Toksikšćnost prethodno zagrijanih kompozita izravnom polimerizacijom i preko CAD / CAM overleja

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

Svjetlosno stvrdnjavajući kompozitni materijali uvelike se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijali zbog estetskih i mehaničkih svojstava te razmjerowo se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijali zbog estetskih i mehaničkih svojstava te razmjerowo se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijali zbog estetskih i mehaničkih svojstava te razmjerowo se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijali zbog estetskih i mehaničkih svojstava te razmjerowo se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijali zbog estetskih i mehaničkih svojstava te razmjerowo se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijali zbog estetskih i mehaničkih svojstava te razmjerowo se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijali zbog estetskih i mehaničkih svojstava te razmjerowo se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijali zbog estetskih i mehaničkih svojstava te razmjerowo se upotrebljavaju u kliničkoj stomatologiji kao restaurativni materijal za zubarsku terapiju.

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 citotoksčnost i do dva tjedna. Ispitivanja u uvjetima in vitro pokazala su da metakrilatni i dimetakrilatni monomeri upotrijebljeni u re-
staurativnim dentalnim materijalima mogu povećati unutar-
stančni razinu reaktivnih kisikovih spojeva, što može pota-
knuti apoptozu. Uz to, suprimiraju mitohondrijsku aktivnost
makrofaga, potičući time njihov ulapni odgovor, umanjjuju-
ći enzimatsku aktivnost te uzrokujući fragmentaciju DNA-e
i ekspresiju čimbenika rasta i citočinka (5 – 9).

Premda je teoretski moguća 100-postotna konverzija mo-
nomera 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 ni-
su reagirale dostupno za interakciju s makromolekulama u bi-
ološkom sustavu (3, 10, 11). Kompozitni materijali ne dosežu
maksimalan stupanj konverzije neposredno nakon polimeri-
zacije (12, 13). Moon i suradnici (5) u svojoj studiji pokaz-
ali su da je potrebno sedam dana da bi stupanj konverzije dose-
gnuo maksimum polimerizacije, na površini i na dnu ispuna.
Nadalje, zaključili su da se količina monomera otpuštena iz
kompozitnog materijala razlikuje s obzirom na vrstu polime-
rizatora 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 emitirane iz uređaja za polimerizaciju manja od 17 J/ cm².
Nasuprot tomu, ako je količina emitirane energije veća od 17 J/cm²
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 mehanič-
kih svojstava, a bez negativnog učinka na marginalno brtvlje-
nje (16, 17). Korištenje zagrijanih kompozita postaje sve po-
pularnije i kao sredstvo cementiranja CAD/CAM restauracija
(18 – 20).

Darnoch i suradnici (17) pokazali su da kompozitni ma-
terijali zagrijani do 60 °C mogu poboljšati stupanj konverzi-
j na površini i na dubini ispuna do 2 mm. No Froes-Salga-
do i njegovi kolege (21) nisu pronašli značajno poboljšanje
mehaničkih svojstava ni stupnja konverzije na unaprijed zagri-
janim kompozitnim materijalima, ali su pokazali pobolj-
šanje adaptacije kompozitnih materijala za stijenku kavite.
Lohbauer i suradnici (22) istakнуli su da unaprijed zagrija-
ni 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 zagrija-
nog kompozita polimeriziranog preko CAD/CAM uzorka
kada je riječ o citotoksčnosti i genotoksčnosti. Zato je svrha
ove studije procijeniti citotoksčnost i genotoksčnost dvaju
kompozitnih materijala zagrijanih na tri različite temperatu-
re te osvijetljenih izravno i preko CAD/CAM overleja deblji-
ne 2 mm. S obzirom na to, postavljene su sljedeće hipoteze:

1. izravna polimerizacija kompozita pokazuje jednaku citot-
oksčnost i genotoksčnost kao i polimerizacija kompo-
zitnog uzorka preko CAD/CAM overleja

trigger cytotoxicity for up to 2 weeks. In vitro studies indicat-
ed that methacrylate and dimethacrylate monomers used in
restorative dental materials may increase the intracellular lev-
ell of reactive oxygen species which induce apoptosis. In addi-
tion, they suppress the mitochondrial activity of macrophages,
thus altering their inflammatory responses, affect the recruit-
ment of leukocytes and decrease the expression of intercellu-
lar adhesion molecules, induce enzymatic activity, DNA frag-
mentation, expression of growth factors and cytokines (5-9).

Theoretically, a 100% conversion of monomers to poly-
mers is possible, but usually 25-50% of methacrylate mono-
mer 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 bio-
logical 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 monom-
ers 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/cm². In contrast, if the radiant energy
is higher than 17 J/cm², those differences disappear regard-
less 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 ap-
proach in order to obtain a higher degree of conversion and
better mechanical properties without negative effect on mar-
ginal seal (16,17). The use of pre-heated composite as a lut-
ing material for CAD/CAM restorations was also reported
(18-20).

Darnoch et al. (17) reported that pre-heating the com-
posite up to 60 °C may improve the degree of conversion on
both, top and 2 mm deep surface. However, Froes-Salga-
do et al., (21) did not find any improvement in mechanical
properties and the degree of conversion of pre-heated com-
posite, but reported an improvement in composite adapta-
tion to cavity walls. Lohbauer et al. (22) indicated that pre-
heating 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 lut-
ing material for CAD/CAM fabricated restorations are be-
coming more and more popular, it will be interesting to see
the impact of the light curing of heated composite through
CAD/CAM restoration on cytotoxicity/ genotoxicity. There-
fore, the aim of this study was to assess cytotoxicity and gen-
otoxicity 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 follow-
ing null-hypotheses are formed:

1. Direct light-curing of composite resin exhibits similar cy-
totoxicity 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) i litijev disilikatna staklena keramika (LDC, e.max CAD, Ivoclar Vivident, Schaan, Liechtenstajn) 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 pijalinom uz vodeno hlađenje. Veličina overleja bila je 14 x 14 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, Liechtenstajn) 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, USA) na trima različitim temperaturama – 37 °C (T1), 54 °C (T2), 68 °C (T3) prema uputama proizvođača.

Koristi se 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, USA) na trima različitim temperaturama – 37 °C (T1), 54 °C (T2), 68 °C (T3) prema uputama proizvođača.

**Table 1. Materijali korišteni u istraživanju**

| Materijal • Material                  | Proizvođač • Manufacturer | Sastav • Composition                                                                 |
|--------------------------------------|---------------------------|--------------------------------------------------------------------------------------|
| Keramikom pojačan polimer •         | LAVA Ultimate, 3M ESPE,   | - kompozitna nanokeramika sadrži 79 % nanokeramikačkih čestica                       |
| Ceramic-reinforced polymer (CRP)     | St. Paul, MN,             | povezani u smoli materijala • resin nanokeramic containing approximately 79% nanoceramic particles bound in the resin matrix |
| CAD/CAM                              | SAD • USA                 | - kombinacija neaglomeriranog / neagregiranog 20 nm silikatnog punila,               |
|                                      |                           | neaglomeriranog / neagregiranog 4-11 nm cirkonijeva punila i agregiranih punila      |
|                                      |                           | cirkonij/silika (20 nm slike i 4-11 nm čestice cirkonij) • 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 i 4-11 nm zirconia particles). |
| Litij disilikatna staklena keramika  | Ivoclar Vivident,         | - kvarc, litij dioksid, fosforov oksid, aluminiij, kalijijski oksidi te druge komponente • |
| • Lithium disilicate glass-ceramic   | Schaan, Liechtenstein     | quartz, lithium dioxide, phosphor oxide, alumina, potassium oxide and other components |
| (LDC), e.max CAD                     |                           |                                                                                     |
| Z100                                 | 3M ESPE, St. Paul, MN,    | - mikrohibridna kompozitna smola • microhybrid composite resin                     |
|                                      | SAD • USA                 | - matriks; BIS-GMA i TEGDMA • matrix; BIS-GMA and TEGDMA                             |
|                                      |                           | - punilo cirkonija/silika, anorgansko punilo 66 % w, veličina čestica od 3,5 do 0,01 µm • |
|                                      |                           | - 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,    | - nanopunjeni kompozitni materijal • nanofilled composite resin                    |
|                                      | SAD • USA                 | - 100 % nanopunilo, primarne čestice manje su od 100 nm • 100% nanofiller, the primary particles are below 100 nm |
|                                      |                           | - smola: Bis-GMA, UDMA, TEGDMA, te bis-EMA • resin: Bis-GMA, UDMA, TEGDMA, and bis-EMA |
|                                      |                           | - punilo: kombinacija neaglomeriranog/neagregiranog 20 nm silikatnog punila, neaglomeriranog/neagregiranog 4-11 nm cirkonijeva punila i agregiranih punila cirkonija/silika (20 nm slike i 4-11 nm čestice cirkonija) • fillers: combination of non-agglomerated/non-aggregated 20 nm silica filler, non-agglomerated/non-aggregated 4-11 nm zirconia filler, and aggregated zirconia/silica cluster filler (20 nm silica i 4-11 nm zirconia particles) |
|                                      |                           | 100% nanopunilo, primarne čestice manje su od 100 nm • 100% nanofiller, the primary particles are below 100 nm |
|                                      |                           | - 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 dionim uredajem (LED) (Bluephase G2, Vivadent, Schaan, Lihtenštajn) uporabom programa visokog intenziteta (1180 mW/cm²). Primijenjena su tri načina osvjetljavanja – (1) izravno osvjetljavanje, (2) osvjetljavanje preko CRP CAD/CAM veličine i (3) osvjetljavanje preko LDC veličine. 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 svrhi uzimanja krvi te o svrhi testiranja uzetog uzorka krvi.

Venska krv izvijedena 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), jako su poslije oslikane, a omjer kontrole i kompozita polimeriziranog materijala je 7 ml. 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 na 37°C i 5 % CO₂. Nakon 24 sata kulture su pet minuta centrifugirane na 300 g. Supernatant je uklonjen, a talog koji sadržava limfocite paljivo je resuspendiran i korišten za daljnju analizu.
Kvantitativna fluorescencijska metoda za procjenu preživljenja stanica, apoptoze i nekroze

Preživljenje limfocita izoliranih iz perifernih krvi izmjerno je metodom istodobnog bojenja dvjema fluorescencijskim bojama (24). Nakon bojenja etidijevim bromidom i akridinskom narančastoj boji (100 µg/ml) (Sigma-Aldrich, SAD) u jednakim volumnim omjerima (1:1; v/v) iz svake limfocitne kulture mikropipetom je odmjerno 20 µl suspenzije stanica i prebačeno na predmetno mikroskopsko staklo. Na suspenziju stanica pažljivo je pipetirana miješavina fluorescencijskih boja, uzorak je pokriven pokrovnim stakalom i odmah analiziran fluorescencijskim mikroskopom (Olympus BX; povećanje 400 x). Za svaki uzorak učinjena su tri uzastopne testa te je ukupno pregledano 300 stanica po uzorku. Uspoređeno 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, je zgreška im je nakon dovojnog bojenja pod fluorescencijskim mikroskopom zelena. Mrtve nekrotične stanice imaju narančastu crveno obojen kromatin, a apoptotične imaju izrazito zelenu i visoko kondenziranu ili fragmentiranu jezgru.

Komet-test u alkalnim uvjetima

Za pripremu mikrogelova agarose korištenih u komet-testu, 10 µl limfocitne suspenzije pomiješano je sa 100 µl 5-po storne agarose niskog tališta (37 °C; Sigma-Aldrich, MO, SAD). Dobivena suspenzija pipetirana je na pripremljene mikroskopske stubove premašenu slojem agarose normalne temperature tališta (Sigma-Aldrich, MO, SAD) te pokrivena pokrovnim stakalom. Gelovi su 10 minuta držani na ledu radi polimeriziranja. Nakon toga su pažljivo utapljeni i kromatin, a mikrogelovi uronjeni u pufer za lizu (2.5 M NaCl, 0.1 M Na2EDTA, 10 mM Tris-HCl, 1 % N-lauril sarcosnin, 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 Na2EDTA, 300 mM NaOH; Sigma-Aldrich, MO, SAD; pH > 13), te povrnuti 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) povezanom sa sustavom za analizu slike. Tri testa su težili sa uzorcima između živih stanica (stanice s cjelovitim plazmatskim membranom), apoptotičnih i nekrotičnih stanica. Kako žive stanice (stanice s oštećenom 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 odmjerno 20 µl suspenzije stanica i prebačeno na predmetno mikroskopsko staklo. Na suspenziju stanica pažljivo je pipetirana mješavina fluorescencijskih boja, uzorak je pokriven pokrovnim stakalom i odmah analiziran fluorescencijskim mikroskopom (Olympus BX; povećanje 400 x). Za svaki uzorak učinjena su tri uzastopne testa te je ukupno pregledano 300 stanica po uzorku. Uspoređeno 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, je zgreška im je nakon dovojnog bojenja pod fluorescencijskim mikroskopom zelena. Mrtve nekrotične stanice imaju narančastu crveno obojen kromatin, a apoptotične imaju izrazito zelenu i visoko kondenziranu ili fragmentiranu jezgru.

Quantitative fluorescent test 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 etidium bromide and acridine orange.

A mixture of etidium bromide and acridine orange (Sigma-Aldrich, USA) in final concentrations of 100 µg/ml (1:1; v/v) was gently pipetted 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 etidium 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 agarose (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 Na2EDTA, 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 Na2EDTA, 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 H2O2 for 10 min placed on ice.
Statistička analiza

Procjena statističke značajnosti rezultata dobivenih za preživljivost stanica, apoptozu i nekrozou 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 rasipnjada, podatci su najprije logaritmirani (25), a zatim je primijenjena analiza varijance (ANOVA) uz Tukey post hoc test (α < 0,05).

Rezultati

Mikrohibridni kompozit Z100

Rezultati kvantitativnog flourescencijskog testa za istodobnu identifikaciju apoptotičnih i nekrotičnih limfocita u kulturama inkubiranim 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 kontroli 97,7 ± 0,6 % živih limfocita.

Statistical analysis

Comparisons between the values observed for cell viability, apoptosis, and necrosis were performed by Pearson’s χ²-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, StartSoft, 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 (α < 0,05).

| Table 2. Rezultati kvantitativnog flourescencijskog testa za istodobnu identifikaciju apoptotičnih i nekrotičnih limfocita u uzorcima izloženim 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) iliizravno osvjetljavanje.

| Materijal • Material | Z100 | Žive stanice • Viable cells (%) | Statistički značajno u usporedbi uzorkom • Statistically significant compared to | Apoptoza • Apoptosis (%) | Statistički značajno u usporedbi uzorkom • Statistically significant compared to | Nekroza • Necrosis (%) | Statistički značajno u usporedbi 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 | NC | 1.7±0.6 | - | 0.6±0.6 | - |

Bilješka • Note

Analizirano je 300 stanica po uzorku i svakoj ispitivanoj stavki. Statistički značajnost rezultata procijenjena je χ² 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 polimerizacijom materijalu, zagrijan na T3. 300 cells per sample per each experimental point were analysed. Statistical significance of data was evaluated using χ² 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.
Nanopunjeni kompozit Filtek Supreme Ultra
Rezultati kvantitativnog flourescencijskog testa za istodobnu identifikaciju apoptotičnih i nekrotičnih limfocita u uzorcima inkubiranim kompozitom Filtek Supreme Ultra nalaze se u tablici 3. Nakon 24 sata inkubacije s nepolimeriziranim kompozitom Filtek Supreme Ultra ustanovljeno je 89,7 ± 2,1 % živih limfocita, a u negativnom kontroli previđen je 97,7 ± 0,6 % (P < 0,0001). Citotoxiknosti polimeriziranog materijala, bez obzira na postupak osvjetljavaanja (izravan, preko CAD/CAM CRP-a ili LDC overlay) i temperaturu zagrijavanja, u svim slučajevima je značajno veća od 97,7 ± 0,6 % (P < 0,0001). U učestalosti apoptotičnih limfocita bila je više negoli nekrotičnih limfocita. Apoptoza dominira u usporedbi s nekrozom samo u uzorcima Z100 osvijetljenim izravnim postupkom, bez obzira na temperature prethodnog zagrijavanja uzorka. Porast temperaturu zagrijavanja nije značajno utjecao na učestalost nekroze u testiranim uzorcima.

Zagrijavanje na T1 rezultiralo je najvećem postotkom živih stanica u uzorku koji je osvijetljen direktno preko 2 mm LDC CAD/CAM overlay: 90,3 ± 1,5 %. Nešto niže previđenje limfocita uočeno je nakon osvjetljavaanja preko CRP CAD/CAM overlay (86,7 ± 1,5 %), a najniže je zabilježeno nakon izravnog osvjetljavaanja uzorka (84,7 ± 3,2 %). U učestalosti negativne kontrole i nepolimeriziranog kompozita Filtek Supreme Ultra, uočena je nešto veća učestalost apoprototičnih limfocita u odnosu na nekrotične. Apoptoza predominirova u odnosu prema nekrozii 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 osvijetljenim preko LDC CAD/CAM overlay (4,7 ± 0,6 % limfocita u apoptozi). Porast temperaturu 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.

trolj njihov je udjel iznosio 97,7 ± 0,6 % (p < 0,0001). Citotoxiknosti kompozita Z100 zagrijanog na T1 bila je manja u usporedbi s nepolimeriziranim uzorkom, bez obzira na način polimerizacije (izravni, preko CRP-a ili LDC overlay). 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 osvjetljavaanj; 92,3 ± 2,1 % živih stanica pri osvjetljavaņju preko CRP-a; te 91,7 ± 0,6 % pri osvjetljavaņju preko LDC CAD/CAM overlay (tablica 2.). U negativnoj kontroli i nepolimeriziranom Z100, učestalost apoptotičnih limfocita bila je nešto viša negoli nekrotičnih limfocita. Apoptoza dominira u usporedbi s nekrozom samo u uzorcima Z100 osvijetljenima izravnim postupkom, bez obzira na temperature prethodnog zagrijavanja uzorka. Porast temperaturu zagrijavanja nije značajno utjecao na učestalost nekroze u testiranim uzorcima.

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-curing through 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.
Toksičnost prethodno zagrijanih kompozita

Komet-test u alkalnim uvjetima

Rezultat komet-testa u alkalnim uvjetima, primijenjenog za mjerenje razine primarnog oštećenja DNA-e, prikazani su za parametar dužina repa kometa u tablici 4. Primijenjena su indirektna polymerizacija Z100 i Filtek Supreme Ultra i u negativnoj kontroli; analizirano je 300 stanica po uzorku i svakoj ispitivanoj stavki. Eksponiranost uzoraka od nivoa zraka ili obilježenog snopa elektromagnetnog zraka ne značajno pridonose stvaranju primarnih oštećenja u DNA-i. No, učinak nepolimeriziranog materijala značajno je izraženiji kod Z100 negoli kod Filtek Supreme Ultra.

Uzmimajući u obzir indirektnu polymerizaciju Z100, izmjerene vrijednosti intenziteta repa kometa i statistički značajno odstupale s obzirom na temperaturu zagrijavanja i postupak osvjetljanja (tablica 5.). No, dužina repa kometa značajno je smanjena nakon što je materijal zagrijan na 68 °C (T3). Osobito je ista primjerena za Z100 i Filtek Supreme Ultra u nepolimeriziranom obliku.

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 °C (T2) were used in pre-heating (Table 4).
Za Filtek Supreme Ultra, uočene su značajno niže vrijednosti dužine repa kometa pri zagrijavanju materijala na 68 °C (T3) i osvjetljanju preko LDC CAD/CAM barijere u usporedbi s ostalim postupcima (tablica 4.). No izmjerene vrijednosti intenziteta repa nisu pokazale statistički značajnosti.

**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)

| Materijal • Material | Polimerizacijski postupak • Polymerization procedure | Zagrijavanje • Preheating | Dužina repa • Tail length / µm | Statistički značajno u usporedbi s uzorkom • Statistically significant compared to to |
|----------------------|-----------------------------------------------|---------------------------|-------------------------------|-------------------------------------------------|
| Nepolimerizirani • Unpolymerized | Polimerizacija preko CRP CAD/CAM overlay • Polymerization through CRP CAD/CAM overlay T1 | 28.03 25.21 6.43 | NC, Z100 |
| | T2 | 26.38 24.79 5.71 | NC, Z100 |
| | T3 | 27.92 26.46 6.69 | NC, Z100 |
| | Polimerizacija preko LDC CAD/CAM overlay • Polymerization through LDC CAD/CAM overlay T1 | 28.57 27.08 4.09 | NC, Z100 |
| | T2 | 26.80 25.42 4.09 | NC, Z100 |
| | T3 | 25.13 23.33 5.96 | NC, Z100 |
| | Direktna polimerizacija • Directly polymerized T1 | 26.84 25.42 7.43 | NC |
| | T2 | 26.12 24.38 6.31 | NC, Z100 |
| | T3 | 30.17 28.33 6.31 | NC, T1, T2, CoT3, CeT2, CeT3 |
| | Negativna kontrola • Negative control | 22.66 22.08 3.28 | |
| | Pozitivna kontrola 1mM H2O2, 10 min • Positive control 1mM H2O2, 10 min | 52.29 51.30 11.83 | NC |

**Z100**

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 u obziru 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

Co Tx - statistički značajno u usporedbi s rezultatima za polimerizaciju preko CRP CAD/CAM overlay 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

Ce Tx - statistički značajno s obzirom na rezultate polimerizacije preko LDC CAD/CAM overlay 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
Tablica 5. Rezultati komet-testa u alkalnim uvjetima za procjenu genotoksičnosti kompozitnih materijala s obzirom na temperaturu koristenu za zagrijavanje materijala i vrstu polimerizacije; primarno oštećenje DNA-e izraženo je kroz parametar intenzitet repa kometa, koji odgovara postotku genomskih DNA-e koja je migrirala u rep kometa

| Materijal | Polimerizacijski postupak | Zagrijavanje | Intenzitet repa (% DNA u repu kometa) | Statistički značajno u usporedbi s uzorkom (S.D.) |
|-----------|--------------------------|-------------|--------------------------------------|-----------------------------------------------|
|           |                          | Preheating  | Tail intensity (tail % DNA)          |                                               |
|           |                          |             | Mean       | Median       |                                               |
| Z100      | Nepolimerizirani         | /           | 6.04       | 4.55         | 5.80 NC                                      |
|           | Unpolymerized            |             |            |              |                                               |
|           | Polimerizacija preko CRP CAM overlay | T1 | 3.47       | 0.89         | 5.76 NC, Un                                  |
|           | Polimerizacija preko CRP CAD/CAM overlay | T2 | 1.86       | 0.36         | 3.13 Un                                      |
|           | Polimerizacija preko LDC CAM overlay | T3 | 1.96       | 0.48         | 2.77 Un                                      |
|           | Polimerizacija preko LDC CAD/CAM overlay | T1 | 3.01       | 0.97         | 4.35 NC, Un                                  |
|           | Polimerizacija preko LDC CAD/CAM overlay | T2 | 2.70       | 0.82         | 3.92 NC, Un                                  |
|           | Polimerizacija preko LDC CAD/CAM overlay | T3 | 2.57       | 0.68         | 3.29 NC, Un                                  |
| Zilek Supreme Ultra | Nepolimerizirani | /           | 2.51       | 0.79         | 3.43 NC, Z100                               |
|           | Unpolymerized            |             |            |              |                                               |
|           | Polimerizacija preko CRP CAM overlay | T1 | 1.94       | 0.63         | 3.25                                         |
|           | Polimerizacija preko CRP CAD/CAM overlay | T2 | 2.55       | 0.62         | 3.92 NC                                      |
|           | Polimerizacija preko LDC CAD/CAM overlay | T3 | 3.06       | 0.70         | 4.28 NC                                      |
|           | Polimerizacija preko LDC CAD/CAM overlay | T1 | 2.83       | 1.81         | 2.93 NC                                      |
|           | Polimerizacija preko LDC CAD/CAM overlay | T2 | 2.35       | 0.99         | 2.95 NC                                      |
|           | Polimerizacija preko LDC CAD/CAM overlay | T3 | 1.85       | 0.13         | 3.56                                         |
|           | Direktna polimerizacija | T1 | 1.84       | 0.13         | 5.15                                         |
|           | Directly polymerized      | T2 | 2.49       | 0.74         | 4.02 NC                                      |
|           | Direktna polimerizacija | T3 | 3.94       | 2.38         | 4.21 NC, T1,T2,CoT2,CoT3,CeT3               |
| Negativna kontrola | Negative control | 1.15       | 0.06       | 2.91                                           |
| Pozitivna kontrola | 1mM H₂O₂ 10 min | 32.65       | 30.15       | 20.67 NC                                      |

DNA damage measured as tail length, the one measured in terms of % of DNA that migrated into the comet tail did 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 oslikavala je kliničke uvjete jer je inkubacija staničnih kultura s kompozitnim uzorcima provedena neposredno nakon pripreme uzorka. Maksimalno vrijeme osvjetljavanja koje je preporučljivo proizvođač za program visokog intenziteta LED uređaja rabiljenog u eksperimentu (Bluephase G2) jest 15 sekunda, no u ovoj studiji vrijeme polimerizacije iznosilo je 40s. Razlog tomu je što se tijekom adhezijskog cementiranja CAD/CAM restauracija svjetlo loži i raspušta prelazeci 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 citotoksičnosti 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 limfocitinu 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 smešten u svježu kulturu stanica, zagrijani uzorak kompozitnog materijala može uzrokovati slabije preživljavanje stanica. Razlog za to nisu komponente kompozitnog materijala koje nisu reagirale nego temperatura uzorka. Naše prijašnje studije pokazale su da je ključan porast obaju parametara komet-testa.

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 zagrijan do 60 °C i izvan iz uredaja za zagrijavanje, pokazuju pad temperature kompozitnog materijala od 35 do 40 % nakon 40 sekunda, do 50 % nakon 2 minute te do 90 % nakon 5 minuta. No, aplikacije topline može utjecati na intramolekularnu kemijsku vezu. Tijekom indirektnih postupaka osvjetljavanja rezultati dobiveni za zagrijavanje na temperatura od 54 °C (T2), koju imaće 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 monomerne 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 °C. Danroch et al. (29) concluded that when composite material is heated up to 60 °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 °C, which is recommended by composite manufacturers, did not differ compared to the results obtained with pre-heating at T3 - 68 °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 na-
šoj studiji korištena Mylar folija kako bi se spriječilo stvaranje
slota atmosferskog kisika na površini uzorka te tako spriječilo
stvaranje nepolimerizirane površine sloja kompozitnog mate-
rijala s posljednjim citotoksičnim učinkom.
Darmani i suradnici (32) proučavali su citotoksičnost razli-
čitih kompozitnih materijala i ustanovili pad preživljenja sta-
nica do 48 % u slučaju njihova izlaganja kompozitnom ma-
terijalu Z100. U našoj studiji, smanjenje preživljenja stanica
nakan kontaktu sa Z100, nije bilo toliko izraženo kao u spo-
menutoj studiji (tablice 2. i 3.). Statistička usporedbna rezulta-
ta citotoksičnosti između Z100 i Filtek Supreme Ultra pokaz-
ala je da nakon polimerizacije obaju kompozitnih materijala
preko CRP CAD/CAM overleja debljine 2 mm i pri tempe-
raturama T1 i T3, bolje rezultate (veće preživljenje limfocita)
pokazuje kompozit Z100. No nakon zagrijavanja na tempe-
raturi 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 kompo-
zita Z100 bila je nešto niža ili jednaka citotoksičnosti kompo-
zita Filtek Supreme Ultra, ali bez statistički 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 direktno polimerizacije i polimerizacije preko CRP
CAD/CAM overleja.
Uzimajući u obzir oba parametra komet-testa, duljinu
repa i intenzitet repa, nepolimerizirani Z100 pokazao je ve-
lik potencijal za izazivanje primarnih oštećenja DNA-e u us-
poredbi s kompozitnim materijalom Filtek Supreme Ultra. Uzrok za to mogao bi se povezati sa sastavom tih dvaju mate-
rijala. 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
monomeri iz nepolimeriziranog Z100 i njihov toksični uči-
nak spominju se u nekoliko studija (32 – 34). Darmani i sur-
adnici (32) navode otpuštanje velike količina TEGDMA-e i
manje količine Bis-GMA-e iz testiranih kompozita, ukluču-
jujući 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 za-
štitnom funkcijom i tako uništiti stanice. Ustanovljeno je da
Filtek Supreme Ultra sadržava znatnu količinu cirkonjevih
čestica, a dokazano je da taj element ima značajnu antioksid-
aativnu 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 dogada
u prva 24 sata nakon što je restaurativni materijal postav-
ljen u kavitet (38). Dobiveni podatci o razinama primarnih
oštećenja genoma pokazuju da bi se CRP CAD/CAM mog-
gao smatrati bolijim materijalom s obzirom na to da su i du-
žina i intenzitet repa komet bili manji u usporedbi s vrijed-
nostima dobivenima pri osvjetljavanju preko LDC overleja.
Postotak DNA-e u repu kometa općenito se smatra točnijim
bimerkom 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 viabili-
ties up to 48 % when the cells were exposed to Z100 com-
posite material. In our study viability was not affected to such
extent (Table 2,3). Statistical comparison of cytotoxicity re-
results between Z100 and Filtek Supreme Ultra showed that af-
ter 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. How-
ever, after preheating at temperature T2, Filtek Supreme Ul-
tra had lower cytotoxicity (P=0.0228). Also, after light-cur-
ing 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, howev-
ner none of the differences was statistically significant. In gen-
eral, 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 unpolymorized form of Z100 exhibited
higher potency of inducing primary damage to DNA com-
pared to Filtek Supreme Ultra. The observed effect is mediat-
ed by a significant difference in the composition of two evalu-
ated composite materials. Z100 is TEGDMA and Bis-GMA
based material, while Filtek Supreme Ultra besides TEGD-
MA 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 TEGD-
MA and comparatively smaller amounts of Bis-GMA were
released from tested composite materials. As Engelmann et
(35) explains, TEGDMA forms adducts with glutathione
(which has protective functions to the cells) and may inter-
ference with its protective function leading to the cell destruc-
In our 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 indicat-
ed 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 polymer-
ization 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 ge-
tomic DNA affected by the adverse biological effects of the
substance (39,40). The obtained values for that parameter in-
dicates that there is no effect on the primary damage to DNA
if preheating is performed at 54 °C (T2) or 68 °C (T3) (Ta-
bles 4,5). Although there is no statistically significant differ-
Toxicity of Pre-heated Composite

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Zaključak

Za kompozitni materijal Z100, najveći postotak živih stanica zabilježen je nakon izravnog osvjetljavanja, dok se za materijal Filtek Supreme Ultra najveći postotak živih stanica zabilježen je nakon zagrijavanja preko CRP-a. Razlike 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. Ovo može uzrokovati manju transmisiju svjetla i time manje polimerizirani uzorak, što pokazuje preživljenje stanica.

Pri izravnoj polymerizaciji 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 vjerojatno jedini pokazatelj razine primarnog oštećenja DNA-e negoli dužina repa i B) da se do 10 % DNA-e u repu smatra prihvatljivom razono oštećenja bez značajnijeg narušavanja cjelovitosti genom (25, 42), može se pretpostaviti da u uvjetima korištenim u ovom istraživanju, zadnjih šest sati izjavnih materijala ne bi bilo statistički značajnijeg rizika primarnog oštećenja dB. Izvršena istraživanja pokazuju više negoli smjernice za očuvanje uzorka u pozadini i genotoksičnost. Prema tome, promjene u razini primarnog oštećenja DNA-e nakon izlaganja limfocita kompozitnim materijalima, bez obzira na to što su neki rezultati pokazali statistički značajne razlike, može da bih biološki relevantne. Važnost prikazanih rezultata odlučuje se u pogledu izvršenja ispitivanja kompozitnih materijala u različitim uvjetima izravnog osvjetljavanja. Za oba testirana kompozitna materijalna, prva je hipoteza prihvaćena, a druga je odbaćena. Rezultati dobiveni na staničnim kulturama ne mogu se izravno primijeniti za objašnjenja mogućih scenarija u uvjetima in vivo. No dobiveni rezultati su u skladu sa očekivanim rezultatima i pokazuju trošak energije, čak i u smislu intenziteta repa.

Za materijal Z100 najveći postotak živih stanica zabilježen je nakon osvjetljavanja na T1 za CRP, LDC te za izravno osvjetljavanje uzoraka. Za kompozit Filtek Supreme Ultra, najveći postotak živih stanica zabilježen je nakon osvjetljavanja na T2 za CRP te nakon izravnog osvjetljavanja na T1/T3 za LDC. U negativnoj kontroli i nepolimeriziranim uzorcima obaju kompozitnih materijala –Z100 i Filtek Supreme Ultra–, even considering tail intensity parameter CRP-through polymerization may be preferable procedure (Table 5). The difference in results when polymerized through CRP or LDC CAD/CAM overlay may be explained with different material composition. Illie and Hickel (41) showed in their study that lithium disilicate glass-ceramic due to its 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 57 °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 than 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 cytotoxicity 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 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 necrotic lymphocytes. For
Toksičnost prethodno zagrijanih kompozita

Knežević i sur.

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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 culture 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.

Sukob interesa

Nije ga bilo

References

1. Durner J, Walther U, Zaspel J, Hickel R, Reichl FX. Metabolism of TEGDMA and HEMA in human cells. Biomaterials. 2010 Feb;31(5):818-23.
2. Schweikl H, Hiller KA, Bolay C, Kreismann W, Nusser A, et al. Cytoxic and mutagenic effects of dental composite materials. Biomaterials. 2005 May;26(14):1713-9.
3. Ferracane JL, Condon JR. Rate of elution of leachable components from composites. Dent Mater. 1990 Oct;6(4):282-7.
4. Wattha JC, Rueggeberg FA, Lapp CA, Lewis JB, Lockwood PE, Ergle JW, et al. In vitro cytotoxicity of resin-containing restorative materials after aging in artificial saliva. Clin Oral Investig. 1999 Sep;3(3):144-9.
5. Moon HJ, Lee YK, Lim BS, Kim CW. Effects of various light curing methods on the leachability of uncurable substances and hardness of a composite resin. J Oral Rehabil. 2004 Mar;31(3):258-64.
6. Shehata M, Durner J, Eldzen A, Van Landuyt K, Styliou P, Rothmund L, et al. Cytotoxicity and induction of DNA double-strand breaks by components leached from dental composites in primary human gingival fibroblasts. Dent Mater. 2013 Sep;29(9):971-9.
7. Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. J Dent Res. 2006 Oct;85(10):870-7.
8. Ruiz-De-Castaneda E, Gaton-Hernandez P, Rodriguez EG, Silva RAB, Nelson-Filho P, Silva LAB. Pulpal and periapical response after restoration of deep cavities in dogs’ teeth with Filtek Silorane and Filtek Supreme XT system. Oper Dent. 2013 Jan-Feb;38(1):73-81.
9. Gregson KS, Terrence O’Neill, Platt JA, Windsor JL. In vitro induction of hydrolytic activity in human gingival and pulp fibroblasts by triethylene glycol dimethacrylate and monocyte chemotactic protein-1. Dent Mater. 2008 Nov;24(11):1461-7.
10. Tabatabaee MH, Mahmavi H, Zandi S, Kharrazi MJ. HPLC analysis of eluted monomers from two composite resins cured with LED and halogen curing lights. J Biomed Mater Res B Appl Biomater. 2009 Jan;88(1):191-6.
11. Imazato S, McCabe JF, Tarumi H, Ehara A, Ebisu S. Degree of conversion of composites measured by DTA and FTIR. Dent Mater. 2001 Mar;17(0):178-83.
12. Par M, Gamulin O, Marovic D, Klaric E, Tarle Z. Effect of temperature on post-cure polymerization of bulk-fill composites. J Dent. 2014 Oct;42(10):1255-60.
13. Par M, Lapas-Barisic M, Gamuli O, Panduric V, Spanovic N, Tarle Z. Long term degree of conversion of two bulk-fill composites. Acta Stomatol Croat. 2016 Dec;50(4):292-300.
14. Moon HJ, Lee YK, Lim BS, Kim CW. Effect of various light curing modes on the leachability of cured substances and hardness of a composite resin. J Oral Rehabil. 2004 Mar;31(3):258-64.

15. Yap AU, Seneviratne C. Influence of light energy density on effectiveness of compositecure. Oper Dent. 2001 Sep-Oct;26(5):460-6.

16. Daronch M, Rueggeberg FA, De Goes MF. Monomer conversion of pre-heated composite. J Dent Res. 2005 Jul;84(7):663-7.

17. Daronch M, Rueggeberg FA, De Goes MF, Giudici R. Polymerization kinetics of pre-heated composite. J Dent Res. 2006 Jan;85(1):38-43.

18. Frankenberger R, Hartmann VE, Krech M, Kramer N, Reich S, Braun A et al. Adhesive luting of new CAD/CAM materials. Int J Comput Dent. 2015;18(1):9-20.

19. Lohbauer U, Pelka M, Bell R, Schmitt J, Mockler E, Jandt KD et al. Degree of conversion of luting resins around ceramic inlays in natural deep cavities: A Micro-Raman spectroscopy analysis. Oper Dent. 2010 Sep-Oct;35(5):579-86.

20. Kramer N, Lohbauer U, Frankenberger R. Adhesive luting of indirect restorations. Am J Dent. 2000 Nov;13(Spec No):60D-70D.

21. Froes-Salgado NR, Silva LM, Kawano Y, Franci C, Reis A, Lougerrcio AD. Composite pre-heating: Effects of marginal adapta tion, degree of conversion and mechanical properties. Dent Mater. 2010 Sep;26(9):908-14.

22. Lohbauer U, Zinelis S, Rahiotis S, Petschelt A, Eliades G. The effect of resin composite pre-heating of monomer conversion and polymerization shrinkage. Dent Mater. 2009 Apr;25(4):514-9.

23. Kopjar N, Zeljezic D, Lucic Vrdoljak A, Radic B, Ramic S, Milic M, et al. Irinotecan toxicity to human blood cells in vitro — relationships between various biomarkers. Basic Clin Pharmacol Toxicol. 2007;100(6):403-413.

24. Duke, RC; Cohen, JJ. Morphological and biochemical assays of apoptosis. In: Current Protocols in Immunology. Green/Wiley: New York; 1992.

25. Lovell DP, Omori T. Statistical issues in the use of the comet assay. In: Lovell DP, Omori T. Statistical issues in the use of the comet assay. Mutagenesis. 2008;23(3):171-182.

26. Knezevic A, Zeljezic D, Kopjar N, Tarle Z. Influence of curing mode intensities on cell culture cytotoxicity/genotoxicity. Am J Dent. 2009 Feb;22(1):43-8.

27. Knezevic A, Zeljezic D, Kopjar N, Tarle Z. Cytotoxicity of composite materials polymerized with LED curing units. Oper Dent. 2008 Jan-Feb;33(1):23-30.

28. Spanovic N, Par M, Skendrovic H, Bjelovucic R, Pskalo K, Tarle Z. Real-time temperature monitoring during light-curing of experimental composites. Acta Stomatol Croat. 2018;52(2):87-96.

29. Daronch M, Rueggeberg FA, Moss L, De Goes MF. Clinically relevant issues related to pre-heating composites. J Esthet Restor Dent. 2006;18(6):340-50; discussion 351.

30. Farracane JL. Hygrosopic and hydrolytic effects in dental polymer networks. Dent Mater. 2006 Mar;22(3):211-22.

31. Schmalz G. The biocompatibility of nonamalgam dental filling materials. Eur J Oral Sci. 1998 Apr;106(2 Pt 2):696-706.

32. Darmani H, Al-Hiyasat AS, Milhem MM. Cytotoxicity of dental composites and their leached components. Quintessence Int. 2007 Oct;38(9):789-95.

33. Lee SY, Huang HM, Lin CY, Shih YH. Leached components from dental composites in oral simulating fluids and the resultant com- posite strengths. J Oral Rehabil. 1998 Aug;25(8):575-88.

34. Milhem MM, Al-Hiyasat AS, Darmani H. Toxicity testing of restorative dental materials using brine shrimp larvae (Artemia salina.). J Appl Oral Sci. 2008 Jul-Aug;16(4):297-301.

35. Engelman J, Leyhausen G, Leibfried D, Geurtsen W. Effect of TEGDMA on the intracellular glutathione concentration of human gingival fibroblasts. J Biomed Mater Res. 2002;63(6):746-51.

36. Karunakaran G, Suriyaprabha R, Manivasakan P, Yuvakkumar R, Rajendran V, Kannan N. Screening of in vitro cytotoxicity, anti- oxidant potential and bioactivity of nano- and micro-ZrO2 and -TiO2 particles. Ecotoxicol Environ Saf. 2013 Jul;93:191-7.

37. Craig, RG. Polymers and polymerization. In: Robert, G; Powers, M — editors. Restorative Dental Materials. 11Ed.St. Louis; Mosby: 2002. p.185-198.

38. Farracane JL. Elution of leachable components from composites. J Oral Rehabil. 1994 Jul;21(4):441-52.

39. Hartman A, Agurell E, Beevers C, Breindler-Schwaab S, Burlinson B, Clay P et al. Recommendations for conducting the in vivo alka line Comet assay. Mutagenesis. 2003 Jan;18(1):45-51.

40. Kumaravel TS, Jha AN. Reliable comet assay measurements for detecting DNA damage induced by ionising radiation and chemi cals. Mutat Res. 2006 Jun 16;605(1-2):7-16.

41. Ille N, Hickell R. Correlation between ceramics translucency and polymerization efficiency through ceramics. Dent Mater. 2008 Jul;24(7):908-14.

42. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol. 2004 Mar;26(3):249-61.