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IZVORNI ZNANSTVENI RAD
ORIGINAL SCIENTIFIC PAPER

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In vitro kompatibilnost prethodno zagrijanoga giomera i mikropunjenoga hibridnog kompozita

In Vitro Biocompatibility of Preheated Giomer and Microfilled-Hybrid Composite

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Sažetak

Svrha: Svrha rada bila je ispitati citotoksičnost dvaju svjetlosno stvrdnjavajućih kompozitnih materijala nakon zagrijavanja na različitim temperaturama te osvjetljivanja izravno i preko CAD/CAM overleja. **Materijali i postupci:** Kompozitni materijali (Gradia Direct Posterior i Beautifill II) zagrijani su u uređaju za zagrijavanje Calset na tri različite temperature (T1: 37 °C, T2: 54 °C, T3: 68 °C). Zatim je mala količina postavljena na okrugli kalup (promjera 6 mm; debljine 0,65 mm), prekrivena Mylar folijom, te sprešana i polimerizirana LED uređajem Bluephase. Jedna skupina uzoraka polimerizirana je izravno, a ostale preko CAD/CAM polimera pojačanog keramikom (CRP) i CAD/CAM litijeva disilikatnog keramičkog onleja debljine 2 mm u trajanju 20 i 40 sekunda. Polimerizirani uzorci postavljeni su odmah nakon osvjetljivanja u staničnu kulturu limfocita izoliranih iz periferne krvi. Preživljenje limfocita određivalo se postupkom dvojnog bojenja etidijevim bromidom i akridinskom narančastom bojom. Kvantitativna procjena učinjena je određivanjem postotka živih, apoptotičnih i mrtvih stanica. Za statističku analizu korišten je Pearsonov hi-kvadrat test. **Rezultati:** Tijekom 20-sekundne polimerizacije najveći broj živih stanica zabilježen je pri zagrijavanju materijala na 37 °C (T1), a za polimerizaciju od 40 sekunda najveći broj živih stanica zabilježen je pri zagrijavanju materijala na 54 °C (T2). Uzorci polimerizirani preko CAD/CAM overleja pokazali su manju citotoksičnost negoli oni izravno polimerizirani. **Zaključak:** Osim sastavom kompozitnog materijala, citotoksičnost je uvjetovana načinom polimerizacije i vremenom osvjetljivanja te temperaturom prethodnog zagrijavanja kompozitnog materijala.

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Ključne riječi
citotoksičnost, prethodno zagrijavanje kompozita, temperatura, kompozitni materijal

Uvod

Kompozitni materijali uspješno se koriste kao stomatološki restaurativni materijal već godinama zbog svojih mehaničkih i estetskih svojstava. U svakodnevnoj kliničkoj praksi nisu samo materijal za nadomjestak izgubljenoga tvrdog zubnog tkiva nego i svojevrsna podloga te sredstvo za adhezijsko cementiranje inleja, onleja, krunica, ljuskica te ortodontskih bravica (1).

Pojavom CAD/CAM restauracija, upotreba kompozitnih materijala kao sredstva za adhezijsko cementiranje postaje sve popularnija. Besek i suradnici (2) uveli su postupak upotrebe kompozitnih materijala kao sredstva za adhezijsko cementiranje sa svrhom preveniranja mikropukotine, postoperativne preosjetljivosti, rekurentnog karijesa te općenito za poboljšanje estetskog izgleda cementirane restauracije. Pri korištenju kompozitnog materijala kao sredstva za adhezijsko cementi-

Introduction

Composite materials have been successfully used for many years as dental restorative materials due to their mechanical and excellent esthetic properties. They are used in everyday clinical practice not only as restorative materials but also as liners or as luting agents for cementation of inlays, onlays, crowns, veneers, and orthodontic brackets (1). With CAD/CAM appearance on the market, the use of composite materials as a luting material becomes more popular. Besek *et al.* (2) first introduced the use of composite materials for CAD/CAM bonding at the time when CAD/CAM system was not so accurate. Due to mechanical properties of composite materials and extended curing time Besek *et al.* (2) introduced composite materials as a luting agent with the aim to prevent microleakage, postoperative sensitivity, recurrent caries and to improve overall esthetic appearance of a cemented restora-

ranje, snaga vezanja određena je postizanjem odgovarajućeg stupnja polimerizacije smolastog materijala. Daronch i suradnici (3) utvrdili su da porast temperature kompozitnog materijala do 60 °C može poboljšati stupanj konverzije na površini kompozitnog materijala te na dubini od 2 mm. Prethodno zagrijavanje kompozitnog materijala u izotermnim uvjetima omogućuje porast konverzije monomera, što za posljedicu ima smanjenje slobodnog volumena unutar polimerne mreže i smanjenje degradacije materijala, a samim time i smanjenje toksičnosti polimeriziranog materijala (3 – 7). No u nekim studijama istaknuto je da prethodno zagrijavanje kompozitnog materijala može negativno utjecati na rubove kompozitne restauracije zbog porasta polimerizacijskog skupljanja u zagrijanom kompozitnom materijalu (7). Suprotno tomu, neka su ispitivanja pokazala da prethodno zagrijavanje kompozitnih materijala prije izlaganja svjetlu za polimerizaciju ne utječe na promjenu mehaničkih svojstava ili konverziju monomera, ali je utvrđena bolja prilagodba kompozitnog materijala na stijenke kaviteta (4). Deb i suradnici (8) u svojem su radu pokazali da prethodno zagrijani kompozitni materijal uzrokuje veće polimerizacijsko skupljanje negoli onaj primijenjen u standardnim uvjetima, tj. nezagrijan, ali je zabilježeno još manje skupljanje negoli za tekuće kompozitne materijale. Isti su autori pokazali da nema razlike između citotoksičnosti zagrijanih i nezagrijanih kompozitnih materijala.

Komponente oslobođene iz nedostatno polimeriziranog kompozitnog materijala, bez obzira na to je li se radilo o prethodno zagrijanom ili nezagrijanom kompozitnom materijalu, mogu uzrokovati toksični ili upalni odgovor tkiva, ovisno o njihovoj agresivnosti te o debljini zaostatnog dentina na dnu prepariranog kaviteta (9, 10). Zato je svrha ovoga rada bila odrediti i usporediti citotoksičnost mikrohibridnog kompozitnog materijala (Gradia Direct Posterior) i giomer kompozitnog materijala (Beautifill II) nakon zagrijavanja materijala na različitim temperaturama te osvjetljivanju izravno ili preko CAD/CAM overleja 20 i 40 sekunda. Kao modelni sustav korišteni su izolirani limfociti periferne krvi. U svrhu ispitivanja postavljene su sljedeće hipoteze:

1. materijal zagrijan na nižoj temperaturi uzrokovat će manju citotoksičnost bez obzira na vrijeme osvjetljivanja i način polimerizacije (izravna polimerizacija ili polimerizacija preko CAM/CAM overleja)
2. dulje osvjetljivanje uzrokovat će manju citotoksičnost bez obzira na temperature zagrijavanja i postupak polimerizacije (izravna polimerizacija ili polimerizacija preko CAM/CAM overleja)
3. izravno polimeriziranje uzoraka izazvat će manju citotoksičnost negoli uzorci polimerizirani preko CAD/CAM overleja, bez obzira na temperature zagrijavanja.

Materijali i postupci

Priprema CAD/CAM keramički ojačanog polimera (CRP) i CAD/CAM litijeva disilikatno keramičkog (LDC) onleja, giomera i kompozitnog materijala

Za izradu overleja korištena su dva bloka CRP CAD/CAM-a (boja A2, veličina bloka 14L, 3M ESPE, LAVA Ultimate, St. Paul, MN, SAD), te LDC CAD/CAM (boja A2,

tion. When using composite materials as a luting agent, the bond strength is determined by achieving appropriate resin polymerization. Daronch *et al.* (3) reported in their study that increasing composite temperature up to 60° C might enhance the degree of conversion on the top of the composite material and in 2 mm of the bottom surfaces as well. Preheating of a composite material under an isothermal condition allows increasing monomer conversion which as a consequence has reduction of the free volume within polymer network, solvent uptake and material degradation, which can lead to less cytotoxicity of the polymerized material (3 – 7). However, some studies reported that pre-heating of a composite material may cause negative effects on the composite restoration margins due to the increased polymerization shrinkage in heated composite resin (7). On the contrary, some studies showed that pre-heating of a composite material before light-curing did not alter mechanical properties and monomer conversion but did provide enhanced adaptation of composite materials to the cavity walls (4). Deb *et al.* (8) confirmed in their study that a pre-heated composite material produced more shrinkage than non-heated composite, but still less than flowable composite materials. The same authors also concluded that there is no difference between cytotoxicity of heated and non-heated composite materials. Reactive components released from unpolymerized or underpolymerized composite resins may induce toxicity or inflammatory tissue responses depending on their aggressiveness and the thickness of the remaining dentin on the cavity floor (9, 10).

The aim of the present investigation has been to evaluate and compare cytotoxic potencies of one micro hybrid composite material (Gradia Direct Posterior) and one giomer composite material (Beautifill II) after heating at different temperatures and cured directly and through CAD/CAM overlay for 20 and 40 seconds. Isolated human peripheral lymphocytes were used as a model system. For the purpose of the study following null hypotheses were established:

1. Materials heated on lower temperature will cause less cytotoxicity regardless of the curing time and polymerization pattern (directly polymerized or through CAD/CAM overlay)
2. Longer curing time will cause less cytotoxicity regardless of the curing time and polymerization pattern (directly polymerized or through CAD/CAM overlay)
3. Samples polymerized directly will show less cytotoxicity than the samples polymerized through CAD/CAM overlay regardless of the temperature used.

Materials and methods

Preparation of CAD/CAM ceramic-reinforced polymer (CRP), CAD/CAM lithium disilicate ceramic (LDC), giomer and composite samples

Two blocks of the CRP CAD/CAM (shade A2, block size 14L, 3M ESPE, LAVA Ultimate, St. Paul, MN, USA), and LDC CAD/CAM (shade A2, block size 14L, e.max, Ivoclar

veličina bloka 14L, e.max, Ivoclar Vivadent, Amherst, NY, SAD). CRP i blokovi LDC CAD/CAM-a izrezani su dijamantnom pilom na uzorke debljine 2 mm. Uzorci su zatim polirani pod vodenim hlađenjem s obje strane u uređaju za poliranje (11). Svaki LDC CAD/CAM uzorak poprskan je sprejom za glazuru (Crystall/Glaze Spray; Ivoclar/Vivadent, Schaan, Liechtenstein) te stavljen u uređaj Pro 100 (Whip-Mix; Louisville, KY) prema uputama proizvođača.

Beautifill II (SHOFU Dental GmbH; Ratingen, Germany) jest nanohibridni kompozitni materijal koji je uvršten u skupinu giomera. Prema podatcima proizvođača, sadržava tzv. površinske čestice stakla koje su već reagirale i mogu otpuštati fluoridne, natrijeve, stroncijeve, aluminijske, silikatne i boratne ione, te bisfenol glicidil dimetakrilat (Bis-GMA) i trietilen glikol dimetakrilat (TEGDMA).

Gradia Direct Posterior (GC, Europe N.V.; Leuven, Belgium) mikropunjeni je hibridni kompozitni materijal. Prema podatcima proizvođača sadržava mikrofine prepolimerizirane čestice punila (silika 19 % težinskog udjela, prosječna veličina čestica 0,85 µm; prepolimerizirano punilo 20 % težinskog udjela). Organski matriks mješavina je uretan-dimetakrilata (UDMA) i dimetakrilatnih komonomera (23 % težinskog udjela). Zbog radiopaktnosti dodano je fluoro-aluminosilikatno staklo (38 % težinskog udjela).

Za zagrijavanje kompozitnih materijala korišten je uređaj Calaset (AdDent INc., Danbury, Connecticut, SAD). Proizvođač preporučuje zagrijavanje kompozita na temperaturu od 68 °C. U ovom radu korištene su tri različite temperature – T1: 37 °C, T2: 54 °C i T3: 68 °C. Ampule kompozitnog materijala stavljaju se u kućište s poklopcem te je potrebno otprilike 10 minuta da se kompozit zagrije na željenu temperaturu.

Uzorci za ispitivanje citotoksičnosti pripremljeni su na sljedeći način: mala količina kompozitnog materijala, prije toga zagrijana na T1, T2 ili T3, stavljena je u kalup u obliku prstena promjera 6 mm i debljine 0,65 mm. Kalup je zatim stavljen na okruglu pločicu od plemenitog čelika debljine 5 mm prekrivenu Mylar folijom. Nakon toga ispunjen je kompozitnim materijalom te prekriven Mylar folijom. Zatim je uzorak pritisnut drugom pločicom od plemenitog čelika istih dimenzija kao i prva pločica kako bi se dobio homogeni uzorak iste debljine kao i kalup (0,65 mm) (11). Mylar folija korištena je između ostalog i da bi se spriječilo nastajanje sloja inhibiranog kisikom na površini polimeriziranoga kompozitnog materijala. Nakon što je uzorak kompozitnog materijala sprešan, polimeriziran je uređajem za polimerizaciju Bluephase (Vivadent, Schaan, Lichtenstein) upotrebom programa visokog intenziteta (1180 mW/cm^2) u trajanju 20 i 40 sekunda. Pripremljeno je po sedam uzoraka za svaku ispitivanu skupinu. Korištena su tri načina polimerizacije uzoraka kompozitnog materijala:

(1) izravna polimerizacija preko Mylar folije
(2) polimerizacija preko Mylar folije iznad koje je postavljen CAD/CAM CRP overlej debljine 2 mm
(3) polimerizacija preko Mylar folije iznad koje je postavljen CAD/CAM LDC overlej debljine 2 mm.

Tako pripremljeni uzorci nakon polimerizacije i uklanjanja Mylar folije uronjeni su izravno u staničnu kulturu limfocita.

Vivadent, Amherst, NY, USA) were used for preparing overlays for polymerization of composite materials.

CRP and LDC CAD/CAM blocks were cut using a low speed diamond saw in 2 mm thick slices. Thereafter, the samples were polished under water-cooling on both sides with a polishing machine (11). For LDC CAD/CAM samples, each sample was coated with glaze (Crystall/Glaze Spray; Ivoclar/Vivadent, Schaan, Liechtenstein) and placed in in the oven Pro 100 (Whip-Mix; Louisville, KY) according to the manufacturer's instructions.

Beautifill II (SHOFU Dental GmbH; Ratingen, Germany) is a nano-hybrid composite which belongs to the Giomer group. According to the manufacturer's data, it contains Surface Pre-Reacted Glass (S-PRG) filler particles which are capable of releasing fluoride, sodium, strontium, aluminum, silicate and borate ions, bisphenol A-diglycidyl dimethacrylate (Bis-GMA) and tryethyleneglycol dimethacrylate (TEGDMA).

Gradia Direct Posterior (GC, Europe N.V.; Leuven, Belgium) is a micro-filled hybrid resin composite. According to the manufacturer's data, it contains microfine pre-polymerized resin fillers (silica 19 wt % average particle size 0.85 µm; pre-polymerized filler 20 wt %). The matrix consists of a mixture of urethane dimethacrylate (UDMA) and dimethacrylate co-monomers (23 wt %). Fluoro-Alumino-Silicate glass is added to the posterior formulation to obtain radiopacity (38 wt %).

The Calset Composit Warmer (AdDent INc., Danbury, Connecticut, USA) was used for heating of tested composite materials. Manufacturer suggested temperature for warming composite materials in syringe is 68 °C. In this experiment three temperature levels were used: temperature T1: 37 °C, T2: 54 °C and T3: 68 °C. A tray placed on the top of the heating unit contains slots to place the composite compules. The desired temperature is reached in approximately 10 minutes and the composite is ready to use.

The samples for cytotoxicity testing were prepared as follows: small amount of tested material, pre-heated at T1, T2 or T3, was placed in a mold of 6 mm in diameter and 0.65 mm in thickness. The mold was positioned on stainless steel round 5 mm thick disc, which was covered with Mylar sheet. The mold was then filled with the composite material. The sample was covered with another Mylar sheet and pressed with another stainless steel round 5 mm thick disc to obtain homogenous and exact thickness of the sample (0.65 mm) (11). The Mylar sheet was used in order to prevent the formation of an oxygen inhibited layer on the surface of polymerized composite material. After the sample had been pressed with the hand, the stainless steel was removed and Gradia Direct Posterior or Beautifil II samples were polymerized with Bluephase light curing unit (Vivadent, Schaan, Lichtenstein) using high intensity polymerization mode (1180 mW/cm^2) for 20 and 40 seconds, respectively. Seven samples were made for each single tested group.

Three modes of sample polymerization were used:

(1) Direct polymerization through the Mylar sheet,
(2) Polymerization through the Mylar sheet overlaid with 2 mm CAD/CAM CRP overlay,

(3) Polymerization through the Mylar sheet overlaid with 2 mm thick CAD/CAM LDC overlay.

So prepared samples were removed from the mold and the Mylar sheet and placed directly into the lymphocyte cell cultures.

Kultura ljudskih limfocita izoliranih iz periferne krvi

Ovo istraživanje odobrilo je Etičko povjerenstvo Stomatološkog fakulteta u Zagrebu, Hrvatska. Kako bi se izbjegle moguće interindividualne razlike u odgovoru na tretman, u istraživanju je korišten uzorak venske krvi jednoga zdravog muškog donora (39 godina, nepušač) bez povijesti kroničnе ili akutne bolesti. Prije uzimanja uzorka krvi, donor je bio obaviješten o postupku i svrsi uzimanja krvi, zatim o svrsi testiranja uzetoga uzorka te je potpisao informirani pristanak.

Venska krv izvađena je sterilnim priborom za jednokratnu upotrebu u spremnik s litijevim heparinom (Becton Dickinson, UK). Odmah je obavljena izolacija limfocita u skladu s uputama proizvođača reagensa Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, SAD).

Suspenzija izoliranih limfocita podijeljena je na manje volumene koji su prebačeni 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. U kulture nije dodavan ni mitogen ni teleći serum.

Kulture limfocita tretirane su testiranim materijalom u polimeriziranom i nepolimeriziranom stanju (0,06 g) koji je stavljen 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. Isti postupak korišten je i u ranijim istraživanjima (11 – 13).

Kvantitativna fluorescencijska metoda za procjenu preživljenja stanica, apoptoze i nekroze

Nakon 24-satnog tretmana kulture su centrifugirane 10 minuta brzinom od 600 okr./min. Supernatant je uklonjen, a talog koji sadržava limfocite pažljivo je resuspendiran i korišten za daljnju analizu.

S pomoću mikropipete uzorci stanične suspenzije (V=20 µl) preneseni su na predmetno staklo i pomiješani s jednakim volumenom boja – etidijeva bromida i akridinske narančaste boje (Sigma-Aldrich, SAD) koje su pripremljene u koncentracijama od 100 µg/ml (1 : 1; v/v). Neposredno nakon bojenja, na fluorescencijskom mikroskopu (Olympus BX 51; povećanje 400 x; Olympus, Tokyo, Japan) analizirano je preživljenje limfocita primjenom metode dvojnog bojenja (14). Kvantitativna procjena obavljena je određivanjem postotka živih, apoptotičnih i nekrotičnih stanica. Kako žive stanice u svoj DNK ne ugrađuju etidijev bromid, jezgra im je nakon dvojnog bojenja pod fluorescencijskim mikroskopom zelena. Mrtve nekrotične stanice imaju narančasti do crveno obojeni kromatin, a apoptotične su izrazito zelene i imaju visoko kondenziranu ili fragmentiranu jezgru.

Za svaki uzorak obavljena su tri uzastopna testa, te je ukupno pregledano 300 stanica po uzorku. Usporedo s testiranim uzorcima, u istim je uvjetima držan i kontrolni uzorak,

Primary lymphocyte cultures

This study was approved by the Ethical Committee, School of Dental Medicine, University of Zagreb, Croatia. To overcome possible inter-individual differences in response to the treatment, a blood sample was obtained from one healthy male donor (age 39 years, non-smoker), with no medical records of chronic or acute adverse health conditions. Prior to blood sampling, the donor was acquainted with the procedure, purpose of blood donation, and the aim of blood testing. He signed an informed consent. Venous blood (40 ml) was collected under sterile conditions in heparinized vacutainer tubes (Becton Dickinson, UK) containing lithium heparin as anticoagulant. Lymphocytes were freshly isolated using the Histopaque-1077 reagent (Sigma Chemical Co., St. Louis, MO, USA) according to the manufacturer's instructions. Following the isolation, 50,000 lymphocytes were seeded in sterile tubes (Nange Nunc Int, Naperville, IL, USA) in RPMI 1640 culture medium with penicillin and streptomycin (Gibco Invitrogen, Paisley, UK). The final culture volume was 7 ml. No newborn calf serum or mitogen was added. Each culture was treated for 24 h with 0.06 g of unpolymerized or polymerized tested material at 37 °C in a 5 % CO_2 atmosphere (Heraeus Hera Cell 240 incubator, Langenselbold, Germany). The same study design has been proved in our previous investigation (11 – 13).

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

After 24 hours of treatment, the cultures were centrifuged at 600 rpm for 10 minutes, supernatant was removed and the remaining pellet was gently re-suspended. Aliquots of lymphocyte suspension (V=20 µl) were pipetted, put on the microscope slide and mixed with the same volume of ethidium bromide and acridine orange dyes (Sigma-Aldrich, USA), prepared in final concentrations of 100 µg/ml (1:1; v/v). After covering the preparation with a coverslip, lymphocyte viability was immediately evaluated under a fluorescence microscope (Olympus BX 51; 400 x magnification; Olympus, Tokyo, Japan), by applying a dye exclusion method (14). Quantitative assessments were made by determination of the percentage of viable, apoptotic and necrotic cells. Viable cells with intact plasma membrane 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. Three tests with aliquots of the same sample were performed and a total of 300 cells per sample were counted. The untreated lymphocyte culture was studied in parallel as a control group.

tj. netretirana limfocitna kultura.

Procjena statističke značajnosti rezultata dobivenih za preživljenje stanica, apoptozu i nekrozu u tretiranim i kontrolnim uzorcima učinjena je Pearsonovim hi-kvadrat testom. Kao prag statističke značajnosti korišten je $p < 0,05$.

Rezultati

Rezultate kvantitativne fluorescencijske metode za procjenu preživljenja stanica, apoptoze i nekroze u uzorcima limfocita inkubiranih nepolimeriziranim i polimeriziranim kompozitnim materijalima Beautifil II i Gradia Direct Posterior, vidi u tablicama 1. i 2.

Polimerizacija 20 sekunda

Profil citotoksičnosti za oba ispitivana materijala prikazan je na slici 1. Nepolimerizirani Beautifil II ima značajno veću citotoksičnost negoli Gradia Direct Posterior ($P < 0,0001$). Nakon izravne polimerizacije na temperaturama T1 i T3, Beautifil II pokazuje nižu citotoksičnost negoli Gradia Direct Posterior, no razlika je bila statistički značajna samo za T3 ($P = 0,0013$). Na toj temperaturi za Gradia Direct Posterior uočena je statistički značajno veća učestalost nekroze limfocita

Comparisons between values obtained for the cell viability treated and control samples were made by the Pearson's χ^2 test for two-by-two contingency tables. Statistical decisions were made at a significance level of $p < 0.05$.

Results

The results of the quantitative fluorescent assay for simultaneous identification of apoptotic and necrotic cells in lymphocyte samples incubated with non-polymerized and polymerized Beautifil II are shown in Table 1 and 2.

Polymerization time 20 seconds

Cytotoxicity profiles of both tested materials are shown in Figure 1. Unpolymerized Beautifil II had a significantly higher cytotoxicity than Gradia Direct Posterior ($P<0.0001$). After direct polymerization at T1 and T3, Beautifil II had lower cytotoxicity than Gradia Direct Posterior, but the difference was statistically significant only at T3 ($P=0.0013$). At this polymerization temperature, Gradia Direct Posterior caused a significantly higher frequency of lymphocyte necrosis than

Tablica 1. Rezultati kvantitativne fluorescencijske metode za procjenu preživljenja stanica, apoptoze i nekroze; limfociti su tretirani u uvjetima *in vitro* 24 sata nepolimeriziranim i polimeriziranim kompozitnim materijalom Beautifill II; usporedo je promatrana kontrolna skupina netretiranih stanica

Table 1 Results of the quantitative fluorescent assay for simultaneous identification of apoptotic and necrotic cells. Lymphocytes were treated *in vitro* for 24 hours with unpolymerized and polymerized composite material Beautifill II. Control, non-treated cells were studied in parallel.

Materijal • Material – *Beautifil II*	Žive stanice • Viable cells (%)	Mrtve stanice • Non-viable cells (%)		
		Σ	Apoptoza • Apoptosis	Nekroza • Necrosis
Kontrola • Control	97.3±1.5	2.7±1.5	2.0±1.0	0.7±1.2
Nepolimerizirani • Unpolymerized	16.0±6.6	84.0±6.6	24.3±3.5	59.7±8.7
Polimerizirani 20 sekunda • Polymerized for 20 seconds				
D–T1	87.7±2.1	12.3±2.1	8.0±1.0	4.3±1.2
D–T2	56.7±10.7	43.0±11.1	22.3±11.1	20.7±0.6
D–T3	78.7±4.2	21.3±4.2	17.0±4.6	4.3±2.5
CRP–T1	92.0±1.0	8.0±1.0	5.7±2.1	2.3±1.2
CRP–T2	82.7±8.6	17.3±8.6	14.0±6.2	3.3±2.5
CRP–T3	68.3±8.0	31.8±8.0	15.0±9.5	16.7±3.8
LDC–T1	90.3±1.5	9.7±1.5	4.3±3.2	5.3±2.1
LDC–T2	64.3±3.2	39.0±8.2	22.7±1.5	13.0±2.0
LDC–T3	81.0±7.5	19.0±7.5	13.3±7.5	5.7±1.2
Polimerizirani 40 sekunda • Polymerized for 40 seconds				
D–T1	83.3±2.1	16.7±2.1	9.7±2.3	7.0±1.7
D–T2	67.0±6.6	33.0±6.6	11.7±4.7	21.3±8.4
D–T3	72.7±5.0	27.3±5.0	17.3±6.5	10.0±6.2
CRP–T1	75.7±3.8	24.3±3.8	14.3±1.5	10.0±3.0
CRP–T2	81.0±1.0	19.0±1.0	11.3±3.5	7.7±4.2
CRP–T3	74.0±7.8	26.0±7.8	11.0±3.6	15.0±4.4
LDC–T1	75.7±2.5	24.3±2.5	12.3±0.6	12.0±2.0
LDC–T2	84.3±1.5	15.7±1.5	11.3±0.6	4.3±1.5
LDC–T3	77.0±4.6	23.0±4.6	12.0±1.7	11.0±4.6

Bilješka • Note:
D – izravna polimerizacija • directly polymerized; T1, T2, T3 – temperature zagrijavanja • polymerization temperatures;
CRP - CRP CAD/CAM overlej • overlay; LDC - LDC CAD/CAM e.max overlej • e.max overlay
Analizirano je 300 stanica po uzorku za svaku ispitivanu točku. • 300 cells per sample per each experimental point were analysed.
Statističke značajnosti utvrđene su primjenom Pearsonova χ^2-testa • Statistical significance of data was evaluated using Pearson χ^2 test.

Tablica 2. Rezultati kvantitativne fluorescencijske metode za procjenu preživljenja stanica, apoptoze i nekroze, limfociti su tretirani u uvjetima *in vitro* 24 sata nepolimeriziranim i polimeriziranim kompozitnim materijalom Gradia Direct Posterior, usporedo je promatrana kontrolna skupina netretiranih stanica

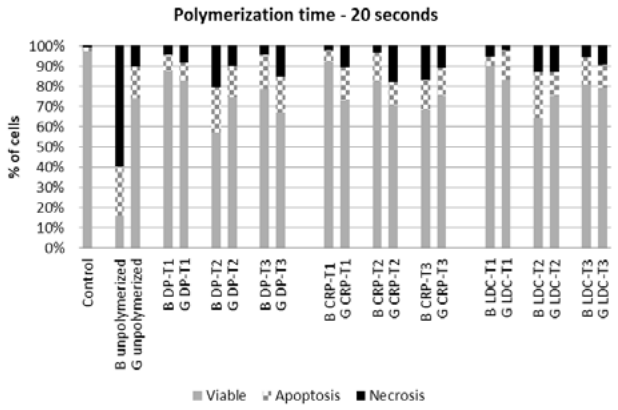
Table 2 Results of the quantitative fluorescent assay for simultaneous identification of apoptotic and necrotic cells. Lymphocytes were treated *in vitro* for 24 hours with unpolymerized and polymerized composite material Gradia Direct Posterior. Control, non-treated cells were studied in parallel.

Material/Materijal – *Gradia Direct Posterior*	Viable cells Žive stanice (%)	Non-viable cells/Mrtve stanice (%)		
		Σ	Apoptosis Apoptoza	Necrosis Nekroza
Kontrola • Control	97.3±1.5	2.7±1.5	2.0±1.0	0.7±1.2
Nepolimerizirani • Unpolymerized	74.0±11.1	26.0±11.1	15.7±11.1	10.3±0.6
Polimerizacija 20 sekunda • Polymerized for 20 seconds				
D–T1	83.0±1.0	17.0±1.0	8.7±1.5	8.3±0.6
D–T2	74.3±6.1	25.7±6.1	15.7±5.1	10.0±1.0
D–T3	67.0±9.5	33.0±9.5	17.7±9.6	15.3±4.0
CRP–T1	73.0±2.6	27.0±2.6	16.3±3.5	10.7±5.7
CRP–T2	70.7±3.8	29.3±3.8	11.3±2.3	18.0±3.0
CRP–T3	75.3±5.5	24.7±5.5	13.7±1.2	11.0±4.4
LDC–T1	83.3±2.3	16.7±2.3	14.7±2.9	2.0±2.6
LDC–T2	75.3±5.5	24.7±5.5	11.7±3.1	13.0±4.4
LDC–T3	79.0±3.6	21.0±3.6	11.7±3.8	9.3±0.6
Polimerizacija 40 sekunda • Polymerized for 40 seconds				
D–T1	68.3±1.5	31.7±1.5	12.0±2.6	19.7±3.1
D–T2	78.7±7.8	21.3±7.8	13.3±2.3	8.0±8.7
D–T3	75.7±5.1	24.3±5.1	11.7±4.2	12.7±4.9
CRP–T1	81.0±2.6	19.0±2.6	10.7±1.5	8.3±1.5
CRP–T2	90.0±2.0	10.3±1.5	7.0±1.0	3.0±2.6
CRP–T3	79.7±5.0	20.3±5.0	10.3±4.0	10.0±1.0
LDC–T1	83.0±1.7	17.0±1.7	12.7±2.1	4.3±0.6
LDC–T2	84.0±2.0	16.0±2.0	9.0±2.0	7.0±0.0
LDC–T3	81.0±6.9	19.0±6.9	13.0±4.4	6.0±2.6

Bilješka • Note:
D – izravna polimerizacija • directly polymerized; T1, T2, T3 – temperature zagrijavanja • polymerization temperatures;
CRP - CRP CAD/CAM overlej • overlay; LDC - LDC CAD/CAM e.max overlej • e.max overlay
Analizirano je 300 stanica prema uzorku za svaku ispitivanu točku. • 300 cells per sample per each experimental point were analysed.
Statističke značajnosti utvrđene su primjenom Pearsonova χ^2-testa • Statistical significance of data was evaluated using Pearson χ^2 test.

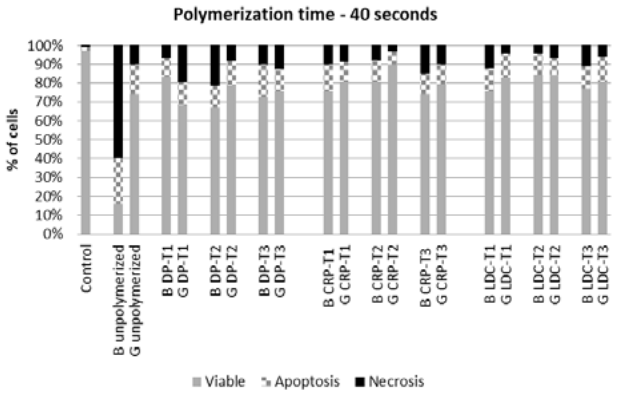
negoli za Beautifil II (P < 0,0001). Suprotno je ustanovljeno za temperaturu zagrijavanja T2, kada je Gradia Direct Posterior pokazao značajno nižu citotoksičnost negoli Beautifil II (P < 0,0001, zbog manjeg udjela nekrotičnih stanica – P = 0,0003). Nakon polimerizacije preko CRP CAD/CAM overleja na temperaturi T1, Gradia Direct Posterior pokazao je veću citotoksičnost negoli Beautifil II (P < 0,0001, zbog porasta udjela i apoptotičnih i nekrotičnih stanica – P < 0,0001). Slično je uočeno i pri temperaturi zagrijavanja T2 – Gradia Direct Posterior pokazao je veću citotoksičnost negoli Beautifil II (P = 0,0005, zbog većeg udjela nekrotičnih stanica – P = 0,0001). Na temperaturi zagrijavanja T3, Beautifil II pokazao je veću citotoksičnost negoli Gradia Direct Posterior, no razlika nije bila statistički značajna. Nakon polimerizacije preko LDC CAD/CAM overleja na temperaturi zagrijavanja T1, Gradia Direct Posterior pokazao je veću citotoksičnost negoli Beautifil II (P = 0,0112, zbog porasta udjela apoptotičnih stanica – P < 0,0001 i nekrotičnih stanica – P = 0,0298). Suprotno je uočeno pri temperaturi T2, kada je Gradia Direct Posterior pokazao značajno nižu citotoksičnost negoli Beautifil II (P = 0,0112). Pri temperaturi zagrijavanja T3, za Gradia Direct Posterior uočena je nešto veća citotoksičnost negoli za Beautifil II, no razlika nije bila statistički značajna.

Beautifil II (P<0.0001). Quite opposite results were obtained at T2, when Gradia Direct Posterior showed a significantly lower cytotoxicity than Beautifil II (P<0.0001, due to lower frequency of necrotic cells, P=0.0003). After polymerization through CRP CAD/CAM overlay at T1, Gradia Direct Posterior was more cytotoxic than Beautifil II (P<0.0001) due to increased frequencies of both apoptotic and necrotic cells P<0.0001). Similar results were obtained at T2: Gradia Direct Posterior was more cytotoxic than Beautifil II (P=0.0005) due to higher frequency of necrotic cells, P=0.0001). At T3, Beautifil II was more cytotoxic than Gradia Direct Posterior, but the difference was not statistically significant. After polymerization through LDC CAD/CAM overlay at T1, Gradia Direct Posterior was more cytotoxic than Beautifil II (P=0.0112) due to increased frequencies of apoptotic cells, P<0.0001 and necrotic cells P=0.0298). Quite opposite was found at T2, when Gradia Direct Posterior showed a significantly lower cytotoxicity than Beautifil II (P=0.0112). At T3, Gradia Direct Posterior was slightly more cytotoxic than Beautifil II, but the difference was not statistically significant.



Slika 1. Usporedba citotoksičnosti materijala Beautifil II i Gradia Direct Posterior polimeriziranih 20 sekunda; postotak živih, apoptotičnih i nekrotičnih stanica određen je kvantitativnim fluorescencijskim testom nakon istodobnog bojenja etidijevim bromidom i akridinskom narančastom bojom. DP – izravna polimerizacija; P – polimerizacija na temperaturama T1-T3; CRP-CRP CAD/CAM overlej; LDC-LDC CAD/CAM overlej

Figure 1 Comparison of cytotoxicity between Beautifil II and Gradia Direct Posterior polymerized for 20 seconds. Percentages of viable, apoptotic and necrotic cells were determined using the quantitative fluorescent assay after simultaneous staining with ethidium bromide and acridine orange. DP-directly polymerized; P-polymerized at temperatures T1-T3; CRP-CRP CAD/CAM overlay; LDC-LDC CAD/CAM overlay



Slika 2. Usporedba citotoksičnosti materijala Beautifil II i Gradia Direct Posterior polimeriziranih 40 sekunda; postotak živih, apoptotičnih i nekrotičnih stanica određeni su kvantitativnim fluorescencijskim testom nakon istodobnog bojenja etidijevim bromidom i akridinskom narančastom bojom. DP – izravna polimerizacija; P – polimerizacija na temperaturama T1-T3; CRP-CRP CAD/CAM overlej; LDC-LDC CAD/CAM overlej

Figure 2 Comparison of cytotoxicity between Beautifil II and Gradia Direct Posterior polymerized for 40 seconds. Percentages of viable, apoptotic and necrotic cells were determined using the quantitative fluorescent assay after simultaneous staining with ethidium bromide and acridine orange. DP-directly polymerized; P-polymerized at temperatures T1-T3; CRP-CRP CAD/CAM overlay; LDC-LDC CAD/CAM overlay

Polimerizacija 40 sekunda

Citotoksičnost obaju ispitivanih materijala nakon 40-sekundne polimerizacije prikazana je na slici 2. Nepolimerizirani Beautifil II ima značajno veću citotoksičnost negoli Gradia Direct Posterior (P < 0,0001). Nakon izravne polimerizacije na temperaturi zagrijavanja T1, Beautifil II pokazao je značajno nižu citotoksičnost negoli Gradia Direct Posterior (P < 0,0001). To je većinom uzrokovano porastom udjela limfocita u nekrozi. Na ostalim temperaturama zagrijavanja Gradia Direct Posterior pokazao je nižu citotoksičnost negoli Beautifil II, uz statističku značajnost na temperaturi T2 (P = 0,0013, zbog porasta udjela nekroticnih stanica – P < 0,0001). Polimerizacija preko obaju overleja smanjila je citotoksičnost, s tim da su bolji rezultati u oba slučaja zabilježeni za Gradia Direct Posterior. Polimerizacija Beautifil II i Gradia Direct Posterior preko CRP CAD/CAM overleja na temperaturama zagrijavanja T1 i T2, rezultirala je većim preživljenjem limfocita, u usporedbi s rezultatima pri zagrijavanju na temperaturi T3. Statistički značajna razlika zabilježena je između ispitivanih materijala jedino na temperaturi T2 (P = 0,0017). U ovom slučaju Beautifil II uzrokovao je veću smrtnost limfocita u odnosu prema Gradia Direct Posterioru (P = 0,0082). Nakon polimerizacije preko LDC CAD/CAM overleja pri temperaturi zagrijavanja T1, Beautifil II pokazao je veću citotoksičnost negoli Gradia Direct Posterior (P = 0,0265, zbog porasta udjela nekrotičnih stanica – P = 0,0006). Na temperaturi zagrijavanja T2 citotoksičnost je bila gotovo podjednaka za oba materijala. Pri temperaturi T3 Beautifil II bio je citotoksičniji negoli Gradia Direct Posterior, ali razlika nije bila statistički značajna.

Polymerization time 40 seconds

Cytotoxicity profiles of both tested materials are shown in Figure 2. Unpolymerized Beautifil II had significantly higher cytotoxicity than Gradia Direct Posterior (P<0.0001). After direct polymerization at T1, Beautifil II had a significantly lower cytotoxicity than Gradia Direct Posterior (P<0.0001). This was mostly influenced by an increased frequency of lymphocyte necrosis. At two higher polymerization temperatures, Gradia Direct Posterior showed lower cytotoxicity than Beautifil II, which was statistically significant at T2 (P=0.0013) due to an increased frequency of necrotic cells, P<0.0001). Polymerization through both overlays contributed to lowering of cytotoxicity, and better results in both cases were obtained for Gradia Direct Posterior. Polymerization of Beautifil II and Gradia Direct Posterior through CRP CAD/CAM overlay at T1 and T2 resulted with higher lymphocyte viability, as compared with T3. A statistically significant difference was recorded only between two tested materials at T2 (P=0.0017). In this case, Beautifil II caused more lymphocyte necrosis as compared to Gradia Direct Posterior (P=0.0082). After polymerization through LDC CAD/CAM overlay at T1, Beautifil II was more cytotoxic than Gradia Direct Posterior (P=0.0265) due to increased frequency of necrotic cells P=0.0006). At T2 their cytotoxicity was similar. At T3 Beautifil II was again more cytotoxic than Gradia Direct Posterior, but the difference was not statistically significant.

Rasprava

Ovo istraživanje provedeno u uvjetima *in vitro* procjenjuje citotoksičnost mikrohibridnog kompozitnog materijala Gradia Direct Posterior te giomera Beautifill II, prethodno zagrijanih na tri različite temperature te polimeriziranih tijekom 20 i 40 sekunda izravnom polimerizacijom ili polimerizacijom preko CAD/CAM overleja. Kao modelni sustav korišteni su izolirani limfociti periferne krvi. Taj se sustav često primjenjuje u toksikološkim ispitivanjima u uvjetima *in vitro*. Limfociti su lako dostupni i dobar su model surogatnih stanica u različitim uvjetima testiranja. Ipak, najveća im je prednost što su primarne stanice. Takav eksperimentalni postav na primarnim kulturama limfocita prihvatljiv je za procjenu biokompatibilnosti kompozitnih materijala, a na osnovi njegove uspješne primjenjivosti u drugim studijama (11, 14, 15) korišten je i u ovom istraživanju.

Iako oba ispitivana materijala imaju određeni citotoksični potencijal, naši rezultati pokazuju da u određenim eksperimentalnim uvjetima Gradia Direct Posterior ima bolju biokompatibilnost negoli Beautifill II. Dobiveni rezultati također pokazuju da, uz sastav materijala, na preživljenje stanica značajno utječe vrijeme polimerizacije, temperatura na koju je kompozit prethodno zagrijan te način na koji je obavljena polimerizacija. Treba istaknuti da je veće preživljenje stanica zabilježeno nakon polimerizacije uzoraka preko CAD/CAM overleja kod obaju ispitivanih materijala.

Kako bismo procijenili preživljenje limfocita, primijenili smo brzi test dvojnog bojenja etidijevim bromidom i akridinskom narančastom bojom koji omogućuje određivanje udjela živih, apoptotičnih i nekrotičnih stanica na osnovi stanične morfologije te dezintegracije jezgre i kromatina. Dok je u kontrolnim limfocitima morfologija jezgre sačuvana, stanice u kasnoj apoptozi imaju znakove promjena na membranama, fragmentacije jezgre i stvaranja apoptotičkih tijela. Propadanje kromatina u apoptotičnim stanicama većinom je neorganizirano i praćeno nastankom vakuola u citoplazmi. Zbog narušavanja integriteta membrane, u nekrotičnim se stanicama nakuplja etidijev bromid i zato je njihov kromatin obojen crveno (14). Gledano u cjelini, nalazi dobiveni analizama provedenima s pomoću fluorescencijskog mikroskopa upućuju na to da su citotoksični učinci nakon tretmana s oba ispitivana materijala u polimeriziranom stanju većinom posredovani apoptozom. Takva su zapažanja važna s kliničkog gledišta jer je apoptoza dobro kontroliran i visoko usklađen fiziološki proces koji ne izaziva upalne promjene oko umiruće stanice, za razliku od nekroze (16 – 19). Najveći postotak nekrotičnih stanica (oko 60 %) zabilježen je nakon inkubacije uzoraka nepolimeriziranim materijalom Beautifill I, što je rezultiralo s 84 % mrtvih stanica nakon 24 sata izlaganja u uvjetima *in vitro*.

Iz perspektive kliničara, te kao što je dokumentirano u nekim kliničkim studijima, postupak upotrebe prethodno zagrijanih kompozitnih materijala kao sredstva za adhezijsko cementiranje inlej i onlej restauracija, ima značajne prednosti kao što su produženo vrijeme rukovanja, odnosno obavljanja postupka adhezijskog cementiranja, lakše uklanjanje viška materijala nakon cementiranja te bolje rubno brtvljenje

Discussion

The present study reports results regarding the *in vitro* assessment of cytotoxic potencies of one micro hybrid composite material (Gradia Direct Posterior) and one giomer composite material (Beautifill II) polymerized at three temperatures for two time periods. Peripheral blood lymphocytes were used as a model system in the present study. This test system is well-established in *in vitro* toxicology. Lymphocytes are easily available and proven to be good surrogate cells in different testing conditions. The most important fact is that lymphocytes are primary cells. Since an *in vivo* situation is generally better-simulated by primary cultures (11, 14, 15), such experimental design seems to be appropriate for the assessment of biocompatibility of composite materials as it was performed in the present study.

Although both studied materials possessed certain degrees of cytotoxicity, our results suggest that in the experimental conditions as applied here, Gradia Direct Posterior was more biocompatible material than Beautifill II. The obtained results have also shown that, apart from material composition, cell viability was also influenced by curing time, temperature of pre-heating and the polymerization pattern. It has to be stressed that greater cell viability was observed after polymerization of both materials through CAD/CAM overlays, as compared to direct polymerization.

To assess the lymphocyte viability in this study, we applied a rapid viability assay with acridine orange and ethidium bromide, which allowed for counting the fractions of viable, apoptotic and necrotic cells based on the cell morphology, nuclear and chromatin disintegration. While control lymphocytes showed intact morphology, in late apoptotic cells we observed membrane blebbing, fragmentation of nuclei and formation of apoptotic bodies. In necrotic cells, on the other hand, more irregular chromatin destruction was noticed, along with vacuole formation in the cytoplasm. Due to breakdown of the plasma membrane, necrotic cells accumulated ethidium bromide and their chromatin thus was stained bright red, as reported in the literature (14). On the whole, our fluorescent microscopic findings suggest that the cytotoxic effects of both tested materials in their polymerized forms have been predominantly mediated by apoptosis. Such results are important from the clinical point of view as apoptosis represents a well-controlled, and tightly-regulated physiological process, which does not result in inflammation around the dying cell, in contrast to necrosis (16 – 19). The highest percentage of necrotic cells (about 60%) was found in the sample incubated with unpolymerized Beautifill II, which resulted in 84 % of dead cells after 24 hours of *in vitro* exposure. The most important are induction of lipid peroxidation, glutathione depletion, downregulation of glutathione peroxidase levels and increase of intracellular calcium levels.

From the dentists' point of view, using heated composite material as a luting agents for inlay and onlay restorations has advantage regarding prolonged handling time, easier removal of excess of the material, better sealing of unideal fitting of the restoration as it is documented in some clinical studies (9, 20 – 22). Dab *et al.* (8) measured shrinkage in pre-heated and

restauracija koje ne priliježu idealno uz rubove kaviteta (9, 20 – 22). Dab i suradnici (8) ispitivali su skupljanje prethodno zagrijanih i nezagrijanih kompozita te zaključili da, bez obzira na porast skupljanja, ono još ne mora biti značajno u kliničkim uvjetima. Razlog za to može biti i pad temperature materijala tijekom prijenosa iz uređaja za zagrijavanje u kavitet. Daronch i suradnici (22) promatrali su ponašanje kompozitnih materijala zagrijanih u uređaju za zagrijavanje Calset na različitim temperaturama te su uočili nagli pad temperature kompozita nakon što je izvađen iz uređaja za zagrijavanje. U rezultatima njihove studije navodi se pad temperature materijala i do 50 % samo dvije minute nakon što je izvađen iz uređaja za zagrijavanje (22). Bez obzira na to je li korišten prethodno zagrijan ili nezagrijan kompozitni materijal, iznimno je važno da je kompozit odgovarajuće polimeriziran. Ranija studija (3) pokazala je da prethodno zagrijani kompozit dopušta kraću polimerizaciju, uz isti stupanj konverzije kao kompozit polimeriziran dulje na sobnoj temperaturi.

Prva radna hipoteza da će kompozitni materijali zagrijani na nižoj temperaturi uzrokovati manju citotoksičnost bez obzira na vrijeme osvjetljivanja i način polimerizacije (izravna polimerizacija ili polimerizacija preko CAD/CAM overleja), prihvaćena je za sve slučajeve, osim za 20-sekundnu polimerizaciju kompozita Gradia Direct Posterior preko CRP overleja. Za kompozit Beautifil II najbolje preživljenje stanica zabilježeno je u slučaju polimerizacije od 20 sekunda i pri temperaturi zagrijavanja T1 tijekom izravne polimerizacije i polimerizacije preko obaju CAD/CAM overleja te tijekom izravne polimerizacije u trajanju od 40 sekunda. Tijekom polimerizacije materijala Beautifil II preko CAD/CAM overleja u trajanju od 40 sekunda, uzorci zagrijani na temperaturi T2 pokazuju najveći broj živih stanica. Kod kompozitnog materijala Gradia Direct Posterior svi uzorci polimerizirani 40 sekunda pokazuju najveći broj živih stanica kada je materijal zagrijan na temperaturi T2. Tijekom polimerizacije u trajanju od 20 sekunda za isti spomenuti materijal zabilježen je najveći broj živih stanica kada je materijal prethodno bio zagrijan na temperaturi T1, osim u slučaju polimerizacije preko CRP CAD/CAM overleja, kao što je već navedeno.

Druga hipoteza, da dulje osvjetljivanje uzrokuje manju citotoksičnost bez obzira na temperature osvjetljivanja i polimerizacijski postupak (izravna polimerizacija ili polimerizacija preko CAD/CAM overleja), prihvaćena je za kompozit Gradia Direct Posterior. Za Beautifil II ta je hipoteza prihvaćena u slučaju zagrijavanja materijala na najvišu temperaturu – T3 (68 °C), ali odbijena je za dvije niže temperature jer je uočen veći broj živih stanica u slučaju osvjetljivanja uzoraka 20 sekunda.

Treća radna hipoteza da će izravno polimerizirani uzorci pokazati manju citotoksičnost negoli oni polimerizirani preko CAD/CAM overleja bez obzira na temperature zagrijavanja, odbijena je za oba materijala i za oba vremena osvjetljivanja. Moglo se očekivati da će materijal osvijetljen izravnim postupkom biti bolje polimeriziran te da će nakon završetka polimerizacije ostati manje monomera koji je već reagirao. No naši rezultati ne idu u prilog spomenutoj tvrdnji. Očito postoji drugi razlog koji bi mogao utjecati na broj živih stanica u staničnoj kulturi inkubiranoj uzorcima polimeriziranima non-heated composite and concluded that despite a shrinkage increase, this increase may not be significant in clinical scenarios. This may also occur due to drop of the material temperature, while the material is taken from the heating unit and placed in the cavity. Daronch *et al.* (22) observed the behavior of composite materials heated in Calset heating unit at different temperatures and noticed a rapid decrease in composite temperature after the removal from the heating unit. They reported a drop of 50 % in material temperature 2 minutes after the removal from the heating unit (22). Regardless of whether the pre-heated or non-heated composite materials are used, it is essential to cure composite material properly. A previous study (3) demonstrated that pre-heated composite allows for a shorter time of light exposure with a similar degree of conversion rate than when the composite is irradiated for a longer exposure time at a room-temperature.

The first working hypothesis of this study stating that materials heated on lower temperature will cause less cytotoxicity regardless of the curing time and polymerization pattern (directly polymerized or through CAD/CAM overlay) was accepted in all cases except in the case of polymerization of Gradia Direct Posterior composite through CRP overlay for 20 seconds. For Beautifil II composite, the highest number of viable cells was recorded in the case of 20 seconds polymerization and heating temperature T1 for direct polymerization and polymerization through both CAD/CAM overlays and for 40 seconds direct polymerization. For 40 seconds, Beautifil II polymerization through CAD/CAM overlays samples heated at temperature T2 showed the highest number of viable cells. For Gradia Direct Posterior composite material, all samples polymerized for 40 seconds showed the highest number of viable cells when the material was heated at temperature T2, while for 20 seconds polymerization, the highest number of viable cells were recorded when the material was heated at temperature T1, apart from polymerization through CRP CAD/CAM overlay as stated before.

The second hypothesis stating that longer curing time causes less cytotoxicity regardless of the curing time and polymerization pattern (directly polymerized or through CAD/CAM overlay) was accepted for Gradia Direct Posterior composite. For Beautifil II, this hypothesis was accepted in the case of material heating at the highest temperature, T3 (68 °C), but it was rejected at two lower temperatures where higher numbers of viable cells were recorded when the samples were polymerized for 20 seconds.

The third working hypothesis was that samples polymerized directly would show less cytotoxicity than the samples polymerized through CAD/CAM overlay regardless of the temperature used was rejected for both materials and both curing times. It would be expected that the material which is cured directly will possess better curing quality and less unreacted components left after curing. Our results, however, did not speak in favor of this assumption. Obviously, there is another reason that might influence the number of viable cells in cultures that were incubated with directly polymerized material, and that is most likely the temperature. If this was the case, a higher temperature produced from the curing unit in direct contact with the Mylar sheet which was cover-

izravnim postupkom, a to je najvjerojatnije temperatura. Ako je to točno, viša temperatura emitirana tijekom osvjetljivanja iz uređaja za polimerizaciju u izravnom kontaktu s Mylar folijom koja prekriva materijal, može pridonijeti povećanju citotoksičnosti.

Rezultati dobiveni u našim ranijim ispitivanjima o utjecaju intenziteta programa polimerizacijskog uređaja na citotoksičnost kompozitnog materijala (12, 13) u skladu su s rezultatima ove studije. Rasvjetljavanje uzroka smrtnosti limfocita nakon inkubacije s uzorcima materijala polimeriziranih različitim polimerizacijskim postupcima svakako bi trebalo biti predmet budućih istraživanja.

Rezultati su također pokazali da polimerizacija preko oba CAD/CAM overleja pridonosi smanjenju citotoksičnosti ispitivanog materijala te su bolji rezultati u oba slučaja zabilježeni za kompozitni materijal Gradia Direct Posterior pri polimerizaciji od 40 sekunda.

Objašnjenje za to može biti u različitom organskom sastavu svakog materijala, kompozitnih materijala korištenih u studiji, a i CAD/CAM overleja. Zbog svojeg različitog sastava oba CAD/CAM materijala posjeduju različita svojstva loma i propuštanja svjetla iz uređaja za polimerizaciju, što uvelike utječe na stupanj konverzije materijala koji se polimerizira preko CAD/CAM overleja. Uz to, Gradia Direct Posterior u svojem sastavu ima UDMA-u koja bi trebala uzrokovati nižu citotoksičnost u usporedbi s Bis-GMA-om koja je u sastavu materijala Beautifil II i, prema navodima u literaturi, uzrokuje veću citotoksičnost (23). Tadin i suradnici (24) ispitivali su genotoksičnost Gradia Direct Posteriora i ustanovili da taj materijal pokazuje veću toksičnost nakon pet dana negoli nakon prvog dana, što objašnjavaju kao postupno otpuštanje i biodegradaciju UDMA-e.

Prema podatcima proizvođača, Beautifil II sadržava površinsko stakleno (S-PRG) punilo koje je već reagiralo i pokazalo se da ima svojstvo neutraliziranja kiseline i inhibiciju formiranja plaka. Glasionomeri i kompomeri zahtijevaju apsorpciju vode nakon osvjetljivanja kako bi mogli otpuštati fluoridne ione. Suprotno tomu, giomeri sadržavaju multifunkcionalnu jezgru stakla koja podliježe acido-baznoj reakciji tijekom postupka proizvodnje te je zaštićena površinskim modificiranim slojem. Restaurativni materijal sa svojstvom otpuštanja fluorida nakon što je postavljen u kavitet, može služiti kao pričuva fluorida te potaknuti lagano otpuštanje fluorida, povećavajući tako njihovu razinu u oralnim tekućinama i sprječavajući nastanak zubnog karijesa. Madhyastha i suradnici (25) ispitivali su otpuštanje fluorida na različitim temperaturama te zaključili da je intenzivnije na temperaturi od 55 °C. Zanimljivo je da je u ovoj studiji Beautifil II utjecao na pojavu najvećeg broja živih stanica kada je bio zagrijan na temperature T1 (37 °C) i T2 (54 °C). Isti autori također su zaključili da se intenzivnije i češće otpuštanje fluorida dogodilo prvog dana nakon postavljanja i polimerizacije nakon čega slijedi otpuštanje fluorida poslije 7 i 14 dana, da bi najslabije otpuštanje bilo zabilježeno nakon 28 dana. Neka druga ispitivanje također su pokazala da citotoksičnost opada s obzirom na vrijeme prema istoj spomenutoj shemi (25). Rezultati Madahystha i suradnika (25) upućuju na prethodno zagrijavanje ovog materijala prije negoli se postavi uz stiing the material could contribute to an increase in lymphocyte cytotoxicity. Findings of our previous study about the influence of curing mode intensity on cytotoxicity of composite materials (12, 13) are in agreement with the results of the present study. Nevertheless, the reasons behind lymphocyte death following incubation with samples that were polymerized using different modes should be investigated more thoroughly in future studies.

The results also showed that polymerization through both CAD/CAM overlays contributed to lowering of cytotoxicity of the tested material, and better results in both cases were obtained for Gradia Direct Posterior composite material in case of 40 seconds polymerization. Explanation for this may be in the different organic structure of each material: Gradia Direct Posterior has UDMA in its composition which should lead to less cytotoxicity, while Bis-GMA, which is present in Beautifil II material according to the literature, causes more cytotoxicity (23). Tadin *et al.* (24) tested genotoxicity of Gradia Direct Posterior and found that Gradia Direct Posterior show higher toxicity after five days than after first day which was explained by gradual release and biodegradation of UDMA.

Beautifil II contains a surface pre-reacted glass (S-PRG) filler that has been shown to possess acid neutralization capabilities and inhibition of plaque formation according to the manufacturer data. Glass-ionomers and compomers require water absorption after light curing in order to release fluoride ions. Conversely, giomers contain a multifunctional glass core which undergoes an acid-base reaction during manufacturing procedure and is protected by surface modified layer. Restorative materials with potential fluoride release when placed in a cavity may serve as a fluoride reservoir and lead to low fluoride release, thus increasing the fluoride level in oral fluids and preventing the dental caries. Madhyastha *et al.* (25) tested the fluoride release at different temperatures and concluded that it is highest at the temperature of 55 °C. It is interesting that Beautifil II in this study showed the highest number of viable cells at temperature T1 (37 °C) and T2 (54 °C). The same authors also concluded that the highest amount of fluoride release occurs the first day after application followed by the days 7 and 14 with least release after 28 days. Some other studies also showed that the cytotoxicity level drops with time in the same manner (25). The findings of Madahystha *et al.* (25) suggest that pre-heating of this material prior to placement in the cavity will accelerate and facilitate the fluoride release. Potentially, the use of this material for luting of CAD/CAM may be beneficial due to fluoride release of this material. However, although the results of this study have clinical implications, further clinical research is needed to implement this material as a potential luting agent for CAD/CAM restorations.

jenke kaviteta, što će pak ubrzati otpuštanje fluorida. Upotreba ovog materijala za vezivanje CAD/CAM restauracija može biti korisna upravo zbog otpuštanja fluorida. Iako rezultati ovog ispitivanja imaju moguću kliničku primjenu, u budućnosti je potrebno nastaviti s kliničkim ispitivanjima, a trebala bi se usredotočiti na to kako najbolje primijeniti materijal te potencijalno sredstvo adhezijskog cementiranja CAD/CAM restauracija.

Zaključak

Uzimajući u obzir da dosad nije bilo mnogo studija sličnih ovoj, naše spoznaje daju preliminarni uvid u citotoksičnost mikrohibridnoga kompozitnog materijala (Gradia Direct Posterior) i giomernoga kompozitnog materijala (Beautifill II) prema ljudskim neciljnim stanicama. Budući da su rezultati ovog istraživanja dobiveni na sustavu stanične kulture, ne mogu se izravno preslikati na uvjete *in vivo*. No dobiveni rezultati čvrst su okvir za iduća istraživanja istih materijala kako bi se objasnili mehanizmi uključeni u njihovu citotoksičnost.

Sukob interesa

Autori nisu bili u sukobu interesa.

Conclusion

Bearing in mind the fact that similar studies on this topic have been rare, our findings provide a preliminary insight into the cytotoxicity of micro hybrid composite material (Gradia Direct Posterior) and giomer composite material (Beautifill II) toward human non-target cells. Since these findings are observed on a cell culture system, they cannot be directly extrapolated to *in vivo* situations. However, our results make a solid frame for designing future studies with the same materials aiming to further clarify mechanisms involved in their cytotoxic action.

Conflict of interest

None declared

Abstract

Objective: The aim of this study was to evaluate cytotoxic potencies of two light cured composite materials after heating on different temperatures and cured directly and through CAD/CAM overlay. **Materials and methods:** Composite materials (microfilled-hybrid Gradia Direct Posterior and Beautifil II) were heated in a Calset warming unit at three different temperatures (T1:37°C, T2:54°C, T3:68°C). A small amount of heated composite material was placed in a round mold (diameter 6mm; 0.65mm thick), covered with Mylar sheet, pressed and polymerized with Bluephase LED unit. One group of samples were polymerized directly, and the other group through 2mm thick CAD/CAM ceramic-reinforced polymer (CRP) and CAD/CAM lithium disilicate ceramic (LDC) overlay for 20 and 40 seconds. The polymerized samples were placed immediately after curing in a lymphocyte cell culture. The viability of peripheral blood lymphocytes was evaluated using a dye exclusion technique by simultaneous staining with ethidium bromide and acridine orange. Quantitative assessments were made by determination of the percentage of viable, apoptotic and necrotic cells. The Pearson chi-square test was used for statistical analysis. **Results:** In case of 20 seconds polymerization, the highest number of viable cells polymerization were recorded when materials were heated at 37°C (T1), while in case of 40 seconds polymerization, the highest number of viable cells were recorded when the materials were heated at 54°C (T2). The samples polymerized through CAD/CAM overlays showed less cytotoxicity than samples polymerized directly. **Conclusion:** Apart from composite material composition, the cell viability was also influenced by curing time, temperature of pre-heating and polymerization pattern.

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References

1. Darmani H, Al-Hiyasat AS, Milhem MM. Cytotoxicity of dental composites and their leached components. Quintessence Int. 2007 Oct;38(9):789-95.
2. Schweiz Monatsschr Zahnmed. 1995;105(9):1123-8. The curing of composites under Cerec inlays. Schweiz Monatsschr Zahnmed. 1995;105(9):1123-1128.
3. Daronch M, Rueggeberg FA, De Goes MF. Monomer conversion of pre-heated composite. J Dent Res. 2005 Jul;84(7):663-7.
4. Froes-Salgado NR, Silva LM, Kawano Y, Francci Ca, Reis A, Lougerico AD. Composite pre-heating: Effects on marginal adaptation, degree of conversion and mechanical properties. Dent Mater. 2010 Sep;26(9):908-14.
5. Daronch M, Rueggeberg FA, De Goes MF. Polymerization kinetics of pre-heated composite. J Dent Res. 2006 Jan;85(1):38-43.
6. Asmussen E, Peutzfeldt A. Influence of pulse-delay curing on softening of polymer structures. J Dent Res. 2001 Jun;80(6):1570-3.
7. Lohbauer U, Zinelis S, Rahiotis C, 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.
8. Deb S, Di Silvio L, Mackler HE, Millar BJ. Pre-warming of dental composites. Dent Mater. 2011 Apr;27(4):e51-9.
9. Tabatabaee MH, Mahdavi 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.
10. Ruix-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 systems. Oper Dent. 2013 Jan-Feb;38(1):73-81.
11. Knezevic A, Zeljezic D, Kopjar N, Duarte S Jr, Par M, Tarle Z. Toxicity of Pre-heated Composites Polymerized Directly and Through CAD/CAM Overlay. Acta Stomatol Croat 2018;52(3):203-217.
12. Knežević A, Želježić D, Kopjar N, Tarle Z. Cytotoxicity of composite materials polymerized with LED curing units. Oper Dent. 2008 Jan-Feb;33(1):23-30.
13. 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.

14. Duke, RC; Cohen, JJ. Morphological and biochemical assays of apoptosis. In: Coligan, JE; Kruisbeal, AM – editors. Current Protocols in Immunology. John Willey and Sons: New York; 1992. p. 1–3.
15. Geurtsen W. Biocompatibility of Resin-Modified Filling Materials. Crit Rev Oral Biol Med. 2000;11(3):333-55.
16. Edinger AL, Thompson CB. Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol. 2004 Dec;16(6):663-9.
17. Pinna L, Brackett MG, Lockwood PE, Huffman BP, Mai S, Cotti E et al. In vitro cytotoxicity evaluation of a self-adhesive, methacrylate resin-based rooth canal sealer. J Endod. 2008 Sep;34(9):1085-8.
18. Matsui S, Takahashi C, Tsujimoto Y, Matsushima K. Simulatory effects of low-concentration reactive oxygen spacies on calcification ability of human dental pulp cells. J Endod. 2009 Jan;35(1):67-72.
19. Camargo Se, Camargo CH, Hiller KA, Rode SM, Schweikl H, Schmalz G. Cytotoxicity and genotoxicity of pulp cappig materials in two cell lines. Int Endod J. 2009 Mar;42(3):227-37.
20. Krämer N, Lohbauer U, Frankenberger R. Adhesive luting of indirect restorations. Am J Dent. 2000 Nov;13(Spec No):60D-76D.
21. Krämer N, Frankenberger R. Clinical performance of bonded leucite-reinforced glass ceramic inlays and onlays after 8 years. Dent Mater. 2005 Mar;21(3):262-71.
22. Daronch M, Rueggeberg FA, Moss L, de Goes MF. Clinically relevant issues related to preheating composites. J Esthet Restor Dent. 2006;18(6):340-50; discussion 351.
23. Ferracane JL. Hygroscopic and hydrolytic effects in dental polymer networks. Dent Mater. 2006 Mar;22(3):211-22.
24. Tadin A, Marovic D, Galic N, Milevoj A, Medvedec Mikic I, Zeljezic D. Genotoxic biomonitoring of flowable and non-flowable composite resins in peripheral blood leukocytes. Acta Odontol Scand. 2013 May-Jul;71(3-4):923-9.
25. Madhyastha P, Kotain R, Vivekananda P, Khader AMA. Fluoride release from glass-ionomer cements: Effect of temperature, time interval and storage condition. J Contemp Dent. 2013;3:68-73.