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conversion of monomers to polymers is influenced by various parameters, such as the intensity of the light around the wavelength triggering level, the photoinitator system, the duration of irradiation, concentrations, types and mixtures of photoinitators, co-initators, stabilizers and inhibitors, as well as the types and proportions of monomers and fillers (Moszner *et al.* 2005). Adequate polymerization is the most important factor in maximizing physical properties and clinical performance. Problems associated with inadequate polymerization include inferior physical properties, solubility in the oral environment and increased microleakage with resultant recurrent decay and pulpal irritation. On the other hand, the amounts of leachable residual monomers may vary with the light source used for curing (Jandt *et al.* 2000, Stahl *et al.* 2000, Kurachi *et al.* 2001). Amongst the different LCUs available in dental practice, halogen lamps are the most frequently used, although recently the light-emitting diode technology has been successfully proposed (Jandt *et al.* 2000, Stahl *et al.* 2000, Knezevic *et al.* 2001, Kurachi *et al.* 2001). Compared with quartz tungsten halogen lights, light-emitting diodes convert electricity into light more efficiently, produce less heat, and are more robust. Light-emitting diodes also last for thousands of hours in contrast to the 30- to 50-h life span of a conventional quartz tungsten halogen light bulb (Price *et al.* 2005).

Several authors have demonstrated the possibility of using light-emitting diodes as an alternative to conventional halogen lamps in their studies on the depth of cure (Jandt *et al.* 2000), flexibility (Stahl *et al.* 2000), Knoop or Vickers hardness (Jandt *et al.* 2000, Kurachi *et al.* 2001, Mills *et al.* 2002, Uhl *et al.* 2002) and degree of conversion (Jandt *et al.* 2000). Nomura *et al.* (2002) have reported that the resins cured with light-emitting diode units have a more stable internal structure than those cured with conventional LCUs based on a thermal analysis.

Numerous investigators of DBAs have focused on their chemistry on bonding strength, or on their effects on microleakage (Huang *et al.* 2003). Biological compatibility is one of the most important requirements for DBAs, because the bonding agents usually remain in close contact with living dental tissues over a long period of time. The elution of unpolymerized resin components becomes significant when these materials diffuse across dentine from a fresh cavity preparation and are of concentrations high enough to produce a biological effect upon the dental pulp (Jontell *et al.* 1995). The toxic potential of components of DBAs has been shown in both *ex vivo* (Ratanasathien *et al.* 1995, Bouillaguet *et al.* 1998, Costa *et al.* 1999, Demarco *et al.* 2001, Szep *et al.* 2002, Huang *et al.* 2003) and *in vivo* (Demarco *et al.* 2001) studies. It was found that hydrophilic monomers, such as 2-hydroxyethyl methacrylate (HEMA) or Triethylene glycol dimethacrylate (TEGDMA), were cytotoxic but to a lesser degree than the more hydrophobic monomers bisphenol glycidylmethacrylate (bis-GMA) or urethane dimethacrylate (UDMA). Interaction of different monomers has been demonstrated, with the potential of increasing the toxicity of the single components (Ratanasathien *et al.* 1995). Less toxic hydrophilic monomers may act as carriers for more toxic hydrophobic monomers. Dentine-bonding components, such as HEMA or TEGDMA, may also have an influence on the immune system, leading to both immunosuppression and immunostimulation (Rakich *et al.* 1999).

There is little information on biocompatibility tests, especially on the effect of light curing type on cytotoxicity for DBAs, which is directly related to clinical success of the dental restorative materials.

The aim of this study was to compare the cytotoxic effects of a series of DBAs that were polymerized with conventional quartz tungsten halogen and light-emitting diode on L–929 mouse fibroblast cells over 24 h and 72 h periods.

Material and Methods

Cells

The cells used for the experiments were L-929 mouse fibroblasts (L-929 An_2 HÜKÜK 95030802; Ankara Şap Enstitüsü, Ankara, Turkey). The cells were grown as monolayer cultures in T-25 flasks (Costar, Cambridge, MA, USA), subcultured three times a week at 37 °C in an atmosphere of 5% CO_2 in air and 100% relative humidity and maintained at third passage. The culture medium was Dulbecco's modified Eagle medium (DMEM)/Ham's F12 nutrient mixture (1 : 1; Sigma, St Louis, MO, USA) supplemented with 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany) without antibiotics. Adherent cells at a logarithmic growth phase were controlled under an inverted tissue culture microscope (Olympus CK40, Japan) and detached with a mixture of 0.025% trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid (EDTA; Sigma), incubated for 2–5 min at 37 °C and used for cell inoculation.

Sample preparation

The test materials are listed in Table 1. Three dentine-bonding systems were used. One bottle adhesive systems: Adper Single Bond 2 (SB2, 3 M Espe, St Paul, MN, USA), SwisstecSL Bond (SLB, Coltene, Whaladent AG, Altstatten, Switzerland) and Pentron Bond 1 (PB1, Pentron Clin. Tech., LLC, Wallingford USA); self-etching adhesive systems: Xeno III (XeIII, Dentsply De Trey Gmbh, Germany) and Clearfil SE Bond (CSB, Kuraray Med. Inc., Okuyama, Japan); an All-in-one adhesive system (one-step adhesive system): Adper Prompt L-Pop (PLP, Espe Dental-Medizin, Seefeld, Germany).

Fourty disc-shaped samples (6 mm diameter × 1 mm thickness) were prepared for each test material; 20 discs were prepared using the quartz tungsten halogen and 20 discs using the light-emitting diode. All samples were prepared by the same operator.

Photoactivation was performed with the quartz tungsten halogen LCU (Hilux Curing Light-Optimax, Benlioğlu Dental Inc., 1071031 Ankara, Turkey) and the light-emitting diode LCU (Elipar Freelight 2, 3 M ESPE, 939820000601, Seefeld, Germany). Standard exposure mode, providing full light intensity for the entire exposure period was chosen with both LCUs. The quartz tungsten halogen LCU, that was used in the study had a light guide tip diameter of 11 mm with an irradiance of 650 mW cm^{-2} and wavelength of 450–520 nm. The light-emitting diode LCU had a light guide tip diameter of 8 mm with irradiance of 1000 mW cm^{-2} with a wavelength of 430–480 nm according to the manufacturer.

The DBAs were shaken and then poured into sterile circular polytetrafluoroethylene moulds. The light tip (cleaned with ethanol) was applied directly on the mould edge (Spagnuolo *et al.* 2004) and the DBAs were

Table 1 Test materials and their composition according to manufacturers

Trade name	Composition	Manufacturer's instructions	LOT number	Manufacturer
Swisstec SL Bond (SLB)	HEMA, Hydroxpropyl methacrylate, Glycerol dimethacrylate, Polyalkenoate methacrylized, UDMA	30 s	NH102	Coltene Whaledent AG, Altstätten, Switzerland
Clearfil SE Bond (CSB)	MDP, bis-GMA, HEMA, hydrophobic dimethacrylate DL-camphorquinone *N,N*-diethanol-P-toluidine silanated colloidal silica	10 s	41222	Kuraray Medical INC, Okayama, Japan
Adper Single Bond 2 (SB 2)	bis-GMA, HEMA, dimethacrylates, ethanol, water, photoinitiator system, methacrylate functional copolymer of polyacrylic	10 s	20050308	3M ESPE Dental Products, St Paul, MN, USA
Adper Prompt L-Pop (PLP)	Methacrylic phosphates, initiator, stabilizer, fluoride, water	10 s	L2 201130	ESPE Dental-Medizin, Seefeld, Germany
Xeno III (Xe III)	Liquid A: HEMA, purified water, ethanol, BHT, highly dispersed silicon dioxide Liquid B: Pyro-EMA, Pem-F, UDMA, BHT, CQ, ethyl-4-dimethylaminobenzoate	10 s	304001675	Dentsply DeTrey GmbH, Germany
Pentron Bond 1 (PB 1)	PMGDM, HEMA, light curing initiator, phosphoric acid, nonsilicate based, acetone, water soluble polymer, thickening agent	10 s	99490	Pentron Clinical Technologies, L.L.C, Wallingford, CT, USA

bis-GMA: bisphenol glycidylmethacrylate.
HEMA: 2-hydroxyethyl methacrylate.
BHT: butylated hydroxy toluene.
UDMA: urethane dimethacrylate.
MDP: 10-methacryloyloxydecyl dihydrogen phosphate.
Pyro-EMA: phosphoric acid modified methacrylacte resin.
Pem-F: mono fluoro phosphazene modified polymethacrylate resin.
CQ: camphorquinone.
PMGDM: pyromellitic acid diethyl methacrylate.

cured according to the manufacturer's curing time as shown in Table 1, under aseptic conditions at laminar flow (Holten, Class II, Denmark). The freshly prepared DBA samples were placed immediately at the bottom of six-well plates (Costar, Cambridge MA, USA).

The ratio of the surface area of the disc samples to the extraction volume was 0.5 cm^2 mL^{-1} in this study, which is in line with ISO 10993-12:1996. The samples were placed in DMEM/F12 with 10% FBS and incubated at 37 °C in an atmosphere of 5% CO_2 in air without agitation for 24 and 72 h periods. After the incubation, the extracts were filtered through 0.22-μm cellulose acetate filters (Millipore; Sigma) and then they were used to evaluate cytotoxicity.

Cytotoxicity testing (MTT assay)

The L-929 cell suspension with DMEM/F12 with 10% FBS and 1% antibiotic was prepared at a concentration of 3×10^4 cells mL^{-1} and inoculated onto 96-well cluster cell culture plates (100 μL per well). The multiwell plates were incubated at 37 °C, 5% CO_2 in air for 24 h. After 24 h, the culture medium was removed from the wells and equal volumes (100 μL) of the extracts were added into each well. In control wells, 100-μL DMEM/F12 with 10% FBS and 1% antibiotic was added. Then 96-well cluster cell culture plates were incubated for 24 h at 37 °C. After the 24 and 72 h incubation period test extracts were removed. Following removal of the test extracts, 100 μL per well DMEM/F12 with 10% FBS and 1% antibiotic and 12-μL MTT (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were added to each well and incubated in a dark environment for 4 h at 37 °C. After incubation 96 wells were checked for formazan crystals with inverted tissue culture microscope. MTT was aspirated and 100 μL per well of isopropanol (Merck, Darmstadt, Germany) was added to each well. Subsequently, the absorbance at 570 nm was measured using a UV–visible spectrophotometer (LPB Pharmacia, Bromma, Sweden).

Then the viable cells were counted under a light microscope and calculated as a percentage of the controls. Triplicate experiments were performed throughout this study.

Statistical analysis

The cytotoxic effects of the DBAs, the LCUs and the exposure times on the fibroblast cells' survival rates were evaluated by a three-way analysis of variances. Then, two independent samples were compared by the nonparametric two-independent sample Mann–Whitney *U*-test. All statistical analyses were performed with the SPSS 11.5 statistical software package (SPSS Inc., Chicago, IL, USA).

Results

The results in Fig. 1 demonstrated that all freshly prepared materials (cured with the quartz tungsten halogen or the light-emitting diode) reduced cell numbers compared with the control (culture without sample).

According to the three-way ANOVA, there was no significant three-factor interaction amongst the LCUs, the DBAs and the time factors ($F = 0.214$, $P = 0.955$). The LCUs and the experimental time factors had no two-factor interaction ($F = 0.828$, $P = 0.367$). Likewise the DBAs and the experimental time (24–72 h) factors had no two-factor interaction ($F = 0.589$, $P = 0.708$). However, the LCUs and DBAs had a significant two-factor interaction ($F = 5.662$, $P < 0.001$). Therefore, the effects of LCUs were evaluated for each DBA (Table 2).

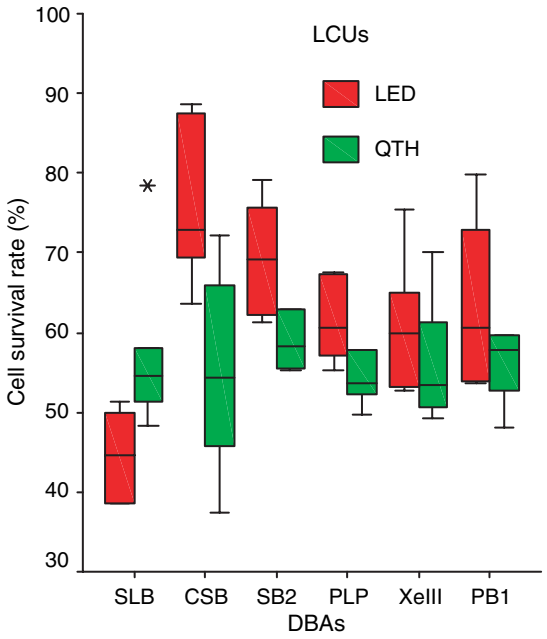

Figure 1 The distribution of cell survival rates (%) on light curing units (LCUs) in each dentine-bonding agents (DBAs). Cell survival rates were expressed as a percentage of controls (cultures without samples). Bars show the mean and standard deviations of three independent experiments.

Table 2 The comparisons cell survival rates between light curing units (LCUs) in each dentine-bonding agents (DBAs)

Tested materials	LCUs								Mann–Whitney *U*-test	
	LED				QTH					
	CSR%				CSR%					
DBAs	24 h	72 h	Mean	SD	24 h	72 h	Mean	SD	*z*-value	*P*-value
SLB	46.66	42.61	44.63	5.45	62.55	52.52	57.53	10.76	−2.402	0.015*
CSB	81.84	69.80	75.81	10.28	64.99	45.05	55.01	12.79	−2.242	0.026*
SB2	71.84	67.05	69.44	7.96	67.33	56.09	61.70	9.39	−1.601	0.132
PLP	65.53	57.27	61.39	5.19	59.54	53.15	56.34	7.67	−1.601	0.132
XeIII	66.51	55.56	61.03	8.48	61.10	51.57	56.33	7.97	−1.121	0.310
PB1	68.85	58.29	63.57	10.73	65.93	52.29	59.10	10.57	−0.801	0.485

There is significant difference at $*P < 0.01$. The cell survival rates (CSR%) of tested materials at experimental times.

In general, the test materials cured with the light-emitting diode LCU (CSB, SB2, PLP, XeIII and PB1) demonstrated higher cell survival rates when compared with the ones cured with the quartz tungsten halogen. The CSB which was cured with light-emitting diode had the least effect on cell survival rate ($z = -2.242$, $P = 0.026$) amongst the materials that were polymerized with the light-emitting diode LCU. However, different from the other tested materials, the SLB cured with quartz tungsten halogen had a significant greater effect on cell survival rates than the SLB cured with light-emitting diode ($z = -2.402$, $P = 0.015$).

Discussion

The analysis of the effect on cell survival and cell growth of substances leached or dissolved from DBAs showed that, at least during the polymerization of the bonding system, these substances are present and lead to cell death and to a loss of proliferation ability of cells in culture (Cavalcanti *et al.* 2005).

In the present study, the effect of DBAs cured with the light-emitting diode or quartz tungsten halogen on L-929 fibroblasts were investigated with the MTT assay. The dimetylthiazol diphenyltetrazolium (MTT) test can be used to indicate cytotoxic effects by assessing the functional state of the cell mitochondria after exposure to chemicals or devices. Mitochondrial dehydrogenases in living cells reduce the yellow tetrazolium salt, MTT to blue MTT formazan, which is then retained in the cell [ISO 10993-12: 1996 (E), Spagnuolo *et al.* 2004]. Formation of the formazan product has been found to correlate well with the number of viable cells (Lonnroth & Dahl 2003).

In the present study, the freshly prepared samples were placed in medium immediately. It is important for the materials to be tested immediately after mixing/curing to avoid the loss of toxic substances released from the tested materials at this initial stage.

The health of dental pulp tissue following operative procedures and tooth restoration may depend on the severity of inflammatory response induced by DBA that can be a reversible or irreversible event. This is of major concern during clinical operative procedures because if DBAs induce irreversible pulpal damage, subsequent endodontic treatment will become inevitable (Chen *et al.* 2003).

The cytotoxicity of DBAs has been studied previously (Ratanasathien *et al.* 1995, Costa *et al.* 1999, de Souza Costa *et al.* 2002, Chen *et al.* 2003, Huang *et al.* 2003). In these studies, it has been shown that different LCUs can affect the release of resin monomers (Munksgaard *et al.* 2000, Spagnuolo *et al.* 2004). Release of monomers has a potential impact on the biocompatibility of dental materials (Schweikl *et al.* 2001, Kleinsasser *et al.* 2004, Schwengberg *et al.* 2005). Light curing of DBAs will polymerize resins in a solid phase hence significantly diminishing the amount of free monomer and substantially reducing the potential for noxious stimuli. Complete polymerization would suppress all stimuli. Forever-cured resins are never fully polymerized, they will degrade with time (Koliniotou-Koubia *et al.* 2001).

Usually DBAs are polymerized by photo-activation and free monomer may be released from resinous materials before and after polymerization. These monomers may pass through dentinal tubules and reach the pulp tissue causing pulpal irritation. An insufficient photo-activation can contribute to an increase in the level of unreacted monomers through a reduced degree of polymerization and cross linking. When light curing DBAs are not fully polymerized,

leachable components, such as bis-GMA, UDMA, camphorquinone and HEMA, may penetrate through dentinal tubules, exert potential pulpal injury and inhibit pulp tissue repair (al-Dawood & Wennberg 1993, Ferracane 1994, Spahl *et al.* 1998, Spagnuolo *et al.* 2004). A recent series of papers and data demonstrates the importance of the cytotoxicity of DBAs and therefore the importance of the sufficient polymerization with the LCUs (Costa *et al.* 1999, Spagnuolo *et al.* 2004, Cavalcanti *et al.* 2005).

Various studies have addressed the application of light-emitting diode technology to cure restorative materials (Stahl *et al.* 2000, Mills *et al.* 2002, Uhl *et al.* 2002). Typically, the advantages claimed for second generation light-emitting diode LCUs are more efficient curing, decreased heat from the light tip, consistent output over time without degradation and significantly longer useful life of the diodes compared with quartz tungsten halogen bulbs (Lonnroth & Dahl 2003). Furthermore, with the introduction of light-emitting diode LCUs, it was asserted that the emission of blue light-emitting diode LCUs is the ideal spectra for the conversion of dental materials containing monomer. In this way, fewer toxic substances may leach into the environment (Chen *et al.* 2001, 2003). Biocompatibility of light-cured dental materials may be affected by the quality of the LCU used. Therefore, in this study the cytotoxicity of different types of light-cured DBAs were compared with curing after the light-emitting diode or the quartz tungsten halogen LCUs.

In the present study, the cytotoxicity of one bottle, self-etching and all-in-one adhesive systems were evaluated after polymerization. It was clear that all sufficient quantities of test materials cured with either the quartz tungsten halogen or light-emitting diode leached a variety of components into cell culture medium and affected cell activity. After testing the cytotoxicity of polymerized test samples, statistically significant differences were found regarding cell survival rates for the different LCUs and DBAs. When comparing the control cultures, SLB cured with the light-emitting diode had resulted in the lowest survival rate of 44.63%. However, CSB cured with light-emitting diode revealed the highest cell survival rate of 75.81% ($P < 0.05$). The difference between these groups suggest a relationship with its chemical composition or to the fact that SLB is cured more fully with quartz tungsten halogen compared with light-emitting diode. In this way, it is possible that these characteristics of the SLB cured with quartz tungsten halogen reduce the release of toxic substances into the culture medium.

Generally, when the results of all the test samples were evaluated, materials cured with the quartz tungsten halogen (except SLB) had reduced cell survival rates compared with samples cured with the light-emitting diode.

The results revealed that there might be several possible reasons for different effects of DBAs or LCUs on their cytotoxicity, such as the light transmission characteristics, the released energy during the polymerization of the DBAs and the amount, as well as the type of released toxic substances from the unpolymerized DBAs.

On the other hand, the experimental time had no statistically significant effect on the cytotoxicity of the DBAs cured with the different LCUs.

Spagnuolo *et al.* (2004) investigated the cytotoxicity and oxidative stress of two 'one-bottle' adhesive systems after polymerization with a conventional quartz tungsten halogen LCU or a light-emitting diode LCU and they reported that the production of intracellular reactive oxygen species (ROS) by the adhesive extracts was both light source and time dependent. They claimed that cytotoxicity was light source dependent and declared that when the cell survival rates of the test materials were taken into account, quartz tungsten halogen performed better than light-emitting diode.

Cavalcanti *et al.* (2005) reported that substances leached or dissolved from pulp-capping materials were cytotoxic for human dental pulp fibroblasts in culture. In their study, Single Bond cured with quartz tungsten halogen resulted in a decrease in cell viability. Whereas cell viability was 70% at the beginning, it then was decreased to 10% at the end of 12 h and 7 days. In contrast to the study of Cavalcanti *et al.* (2005), the present study showed that SB2 cured with quartz tungsten halogen resulted in a cell survival rate of 67% at 24 h and 56% at 72 h. Then, the results of the current study do not correlate with those obtained by Cavalcanti *et al.* (2005) who evaluated the cytotoxicity of Single Bond and found severe toxicity.

It is difficult or even impossible to compare the results from different cell culture experiments because of the many variations in experimental conditions, such as the cell type, the cell material contact method and exposure time (Spangberg 1981).

Costa *et al.* (1999) evaluated the cytotoxic effect of three current one-bottle DBAs (Prime and Bond 2.1, Single Bond and Syntac Sprint) cured with quartz

tungsten halogen or uncured. The authors reported that fresh adhesives exhibited more toxic (cytopathic effects) on MDPC-23 cells than polymerized adhesives. They stressed the importance of polymerization and reported that both the acidic and nonacidic components of these unpolymerized adhesive resins were responsible for the high cytopathic effects on odontoblast.

As the results have indicated, the DBAs cured with either quartz tungsten halogen or light-emitting diode may cause harmful effects to the pulp. The findings of the present study revealed that the LCUs had significant effect on the cytotoxicity of the DBAs and the light-emitting diode LCU resulted in better cell survival than the quartz tungsten halogen. On the other hand, most eluted substances are found to be cytotoxic *ex vivo* and therefore the materials may not necessarily be cytotoxic *in vivo*. From a clinical point of view, there are limitations regarding the correlation between *ex vivo* testing and clinical usage tests. However, the *ex vivo* cytotoxicity test is important in understanding the biological risk of these materials at the initial setting stage.

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