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The Effects of Grape Seed Oligomeric Proanthocyanidin and Nisin on Dental Pulp Stem Cells

Utjecaj oligomernih proantocijanidina sjemenki grožđa i nizina na matične stanice zubne pulpe

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Abstract

Objective: This study aimed to evaluate the biological effects of "proanthocyanidin" (PA), and "nisin" (Ni), on dental pulp stem cells (DPSCs) and LPS-induced DPSCs as well as their antimicrobial effects against *S. aureus* and *E. coli*. **Materials and methods:** After characterization of DPSCs, cytotoxicity of PA and Ni on DPSCs were evaluated using a water-soluble tetrazolium salt (WST-1). The cytokines and chemokines released by DPSCs and the expression levels of IL-6, IL-8, and TNF alpha were detected with human Cytokine Array C5 and enzyme-linked immunosorbent assay (ELISA), respectively. The antibacterial activities of PA and Ni were tested using the drop plate method. **Results:** PA at 75 µg/ml increased cell viability, decreased TNF-α expression of DPSCs, did not show any cytotoxic effects on LPS-induced DPSCs, and also showed a tendency to decrease TNF-α expression. PA at 75 µg/ml exhibited higher expressions of TIMP-2, OPG, IL-7, and IL-8 in LPS-induced DPSCs compared to DPSCs. Ni at 100 µg/ml decreased TNF-α expression in DPSCs with no cytotoxic effects. It provided increased cell viability and a downregulation trend of TNF-α expression in LPS-induced DPSCs. Both Ni and PA provided strong antibacterial effects against *S. aureus*. Ni at 200µg/ml had strong antibacterial effects against *E. coli* without affecting negatively the viability of both DPSCs and LPS-induced DPSCs and showed anti-inflammatory activity by decreasing TNF-α expression. PA provided strong antibacterial effects against *E. coli* at 200 µg/ml but affected DPSCs viability negatively. **Conclusion:** PA and Ni at specific concentrations exhibited immunomodulatory activity on DPSCs and LPS-induced DPSCs without any cytotoxic effects and strong antibacterial effects on *S. aureus*.

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Introduction

Dental caries is one of the most common disease globally (1) and its progressing can cause the inflammation of pulp tissue due to bacterial components and byproducts reaching the pulp. The main membrane component of Gram-negative bacteria, lipopolysaccharide (LPS), is one of the most important sources of infection and oxidative stress related to dental caries. LPS elevates blood flow and CO_2, as well as lower pH levels in this environment (2). It has been well known fact that the diseased microenvironment disrupts the functions of mesenchymal stem cells (MSCs) and also influences the fate of dental pulp stem cells (DPSCs), a source of MSC (3).

Uvod

Karijes je jedna od najčešćih bolesti globalno (1), a njegovo napredovanje može prouzročiti upalu pulpnog tkiva zbog bakterijskih komponenti i nusprodukata koji dosežu pulpu. Lipopolisaharid (LPS), glavna membranska komponenta gram-negativnih bakterija, ključan je izvor infekcije i oksidacijskoga stresa povezanog s karijesom. LPS povećava protok krvi i razinu CO_2, a smanjuje pH u tkivnom okruženju (2). Dobro je poznato da bolesno mikrookruženje narušava funkcije mezenhimalnih matičnih stanica (MSC) i utječe na sudbinu matičnih stanica zubne pulpe (DPSC), izvora MSC-a (3).

Therefore, in order to provide the appropriate microenvironment for healing and regeneration of the pulp, many compounds or target molecules either alone or in combination with dental materials have been investigated to date and are currently a matter of interest for obtaining the most favorable outcomes. Grape is a fruit rich in phenolic compounds exhibiting antioxidant, anti-microbial, anti-inflammatory, anti-carcinogenic, cardioprotective and anti-aging benefits for human health. Proanthocyanidin (PA) is the most prevalent phenolic compound in grape seeds. It has been reported that PA is an antioxidant, free-radical scavenger, and cardiovascular protector (4, 5). Grape seed compounds have also been evaluated in the field of dentistry in regard to their anti-microbial efficiency as intracanal irrigant in endodontics (6), antioxidant properties against LPS from periodontopathogens in periodontology (7), as a natural collagen crosslinking agent in restorative dentistry (8), and as a cross linker of tissue engineering scaffolds (9). Protective effects of grape seed extracts against LPS induced inflammatory responses and their potent anti-inflammatory impacts in experimental inflammation have been demonstrated in numerous studies (7, 10, 11). The challenges posed by antibiotic-resistant pathogens and infections associated with biofilms have promoted the search of new therapeutic strategies. Natural anti-microbial peptides (AMP) and their peptidomimetics have attracted attention due to their low bacterial resistance and potent anti-microbial activities. Nisin, derived from *Lactococcus lactis* bacteria, is the only FDA approved (for inhibiting pathogens in food manufacturing) natural anti-microbial peptide. In medical applications, nisin showed efficacy in the treatment of *Staphylococcal mastitis* and atopic dermatitis, as well as inhibition of bacterial adhesion on implantable materials (12, 13). The inhibitor effects of nisin against intracanal pathogens *Streptococcus gordonii, Enterococcus faecalis* and dental caries associated microorganism, were also reported for dental applications (14, 15, 16). The dental adhesive incorporating nisin demonstrated inhibitory effect on *Streptococcus mutans* (17). AMPs do not only exhibit antibacterial effects but also regulate immuno-inflammatory responses through their immunomodulatory and wound-healing potential. They stimulate the production of pro-inflammatory cytokines and recruit host defense cells (18). Nisin at certain concentrations demonstrated anti-biofilm effects against saliva derived multi-species biofilms with no cytotoxic effects on the human oral cells (15). Kindrachuk et al. (19) showed that purified nisin induced immunomodulatory responses within both *ex vivo* and *in vivo* infection models. Baskaran et al. (20) synthesized Alpha Tricalcium Phosphate (NTCP) incorporated with nisin and assessed the release of nisin from NTCP when it was employed as a pulp capping agent *in vitro*. Nisin incorporated dental adhesive against *Streptococcus mutans* has also been developed and exhibited inhibitory effect without no adverse effects (17). Although many beneficial effects of both grape seed oligomeric proanthocyanidins and nisin were reported in the literature, their effects on cell viability and cytokine expressions in both DPSCs and LPS-induced DPSCs have not yet been investigated. The aim of the present study was to evaluate the effects of different concentrations of pro-

Kako bi se osiguralo odgovarajuće mikrookruženje za cijeljenje i regeneraciju pulpe, istraživali su se mnogi spojevi ili ciljane molekule, samostalno ili u kombinaciji s dentalnim materijalima. Grožđe je voće bogato fenolnim spojevima koji pokazuju, kad je riječ o ljudskom zdravlju, antioksidacijska, antimikrobna, protuupalna, antikarcenogena, kardioprotektivna i antiaging svojstva. Proantocijanidin (PA) najzastupljeniji je fenolni spoj u sjemenkama grožđa, a poznat je po svojim antioksidacijskim i kardioprotektivnim svojstvima (4, 5).

Spojevi sjemenki grožđa istraživali su se u stomatologiji zbog njihove antimikrobne učinkovitosti kao intrakanalni iriganasi u endodonciji (6), zbog antioksidacijskih svojstava protiv LPS-a iz parodontopatogena u parodontologiji (7), kao prirodni agensi za umrežavanje kolagena u restauratívnoj stomatologiji (8) i kao sredstvo za umrežavanje u sklopu tkivnoga inženjerstva (9). Autori mnogobrojnih istraživanja istaknuli su zaštitne učinke ekstrakata sjemenki grožđa kad je riječ o upalnim odgovorima izazvanima LPS-om i njihov izraženi protuupalni učinak u eksperimentalnim upalama (7, 10, 11).

Izazovi prouzročeni patogenima otpornima na antibiotike i infekcijama povezanima s biofilmom potaknuli su traženje novih terapijskih strategija. Prirodni antimikrobni peptidi (AMP) i njihovi peptidomimeti privukli su pozornost zbog niske bakterijske rezistencije i snažnih antimikrobnih svojstava. Nizin, dobiven iz bakterije *Lactococcus lactis*, jedini je prirodni antimikrobni peptid koji je odobrila FDA (odobren za inhibiranje patogena u proizvodnji hrane). U medicinskim primjenama pokazao je učinkovitost u liječenju stafilokoknog mastitisa, atopijskog dermatitisa te u inhibiciji bakterijskog prianjanja na implantabilne materijale (12, 13).

Inhibicijski učinci nizina, kad je riječ o intrakanalnim patogenima *Streptococcus gordonii, Enterococcus faecalis* i mikroorganizama povezanih s karijesom, također su navedeni u stomatološkim primjenama (14, 15, 16). Dentalni adheziv koji sadržava nizin pokazao je inhibicijski učinak na *Streptococcus mutans* (17). AMP ne pokazuje samo antibakterijski učinak, nego regulira imunoupalni odgovor putem njihova imunomodulacijskog potencijala i potencijala za zacjeljivanje rana. Potiče proizvodnju proupalnih citokina i privlači stanice obrane domaćina (18).

Nizin je u određenim koncentracijama pokazao da utječe na formiranje biofilma višestrukih vrsta u slini bez citotoksičnog učinka na oralne stanice čovjeka (15). Kindrachuk i suradnici (19) pokazali su da pročišćeni nizin izaziva imunomodulacijski odgovor u modelima infekcije *ex vivo* i *in vivo*. Baskaran i suradnici (20) sintetizirali su alfa trikalcijev fosfat (NTCP) koji sadržava nizin i procijenili otpuštanje toga peptida iz NTCP-a kada je upotrijebljen kao agens za prekrivanje pulpe *in vitro*. Razvijen je i dentalni adheziv koji sadržava nizin protiv bakterije *Streptococcus mutans* i pokazao je inhibicijski učinak bez nuspojava (17).

Iako se mnogi korisni učinci sjemenki grožđa i nizina mogu pronaći u literaturi, njihovi učinci na vijabilnost stanica i izražavanje citokina u DPSC-u i LPS-izazvanim DPSC-ima još nisu istraženi.

Cilj ovog istraživanja bio je procijeniti učinke različitih koncentracija proantocijanidina i nizina na DPSC te prona-

anthocyanidin and nisin on DPSCs and also to find the optimal concentration which could enhance tissue regeneration.

Materials and methods

Isolation and Characterization of Dental Pulp Stem Cells

The principles of the Declaration of Helsinki were followed in this study. Dental pulp samples were obtained with the approval of Hacettepe University Non-Interventional Clinical Research Ethics Committee (*GO22-431*). The surgically removed five impacted third molars were gathered from five healthy donors with no history of medicine within the last two weeks and non-smokers aged 18-24. Informed and signed consent was obtained from all individual participants included in the study. After the surgical extractions were conducted in the operating rooms of the Hacettepe University School of Dentistry, teeth were gently wiped with 70% alcohol and were irrigated with distilled water. Teeth were transferred within the storage medium immediately to the Center for Stem Cell Research and Development of Hacettepe University. Dental pulp tissue was extracted by carefully separating it from the pulp chamber using sterile instruments and diamond burs while constantly cooling it with sterile saline solution. The collected tissue was placed in a transport medium containing DMEM-LG (Gibco) with 5% Penicillin-Streptomycin (Pen-Strep, Sigma) and 5% Amphotericin B (Amf B, Biological Industries), and transported to the laboratory. Subsequently, the pulp tissue was transferred to a 35 mm transparent petri dish and rinsed with DMEM-LG (Gibco) containing 2 mL of 5% Pen-Strep and 5% Amf B. The pulp tissue was then finely minced using a scalpel, and enzymatically digested using 0.3 mg/mL collagenase type I (Sigma-Aldrich) for a period of 2 hours at 37°C. It was then centrifuged at 500g for 6 minutes, and subsequently passed through a 40 µm filter (Cell Strainer, BD Biosciences Discovery Labware). The filtered cells were then cultured at 37°C in Alpha-MEM (Biological Industries) supplemented with 20% FBS (BioWest). The culture medium was refreshed every three days. When the cells reached 80.0%-85.0% confluence, adherent DPSCs were detached with trypsin (Grisp) and trypan blue dye exclusion for cell viability. The flow cytometry (Accuri, Becton Dickinson Biosciences) was used for analyzing the surface markers of DPSCs at passage 2 (P2). Cells were incubated in the dark with anti-human antibodies (Becton Dickinson Pharmingen), including CD34-phycoerythrin (PE), CD105-PE, CD45-allophycocyanin (APC), CD90-fluorescein isothiocyanate (FITC), and CD73-FITC. The analysis was performed using BD CSampler Plus Analysis Software (Becton Dickinson Biosciences). To assess their differentiation potential, DPSCs at P2 were seeded in 24-well culture plates (10 000 cells/mL) and cultured until reaching confluence. Osteogenic differentiation was induced by incubating the cells for 21 days in an osteogenic differentiation medium containing (DMEM-LG (Gibco), 10% Fetal Bovine Serum (FBS), 10^{-7} M dexamethasone (Sigma), 0.2 mM ascorbic acid (Sigma) and 10 mM glycerol 2-phosphate (Sigma), 1% Penicillin-Streptomycin, 1% L-glutamine).

ći optimalnu koncentraciju koja bi mogla pospješiti regeneraciju tkiva.

Materijali i metode

Izolacija i karakterizacija matičnih stanica zubne pulpe

U ovom istraživanju poštovala su se načela Helsinške deklaracije. Uzorci zubne pulpe prikupljani su uz odobrenje Etičkog povjerenstva za klinička istraživanja Sveučilišta Hacettepe (GO22-431). Pet kirurški uklonjenih impaktiranih umnjaka dobiveno je od pet zdravih donora bez povijesti uzimanja lijekova u posljednja dva tjedna i nepušača u dobi od 18 do 24 godine. Informirani pristanak potpisali su svi sudionici uključeni u istraživanje. Poslije kirurških ekstrakcija provedenih u operacijskim dvoranama Stomatološkog fakulteta Sveučilišta Hacettepe, zubi su pažljivo obrisani 70-postotnim alkoholom i isprani destiliranom vodom te su odmah preneseni u sredstvu za čuvanje u Centar za istraživanje i razvoj matičnih stanica Sveučilišta Hacettepe. Tkivo zubne pulpe ekstrahirano je pažljivim odvajanjem s pomoću sterilnih instrumenata i dijamantnih svrdala uz stalno hlađenje sterilnom fiziološkom otopinom. Skupljeno tkivo stavljeno je u transportni medij koji sadržava DMEM-LG (Gibco) s 5 % penicilin-streptomicina (Pen-Strep, Sigma) i 5 % amfotericina B (Amf B, Biological Industries) te transportirano u laboratorij. Zatim je pulpno tkivo stavljeno u prozirnu Petrijevu zdjelicu promjera 35 mm i isprano DMEM-LG-om (Gibco) s 2 mL 5 % Pen-Strepa i 5 % Amf B-a. Pulpno tkivo je nakon toga sitno narezano skalpelom i enzimatski obrađeno s 0,3 mg/mL kolagenaze tip I (Sigma-Aldrich) tijekom 2 sata na 37 °C. Slijedilo je centrifugiranje pri 500 g tijekom 6 minuta te filtriranje kroz filtar od 40 µm (Cell Strainer, BD Biosciences Discovery Labware). Filtrirane stanice zatim su kultivirane na 37 °C u Alpha-MEM-u (Biological Industries) s dodatkom 20 % FBS-a (BioWest). Sredstvo za kultivaciju obnavljalo se svaka tri dana. Kad su stanice dosegnule konfluenciju od 80 do 85 %, adhezivni DPSC odvojen je tripsinom (Grisp) i obojen tripanskim plavilom za procjenu vijabilnosti stanica. Protokolorija (Accuri, Becton Dickinson Biosciences) koristila se za analizu površinskih markera DPSC-a u prolazu 2 (P2). Stanice su inkubirane u mraku antihumanim antitijelima (Becton Dickinson Pharmingen), uključujući CD34-fikoeritrin (PE), CD105-PE, CD45-alofikocijanin (APC), CD90-fluorescein izotiocijanat (FITC) i CD73-FITC. Analiza je obavljena korištenjem BD CSampler Plus Analysis Softwarea (Becton Dickinson Biosciences). Za procjenu njihova diferencijacijskog potencijala, DPSC u prolazu 2 zasijan je na 24-ćelijske kultivacijske podloge (10 000 stanica/mL) i kultiviran do konfluencije. Osteogena diferencijacija inducirana je 21 dan inkubacijom stanica u osteogenom diferencijacijskom mediju koji sadržava (DMEM-LG (Gibco), 10 % fetalnoga goveđega seruma (FBS), 10^{-7} M deksametazona (Sigma), 0,2 mM askorbinske kiseline (Sigma) i 10 mM glicerol 2-fosfata (Sigma), 1 % penicilin-streptomicina, 1 % L-glutamina). Stanice su 21 dan adipogeno inducirane kultivacijom u adipogenom diferencijacijskom mediju koji sadržava (DMEM-LG (Gibco), 10 mM indometacina (Santa Cruz Biotechnology, Oregon, SAD), 0,5 mM

The cells were induced adipogenically by culturing them for 21 days in adipogenic differentiation medium containing (DMEM-LG (Gibco), 10 mM indomethacin (Santa Cruz Biotechnology, Oregon, USA), 0.5 mM 3-isobuthylmethyl-xanthine (Sigma), 1 μM dexamethasone (Sigma), 10 μg/mL insulin (Santa Cruz Biotechnology), 1% Penicillin-Streptomycin, 1% L-glutamine). All cultures were maintained at 37°C in a 5% CO_2 incubator and medium replacement was performed three times per week. Osteogenic and adipogenic differentiation of DPSCs were assessed by microscopic examination after staining with Alizarin red (Sigma) and Oil red-O (Sigma), respectively.

Evaluation the Cytotoxic Effect of Proanthociyanidin and Nisin

DPSCs (P2) were harvested in 96-well plate at concentration of 10.000 cells/ml. After cell adherence, LPS (lipopolysaccharides from Escherichia coli O111:B4; Sigma-Aldrich, St. Louis, MO, USA) was replaced with a medium containing 2 µg/mL and cultured at 37°C in a 5% CO2 incubator for 24h. A culture condition without LPS was prepared as a control group. Different concentration of Nisin (Ni, from *Lactobacillus lactis*, Sigma Aldrich, USA) and Proanthocyanidin (PA, Sigma Aldrich, USA) were applied to the cell culture plate at 25-50-75-100-200 µg/ml for 24h to evaluate cytotoxicity. A water-soluble tetrazolium-based assay [10% WST-1,4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzenedisulfonate] was used for evaluating the cell metabolic activity for 2 hours. Subsequently, 100 µl of the medium from each condition was transferred to enzyme-linked immunosorbent assay (ELISA) microplates (96-well plates, Corning Life Sciences). The absorbance values of the solutions at 450 nm were determined spectrophotometrically (n=3).

Cytokine Array and ELISA

Detection of cytokines and chemokines released in conditioned medium was performed with the human Cytokine Array C5 (AAH-CYT-5-8) by following the manufacturer's instructions (RayBiotech, Norcross GA). Densitometry analyses were carried out utilizing the Gel Doc 2000 imaging apparatus and ImageLab software (Bio-Rad, Mississauga, ON). All values were normalized with respect to the mean intensity of the positive and negative controls (n=3). The concentrations of IL-6, IL-8 and TNF alpha in conditioned medium were measured using the human IL-6, IL-8 and TNF alpha ELISA kits following the manufacturer's instructions (Nephente, Kocaeli, Turkey). The absorbance of the solutions was determined spectrophotometrically at 450 nm (n=3).

Evaluation of Antibacterial Activity of Proanthocyanidin and Nisin

Antibacterial activity of PA and Ni were tested against a Gram (+) strain *Staphylococcus aureus* (*S. aureus*, ATTC 6338), and a Gram (-) strain *Escherichia coli* (*E.coli*, ATTC 8739) using drop plate method (21). The cultures of bacteria were grown in Nutrient Broth (NB) medium at 37 °C for overnight incubation at 100 rpm. Different concentrations of PA and Ni (25, -50, -75, -100, -200 µg/ml) were added in-

3-izobutilmetil- ksantina (Sigma), 1 µM deksametazona (Sigma), 10 µg/mL inzulina (Santa Cruz Biotechnology), 1 % penicilin-streptomicina, 1 % L-glutamina. Sve kulture održavane su na 37 °C u inkubatoru s 5 % CO_2, a zamjena medija obavljana je tri puta na tjedan. Osteogena i adipogena diferencijacija DPSC-a procijenjena je mikroskopskim pregledom poslije bojenja crvenilima alizarin (Sigma) i Oil red-O (Sigma).

Procjena citotoksičnog učinka proantocijanidina i nizina

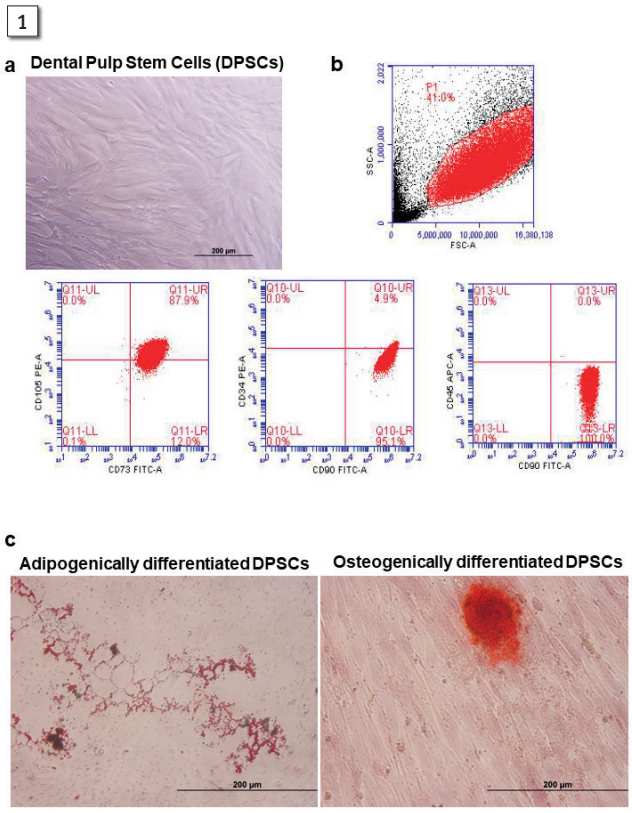
DPSC (P2) je prikupljen na 96-ćelijskoj podlozi u koncentraciji od 10 000 stanica/mL. Nakon adherencije stanica, LPS (lipopolisaharidi iz bakterije *Escherichia coli* O111:B4; Sigma-Aldrich, St. Louis, MO, SAD) zamijenjen je medijem koji sadržava 2 µg/mL i kultiviran je na 37 °C u inkubatoru s 5 % CO_2 tijekom 24 sata. Kao kontrola pripremljena je kultivacija bez LPS-a. Različite koncentracije nizina (Ni iz *Lactobacillus lactis*, Sigma Aldrich, SAD) i proantocijanidina (PA, Sigma Aldrich, SAD) primijenjene su na staničnu kulturu u koncentracijama od 25-50-75-100-200 µg/mL tijekom 24 sata radi procjene citotoksičnosti. Za procjenu metaboličke aktivnosti stanica korišten je test temeljen na tetrazoliju topljivom u vodi [10 % WST-1,4-[3-(4-jodofenil)-2-(4-nitrofenil)-2H-5-tetrazolio]-1, 3-benzenedisulfonat] tijekom 2 sata. Poslije toga je 100 µL medija za svaki uvjet preneseno u mikrolopatice za enzimski imunosorbentni test (ELISA) (96-ćelijske podloge, Corning Life Sciences). Apsorpcijske vrijednosti otopina na 450 nm određivane su spektrofotometrijski (n = 3).

Niz citokina i ELISA

Za detekciju citokina i kemokina oslobođenih u kondicioniranom mediju korišten je humani niz citokina C5 (AAH-CYT-5-8) slijedeći upute proizvođača (RayBiotech, Norcross GA). Denzitometrijske analize provedene su s pomoću uređaja za slikanje Gel Doc 2000 i softvera ImageLab (Bio-Rad, Mississauga, ON). Sve vrijednosti normalizirane su u odnosu na srednju jakost pozitivnih i negativnih kontrola (n = 3). Koncentracije IL-6, IL-8 i TNF alfa u kondicioniranom mediju mjerene su s pomoću ELISA setova za humani IL-6, IL-8 i TNF alfa prema uputama proizvođača (Nephente, Kocaeli, Turska). Apsorpcijske vrijednosti otopina određene su spektrofotometrijski na 450 nm (n = 3).

Procjena antibakterijskog djelovanja proantocijanidina i nizina

Antibakterijska aktivnost PA-e i Ni-a testirana je protiv gram (+) soja *Staphylococcus aureus* (*S. aureus*, ATTC 6338) i gram (-) soja *Escherichia coli* (*E. coli*, ATTC 8739) korištenjem metode kapanja na ploču (21). Kulture bakterija uzgajane su u hranjivom bujonu (NB) na 37 °C tijekom noćne inkubacije pri 100 o/min. Različite koncentracije PA-e i Ni-a (25, -50, -75, -100, -200 µg/ml) dodane su u epruvete s kul-



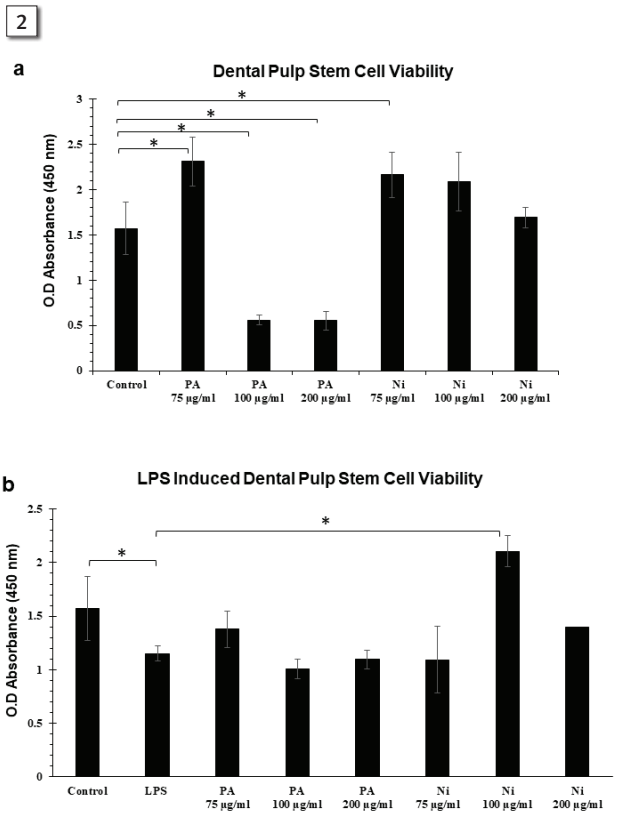


Figure 1 Morphology and characterization of dental pulp stem cells. Phase-contrast microphotographs showing dental pulp stem cells (a), scale bar = 200 µm. Representative FACS analysis of dental pulp stem cells (b). Cells highly expressed CD90, CD73 and CD 105 (>87.0%) and lacked CD34 and CD45 (<5.0%) markers.
Determination of adipogenic and osteogenic differentiation capacities of human dental pulp stem cells by Oil Red-O and Alizarin Red staining methods (c). 21st day of the culture demonstrating the formation of lipid droplets, bone like mineralization was observed with red staining at day -21. Scale bar: 200 µm

Slika 1. Morfologija i karakterizacija matičnih stanica zubne pulpe; mikrofotografije u faznom kontrastu prikazuju matične stanice zubne pulpe (a), mjerna skala = 200 µm; reprezentativna FACS analiza stanica matičnih stanica zubne pulpe (b); stanice s visokom ekspresijom CD90, CD73 i CD105 (> 87,0 %) i nedostatkom markera CD34 i CD45 (< 5,0 %)

Figure 2 The cell viability analysis of DPSCs (a) and LPS-induced dental pulp stem cells (DPSCs) (b) which were conditioned with different concentrations of Proanthocyanidin (PA) and Nisin (Ni) for 24h. The control group is DPSCs, and the LPS group is LPS-induced DPSCs (2 µg/ml for 24h). Significant differences were found by paired t test * $p<0.05$ (mean ± std, n=3).

Slika 2. Analiza vijabilnosti matičnih stanica zubne pulpe (DPSC) (a) i LPS-induciranih matičnih stanica zubne pulpe (DPSCs) (b) koje su uvjetovane različitim koncentracijama proantocijanidina (PA) i nizina (Ni) tijekom 24 sata; kontrolna skupina su DPSCi, a LPS skupina je LPS-inducirani DPSC (2 µg/mL tijekom 24 sata); značajne razlike utvrđene su s pomoću uparenog t-testa * $p < 0,05$ (srednje ± SD, n = 3)

to culture tubes of *E. coli* and *S. aureus* (0.5 McFarland) and incubated at 37 °C with continuous shaking at 100 rpm for 24 h. After incubation time, the samples were taken, serially diluted in PBS buffer, subsequently 10 µL were dropped on Nutrient Agar (NA) agar plates. The plates were incubated at inverted position overnight at 37 °C for 24 h. After incubation, the colonies on agar plates were counted. The logarithm to the base 10 (log) of the cell counts was taken for statistical evaluation (n=3).

Statistical Analysis

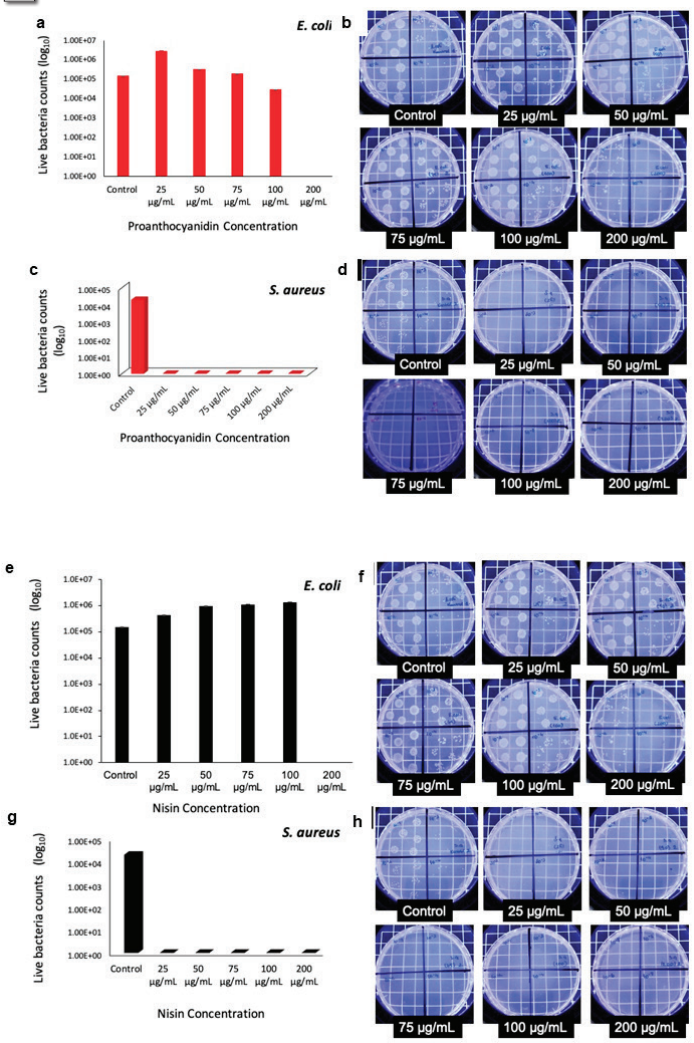
All experiments were conducted three times (mean ± standard deviation) and analyses were done with SPSS 16.0 (SSP Inc., Chicago, IL, USA). Comparisons between experimental groups were conducted using the Student's t-test. Statistical significance was determined for results with a p-value < *0.05*.

turama *E. coli* i *S. aureus* (0,5 McFarland) te inkubirane na 37 °C uz kontinuirano miješanje pri 100 o/min. tijekom 24 sata. Poslije inkubacije uzorci su uzeti i serijski razrijeđeni u PBS puferu, zatim je kapljicom naneseno 10 µL na hranjivi agar (NA) na agar-pločama. Ploče su inkubirane u invertiranom položaju preko noći na 37 °C tijekom 24 sata. Poslije inkubacije kolonije na agar-pločama su prebrojene. Logaritam broja stanica (log) uzet je za statističku evaluaciju (n = 3).

Statistička analiza

Sva ispitivanja provedena su tri puta (srednja vrijednost ± standardna devijacija), a analize su obavljene u SPSS-u 16.0 (SSP Inc., Chicago, IL, SAD). Usporedbe između eksperimentalnih skupina provedene su s pomoću Studentova t-testa. Statistička značajnost utvrđena je za rezultate s vrijednošću $p < 0,05$.

3



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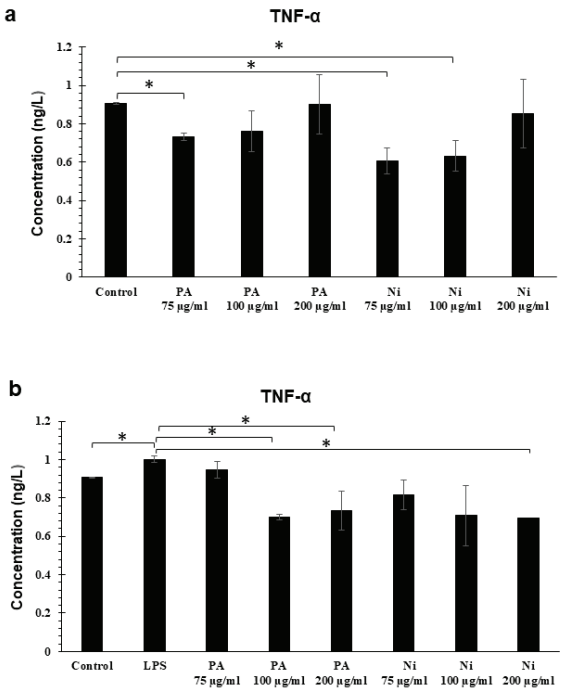

Figure 3 Antibacterial activity of Proanthocyanidin (a, b, c, d) and Nisin (e, f, g, h) against a Gram (-) strain *Escherichia coli* and a Gram (+) strain *Staphylococcus aureus*, using drop plate method. Proanthocyanidin and nisin showed a strong antibacterial property against *E. coli* at 200 µg/mL concentration (a) and against *S. aureus* at all concentrations (c).

Slika 3. Antibakterijska aktivnost proantocijanidina (a, b, c, d) i nizina (e, f, g, h) protiv gram-negativnog soja *Escherichia coli* i gram-pozitivnog soja *Staphylococcus aureus*, korištenjem metode kapanja na ploču; proantocijanidin i nizin pokazali su snažna antibakterijska svojstva protiv *E. coli* pri koncentraciji od 200 µg/mL (a) i *S. aureus* u svim koncentracijama (c)

Figure 4 Effects of Proanthocyanidin (PA) and Nisin (Ni) on the expression of TNF-α in DPSCs and LPS induced-DPSCs (a,b). DPSCs treated with 2 µg/ml LPS for 24 h are the LPS induced DPSCs (b). Subsequently, DPSCs were conditioned with different concentrations of PA and Ni for 24 h. TNF-α expression levels in the culture medium of PA and Nisin conditioned DPSCs/ LPS induced DPSCs were measured using ELISA. Significant differences were determined by paired t test. * $p<0.05$ (mean ± std, n=3).

Slika 4. Učinci proantocijanidina (PA) i nizina (Ni) na izražavanje TNF-α u DPSC-u i LPS induciranom DPSC-u (a, b); DPSC tretiran s 2 µg/mL LPS tijekom 24 sata predstavlja LPS-inducirani DPSC (b); poslije toga DPSC je 24 sata kondicioniran različitim koncentracijama PA-e i Ni-e; razina ekspresije TNF-α u mediju kulture DPSC-a kondicioniranoga s PA-e i Ni-e /LPS induciranoga DPSC-a mjerena je s pomoću ELISA-e; značajne razlike utvrđene su uparenim t-testom; * p < 0,05 (srednje ± SD, n = 3).

Results

Characterization of Dental Pulp Stem Cells

Typical spindle-shaped morphologic characteristics of cultured DPSCs were shown in Figure 1a. Flow cytometry analysis revealed that DPSCs exhibited positive staining for mesenchymal stem cell markers CD73, CD105, CD90 (>87%) and negative staining for hematopoietic cell markers CD34, CD45 (<5%), as illustrated in Figure 1b. The multilineage differentiation of DPSCs was confirmed using Alizarin red staining for osteogenesis and Oil red staining for adipogenesis on the 21st days of the culture (Figure 1c).

Rezultati

Karakterizacija matičnih stanica zubne pulpe

Uobičajene morfološke karakteristike kultiviranoga DPSC-a prikazane su na slici 1. a. Analiza protoka citometrijom otkrila je da je DPSC pokazao pozitivno bojenje na markere mezencimalnih matičnih stanica CD73, CD105, CD90 (> 87 %) i negativno bojenje na markere hematopoetskih stanica CD34, CD45 (<5 %), kako se vidi na slici 1. b. Višelinijska diferencijacija DPSC-a potvrđena je primjenom crvenila alizarina za osteogenezu i oil-crvenila za adipogenezu 21. dana kulture (slika 1. c).

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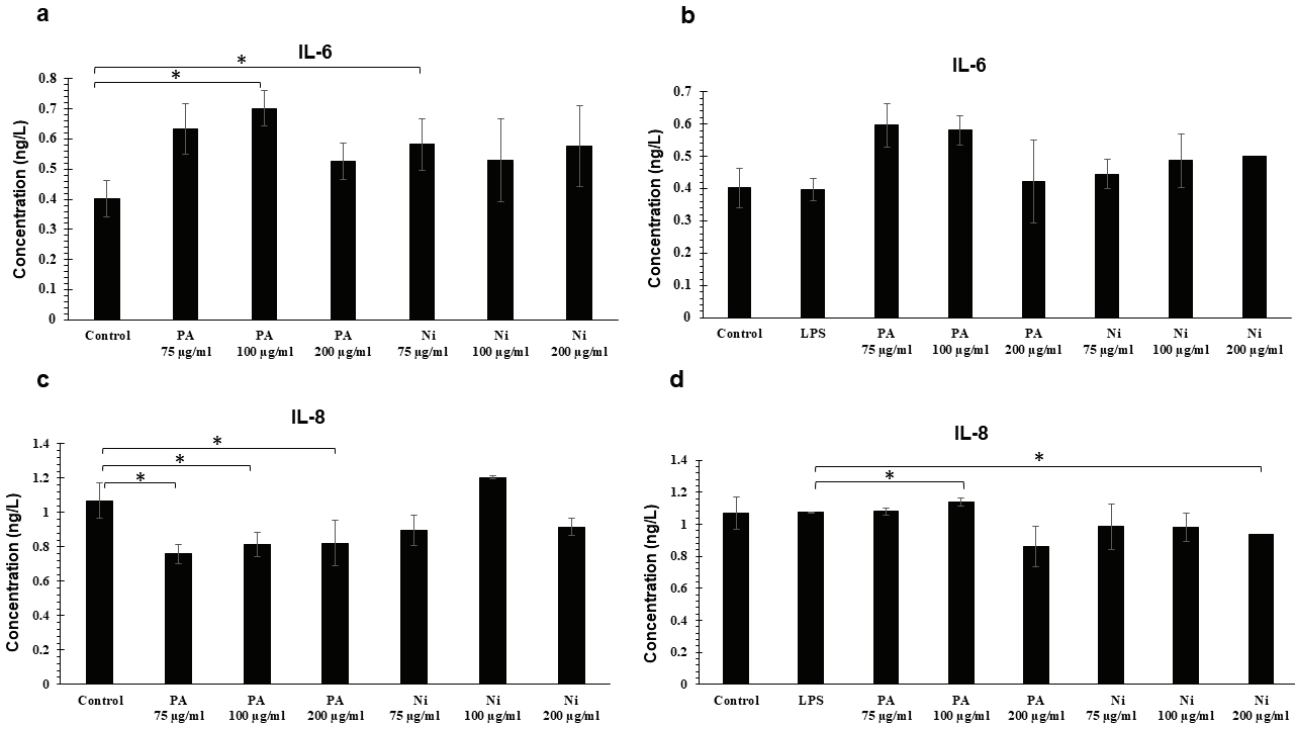

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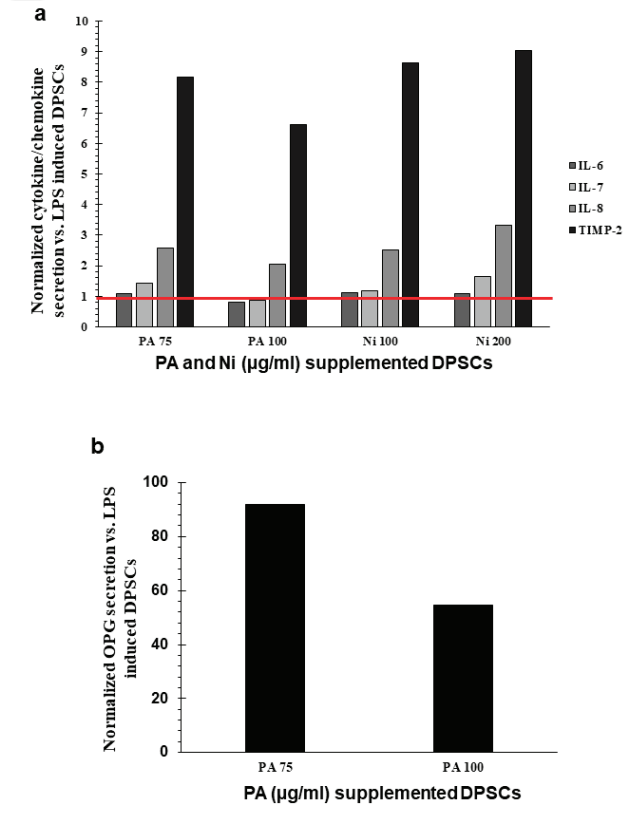

Figure 5 Effects of Proanthocyanidin (PA) and nisin on the expression of IL-6 (a,b) and IL-8 (c,d) in DPSCs and LPS induced-DPSCs. DPSCs treated with 2 µg/ml LPS for 24 h are the LPS induced DPSCs (b,d). Subsequently, DPSCs were conditioned with different concentrations of PA and Ni for 24h. IL-6 and IL-8 expression levels in the culture medium of PA and nisin conditioned DPSCs/ LPS induced DPSCs were measured using ELISA. Significant differences were found by paired t test. * $p<0.05$ (mean ± std, n=3).

Slika 5. Učinci proantocijanidina (PA) i nizina na ekspresiju IL-6 (a, b) i IL-8 (c, d) u DPSC-u i DPSC-u induciranom LPS-om; DPSC tretiran s 2 µg/mL LPS-a tijekom 24 sata predstavlja LPS-inducirani DPSC (b, d); poslije toga DPSC je kondicioniran različitim koncentracijama PA-e i nizina tijekom 24 sata; razine ekspresije IL-6 i IL-8 u mediju kulture DPSC-a kondicioniranoga s PA-om i nizinom/LPS induciranom DPSC-u mjerene su s pomoću ELISA-e; značajne razlike utvrđene su uparenim t-testom; * $p < 0,05$ (srednje ± SD, n = 3)

Figure 6 Cytokine/chemokine secretions of LPS-induced DPSCs which were conditioned with Proanthocyanidin (PA) and nisin (Ni) after normalization based on PA and Ni-supplemented conditions. Different secretion profile of IL-6, IL-7, IL-8 and TIMP-2 in LPS induced DPSCs at 75, 100 µg/ml PA and 100,200 µg/ml Ni concentrations (a). OPG secretion profile of LPS induced DPSCs at 75, 100 µg/ml PA (b), (mean ± std, n=3).

Slika 6. Sekrecija citokina/kemokina LPS-induciranog DPSC-a koje su kondicionirane proantocijanidinom (PA) i nizinom (Ni) nakon normalizacije temeljene na uvjetima s dodatkom PA-e i Ni-e; različiti profil sekrecije IL-6, IL-7, IL-8 i TIMP-2 u LPS induciranom DPSC-u pri koncentracijama nizina od 75, 100 µg/mL i protoantocijanidina od 100, 200 µg/mL (a); profil sekrecije OPG-a u LPS induciranom DPSC-u pri 75, 100 µg/mL PA-e (b) (srednje ± SD, n = 3)

Cell Viability

The cytotoxic effect of PA and Ni on LPS induced or control DPSCs was determined by a water-soluble tetrazolium-based assay. Since 25, 50 µg/ml concentrations of PA and Ni did not show any effects on viability of DPSCs (data not shown), PA and Ni at 75, 100 and 200 µg/ml concentrations were evaluated (Figure 2). DPSCs conditioned with 75 µg/ml PA (1.48-fold, $p<0.05$) and 75 µg/ml Ni (1.37-fold, $p<0.05$) showed significantly higher cell viability results compared to DPSCs alone condition (Control). DPSCs showed significantly lower cell viability when conditioned with increasing concentrations of PA (2.80-fold and 2.85-fold for 100 and 200 µg/ml PA respectively, $p<0.05$). LPS induction of DPSCs for 24h resulted in significantly lower cell viability compared to DPSCs control (1.37- fold, $p<0.05$). At 100 µg/ml Ni containing condition, LPS-induced DPSCs showed significantly higher cell viability results than control DPSCs (1.83 – fold, $p<0.05$). Cell viability of LPS induced DPSCs was tended to increase after conditioned with 75 µg/ml PA and 200 µg/ml Ni (1.2- fold, $p>0.05$).

Antibacterial Activity of Proanthocyanidin and Nisin

Antibacterial properties of PA and Ni were also evaluated. Figure 3a and c shows the bacteria colony counts results and Figure 3b and 3d shows representative photographs of agar plates with the counts of bacteria after 24 h incubation time for different concentrations of PA. Firstly, 25-50 and 75 µg/ml of PA concentrations did not show antibacterial activity against *E. coli*. However, in 100 µg/ml PA concentration, live bacteria number began to decrease compared to control bacteria. In the end, 200 µg/mL PA completely killed *E. coli.* Conversely, PA exhibited significant antibacterial activity against *S. aureus* at all concentrations. Figure 3e and Figure 3g show the bacteria colony counts results as well as Figure 3f, and Figure 3h shows representative photographs of agar plates with the counts of bacteria after 24 h incubation time for different concentrations of Ni. As shown in Figure 3e and 3f, Ni did not show antimicrobial activity at the 25-50-75-100 µg/ml concentration for *E. coli*. The number of *E. coli* bacteria increased until all bacteria were killed at 200 µg/ml concentration. However, as illustrated in Figure 3g and 3h, Ni showed a strong antibacterial property against *S. aureus* at all concentrations.

TNF-α, IL-6 and IL-8 Expressions of DPSCs

To determine the expression of TNF-α, IL-6 and IL-8 from DPSCs, DPSCs were cultured with or without 2 µg/ml LPS for 24h and were conditioned for 24 hours with 0 (control), 75, 100, and 200 µg/ml PA and Ni concentrations. Figure 4, and 5 shows the expression levels of TNF-α, IL-6 and IL-8 of DPSCs and LPS-induced DPSCs, respectively. According to the results detected by ELISA methods, for the groups of DPSCs, TNF-α expression of DPSCs significantly decreased at 75 µg/ml PA, 75 and 100 µg/ml Ni concentrations compared to control condition (1.24-fold, 1.49-fold and 1.44-fold, respectively, $p<0.05$). LPS induction of DPSCs significantly upregulated the TNF-α expression com-

Vijabilnost stanica

Citotoksični učinak PA-e i Ni-a na DPSC potaknut LPS-om, ili kontrolni DPSC određen je s pomoću testa temeljenog na tetrazoliju topljivom u vodi. Budući da koncentracije PA-e i Ni-a od 25 i 50 µg/mL nisu pokazivale nikakve učinke na vijabilnost DPSC-a (podatci nisu prikazani), procijenjeni su pri koncentracijama od 75, 100 i 200 µg/mL (slika 2.). DPSC kondicioniran sa 75 µg/mL PA-e (1,48 puta, $p < 0,05$) i 75 µg/mL Ni-a (1,37 puta, $p < 0,05$) pokazao je značajno veće rezultate vijabilnosti stanica u usporedbi s DPSC-om bez kondicioniranja (kontrola). Vijabilnost DPSC-a značajno se smanjivala s porastom koncentracije PA-e (2,80 puta i 2,85 puta za 100 i 200 µg/ml PA, $p < 0,05$). Indukcija DPSC-a LPS-om tijekom 24 sata rezultirala je značajno smanjenom vijabilnošću stanica u usporedbi s kontrolom DPSC-a (1,37 puta, $p < 0,05$). U uvjetima sa 100 µg/mL nizina, DPSC-i inducirani LPS-om pokazali su značajno veće rezultate vijabilnosti stanica od kontrolnoga DPSC-a (1,83 puta, $p < 0,05$). Vijabilnost stanica DPSC-a induciranog LPS-om imala je tendenciju povećanja poslije kondicioniranja sa 75 µg/mL PA-e i 200 µg/mL Ni-a (1,2 puta, $p > 0,05$).

Antibakterijsko djelovanje proantocijanidina i nizina

Također su procijenjena antibakterijska svojstva PA-e i Ni-a. Na slikama 3. a i c rezultati su broja bakterijskih kolonija, a slike 3. b i 3. d prikazuju reprezentativne fotografije agar-ploča s brojem bakterija poslije 24 sata inkubacije za različite koncentracije PA-e. Najprije koncentracije PA-e od 25 do 50 i 75 µg/mL nisu pokazale da antibakterijski djeluju na *E. coli*. No pri koncentraciji od 100 µg/mL PA-e broj živih bakterija počeo je opadati u usporedbi s kontrolnim bakterijama. Na kraju je 200 µg/mL PA-e potpuno uništilo *E. coli*. Nasuprot tomu, PA je pokazala značajnu antibakterijsku aktivnost protiv *S. aureus* u svim koncentracijama. Na slikama 3. e i 3. g rezultati su broja bakterijskih kolonija, a slike 3. f i 3. h prikazuju reprezentativne fotografije agar-ploča s brojem bakterija poslije 24-satne inkubacije za različite koncentracije nizina. Kao što se vidi na slikama 3. e i 3. f, nizin nije pokazao antimikrobno djelovanje u koncentracijama od 25-50-75-100 µg/mL *za E. coli.* Broj bakterija *E. coli* povećao se, a sve su bakterije bile uništene pri koncentraciji od 200 µg/mL. Međutim, kako je ilustrirano na slikama 3. g i 3. h, nizin je pokazao snažna antibakterijska svojstva protiv *S. aureus* u svim koncentracijama.

Ekspresije TNF-α, IL-6 i IL-8 DPSC-a

Da bi se odredila ekspresija TNF-α, IL-6 i IL-8 iz DPSC-a, DPSC je kultiviran 24 sata s 2 µg/mL LPS-a ili bez njega te kondicioniran tijekom 24 sata s koncentracijama PA-e i Ni-a od 0 (kontrola), 75, 100 i 200 µg/mL. Slike 4. i 5. pokazuju razine ekspresije TNF-α, IL-6 i IL-8 DPSC-a i LPS-induciranoga DPSC-a. Prema rezultatima dobivenima ELISA metodom, za skupine DPSC-a ekspresija TNF-α značajno se smanjila pri koncentracijama od 75 µg/mL PA-e, 75 i 100 µg/mL Ni-a u usporedbi s kontrolnim uvjetima (1,24 puta, 1,49 puta i 1,44 puta, $p < 0,05$). LPS indukcija DPSC-a značajno je povećala ekspresiju TNF-α u usporedbi s kontrolnim DPSC-om (1,24 puta, $p < 0,05$). Za DPSC induciran LPS-om ek-

pared to control DPSCs (1.24-fold, $p<0.05$). For LPS induced DPSCs, TNF-α expression significantly decreased at 100 and 200 µg/ml PA and at 200 µg/ml Ni compared to LPS-induced DPSCs (1.42-fold, 1.36-fold, and 1.44-fold respectively, $p<0.05$). The TNF-α expression tended to decrease at concentrations of 75 µg/ml PA and at 75-, 100 µg/ml Ni. IL-6 expression of DPSCs increased after PA and Ni conditioning, but it was only statistically significant at 100 µg/ml PA and 75 µg/ml Ni concentrations (1.74- fold and 1.45- fold, respectively, $p<0.05$). IL-6 and IL-8 expression of DPSCs did not change after LPS stimulation ($p>0.05$). IL-6 expression of LPS induced DPSCs increased after PA and Ni stimulation but only statistically significant at 100 and 75 µg/ml PA (1.5- fold and 1.46- fold, respectively, $p<0.05$). IL-8 expression of DPSCs significantly decreased at 75, 100 and 200 µg/ml PA conditioned concentrations compared to controls (1, 41-fold, 1.31-fold and 1.30-fold, respectively, $p<0.05$). IL-8 expression of LPS induced DPSCs increased significantly at 100 µg/ml PA and decreased at 200 µg/ml Ni conditioned concentration compared to only LPS induced DPSCs (1.06-fold and 1.15- fold, respectively, $p<0.05$).

Cytokine Array Analysis

Finally, our objective was to determine the pattern of 80 cytokines/chemokines/growth factors released by the LPS induced, PA or Ni conditioned DPSCs and compared it with the condition of LPS induced DPSCs. This was accomplished using a semiquantitative antibody-based cytokine array (RayBiotech, AAHCYT-5-8). The levels of IL-6, IL-7, IL-8, tissue inhibitor matrix metalloproteinases (TIMP-2) and osteoprotegerin (OPG) of LPS induced DPSCs conditioned with 0, 75 and 100 µg/ml PA, 0, 100 and 200 µg/ml Ni normalized versus those of LPS induced DPSCs control condition (Figure 6). OPG secretion of LPS-induced DPSCs conditioned with 75 and 100 µg/ml PA concentrations was at higher levels (92-fold and 55-fold respectively). IL-8 and TIMP-2 secretion was higher at PA and Ni conditioned DPSCs compared to LPS induced DPSCs condition. IL-7 secretion was higher at 200 and 100 µg/ml Ni, 75 µg/ml PA concentrations (1.63- fold, 1.18- fold and 1.42-fold, respectively).

Discussion

DPSCs are promising candidates for stem cell-based regenerative therapies, from regenerative endodontic applications to central nervous disorders, due to ease of isolation, immunomodulatory, and anti-inflammatory capacities (22). Conditioning stem cells with stress-forming inducers such as LPS, hypoxia, and reactive oxygen radicals for mimicking the possible clinical environment of damaged tissue, and then inducing with a molecule/extract/material to improve cell proliferation, paracrine ability and therapeutic potential is among the stem cell-based studies (23). Using biologically compatible materials and the minimization of the possible adverse impacts on biological tissues is essential for obtaining the most favorable results in biomedical applications, and that may be exerted based on such basic/preclinic research results. Trends for using natural products as a biomaterial in the biomedical field have increased dramatically during the

spresija TNF-α značajno se smanjila pri koncentracijama od 100 i 200 µg/mL PA-e te pri 200 µg/mL Ni-a u usporedbi s DPSC-om induciranim LPS-om (1,42 puta, 1,36 puta i 1,44 puta, $p < 0,05$). Ekspresija TNF-α tendenciozno se smanjivala pri koncentracijama od 75 µg/mL PA-e te pri 75 i 100 µg/mL Ni-a. Ekspresija IL-6 DPSC-a povećala se nakon kondicioniranja PA-e i Ni-a, ali bila je statistički značajna samo pri koncentracijama od 100 µg/mL PA-e i 75 µg/mL Ni-a (1,74 puta i 1,45 puta, $p < 0,05$). Ekspresija IL-6 i IL-8 DPSC-a nije se promijenila poslije stimulacije LPS-om ($p > 0,05$). Ekspresija IL-6 LPS-induciranoga DPSC-a povećala se nakon stimulacije PA-e i Ni-a, ali bilo je statistički značajno samo pri koncentracijama od 100 µg/mL PA-e (1,5 puta) i 75 µg/mL Ni-a (1,46 puta, $p < 0,05$). Ekspresija IL-8 DPSC-a značajno se smanjila pri koncentracijama od 75, 100 i 200 µg/mL PA-e u usporedbi s kontrolama (1,41 puta, 1,31 puta i 1,30 puta, $p < 0,05$). Ekspresija IL-8 LPS-induciranoga DPSC-a značajno se povećala pri 100 µg/mL PA-e i smanjila pri 200 µg/mL Ni-a u usporedbi sa samo LPS-induciranim DPSC-om (1,06 puta i 1,15 puta, $p < 0,05$).

Analiza niza citokina

Konačno, naš je cilj bio odrediti uzorak od 80 citokina/kemokina/faktora rasta koji se oslobađaju iz LPS- induciranih UV-UVPA ili Ni-kondicioniranoga DPSC-a i usporediti ga s LPS-induciranim DPSC-om. To je postignuto korištenjem polukvantitativnoga citokinskog niza na temelju antitijela (RayBiotech, AAHCYT-5-8). Razine IL-6, IL-7, IL-8, tkivnog inhibitora metaloproteinaza matrice (TIMP-2) i osteoprotegerina (OPG) LPS-induciranih DPSC-a kondicioniranih s 0, 75 i 100 µg/mL PA-e, 0, 100 i 200 µg/mL Ni-a, normalizirane su u odnosu na kontrolni LPS-inducirani DPSC (slika 6.). Sekrecija OPG-a LPS-induciranoga DPSC-a kondicioniranoga s koncentracijama od 75 i 100 µg/mL PA-e bila je na višim razinama (92 puta i 55 puta). Sekrecija IL-8 i TIMP-2 bila je veća kod DPSC-a kondicioniranoga s PA-e i Ni-a u usporedbi s LPS-induciranim DPSC-om. Sekrecija IL-7 bila je veća kod koncentracija 200 i 100 µg/mL Ni-a, te 75 µg/mL PA-e (1,63 puta, 1,18 puta i 1,42 puta).

Rasprava

DPSC pokazuje obećavajuće rezultate u regeneracijskoj terapiji temeljenoj na matičnim stanicama – od primjene u regeneracijskoj endodonciji do liječenja poremećaja središnjega živčanog sustava, zbog jednostavnosti izolacije te imunomodulacijskih i protuupalnih svojstava (22). Unaprjeđenje stanične proliferacije, parakrine sposobnosti i terapijskog potencijala provodi se kondicioniranjem matičnih stanica stresom, poput LPS-a, hipoksije i reaktivnih kisikovih radikala kako bi se oponašalo moguće kliničko okruženje oštećenog tkiva, a zatim induciralo molekulom/ekstraktom/materijalom (23). Upotreba biološki kompatibilnih materijala i minimiziranje mogućih negativnih učinaka na biološka tkiva ključno je za postizanje najpovoljnijih rezultata u biomedicinskim primjenama, a to se može ostvariti na temelju takvih osnovnih/pretkliničkih istraživanja. Trend korištenja prirodnih proizvoda kao biomaterijala u biomedicinskom polju

last decade. Natural products are usually either of prebiotic-derived or derived from microbes, plants, and animal sources. In the present study, biological effects of various concentrations of grape seed-derived "proanthocyanidin" and *Lactobacillus lactis*-derived "nisin" on DPSCs, and also antibacterial effects against gram-positive S. *aureu*s and gram-negative E. *coli* were evaluated. LPS from *E coli* was used to as a bacterial inducer for pulpitis because it has been found to be most frequent inducer in the literature (24), although it is not a microorganism involved in caries formation.

Oligomeric proanthocyanidins (OPCs), a group of polyphenols in plants such as grapes and cranberries, are well known for their naturally occurring antioxidant activity and the scavenging of free radicals directly (25). Dos Santos et al. (26) evaluated the metabolism of pulp cells following both direct and indirect contact with the different concentrations of grape extract (0.0065–6.5%) for 1 h. They confirmed the non-cytotoxicity of OPCs in both direct or transdentinal conditions and recommended further experiments for establishing its stimulating potential. Tissue regeneration capacity of OPCs (as a crosslinking agent in Type I collagen membrane) and antibacterial properties have also been evaluated and it has been reported that 10% OPCs-Col membrane was non-toxic, and it stimulated the proliferation of L929 and MG-63 cells in the *in vitro* study. Cardoso et al. (27) evaluated the cytotoxic and genotoxic effects of various concentrations (100, 50, 10, and 5 µg/mL) of grape seed extract (GSE) on human gingival fibroblasts (HGF). They reported that the highest GSE concentrations (100 and 50 µg/mL) were both genotoxic and cytotoxic on HGF and GSE, while GSE at concentrations of 10 and 5 µg/mL enhanced cell viability after a 24h period. In our study, we found that PA at 75 µg/ml concentration increased the cell viability of both DPSCs (significantly) and LPS-induced DPSCs (not significantly). Ni is considered as a safe, natural food preservative and it has also attention as a potential therapeutic alternative to antibiotics. To date, immunomodulatory role of Ni and effects of this peptide on some oral bacteria species have been evaluated (15, 28). Although its usage together with dental materials was studied or recommended, so far, there has been no available study regarding the biologic effects of Ni to DPSCs. In a study by Eftekhari et al. (29), nisin was found to enhance the differentiation of induced pluripotent stem cells into neuronal lineages. Namjoo et al. (30) showed that preconditioning with Ni improved the viability and the anti-apoptotic capacity of MSCs. Similarly, in our study Ni at 75 and at 100 µg/ml elevated the cell viability of DPSCs and LPS-induced DPSCs significantly.

TNF-α, IL-6 and IL-8 expressions of DPSCs and LPS-induced DPSCs after exposed to distinct concentrations of PA and Ni were analyzed by ELİSA. TNF-α is a prominent proinflammatory cytokine known to elevate protein expressions associated with cellular response to stress and apoptosis (31). It has been reported that oligomerized grape seed polyphenols at 20 µg/ml showed decreased inflammatory changes by lower TNF-α expression in adipocyte and macrophage co-culture condition (32). Another study, it was highlighted that decreased IL-6, IL-8 and TNF-α expression was ob-

drastično je porastao tijekom posljednjeg desetljeća. Prirodni proizvodi obično se dobivaju od prebiotika ili od mikroba, biljaka i životinjskih izvora. U ovom istraživanju procijenjeni su biološki učinci različitih koncentracija proantocijanidina dobivenog iz sjemenki grožđa i nizina izvedenoga iz *Lactobacillusa lactis* na DPSC, te antibakterijski učinci na gram-pozitivni *S. aureus* i gram- negativnu *E. coli*. LPS iz *E. coli* korišten je kao bakterijski induktor pulpitisa jer je u literaturi utvrđen kao najčešći induktor (24), iako nije mikroorganizam koji sudjeluje u nastanku karijesa.

Oligomerni proantocijanidini (OPC), skupina polifenola u biljkama kao što su grožđe i brusnice, dobro su poznati po svojem prirodnom antioksidacijskom djelovanju i izravnom hvatanju slobodnih radikala (25). Dos Santos i suradnici (26) procijenili su metabolizam stanica pulpe poslije izravnoga i neizravnoga kontakta s različitim koncentracijama ekstrakta grožđa (0,0065 – 6,5 %) tijekom 1 sata. Potvrdili su necitotoksičnost OPC-a u izravnim ili transdentinalnim uvjetima i preporučili daljnje pokuse za utvrđivanje njegova stimulativnog potencijala. Kapacitet regeneracije tkiva OPC-a (kao sredstva za umrežavanje u kolagenskoj membrani tip I) i antibakterijska svojstva također su procijenjeni te je objavljeno da je 10 % OPCs-Col membrana netoksična i stimulirala je proliferaciju L929 i MG 63 stanice u istraživanju *in vitro*. Cardoso i suradnici (27) procijenili su citotoksične i genotoksične učinke različitih koncentracija (100, 50, 10 i 5 µg/mL) ekstrakta sjemenki grožđa (GSE) na ljudske gingivne fibroblaste (HGF). Izvijestili su da su najviše koncentracije GSE-a (100 i 50 µg/mL) bile i genotoksične i citotoksične na HGF i GSE, dok je GSE u koncentracijama od 10 i 5 µg/mL povećao vitalnost stanica poslije 24 sata. U našem smo istraživanju otkrili da je PA u koncentraciji od 75 µg/mL povećala vitalnost stanica i DPSC-a (značajno) i DPSC-a induciranog LPS-om (beznačajno). Nizin se smatra sigurnim, prirodnim konzervansom za hranu, a privlači pozornost i kao potencijalna terapijska alternativa antibioticima. Do danas je procijenjena njegova imunomodulacijska uloga i učinci na neke vrste oralnih bakterija (15, 28). Iako je uporaba toga peptida zajedno sa stomatološkim materijalima proučavana ili preporučena, dosad nije bilo dostupnih istraživanja o biološkim učincima Ni-a na DPSC. U istraživanju Eftekharija i suradnika (29) utvrđeno je da nizin pojačava diferencijaciju induciranih pluripotentnih matičnih stanica u neuronske loze. Namjoo i suradnici (30) pokazali su da predkondicioniranje s nizinom poboljšava vijabilnost i antiapoptotski kapacitet MSC-a. Slično tomu, u našem istraživanju je Ni pri 75 i pri 100 µg/mL značajno povećao vijabilnost stanica DPSC-a i DPSC-a induciranog LPS-om.

Ekspresije TNF-α, IL-6 i IL-8 DPSC-a i DPSC-a induciranog LPS-om nakon izlaganja različitim koncentracijama PA-e i Ni-a analizirane su ELİSA-om. TNF-α istaknuti je proupalni citokin za koji se zna da povećava ekspresiju proteina povezanu sa staničnim odgovorom na stres i apoptozu (31). Zabilježeno je da su oligomerizirani polifenoli sjemenki grožđa pri 20 µg/mL pokazali smanjene upalne promjene manjom ekspresijom TNF-α u uvjetima subkulture adipocita i makrofaga (32). U drugom istraživanju istaknuto je da je smanjena ekspresija IL-6, IL-8 i TNF-α dobivena u staničnoj

tained in human colorectal adenocarcinoma cell line Caco-2 after treatment of grape seed extract (GSE), with or without LPS (25 μg/mL). As for the effect of Ni on TNF-α expression, it was exhibited in a previous study (19) that Ni Z, a class of lantibiotics, at 50 μg/ml induced the secretion of IL-8 and reduced TNF-α expression in response to bacterial LPS in the human peripheral blood mononuclear cells. Ni Z may selectively modulate host immune responses and contribute to protective host immunity with its immunomodulatory activities (19). Similar to what has been found in the literature, PA at 75μg/ml and Ni at 75 and 100μg/ml decreased TNF-α expression of DPSCs significantly in our study. As for LPS-induced DPSCs conditions, PA at 100 and 200 μg/ml and Ni at 200 μg/ml decreased TNF-α significantly. Ni at 75 μg/ml, PA at 75 and 100 μg/ml have significantly increased IL-6 synthesis in DPSCs, and an upregulation trend in LPS-induced DPSCs was also observed, although not significantly. This may be explained by the immunoregulatory role of IL-6, a dual function that possess both pro-inflammatory and anti-inflammatory (or regenerative) properties (33). It is a biologically active factor secreted by MSCs and has many biological functions such as regulating migration as well as stimulating mitosis and angiogenesis (34). It was observed that osteogenically differentiated DPSCs showed remarkably higher IL-6 expression than undifferentiated DPSCs (35). Similarly, IL-6-stimulated DPSCs exhibited higher osteogenic differentiation and stronger osteogenic markers than non-stimulated DPSCs (36). In our study, although LPS induction did not alter the IL-6 expression in DPSCs, higher expression of IL-6 due to PA may be explained by the induction of the regenerative process, which increased TIMP-2, and OPG. Besides, after material induction, increased cytokine releasing such as IL-1α, IL-1β, IL-6, and IL-8 from mineralizing cells, mild and acute inflammatory responses may also contribute to pulp , and hence the clinical repair (37). IL-8 is one of the pro-inflammatory and immunomodulatory mediators that were defined as a chemoattractant of neutrophils, recruited in acute inflammation, as well as chemotactic of endothelial cells with a major role in angiogenesis. It has also a major role in odontoblast defense against dentin-invading bacteria. Increasing IL-8 expression was reported in osteogenically differentiated MSCs and also in LPS-stimulated odontoblasts (37). The results obtained in our study show that IL-8 expression in DPSCs decreased at all PA concentrations, but after LPS stimulation they increased at PA 100 significantly, and at Ni 200 vice versa. However, they did not change at other concentrations. Previous studies (38, 39) reported anti-inflammatory effects of bioflavonoids due to lower IL-8 expression but is difficult to compare the studies since there were differences in doses, cells and types of bioflavonoid source. Kindrachuk et al. (19) reported that Ni Z at 50 μg/ml induced the IL-8 expression in PBMCs, hence it may modulate the host immune response. Only Ni at 100 μg/ml (not significantly) increased IL-8 expression of DPSCs in our study. IL-8 expressions regulate in stimulus-specific and cell type-specific manner (40). PBMCs, hematopoietic stem cell-derived, and DPSCs may show distinct expression patterns to Ni. It has been stated in a review regarding the immunomodulatory

liniji ljudskoga kolorektalnog adenokarcinoma Caco-2 poslije tretmana ekstraktom sjemenki grožđa (GSE), s LPS-om ili bez njega (25 μg/mL). Kad je riječ o učinku Ni-a na ekspresiju TNF-α, istaknuto je u prethodnoj studiji (19) da Ni Z, klasa antibiotika, pri 50 μg/mL, inducira izlučivanje IL-8 i smanjuje ekspresiju TNF-α u odgovoru na bakterijski LPS u mononuklearnim stanicama periferne krvi čovjeka. Nizin Z može selektivno modulirati imunosni odgovor domaćina i pridonijeti zaštitnoj imunosti domaćina svojim imunomodulacijskim djelovanjem (19). Slično onomu što je pronađeno u literaturi, PA pri 75 μg/mL i Ni pri 75 i 100 μg/mL značajno su smanjili ekspresiju TNF-α DPSC-a u našem istraživanju. Kad je riječ o stanju DPSC-a induciranoga LPS-om, PA pri 100 i 200 μg/mL i Ni pri 200 μg/mL značajno su smanjili TNF-α. Nizin pri 75 μg/mL, PA pri 75 i 100 μg/mL značajno su povećali sintezu IL-6 u DPSC-u, a uočen je i trend regulacije u DPSC-u induciranom LPS-om, iako ne značajno. To se može objasniti imunoregulacijskom ulogom IL-6, dvostrukom funkcijom koja posjeduje i proupalna i protuupalna (ili regeneracijska) svojstva (33). To je biološki aktivan čimbenik koji izlučuje MSC i ima mnoge biološke funkcije poput regulacije migracije i poticanja mitoze i angiogeneze (34). Uočeno je da je osteogenski diferencirani DPSC pokazao značajno veću ekspresiju IL-6 nego nediferencirani DPSC (35). Slično tomu, DPSC stimuliran s IL-6 pokazao je veću osteogenu diferencijaciju i jače osteogene markere od nestimuliranoga DPSC-a (36). U našem istraživanju, iako indukcija LPS-a nije promijenila ekspresiju IL-6 u DPSC-ima, veća ekspresija IL-6 zbog PA-e može se objasniti indukcijom regeneracijskoga procesa koji je povećao TIMP-2 i OPG. Osim toga, nakon materijalne indukcije, povećanog otpuštanja citokina kao što su IL-1α, IL-1β, IL-6 i IL-8 iz mineralizirajućih stanica, blagi i akutni upalni odgovori također mogu pridonijeti pulpi, a time i kliničkoj reparaciji (37). IL-8 jedan je od proupalnih i imunomodulacijskih medijatora koji su definirani kao kemoatraktant neutrofila, regrutiran u akutnoj upali, te kemotaksik endotelnih stanica s glavnom ulogom u angiogenezi. Također ima važnu zadaću u obrani odontoblasta od bakterija koje napadaju dentin. Povećanje ekspresije IL-8 zabilježeno je u osteogeno diferenciranim MSC-ima te također u odontoblastima stimuliranima LPS-om (37). Rezultati dobiveni u našem istraživanju pokazuju da se ekspresija IL-8 u DPSC-ima smanjila u svim koncentracijama PA-e, ali nakon stimulacije LPS-om značajno se povećala kod PA-e 100, a kod Ni-a 200 dogodilo se obrnuto. Međutim, nisu se promijenile u drugim koncentracijama. U dosadašnjim istraživanjima (38, 39) autori su izvijestili o protuupalnim učincima bioflavonoida zbog niže ekspresije IL-8, ali teško ih je uspoređivati jer su postojale razlike u dozama, stanicama i vrstama izvora bioflavonoida. Kindrachuk i suradnici (19) istaknuli su da Ni Z pri 50 μg/mL inducira ekspresiju IL-8 u PBMC-ima, stoga može modulirati imunosni odgovor domaćina. Samo je Ni pri 100 μg/mL (ne značajno) povećao ekspresiju IL-8 DPSC-a u našem istraživanju. Ekspresija IL-8 regulira se na način specifičan za podražaj i tip stanice (40). PBMC, hematopoetske matične stanice, i DPSC mogu pokazivati različite obrasce ekspresije za Ni. U pregledu imunomodulatornih svojstava nizina navedeno je da protuupalno djeluje na zaraženi orga-

properties of Ni that it has anti-inflammatory effects on the infected organism. However, the discrepancies in the results of studies with regard to the effect of Ni on cytokine production were reported as resulting in the differences in the experimental models (different types of cells, concentrations of nisin, or incubation times) (41). To our knowledge, our study represents the first report on the effects of Ni on DPSCs.

Matrix metalloproteinases (MMPs), a group of host-derived proteolytic enzyme are responsible for breaking down extracellular matrix (ECM) components in both physiologic and pathologic conditions (42). The activation of MMPs from proenzymes and their tissue inhibitors (TIMP) control the catalytic activity of MMPs. Inhibiting the secretion of MMPs could be an effective strategy to prevent and manage pathological tissue damage (42). La et al. (11) observed no obvious cytotoxic effects (cell viability up to 90%) after the 24h induction of macrophages with up to 100 μg/ml GSE that contains 52% PA. They treated macrophages with different concentrations of GSE (0, 25, 50, and 100 μg/ml) for 2h and then stimulated the cells with LPS (1μg/ml) for 24h to assess the impact of this extract on MMP secretion. Their findings indicated that non-toxic concentrations of GSE inhibited the secretion of MMP-1, -3, -7, -8, -9, and -13 by LPS-stimulated macrophages, thus indicating that the polyphenols in GSE may assist in preventing the excessive accumulation of MMPs. According to our cytokine array results, both Ni at 100, 200 and PA at 75, 100 μg/ml concentrations expressed higher TIMP-2. TIMPs are specialized inhibitors that form a stable complex with MMPs. TIMPs can directly promote ECM deposition by inhibiting the MMPs, hence ECM proteolysis, and indirectly regulate ECM turnover (43). In the present study, it was observed that when DPSCs are exposed to stressful conditions, they secrete greater amounts of neurotrophic factors, such as BDNF, GDNF, as well as TIMP-2 which helps protect the tissue integrity by inhibiting MMP activity (44). Higher expressions of TIMP-2 were also observed in inflamed dental pulp compared to the healthy dental pulp (45). Similarly, DPSCs exposed to LPS are under stressful conditions and have less cell viability and more TNF-α expression in our study. Higher TIMP-2 expression of LPS-induced DPSCs after exposure to Ni at 100, 200, and PA at 75, 100 μg/ml concentrations may show increasing ECM accumulation by which these molecules provide a favorable environment. Interleukin-7 (IL-7) is a multipotent cytokine that plays a vital role in maintaining the homeostasis of the immune system by regulating T-cell development, proliferation, differentiation and B-cell maturation (46). Its inhibitor effect on osteoclastogenesis was also shown in previous studies (47, 48). The human IL-7 overexpression in the osteoblast lineage of mice resulted in higher trabecular bone volume by mCT in vivo and lower osteoclast formation (48). Trubiani et al. (49) showed that IL-7 expression increased during osteogenic differentiation of the periodontal ligament-derived MSCs, and they highlighted its function in promoting autocrine growth and delivering survival and differentiation signals to the adjacent odontogenic structures. However, controversial results are present regarding the effects of IL-7 in bone metabolism. There are studies reporting

nizam. Međutim, prijavljeno je da odstupanja u rezultatima istraživanja, s obzirom na učinak nizina na proizvodnju citokina, rezultiraju razlikama u eksperimentalnim modelima (različiti tipovi stanica, koncentracije nizina ili vremena inkubacije) (41). Koliko znamo, naše istraživanje prvo je izvješće o učincima Ni-a na DPSC.

Matrične metaloproteinaze (MMP), skupina proteolitičkih enzima podrijetlom iz domaćina, odgovorne su za razgradnju komponenti izvanstaničnoga matriksa (ECM) u fiziološkim i patološkim stanjima (42). Aktivacija MMP-a iz proenzima i njihovih tkivnih inhibitora (TIMP) kontrolira katalitičku aktivnost MMP-a. Inhibicija izlučivanja MMP-a mogla bi biti učinkovita strategija za prevenciju i upravljanje patološkim oštećenjem tkiva (42). La i suradnici (11) nisu uočili očite citotoksične učinke (preživljavanje stanica do 90 %) poslije 24-satne indukcije makrofaga s do 100 μg/mL GSE-a koji sadržava 52 % PA-e. Tretirali su 2 sata makrofage različitim koncentracijama GSE-a (0, 25, 50 i 100 μg/mL), a zatim stimulirali stanice LPS-om (1 μg/mL) tijekom 24 sata kako bi procijenili utjecaj toga ekstrakta na izlučivanje MMP-a. Njihovi nalazi pokazali su da netoksične koncentracije GSE-a inhibiraju izlučivanje MMP-1, -3, -7, -8, -9 i -13 iz LPS-stimuliranih makrofaga, što upućuje na to da polifenoli u GSE-u mogu pomoći u sprječavanju prekomjernog nakupljanja MMP-a. Prema našim rezultatima niza citokina, i nizin u koncentracijama od 100, 200 i PA u koncentracijama od 75, 100 μg/mL pokazali su viši TIMP-2. TIMP-i su specijalizirani inhibitori koji tvore stabilni kompleks s MMP-om. TIMP-ovi mogu izravno pospješiti taloženje ECM-a inhibicijom MMP-a, a time i proteolizu ECM-a, te neizravno regulirati promet ECM-a (43). U ovom je istraživanju uočeno da, kada su DPSC-i izloženi stresnim uvjetima, izlučuju veće količine neurotrofnih čimbenika, kao što su BDNF, GDNF, te TIMP-2 koji pomaže u zaštiti integriteta tkiva inhibicijom aktivnosti MMP-a (44). Veća ekspresija TIMP-2 također je primijećena u upaljenoj zubnoj pulpi u usporedbi sa zdravom (45). Slično tomu, DPSC izložen LPS-u u stresnim je uvjetima i ima manju vitalnost stanica i veću ekspresiju TNF-α u našem istraživanju. Veća ekspresija TIMP-2 u LPS-induciranom DPSC-u nakon izlaganja nizinu pri koncentracijama od 100, 200 te PA-e pri koncentracijama od 75, 100 μg/mL može pokazati povećanje nakupljanja ECM-a kojim te molekule osiguravaju povoljno okruženje. Interleukin-7 (IL-7) multipotentni je citokin koji je vitalan u održavanju homeostaze imunosnog sustava reguliranjem razvoja T-stanica, proliferacije, diferencijacije i sazrijevanja B-stanica (46). Njegov inhibitorni učinak na osteoklastogenezu također je prikazan u prethodnim istraživanjima (47, 48). Prekomjerna ekspresija ljudskoga IL-7 u lozi osteoblasta miševa rezultirala je većim volumenom trabekularne kosti na mCT-u *in vivo* i manjim stvaranjem osteoklasta (48). Trubiani i suradnici (49) pokazali su da se ekspresija IL-7 povećala tijekom osteogene diferencijacije MSC-a izvedenih iz parodontnog ligamenta te su istaknuli njegovu funkciju u promicanju autokrinog rasta i isporuci signala preživljavanja i diferencijacije susjednim odontogenim strukturama. Međutim, zabilježeni su proturječni rezultati u vezi s učinkom IL-7 na metabolizam kosti. Postoje istraživanja koja govore o njegovoj ulozi u aktivno-

its roles in osteoclastogenesis activity or in suppressing osteogenic differentiation (50, 51). As for the expression of IL-7 in dental pulp, Elmeguid et al. (52) observed lower IL-7 expression in both pulp tissues with reversible and irreversible pulpitis compared to that in healthy controls. In the present study, higher IL-7 expression was observed in Ni at 200, PA at 75 µg/ml compared to only LPS-induced DPSCs. The effect of OPCs and Ni on IL-7 expression needs to be investigated in a greater number of studies. Higher OPG expression of LPS-induced DPSCs when conditioned with PA 75 and 100 µg/ml in our study may be attributed to the potential of PA promoting the odontoblastic differentiation and mineralization process. It has been reported that OPG inhibits osteoclast differentiation through binding to the receptor activator of nuclear factor-kB ligand (RANKL) and preventing RANKL from interacting with RANK, and thereby the OPG/RANKL ratio is an indicator of bone health and reflects the balance between bone formation and resorption (53). High expression of OPG in the odontoblastic layer of healthy and inflamed peripheral pulp samples has been shown (54). OPG and ALP expressions are commonly considered markers of odontoblastic differentiation. Huang et al. (55) reported that platelet-rich fibrin enhanced the cell proliferation and differentiation of DPSCs by up-regulating OPG and ALP expression. As a result, it might have potential in reparative dentin formation. Similarly, OPG expression slightly increased in DPSCs under tension (56). Belisibakis et al. (57) suggested that the increased expression of OPG could provide protection against dentine or bone resorption, and homeostatic balance might shift to tissue formation. Kwak et al. (10) found that grape seed proanthocyanidin significantly descends (RANKL)-induced osteoclast differentiation, hence the activity of mature osteoclasts in bone resorption. They also showed that GSPE protects against LPS-induced bone loss in mice. According to the results of our study, PA at 75 µg/ml concentrations may offer a favorable environment for dentin formation by inducing OPG and TIMP-2 expression and increasing the proliferation of LPS-induced DPSCs.

When an antimicrobial agent confronts its target, it must initially break down through the microbial cell wall. Considering the differences in the cell wall structures, the effect of the antimicrobial agent to be applied can be different due to these two different cell wall characteristics (58). From this point of view, in this study one Gram (+) bacterium strain, *Staphylococcus aureus* and one Gram (-) bacterium strain, *Escherichia coli* were evaluated in antibacterial activity. PA at 75 and 100 µg/ml concentrations showed antibacterial activity against *E. coli* and both PA and Ni at 200µg/ml concentrations completely killed *E. coli*. However, the number of bacteria increased when exposed to Ni at concentrations lower than 200 µg/ml. Since Ni is well known for its potent antibacterial activity against Gram-positive bacteria, but not against Gram-negative bacteria, the number of *E. coli* bacteria increased until all bacteria were killed at 200 µg/ml concentration. The reason for the insensitivity of Gram-negative bacteria to Ni could be attributed to the relatively large size (1.8–4.6 kDa) of Ni, hindering its passage through the outer membrane of Gram-negative bacteria. The polyphenols, like-

sti osteoklastogeneze ili u suzbijanju osteogene diferencijacije (50, 51). Kad je riječ o ekspresiji IL-7 u zubnoj pulpi, Elmeguid i suradnici (52) primijetili su manju ekspresiju IL-7 u tkivu pulpe s reverzibilnim i ireverzibilnim pulpitisom u usporedbi s onom kod zdravih kontrola. U ovom istraživanju veća ekspresija IL-7 uočena je u nizinima pri 200, PA-i pri 75 µg/mL u usporedbi samo s DPSC-om induciranim LPS-om. Učinak OPC-a i Ni-a na ekspresiju IL-7 potrebno je istražiti u većem broju istraživanja. Veća ekspresija OPG-a u DPSC-u izazvanom LPS-om kada je kondicioniran s PA-e 75 i 100 µg/mL u našem istraživanju može se pripisati potencijalu PA-e da potiče odontoblastičnu diferencijaciju i proces mineralizacije. Zabilježeno je da OPG inhibira diferencijaciju osteoklasta vezanjem na aktivator receptora liganda nuklearnog faktora-kB (RANKL) i sprječavanje interakcije RANKL-a s RANK-om, te je zato omjer OPG/RANKL pokazatelj zdravlja kostiju i odražava ravnotežu između formiranja i resorpcije kosti (53). Utvrđena je visoka ekspresija OPG-a u odontoblastičnom sloju uzoraka zdrave i upaljene periferne pulpe (54). Ekspresije OPG-a i ALP-a obično se smatraju markerima odontoblastične diferencijacije. Huang i suradnici (55) izvijestili su da fibrin bogat trombocitima pojačava staničnu proliferaciju i diferencijaciju DPSC-a snažnijom regulacijom ekspresije OPG-a i ALP-a. Rezultat toga mogao bi imati potencijal za reparacijsko stvaranje dentina. Slično, ekspresija OPG-a blago se povećala u DPSC-u pod stresom (56). Belisibakis i suradnici (57) sugerirali su da povećana ekspresija OPG-a može osigurati zaštitu od resorpcije dentina ili kosti, a homeostatska ravnoteža mogla bi se pomaknuti u korist stvaranja tkiva. Kwak i suradnici (10) otkrili su da proantocijanidin iz sjemenki grožđa značajno smanjuje (RANKL)-induciranu diferencijaciju osteoklasta, a time i aktivnost zrelih osteoklasta u resorpciji kosti. Također su pokazali da GSPE štiti od LPS-induciranog gubitka kosti kod miševa.

Prema rezultatima našeg istraživanja, PA u koncentracijama od 75 µg/mL može ponuditi povoljno okruženje za stvaranje dentina inducirajući ekspresiju OPG-a i TIMP-2 i povećavajući proliferaciju DPSC-a induciranoga LPS-om.

Kada se antimikrobni agens suoči sa svojom metom, najprije se mora probiti kroz staničnu stijenku mikroba. S obzirom na razlike u strukturi stanične stijenke, učinak antimikrobnog sredstva koje se primjenjuje može biti različit zbog tih dvaju različitih svojstava stanične stijenke (58). S te točke gledišta, u ovom istraživanju jedan *gr*am (+) soj bakterije *Staphylococcus aureus* i jedan gram (-) soj bakterije *Escherichia coli* procijenjeni su prema antibakterijskom djelovanju. PA u koncentracijama od 75 i 100 µg/mL pokazala je antibakterijsko djelovanje na *E. coli*, a i PA i Ni u koncentracijama od 200 µg/mL potpuno su uništili *E. coli*. Međutim, broj bakterija povećao se pri izlaganju Ni-a u koncentracijama nižima od 200 µg/mL. Budući da je Ni dobro poznat po snažnom antibakterijskom djelovanju na gram-pozitivne bakterije, ali ne i na gram-negativne, broj bakterija *E. coli* povećavao se sve dok sve nisu ubijene pri koncentraciji od 200 µg/mL. Razlog za neosjetljivost gram-negativnih bakterija na nizin mogao bi se pripisati razmjerno velikoj veličini (1,8 – 4,6 kDa) nizina koja ometa njegov prolazak kroz vanjsku membranu gram-negativnih bakterija. Polifenoli, kao i proantocijanidin unu-

wise proanthociyanidin within the GSE, can eradicate Gram-positive and Gram-negative bacteria by modifying the microbial cell permeability and thereby affecting pathways such as nucleic acid synthesis, cell cytoplasmic membrane function, and bacterial metabolism (59). Grape seed extract was used as an endodontic irrigant solution in a previous study (60), and antibacterial activity of 6.5% GSE was exhibited against Gram positive bacteria *E. faecalis* biofilm through confocal laser scanning microscopy. In a recent review on the antimicrobial activity of GSE in endodontic disinfection, it has been reported that GSE exhibited a noteworthy antimicrobial activity, and the effectiveness of GSE was found to be associated with factors such as concentration, physical state, and exposure duration (59).

The present study has several limitations: it might have been more beneficial to evaluate the expression levels of IL-6, IL-8, and TNF-α by normalizing to the cell counts and to assess the cytokines with anti-inflammatory properties. In addition to *S. aureus* and *E. coli*, investigating more predominant bacterial species associated with caries and endodontic infections (61, 62) would allow for a more comprehensive assessment of the antibacterial activity of PA and Ni.

Conclusion

The impacts of PA and Ni on both healthy DPSCs and on LPS-induced DPSCs, with the aim of mimicking the harsh conditions of inflamed pulp, were evaluated in this study. According to the results, PA at 75 µg/ml increased cell viability, decreased TNF-α expression of DPSCs, did not show any cytotoxic effects on LPS-induced DPSCs, and also showed a tendency to decrease TNF-α expression. Besides, it exhibited higher expressions of TIMP-2, OPG, and IL-7 in LPS-induced DPSCs, thus suggesting that PA at 75µg/ml may induce DPSCs toward osteoblastic/odontoblastic differentiation and further hard tissue formation. Ni at 100 µg/ml showed a tendency to increase DPSCs viability and it decreased TNF-α expression. As for the LPS-induced DPSCs, it increased cell viability and showed a tendency to decrease TNF-α expression. Also, Ni at 200 µg/ml decreased TNF-α expression significantly. As natural compounds, both Ni and PA have strong antibacterial effects on Gram-positive bacteria, which could provide a decrease the microbial load. Ni at 200µg/ml provided strong antibacterial effects against Gram-negative bacteria without negatively affecting the viability of both DPSCs and LPS-induced DPSCs. Also, it showed anti-inflammatory activity by decreasing TNF-α expression. Although PA at 200 µg/ml provided strong antibacterial effects against Gram-negative bacteria, its negative effect on DPSCs viability should be considered. Further study is needed to evaluate the osteogenic/odontogenic properties of PA stimulated DPSCs by checking the odontogenic and osteogenic gene expression profiles.

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tar GSE-a, mogu iskorijeniti gram-pozitivne i gram-negativne bakterije modificirajući propusnost mikrobnih stanica i tako utjecati na puteve kao što su sinteza nukleinske kiseline, funkcija stanične citoplazmatske membrane i bakterijski metabolizam (59). Ekstrakt sjemenki grožđa korišten je kao otopina za endodontsko ispiranje u prethodnom istraživanju (60), a antibakterijsko djelovanje od 6,5 % GSE-a prikazano je protiv biofilma gram-pozitivne bakterije *E. faecalis* putem konfokalne laserske skenirajuće mikroskopije. U nedavnom pregledu antimikrobne aktivnosti GSE-a u endodontskoj dezinfekciji, objavljeno je da je GSE pokazao značajnu antimikrobnu aktivnost, a utvrđeno je da je njegova učinkovitost povezana s čimbenicima kao što su koncentracija, fizičko stanje i trajanje izloženosti (59).

Ovo istraživanje ima nekoliko ograničenja: možda bi bilo korisnije procijeniti razine ekspresije IL-6, IL-8 i TNF-α normalizacijom na broj stanica i procijeniti citokine s protuupalnim svojstvima. Uz *S. aureus* i *E. coli*, istraživanje dominantnijih bakterijskih vrsta povezanih s karijesom i endodontskim infekcijama (61, 62) omogućilo bi sveobuhvatniju procjenu antibakterijskog djelovanja PA-e i Ni-a.

Zaključak

U ovom istraživanju procijenjen je utjecaj PA i Ni na zdravi DPSC i na DPSC induciran LPS-om, sa svrhom oponašanja teških uvjeta upaljene pulpe. Prema rezultatima, PA je pri 75 µg/mL povećala vitalnost stanica, smanjila ekspresiju TNF-α DPSC-a i nije pokazala nikakve citotoksične učinke na DPSC-e inducirane LPS-om, a također je pokazala tendenciju smanjenja ekspresije TNF-α. Uz to, pokazala je veću ekspresiju TIMP-2, OPG i IL-7 u DPSC-u induciranom LPS-om, što sugerira da PA pri 75 µg/mL može inducirati DPSC prema osteoblastičnoj/odontoblastičnoj diferencijaciji i daljnjem stvaranju tvrdoga tkiva. Nizin je pri 100 µg/mL pokazao tendenciju povećanja vijabilnosti DPSC-a i smanjio ekspresiju TNF-α. Kad je riječ o DPSC-u induciranom LPS-om, on je povećao vitalnost stanica i pokazao tendenciju smanjenja ekspresije TNF-α. Također, nizin je pri 200 µg/mL značajno smanjio ekspresiju TNF-α. Kao prirodni spojevi, i Ni i PA snažno antibakterijski djeluju na gram-pozitivne bakterije, što može smanjiti mikrobno opterećenje. Nizin u koncentraciji od 200 µg/mL omogućuje snažne antibakterijske učinke na gram-negativne bakterije, bez negativnog utjecaja na vijabilnost DPSC-a i DPSC-a induciranog LPS-om. Također je pokazao protuupalno djelovanje smanjenjem ekspresije TNF-α. Iako je PA od 200 µg/mL imala snažne antibakterijske učinke na gram-negativne bakterije, treba uzeti u obzir njezin negativni učinak na vijabilnost DPSC-a. Potrebna su daljnja istraživanja da bi se procijenila osteogena/odontogena svojstva PA-om stimuliranih DPSC-a provjerom profila ekspresije odontogenih i osteogenih gena.

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Sažetak

Cilj: Željelo se procijeniti biološke učinke proantocijanidina (PA) i nizina (Ni) na matične stanice zubne pulpe (DPSC) te na DPSC-e inducirane LPS-om i njihove antimikrobne učinke na *S. aureus* i *E. coli*. **Materijali i metode:** Nakon karakterizacije DPSC-a, citotoksičnost PA i Ni na DPSC evaluirana je korištenjem soli tetrazolija (WST-1) topljive u vodi. Citokini i kemokini koje otpušta DPSC, te ekspresija razine IL-6, IL-8 i TNF-alfa, detektirani su s pomoću ljudskoga citokinskog niza C5 i enzimskoga imunološkog testa vezanoga za imunosni sustav (ELISA). Antibakterijske aktivnosti PA-e i Ni-a testirane su metodom kapanja na ploču. **Rezultati:** PA je pri 75 µg/mL povećala vijabilnost stanica, smanjila ekspresiju TNF-α u DPSC-u, nije pokazala citotoksične učinke na LPS-inducirani DPSC te je pokazala trend smanjenja ekspresije TNF-α. Također je zabilježeno povećanje ekspresije TIMP-2, OPG-a, IL-7 i IL-8 u LPS-induciranom DPSC-u u usporedbi s DPSC-om. Nizin je pri 100 µg/mL smanjio ekspresiju TNF-α u DPSC-u bez citotoksičnih učinaka. Doveo je do povećanja vijabilnosti stanica i trenda smanjenja ekspresije TNF-α u LPS-induciranom DPSC-u. I Ni i PA imali su snažan antibakterijski učinak na *S. aureus*. Tako je Ni pri 200 µg/mL snažno antibakterijski djelovao na *E. coli,* bez negativnog utjecaja na vijabilnost DPSC-a i LPS-induciranog DPSC-a, te pokazao protuupalno djelovanje smanjenjem ekspresije TNF-α. PA je imala snažan antibakterijski učinak na *E. coli* pri 200 µg/mL, ali je negativno utjecala na vijabilnost DPSC-a. **Zaključak:** PA i Ni u određenim koncentracijama pokazali su imunomodulatorno djelovanje na DPSC i LPS-inducirani DPSC, bez citotoksičnoga učinka, uz snažan antibakterijski učinak na *S. aureus*.

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