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IZVORNI ZNANSTVENI RAD
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Toksiksičnost prethodno zagrijanih kompozita izravnom polimerizacijom i preko CAD / CAM overleja

Toxicity of Pre-heated Composites Polymerized Directly and Through CAD/CAM Overlay

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Sažetak

Svrha rada: Usporediti citotoksičnost/genotoksičnost zagrijanih kompozita polimeriziranih preko CAD/CAM overleja na kulturi izoliranih ljudskih limfocita. **Materijali i postupci:** Mikrohibridni (Z100, 3M ESPE) i nanopunjeni kompozit (Filtek Supreme Ultra, 3M ESPE) zagrijani su u uređaju za zagrijavanje kompozita (Calset, AdDent Inc.) na temperaturama od 37 °C (T1), 54 °C (T2) i 68 °C (T3). Mala količina zagrijanog kompozita stavljena je u cilindrični kalup (promjera 6 mm, debljine 0.65 mm), prekrivena Mylar folijom, sprešana, te osvjetljena izravno, preko CAD/CAM keramički pojačanog polimera (CRP) (LAVA Ultimate, 3M ESPE) ili CAD/CAM litij disilikatnog keramičkog overlaja (LDC)(e.max, Ivoclar/Vivadent) debljine 2 mm. Odmah nakon osvjetljavanja uzorci su stavljeni u staničnu kulturu limfocita izoliranih iz periferne krvi. Citotoksičnost je izmjerena metodom dvojnog bojenja etidijevim bromidom i akridinskom narančastom bojom koja omogućuje određivanje postotka živih stanica te stanica u apoptozi i nekrozi na osnovi njihovih morfoloških obilježja. Genotoksičnost je procijenjena uporabom komet-testa u alkalnim uvjetima. **Rezultati:** Za Z100, najveći postotak živih stanica zabilježen je na T1 (93,7 %) nakon izravnog osvjetljavanja; slijedi osvjetljavanje preko CRP-a (92,3 %) te LDC-a (91,7 % T1, T3). Za Filtek Supreme Ultra najveći postotak živih stranica zabilježen je nakon osvjetljavanja preko CRP-a (91,2 % T2), LDC-a (90 % T1, T3) te pri izravnom osvjetljavanju (88,7 % T2). **Zaključak:** Za oba ispitivana materijala, zagrijavanje na T1 i T2 postupak je izbora. S obzirom na genotoksičnost, ne preporučuje se zagrijavanje na T3.

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Ključne riječi
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Uvod

Svjetlosno stvrdnjavajući kompozitni materijali uvelike se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijali zbog estetskih i mehaničkih svojstava te razmjerno jednostavnog rukovanja. Ako kompozitni materijal nije adekvatno polimeriziran, može otpuštati pojedine komponente iz punila, ili češće iz nepolimerizirane organske matrice. Čak i odgovarajuće polimerizirani kompozitni materijal sadržava određenu količinu zaostatnog monomera koji, ako je otpušten, može toksično djelovati (1). Čimbenici kao što su sastav i vrsta punila te vrsta i površina čestica punila, mogu utjecati na količinu otpuštenog monomera (1, 2). Ferracane i Condon (3) istaknuli su da se najveća citotoksičnost uzrokovana nepolimeriziranim kompozitom događa tijekom prva 24 sata. No Wattha i suradnici (4) ispitivali su citotoksičnost smolastih restaurativnih materijala podvrgnutih procesu starenja u umjetnoj slini i zaključili da oni mogu otpuštati zaostat-

Introduction

Light cured composite materials are widely used in clinical dentistry as restorative materials due to their esthetic, mechanical and handling properties. If a composite material is not polymerized properly, it can lead to the leaching of components either from filler or mostly, from unpolymerized organic matrix. Even properly cured composite materials contain a certain amount of residual monomers that can be eluted and exert a toxic effect (1). Factors like filler composition, filler content, filler surface area and type of filler particle treatment process can influence the amount of leached monomers (1,2). Ferracane and Condon (3) showed in their study that the highest cytotoxicity induced by unpolymerized composites occurs during the first 24 h. However, Wattha et al., (4) tested cytotoxicity of resin-containing restorative materials after aging in artificial saliva and concluded that resin-based restorative materials may release residual components, which

ne komponente koje, pak, mogu uzrokovati citotoksičnost i do dva tjedna. Ispitivanja u uvjetima *in vitro* pokazala su da metakrilatni i dimetakrilatni monomeri upotrijebljeni u restaurativnim dentalnim materijalima mogu povećati unutarstaničnu razinu reaktivnih kisikovih spojeva, što može potaknuti apoptozu. Uz to, suprimiraju mitohondrijsku aktivnost makrofaga, potičući time njihov upalni odgovor, umanjujući enzimatsku aktivnost te uzrokujući fragmentaciju DNA-e i ekspresiju čimbenika rasta i citokina (5 – 9).

Premda je teoretski moguća 100-postotna konverzija monomera u polimer, obično od 25 do 50 % dvostrukih veza metakrilatnog monomera ne reagira te se procjenjuje da je 5 do 10 % ukupne količine dvostrukih ugljikovih veza koje nisu reagirale dostupno za interakciju s makromolekulama u biološkom sustavu (3, 10, 11). Kompozitni materijali ne dosežu maksimalan stupanj konverzije neposredno nakon polimerizacije (12, 13). Moon i suradnici (5) u svojoj su studiji pokazali da je potrebno sedam dana da bi stupanj konverzije dosegnuo maksimum polimerizacije, na površini i na dnu ispuna. Nadalje, zaključili su da se količina monomera otpuštena iz kompozitnog materijala razlikuje s obzirom na vrstu polimerizatora i postupka polimerizacije. U drugoj pak studiji isti su autori (14) istaknuli da se razlike između količine otpuštenog monomera i mehaničkih svojstava događaju kada je količina energije emitirana iz uređaja za polimerizaciju manja od 17 J/cm^2. Nasuprot tomu, ako je količina emitirane energije veća od 17 J/cm^{2,} razlike nestaju bez obzira na to koji je program polimerizacije ili polimerizacijski uređaj upotrijebljen (15).

Nedavno je zagrijavanje kompozitnih materijala prije unošenja u kavitet postalo popularan i prihvatljiv pristup u svrhu postizanja većeg stupnja konverzije te boljih mehaničkih svojstava, a bez negativnog učinka na marginalno brtvljenje (16, 17). Korištenje zagrijanih kompozita postaje sve popularnije i kao sredstvo cementiranja CAD/CAM restoracija (18 – 20).

Daronch i suradnici (17) pokazali su da kompozitni materijali zagrijani do 60 °C mogu poboljšati stupanj konverzije na površini i na dubini ispuna do 2 mm. No Froes-Salgado i njegovi kolege (21) nisu pronašli značajno poboljšanje mehaničkih svojstava ni stupnja konverzije na unaprijed zagrijanim kompozitnim materijalima, ali su pokazali poboljšanje adaptacije kompozitnih materijala za stijenku kaviteta. Lohbauer i suradnici (22) istaknuli su da unaprijed zagrijani kompozitni materijali mogu negativno utjecati na rubove kompozitnog ispuna zbog većeg polimerizacijskog skupljanja kao posljedice višeg stupnja konverzije.

Kako upotreba zagrijanih kompozitnih materijala kao sredstva za cementiranje CAD/CAM restauracija postaje sve popularnija, zanimljivo će biti istražiti utjecaj tako zagrijanog kompozita polimeriziranog preko CAD/CAM uzorka kad je riječ o citotoksičnosti i genotoksičnosti. Zato je svrha ove studije procijeniti citotoksičnost i genotoksičnost dvaju kompozitnih materijala zagrijanih na tri različite temperature te osvijetljenih izravno i preko CAD/CAM overleja debljine 2 mm. S obzirom na to, postavljene su sljedeće hipoteze:

1. izravna polimerizacija kompozita pokazuje jednaku citotoksičnost i genotoksičnost kao i polimerizacija kompozitnog uzorka preko CAD/CAM overleja

trigger cytotoxicity for up to 2 weeks. In vitro studies indicated that methacrylate and dimethacrylate monomers used in restorative dental materials may increase the intracellular level of reactive oxygen species which induce apoptosis. In addition, they suppress the mitochondrial activity of macrophages, thus altering their inflammatory responses, affect the recruitment of leukocytes and decrease the expression of intercellular adhesion molecules, induce enzymatic activity, DNA fragmentation, expression of growth factors and cytokines (5-9).

Theoretically, a 100% conversion of monomers to polymers is possible, but usually 25-50% of methacrylate monomer double-bonds remain unreacted and it is estimated that 5-10% of the total amount of unreacted C=C bonds are available for interaction with macromolecules in a biological system (3,10,11). Composite materials do not reach the maximum degree of conversion immediately after light curing (12,13). Moon et al.,(5) reported that a period of 7 days is needed for the degree of conversion of the materials to reach maximum polymerization on both bottom and top surfaces. Further, they concluded that the amount of monomers leached from the same composite material differed in regards to the type of the curing unit and the curing method. In another study, Moon (14) shows that differences between the amount of leached monomers and mechanical properties occurred when the radiant energy emitted from the curing units is lower than 17 J/cm^2. In contrast, if the radiant energy is higher than 17 J/cm^2, those differences disappear regardless of the irradiation programs and curing units used (15).

Recently, the pre-heating of composite materials before their application in the oral cavity became an acceptable approach in order to obtain a higher degree of conversion and better mechanical properties without negative effect on marginal seal (16,17). The use of pre-heated composite as a luting material for CAD/CAM restorations was also reported (18-20).

Daronch et al. (17) reported that pre-heating the composite up to 60 °C may improve the degree of conversion on both, top and 2 mm deep surface. However, Froes-Salgado et al., (21) did not find any improvement in mechanical properties and the degree of conversion of pre-heated composite, but reported an improvement in composite adaptation to cavity walls. Lohbauer et al. (22) indicated that preheating of composite materials may have a negative effect on the restoration margins because of the higher polymerization shrinkage due to a higher degree of conversion.

Since the use of pre-heated composite materials as a luting material for CAD/CAM fabricated restorations are becoming more and more popular, it will be interesting to see the impact of the light curing of heated composite through CAD/CAM restoration on cytotoxiticy/ genotoxicity. Therefore, the aim of this study was to assess cytotoxicity and genotoxicity of two different composite resins pre-heated at three different temperatures and light-cured directly and through 2 mm thick CAD/CAM onlays. For that purpose, the following null-hypotheses are formed:

1. Direct light-curing of composite resin exhibits similar cytotoxicity and genotoxicity as in specimens polymerized through CAD/CAM overlays

2. različite temperature zagrijavanja kompozita ne utječu na citotoksičnost i genotoksičnost kompozitnog uzorka.

Materijali i postupci

Priprema uzoraka

Keramički pojačan polimer (CRP, LAVA Ultimate, 3M ESPE, St. Paul, MN, SAD) debljine 2 mm, boje A2 (veličina bloka 14; 14 x 14 x 18 mm) i litijeva disilikatna staklena keramika (LDC, e.max CAD, Ivoclar Vivadent, Schaan, Lihtenštajn) boje A2 (veličina bloka 14; 14 x 12 x 17 mm), upotrijebljeni su kao overleji pri polimerizaciji kompozitnih uzoraka. CRP i LDC blokovi izrezani su preciznom dijamantnom pilom, uz vodeno hlađenje. Veličina overleja bila je 14 x 14 x 2 mm za CRP i 14 x 12 x 2 mm za LDC, zbog razlika u veličini blokova. Nakon toga uzorci su polirani do visokog sjaja (#600, #800, #1200 finoće). LDC uzorci premazani su glazurom (Crystall/Glaze Spray; Ivoclar/Vivadent, Schaan, Lihtenštajn) prema uputama proizvođača.

Korištena su dva različita kompozitna materijala – mikrohibridni kompozit (Z100, 3M ESPE, St. Paul, MN, SAD) i nanopunjeni kompozitni materijal (Filtek Supreme Ultra, 3M, ESPE). Sastav materijala upotrijebljenog u eksperimentu prikazan je u tablici 1. Kompozitni materijali zagrijani su u uređaju za zagrijavanje kompozita (Calset, AdDent Inc., Danbury, CT, SAD) na trima različitim temperaturama – 37 °C (T1), 54 °C (T2), 68 °C (T3) prema uputama proizvođača.

2. Different pre-heating temperatures do not affect cytotoxicity and genotoxicity

Materials and methods

Specimen Preparation

A two mm thick layer of a ceramic-reinforced polymer (CRP, LAVA Ultimate, 3M ESPE, St. Paul, MN, USA) CAD/CAM of shade A2 (block size 14; 14x14x18 mm) and lithium disilicate glass-ceramic (LDC, e.max CAD, Ivoclar Vivadent, Schaan, Liechtenstein) of shade A2 (block size 14; 14x12x17 mm), were used as overlays for light-curing of composite samples. CRP and LDC blocks were sectioned using a water-cooled precision diamond saw. The overlay sample size was 14x14x2 mm for CRP and 14x12x2 mm for LDC. Thereafter, the samples were metallurgically polished to high gloss (#600, #800, #1200 grit). For LDC samples, each sample was glazed (Crystall/Glaze Spray; Ivoclar/Vivadent, Schaan, Liechtenstein) according to the manufacturer instructions.

Two different composite resins were used: a microhybrid composite (Z100, 3M ESPE, St. Paul, MN, USA) and a nanofilled composite resin (Filtek Supreme Ultra, 3M ESPE). The composition of these materials used in this study is given in Table 1. The composite resins were pre-heated using a heating unit (Calset, AdDent Inc., Danbury, CT, USA) at three different temperatures: 37 °C (T1), 54 °C (T2), and 68 °C (T3) ac-

Tablica 1. Materijali korišteni u istraživanju
Table 1 Materials used in the study

Materijal • Material	Proizvođač • Manufacturer	Sastav • Composition
Keramikom pojačan polimer • Ceramic-reinforced polymer (CRP) CAD/CAM	LAVA Ultimate, 3M ESPE, St. Paul, MN, SAD • USA	- kompozitna nanokeramika sadrži otprilike 79 % (%w) nanokeramičkih čestica povezanih u smoli materijala • resin nanoceramic containing approximately 79% (%w) nanoceramic particles bound in the resin matrix - kombinacija neaglomeriranog / neagegiranog 20 nm silikatnog punila, neaglomeriranog /neagregiranog 4-11 nm cirkonijeva punila i agregiranih punila cirkonij/ nakupina silika (20 nm silike i 4-11 nm čestice cirkonija) • combination of non-agglomerated/non-aggregated 20 nm silica filler, non-agglomerated/nonaggregated 4-11 nm zirconia filler, and aggregated zirconia/silica cluster filler (20 nm silica and 4-11 nm zirconia particles).
Litij disilikatna staklena keramika • Lithium disilicate glass-ceramic (LDC), e.max CAD	Ivoclar Vivadent, Schaan, Liechtenstein	- kvarc, litijev dioksid, fosforov oksid, aluminij, kalijevi oksidi te druge komponente • quartz, lithium dioxide, phosphor oxide, aluminia, potassium oxide and other components
Z100	3M ESPE, St. Paul, MN, SAD • USA	- mikrohibridna kompozitna smola • microhybrid composite resin - matriks; BIS-GMA i TEGDMA • matrix: BIS-GMA and TEGDMA - punilo cirkonija/silika, anorgansko punilo 66 %w, veličina čestica od 3,5 do 0,01µm • filler: zirconia/silica; inorganic filler loading is 66% w, particle size range of 3.5 to 0.01 µm
Filtek Supreme Ultra	3M ESPE, St. Paul, MN, SAD • USA	- nanopunjeni kompozitni materijal • nanofilled composite resin - 100 % nanopunilo, primarne čestice manje su od 100 nm • 100% nanofiller, the primary particles are below 100 nm - smola: BisGMA, UDMA, TEGDMA, te bis-EMA • resin: Bis-GMA, UDMA, TEGDMA, and bis-EMA - punilo: kombinacija neaglomeriranog/ne-agregiranog 20 nm silika punila, ne-aglomeriranog/neagregiranog 4-11 nm cirkonijeva punila te cirkonij/silika punilo (20 nm silika, 4-11nm čestice cirkonija). • fillers: combination of non-agglomerated/non-aggregated 20 nm silica filler, non-agglomerated/non-aggregated 4 to 11 nm zirconia filler, and aggregated zirconia/silica cluster filler (20 nm silica and 4 to 11 nm zirconia particles) - anorgansko punilo 78,5 % w, (63,3 % vol.) • inorganic filler loading is 78.5% w (63.3% vol)

Potrebno je oko deset minuta da bi se postigla željena temperatura i da bi kompozit bio spreman uz uporabu.

Uzorci za ispitivanje cito-/genotoksičnosti pripremljeni su na sljedeći način: kalup promjera 6 mm i debljine 0,65 mm pozicioniran je na okrugli disk od plemenitog čelika (promjera 6 mm, debljine 5 mm) i prekriven Mylar folijom. Nakon toga kalup je oprezno ispunjen nepolimeriziranim kompozitnim materijalom, izbjegavajući inkorporaciju mjehurića zraka. Uzorci kompozitnog materijala prekriveni su Mylar folijom i sprešani s pomoću drugog diska od plemenitog čelika (promjera 6 mm, debljine 5 mm) kako bi se dobila homogena debljina kompozitnog uzorka (0,65 mm). Disk od plemenitog čelika nakon toga je uklonjen, a Mylar folija ostavljena je na uzorku kako bi se spriječilo stvaranje sloja inhibiranog kisikom na površini polimeriziranoga kompozitnog materijala. Svi uzorci kompozitnog materijala polimerizirani su 40 sekunda diodnim uređajem (LED) (Bluephase G2, Vivadent, Schaan, Lihtenštajn) uporabom programa visokog intenziteta (1180 mW/cm^2). Primijenjena su tri načina osvjetljavanja – (1) izravno osvjetljavanje, (2) osvjetljavanje preko CRP CAD/CAM overleja i (3) osvjetljavanje preko LDC overleja. Nakon polimerizacije, Mylar folija je uklonjena te su uzorci uronjeni u kulturu stanica.

Za pripremu nepolimeriziranih uzoraka korišteno je 0,06 g kompozitnog materijala i uronjeno izravno u staničnu kulturu.

Kultura ljudskih limfocita izoliranih iz periferne krvi

Ovu studiju odobrilo je Etičko povjerenstvo Stomatološkog fakulteta u Zagrebu, Hrvatska. Kultura primarnih limfocita dobivena je od izoliranih limfocita 39-godišnjeg muškarca, nepušača, bez kronične ili akutne bolesti u anamnezi. U istraživanju je korišten model jednog donora (engl. *single donor approach*) da bi se izbjegle moguće interindividualne razlike u odgovoru na tretman. Prije nego što je uzet uzorak krvi, donor je bio obaviješten o postupku i svrsi uzimanja krvi te o svrsi testiranja uzetog uzorka krvi.

Venska krv izvađena je sterilnim priborom za jednokratnu upotrebu u heparinizirani spremnik (Becton Dickinson, UK). Odmah je obavljena izolacija limfocita, u skladu s postupkom opisanim u studiji Kopjara i suradnika (23). Suspenzija izoliranih limfocita podijeljena je na manje volumene koji su premješteni u sterilne epruvete (Nange Nunc Int, Naperville, IL, SAD) napunjene hranjivim medijem za stanične kulture RPMI 1640 (Gibco Invitrogen, UK) kako bi se postigla gustoća od 50 000 limfocita po kulturi. Ukupni volumen tako pripremljenih kultura iznosio je 7 ml.

Svaki testirani materijal (0,06 g), polimerizirani i nepolimerizirani, stavljen je u limfocitnu kulturu i držan 24 sata u inkubatoru za uzgoj staničnih kultura (Heraeus Hera Cell 240 Incubator, Langenselbold, Njemačka) na temperaturi od 37 °C i 5 % CO_2. Nakon 24 sata kulture su pet minuta centrifugirane na 300 *g*. Supernatant je uklonjen, a talog koji sadržava limfocite pažljivo je resuspendiran i korišten za daljnju analizu.

cording to the manufacturer instructions. A tray placed on the top of the heater contains slots to place the composite compules. In approximately 10 minutes the desired temperature is reached and the composite is ready to use.

The samples for cyto-/genotoxicity testing were prepared as follows: A mold of 6 mm in diameter and 0.65 mm in thickness was positioned on a round stainless steel disc (diameter 6 mm, thickness 5 mm), which was covered with a clear Mylar matrix. The mold was then carefully filled with the uncured composite material ensuring that no air bubbles were left. The composite specimens were covered with another Mylar sheet and pressed with another round stainless steel disc (diameter 6 mm, thickness 5 mm) to obtain a homogenous thickness of the composite sample (0.65 mm). The stainless steel disc was removed and Mylar matrix remained on the sample to prevent formation of the oxygen-inhibited layer on the surface of polymerized composite material. All tested composite resin samples were polymerized with a light-emitting diode (LED) light curing unit (Bluephase G2, Vivadent, Schaan, Liechtenstein) using high intensity mode (1180 mW/cm^2) for 40 sec. Three light-curing procedures were used: (1) direct curing; (2) curing through CAD/CAM CRP overlay, and (3) curing through LDC overlay. Once the light-curing was completed, the specimens were removed from the mold and introduced into cell cultures.

For the preparation of unpolymerized sample, 0.06 g of the composite material was taken and introduced directly into the cell culture.

Primary lymphocyte cultures

This study was approved by the Ethical Committee, School of Dental Medicine, University of Zagreb, Croatia. Primary lymphocyte cultures were set from the cells of a single donor, 39 year old male, non-smoker, with no medical records of chronic or acute adverse health conditions. To overcome possible inter-individual differences in response to the treatment we applied a single donor sampling approach. Prior to blood sampling, the donor was acquainted with the procedure, purpose of blood donation, and aims of the testing the blood is to be used for.

Blood was collected by antecubital venipucture in heparinized vacutainers (Becton Dickinson, UK). Lymphocytes were isolated as described previously by Kopjar et al. (23). Following the isolation, 50,000 lymphocytes were seeded in sterile tubes (Nange Nunc Int, Naperville, IL, USA) in RPMI 1640 culture medium (Gibco Invitrogen, UK), with a final culture volume to be 7 ml.

Each tested material (0.06 g), both in unpolymerized and polymerized form, was placed in the lymphocyte culture. Treatments lasted for 24 h at 37 °C in 5 % CO_2 atmosphere (Heraeus Hera Cell 240 incubator, Langenselbold, Germany). Following the 24 h of treatment, cultures were centrifuged at 300 g, 5 min. Supernatant was removed and pellet containing the lymphocytes was resuspended and used for further analyses.

Kvantitativna fluorescencijska metoda za procjenu preživljenja stanica, apoptoze i nekroze

Preživljenje limfocita izoliranih iz periferne krvi izmjereno je metodom istodobnog bojenja dvjema fluorescencijskim bojama (24). Nakon bojenja etidijevim bromidom i akridinskom narančastom bojom na osnovi morfoloških obilježja, može se pod fluorescencijskim mikroskopom razlikovati žive stanice (stanice s cjelovitom plazmatskom membranom) i mrtve stanice (stanice s oštećenom plazmatskom membranom).

Za bojenje je korištena mješavina etidijeva bromida (100 µg/ml) i akridinske narančaste boje (100 µg/ml) (Sigma-Aldrich, SAD) u jednakim volumnim omjerima (1 : 1; v/v). Iz svake limfocitne kulture mikropipetom je odmjereno 20 µl suspenzije stanica i prebačeno na predmetno mikroskopsko staklo. Na suspenziju stanica pažljivo je pipetirana mješavina fluorescencijskih boja, uzorak je pokriven pokrovnim stakalcem i odmah analiziran fluorescencijskim mikroskopom (Olympus BX; povećanje 400 x). Za svaki uzorak učinjena su tri uzastopna testa te je ukupno pregledano 300 stanica po uzorku. Usporedno s testiranim uzorcima, u istim je uvjetima držan i kontrolni uzorak, tj. netretirana limfocitna kultura. Kvantitativna procjena određivala se prema postotku živih, apoptotičnih i nekrotičnih stanica. Kako žive stanice u svoju DNA-u ne ugrađuju etidijev bromid, jezgra im je nakon dvojnog bojenja pod fluorescencijskim mikroskopom zelena. Mrtve nekrotične stanice imaju narančast do crveno obojen kromatin, a apoptotične imaju izrazito zelenu i visoko kondenziranu ili fragmentiranu jezgru.

Komet-test u alkalnim uvjetima

Za pripremu mikrogelova agaroze korištenih u komet-testu, 10 µl limfocitne suspenzije pomiješano je sa 100 µl 5-postotne agaroze niskog tališta (37 °C; Sigma-Aldrich, MO, SAD). Dobivena suspenzija pipetirana je na pripremljena mikroskopska stakla premazana slojem agaroze normalne temperature tališta (Sigma-Aldrich, MO, SAD) te pokrivena pokrovnim stakalcem. Gelovi su 10 minuta držani na ledu radi polimeriziranja. Nakon toga su pažljivo uklonjene pokrovnice, a mikrogelovi uronjeni u pufer za lizu (2,5 M NaCl, 0,1 M Na2EDTA, 10 mM Tris-HCL, 1 % N-lauroil sarkozin, 10 % DMSO, 1 % Triton X-100; Sigma-Aldrich, MO, SAD; pH 10) na 4 °C. Zatim su mikrogelovi 20 minuta denaturirani u puferu za denaturaciju i elektroforezu (1 mM Na_2EDTA, 300 mM NaOH; Sigma-Alrich, MO, SAD; pH > 13), te podvrgnuti elektroforezi u istom puferu. Elektroforeza je trajala 20 minuta na 0,7 V/cm. Analiza preparata provedena je pod epifluorescencijskim mikroskopom Olympus BX 51 (Olympus, Japan) povezanim sa sustavom za analizu slike Comet Assay IV (Perceptive Instruments, UK). Za svaki uzorak izmjereno je ukupno 50 kometa po tretmanu, na dva paralelna preparata. Kao parametri za procjenu oštećenja DNA-e korišteni su dužina repa kometa (izražena u µm) i intenzitet repa kometa (% DNA-e u repu), prezentirani srednjom vrijednošću, medijanom i standardnom devijacijom dvaju neovisnih mjerenja.

Kako bi se pripremila pozitivna kontrola, uzorak netretiranih limfocita prije uranjanja u pufer za lizu tretiran je 10 minuta na ledu sa 60 µl 1mM H_2O_2.

Quantitative fluorescent assay for the assessment of cell viability, apoptosis and necrosis

The viability of peripheral blood lymphocytes was assessed using a dye exclusion method (24). In this assay, viable (intact plasma membrane) and dead (damaged plasma membrane) cells can be visualized after simultaneous staining with the fluorescent DNA-binding dyes ethidium bromide and acridine orange.

A mixture of ethidium bromide and acridine orange (Sigma-Aldrich, USA) in final concentrations of 100 µg/ml (1:1; v/v) was gently pippeted onto the lymphocyte suspension (V=20 µL) placed on a microscope slide, covered with a coverslip and immediately analyzed under a fluorescence microscope (Olympus BX; 400 x magnification). Three tests with aliquots of the same sample were performed and a total of 300 cells per sample were counted. Control, untreated lymphocyte culture was studied in parallel. Quantitative assessments were made by the determination of the percentage of viable, apoptotic and necrotic cells. Viable cells excluded ethidium bromide and the appearance of their nuclei with an intact structure was bright green. Non-viable necrotic cells had orange to red colored chromatin with organized structure while apoptotic cells were bright green with highly condensed or fragmented nuclei.

Alkaline comet assay

Ten µl of lymphocyte resuspension was mixed with 100 µl of 5 % low melting point agarose (37 °C; Sigma–Aldrich, MO, USA) and placed onto normal melting point (Sigma–Aldrich, MO, USA) precoated microscope slides, covered with a slip cover, and let to polymerize. Slides were immersed into lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris–HCl, 1% N-lauroylsarcosine, 10% DMSO, 1% Triton X-100; Sigma-Aldrich, MO, USA; pH 10) for 20 min at 4 °C. Slides were denaturated in buffer (1 mM Na_2EDTA, 300 mM NaOH; Sigma-Aldrich, MO, USA; pH > 13 for 20 min) and subjected to electrophoresis using a buffer of the same composition as the one used for denaturation. Electrophoresis lasted for 20 min at 0.7 V/cm. Slides were analysed under epifluorescent microscope Olympus BX 51 (Olympus, Japan) connected to Comet Assay IV analysis system (Perceptive Instruments, UK). A total of 50 comets per treatment were scored in duplicate. Results are expressed using tail length (µm) and tail intensity (% of DNA in comet tail) and presented as mean and median and S.D. of two scorings.

Prior to immersion in lysis buffer, as the positive control, slides obtained from untreated lymphocyte cultures were treated with 60 µl of 1 mM H_2O_2 for 10 min placed on ice.

Statistička analiza

Procjena statističke značajnosti rezultata dobivenih za preživljenje stanica, apoptozu i nekrozu učinjena je uporabom Pearsonova hi-kvadrat testa. Podatci dobiveni komet-testom najprije su obrađeni primjenom deskriptivne statistike (Microsoft Excel). Detaljnija statistička analiza obavljena je statističkim softverom (Statistica 10, StartSoft, OK, SAD). Da bi se postigla normalna raspodjela, podatci su najprije logaritmirani (25), a zatim je primijenjena analiza varijance (ANOVA) uz Tukey *post hoc* test ($\alpha < 0,05$).

Rezultati

Mikrohibridni kompozit Z100

Rezultati kvantitativnog flourescencijskog testa za istodobnu identifikaciju apoptotičnih i nekrotičnih limfocita u kulturama inkubiranima sa Z100, prikazani su u tablici 2. Nakon 24 sata inkubacije s nepolimeriziranim Z100, ustanovljeno je 88,7 ± 2,1 % živih limfocita, a u negativnoj kon-

Statistical analysis

Comparisons between the values observed for cell viability, apoptosis, and necrosis were performed by Pearson's c^2-test for two-by-two contingency tables. The data acquired by alkaline comet assay were first evaluated using descriptive statistics (Microsoft Excel). More detailed statistical analysis was performed with the statistical software (Statistica 10, StatSoft, OK, USA). The data were first transformed by applying log transformation to normalize the distribution (25). A one-way analysis of variance (ANOVA) was computed, followed by a Tukey post hoc test ($\alpha < 0.05$).

Results

Microhybrid Composite: Z100

Results of the quantitative fluorescent assay for simultaneous identification of apoptotic and necrotic cells in lymphocyte samples incubated with Z100 are reported in Table 2. After 24 h of incubation with unpolymerized Z100 there were 88.7±2.1 % viable lymphocytes, while in the negative

Tablica 2. Rezultati kvantitativnog flourescencijskog testa za istodobnu identifikaciju apoptotičnih i nekrotičnih limfocita u uzorcima izloženima nepolimeriziranom i polimeriziranom kompozitnom materijalu Z100 te u negativnoj kontroli; polimerizacija materijala zagrijanog na temperaturama T1-T3 (37 °C, 54 °C, 68 °C) iznosila je 40 sekunda preko overleja (CRP i LDC) ili izravno

Table 2 Results of the quantitative fluorescent assay for simultaneous identification of apoptotic and necrotic cells in lymphocyte samples exposed to unpolymerized and polymerized Z100 material as well as in the negative control. Light-curing of material preheated at temperatures T1-T3 (37 oC, 54 oC, and 68 oC) lasted for 40 seconds, through overlays (CRP and LDC) or directly.

Materijal • Material Z100	Žive stanice • Viable cells (%)	Statistički značajno u usporedbi uzorkom • Statistically significant compared to	Apoptoza • Apoptosis (%)	Statistički značajno u usporedbi s uzorkom • Statistically significant compared to	Nekroza • Necrosis (%)	Statistički značajno u usporedbi s uzorkom • Statistically significant compared to
Nepolimeriziran • Unpolymerized	88.7±2.1	NC	6.3±2.1	NC	5.0±0.0	NC
Osvjetljavan preko CRP-a – T1 • Light-cured – through CRP – T1	92.3±2.1	NC	1.7±2.1	UN	6.0±1.0	NC; DIR-T1
Osvjetljavan preko CRP-a – T2 • Light-cured – through CRP – T2	84.7±1.5	NC, T1, T3; LDC-T2	5.7±2.1	NC, T1	9.7±0.6	NC
Osvjetljavan preko CRP-a – T3 • Light-cured – through CRP – T3	91.0±1.0	NC	5.3±1.5	NC, T1	3.7±0.6	NC
Osvjetljavan preko LDC-a – T1 • Light-cured – through LDC – T1	91.7±0.6	NC	3.0±1.0	-	5.3±0.6	NC; DIR-T1
Osvjetljavan preko LDC-a – T2 • Light-cured – through LDC – T2	91.3±1.5	NC	3.0±1.0	-	5.7±1.2	NC
Osvjetljavan preko LDC-a – T3 • Light-cured – through LDC – T3	90.7±3.1	NC	5.3±3.2	NC	4.0±1.7	NC
Osvjetljavan direktno – T1 • Light-cured – directly T1	93.7±0.6	NC,UN	5.3±0.6	NC; CRP-T1	1.0±0.0	UN
Osvjetljavan direktno – T2 • Light-cured – directly T2	85.7±1.5	NC, T1; LDC-T2	8.7±0.6	NC; LDC-T2	5.7±1.2	NC, T1, T3
Osvjetljavan direktno – T3 • Light-cured – directly T3	88.3±3.1	NC, T1	10.0±3.6	NC, T1; CRP-T3, LDC-T3	1.7±2.1	UN
Negativna kontrola • Negative control	97.7±0.6		1.7±0.6		0.6±0.6	

Bilješka • Note

Analizirano je 300 stanica po uzorku i svakoj ispitivanoj stavki.

Statistička značajnost rezultata procijenjena je $\chi 2$ testom. Statistički značajne razlike ($p < 0,5$) prikazane su u tablici; NC-vs. negativna kontrola; UN vs. nepolimerizirani materijal; T1 vs. uzorak eksponiran istom materijalu, zagrijan na T1; T2 vs. uzorak eksponiran istom polimeriziranom materijalu, zagrijan na T2; T3 vs. uzorak izložen istom polimerizacijskom materijalu, zagrijan na T3.

300 cells per sample per each experimental point were analysed.

Statistical significance of data was evaluated using χ^2 test. Significant differences ($P<0.05$) are indicated in the table; NC – *vs.* negative control; UN – *vs.* unpolymerized material; T1 – *vs.* sample exposed to the same polymerized material, preheated at T1; T2 – *vs.* sample exposed to the same polymerized material, preheated at T2; T3 – *vs.* sample exposed to the same polymerized material, preheated at T3.

troli njihov je udjel iznosio 97,7 ± 0,6 % (p < 0,0001). Citotoksičnost kompozita Z100 zagrijanog na T1 bila je manja u usporedbi s nepolimeriziranim uzorkom, bez obzira na način polimerizacije (izravni, preko CRP-a ili LDC overleja). Statistički značajna razlika utvrđena je između nepolimeriziranog i izravno osvijetljenog uzorka Z100 (p = 0,0309). Najbolji rezultati s najvećim udjelom živih stanica, uočeni su nakon zagrijavanja na temperaturi T1 – 93,7 ± 0,6 % živih stanica pri direktnom osvjetljavanju; 92,3 ± 2,1 % živih stanica pri osvjetljavanju preko CRP-a; te 91,7 ± 0,6 % pri osvjetljavanju preko LDC CAD/CAM overleja (tablica 2.). U negativnoj kontroli i nepolimeriziranom Z100, učestalost apoptotičnih limfocita bila je nešto viša negoli nekrotičnih lifmfocita. Apoptoza dominira u usporedbi s nekrozom samo u uzorcima Z100 osvijetljenima izravnim postupkom, bez obzira na temperature prethodnog zagrijavanja uzorka. Porast temperature zagrijavanja nije značajno utjecao na učestalost nekroze u testiranim uzorcima.

Nanopunjeni kompozit Filtek Supreme Ultra

Rezultati kvantitativnog flourescencijskog testa za istodobnu identifikaciju apoptotičnih i nekrotičnih limfocita u uzorcima inkubiranima kompozitom Filtek Supreme Ultra nalaze se u tablici 3. Nakon 24 sata inkubacije s nepolimeriziranim kompozitom Filtek Spureme Ultra ustanovljeno je 89,7 ± 2,1 % živih limfocita, a u negativnoj kontroli preživljenje je iznosilo 97,7 ± 0,6 % (p < 0,0001). Citotoksičnost polimeriziranog materijala, bez obzira na postupak osvjetljavanja (izravan, preko CAD/CAM CRP-a ili LDC overleja) i temperaturu zagrijavanja, u svim slučajevima je značajno veća u usporedbi s negativnom kontrolom (p < 0,01). Iako je uočena vrlo mala razlika u postotku živih stanica u uzorcima zagrijanima na tri različite temperature i različitim načinima osvjetljavanja, ni jedna od njih nije statistički značajna u usporedbi s nepolimeriziranim uzorkom.

Zagrijavanje na T1 rezultiralo je najvećim postotkom živih stanica u uzorku koji je osvijetljen preko 2 mm LDC CAD/CAM overleja (90,3 ± 1,5 %). Nešto niže preživljenje limfocita uočeno je nakon osvjetljavanja preko CRP CAD/CAM overleja (86,7 ± 1,5 %), a najniže je zabilježeno nakon izravnog osvjetljavanja uzorka (84,7 ± 3,2%). U uzorcima negativne kontrole i nepolimeriziranog kompozita Filtek Supreme Ultra, uočena je nešto veća učestalost apopototičnih limfocita u odnosu na nekrotične. Apoptoza predominira u odnosu prema nekrozi u gotovo svim polimeriziranim uzorcima, bez obzira na temperaturu njihova zagrijavanja. Najniža učestalost apoptoze uočena je nakon zagrijavanja na T2; s najboljim rezultatima na uzorcima osvijetljenima preko LDC CAD/CAM overleja (4,7 ± 0,6 % limfocita u apoptozi). Porast temperature zagrijavanja u većini uzoraka nije značajno utjecao na pojavnost nekroze u usporedbi s nepolimeriziranim materijalom. No u gotovo svim uzorcima postotak nekrotičnih stanica bio je značajno veći negoli u negativnoj kontroli.

control lymphocyte viability was 97.7±0.6 % (P<0.0001). Cytotoxicity of the Z100 subjected to T1sec preheating was generally lower when compared with that of the unpolymerized Z100, irrespective of the light-curing procedure (direct, through CRP or LDC). Statistically significant differences were found between unpolymerized and directly light-cured Z100 (P=0.0309). The best results with the highest percentages of viable cells were observed for T1 preheating: 93.7±0.6 % of viable cells after direct light-curing; 92.3±2.1 % of viable cells after light-curingthrough CRP, and 91.7±0.6 % of viable cells after light-curing through LDC CAD/CAM overlay (Table 2). In the negative control and unpolymerized Z100 the frequency of apoptotic lymphocytes was slightly higher than the frequency of necrotic lymphocytes. Apoptosis predominated over necrosis only in samples of Z100 prepared with direct light-curing regardless of the temperature applied for preheating. Increase of preheating temperature did not significantly influence the frequency of necrosis in tested samples. The only significant differences were found at T1, where in directly light-cured sample the lowest percentage of necrotic cells was found.

Nanofilled Composite: FILTEK SUPREME ULTRA

Results of the quantitative fluorescent assay for the simultaneous identification of apoptotic and necrotic cells in lymphocyte samples incubated with Filtek Supreme Ultra are reported in Table 3. After 24 hours of incubation with unpolymerized Filtek Supreme Ultra there were 89.7±2.1 % viable lymphocytes, while in the negative control lymphocyte viability was 97.7±0.6 % (P<0.0001). Cytotoxicity of the polymerized material, regardless of the light-curing procedure (direct, through 2 mm thick CRP CAD/CAM overlay and through 2 mm thick LDC CAD/CAM overlay) and temperatures of preheating, in all cases was significantly higher compared with the negative control (P<0.01). Although the minor differences in the percentages of viable cells between samples preheated at three temperatures using different light-curing procedures were observed, none of them was statistically significant compared to unpolymerized material.

Preheating at T1 resulted with the highest percentage of viable cells in sample which was overlayed with 2 mm thick LDC CAD/CAM overlay: 90.3±1.5 %. Slightly lower viability was observed after light-curing through 2 mm thick CRP CAD/CAM overlay (86.7±1.5 %), while the lowest lymphocyte viability (84.7±3.2 %) was observed after direct light-curing. In the negative control and unpolymerized Filtek Supreme Ultra, the frequency of apoptotic lymphocytes was slightly higher than the frequency of necrotic lymphocytes. Apoptosis predominated over necrosis in almost all polymerized samples, regardless of the temperature applied for preheating. The lowest frequency of apoptosis was observed after preheating at T2; with the best result in sample light-cured through 2 mm thick LDC CAD/CAM overlay (4.7±0.6 % of apoptotic cells). An increase of preheating the temperature in a majority of the tested samples did not significantly influence the frequency of necrosis, as compared to unpolymerized material. However, in almost all samples, the percentages of necrotic cells were significantly higher than in the negative control.

Tablica 3. Rezultati kvantitativnog flourescencijskog testa za istodobnu identifikaciju apoptotičnih i nekrotičnih limfocita u uzorcima izloženima nepolimeriziranom i polimeriziranom kompozitnom materijalu Filtek Supreme Ultra te u negativnoj kontroli; polimerizacija materijala zagrijanog na temperaturama T1-T3 (37 °C, 54 °C, 68 °C) iznosila je 40 sekunda preko overleja (CRP i LDC) ili izravno

Table 3 Results of the quantitative fluorescent assay for simultaneous identification of apoptotic and necrotic cells in lymphocyte samples exposed to unpolymerized and polymerized Filtek Supreme Ultra material as well as in the negative control. Light-curing of material preheated at temperatures T1-T3 (37 oC, 54 oC, and 68 oC) lasted for 40 seconds, through overlays (CRP and LDC) or directly.

Materijal • Material Filtek Supreme Ultra	Žive stanice • Viable cells (%)	Statistički značajno u usporedbi uzorkom • Statistically significant compared to	Apoptoza • Apoptosis (%)	Statistički značajno u usporedbi s uzorkom • Statistically significant compared to	Nekroza • Necrosis (%)	Statistički značajno u usporedbi s uzorkom • Statistically significant compared to
Nepolimeriziran • Unpolymerized	89.7±2.1	NC	5.7±1.2	NC	4.7±1.5	NC
Osvjetljavan preko CRP-a – T1 • Light-cured – through CRP – T1	86.7±1.5	NC	7.0±2.0	NC	6.3±1.5	NC
Osvjetljavan preko CRP-a – T2 • Light-cured – through CRP – T2	91.0±3.6	NC	5.0±2.0	NC	4.0±2.6	NC
Osvjetljavan preko CRP-a – T3 • Light-cured – through CRP – T3	89.0±0.0	NC	7.0±1.0	NC	4.0±1.0	NC
Osvjetljavan preko LDC-a – T1 • Light-cured – through LDC – T1	90.3±1.5	NC	5.0±1.7	NC	4.7±1.2	NC
Osvjetljavan preko LDC-a – T2 • Light-cured – through LDC – T2	88.0±1.0	NC	4.7±0.6	NC	7.3±1.2	NC; T3
Osvjetljavan preko LDC-a – T3 • Light-cured – through LDC – T3	90.7±3.2	NC	7.0±2.6	NC	2.3±1.2	-
Osvjetljavan direktno – T1 • Light-cured – directly T1	84.7±3.2	NC; LDC-T1	9.3±3.1	NC; LDC-T1	6.0±1.7	NC
Osvjetljavan direktno – T2 • Light-cured – directly T2	88.7±3.1	NC	6.0±2.0	NC	5.3±1.2	NC
Osvjetljavan direktno – T3 • Light-cured – directly T3	85.7±1.2	NC	8.0±1.7	NC	6.3±2.9	NC; LDC-T3
Negativna kontrola • Negative control	97.7±0.6		1.7±0.6		0.6±0.6	

Bilješka • Note

Analizirano je 300 stanica po uzorku i svakoj ispitivanoj stavki.
Statistička značajnost rezultata procijenjena je $\chi 2$ testom. Statistički značajne razlike ($p < 0,05$) prikazane su u tablici; NC-vs. negativna kontrola; UN vs. nepolimerizirani materijal; T1 vs. uzorak eksponiran istom materijalu, zagrijan na T1; T2 vs. uzorak eksponiran istom polimeriziranom materijalu, zagrijan na T2; T3 vs. uzorak izložen istom polimerizacijskom materijalu, zagrijan na T3.
300 cells per sample per each experimental point were analysed.
Statistical significance of data was evaluated using χ^2 test. Significant differences ($P<0.05$) are indicated in the table; NC – *vs.* negative control; UN – *vs.* unpolymerized material; T1 – *vs.* sample exposed to the same polymerized material, preheated at T1; T2 – *vs.* sample exposed to the same polymerized material, preheated at T2; T3 – *vs.* sample exposed to the same polymerized material, preheated at T3.

Komet-test u alkalnim uvjetima

Rezultati komet-testa u alkalnim uvjetima, primijenjenog za mjerenje razine primarnog oštećenja DNA-e, prikazani su za parametar dužina repa kometa u tablici 4. te za parametar intenzitet repa kometa u tablici 5. Uzimajući u obzir izmjerene vrijednosti obaju parametara komet-testa, uočeno je da i Z100 i Filtek Supreme Ultra u nepolimeriziranom obliku značajno pridonose stvaranju primarnih oštećenja u DNA-i u usporedbi s kontrolnom skupinom. No učinak nepolimeriziranih uzoraka značajno je izraženiji kod Z100 negoli kod Filtek Supreme Ultra.

Uzimajući u obzir indirektnu polimerizaciju Z100, izmjerene vrijednosti intenziteta repa nisu statistički značajno odstupale s obzirom na temperaturu zagrijavanja i postupak osvjetljavanja (tablica 5.). No dužina repa kometa značajno je smanjena nakon što je materijal zagrijan na 68 °C (T3). Osvjetljavanje preko CRP CAD/CAM overleja potaknulo je nešto manju migraciju DNA-e u usporedbi s osvjetljavanjem preko LDC overleja. Statistički značajna razlika zabilježena je

Alkaline comet assay

Results of the alkaline comet assay were used to evaluate primary damage to DNA are presented as a tail length parameter in Table 4 and tail intensity in Table 5. Considering both comet assay parameters, Z100 and Filtek Supreme Ultra in unpolymerized form significantly elevated primary lesions in DNA compared to the control. However, the effect of unpolymerized Z100 is significantly more pronounced than the one of Filtek Supreme Ultra.

Considering the indirect polymerization of Z100, tail intensity did not significantly differ in regards to the preheating temperature and polymerization-barrier used (Table 5). However, the tail length was significantly decreased when the material was preheated at 68 °C (T3). Further, light-curing through the CRP CAD/CAM overlay induced slightly lower DNA migration compared to light-curing through LDC CAD/CAM overlay. Statistically significant differences were recorded when temperatures of 54 ^{0}C (T2) were used in preheating (Table 4).

Tablica 4. Rezultati komet-testa u alkalnim uvjetima za procjenu genotoksičnosti kompozitnih materijala s obzirom na temperaturu korištenu za zagrijavanje materijala i vrstu polimerizacije, primarno oštećenje DNA-e izraženo je kroz parametar dužine repa kometa (u mikrometrima)
Table 4 Results of genotoxicity evaluation of composite materials in regards to temperature used to preheat material and type of polymerization-through barrier by applying alkaline comet assay. Primary damage to DNA is expressed in terms of tail length parameter.

Materijal • Material	Polimerizacijski postupak • Polymerization procedure	Zagrijavanje • Preheating	Dužina repa • Tail length / μm			Statistički značajno u usporedbi s uzorkom • Statistically significant compared to
			Srednja vrijednost • Mean	Medijan • Median	S.D.	
Z100	Nepolimerizirani • Unpolymerized	/	32.45	32.08	7.97	NC
	Polimerizacija preko CRP CAD/CAM overleja • Polymerization through CRP CAD/CAM overlay	T1	28.26	25.83	7.77	NC,Un
		T2	26.70	25.00	5.00	NC,Un
		T3	24.53	23.54	4.38	NC,Un,T1,T2
	Polimerizacija preko LDC CAD/CAM overleja • Polymerization through LDC CAD/CAM overlay	T1	28.53	26.67	7.51	NC,Un
		T2	29.48	28.33	4.47	NC,Un,Co
		T3	25.28	23.75	4.71	NC,Un,T1,T2
	Direktna polimerizacija • Directly polymerized	T1	25.82	24.17	4.21	NC,Un,CoT1,CeT1,CeT2
		T2	27.27	24.38	6.33	NC,Un,CoT3
		T3	30.03	30.42	6.99	NC,T1,T2,CoT2,CoT3,CeT3
Filtek Supreme Ultra	Nepolimerizirani • Unpolymerized	/	28.77	26.25	6.41	NC,Z100
	Polimerizacija preko CRP CAD/CAM overleja • Polymerization through CRP CAD/CAM overlay	T1	28.03	25.21	6.43	NC
		T2	26.38	24.79	5.71	NC,Un
		T3	27.92	26.46	6.69	NC,Z100
	Polimerizacija preko LDC CAD/CAM overleja • Polymerization through LDC CAD/CAM overlay	T1	28.57	27.08	4.65	NC
		T2	26.80	25.42	4.09	NC,Z100
		T3	25.13	23.33	5.96	NC,Un,T1, CoT3
	Direktna polimerizacija • Directly polymerized	T1	26.84	25.42	7.43	NC
		T2	26.12	24.38	6.31	NC,Un
		T3	30.17	28.33	6.33	NC,T1,T2,CoT2,CoT3,CeT2,CeT3
Negativna kontrola • Negative control			22.66	22.08	3.28	
Pozitivna kontrola 1mM H_2O_2 10 min • Positive control 1mM H_2O_2 10 min			52.29	51.30	11.83	NC

NC - statistički značajno u usporedbi s negativnom kontrolom • statistically significant compared to the negative control
Un - statistički značajno u usporedbi s rezultatima za nepolimerizirani oblik materijala • statistically significant compared to the results for unpolymerized form of the material of concern
Z100 - statistički značajno u usporedbi s rezultatima za Z100 s obzirom na polimerizacijski oblik • statistically significant compared to the results for Z100 in the state and polymerization mode of concern
Co - statistički značajno u usporedbi s rezultatima za polimerizaciju preko CRP-a • statistically significant compared to the results for polymerization through CRP CAD/CAM overlay of the material of concern
Ce - statistički značajno u usporedbi s rezultatima za polimerizaciju preko LDC CAD/CAM-a s obzirom na materijal • statistically significant compared to the results for polymerization through LDC CAD/CAM overlay of the material of concern
T1 - statistički značajno s obzirom na rezultate za T1 zagrijavanje prije polimerizacije materijala • statistically significant compared to the results for T1 preheating prior to the polymerization of the material of concern
T2 - statistički značajno u usporedbi s rezultatima za T2 zagrijavanje prije polimerizacije materijala • statistically significant compared to the results for T2 preheating prior to the polymerization of the material of concern
CoTx - statistički značajno u usporedbi s rezultatima za polimerizaciju preko CRP CAD/CAM overleja s obzirom na zagrijani materijal • statistically significant compared to the results for polymerization through CRP CAD/CAM overlay of the material of concern preheated at indexed temperature
CeTx - statistički značajno s obzirom na rezultate polimerizacije preko LDC CAD/CAM overleja s obzirom na zagrijani materijal • statistically significant compared to the results for polymerization through LDC CAD/CAM overlay of the material of concern preheated at indexed temperature

pri zagrijavanju uzoraka na temperaturu od 54 °C (T2) (tablica 2.).

Za Filtek Supreme Ultra, uočene su značajno niže vrijednosti dužine repa kometa pri zagrijavanju materijala na 68 °C (T3) i osvjetljavanja preko LDC CAD/CAM barijere u usporedbi s ostalim postupcima (tablica 4.). No izmjerene vrijednosti intenziteta repa nisu pokazale statistički značaj-

For Filtek Supreme Ultra, a significantly lower tail length value compared to other light-curing procedures was obtained by preheating the material at 68 ^{0}C (T3) and light-curing it through the LDC CAD/CAM barrier (Table 4). However, the tail intensity parameter did not reveal any statistical significance regarding the preheating temperature or light-curing barrier used (Table 5). Contrary to primary

Tablica 5. Rezultati komet-testa u alkalnim uvjetima za procjenu genotoksičnosti kompozitnih materijala s obzirom na temperaturu korištenu za zagrijavanje materijala i vrstu polimerizacije; primarno oštećenje DNA-e izraženo je kroz parametar intenzitet repa kometa, koji odgovara postotku genomske DNA-e koja je migrirala u rep kometa
Table 5 Results of genotoxicity evaluation of composite materials in regards to temperature used to preheat material and type of polymerization-through barrier by applying alkaline comet assay. Primary damage to DNA is expressed in terms of tail intensity parameter, corresponding to the extent of genomic DNA migrating into the comet tail.

Materijal • Material	Polimerizacijski postupak • Polymerization procedure	Zagrijavanje • Preheating	Intenzitet repa (% DNA u repu kometa) • Tail intensity (tail % DNA)			Statistički značajno u usporedbi s uzorkom • Statistically significant compared to
			Srednja vrijednost • Mean	Medijan • Median	S.D.	
Z100	Nepolimerizirani • Unpolymerized	/	6.04	4.55	5.80	NC
	Polimerizacija preko CRP CAD/CAM overleja • Polymerization through CRP CAD/CAM overlay	T1	3.47	0.89	5.76	NC,Un
		T2	1.86	0.36	3.13	Un
		T3	1.96	0.48	2.77	Un
	Polimerizacija preko LDC CAD/CAM overleja • Polymerization through LDC CAD/CAM overlay	T1	3.01	0.97	4.35	NC,Un
		T2	2.70	0.82	3.92	NC,Un
		T3	2.57	0.68	3.29	NC,Un
	Direktna polimerizacija • Directly polymerized	T1	1.62	0.27	2.58	Un
		T2	2.53	0.54	3.82	Nc,Un
		T3	4.16	2.55	3.99	NC,T1,T2,CoT2,CoT3,CeT3
Filtek Supreme Ultra	Nepolimerizirani • Unpolymerized	/	2.51	0.79	3.43	NC,Z100
	Polimerizacija preko CRP CAD/CAM overleja • Polymerization through CRP CAD/CAM overlay	T1	1.94	0.63	3.25	
		T2	2.55	0.62	3.92	NC
		T3	3.06	0.70	4.28	NC
	Polimerizacija preko LDC CAD/CAM overleja • Polymerization through LDC CAD/CAM overlay	T1	2.83	1.81	2.93	NC
		T2	2.35	0.99	2.95	NC
		T3	1.85	0.13	3.56	
	Direktna polimerizacija • Directly polymerized	T1	1.84	0.13	5.15	
		T2	2.49	0.74	4.02	NC
		T3	3.94	2.38	4.21	NC,T1,CoT1,CoT2,CeT2,CeT3
Negativna kontrola • Negative control			1.15	0.06	2.91	
Pozitivna kontrola 1mM H_2O_2 10 min • Positive control 1mM H_2O_2 10 min			32.65	30.15	20.67	NC

NC - statistički značajno u usporedbi s negativnom kontrolom • statistically significant compared to the negative control
Un - statistički značajno u usporedbi s rezultatima za nepolimerizirani oblik materijala • statistically significant compared to the results for unpolymerized form of the material of concern
Z100 - statistički značajno u usporedbi s rezultatima za Z100 s obzirom na polimerizacijski oblik • statistically significant compared to the results for Z100 in the state and polymerization mode of concern
Co - statistički značajno u usporedbi s rezultatima za polimerizaciju preko CRP-a • statistically significant compared to the results for polymerization through CRP CAD/CAM overlay of the material of concern
Ce - statistički značajno u usporedbi s rezultatima za polimerizaciju preko LDC CAD/CAM-a s obzirom na materijal • statistically significant compared to the results for polymerization through LDC CAD/CAM overlay of the material of concern
T1 - statistički značajno s obzirom na rezultate za T1 zagrijavanje prije polimerizacije materijala • statistically significant compared to the results for T1 preheating prior to the polymerization of the material of concern
T2 - statistički značajno u usporedbi s rezultatima za T2 zagrijavanje prije polimerizacije materijala • statistically significant compared to the results for T2 preheating prior to the polymerization of the material of concern
CoTx - statistički značajno u usporedbi s rezultatima za polimerizaciju preko CRP CAD/CAM overleja s obzirom na zagrijani materijal • statistically significant compared to the results for polymerization through CRP CAD/CAM overlay of the material of concern preheated at indexed temperature
CeTx - statistički značajno s obzirom na rezultate polimerizacije preko LDC CAD/CAM overleja s obzirom na zagrijani materijal • statistically significant compared to the results for polymerization through LDC CAD/CAM overlay of the material of concern preheated at indexed temperature

no odstupanje s obzirom na temperaturu zagrijavanja ili postupak osvjetljavanja (tablica 5.). Nasuprot primarnom oštećenju DNA-e iskazanom parametrom dužine repa, mjerenja iskazana postotkom DNA-e koja je migrirala u rep kometa, nisu pokazala značajnu razliku između genotoksičnog potencijala dvaju ispitivanih kompozitnih materijala. Pri izravnom

DNA damage measured as tail length, the one measured in terms of % of DNA that migrated into the comet tail did not indicate any difference between the genotoxic potential of two tested composite materials.

In regards to direct light-curing, both materials exhibited a significant genotoxic effect when preheated at 68 °C (T3)

osvjetljavanju, oba materijala pokazuju značajan genotoksični učinak kada su zagrijani na 68 °C (T3) prije osvjetljavanja u usporedbi s ostalim uzorcima. Učinak je zabilježen kao značajan porast obaju parametara komet-testa.

Rasprava

Budući da su kompozitne restauracije izložene izravnom utjecaju oralne sredine samo nekoliko minuta nakon polimerizacije, naša studija oslikava kliničke uvjete jer je inkubacija staničnih kultura s kompozitnim uzorcima provedena neposredno nakon pripreme uzorka. Maksimalno vrijeme osvjetljavanja koje je preporučio proizvođač za program visokog intenziteta LED uređaja rabljenog u eksperimentu (Bluephase G2) jest 15 sekunda, no u ovoj studiji vrijeme polimerizacije iznosilo je 40 sekunda. Razlog tomu jest što se tijekom adhezijskog cementiranja CAD/CAM restauracija svjetlo lomi i raspršuje prolazeći kroz restauraciju te je zato potrebno dulje osvjetljavanja kako bi se kompozit korišten za adhezijsko cementiranje mogao potpuno polimerizirati. Prema rezultatima ove studije, nije utvrđena statistički značajna razlika u cito-/genotoksičnosti između osvjetljavanja kompozita izravno ili preko CRP ili LDC CAD/CAM overleja debljine 2 mm. No takvi rezultati mogu se povezati s činjenicom da su uzorci stavljeni u limfocitnu staničnu kulturu neposredno nakon polimerizacije. U slučaju izravne polimerizacije, kompozitni uzorak prima više topline negoli onaj polimeriziran preko CRP ili LDC CAD/CAM overleja debljine 2 mm. Zato, kada je smješten u svježu kulturu stanica, zagrijani uzorak kompozitnog materijala može uzrokovati slabije preživljenje stanica. Razlog za to nisu komponente kompozitnog materijala koje nisu reagirale nego temperatura uzorka. Naše prijašnje studije pokazale su da je ključan porast temperature tijekom polimerizacije (26, 27, 29). U ovoj studiji zabilježen je i porast temperature zbog zagrijavanja uzorka, a ne isključivo zbog polimerizacije. Treba imati na umu da temperatura zagrijanog kompozita opada vrlo brzo nakon što je izvađen iz uređaja za zagrijavanje.

Porast razine primarnih oštećenja DNA-e uočen je u ispitivanjima obaju kompozitnih materijala. Pretpostavljamo da je oštećenje DNA-e vjerojatno uzrokovano porastom temperature materijala zagrijanog pri višim temperaturama (68 °C). Danroch i suradnici (29) utvrdili su da kompozitni materijal zgrijan do 60 °C i izvađen iz uređaja za zagrijavanje, pokazuje pad temperature kompozitnog materijala od 35 do 40 % nakon 40 sekunda , do 50 % nakon 2 minute te do 90 % nakon 5 minuta. No apliciranje topline može utjecati na intramolekularnu kemijsku vezu. Tijekom indirektnih postupaka osvjetljavanja rezultati dobiveni za zagrijavanje na temperaturi od 54 °C (T2), koju inače preporučuju proizvođači, nisu odstupali u odnosu na rezultate dobivene pri zagrijavanju uzoraka na 68 °C (T3).

U nekim studijama upozorava se na činjenicu da kisik tijekom polimerizacije kompozitnog materijala može inhibirati polimerizaciju. Posljedica je nastanak nepolimeriziranog monomernog sloja na površini kompozita koji, ako se ne ukloni nakon polimerizacije, može uzrokovati porast citotoksičnosti materijala (30, 31). Citotoksični učinak manje

prior to light-curing compared to other procedures tested. The effect was recorded as a significant increase in comet assay parameters; both tail length and tail intensity.

Discussion

Since composite restorations are commonly exposed to the oral environment only a few minutes after light-curing, our study mimicked the clinical conditions by incubating cell cultures with composite samples immediately after the sample preparation. The maximum curing time recommended by the manufacturer for the high intensity mode of the LED light curing unit (Bluephase G2) is 15 s, in this study the curing time of 40s was used. The reason for that is when bonding the CAD/CAM restoration, the curing light is attenuated while passing through the restoration and longer exposures have to be done in order to cure the luting composite completely. According to the results of this study, there was no statistically significant difference in cyto-/genotoxicity between light-curing of the composites directly or through 2 mm thick CRP or LDC CAD/CAM overlay. However, the explanation for the results given in this study may be due to the fact that the samples were placed in a lymphocyte cell culture immediately after polymerization. In case of the direct polymerization, the composite sample received more heat than the sample polymerized through 2 mm thick CRP or LDC CAD/CAM sample. Therefore, the heated composite sample when placed in a fresh cell culture may cause less viable cells. This is not because there are unreacted components from the material but because of the temperature of the sample. Our former studies show that the temperature during polymerization plays a crucial role (26,27,29) but still stays a question since the temperature drops off quickly after removing the composite from the heating unit.

The increase in the level of primary DNA damage was observed for both tested materials. It is unlikely though that the observed effect was mediated by increased temperature of material samples placed in the culture due to pre-heating at 68 ^{0}C. Danroch et al. (29) concluded that when composite material is heated up to 60 ^{o}C and removed from the heating unit, the temperature of the composite material decreased 35-40% after 40s, up to 50% after 2 min, and up to 90% after 5 min. However, applied heat may affect intramolecular chemical bonds. The radiation absorbed by molecules causes increased vibration. When indirect curing procedures were applied, results obtained for pre-heating temperatures of T2 of 54 ^{o}C, which is recommended by composite manufacturers, did not differ compared to the results obtained with pre-heating at T3 - 68 ^{o}C.

Some studies reported that the presence of oxygen during the light curing of materials might inhibit polymerization. This leads to the formation of unpolymerized monomeric surface layer which, if not removed after curing, increases cytotoxicity of materials (30,31). The cytotoxic effect was less exhibited if that surface layer was removed. Therefore, in the present study we used a Mylar sheet to prevent the access of material surface to atmospheric oxygen and to avoid the for-

je uočljiv ako je taj površinski sloj odstranjen. Zato je u našoj studiji korištena Mylar folija kako bi se spriječilo stvaranje sloja atmosferskog kisika na površini uzorka te tako spriječilo stvaranje nepolimerizirane površine sloja kompozitnog materijala s posljedičnim citotoksičnim učinkom.

Darmani i suradnici (32) proučavali su citoksičnost različitih kompozitnih materijala i ustanovili pad preživljenja stanica do 48 % u slučaju njihova izlaganja kompozitnom materijalu Z100. U našoj studiji, smanjenje preživljenja stanica nakon kontakta sa Z100, nije bilo toliko izraženo kao u spomenutoj studiji (tablice 2. i 3.). Statistička usporedba rezultata citotoksičnosti između Z100 i Filtek Supreme Ultra pokazala je da nakon polimerizacije obaju kompozitnih materijala preko CRP CAD/CAM overleja debljine 2 mm i pri temperaturama T1 i T3, bolje rezultate (veće preživljenje limfocita) pokazuje kompozit Z100. No nakon zagrijavanja na temperaturi T2, Filtek Supreme Ultra bio je manje citotoksičan (P = 0,0228). Također, nakon polimerizacije obaju kompozitnih materijala preko LDC CAD/CAM overleja debljine 2 mm na svim temperaturama zagrijavanja, citotoksičnost kompozita Z100 bila je nešto niža ili jednaka citotoksičnosti kompozita Filtek Supreme Ultra, ali bez statističke značajnosti. Općenito, čini se da je Filtek Supreme Ultra manje osjetljiv na postupak osvjetljavanja, izravno ili preko CRP ili LDC CAD/CAM onleja debljine 2 mm. Za oba materijala najniža citotoksičnost uočena je pri zagrijavanju na temperaturi T1 tijekom direktne polimerizacije i polimerizacije preko CRP CAD/CAM overleja.

Uzimajući u obzir oba parametra komet-testa, duljinu repa i intenzitet repa, nepolimerizirani Z100 pokazao je velik potencijal za izazivanje primarnih oštećenja DNA-e u usporedbi s kompozitnim materijalom Filtek Supreme Ultra. Uzrok za to mogao bi se povezati sa sastavom tih dvaju materijala. Za Z100 temeljni su materijali TEGDMA i Bis-GMA, a Filtek Supreme Ultra, osim TEGDMA-e i Bis-GMA-e sadržava UDMA i Bis-EMA monomer. Otpuštanje rezidualnih monomera iz nepolimeriziranog Z100 i njihov toksični učinak spominju se u nekoliko studija (32 – 34). Darmani i suradnici (32) navode otpuštanje velike količina TEGDMA-e i manje količine Bis-GMA-e iz testiranih kompozita, uključujući Z100. Kao što su objasnili Engelmann i suradnici (35), TEGDMA formiranjem veza s glutationom (koji inače ima zaštitni učinak za stanice) može interferirati s njegovom zaštitnom funkcijom i tako uništiti stanicu. Ustanovljeno je da Filtek Supreme Ultra sadržava znatnu količinu cirkonijevih čestica, a dokazano je da taj element ima značajna antioksidativna svojstva (1, 36, 37).

U našoj se studiji izbor 24-satnog tretmana temelji na spoznajama dobivenima iz ranijih studija, pokazujući da se najveće otpuštanje komponenata koje nisu reagirale događa u prva 24 sata nakon što je restaurativni materijal postavljen u kavitet (38). Dobiveni podatci o razinama primarnih oštećenja genoma pokazuju da bi se CRP CAD/CAM mogao smatrati boljim materijalom s obzirom na to da su i dužina i intenzitet repa kometa bili manji u usporedbi s vrijednostima dobivenima pri osvjetljavanju preko LDC overleja. Postotak DNA-e u repu kometa općenito se smatra točnijim biomarkerom genotoksičnosti jer je izravno razmjeran udjelu

mation of unpolymerized surface layer and consequently its effect on the cytotoxicity results.

Darmani et al. (32) evaluated cytotoxicity of different composite materials and reported a decrease in cell viabilities up to 48 % when the cells were exposed to Z100 composite material. In our study viability was not affected to such extent (Table 2,3). Statistical comparison of cytotoxicity results between Z100 and Filtek Supreme Ultra showed that after light-curing of both materials through 2 mm thick CRP CAD/CAM overlay at temperature T1 and T3, better results (higher lymphocyte viability) were obtained for Z100. However, after preheating at temperature T2, Filtek Supreme Ultra had lower cytotoxicity (P=0.0228). Also, after light-curing of both materials through 2 mm thick LDC CAD/CAM overlay at all preheating temperatures, cytotoxicity of Z100 was slightly lower or similar to Filtek Supreme Ultra, however none of the differences was statistically significant. In general, Filtek Supreme Ultra appears less sensitive on how the light-curing is conducted – directly, or through 2 mm thick CRP CAD/CAM sample or 2 mm thick LDC CAD/CAM sample. For both studied materials regardless whether they were cured through CRP CAD/CAM overlay or directly, the lowest cytotoxicity was observed with pre-heating at T1.

Considering both comet assay parameters tail length and tail intensity, the unpolymerized form of Z100 exhibited higher potency of inducing primary damage to DNA compared to Filtek Supreme Ultra. The observed effect is mediated by a significant difference in the composition of two evaluated composite materials. Z100 is TEGDMA and Bis-GMA based material, while Filtek Supreme Ultra besides TEGDMA and Bis-GMA contains also UDMA and Bis-EMA resin monomers. Leaching of residual monomers from Z100 and their toxic effects were reported by several studies (32-34). Darmani et al. (32) concluded that high amount of TEGDMA and comparatively smaller amounts of Bis-GMA were released from tested composite materials. As Engelmann et al. (35) explains, TEGDMA forms adducts with glutathione (which has protective functions to the cells) and may interfere with its protective function leading to the cell destruction. However, Filtek Supreme Ultra contains a considerable amount of zirconia particles, and zirconia is shown to exhibit significant antioxidative properties (1,36,37).

In the present evaluation, 24 hours of treatment has been used based on the knowledge gained from previous studies indicating that highest leaching occurs within first 24 hours following restorative material placement in oral cavity (38). Evaluation of the level of primary genomic lesions indicated that CRP CAD/CAM might be a preferable material to perform indirect polymerization, since both tail length and % of DNA in tail were lower compared to LDC polymerization procedure. The percentage of DNA (tail intensity) in the comet tail that is accepted as a more reliable biomarker of genotoxicity directly corresponds to the proportion of genomic DNA affected by the adverse biological effects of the substance (39,40). The obtained values for that parameter indicates that there is no effect on the primary damage to DNA if preheating is performed at 54 °C (T2) or 68 °C (T3) (Tables 4,5). Although there is no statistically significant differ-

genomske DNA-e u repu kometa, na čiji porast utječu štetni biološki učinci različitih agensa (39, 40). Dobivene vrijednosti za taj parametar pokazuju da zagrijavanje kompozita na 54 °C (T2) ili 68 °C (T3) ne utječe na razinu primarnog oštećenja DNA-e (tablice 4. i 5.). Iako nema statistički značajne razlike, čak ako se uzme u obzir intenzitet repa, polimerizacija preko CRP-a može biti postupak izbora (tablica 5.). Razlika u rezultatima uočenima nakon polimerizacije preko CRP-a ili LDC CAD/CAM overleja može se objasniti različitim sastavom materijala. Illie i Hickel (41) pokazali su da je litijeva disilikatna staklena keramika više opakna zbog kristalne strukture. To može uzrokovati manju transmisiju svjetla i time manje polimerizirani uzorak, što pak smanjuje preživljenje stanica.

Pri izravnoj polimerizaciji obaju ispitivanih materijala, zagrijavanje na 37 °C (T1) ili 54 °C (T2) može biti postupak izbora. Nadalje, s obzirom na genotoksičnost, ne preporučuje se prethodno zagrijavanje na 68 °C (T3).

Općenito, uzimajući u obzir dva čimbenika: A) da je intenzitet repa vjerodostojniji pokazatelj razine primarnog oštećenja DNA-e negoli dužina repa i B) da se do 10 % DNA-e u repu smatra prihvatljivom razinom oštećenja bez značajnijeg narušavanja cjelovitosti genoma (25, 42), može se pretpostaviti da u uvjetima korištenima u ovoj studiji obaju ispitivanih materijala pokazuju više negoli prihvatljivu razinu biokompatibilnosti s obzirom na cito- i genotoksičnost. Prema tomu, promjene u razini primarnog oštećenja DNA-e nakon izlaganja limfocita kompozitnim materijalima, bez obzira na to što su neki rezultati pokazali statistički značajne razlike, možda nisu biološki relevantne. Važnost prikazanih rezultata ogleda se u pokušaju iznalaženja najprihvatljivijeg postupka za polimerizaciju kompozita i najpovoljnijih uvjeta zagrijavanja. Za oba ispitivana kompozitna materijala, prva je hipoteza prihvaćena, a druga je odbačena. Rezultati dobiveni na staničnim kulturama ne mogu se izravno primijeniti za objašnjenja mogućih scenarija u uvjetima *in vivo*. No dobiveni rezultati upućuju na to da se neprestano trebaju pronalaziti napredniji postupci te modificirati sastavi kompozitnih materijala kojima bi se poboljšala polimerizacija kompozita te umanjio potencijalni rizik za pacijenta i osoblje koje radi s takvim materijalima.

Zaključak

Za kompozitni materijal Z100, najveći postotak živih stanica zabilježen je nakon izravnog osvjetljavanja, nakon čega slijedi osvjetljavanje preko CRP-a te LDC overleja. Za kompozit Filtek Supreme Ultra najveći postotak živih stanica zabilježen je pri polimerizaciji preko CRP overleja, nakon čega slijedi polimerizacija preko LDC overleja i izravna polimerizacija.

Za materijal Z100 najveći postotak živih stanica zabilježen je nakon zagrijavanja na T1 za CRP, LDC te za izravno osvjetljavanje uzoraka. Za kompozit Filtek Supreme Ultra, najveći postotak živih stanica zabilježen je pri zagrijavanju na T2 za CRP te pri izravnom osvjetljavanju na T1/T3 za LDC.

U negativnoj kontroli i nepolimeriziranim uzorcima obaju kompozitnih materijala –Z100 i Filtek Supreme Ultence, even considering tail intensity parameter CRP-through polymerization may be preferable procedure (Table 5). The difference in results when polymerized through CPR or LDC CAD/CAM overlay may be explained with different material composition. Ilie and Hickel (41) showed in their study that lithium disilicate glass-ceramic due to is crystalline structure shows more opacity. This can cause less light transmission and therefore less polymerized sample leading to less viable cells.

Regarding the direct polymerization of both tested materials, preheating the procedure at 37 °C (T1) or 54 °C (T2) may be the procedure of choice. Furthermore, in terms of genotoxicity, preheating at 68 °C (T3) may not be recommended.

In general, considering two facts: A) that tail intensity is a more reliable parameter of primary damage to DNA over tail length, and B) that up to 10% of DNA in tail is considered baseline level with no significant effect on genome integrity (25,42), it may be suggested that under conditions used in the present study both tested materials showed more than acceptable level of biocompatibility in terms of cyto- and genotoxicity. Thus, observed changes in the level of the primary damage to DNA due to both composite treatments, though some of them showed statistical significance, may be of no biological relevance. Importance of the presented results lies in the attempt to indicate the most suitable procedure for their polymerization and preheating options. For both tested composite materials, the first null-hypothesis was accepted and the second one was rejected. These results with a cell culture cannot be directly used for explanation of the in vivo scenario. However, this does indicate a constant need for finding more advanced procedures and composite resin modifications which would improve polymerization of composite materials and minimize the potential risk for patients and dental personnel.

Conclusion

For the Z100, the highest percentage of viable cells was recorded after direct light curing, followed by light curing through CRP and through LDC. For Filtek Supreme Ultra highest percentage of viable cells is recorded while curing through CRP followed by LDC and direct light curing.

For Z100, the highest percentage of viable cells is recorded after preheating on T1 for CRP, LDC and direct light curing. For Filtek Supreme Ultra, highest percentage of viable cells is recorded while preheating composite at T2 for CRP and direct light curing and T1/T3 for LDC.

In the negative control and unpolymerized samples of both tested composite materials, Z100 and Filtek Supreme Ultra, the frequency of apoptotic lymphocytes was slightly higher than the frequency of nectotic lymphocytes. For

tra – učestalost apoptotičnih limfocita nešto je veća negoli nekrotičnih limfocita. Za Filtek Supreme Ultra, apoptoza predominira nad nekrozom u gotovo svim polizmeriziranim uzorcima bez obzira na temperaturu zagrijavanja. No za kompozitni materijal Z100 apoptoza predominira nad nekrozom samo u uzorcima polimeriziranima izravnim postupkom osvjetljavanja, bez obzira na temperaturu zagrijavanja.

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Sukob interesa

Nije ga bilo

Filtek Supreme Ultra, apoptosis predominated over necrosis in almost all polymerized samples regardless of the preheating temperature. However, for Z100 composite material apoptosis predominated over necrosis only in samples prepared with direct light curing, regardless of the heating tempaerature.

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Disclosure statement

The authors report no conflicts of interest.

Abstract

Objectives: The aim was to compare cytotoxicity/genotoxicity of pre-heated composites polymerized through CAD/CAM overlays on isolated human peripheral blood lymphocytes. **Material and Methods:** A microhybrid (Z100, 3M ESPE) and nanofilled composite (Filtek Supreme Ultra, 3M ESPE) were heated in a heating unit (Calset, AdDent Inc.) at different temperatures: 37 °C, 54 °C, and 68 °C. A small amount of heated composite was placed in a cylindrical mold (6mm diameter; 0.65mm thick), covered with a Mylar sheet, pressed and light-cured directly and through 2 mm thick CAD/CAM ceramic-reinforced polymer (CRP)(LAVA Ultimate, 3M ESPE) or CAD/CAM lithium disilicate ceramic (LDC)(e.max, Ivoclar/Vivadent) overlay. After curing, the specimens were immediately placed in a prepared lymphocyte cell culture. Cytotoxicity was assessed using a dye exclusion method by simultaneous staining with ethidium bromide and acridine orange, aimed to determine percentages of viable, apoptotic and necrotic cells. Genotoxicity was studied using alkaline comet assay. **Results:** For Z100, the highest percentage of viable cells is recorded at T1 (93.7%) after direct light curing, followed by light curing through CRP (92.3%) and through LDC (91.7%T1,T3). For Filtek Supreme Ultra, the highest percentage of viable cells is recorded while curing through CRP (91.0% T2), followed by LDC (90% T1,T3) and direct light curing (88.7%T2). **Conclusion:** For both tested materials, preheating the procedure at T1 and T2 may be the procedure of choice. In terms of genotoxicity, preheating at T3 may not be suggested.

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Key words
Composite resins, cytotoxicity, genotoxicity, dental light curing units

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