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Genotoxicity evaluation of dentine bonding agents by comet assay

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Abstract

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Aim To evaluate the genotoxicity of four different adhesives, Clearfil SE Bond, SL Bond, i Bond and Clearfil Protect Bond and the primers of Clearfil SE Bond and Clearfil Protect Bond.

Methodology Genotoxicity assessment of the adhesives and primers was carried out *in vitro* in human lymphocytes at different elution concentrations, using the alkaline single-cell gel electrophoresis technique (comet assay). After the incubation of lymphocytes with varying volumes of the test agent, cells were embedded in a low-melting-point agarose suspension and then lysed in alkaline (pH > 13) conditions. Electrophoresis was performed on the suspended lysed cells followed by visual analysis with staining of DNA. Fluorescence was than calculated to determine the extent of DNA damage using imaging software. Statistical comparison of the results was carried out by one-way analysis of variance (ANOVA).

Results A significant increase ($P < 0.001$) compared to untreated controls in DNA damage was observed with 'Clearfil Protect Bond' and 'Clearfil SE Bond' primer in human lymphocytes at concentrations of 2.5 and 5.0 mg mL^{-1}. Clearfil Protect Bond and Clearfil SE Bond adhesives induced significant ($P < 0.001$) DNA damage only at the higher concentration of 5.0 mg mL^{-1}. No significant increase in DNA damage was observed with SL Bond and i Bond. No significant DNA damage was observed with any dentine bonding agents at the lower concentration of 1.25 mg mL^{-1}.

Conclusions 'Clearfil Protect Bond' and 'Clearfil SE Bond' primers/adhesives increased DNA damage in human peripheral lymphocytes in high doses.

Keywords: Clearfil Protect Bond, Clearfil SE Bond, Comet assay, Dentine bonding agents, SL Bond, i Bond, DNA damage.

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Introduction

Over the last 30 years, increasing numbers of resinous dentine bonding materials and adhesives have been introduced in restorative dentistry. These resinous monomers are formed by different organic molecules, such as glycidyl methacrylate (GMA), bisphenol A-glycidyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA), dipentaerythritol penta acrylate monophosphate, hydroxyethyl methacrylate (HEMA) and 4-metacryloxyethyl trimellitate anhydride (4-META), which function as copolymeric chains (Peutzfeldt 1997). However, the question whether dentine bonding agents have adverse effects on the health is of concern (Geurtsen 2001, 2003).

Incomplete polymerization of dental resin composites and resin-based bonding agents under clinical conditions results in free resin monomers of the bonding materials being released from the resin matrix into the aqueous environment of the oral cavity or into the dentine–pulp complex; some components may be released even after polymerization (Gerzina & Hume 1994, Ortengren *et al.* 2001,

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Schwengberg *et al.* 2005, Sutow *et al.* 2006). Monomers eluted from dentine adhesive systems and bacterial microleakage have been implicated as possible causes of pulpal irritation after placement of composite resin restorations (Akimoto *et al.* 1998, Cox *et al.* 1998). Some studies indicated that dentine bonding agents are not appropriate for direct pulp capping because of persistent inflammation (Cehreli *et al.* 2000, Costa *et al.* 2000).

Allergic responses to such materials following skin contact have been reported by dentists (Tosic 2004). In addition to reports on hypersensitivity reactions to composites (Hamann *et al.* 2004, Isaksson *et al.* 2005) because of incomplete polymerization and to degradation processes in the oral environment (Cavalcanti *et al.* 2005, Bakopoulou *et al.* 2006), a number of reports have identified the cytotoxicity of these compounds (Al-Hiyasat *et al.* 2005, Cao *et al.* 2005, Cimpan *et al.* 2005, Reichl *et al.* 2006, Brzovic *et al.* 2009).

In general, cytotoxicity of dentine bonding agents depends on dentine permeability, adhesive composition and time passed after their placement (Prica *et al.* 2006). The highest toxicity was observed within the first 24 h after the placement (Bouillaguet *et al.* 1998). Although it was suggested that low amounts of compounds released into aqueous solutions by resin-based materials have not been sufficient to cause acute cytotoxicity, Demirci *et al.* (2008) reported that dentine primers and bonding agents of Clearfil SE Bond, Clearfil Protect Bond (Kuraray Medical, Okayama, Japan), Prompt L-Pop (3M ESPE, Seefeld, Germany), AdheSE and Excite (Vivadent-Ivoclar AG, Schaan, Liechtenstein) decreased cell survival in a dose-related manner in human pulp-derived cells exposed to extracts of primers for 24 h. Other studies indicate that these materials have been sufficiently high in concentration to modify essential cell functions such as induction of heat-shock proteins, modifications of cell-mediated immune responses and genetic effects such as gene mutations or chromosomal aberrations (Gerzina & Hume 1994, Geurtsen 2000, Noda *et al.* 2002, Schweikl *et al.* 2006).

On the other hand, there are only limited number of studies on genotoxicity of dentine bonding agents. Experimental data show that resin-based dental materials enhance intracellular reactive oxygen species (ROS), which are a well-known potential genotoxic factor implicated in many human chronic degenerative diseases including cancer and cause oxidative DNA damage (Chang *et al.* 2005, Schweikl *et al.* 2006, Valko *et al.* 2006, Demirci *et al.* 2008). Dentine bonding systems that contain HEMA and Bis-GMA have been shown to exert genotoxic effects on human gingival fibroblasts (Huang *et al.* 2003). Dose-related increases in the number of ROS were observed in human gingival epithelial S-G cells and pulp fibroblasts with HEMA (Chang *et al.* 2005), in human fibroblasts with TEGDMA (Stanislawski *et al.* 2003, Goldberg 2008) and also a dose-related increase in the number of micronuclei was observed in V79 cells with Adhe SE Primer indicating clastogenic activity of these chemicals *ex vivo* (Demirci *et al.* 2008). However, there was no obvious relation between ROS production by the dental adhesives tested in their study and genotoxicity as indicated by the formation of micronuclei (Demirci *et al.* 2008).

Prica *et al.* (2006) have also evaluated possible genotoxicity of Adper Single Bond (Bis-GMA, HEMA, Dimethacrylate, methacrylic copolymer of polyacrylic and polyitaconic acid, and photoinitiators), Adper Single Bond2 (Bis-GMA, HEMA, Dimethacrylate, silica, methacrylate copolymer, polyacrylic and polyitaconic acid, and photoinitiators), Prompt L-pop (Bis-GMA, HEMA, methacrylic phosphoesters, camphorquinone and polyalcenoic acid) (3M ESPE, St Paul, MN, USA), Excite (Bis-GMA, HEMA, glycerine dimethacrylate, phosphoric acrylates, silica, initiators and stabilizers) (Vivadent-Ivoclar AG), Optibond Solo Plus (HEMA, dimethacrylate, silica, initiators and stabilizers) (Kerr S.p.a, Salerno, Italy) using *ex vivo* chromosomal aberration analysis in human lymphocytes. Slight but significant increase in the number of chromatid breaks was observed after 24-h elution periods for adhesives Adper Single Bond2, Excite, Optibond Solo Plus at dilutions $1 : 10^6$ and $1 : 10^5$ and for Adper Single Bond and Prompt L-pop only at dilutions of $1 : 10^5$.

Genotoxic damage may significantly diminish the self-repairing potential of tissue or cause the development of neoplasia in the long term. This study was carried out to further investigate the potential genotoxicity of different resin and polymer-based dental restorative materials; Clearfil SE Bond adhesive (HEMA, bisphenol A, colloidal silica), Clearfil Protect Bond adhesive (Bis-GMA, MDP, HEMA, colloidal silica), Clearfil SE Bond primer (HEMA, *N*,*N*-diethanol-*p*-toluidine, D,L-camphor-quinone dimethacrylate), Clearfil Protect Bond primer (MDPB, MDP, HEMA, hydrophobic monomer dimethacrylate), SL Bond (Bis-GMA, BPDM and HEMA), i Bond (UDMA, 4-META) in human peripheral lymphocytes by comet assay, which is considered a reliable and easy test for the assessment of DNA damage.

Materials and methods

Chemicals

The composition of the dentine bonding agents and the other chemicals used in the comet assay are shown in Table 1a,b, respectively.

Blood samples and cell preparation

For each experiment, 5 mL heparinized (50 units mol^{-1} sodium heparin) whole blood was collected by venepuncture from one 33-year-old non-smoking female donor not exposed to radiation or drugs. The donor participated voluntarily and provided oral consent, which was reviewed and approved by the local Institutional Review Board before the blood sample was drawn from her. Lymphocytes were isolated by Ficoll-Hypaque density gradient (Boyum 1976) and washed with phosphate buffered saline (PBS). Cell concentrations were adjusted to approximately 2×10^5 mL in the buffer. The cells were suspended in a total volume of 1 mL, and each reaction contained 50 μL suspension ($\approx 10^4$ cells), varying microlitre amounts of the test agent (i Bond, SL Bond, Clearfil SE Bond primer, Clearfil SE Bond adhesive, Clearfil Protect Bond primer and Clearfil Protect Bond adhesive) and PBS buffer in a total volume of 1 mL; 1.25, 2.50 and 5.00 mg mL^{-1} concentrations of the dentine bonding agents were examined. The cells were incubated for 1 h at 37 °C in an incubator together with untreated control samples. Each experiment included a positive control, which was hydrogen peroxide at the concentration of 50 μmol L^{-1}. All test substances were dissolved in PBS with concentration of 50 mg mL^{-1} and incubated for 24 h at 37 °C. After incubation, the lymphocytes were harvested by centrifugation at 800 g for 3 min at 4 °C, and the cells were suspended in 75 μL low melting agarose (LMA) for embedding on slides. The replicate experiments were carried out with blood samples from the same donor collected at different time intervals. An aliquot of cells was used to check for viability by trypan blue exclusion. In trypan blue exclusion test of cell viability, a cell suspension is simply mixed with the dye (trypan blue) and then visually examined to determine whether cells take up or exclude dye. A viable cell will have clear cytoplasm, whereas a non-viable cell will have a blue cytoplasm (Strober 2001).

Slide preparation

The basic alkaline technique of Singh *et al.* (1988), as further described by Collins *et al.* (1997), was followed. Microscopic slides had been pre-coated with 1% normal melting agarose at about 45 °C in Ca^{2+}- and Mg^{2+}-free PBS before the experiment. This layer was used to promote the attachment of the second layer. For the second layer, around 10 000 cells mixed with 80 μL of 1% LMA (pH 7.4) were rapidly pipetted onto this slide, spread using a cover slip and maintained on an ice-cold flat tray for 5 min to solidify. After removal of the cover slip, the slides were immersed in cold lysing solution (2.5 mol L^{-1} NaCl, 100 mmol L^{-1} Na_2EDTA, 10 mmol L^{-1} Tris, 1% sodium sarcosinate, pH 10) with 1% Triton X-100 and 10% dimethylsulfoxide added just before use, for a minimum of 1 h at 4 °C.

Electrophoresis

The slides were removed from the lysing solution, drained and placed in horizontal gel electrophoresis tank side by side, avoiding spaces and with the agarose ends facing each other, nearest the anode. The tank was filled with fresh electrophoresis solution (1 mmol L^{-1} Na_2EDTA and 300 mmol L^{-1} NaOH, pH 13) to a level

Table 1 (a) Composition of dentine bonding agents

Dentin bonding agents	Manufacturer	Composition
Clearfil SE Bond Adhesive (SE Bond)	Kuraray Dental Ltd., İzmir, Turkey	HEMA, Bisphenol A, colloidal silica
Clearfil Protect Bond Adhesive (PB)	Kuraray Dental Ltd., İzmir, Turkey	Bis-GMA, MDP, HEMA, colloidal silica
Clearfil SE Bond Primer (SE Primer)	Kuraray Dental Ltd., İzmir, Turkey	HEMA, *N*,*N*-diethanol-*p*-toluidine, D,L-camphor-quinone dimethacrylate
Clearfil Protect Bond Primer (PP)	Kuraray Dental Ltd., İzmir, Turkey	MDPB, MDP, HEMA, hidrofobic monomer dimethacrylate
SL Bond (SL)	Swiss-Tec, Altstätten, Switzerland	Bis-GMA, BPDM and HEMA
İ-Bond	Heraeus Kulzer, Hanau, Germany	UDMA, 4-META

HEMA, hydroxyethyl methacrylate; UDMA, urethane dimethacrylate; Bis-GMA, bisphenol A-glycidyl methacrylate; 4-META, 4-metacryloxyethyl trimellitate anhydride.

Table 1 (b) Chemicals used in the comet assay

Chemicals	Manufacturer
Normal melting agarose	Boehringer Manheim, Germany
Low melting agarose	Boehringer Manheim, Germany
Sodium chloride	Merck Chemicals, Darmstadt, Germany
Sodium hydroxide	Merck Chemicals, Darmstadt, Germany
Dimethylsulfoxide	Sigma-Aldrich, St Louis, MO, USA
Ethidium bromide	Sigma-Aldrich, St Louis, MO, USA
Hydrogen peroxide (H_2O_2) 30% w/w	Sigma-Aldrich, St Louis, MO, USA
Triton X-100	Sigma-Aldrich, St Louis, MO, USA
Phosphate buffered saline tabletes	Sigma-Aldrich, St Louis, MO, USA
Ethylenediamine tetraacetic acid disodium salt dihydrate	ICN Biochemicals, Aurora, OH, USA
N-lauroyl sarcosinate	ICN Biochemicals, Aurora, OH, USA
Tris	ICN Biochemicals, Aurora, OH, USA

approximately 0.25 cm above the slides. Before electrophoresis, the slides were left in the solution for 20 min at 4 °C to allow the unwinding of the DNA and expression of alkali labile damage. Electrophoresis was conducted at a low temperature (4 °C) for 20 min using 24 V and adjusting the current to 300 mA by raising or lowering the buffer level and using a compact power supply (Power Pack P 25 Biometra Analytic GmbH). All of these steps were conducted under dimmed light to prevent the occurrence of additional damage. After electrophoresis, the slides were taken out of the tank, washed in distilled water. Tris buffer (0.4 mol L^{-1} Tris, pH 7.5) was added dropwise and gently to neutralize the excess alkali, and the slides were allowed to sit for 5 min. The neutralizing procedure was repeated thrice. After waiting the slides each for 5 min in distilled water, 50%, 75% and 99% ethanol, they were allowed to dry at room temperature.

Staining and slide scoring

To each slide, 30 μL of EtBr (20 μL mL^{-1}) was added. For visualization of DNA damage, slides were examined at a 1000× magnification using a 40× objective on a fluorescence microscope Leica (Wetzlar, Germany). Measurements were made by a computer-based image analysis system 'Comet Assay III' Perceptive Instruments (Norwich, England). Images of 100 randomly selected lymphocytes, i.e. 50 cells from each of two replicate slides, were analysed from each sample. Breaks in the DNA molecule disturb its complex supercoiling, allowing liberated DNA to migrate towards the anode. Staining shows the DNA as 'comets'. The mean value of the tail length, tail intensity and tail moment was calculated and used for the evaluation of DNA damage.

Statistical analysis

The SPSS for Windows 10.0 computer program (IBM Company, Somers, NY, USA) was used for statistical analysis. Distribution of data was checked for normality by Kolmogorov–Smirnov test. Statistical comparison of the results from controls, i Bond, SL Bond, Clearfil SE Bond primer, Clearfil SE Bond adhesive, Clearfil Protect Bond primer and Clearfil Protect Bond adhesive groups was carried out by one-way analysis of variance (ANOVA) test, and post hoc analysis of group differences was performed by LSD test. Results are expressed as mean ± SD.

Results

Cell viability, as tested using trypan blue dye exclusion of each treated group, was more than 90%. The DNA damage expressed as tail length, tail intensity and tail moment in the lymphocytes following *ex vivo* 1 h treatment with 1.25, 2.5 and 5.0 mg mL^{-1} concentrations of the dentine bonding agents tested is given in Figs 1–3. Images of cells with increasing levels of DNA damage in comet assay are shown in Fig. 4. According to the data obtained from three separate experiments, tail length and tail intensity were significantly increased ($P < 0.001$) at concentrations of 2.5 and 5.0 mg mL^{-1} of Clearfil SE Bond primer, 5.0 mg mL^{-1} of Clearfil SE Bond adhesive, 5.0 mg mL^{-1} of Clearfil Protect Bond primer and 5.0 mg mL^{-1} of Clearfil Protect Bond adhesive, as compared with untreated cells (Figs 1 and 2). The tail moment was significantly increased ($P < 0.001$) above the control values at 2.5 and 5.0 mg mL^{-1} concentrations of Clearfil SE Bond primer, 5.0 mg mL^{-1} of Clearfil Protect Bond primer and 5.0 mg mL^{-1} of Clearfil Protect Bond adhesive, as compared with untreated cells (Fig. 3). The DNA damage observed with Clearfil Protect Bond Primer was 3.7-fold higher (1.25 mg mL^{-1} dose); 4.6-fold higher (2.5 mg mL^{-1} dose) and 13.3-fold higher (5 mg mL^{-1} dose) compared with untreated cells in tail intensity. No significant increase in DNA damage in the lymphocytes was observed with all the concentrations of i bond and SL bond.

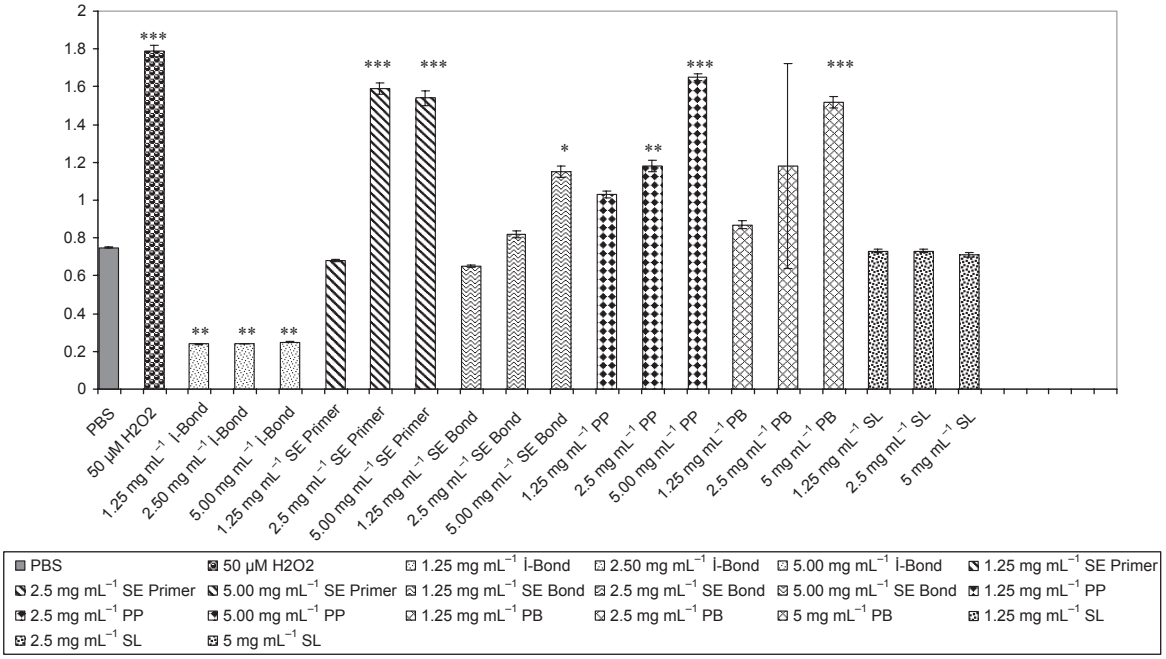

Figure 1 Tail length of İ Bond-, SE Primer-, SE Bond-, PP-, PB- and SL-treated human peripheral lymphocytes[#]. [#]Results as shown mean ± SD (N = 300 for each dose). *P < 0.05; **P < 0.005; ***P < 0.001 compared to phosphate buffered saline.

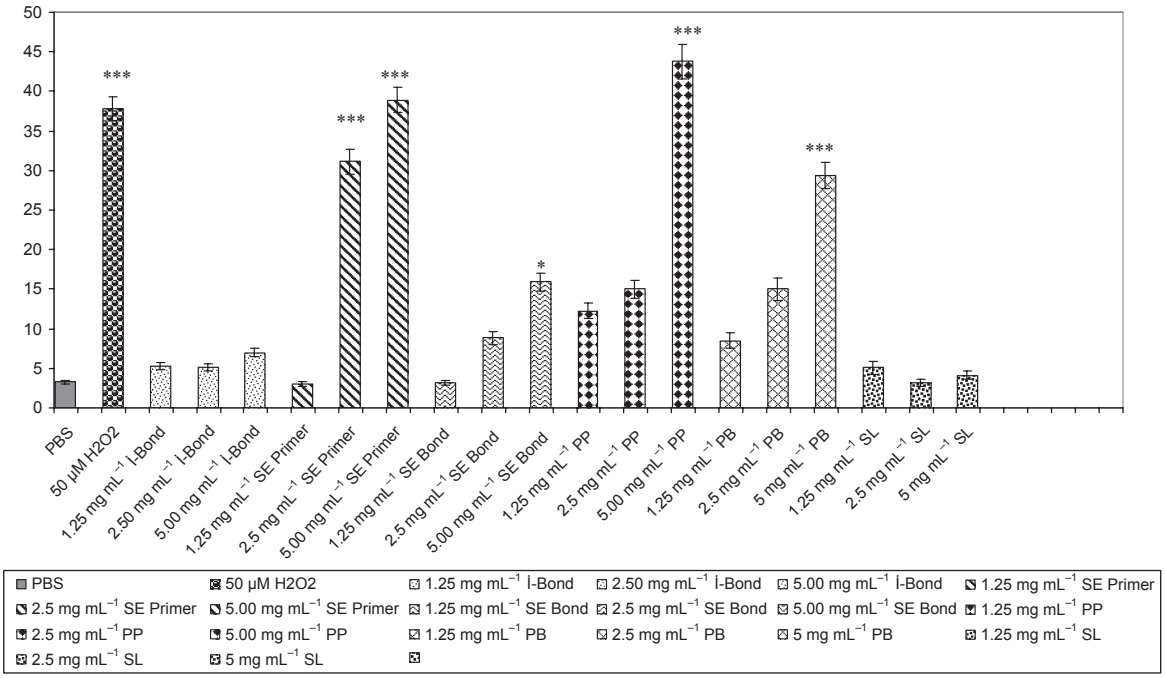

Figure 2 Tail intensity of İ Bond-, SE Primer-, SE Bond-, PP-, PB- and SL-treated human peripheral lymphocytes[#]. [#]Results as shown mean ± SD (N = 300 for each dose). *P < 0.05; **P < 0.005; ***P < 0.001 compared to phosphate buffered saline.

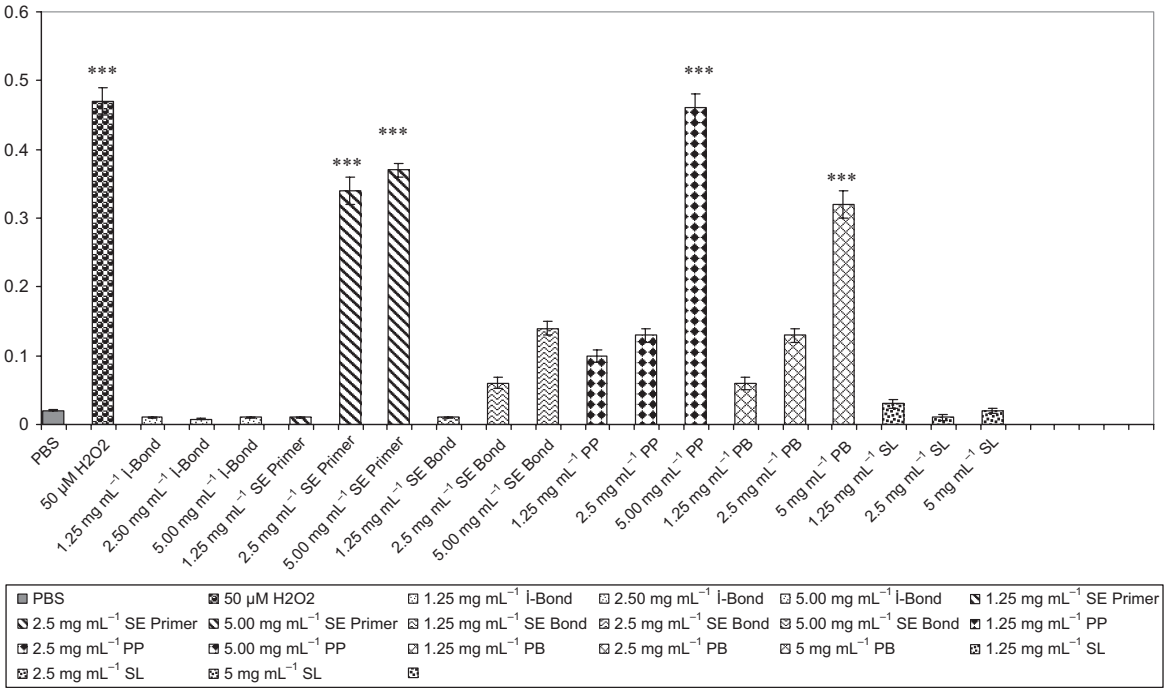

Figure 3 Tail moment of İ Bond-, SE Primer-, SE Bond-, PP-, PB- and SL-treated human peripheral lymphocytes#. #Results as shown mean ± SD (N = 300 for each dose). *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$ compared to phosphate buffered saline.

Discussion

Components of composites and bonding materials have been suggested to exert adverse effects because they may be released into the saliva during implantation and even after polymerization and diffuse into the tooth pulp or gingiva, mucosa and salivary glands. Polymerized dental resin materials may release residual monomers that may interact with pulp tissue and cause cytotoxicity in pulp cells via the generation of reactive oxygen species that may also contribute to genotoxic effects. Other studies suggest the cytotoxic potential of dental restorative materials such as TEGDMA, UDMA and 2-HEMA (Schedle *et al.* 1998, Huang & Chang 2002, Szep *et al.* 2002, Walther *et al.* 2002, Janke *et al.* 2003, Becher *et al.* 2005, Kleinsasser *et al.* 2006). Dental adhesives such as Clearfil SE Bond, Clearfil Protect Bond, AdheSe, Prompt L-Pop and Excite were reported to increase ROS levels in pulp cells in a dose-related manner. They also disturbed the cellular redox state of pulp cells in monolayer cultures (Demirci *et al.* 2008).

The existing data on the genotoxic effects of dentine bonding agents on human cells are limited and controversial. Brzovic *et al.* (2009), using both chromosomal aberration analysis and comet assay, reported no genotoxic potential was noted for Epiphany that contains UDMA, PEGDMA, EBPADMA and Bis-GMA or GuttaFlow, whereas zinc oxide–eugenol-based sealers, such as Hermetic, and SuperEBA exhibited limited genotoxic activity on peripheral blood lymphocytes *ex vivo* (3M ESPE).

A dose-related increase in the numbers of micronuclei was also observed with TEGDMA, HEMA and GMA, suggesting a clastogenic activity of these chemicals. The very low activity of Bis-GMA and UDMA and the elevated numbers of micronuclei caused by high concentrations of methyl methacrylate and bisphenol A were associated with cytotoxicity. It has been shown that TEGDMA also caused gene mutations and DNA sequence deletions in mammalian cells (Schweikl *et al.* 2001). Dimethacrylates used as a monomer in dental resinous materials such as TEGDMA and UDMA were found to be genotoxic in human lymphocytes, L5178Y mouse lymphoma cells and V79 cells (Müller *et al.* 2003, Arossi *et al.* 2009); however, no specific component was identified as the causative agent in these studies (Fredericks 1981, Cross *et al.* 1983, Miller *et al.* 1984, Schweikl *et al.* 2001, Müller *et al.* 2003). Drosophila wing spot test showed that Adper Single

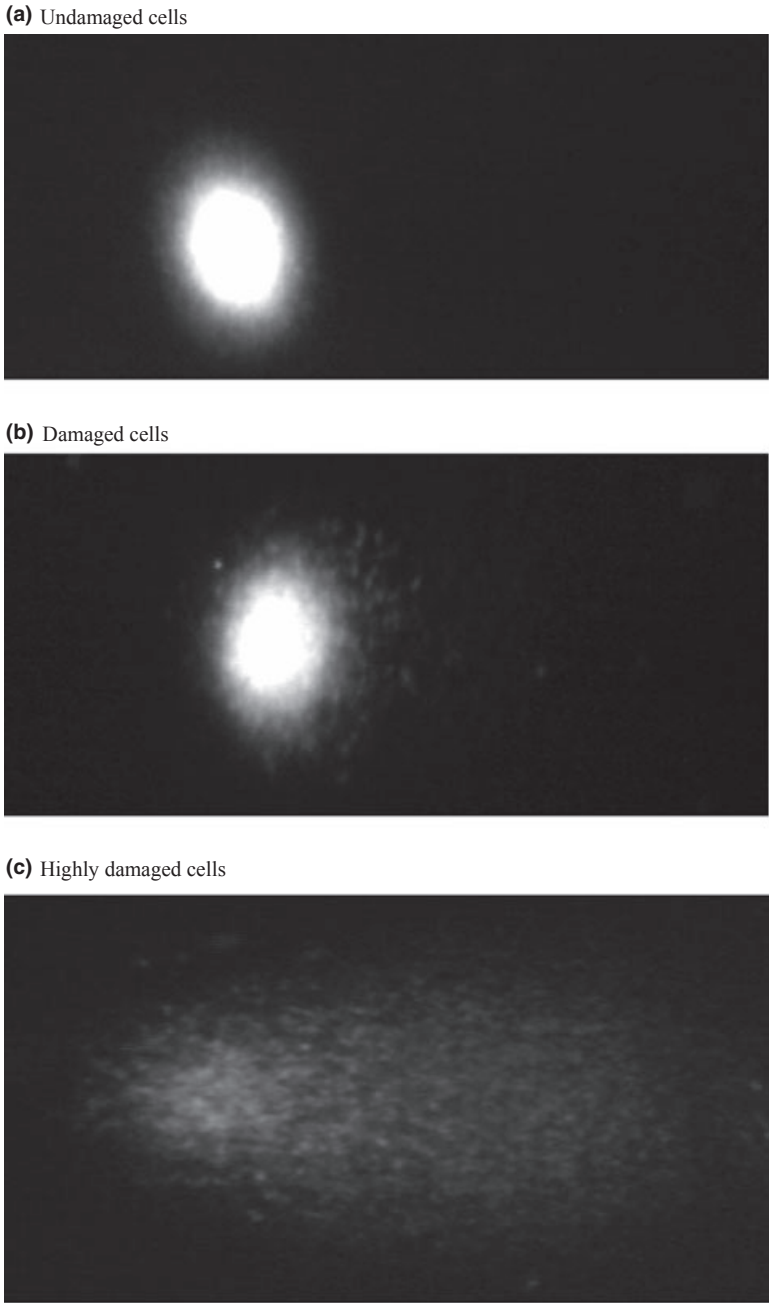

Figure 4 Images of cells with increasing levels of DNA damage in comet assay. #Results as shown mean ± SD (N = 300 for each dose). *P < 0.05; **P < 0.005; ***P < 0.001 compared to phosphate buffered saline.

Bond Plus (Bis-GMA, HEMA and UMA) promoted homologous mitotic recombination, although Prime&-Bond 2.1 (UDMA and HEMA) induced recombinagenic and, to a lesser extent, mutational events (Arossi *et al.* 2009).

In the present study, possible genotoxicity of dental bonding adhesives and primers that contain nearly the same resin monomers previously studied were tested in human lymphocytes *ex vivo* at different elution concentrations by alkaline comet assay, which is a generally accepted technique used for the evaluation of the genotoxic potential of chemicals (Singh *et al.* 1988, Collins *et al.* 1997, Dhawan & Anderson 2009).

In the present study, Clearfil SE Bond primer caused DNA damage at concentrations of 2.5 and 5.0 mg mL^{-1} in human lymphocytes, whereas Clearfil

Protect Bond primer induced DNA damage only at the higher concentrations of 5.0 mg mL^{-1} compared to controls. A significant increase in the DNA damage was also observed with the adhesives 'Clearfil Protect Bond' (Bis-GMA, MDP, HEMA and colloidal silica) and 'Clearfil SE Bond adhesive' (HEMA, bisphenol A and colloidal silica) in human lymphocytes at the higher concentration of 5.0 mg mL^{-1} ($P < 0.001$ and $P < 0.05$, respectively). However, no significant increase in the DNA damage was seen with SL Bond (Bis-GMA, BPDM and HEMA) and i Bond (UDMA, 4-META). H_2O_2 is used as positive control because it induces DNA damage in concentration of 50 μmol L^{-1} without cytotoxic effect. The DNA damage observed with the dental bonding agents was not higher than the damage induced by H_2O_2. As a result of the various components in the formulas, differences in the genotoxic effects have also been observed but from the results of this study, it is clear that there is a possibility of a genotoxic effects of dentine bonding materials in a dose manner such as with Clearfil Protect and Clearfil SE Bond adhesives; however, it is impossible to find the causative agents. These results are consistent with some previous studies that have examined the genotoxic effects of methacrylates *ex vivo* and *in vivo* by comet assay (Kleinsasser *et al.* 2004, 2006).

Similar to the present findings, the methacrylates TEGDMA, UDMA, Bis-GMA and HEMA induced significant DNA migration in high concentrations in human salivary glands and lymphocytes as human target cells of carcinogenesis. In higher concentrations, all tested substances induced significant but minor enhancement of DNA migration in the comet assay as a possible sign of limited genotoxic effects. At concentrations possibly relevant for the *in vivo* situation ($<10^{-4}$ mol L^{-1}), there was no significant enhancement of DNA migration in the comet assay. No information on the quantity of dimethacrylate monomers contained in dental adhesive systems has been released by manufacturers (Kleinsasser *et al.* 2004, 2006). Prica *et al.* (2006) investigated the *ex vivo* genotoxicity of five different adhesives, Adper Single Bond, Adper Single Bond 2, Excite, Optibond Solo Plus and Prompt L-Pop, on human lymphocytes in association with several elution periods and concentrations. The genotoxic effect was established after 24-h elution period at low dilution, $1 : 10^5$. However, only Adper Single Bond 2, Excite and Optibond Solo Plus induced DNA damage after the same period, at $1 : 10^6$ dilution. The genotoxic effects of that bonding agents are thought to be dose independent. Miletic *et al.* (2000) also found that GuttaFlow, Epihany, Diaket, IRM, SuperEBA and Hermetic had acceptable biocompatibility in terms of genotoxicity.

Conclusion

Dentine bonding agents 'Clearfil Protect Bond' and 'Clearfil SE Bond' primers/adhesives increased DNA damage in human peripheral lymphocytes.

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