EFFECTS OF DENTAL ADHESIVES ON MICRONUCLEUS FREQUENCY IN PERIPHERAL BLOOD LYMPHOCYTES *IN VITRO*

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SUMMARY – Dental adhesives come into direct contact with oral tissues. Due to this close and long-term contact, the materials should exhibit a high degree of biocompatibility. The aim of this study was to evaluate the genotoxic effect of dental adhesives on human lymphocytes *in vitro*. Polymerized dental adhesives (Excite, Adper Single Bond 2, Prompt L-pop and OptiBond Solo Plus) were eluted in dimethyl sulfoxide for 1 hour, 24 h and 120 h (5 days). Thereafter, lymphocyte cultures were treated with different concentrations of eluates (0.2 μg/mL, 0.5 μg/mL and 5 μg/mL) obtained from each of the tested materials. Genotoxicity was evaluated by micronucleus test. The χ^2-test was used on statistical analysis ($p<0.05$). After elution period of 1 h, only the highest dose of all tested materials affected the measured cytogenetic parameters. After 24 h, genotoxicity was demonstrated only in cultures treated with eluates in concentrations of 0.5 μg/mL and 5 μg/mL. Based on the results, it is concluded that the use of dental adhesives causes genotoxic effects in human lymphocytes. Toxic effect of these dental adhesives increases with the tested material concentration and decreases with the length of elution period.

Key words: *Dental adhesives; Mutagenicity tests; Lymphocytes; Micronucleus tests*

Introduction

Biocompatibility is a major requirement for safe use of dental materials and it includes physical, mechanical and chemical properties of materials, as well as the potential cytotoxic, genotoxic, mutagenic and allergenic effects[1-4]. One of the most commonly used dental restorative materials as a substitution for lost hard dental tissues are composite resins. Composite materials have no ability of adhesion to hard dental tissue, so the use of dental adhesives is necessary. The primary purpose of dental adhesives is based on a two-fold adhesion, first is adhesion to composite fillings, and second is bonding to enamel and dentin[5,6]. Today's adhesives either follow an "etch-and-rinse" or a "self-etch" approach[7]. The main difference between them is that the etch-and-rinse adhesives use 37% phosphoric acid for pretreatment of hard dental tissues before application of dental bonding agent, whereas the self-etch adhesives do not require a separate etching step, as they contain acidic monomers that simultaneously condition and prime the dental substrate[8,9].

Dental adhesive system that makes s connection with the biological tissue and allows healing and tissue differentiation is considered biocompatible[10,11]. The opinions about the dentin bonding agents are divided. While some researchers suggest that they are safe (biocompatible) and can be used for direct pulp capping[12,13], others believe that dentin bonding agents are

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hazardous because of constant inflammation of pulp cells that does not heal[14,15]. The usual composition of bonding agents includes resin monomers, initiators, inhibiters or stabilizers, solvents, and sometimes inorganic fillers[5]. It has been reported that dental adhesives release substances[16,17] that have biological effects and toxic potencies[18,19]. After application of bonding agents on the conditioned dentin, uncured residual resin components may diffuse across the subjacent dentinal tubules and reach the pulp[20]. There are a limited number of studies that have proven cytotoxic activity of dentin bonding agents on the cells of human origin (lung fibroblasts[21], pulp cells[16], gingival fibroblasts[22] and oral epithelial cells[23]). Genotoxicity was tested on pulp cells[16], lymphocytes[18,24,25] and leukocytes[26]. Commonly used methods to evaluate the genotoxic effects have been comet assay[24,26], micronucleus test[16] and chromosomal aberration analysis[18]. The results of these analyses showed that the differential toxicity of the materials tested could be attributed to the different ingredients, the interactions between them, and the degree of resin polymerization[21].

In the last thirty years, the micronucleus test has been used for assessing the chromosomal damage in biological monitoring of the human population exposed to diverse mutagens and carcinogens, chemical or physical agents. Chromosomal abnormalities lead to the occurrence of micronuclei, which may originate from an acentric chromosome fragments. Furthermore, micronuclei may originate from whole chromosomes lost from the metaphase plate and therefore provide a measure of both chromosome breakage and chromosome loss[27,28]. The advantages of this method are the simultaneous detection of chromosomal and gene mutations, discrimination between clastogen/aneugenic, possible cooperation detection of apoptosis/necrosis, the application of a number of cell types, low cost, and ease of use[28-30].

The aim of this *in vitro* study was to evaluate the possible genotoxicity of four contemporary dental adhesives (Excite, Adper Sngle Bond 2, Prompt L-pop and OptiBond Solo Plus) on human lymphocytes in relation to the duration of the elution period and concentrations of tested material. The potential genetic risk was evaluated by the micronucleus test. Since the composition and the proportions of ingredients vary across the adhesives, the hypothesis tested was that the "self-etch" adhesives had a higher genotoxic profile.

Materials and Methods

Blood sampling

The potential genotoxicity of dental adhesive systems was evaluated on lymphocytes obtained from a young, healthy, nonsmoking voluntary donor. Peripheral blood sample was obtained from a 33-year-old healthy man, not having been exposed to any chemical or physical agent during the last 12 months before sampling. The donor was acquainted with the purpose of the study and signed permission for the blood sample to be used for scientific purposes. A peripheral blood sample (40 mL) was collected under sterile conditions by venepuncture into heparinized tubes (Becton Dickinson, Plymouth, UK). The study was approved by the Ethics Committee of the School of Dental Medicine, University of Zagreb, Zagreb, Croatia.

Preparation of materials

In the present study, four dental adhesives were tested: Excite (EXC; Ivoclar Vivadent, Schaan, Liechtenstein), Adper Single Bond 2 (ASB; 3M ESPE, St. Paul, MN, USA), Prompt L-pop (PLP; 3M ESPE) and OptiBond Solo Plus (OSP; Kerr S.p.a, Salerno, Italy). The composition of these dental adhesives is shown in Table 1. To test the genotoxicity of dental adhesives, each one was polymerized under aseptic conditions in accordance with the manufacturer's instructions using an Elipar TriLight halogen-curing unit (3M ESPE) from a 2-mm distance for 40 seconds. To ensure complete polymerization, only four drops of an adhesive were cured at the same time. After polymerization, dental adhesives were weighted (Sartorius BLG10S, Göttingen, Germany), fragmented and transferred into gamma sterilized plastic tube bottle (Nunc GmbH, Wiesbaden, Germany). We repeated this procedure until 1 g of the polymerized dentin bonding agent was obtained. The elution of dental adhesive (1 g) was performed in 2 mL of dimethylsulfoxide (DMSO; Kemika, Zagreb, Croatia). The tubes were hermetically sealed with accompanying caps. In the same manner, eluates of all tested dental adhesives were set up at 1 hour, 24 h (1 day), and 120 h (5 days).

The elution periods ended simultaneously for each adhesive when blood sampling was performed.

Cytotoxicity testing

Lymphocyte viability was tested using the trypan blue exclusion technique. Fifty microliters of the lymphocyte layer was mixed with 50 mL of 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO, USA), dropped onto a microscope slide, and covered with a cover slip. Specimens were analyzed using an Olympus CX 40 light microscope (Tokyo, Japan) under X100 magnification. For each concentration tested, 500 leukocytes were analyzed by counting unstained (viable) cells. Blue-colored cells were considered to be nonviable. At the end of the cultivation period, pH of each cell culture was additionally checked with a SevenEasy pH meter (Metler-Toledo, Schwertzenbach, Switzerland). Neither change in the medium color nor change in pH of all cell culture was observed[31,32].

The relative cell viability was tested 72 h after culture had been started by using the trypan blue exclusion technique. For all tested adhesives, the cytotoxity of 1-day eluates of 10 μg/mL concentration appeared to be higher than 25%. Therefore, only dilutions of 0.2 μg/mL, 0.5 μg/mL and 5 μg/mL were analyzed. Upon completion of the cultivation period, pH of each cell culture was additionally checked by SevenEasy pH meter. Results showed that pH was 7.13±0.03, which was in accordance with the manufacturer's recommendations for pH values in F10 cell culture medium[18].

Micronucleus test

The micronucleus test was carried out as described by Fenech and Morley[33]. The same blood sample was used for testing dental adhesives after all three elution periods. Therefore, eluates were started 5 days, 24 h and 1 h prior to blood sampling. As the analysis was carried out in duplicates, blood sample of a single donor was divided in 24 aliquots of 0.8 mL for each tested dentin bonding agent. In order to initiate cell cultures, each blood aliquot was introduced into a cell culture flask (Nunc GmbH, Wiesbaden, Germany) containing 8 mL of F-10 medium (Sigma) supplemented with 20% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mL activator phytohemagglutinin (Murex, Dartford, UK) and antibiotics (penicillin 100 IU/mL and streptomycin 100 g/mL; Sigma-Aldrich, St. Louis, MO, USA). The cultures were incubated for 72 hours in 5% CO_2 at 37 °C. After 24 hours in cultures, 3.6 μL, 90.9 μL and 1 mL eluate of each tested adhesive were added to get final concentrations of adhesives in culture of 0.2 μg/mL, 0.5 μg/mL and 5 μg/mL. Simultaneously, negative control cultures were treated with 1 mL DMSO (Kemika, Zagreb, Croatia). Cytochalasin B (6 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added at 44 hours post-culture initiation to arrest cytokinesis of dividing cells. By the end of the cultivation period (72 hours), the cells were harvested by centrifugation (600 rpm, 5 min). Next, the cells were suspended with 10 mL of saline solution at room temperature and recentrifuged (600 rpm, 5 min). The supernatant was removed and the sediment was resuspended and mixed in cold solution (+4 °C) of methanol and acetic acid (3:1). The resuspended cell sediment was applied to coded microscopic slides pre-warmed at 37 °C. After dropping, the slides were stained with 5% Giemsa for 5 min, washed in distilled water and dried at room temperature. For micronucleus identification, all slides were analyzed in accordance with Fenech[28,30] using an Olympus CX 40 (Olympus, Tokyo, Japan) microscope. The induction of micronucleus was evaluated by scoring a total of 500 binucleated cells at X1000 magnification.

Statistical analysis

The micronucleus test results were analyzed by χ^2-test to determine statistical significance. The level of significance was set at 0.05. All calculations were performed using Statistica 7.0 (StatSoft, Tulsa, OK, USA) commercial software. Results were expressed as mean ± SD.

Results

Trypan blue test showed the cytotoxicity of all investigated adhesive systems to be extremely high at the highest concentration of 10 μg/mL with less than 50% of viable cells. Other tested concentrations (0.2 μg/mL, 0.5 μg/mL and 5 μg/mL) showed viability higher than 80%.

Micronucleus test was used to measure the incidence of micronuclei in 500 binuclear cells. After exposure of lymphocytes to 1-hour eluates, only the highest concentration (5 μg/mL) of all adhesives

showed statistically significant difference in comparison to negative control (Fig. 1). OSP and EXC showed higher values than the other two adhesives, but this difference was not statistically significant. The highest number of cells with micronuclei was observed after lymphocyte treatment with 1-day eluates, concentrations of 0.5 μg/mL and 5 μg/mL, again for all tested materials. However, the EXC material was the one with the highest number of micronuclei at this time point, followed by ASB and OSP. The number of micronuclei again decreased to nonsignificant level in cultures exposed to 5-day eluates at all concentrations of all tested adhesives.

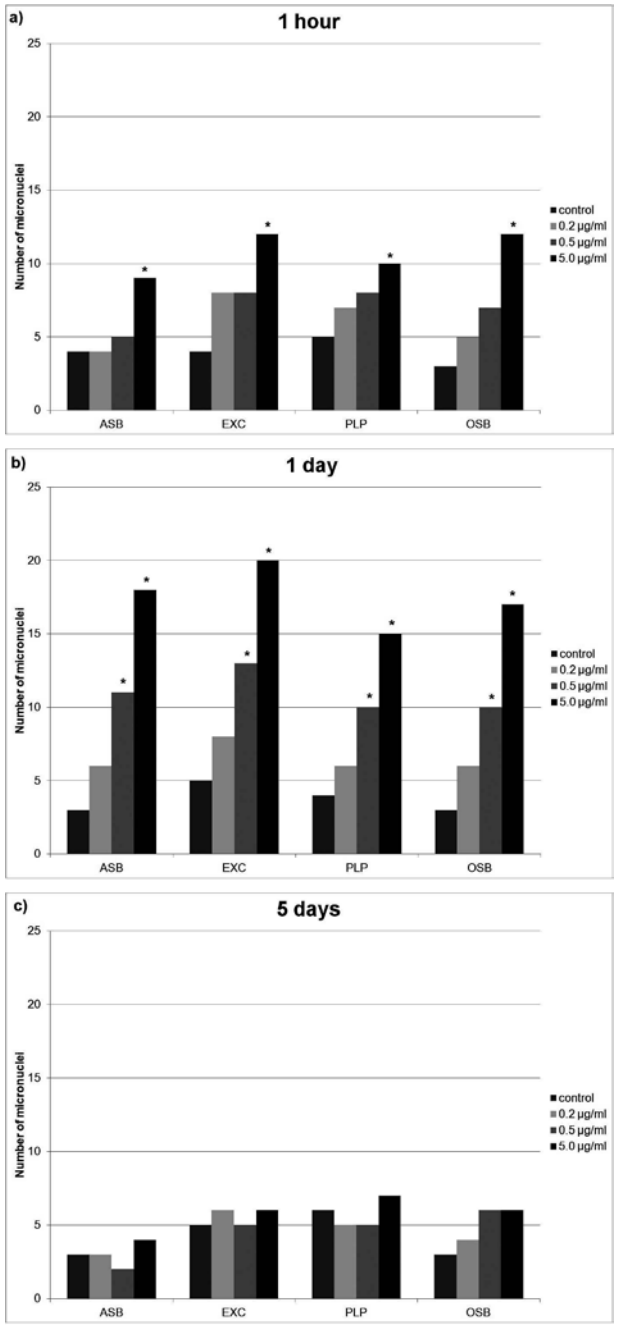

*Fig. 1. Number of micronuclei per 500 lymphocytes exposed to eluates from all tested adhesives: a) 1 hour eluates; b) 1 day eluates; c) 5 day eluates. Statistically significant differences are marked with * ($p<0.05$); Adper Single Bond 2 (ASB), Excite (EXC), Prompt L-pop (PLP), OptiBond Solo Plus (OSP).*

Discussion

This study was performed in order to assess the genotoxic potential of three commonly used etch-and-rinse and one self-etch adhesive system (Excite, Adper Sngle Bond 2, Prompt L-pop and OptiBond Solo Plus) by micronucleus test. The selected scientific method was used because it demonstrates genotoxicity and changes of the cell cycle[16]. Earlier studies showed that the formation of micronuclei may be indicative of chromosomal damage and DNA strand breaks induced by commonly used dental monomers like triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA)[34,35]. The DNA damage arrests cell cycle in G1 and G2/M phases in mammalian cells to allow for repair of the genetic material[34]. It has been shown that these monomers cause disruption of the cellular redox balance by increasing the concentrations of reactive oxygen species and decreasing the levels of the natural radical scavenger gluthatione[36,37]. Intracellular glutathione depletion can activate the pathways leading to apoptosis and it is therefore also responsible for the cytotoxicity. It is suggested that TEGDMA and HEMA are responsible for combined cytotoxic and genotoxic effect of dental resin materials, such as those used here. However, it is known that sub-cytotoxic levels of monomers can modify tissue repair mechanisms and cellular homeostasis[34].

Dental adhesives are light cured materials, which are composed of polymerizable monomers activated by photoinitiators. The transformation from monomers to a cross-linked polymer network is usually incomplete and amounts from 63% to 90%[38]. The residual unpolymerized monomers remain trapped in the polymer network

and can be released when exposed to saliva. Some of them can be hydrolyzed or metabolized by salivary enzymes such as pseudocholinesterase and cholesterolesterase and degraded into even more harmful compounds. Unreacted monomers can also diffuse through dentine tubules into dental pulp despite the internal pulpal pressure and thus reach the blood stream[26,39]. In one *in vitro* study, it was demonstrated that residual HEMA was released from five commercial dental adhesives. Virtually all HEMA was eluted by 24 hours with more than 90% eluted in the first hour and only trace amounts were identifiable after 24 hours[38,39]. The amount of HEMA released from Excite was found to be 25.7 ppm after one hour and additional 1.3 ppm until 24 hours, which amounts to 8.6 wt% of the total amount of HEMA in this adhesive system[38]. Another study measured the monomer release from other adhesives and found that the percentage of HEMA and TEGDMA eluted from polymerized adhesives in 24 hours was 1.5%-2.5% of the total weight, but it was not enough to elicit cytotoxic effects[39].

In the present study, cytotoxic effects of dental adhesives could be evaluated isolated from genotoxicity due to the use of very low eluate concentrations. Surprisingly, results of this study showed that all tested dental adhesives caused similar cytotoxic effects; although the self-etch adhesive PLP was expected to cause greatest genetic damage due to its low pH value (1.1), it was surprising that all of the tested materials performed equally. This is probably related to their composition because HEMA is one of the main ingredients in all of them. The slightly higher incidence of micronuclei for EXC than other materials is attributable to its composition with 30 wt% of HEMA[23]. Our results are in agreement with other *in vitro* studies, which also showed that HEMA induced a large number of micronuclei[18,35,40,41].

The results obtained showed the transitory nature of the genetic breakdown caused by dental adhesives and their dose-dependent behavior. A statistically significantly increased incidence of micronuclei after 1 hour was demonstrated only in the highest eluate concentration, whereas after one day, even the concentration of 0.5 μg/mL was sufficient to cause the appearance of micronuclei and the highest number of micronuclei achieved at 5 μg/mL. Similar results were also obtained in other studies[16,18,34,35].

Our data do not clearly indicate the genotoxic risk posed by currently used dental adhesives. Since they remain in human body for a prolonged period, the genotoxic components release might present a serious clinical problem. Future experiments with dental adhesive systems should be conducted on pulpal or gingival cells, since they are most similar to the conditions in oral cavity.

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Table 1. Composition of tested dental adhesive systems used in the study (manufacturers' data)

Dental adhesive system	Manufacturer (lot no.)	Composition	Solvent
Adper Single Bond 2 (ASB)	3M ESPE, St. Paul, MN, USA (N177065)	Bis-GMA, HEMA, dimethacrylate, silica, methacrylate copolymer, polyacrylic and polyitaconic acid, photoinitiators	Ethanol, water
Excite (EXC)	Ivoclar Vivadent, Schaan, Liechtenstein (M29493)	Bis-GMA, HEMA, glycerin dimethacrylate, phosphoric acrylates, silica, initiators, stabilizers	Ethanol
Prompt L-pop (PLP)	3M ESPE, St. Paul, MN, USA	Bis-GMA, HEMA, methacrylic phosphoesters, CQ and polyalkenoic acid	Water
OptiBond Solo Plus (OSP)	Kerr, Salerno, Italy (4225212)	HEMA, dimethacrylates, silica, initiators and stabilizers	Ethanol

Bis-GMA = bisphenol A-glycidyl methacrylate; CQ = camforquinone; HEMA = 2-hydroxyethyl methacrylate

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Sažetak

UČINAK DENTINSKIH ADHEZIVA NA UČESTALOST MIKRONUKLEUSA U LIMFOCITIMA PERIFERNE KRVI *IN VITRO*

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Stomatološki dentinski adhezivi dolaze u izravan dodir s oralnim tkivima. Zbog ovog bliskog i dugotrajnog kontakta ove materijale treba obilježavati visok stupanj biokompatibilnosti. Svrha ovoga rada bila je procjena genotoksičnog učinka dentinskih adheziva na ljudskim limfocima *in vitro*. Polimerizirani adhezivi (Excite, Adper Sngle Bond 2, Prompt L-pop i OptiBond Solo Plus) eluirani su u dimetil sulfoksidu kroz 1 h, 24 h i 120 h (5 dana). Nakon toga su kulture limfocita tretirane različitim koncentracijama eluata (0,2 μg/mL, 0,5 μg/mL i 5 μg/mL) dobivenim iz ispitivanih materijala. Za procjenu genotoksičnosti koristio se mikronukleus test, dok se χ^2-test koristio za statističku analizu ($p<0,05$). Adhezivi pokazuju genotoksičan učinak u kulturi limfocita nakon jednosatnog tretiranja eluatom koncentracije 5 μg/mL. Nakon 24 h svi su ispitivani adhezivi pokazali genotoksičnost u koncentraciji 0,5 μg/mL i 5 μg/mL. Na temelju rezultata može se zaključiti kako upotreba adheziva izaziva genotoksični učinak u ljudskim limfocitima. Toksični učinak povećava se s koncentracijom ispitivanog materijala, a smanjuje s vremenom trajanja eluacije.

Ključne riječi: *Dentalni adhezivi; Mutageničnost, testovi; Limfociti; Mikronukleus, testovi*