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IZVORNI ZNANSTVENI RAD
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Genotoksični potencijal dentinskih adheziva

Genotoxic Potential of Dentin Bonding Agents

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Sažetak
Svrha rada bila je ispitati genotoksično djelovanje pet dentinskih adheziva: Adper Single Bonda, Adper Single Bonda 2, Prompt L-popa, Excitea i OptiBonda Solo Plus. Ispitivanje genotoksičnosti provedeno je na humanim limfocitima periferne krvi u uvjetima in vitro, ispitane koncentracije adheziva bile su 0,2, 0,5 i 5 µg/ml, a testirana vremena eluacije 1 sat, 24 sata i 5 dana. Genotoksičnost adheziva ispitivala se citogenetičkom metodom - analizom strukturnih aberacija kromosoma, dakle, određivanjem ukupnog broja kromosomskih lomova, kromatidnih lomova i acentričnih fragmenata. Rezultati pokazuju genotoksičnost OptiBonda Solo Plus već u koncentraciji 0,2 µg/ml i to nakon 24-satne eluacije, zatim OptiBonda Solo Plus u koncentraciji 0,5 µg/ml nakon jednosatne eluacije, OptiBonda Solo Plus, Adper Single Bonda 2 i Excitea u koncentraciji 0,5 µg/ml nakon jednodnevne eluacije. U koncentraciji 5 µg/ml nakon jednosatne eluacije genotoksičnost su pokazali OptiBond Solo Plus, Excite, Adper Single Bond 2 i Adper Single Bond, a nakon jednodnevne eluacije svi su ispitivani adhezivi pokazali genotoksičnost. Iz rezultata je jasno da genotoksičnost raste s porastom koncentracije adheziva, a smanjuje se s vremenom. Najveća genotoksičnost zabilježena je nakon 24-satne eluacije.

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Ključne riječi
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Uvod

Današnji dentalni materijali, osim fizičko-kemijskih, moraju zadovoljiti i biološke kriterije. Zato, osobito u posljednja dva desetljeća, sve važnija postaju testiranja citotoksičnosti i genotoksičnosti dentalnih materijala.

Dentinski adhezivi, a oni su prijeko potrebni materijali u današnjoj estetskoj restorativnoj stomatologiji uglavnom zbog vezivanja kompozitnih ili keramičkih materijala za tvrda zubna tkiva, u dubokim kavitetima mogu biti u bliskom kontaktu s pulpnim tkivom. Smatralo se da bi mogli biti učinkoviti i u direktnom prekrivanju pulpe. No, iako se izravno prekrivanje pulpe dentinskim adhezivima pokazalo uspješnim

Introduction

Contemporary dental materials, aside from their physical and chemical properties, must also satisfy biological demands. Therefore, especially in the last two decades, testing for cytotoxicity and genotoxicity in dental materials has become more significant.

Dentin bonding agents, which are necessary materials in current aesthetic restorative dentistry due to their capacity to bond dental materials such as composite and ceramics to hard tooth tissue, may be in close contact with pulp tissue in deep cavities. It was considered they might be effective even as materials used for deep pulp capping. Success was shown in animal teeth – in mice and monkeys

na životinjskim zubima - mišjim i majmunskim (1, 2), na humanima nije bilo uspjeha zbog perzistirajuće upale i izostanka stvaranja dentinskog mosta (3-9), premda neki prikazi slučajeva pokazuju suprotno (10). Peta generacija dentinskih adheziva pokazala je kontrakciju glatke muskulature krvnih žila (11), ali novija studija single-bottle dentinskih adheziva pokazuje njihovo vazodilatirajuće djelovanje (12), što bi moglo još više pospješiti pulpno krvarenje.

U uvjetima *in vitro* dentinski adhezivi već su pokazali citotoksično (13, 14) i genotoksično djelovanje (15-17) na humanim stanicama. Na citotoksičnost, osim samog materijala, utječe debljina dentinskog sloja (18, 19) i njegova permeabilnost (19-21), a time i klinički predtretman dentina (21) te pulpni tlak koji povećava citotoksičnost (22). Citotoksičnost se uglavnom očituje u ranom razdoblju - prvih 24 do 48 sati (21, 23, 24), iako je posljednjih godina poznata i kasna citotoksičnost nastala zbog otpuštanja malih koncentracija tijekom dužeg vremenskog razdoblja (25-27).

Dentinski adhezivi u vodenom se mediju otapaju do određene granice i otpuštaju neke supstancije, kao što su monomeri HEMA (hidroksietil metakrilat) i TEGDMA (trietilen-glikol dimetakrilat) (23, 28, 29). Jedan od glavnih monomera u današnjim dentinskim adhezivima jest HEMA i ona može djelovati citotoksično (28-30), a remeti i normalno funkcioniranje stanica i njihovih organela (31, 32). Monomeri iz dentinskih adheziva također su pokazali genotoksično djelovanje na humanim limfocitima (33, 34), a Bis-GMA (bisfenol-A glicidil metakrilat) u visokim koncentracijama i embriotoksično/teratogeno djelovanje (35).

Svrha ove studije bila je istražiti pokazuju li dentinski adhezivi, koji se u kliničkoj praksi danas najčešće koriste, genotoksični potencijal, tj. dovode li do strukturnih aberacija (kromosomskog loma, kromatidnog loma i acentričnih fragmenata) na kromosomima humanih limfocita periferne krvi te kakva je ovisnost njihove moguće genotoksičnosti o vremenu proteklom od tretmana i o koncentraciji adheziva.

Materijali i metode

Uzorkovanje krvi

Istraživanje potencijalne genotoksičnosti dentinskih adheziva provedeno je na ljudskim limfocitima mladih, zdravih donora iz opće populacije Republike Hrvatske. Na temelju upitnika koje su popunili vidljivo je da ne postoje podaci o njihovoj izloženosti fizikalnim ili kemijskim agensima 12 mjeseci prije uzorkovanja krvi, a mogli bi inducirati oštećenja genoma.

(1, 2), but not so in humans (3-9) due to persistent inflammation and failure in dentin bridging formation, although some case reports showed different results (10). Fifth generation dentin bonding agents showed smooth muscle contraction in blood vessels (11), but a recent study of "single-bottle" dentin bonding agents displayed a vaso-relaxant effect (12), which may promote pulpal bleeding.

Dentin bonding agents showed cytotoxicity (13, 14) and genotoxicity (15-17) within *in vitro* conditions when tested on human cells. It is also reported (25-27) that cytotoxicity, aside from the properties of the material itself, depends upon dentin layer thickness (18, 19), dentin permeability (19-21), and therefore clinical dentin pre-treatment (21), as well as pulpal pressure which increases cytotoxicity (22). It is mostly expressed in the early period, within the first 24-48 hours (21, 23, 24), rather than later, when cytotoxicity results from small concentrations released over prolonged periods of time.

In an aqueous medium, dentin bonding agents melt and yield substances that can be cytotoxic, such as HEMA monomers (hydroxylethyl methacrylate), and TEGDMA (triethylene-glycol dimethacrylate) (23, 28, 29). One of the main monomers in current dentin bonding agents is HEMA, which acts as a cytotoxic agent (28-30), and influences and disturbs normal human cell function and cellular organelles (31, 32). Monomers from dentin bonding agents also showed genotoxicity in human lymphocytes (33, 34), and Bis-GMA (bisphenol-A glycidyl methacrylate) in higher concentrations displayed an embriotoxic/teratogenic effect (35).

The aim of this study was to investigate whether the most frequently used dentin bonding agents have genotoxic potential and whether they cause structural aberrations (chromosome breaks, chomatidee breaks and accentric fragments) on chromosomes from human peripheral blood lymphocytes, and how possible genotoxicity depends upon application time and dentin bonding agent concentration.

Materials and methods

Blood sampling

The research was performed on lymphocytes from young, healthy, non-smoking donors from the general population. According to questionnaire taken, the donors hadn't been exposed to any physical or chemical agents that might have interfered with the results of the genotoxicity testing 12 months prior to blood sampling.

Tijekom uzorkovanja svakom od donora uzeto je 40 ml krvi iz kubitalne vene u heparinizirani vacutainer (Becton Dickenson, Plymouth, Velika Britanija). Odmah nakon toga obavljeno je istraživanje djelovanja adheziva primjenom citogenetičke metode - analizom strukturnih aberacija kromosoma.

Priprava adheziva

Istraživanje je obuhvatilo pet "single-bottle" dentinskih adheziva: Adper Single Bond, Adper Single Bond 2 s nanopunilom, Prompt L-pop (svi 3M ESPE, Seefeld, Njemačka), Excite (Vivadent- Ivoclar, Schaan, Liechtenstein) i OptiBond Solo Plus (Kerr S.p.a, Salerno, Italija).

U sterilnim uvjetima jednake su količine svakog adheziva stavljene na satno stakalce i polimerizirane 40 sekundi halogenom svjetiljkom Elipar TriLight (3M ESPE , Seefeld, Njemačka) na udaljenosti od 2 mm. Nakon polimerizacije svaki je adheziv usitnjen sterilnom špatulom, izvagan (Sartorius BLG10S, Goettingen, Njemačka) i kvantitativno prenesen u penicilinsku bočicu. Eluiranje je rađeno u dimetil-sulfoksidu (Kemika d.o.o, Zagreb, Hrvatska), tako da je na 1g adheziva dodano 2 ml otapala. Testirana su tri vremena eluacije – 1 sat, 24 sata i 5 dana, za svaku koncentraciju adheziva, a testirane koncentracije bile su 0,2 μg/ml, 0,5 μg/ml i 5 μg/ml. One su određene nakon što je koncentracija od 10 μg/ml u pokusnoj studiji pokazala toksičnost veću od 30%. Analizirana su po tri uzorka krvi za svaku ispitivanu koncentraciju adheziva i svako testirano vrijeme.

Analiza strukturnih aberacija kromosoma

Tijekom izrade preparata za potrebe analize kromosomskih aberacija koristila se konvencionalna metoda IAEA (36). Prije kraja svakoga eluacijskog razdoblja (1 sat, 24 sata, 5 dana) uzeta je krv od donora i zatim je slijedila izrada preparata. Za to je 0,8 ml pune krvi kultivirano u 8 ml F-10 medija (Sigma, St. Louis, SAD) obogaćenog s 20% fetalnoga telećeg seruma (Sigma, St. Louis, SAD), 100 IJ penicilina (Sigma, St. Louis, SAD), 100 IJ streptomicina (Sigma, St. Louis, SAD) i 0,5 ml mitogenog aktivatora fitotohemaglutinina (Murex, Dartford, Velika Britanija), na 37^0 C u trajanju od 48 sati. Istodobno s iniciranjem pojedinim je kulturama dodano 3,6 μl, 90,9 μl i 1 ml eluata ispitivanog adheziva, tako da su konačne koncentracije adheziva bile: 5 μg/ml, 0,5 μg/ml i 0,2 μg/ml. Kontrolnoj kulturi limfocita dodan je samo 1 ml dimetilsufoksida (Kemika d.o.o, Zagreb, Hrvatska).

Nakon 45 sati od početka kultivacije svakoj je kulturi dodana otopina kolhicina (Sigma, St. Louis,

From each donor 40 ml of blood was drawn by antecubital venipuncture into heparinized vacutainers (Becton Dickenson, Plymouth, UK). The reseach began immediately after using this the Chomosomal aberration analysis cytogenetic method.

Dentin bonding agent preparation

The study comprised genotoxicity testing of the following five single-bottle adhesives: Adper Single Bond, Adper Single Bond 2 with nanofiller, Prompt L-pop (all 3M ESPE, Seefeld, Germany), Excite (Vivadent-Ivoclar, Schaan, Liechtenstein) and OptiBond Solo Plus (Kerr S.p.a, Salerno, Italy).

In aseptic conditions a sample of each dentin-bonding agent was polymerized in agate mortar for 40 seconds using a halogen source (Elipar, 3M ESPE; Seefeld, Germany) from a 2 mm distance. They were rubbed and chopped up using the agate pastille, weighted (Sartorius BLG10S, Goettingen, Germany) and eluted in dimethyl sulfoxide (DMSO)(Kemika, Zagreb, Croatia): 1g / 2 ml. Each adhesive was eluted respectively, for 1 h, 24 hrs and 5 days. Adhesive elutions were tested at final concentrations 0.2, 0.5 and 5 μg/ml. Concentrations used in the study were determined after the concentration of 10 μg/ml demonstrated cytotixicity higher than 30% in the pilot study. The three specimens for each concentration and each time tested were analysed.

Chromosomal aberration analysis

Slide preparation was done by the conventional IAEA method (36). Just before the end of each elution time (1 h, 24 hrs, 5 days) blood samples were taken and cell cultures started. The 0.8 ml of whole blood was cultivated in an F10 medium (Sigma, St. Louis, USA) supplemented with 20% fetal bovine serum (Sigma, St. Louis, USA), 100 IU of penicillin (Sigma, St. Louis, USA), 100 IU of streptomycin (Sigma, St. Louis, USA) and 10 μg/ml of the mitotic activator phytohemagglutinin (Murex, Dartford, UK), at 37°C for 48 hours. Simultaneously with the culture initiation, volumes of 3.6 μl, 90.9 μl and 1 ml elution of each adhesive were added to get the final concentrations of 0.2, 0.5 and 5 μg/ml. Control cultures were treated with the same volume of DMSO.

Three hours prior to harvesting 0.2 μg/ml of colchicin (Sigma, St. Louis, USA) was added. The cultivation was followed by hypotonic treatment with 0.075M KCl (Kemika, Zagreb, Croatia), fixa-

SAD) u konačnoj koncentraciji od 0,2 μg/ml. Zatim se dodavala hipotonična otopina KCl (Kemika d.o.o, Zagreb) koncentracije 0,075 mol/dm^3, fiksacija u ohlađenoj (4°C) otopini fiksira, koju čine otopina metanola (Kemika d.o.o, Zagreb, Hrvatska) i ledene octene kiseline (Kemika d.o.o, Zagreb, Hrvatska) u omjeru 3:1 te sušenje preparata na sobnoj temperaturi i bojenje 5%-tnom otopinom Giemsae (Sigma, St. Louis, SAD), sve prema IAEA-i (31).

Preparati su analizirani svjetlosnim mikroskopom (Olympus Optical Co, Europa, GMBH, Hamburg, Njemačka) pod ukupnim povećanjem od 1000 puta. Određivao se ukupan broj aberacija, dakle, broj kromosomskih lomova, kromatidnih lomova i acentričnih fragmenata na svih 46 kromosoma u 500 metafaznih limfocita za svaku koncentraciju adheziva i svako testirano vrijeme. Analizirana su po tri uzorka krvi za svaku koncentraciju i svako vrijeme eluacije.

Statistička obrada

Statistička znatnost rezultata testa kromosomskih aberacija između tretiranih i kontrolnih limfocita obavljena je uporabom χ^2 i Fisherova testa. Vjerojatnost je postavljena na 0,05.

Rezultati

Pri koncentraciji 0,2 μg/ml jedino je OptiBond Solo Plus doveo do statistički znatnog povećanja broja strukturnih aberacija kromosoma i to nakon 24-satne eluacije (Tablica 1.).

U koncentraciji 0,5 μg/ml dentinski adhezivi pokazali su nešto veća odstupanja broja izazvanih kromosomskih aberacija od broja aberacija prouzročenih kontrolom, a statistički znatno povećanje pronađeno je samo za OptiBond Solo Plus nakon jednosatne eluacije te za OptiBond Solo Plus, Adper Single Bond 2 i Excite nakon jednodnevne eluacije (Tablica 2.).

Najviša koncentracija adheziva - 5 μg/ml prouzročila je i najviše strukturnih aberacija u kulturi humanih limfocita periferne krvi i najveća odstupanja broja aberacija izazvanih dentinskim adhezivima od broja aberacija prouzročenih kontrolnom otopinom. U tom su slučaju statistički znatno povećanje broja aberacija u odnosu prema kontrolnoj vrijednosti pokazali OptiBond Solo Plus, Excite, Adper Single Bond 2 i Adper Single Bond nakon jednosatne eluacije. Nakon jednodnevne eluacije svi testirani dentinski adhezivi pokazali su statistički znatno povećanje broja aberacija u odnosu prema kontroli, i to najviše OptiBond Solo Plus i Excite, zatim

tion with 3:1 methanol–glacial acetic acid, air-drying and staining with 5% Giemsa (Sigma, St. Louis, USA), according to the IAEA (28).

Preparations were analysed with a light microscope (Olympus Optical Co, Europa, GMBH, Hamburg, Germany), with 1000x magnification. The complete number of aberrations, as well as the number of chromosome breaks, chromatide breaks and acentric fragments, on all 46 chromosomes in 500 metaphase lymphocytes was determined, for each concentration and elution time tested. The three specimens were analysed for each concentration and each time tested.

Statistical analysis

The differences in the number of specific chromosomal aberrations between the treated and control lymphocytes were evaluated using χ^2 and Fisher's PLSD test. The level of significance was set at 0.05.

Results

In the concentration of 0.2 μg/ml only OptiBond Solo Plus showed a statistically significant increase in the number of aberrations after a 24 hour elution period (Table 1).

The concentration of 0.5 μg/ml showed a slightly higher difference in number of aberrations caused by adhesives than in the control, and statistical significance was found only for the OptiBond Solo Plus after a 1 hour elution period and for the OptiBond Solo Plus, Adper Single Bond 2 and Excite after a 1 day elution period (Table 2).

The highest concentration of dentin bonding agents -5 μg/ml caused the most aberrations in peripheral blood lymphocytes and the highest difference between the number of aberrations caused by adhesives and that caused by the control. Here, OptiBond Solo Plus, Excite, Adper Single Bond 2 and Adper Single Bond showed statistically significant increase in number of aberrations after 1 hour elution period with respect to control. After a 1 day elution period all tested dentin bonding agents showed a statistically significant increase in the number of aberrations with respect to the control, and these were: OptiBond Solo Plus and Excite - the most, then Adper Single Bond 2 and

Tablica 1. Broj strukturnih aberacija kromosoma u 500 limfocita tretiranih eluatom adheziva koncentracije 0,2 µg/ml
Table 1 The number of the structural chromosome aberrations in 500 lymphocytes treated with dentin bonding agent elution in concentration 0.2 µg/ml

Vrijeme eluacije • Elution time	Broj uzoraka • Number of specimens	Broj kromosomskih aberacija (medijan) • Number of chromosome aberrations (mean)			SD	min	max
		Kontrola • Control	Adheziv • Dentin bonding agent, c = 0.2 µg/ml				
1 sat • 1 hour	3	1	AdSB	0	0,5774	-0,5774	0,5774
		3	AdSB2	4	1,7321	2,2679	5,7321
		3	Pl-p	1	0,5774	0,4226	1,5774
		1	E	4	1,1547	2,8453	5,1547
		1	OBSP	3	1,1547	1,8453	4,1547
24 sata • 24 hours	3	1	AdSB	2	0,5774	1,4226	2,5774
		2	AdSB2	7	1,7321	5,2679	8,7321
		2	Pl-p	1	0	1	1
		0	E	4	1,1547	2,8453	5,1547
		2	OBSP	8*	2	6	10
5 dana • 5 days	3	1	AdSB	1	0,5774	0,4226	1,5774
		3	AdSB2	4	0,5774	3,4226	4,5774
		3	Pl-p	2	1	1	3
		1	E	3	1	2	4
		2	OBSP	4	0,5774	3,4226	4,5774

* Statistički znatno u odnosu prema kontroli (P < 0,05) • Statistically significant related to control (P < 0.05)

Legenda • Legend:
AdSB = Adper Single Bond
AdSB2 = Adper Single Bond 2
Pl-p = Prompt l-pop
E = Excite
OBSP = Opti Bond Solo Plus
c = koncentracija • concentration
SD = standardna devijacija • standard deviation

Tablica 2. Broj strukturnih aberacija kromosoma u 500 limfocita tretiranih eluatom adheziva koncentracije 0,5 µg/ml
Table 2 The number of the structural chromosome aberrations in 500 lymphocytes treated with dentin bonding agent elution in concentration 0.5 µg/ml

Vrijeme eluacije • Elution time	Broj uzoraka • Number of specimens	Broj kromosomskih aberacija (medijan) • Number of chromosome aberrations(mean)			SD	min	max
		Kontrola • Control	Adheziv • Dentin bonding agent, c = 0.5 µg/ml				
1 sat • 1 hour	3	1	AdSB	2	0	2	2
		3	AdSB2	6	1,5275	4,4725	7,5275
		3	Pl-p	3	1,1547	1,8453	4,1547
		1	E	4	2,6458	1,3542	6,6458
		1	OBSP	8*	1,1547	6,8453	9,1547
24 sata • 24 hours	3	1	AdSB	5	0,5774	4,4226	5,5774
		2	AdSB2	11*	2,6458	8,3542	13,6458
		2	Pl-p	5	1	4	6
		0	E	9*	1	8	10
		2	OBSP	12*	4,5826	7,4174	16,5826
5 dana • 5 days	3	1	AdSB	2	0,5774	1,4226	2,5774
		3	AdSB2	2	1	1	3
		3	Pl-p	1	1,5276	-0,5276	2,5276
		1	E	2	0	2	2
		2	OBSP	4	1,1547	2,8453	5,1547

* Statistički znatno u odnosu prema kontroli (P < 0,05) • Statistically significant related to control (P < 0.05)

Legenda • Legend:
AdSB = Adper Single Bond
AdSB2 = Adper Single Bond 2
Pl-p = Prompt l-pop
E = Excite
OBSP = Opti Bond Solo Plus
c = koncentracija • concentration
SD = standardna devijacija • standard deviation

Tablica 3. Broj strukturnih aberacija kromosoma u 500 limfocita tretiranih eluatom adheziva koncentracije 5 µg/ml
Table 3 The number of the structural chromosome aberrations in 500 lymphocytes treated with dentin bonding agent elution in concentration 5 µg/ml

Vrijeme eluacije • Elution time	Broj uzoraka • Number of specimens	Broj kromosomski aberacija (medijan) • Number of chromosome aberrations (mean)			SD	min	max
		Kontrola• Control	Adheziv • Dentin bonding agent, c = 5 µg/ml				
1 sat • 1 hour	3	1	AdSB	7*	1,1547	5,8453	8,1547
		3	AdSB2	9*	1,1547	7,8453	10,1547
		3	Pl-p	7	1,1547	5,8453	8,1547
		1	E	12*	1	11	13
		1	OBSP	14*	1,7321	12,2679	15,7321
24 sata • 24 hours	3	1	AdSB	12*	3	9	15
		2	AdSB2	16*	0	16	16
		2	Pl-p	12*	0,5774	11,4226	12,5774
		0	E	19*	0,5774	18,4226	19,5774
		2	OBSP	19*	4,3589	14,6411	23,3589
5 dana • 5 days	3	1	AdSB	2	1,1547	0,8453	3,1547
		3	AdSB2	5	2	3	7
		3	Pl-p	2	0,5774	1,4226	2,5774
		1	E	5	2,5166	4,4834	7,5166
		2	OBSP	7	1,5275	4,4725	7,5275

* Statistički znatno u odnosu prema kontroli (P < 0,05) • Statistically significant related to control (P < 0.05)

Legenda • Legend:
AdSB = Adper Single Bond
AdSB2 = Adper Single Bond 2
Pl-p = Prompt l-pop
E = Excite
OBSP = Opti Bond Solo Plus
c = koncentracija • concentration
SD = standardna devijacija • standard deviation

Adper Single Bond 2, i na kraju Adper Single Bond i Prompt L-pop (Tablica 3.).

Petodnevni eluati svih testiranih dentinskih adheziva nisu pokazali statistički znatno odstupanje broja kromosomskih aberacija od kontrolnih vrijednosti ni u jednoj mjerenoj koncentraciji (Tablice 1., 2., 3.).

Rasprava

Metodom analize strukturnih aberacija kromosoma na stanicama sisavaca u uvjetima *in vitro* određuje se genotoksični potencijal kemijskih supstancija. Ovim istraživanjem određen je ukupan broj strukturnih aberacija kromosoma, tj. ukupan broj kromosomskih lomova, kromatidnih lomova i acentričnih fragmenata uzrokovanih različitim koncentracijama pet dentinskih adheziva nakon različitih vremena eluacije. Mjerena pH kultura (indikatorom-fenol crveni i digitalnim pH metrom) ostala je nepromijenjena tijekom eksperimenta, pa isključujemo pojavu genotoksičnosti kao posljedice porasta aciditeta medija.

Najviše kromosomskih aberacija, kako se i moglo očekivati, nađeno je u najvišoj koncentraciji

finally, Adper Single Bond and Prompt L-pop (Table 3).

The five-day-elutes did not show statistically significant increase in number of aberrations for any of the test dentin bonding agents, with respect to the control in any concentration tested (Tables 1, 2, 3).

Discussion

The genotoxic potential of chemical substances is determined by the Structural chromosomal aberration analysis cytogenetic method on mammalian cells within *in vitro* conditions. This study determines the complete number of structural aberrations, and the number of chromosome breaks, chromatidee breaks and acentric fragments caused by different concentrations of five dentin bonding agents after different periods of elution. The pH value of each of the cultures was measured (with the phenol red indicator and a digital pH-meter), and it stayed the same throughout the experiment, which allows us to exclude genotoxicity as a result of increased medium acidity.

The highest number of chromosomal aberrations, as expected, was found in the highest concentration-

adheziva (5 μg/ml) (Tablica 3.). Što se tiče vremena eluacije, najveća genotoksičnost zapažena je nakon 24-satne eluacije (Tablice 1., 2., 3.), što je u skladu s istraživanjem Bouillagueta i suradnika (21), te Geurtsena i suradnika (23). Dentinski adhezivi čak se i nakon polimerizacije u vodenom mediju otapaju i razgrađuju (23, 24), tj. oni tijekom vremena podliježu degradaciji koja počinje već nakon 24 sata i to zbog afiniteta HEMA- e (glavnog ili jednog od glavnih monomera u sastavu adheziva) prema vodi (37-39). Najvjerojatnije se to dogodilo i našim adhezivima. Pritom jednosatni eluati nisu uzrokovali znatnija oštećenja genoma - osim ako se nije radilo o visokim koncentracijama i/ili potencijalno "genotoksičnim" adhezivima (Tablice 3., 1.) najveća genotoksičnost zabilježena je kod 24-satnih eluata kod kojih je već moglo doći do degradacije i otapanja, dok petodnevni eluati ni jednog testiranog dentinskog adheziva nisu pokazali genotoksično djelovanje, čak ni u najvišoj koncentraciji (Tablice 1., 2., 3.). Možemo pretpostaviti da su se kod njih toksične supstancije, koje su se nakon 24 satne eluacije otopile iz adheziva, dalje razgradile u manje toksične spojeve (23, 24, 37, 38).

Pregledom dostupne literature pronađeno je vrlo malo radova o mutagenosti ili genotoksičnosti samih adheziva, odnosno eksperimentalne studije pretežno se bave genotoksičnošću ili citotoksičnošću komponenti u sastavu tih adheziva. Upravo zbog toga nije moguća adekvatna usporedba s našim rezultatima.

Mutagenost dentinskih adheziva, različitih od naših, na stanicama sisavaca u uvjetima in vitro dokazali su i Schweikl i suradnici (15-17). Najveću mutagenost pokazali su adhezivi koji imaju glutaraldehid u svojem sastavu, ali on nije sastojak naših adheziva. U skladu s našim istraživanjem i Huang sa suradnicima (40) pokazuje mutagenost/genotoksičnost dentinskih adheziva (također različitih od naših), ali na humanim gingivnim fibroblastima i to indukcijom ekspresije protoonkogena.

Prema podacima proizvođača, adhezivi korišteni u ovom radu uglavnom su na bazi HEMA-e i Bis-GMA-e (Tablica 4.), a u mnogobrojnim studijama dokazano je da ti monomeri djeluju citotoksično (23, 29-31) i genotoksično (33, 34), a Bis-GMA čak i embriotoksično/ teratogeno (35). Guertsen (24, 41) te Kawahara i suradnici (42) dokazali su da se oba monomera otpuštaju iz polimeriziranih adheziva, a Kleinsasser i suradnici (33) svojim su istraživanjem na humanim limfocitima Comet- testom migracije DNK upozorili na genotoksičnost

5 μg/ml (Table 3). Regarding elution time, the highest genotoxicity was found after a 24-hour elution period (Tables 1, 2, 3), which is in congruence with the study of Bouillaguet et al.(21), and Geurtsen et al. (23). Dentin bonding agents in an aqueous medium, even after polymerization, melt and disintegrate (23, 24), so they yield to degradation even after 24 hours, which is due to HEMA (the main or one of the main monomers in their composition) affinity to water (37-39). The same thing probably happened with our dentin bonding agents. Here, one-hour-elutes did not show significant genotoxicity, except when the high concentrations and/or potentially "genotoxic" agents were involved (Tables 3, 1), the highest genotoxicity was observed after 24-hour elution period where the degradation and melting could have taken place, while five-day-elutes of any dentin bonding agent did not show genotoxicity, even in the highest concentration (Tables 1, 2, 3). It is supposed that the toxic substances, which are probably leached out after 24-hour elution, are transformed into less toxic substances (23, 24, 37, 38).

The review of the literature showed the lack of the studies about mutagenicity/ genotoxicity of the dentin bonding agents, furthermore, the experimental studies are based on examining the genotoxicity or cytotoxicity of the dentin bonding agents' ingredients. So, the adequate correlation with our results is not possible.

Schweikl et al. (15-17) demonstrated the mutagenicity of dentin bonding agents, different from our agents, on mammalian cells within in vitro conditions. The highest mutagenicity was observed in dentin bonding agents with the glutaraldehid ingredient, but it is not found in the composition of our dentin bonding agents. Furthermore, Huang et al. (40) have published a study which is also congruent with our study. They demonstrated the mutagenicity/ genotoxicity of dentin bonding agents (also different from ours) but on human gingival fibroblasts through protooncogen expression.

According to the manufacturer, our dentin bonding agents are based on HEMA and Bis-GMA (Table 4), and many studies have confirmed cytotoxicity of the mentioned monomer (23, 29-31), genotoxicity (33, 34), as well as for Bis-GMA - even an embriotoxic/ theratogenic effect (35). Guertsen (24, 41) and Kawahara et al. (42) have shown that both monomers are leached out from polymerized adhesives and Kleinsasser et al. (33) have shown genotoxicity resulting from HEMA and Bis-GMA in their study on human limphocytes by Comet-assay and DNA migration. Also, Muller et al. (43) showed the geno-

Tablica 4. Sastav dentinskih adheziva korištenih u ovom radu
Table 4 The composition of dentin bonding agents used in this study

Dentinski adheziv • Dentin bonding agent	Sastav • Composition	Otapalo • Solvent
Adper Single Bond	Bis-GMA, HEMA, dimetakrilati, metakrilatni kopolimer poliakrilne i poliitakonske kiseline i fotoinicijatori • Bis-GMA, HEMA, dimethacrylate, methacrylic copolymer of polyacrylic and polyitaconic acid, and photoinitiators	Etanol, voda • Ethanol, water
Adper Single Bond 2	Bis-GMA, HEMA, dimetakrilati, silicij, metakrilatni kopolimer, Poliakrilna i poliitakonska kiselina i fotoinicijatori • Bis-GMA, HEMA, dimethacrylate, silica, methacrylate copolymer, polyacrylic and polyitaconic acid, and photoinitiators	Etanol, voda • Ethanol, water
Prompt L-pop	Bis-GMA, HEMA, metakrilni fosfoesteri, kamforkinon i polialkenoična kiselina • Bis-GMA, HEMA, methacrylic phosphoesters, camphorquinone and polyalcenoic acid	Voda • Water
Excite	Bis-GMA, HEMA, glycerin dimetakrilati, fosforski akrilati, silicij, inicijatori i stabilizatori • Bis-GMA, HEMA, glycerine dimethacrylate, phosphoric acrylates, silica, initiators and stabilizers	Etanol • Ethanol
OptiBond Solo Plus	HEMA, dimetakrilati, silicij, inicijatori i stabilizatori • HEMA, dimethacrylates, silica, initiators and stabilizers	Etanol • Ethanol

HEMA-e i Bis-GMA-e. Također, Muller i suradnici (43) pokazuju genotoksični potencijal staklenoionomernog cementa Vitrebonda koji je na bazi HEMA-e i Bis-GMA-e u uvjetima *in vitro* na stanicama ovarija kineskoga hrčka. Zbog svega toga može se pretpostaviti da su i u našem istraživanju upravo monomeri, koji su se vjerojatno otopili iz adheziva, najzaslužniji za genotoksičnost. Budući da se HEMA s vremenom razgrađuje do manje toksičnog spoja etilen-glikola (23, 24, 37-39), to bi mogao biti razlog zašto petodnevni eluati nisu pokazivali genotoksičnost.

U sastavu naših adheziva su i dimetakrilati (Tablica 4.). Oni se također otpuštaju iz polimeriziranih adheziva (24, 41), a najčešće zastupljeni dimetakrilati u sastavu adheziva su TEGDMA i UDMA (uretan-dimetakrilat). Yoshii (44) je u svojoj studiji pokazao da i oni mogu biti citotoksični. Štoviše, veću citotoksičnost imaju dimetakrilati s manjim brojem oksietilenskih lanaca - 14 ili manje, nego oni s većim brojem lanaca (44). Dimetakrilati također oštećuju genome humanih limfocita, stanice limfoma miša, plućne stanice i stanice ovarija kineskog hrčka (45, 46) te se javlja genotoksičnost u humanim limfocitima (33), pa je moguće da su i dimetakrilati pridonijeli genotoksičnosti naših adheziva - osobito ako su u njihovu sastavu oni s manjim brojem oksietilenskih lanaca (proizvođač ne navodi koji dimetakrilati su zastupljeni).

Inicijator, kamforkinon (Tablica 4.) također je mogao pridonijeti genotoksičnosti, budući da su Atsumi i suradnici (47, 48) dokazali njegovu citotoksičnost na humanim pulpnim fibroblastima. Studije o genotoksičnosti kamforkinona nisu pronađene pregledom dostupne literature.

toxic potential of the glass-ionomer cement Vitrebond which is based on HEMA within *in vitro* conditions on Chinese hamster ovarian cells. Based on the presented evidence, it is supposed that it is precisely the monomers themselves, which are probably melted down from our dentin bonding agents that are responsible for the observed genotoxicity. Over time, HEMA is reverted to its less toxic component, ethylene-glycol (23, 24, 37-39), which could be the reason why none of the five-day-elutes did not show genotoxicity.

Our adhesives also contain dimethacrylates (Table 4). They are also leached out of polymerized adhesives (24, 41), and the common dimethacrylates in dentin bonding agents are TEGDMA and UDMA (urethane dimethacrylate). In his study, Yoshii (44) showed that they can be cytotoxic. Dimethacrylates with a lower number of oxyethylene chains, 14 or less, showed much more citotoxicity then the ones with a higher number of oxyethylene chains (44). Also, dimethacrylates ruin the genes of human lymphocytes, lymphoma cells in mice, pulp fibroblasts and ovarian cells in Chinese hamsters (45, 46) and they expose human lymphocytes to genotoxicity (33), so it is possible that the dimethacrylates from our dentin bonding agents may also behave genotoxically; especially if a lower number of oxyethylene chains is present in their composition (manufacturer does not mention which dimethacrylates are present).

The initiator, camphorquinone (Table 4), also may be one of the reasons behind the observed genotoxicity since Atsumi et al. (47, 48) have proven its cytotoxicity. No studies about the genotoxicity of camphorquinone were found while reviewing the pertinent literature.

Tri adheziva iz naše studije - OptiBond Solo Plus, Excite i Adper Single Bond 2 - pokazala su veću genotoksičnost od preostala dva. Oni u svojem sastavu sadržavaju čestice silicija kao punila jer poboljšava svojstva adheziva (Tablica 4.), ali dokazano je da silicij oštećuje DNK plućnih fibroblasta kineskog hrčka, ali i humanih embrionalnih plućnih fibroblasta nakon izloženosti silicijevoj prašini u *uvjetima in vitro* (49) te djeluje genotoksično uzrokujući pojavu mikronukleusa u istim stanicama (50, 51). Zato je moguće da je silicij uzrok njihove povećane genotoksičnosti.

Također su veću genotoksičnost pokazali adhezivi koji kao otapalo imaju samo etahol - OptiBond Solo Plus i Excite (Tablica 4.). Njihova je citotoksičnost dokazana na humanim stanicama pankreasa (52), hepatocitima i fibroblastima (53) te genotoksičnost na bakterijskim stanicama (54), humanim neuronima (55), limfocitima i stanicama gastrointestinalnog trakta (56) u uvjetima *in vitro*, što znači da je i etanol mogao biti jedan od uzroka genotoksičnosti naših adheziva.

Zaključci

1. Genotoksičnost dentinskih adheziva raste s porastom njihove koncentracije, a smanjuje se s vremenom eluacije, što znači da bi se nakon što se postave u kavitet eventualna genotoksičnost adheziva i/ili njegovih komponenti s vremenom trebala smanjiti.
2. Dentinski adhezivi najveću genotoksičnost pokazuju nakon 24-satne eluacije, tako da bi moguća gentoksična reakcija na adheziv i /ili njegove komponente trebala nastati 1 do 2 dana nakon tretmana.
3. Dentinske adhezive obuhvaćene ovom studijom možemo prema genotoksičnosti razvrstati sljedećim redom: OptiBond Solo Plus > Excite > Adper Single Bond 2 > Adper Single Bond > Prompt L-pop.

Three dentin bonding agents from our study, OptiBond Solo Plus, Excite and Adper Single Bond 2, showed higher genotoxicity than the others. These bonding agents have silica fillers in their composition (Table 4), and it is proven that silica may ruin the DNA of lung fibroblasts in Chinese hamsters and human embrionic pulp fibroblasts after the exposure of silica dust in *in vitro* conditions (49) and acts genotoxically by causing micronucleus in the same cells (50, 51), which is probably the main reason of their higher genotoxicity.

Also, higher genotoxicity was observed in dentin bonding agents with only ethanol as solvent: OptiBond Solo Plus and Excite (Table 4). Its cytotoxicity was shown in human pancreatic acinar cells (52), human hepatocytes and fibroblasts (53), whereas genotoxicity was displayed in bacterial cells (54), human neurons (55), lymphocytes and gastrointestinal mucosal cells (56) in *in vitro* conditions, so it is supposed that ethanol could also contribute to the genotoxicity.

Conclusions

1. Genotoxicity of dentin bonding agents increases with their concentration, and decreases with the elution period length which means that after clinical treatment possible genotoxicity of dentin bonding agents and/or its components should decrease over time.
2. Dentin bonding agents showed the highest genotoxicity after a 24-hour elution period, so the possible genotoxicity reaction to the dentin bonding agent and/or his components should result 1-2 days after the treatment.
3. Dentin bonding agents from this study can be stated in descending order according to their genotoxicity: OptiBond Solo Plus > Excite > Adper Single Bond 2 > Adper Single Bond > Prompt L-pop.

Abstract
The aim of this study was to examine the genotoxic activity of five dentin bonding agents: Adper Single Bond, Adper Single Bond 2, Prompt L-pop, Excite and OptiBond Solo Plus. This in vitro study was performed on human lymphocytes from peripheral blood, and the concentrations of dentin bonding agents tested were 0.2, 0.5 i 5 μg/ml, and elution times tested were 1 hour, 24 hours and 5 days. Genotoxicity testing was done using the Structural chromosomal aberration analysis cytogenic method , which determined the complete number of chromosome breaks, chromatide breaks and acentric fragments. The results showed genotoxicity of OptiBond Solo Plus in the 0.2 μg/ml concentration after a 24-hour elution period , then OptiBond Solo Plus in the 0.5 μg/ml concentration after a 1 hour elution period and OptiBond Solo Plus, Adper Single Bond 2 and Excite in the 0.5 μg/ml concentration after a 1 day elution period. In the 5 μg/ml concentration after 1 hour of elution, genotoxic potential was observed in cultures with OptiBond Solo Plus, Excite, Adper Single Bond 2 and Adper Single Bond, while all dentin bonding agents showed genotoxicity at that highest concentration but after 1 day of elution. From the results it is obvious that genotoxicity increases with the concentration of the dentin bonding agent, and decreases over time. The highest genotoxicity was observed after a 24-hour-elution period.

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