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Antimikrobni učinak ozona stvorenog KP brizgalicom visokofrekvencijskoga generatora ozona

Antimicrobial Effect of Ozone Made by KP Syringe of High-Frequency Ozone Generator

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Sažetak

Svrha: Svrha ovog istraživanja bila je procijeniti *in vitro* učinkovitost ozona na suspenziju triju različitih vrsta bakterija inokluiranih u obrađene korijenske kanale ekstrahiranih ljudskih zubi. **Materijal i metode:** Ozon je proizveden od aspiriranog zraka uporabom specijalne KP brizgalice visokofrekvencijskoga generatora ozona *Ozonytron* (Biozonix, München, Njemačka) s pomoću dielektričnog barijernog izboja, te nastavka na štrcaljki postavljenog u pripremljeni korijenski kanal. Ispitivani su mikroorganizmi *Enterococcus faecalis, Staphylococcus aureus* i *Staphyloccocus epidermidis*. **Rezultati:** Ni jedna metoda nije pokazala 100-postotnu učinkovitost u eliminaciji navedenih bakterija u suspenziji. Primjena ozona značajno je smanjila ukupan broj mikroorganizama (89,3 %), te broj svake vrste bakterija posebno (*Staphyloccocus aureus* – 94,0 %; *Staphyloccocus epidermidis* – 88,6 % i *Enterococcus faecalis* – 79,7 %). Ozon proizveden KP brizgalicom bio je statistički znatno učinkovitiji u eliminaciji *Staphyloccocus aureus* i *Staphyloccocus epidermidis* u usporedbi s NaOCl-om kao pozitivnom kontrolom. **Zaključak:** Ukupan broj *Enterococcus faecalis* bio je statistički manji, no bez značajne statističke razlike između ispitivane i pozitivne kontrolne skupine. Između triju vrsta bakterija u suspenziji, primjena KP štrcaljke pokazala je najmanju učinkovitost u eliminaciji *Enterococcus faecalis*.

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Uvod

Jedan od ciljeva endodontskog liječenja jest ukloniti infekciju iz korijenskoga kanala. Unatoč temeljitoj kemijsko-mehaničkoj obradi, vrlo je teško postići taj cilj, te se preostali mikroorganizmi u korijenskome kanalu smatraju glavnim uzrokom neuspjeha u liječenju (1). Natrijev hipoklorit (NaOCl) danas je najčešće korištena antimikrobna irigacijska otopina pri kemijsko-mehaničkoj obradi korijenskih kanala (2). Ukoliko dođe u kontakt s periapikalnim tkivom ili tkivom oralne sluznice, može izazvati citotoksične učinke (3).

Kako bi se poboljšao antimikrobni učinak, u dezinfekcijski postupak liječenja korijenskog kanala uveden je ozon (2, 4, 5). Dio autora (2) koristi se ozoniziranim NaOCl-om a dio upotrebljava ozoniziranu vodu (4, 6 – 9) ili plinoviti ozon (7 – 9). Antimikrobni učinak testiranog agensa može biti različit s obzirom na stanje u kojemu se nalaze ispitivani mikroorganizmi (mogu biti u obliku planktona gdje ne-

Introduction

one of the main goals of an endodontic therapy is to eradicate infection from the root canal. No matter how meticulously a chemo-mechanical treatment is performed, clinicians sometimes fail to achieve this treatment goal, since residual microorganisms seem to be the main cause of treatment failures (1). Sodium hypochlorite (NaOCl) is today the most common antimicrobial irrigation solution used for chemo-mechanical preparation of root canals (2). However, this endodontic irrigant can evoke a cytotoxic response in contact with periapical or oral mucosal tissues (3).

Ozone was used in root canal disinfection therapy to enhance the antimicrobial effect (2, 4, 5). Some researchers (2) used the ozonated NaOCl solution, while others used ozonated water (4, 6-9) and gaseous ozone (7-9). The antimicrobial effect of tested agent could be different regarding the organization of tested microorganisms: bacteria predominantly grow as either planktonic cells which are unattached and live

vezano i slobodno rastu u suspenziji ili mogu biti ugrađeni u biofilm) (8, 10).

Ozon, troatomna alotropska modifikacija kisika, snažan je oksidans koji se može proizvesti s pomoću različitih vrsta ozonskog generatora. Nikola Tesla je 1896. godine patentirao prvi visokofrekvencijski generator ozona u SAD-u. Visokofrekvencijski generator ozona *Ozonytron* (Biozonix, München, Njemačka) koji je korišten u ovom istraživanju, radi na istom principu.

Ozon djeluje biokemijski na različite načine: inaktivacijom bakterija, virusa, gljivica, kvasaca i protozoa; poboljšanjem cirkulacije; stimuliranjem metabolizma kisika, stvaranjem peroksida; razgradnjom malignih tumora, aktivacijom imunosnog sustava (5).

Ozoniziranje NaOCl-a čini ga još učinkovitijim oksidansom; ozon oksidira i uništava stanične stijenke mikroorganizama, ubrzava razlaganje te skraćuje vrijeme potrebno za rutinsko liječenje korijenskoga kanala (2). Chang i suradnici (11) koristili su se u endodontskoj terapiji ozonom stvorenim u uređaju *HealOzone.* U svojoj metodi rada upotrijebili su kombinaciju ozona i NaOCl-a tijekom kemijsko-mehaničke dezinfekcije korijenskoga kanala. Ozon može znatno poboljšati predvidivost liječenja korijenskoga kanala te skratiti vrijeme potrebno za provedbu zahvata (2).

Ozon je učinkovit ako se primjenjuje u dostatnoj koncentraciji i određenom vremenu te pravilno primijeni nakon provedene uobičajene metode čišćenja, širenja i ispiranja korijenskoga kanala (12).

Svrha ovog istraživanja bila je ispitati *in vitro* antimikrobnu učinkovitost ozona primijenjenog u inficiranim korijenskim kanalima izvađenih zuba, a pritom se koristila posebna KP brizgalica visokofrekvencijskoga generatora ozona *Ozonytron* (Biozonix, München, Njemačka).

Materijal i metode

Priprema uzoraka zuba

Odabrano je četrdeset i pet izvađenih jednokorijenskih ljudskih zuba sličnih po veličini i obliku (gornji sjekutići te gornji i donji očnjaci) koji su bili pohranjeni u sterilnoj izotoničnoj fiziološkoj otopini. Zubi su ekstrahirani iz medicinskih razloga u nekoliko stomatoloških ordinacija u Zagrebu, Hrvatska. Njihovo korištenje za ovo istraživanje odobrili su pacijenti informiranim pristankom te 28. lipnja 2013. Istraživačko etičko povjerenstvo. Nakon mehaničkog čišćenja te sterilizacije u *Euroklavu* 23 VS (Melag, Berlin, Njemačka) na 120 °C i 300 kPa, zubi su ponovno pohranjeni u sterilnu izotoničnu fiziološku otopinu u uvjetima 100-postotne vlage i na temperaturi od 37 °C.

Kliničke krune uzoraka odrezane su, uz vodeno hlađenje, dijamantnim brusnim tijelom *TR-11 ISO199/016* (MANI, INC., Utsunomiya, Tochigi, Japan) u području caklinsko-cementnog spojišta. Svi zubi nakon toga su prerezani 13 mm od apeksa, kako bi se osiguralo da svi primjerci imaju jednaku duljinu, te su uloženi u akrilnu smolu *Palavit G* (Heraeus Kulzer GmbH, Wehrheim, Njemačka). Njihovi kanali obrađeni su *ProTaper* rotacijskom instrumentacijskom tehnikom kori-

freely in suspension, or as microorganisms embedded in biofilms (8, 10).

Ozone, which is an allotropic triatomic form of oxygen, is a powerful oxidant that can be produced in various types of ozone generators. In 1896, Nikola Tesla patented the first high frequency ozone generator in the USA. The mode device of high frequency ozone generator Ozonytron (Biozonix, München, Germany) that was used in this study is based on Tesla's principle.

Ozone acts biochemically in different ways. It inactivates bacteria, viruses, fungi, yeast and protozoa. Ozone enhances circulation, stimulates the oxygen metabolism and forms peroxides. It can dissolve malignant tumors and activate the immune system (5).

After being ozonized, sodium hypochlorite (NaOCl) solution becomes an even more effective oxidant. Ozone oxidizes the cell walls of microorganisms and destroys them. It speeds up the dissolution activity and reduces the time required for a routine root canal therapy (2). Chang et al. (11) used ozone in a root canal treatment. It was delivered from the HealOzone unit. They used a technique aimed to combine ozone with NaOCl during chemo-mechanical root canal disinfection. Ozone dramatically improved the predictability of root canal therapy. Moreover, the authors of the study stated that ozone could shorten the time required for treatment (2).

Ozone is effective when prescribed in sufficient concentration, used for adequate time and delivered correctly into root canals after the traditional cleaning, shaping and irrigation has been completed (12).

The aim of this study was to evaluate *in vitro* the antimicrobial efficacy of ozone delivered to infected root canals of extracted teeth by special KP syringe of Ozonytron - a high frequency ozone generator (Biozonix, München, Germany).

Material and methods

Preparation of samples

Forty-five extracted human single rooted teeth (upper incisors and upper and lower canines) stored in sterile isotonic saline solution were selected according to size and shape similarities. Teeth have been extracted for medical reasons in several dental offices in Zagreb, Croatia. Patients agreed to donate their teeth for purposes of this experiment and genuine informed consent was obtained from research participants after the decision of the Research Ethics Committee was made on June 28th, 2013. After mechanical cleaning, and sterilizing in Euroklav 23 V-S (Melag, Berlin, Germany) at 120° C and 300 kPa, the teeth were stored again in a sterile isotonic saline solution in atmosphere of 100% humidity and at 37^0 C.

Clinical crowns of the specimens were sectioned at the cemento-enamel junction using a diamond bur TR-11 ISO199/016 (MANI, INC., Utsunomiya, Tochigi, Japan) with water-cooling spray. All teeth were subsequently resected 13 mm in length from the apex to ensure that all specimens have the same length and embedded in acrylic *Palavit G* resin (Heraeus Kulzer GmbH, Wertheim, Germany). Tooth canals were instrumented using the ProTaper rotary

štenjem konvencionalnog slijeda (S1, SX, S1, S2, F1, F2, F3) prema uputama proizvođača pri 300 okretaja u minuti (Maillefer, Ballaigues, Švicarska). Zubi su bili podijeljeni u jednu ispitivanu skupinu (35 uzoraka) te dvije kontrolne (pozitivna i negativna kontrolna skupina – u svakoj po pet uzoraka).

Tijekom čišćenja i proširivanja kanali su brizgalicom (Becton Dickinson S.A., Huesca, Španjolska) i iglom 27 (BD Drogheda, Španjolska) isprani s 2,0 mL sterilne fiziološke otopine, između svakog rotacijskog instrumenta. Nakon završetka pripreme, 10 mL sterilne fiziološke otopine korišteno je za završnu irigaciju. Zubi, zajedno sa svim plastičnim potrošnim materijalom, sterilizirani su u plazmatskom sterilizatoru (Sterrad 200, Johnson & Johnson, SAD) i svi daljnji postupci provedeni su u aseptičkim uvjetima u laminarnoj struji zraka.

Test sterilnosti

Sterilnost uzoraka određena je prije početka eksperimenta. Korijenski kanali su brizgalicom (Becton Dickinson SA, Huesca, Španjolska) ispunjeni s 0,02 mL sterilnoga moždano-srčanog bujona (MSB) (BBL Brain Heart infusion broth, Becton Dickinson and Company, Sparks, SAD, Le Pont de Cleux, Francuska). Otvori kanala zatvoreni su sterilnim parafinskim uljem (MDSS, Hannover, Njemačka) i prekriveni sterilnim mikroskopskim pokrovnim stakalcem (Vitrognost, cover glass, Biognost, Zagreb, Hrvatska) te je svaki uzorak smješten u plastičnu posudicu s 3 ml sterilnoga MSB-a. Plastične posudice zatvorene su parafinskim uljem i inkubirane (inkubator: Termo-Medicinski aparati, Dugo Selo, Zagreb, Hrvatska) 24 sata pri temperaturi do 35 °C i u atmosferskim uvjetima (13).

Nakon inkubacije sterilni papirnati štapić (ProTaper F_2, Maillefer, Ballaigues, Švicarska) umočen je 30 sekunda u korijenski kanal i uvaljan na krvni agar. Zatim je stavljan u 3 ml sterilnog MSB-a na dodatnu 48-satnu inkubaciju na temperaturi od 35 °C. Nakon toga MSB je nanesen na krvni agar i inkubiran od 18 do 24 sata na 35 °C. MSB iz plastičnih posudica koji je prilegao uz uzorke također je inkubiran na krvnom agaru od 18 do 48 sati na temperaturi od 35 °C. Svi kanali i plastične posudice ostale su tijekom eksperimenta sterilne.

Mikroorganizmi

Mikroorganizmi korišteni u ispitivanju bili su: *Enterococcus faecalis* ATCC 29212 (LGC-ATCC, Wesel, Germany), *Staphylococcus aureus* ATCC 25922 (LGC-ATCC, Wesel, Germany), *Staphylococcus epidermidis = Coagulase – negative staphylococcus* (CNS) (organizam izoliran iz brisa usta). Logaritamske faze rasta bakterija na krvnom agaru (*Enterococcus faecalis, Staphylococcus aureus*, CNS) korištene su za pripremu suspenzije.

Suspenzija je pripremljena u MSB-u s koncentracijom od 10^8 CFU-a (*Colony Forming Units*). Nakon vrtložnog miješanja (Techo Kartell, Noviglio, Italija) 0,01 ml svake suspenzije inokuliran je kalibriranim nosačem na krvni agar (aerobna inkubacija, 24 sata na 35 °C). Jednaki volumeni (1 ml) suspenzija pomiješani su kako bi se dobila konačna suspenzija za inokulaciju.

instrumentation techniqu222e using conventional sequence (S1, SX, S1, S2, F1, F2, F3) according to the manufacturer's instruction at 300 rpm (Maillefer, Ballaigues, Switzerland). The teeth were divided in one experimental group (35 teeth) and two control groups (positive and negative control groups, five teeth each).

During cleaning and shaping, tooth canals were irrigated with 2.0 mL of sterile saline solution between each reamer, using a syringe (Becton Dickinson S.A., Huesca, Spain) and a 27-gauge needle (BD Drogheda, Spain). After completion of tooth preparation, all canals received a final irrigation with 10 ml sterile saline solution. The teeth were plasma sterilized along with all plastic disposable material (Sterrad 200, Johnson &Johnson, USA) and all further manipulations were performed aseptically in laminar flow.

Sterility testing

Before starting the experiment, sterility of the samples was determined. Root canals were filled with 0.02 ml of sterile Brain-Heart infusion broth (BH) (BBL Brain Heart infusion broth, Becton Dickinson and Company, Sparks, USA, Le Pont de Cleux, France) using syringe (Becton Dickinson S.A., Huesca, Spain). Canal orifices were sealed with sterile paraffin oil (MDSS, Hannover, Germany) and covered with sterile microscope cover slide (Vitrognost, cover glass, Biognost, Zagreb, Croatia) and each specimen was placed in plastic tube containing 3 ml of sterile BHI. Plastic tubes were sealed with paraffin oil and incubated (incubator: Termomedicinski aparati, Dugo Selo, Zagreb, Croatia) for 24 h at 35 °C under athmospheric conditions (13).

After incubation, a sterile paper point (ProTaper F_2, Maillefer, Ballaigues, Switzerland) was dipped into the root canal for 30 seconds and rolled back and forth on a blood agar. The paper point was subsequently placed in a 3 ml sterile BH for further 48-hours incubation at 35 °C. After that, BH was placed on a blood agar and incubated at 35 °C for 18-24 hours. Also, BH from plastic tubes adjacent to the outer surface of specimen was incubated on a blood agar at 35 °C for 18-24 hours. All root canals and plastic dishes remained sterile during testing.

Microorganisms

Microorganisms used in this study were: *Enterococcus faecalis* ATCC 29212 (LGC-ATCC, Wesel, Germany), *Staphylococcus aureus* ATCC 25922 (LGC-ATCC, Wesel, Germany), *Staphylococcus epidermidis = Coagulase – negative staphylococcus* (CNS) (aerobic bacteria isolated from mouth swabs). The log phase of bacteria grown on a blood agar plate (*Enterococcus faecalis, Staphylococcus aureus*, CNS) were used for suspension preparation. Suspension was prepared in Brain-Heart Infusion Broth with concentration of 10^8 CFU (Colony Forming Units). After vortexation (Techo Kartell, Noviglio, Italy), 0.01 ml of each suspension was inoculated by calibrated loop (aerobic incubation at 35^0 C for 24 hours) on a blood agar. Equal volumes (1 ml) of suspensions were mixed forming a final suspension for inoculation.

Inokulacija bakterijske suspenzije

Mješavina 0,02 ml bakterijske suspenzije unesena je brizgalicom u korijenski kanal. Kanali su zapečaćeni sterilnim parafinskim uljem i pokriveni sterilnim mikroskopskim pokrovnim stakalcem. Zubi su uronjeni u plastične posudice ispunjene s 3 ml MSB-a. Plastične posudice zapečaćene su parafinskim uljem i inkubirane 24 sata na temperaturi od 35 °C i u atmosferskim uvjetima.

Primjena ozona

Ozon je proizveden i primijenjen KP brizgalicom visokofrekvencijskoga generatora ozona *Ozonytron* (Biozonix, München, Njemačka). KP štrcaljka specifična je vrsta brizgalice koja se sastoji od središnje postavljene staklene cijevi ispunjene plemenitim plinom i okružene titanijskom mrežicom. Ozon je proizveden od aspiriranoga atmosferskog zraka dielektričnim barijernim izbojem u prostoru između staklene cijevi i titanijske mrežice (14). Prema navodu proizvođača, KP štrcaljka sa spremnikom od 2,2 ml za proizvodnju ozona u koncentraciji od 525 ppm, pogodna je za dezinfekciju i obradu korijenskih kanala. Tijekom potiskivanja ozonom obogaćene mješavine zraka kroz nastavak na vrhu KP brizgalice u korijenskih kanal, staklena cijev ulazi u šuplji prostor klipa brizgalice.

Prije primjene ozona korijenski kanali osušeni su sterilnim papirnatim štapićima (ProTaper F_2, Maillefer, Ballaigues, Švicarska).

Kanali su tretirani ozonom s pomoću KP brizgalice i igle veličine 27 (BD Drogheda, Španjolska). KP brizgalica pripremljena je za uporabu prema uputama proizvođača, a instilacija je rađena 5 sekunda brzinom ≈ 0,4 ml/s.

Šezdeset sekundi nakon primjene ozona korijenski kanali ponovno su brizgalicom ispunjeni s 0,02 ml sterilnoga MSB-a.

Mikrobiološka ispitivanja

Mikrobiološka ispitivanja provedena su neposredno nakon primjene ozona uzimanjem uzorka sterilnim papirnatim štapićem (ProTaper F2, Maillefer, Ballaigues, Švicarska) koji je umočen u MSB u korijenskom kanalu te uklonjen poslije 30 sekunda.

Nakon toga papirnati štapić uronjen je u MSB i inaktivacijski agens SCDLP (pepton, natrijev klorid, kalijev dihidrogenfosfat, glukoza, lecitin, polisorbat 80, voda, pH nakon sterilizacije 7,2), medij u skladu s ISO 20743:2007 (E). Nakon vrtložnog miješanja papirnati štapić je uklonjen i 2 ml ekstrahirane tekućine razrijeđeno je u $\log^{10}$ koracima s MSB + inaktivacijski agens. Konačno je 0,1 ml otopine razrijeđene od 10^{-1} do 10^{-5} nanesen sterilnom pipetom na krvni agar.

Hranjive podloge krvnog agara inkubirane su 24 sata na temperaturi do 35 °C. Osim toga, MSB iz plastičnih posudica koji je bio u doticaju s vanjskom površinom uzorka, inkubiran je od 18 do 24 sata na 35° C. Tijekom ispitivanja plastične posudice ostale su sterilne.

Kolonije narasle na hranjivoj podlozi identificirane su standardnim testovima ispitivanja u mikrobiologiji (testovi: katalaze, DNK-ze, koagulaze, *bile aesculin*).

Usporedo s inokulacijom kanala, u svakom eksperimentu obavljena je kontrola rasta i koncentracije bakterija. Eksperi-

Preparation of the bacterial suspension and inoculation

A total of 0.02 ml of bacterial suspension was inserted into root canals by syringe. The root canals were sealed with sterile paraffin oil and covered with sterile microscopic cover slide. Subsequently, the teeth were submerged into 3 ml of BH broth in plastic tubes. The plastic tubes were sealed with paraffin oil and incubated for 24 h at 35 °C under atmospheric conditions.

Ozone gas delivery

Ozone was generated and applied by special KP syringe of high frequency ozone generator Ozonytron (Biozonix, München, Germany). KP syringe is a specific type of syringe that contains a centrally positioned simple glass tube filled with noble gas surrounded with titanium mash. Ozone was generated from aspirated atmospheric air by dielectric barrier discharge in space between the glass tube and titanium mash (14). According to the manufacturer, KP syringe contains a reservoir of 2.2 ml for ozone production in concentration of 525 ppm and is suitable for disinfection and treatment of root canals. A glass tube slides inside the hollow plunger during the process of instillation thus pushing the ozone enriched air mixture through the tip of the syringe into a prepared root canal.

Before being ozonized, root canals were dried using sterile paper point (ProTaper F_2, Maillefer, Ballaigues, Switzerland).

The root canals were ozonized using a KP syringe and a 27-gauge needle (BD Drogheda, Spain). KP syringe was prepared for application according to the manufacturer's recommendation and applied for 5 seconds at the speed of ≈ 0.4 ml/sec.

Sixty seconds after the ozone application, the root canals were refilled with 0.02 ml of sterile BH using syringe.

Microbiological examinations

After ozonation, a microbiological examination was performed using sterile paper point (ProTaper F_2, Maillefer, Ballaigues, Switzerland) which was dipped into BH inside the root canal and removed after 30 seconds. Subsequently, the paper point was dipped in BH + inactivating agent SCDLP medium (Peptone, sodium chloride, Potassium dihydrogen phosphate, glucose, Lecithin, Polysorbate 80, water, pH after sterilization 7,2) according to the industrial standard method ISO 20743:2007(E). After vortexation, the paper point was removed and two ml of extracted fluid were diluted in log 10 steps with BH + inactivating agent. 0.1 ml of the solution diluted up to 10^{-1} to 10^{-5} was applied to a blood agar with sterile pipette.

Blood agar plates were incubated for 24 h at 35 °C. Subsequently, the BH from plastic tubes adjacent to the outer surface of a specimen was incubated on a blood agar at 35 °C for 18-24 hours. All plastic dishes remained sterile during testing.

The colonies grown on plates were identified using standard microbiology testing (bile esculine, catalase, DNA-ase, coagulase testing).

The control of bacterial growth was performed along with the control of concentration in each experiment in parallel

mentalni uzorci, zajedno sa svim plastičnim potrošnim materijalom, sterilizirani su plazmom između ciklusa i testirani na sterilnost na prije opisan način. Prebrojavanje mikroorganizama obavljeno je pri razrjeđenju koje sadržava od 30 do 200 kolonija, a koncentracije su izražene kao $\log^{10}$ (CFU +1).

Kontrolne skupine

Deset uzoraka u kontrolnim skupinama (pet u pozitivnoj i pet u negativnoj) pripremljeno je i inokulirano mikroorganizmima na isti način kao i uzorci u ispitivanoj skupini. U negativnoj kontrolnoj skupini uzorci nisu bili tretirani. Korijenski kanali u pozitivnoj kontrolnoj skupini irigirani su 5 minuta s 2,0 mL 2,5 % v/v NaOCl-a (8). Mjerenje rasta i koncentracije bakterija provedeno je kao i kod ispitivane skupine.

Statistička analiza

Statitička analiza obavljena je u programu PASW Statistics 17.0. Prema Kolmogorov-Smirnovljevu testu, varijable logaritamskih vrijednosti mikroorganizama prije djelovanja, te logaritamske vrijednosti redukcije mikroorganizama nakon toga, nisu pokazale normalnu distribuciju ($p < 0,005$).

Rezultati

Analiza ukupne redukcije mikroorganizama

Primjena ozona KP brizgalicom pokazala je statistički značajnu redukciju broja mikroorganizama – 7,45 ± 1,38 (89,3 %), ($p = 0,0001$) u usporedi s njihovim početnim brojem. U pozitivnoj kontrolnoj skupini ukupan broj mikroorganizama također je bio statistički značajno manji, no ne 100-postotno (68,7 %). U negativnoj kontrolnoj skupini broj mikroorganizama ostao je nepromijenjen.

Analiza prema vrsti mikroorganizma

Redukcija svakoga ispitivanog mikroorganizma statistički je bila značajna u usporedbi s početnom vrijednošću ($p = 0,0001$) (tablica 1.).

U pozitivnoj kontrolnoj skupini redukcija broja pojedinih mikroorganizama statistički je bila značajna u usporedbi s njihovim početnim brojem, no ne 100-postotna (*Staphylococcus aureus* – 77,0 %, *Staphyococcus epidermidis* – 53,5 % i *Enterococcus faecalis* – 62,5 %).

Kad je riječ o broju *Staphylococcus aureus* i *Staphyococcus epidermidis*, između pozitivne kontrolne skupine i ispitivane skupine nakon primjene ozona, zabilježen je značajno manji broj preživjelih mikroorganizama u ispitivanoj skupini.

Nije bilo značajne razlike u broju mikroorganizma *Enterococcus faecalis* između pozitivne kontrolne skupine (2,5 % NaOCl) i ispitivane skupine.

Kad je riječ o sterilnim uzorcima, nakon primjene ozona stvorenog KP brizgalicom sterilizirano je 20 % uzoraka kontaminiranih *Enterococcus faecalis*, 71 % uzoraka kontaminiranih *Staphyloccocus aureus* i 77 % uzoraka kontaminiranih *Staphyloccocus epidermidis* (tablica 2.) .

with inoculation of canals. Experimental specimens were plasma sterilized between cycles along with all plastic disposable material. All sterility testing assays were performed as described above in the text. The enumeration of microorganisms was performed in dilution containing 30-200 colonies, and concentration counts were expressed as $\log^{10}$ (CFU +1).

Control groups

Ten specimens in control groups (five in the positive control group and five in the negative control group) were prepared and inoculated with microorganisms in the same manner as those belonging to the experimental group. The specimens in the negative control group were not treated at all. The root canals in the positive control group were irrigated with 2.0 mL of 2.5% v/v NaOCl for 5 minutes (8). The measurement of bacterial growth was performed, as well as the measurement of concentration, in the same manner as in the experimental group.

Statistical analysis

Statistical analysis was performed using PASW Statistics 17.0. According to Kolmogorov-Smirnov test, the variables of log values of microorganisms before and after the treatment and log values of reduction of microorganisms were not normally distributed ($p<0,005$).

Results

Analysis regarding a total reduction of microorganisms

KP syringe mode of ozone application showed a statistically significant reduction in a total number of microorganisms 745±1.38 (89.3%), ($p=0.0001$), compared to the baseline number of microorganisms. In the positive control group, the reduction of total number of microorganisms was also statistically significant but it was not 100% (68.7%). In the negative control group, the number of microorganisms remained unchanged.

Analysis regarding the type of microorganisms:

Reduction of each tested microorganism was statistically significant regarding the baseline value ($p=0.0001$) (Table 1).

In the positive control groups, the reduction of the number of each microorganism was statistically significant regarding the baseline count, but it was not 100% (*Staphylococcus aureus* 77.0%, *Staphylococcus epidermidis* 53.5% and *Enterococcus faecalis* 62.5%).

Regarding the number of Staphylococcus *aureus*, and the number of *Staphylococcus epidermidis*, there were significantly less surviving microorganisms in experimental groups after ozonization compared to the positive controls.

After ozonization, there was no difference in the number of *Enterococcus faecalis* between the positive control (2.5% NaOCl) and the experimental group.

Regarding the number of sterile specimens, 20 % specimens were contaminated with Enterococcus *faecalis*, 71% specimens with *Staphyloccocus aureus* and 77% specimens were contaminated with *Staphyloccocus epidermidis* after ozonization (Table 2).

Tablica 1. Srednje vrijednosti redukcije *Staphylococcus aureus*, *Staphyococcus epidermidis* i *Enterococcus faecalis* pri primjeni ozona KP štrcaljkom
Table 1 Mean values of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* reduction for KP syringe mode of ozone application

Skupina • Group	*Staphyococcus aureus* Redukcija mikroorganizama • *Staphylococcus aureus* microorganisms reduction	*Staphyococcus epidermidis* Redukcija mikroorganizama • *Staphylococcus epidermidis* microorganisms reduction	*Enterococcus faecalis* Redukcija mikroorganizama • *Enterococcus faecalis* microorganisms reduction
KP štrcaljka u korijenskom kanalu • KP syringe inside canal	7.87±0.98 (94.0%)	7.97±0.88 (88.6%)	6.74±1.10 (79.7%)

Tablica 2. Sterilizacijski učinak pri primjeni KP štrcaljke
Table 2 Sterilizing specimens by KP probe

Mikroorganizmi • Microorganisms	Broj ispitanih uzoraka • Number of tested specimens	Broj steriliziranih uzoraka (%) • Number of sterilized specimens (%)
Enterococcus faecalis	35	7 (20%)
Staphyloccocus aureus	35	25 (71%)
Staphyloccocus epidermidis	35	27 (77%)

Rasprava

U studiji *in vitro* procijenjena je antimikrobna učinkovitost ozona proizvedenoga KP brizgalicom uređaja *Ozonytron* (Biozonix, München, Njemačka) u suspenziji s trima različitim vrstama mikroorganizama (*Enterococcus faecalis, Staphylococcus aureus*, *Staphyloccocus epidermidis*) kojom su inficirani korijenski kanali izvađenih zuba.

Nekoliko studija, u novijoj literaturi, bavi se učincima različitih oblika primjene ozona na patološke mikroorganizme zastupljene u endodonciji, uključujući i najčešći *Enterococcus faecalis* (15 – 18). *Enterococcus faecalis,* gram-pozitivni fakultativni anaerob, posebno je zanimljiv kao jedan od najčešćih mikroorganizama prisutan u slučajevima revizije. Ta bakterija može opstati kao monoinfekcija u 33 % slučajeva i vrlo je otporna na djelovanje kalcijeva hidroksida (19 – 20). Njezina uloga u neuspjehu liječenja korijenskih kanala, ali i postojećih koncepata terapije dobro je proučena (21 –24).

Wilczińska-Borawska i suradnici (18) ispitali su primjenu ozona s pomoću električnih izboja staklenih elektroda (istog proizvođača), primjenjujući modalitet različit od načina u ovom istraživanju. U njihovu radu prvi je modalitet bio kontinuirano stvaranje ozona u plinovitoj fazi, a drugi stvaranje ozona u plinovitoj fazi kratkotrajnim pulsnim intervalima sa stankama između njih. Autori su zabilježili antimikrobni učinak na bakterijske vrste izolirane iz usne šupljine (između ostalih *Enterococcus faecalis, Staphylococcus aureus* i *Staphylococcus epidermidis*). Naši rezultati također potvrđuju antimikrobi učinak ozona proizvedenog KP štrcaljkom uređaja *Ozonytron* na *Enterococcus faecalis, Staphylococcus aureus* i *Staphylococcus epidermidis*. Novije studije (15, 16) potvrdile su antimikrobni učinak ozona na *Enterococcus faecalis*, no potpuna sterilizacija nije postignuta.

Slične rezultate objavili su Eik i suradnici (25). Oni su ispitivali učinke ozona na patološke mikroorganizme u području parodonta te zabilježili da su četiri potencijalne *superinfektivne* vrste (*Staphylococus aureus, Enterococcus faecalis, Enterobacter cloacae* i *Candida albicans*) djelomično preživjele.

Discussion

In this *in vitro* study, the antimicrobial efficacy of ozone, generated in KP syringe by Ozonytron unit (Biozonix, München, Germany), was evaluated in a mixed suspension of three different species of microorganisms found in the infected root canals of extracted teeth: *Enterococcus faecalis, Staphylococcus aureus*, *Staphylococcus epidermidis*.

In recent time, there have been several studies reported in the literature dealing with the effect of different modes of ozone therapy on endodontic pathogens, including the most prevalent species *Enterococcus faecalis* (15-18). *Enterococcus faecalis,* a gram-positive facultative anaerobe, is of particular interest since it is the most prevalent bacterial species found in retreatment cases. *Enterococcus faecalis* can survive as monoinfection in 33% of cases and is quite resistant to calcium hydroxide (19-20). Its role in root canal treatment failures is well studied along with the current concepts of treatment (21-24).

Wilczińska-Borawska et al. (18) tested the application of ozone through electrical discharge of a glass probe (using a selected probe supplied by a manufacturer). The researchers used the modes which were different from the modes used in this study. In their study, the first mode was a continuous application of ozone gas and the second mode was ozone application through short-lasting pulses, retaining intervals for consecutive exposure. The authors have documented antimicrobial activity against several strains of bacteria isolated from the oral cavity such as *Enterococcus faecalis, Staphylococcus aureus and Staphylococcus epidermidis*. Our results also confirmed the antimicrobial effect of ozone produced by KP syringe of Ozonytron device on *Enterococcus faecalis, Staphylococcus aureus and Staphylococcus epidermidis*. Some recent studies (15, 16) confirmed the antimicrobial effect of ozone gas on *Enterococcus faecalis;* however, a complete sterilization was not achieved.

Similar results were obtained by Eik et al. (25). They studied the effect of ozone on periodontopathogenic species and noticed that four potentially „superinfecting"species,

Case i suradnici (15) reducirali su nakon dvominutnog izlaganja ozonu CFU *Entrococcus faecalis* u biofilmu na 71,6 % u usporedbi kontrolnom skupinom.

U našem ispitivanju ozon stvoren primjenom KP-štrcaljke pokazao je učinkovitiji antimikrobni učinak na mikroorganizme *Staphyloccocus aureus* i *Staphyloccocus epidermidis*, sterilizirajući 71 i 77 % ispitivanih uzoraka, u usporedbi s 20 % steriliziranih uzoraka u skupini *Entrococcus faecalis*. U našoj studiji korištena KP brizgalica stvara koncentraciju ozona od 525 ppm. Schneider (26) navodi da koncentracije ozona između 300 i 800 ppm koje proizvodi uređaj *Ozonytron* imaju baktericidan učinak jer je letalna granica koncentracije ozona za bakterije 296 ppm.

U studiji Hemsa i suradnika (8), ispitana je antimikrobna učinkovitost ozona (proizvedenog stolnim, po narudžbi izrađenim generatorom) na *Enterococcus faecalis* u obliku suspenzije te unutar biofilma. Ozon je antimikrobno djelovao na *Enterococcus faecalis* u suspenziji, no vrlo slab učinak postignut je u biofilmu. Lynch (28) i Huth sa suradnicima (9) navode da su se Hems i suradnici (8) koristili ekstremno malim koncentracijama ozona u svojim eksperimentima (koncentracije ozona koje se spominju u njihovu radu bile su samo 0,68 ppm).

Huth i suradnici (9) u svojoj su studiji procijenili antimikrobne učinke vodene otopine (1,25 – 20 µg/mL) i plinovitog ozona (1 – 53 g/m^3) na endodontske patogene (*Enterococcus faecalis, Candida albicans, Peptostreptococcus micros* i *Pseudomonas aeruginosa)* u modelima suspenzije i biofilma. Koncentracije plinovitog ozona do najniže od 1g/m^3 (gotovo potpuno), te vodene otopine do najniže od 5 µg/mL (potpuno), isto kao i NaOCl-a i klorheksidina, eliminirale su mikroorganizme u suspenziji. Vodikov peroksid i vodene otopine ozona s nižim koncentracijama ozona bile su manje učinkovite. Učinkovitost vodene otopine ozona i plinovitog ozona u uklanjanju bakterija u biofilmu ovisila je o dozi i vrsti bakterija. Bez obzira na različite eksperimentne modele, rezultati ove studije u skladu su s njihovima kad je riječ o ovisnosti, antimikrobnom učinku ozona, o dozi i vrsti bakterija. U našoj studiji primijenjen je samo jedan volumni sadržaj KP brizgalice (2,2 ml). Opetovanim ubrizgavanjem navedene količine u KP štrcaljku vjerojatno bi se mogao postići intenzivniji antimikrobni učinak. Kaptan i suradnici (16) navode pozitivne učinke topikalne primjene plinovitog ozona u ponovljenim dozama pri eliminaciji *Enterococcus faecalis* u biofilmu korijenskih kanala te pojačani antimikrobni učinak u slučaju kombinacije ozona s 2-postotnim NaOCl-om. Terapija ozonom može se primijeniti kao primarna ili kao potpora drugim vrstama liječenja (29). Primjena ozona tijekom kemijsko-mehaničkog čišćenja korijenskoga kanala i nakon toga postupka, te primjena ultrazvučnih uređaja i lasera, svrhovita je metoda za poboljšanje uspješnosti endodontskog postupka (30, 31).

Mogućnost ozona stvorenoga KP brizgalicom uređaja *Ozonytron,* kao i NaOCl, značajno reducira broj mikroorganizama *Enterococcus faecalis* i drugih dviju ispitivanih bakterija i od kliničke je važnosti. No, nužna su daljnja istraživanja radi određivanja optimalne doze i optimalnog vremena djelovanja. Podatci iz pregledane literature o najprikladnijem tra-

Staphylococcus aureus, Enterococcus faecalis, Enterobacter cloacae and *Candida albicans,* can survive for at least part of their life cycle.

Case et al. (15) reported that exposure to O3-enriched air for a total period of 2 min resulted in a 71.6% reduction in viable CFU of *Enterococcus faecalis* in biofilm as compared with the control group.

In our study, KP syringe was more effective in killing *Staphylococcus aureus* and *Staphylococcus epidermidis*, sterilizing 71% and 77% of tested specimens, compared to 20% sterilized specimens in the *Enterococcus faecalis* group. In our study, the concentration of ozone produced by KP syringe was 525 ppm. Schneider (26) reported that concentration of ozone ranging between 300 and 800 ppm produced by Ozonytron device has bactericidal effects because the lethal border of the ozone concentration for bacteria is 296 ppm.

Another study performed by Hems et al. (8) tested the antibacterial efficacy of ozone, produced by custom-made bench generator, against *Enterococcus faecalis* in both broth and biofilm cultures. Ozone had an antibacterial effect on *Enterococcus faecalis* suspended in fluid, but a relatively low effect when embedded in biofilms. Lynch (28) and Huth et al. (9) stated that Hems et al. (8) used an extremely low dose of ozone in their experiments (concentration of ozone in water mentioned in their paper was only 0.68 ppm).

Huth et al. (9) assessed the antimicrobial efficacy of aqueous (1.25-20 µg mL^{-1}) and gaseous ozone (1-53 g m^{-3}) against endodontic pathogens *Enterococcus faecalis, Candida albicans, Peptostreptococcus micros* and *Pseudomonas aeruginosa* in suspension and in a biofilm model. Concentrations of gaseous ozone down to 1 g m^{-3} almost and aqueous ozone down to 5 µg mL^{-1} completely eliminated the suspended microorganisms as did NaOCl and chlorhexidine. Hydrogen peroxide and lower aqueous ozone concentrations were less effective. Aqueous and gaseous ozone were dose- and strain-dependently effective against the biofilm microorganisms. Although our experiments were different from those made by Huth et al., the results of this study are in agreement with their results regarding the dose- and strain-dependence of ozone antimicrobial activity. In our study, only one KP syringe dose was applied (2.2ml). It is most likely that better antimicrobial efficacy could be achieved by establishing the optimal dosage of ozone by KP syringe. Kaptan et al. (16) reported positive effects of topical gaseous ozone in recurrent doses in eradication of *Enterococcus faecalis* biofilm from the root canals. They noticed that ozone had a greater antimicrobial effect if combined with 2% NaOCl. Ozone therapy can be utilized as primary therapy or to support other types of therapies (29). The use of ozone during and after chemo-mechanical treatment, as well as the application of ultrasonic devices and laser, can result in a purposeful method of improving the success of endodontic procedure (30, 31).

Ability of ozone generated by KP syringe of Ozonytron unit, as well as NaOCl, to significantly reduce number of *Enterococcus faecalis* and two other tested bacteria, is of clinical importance. However, further effort should be made in order to evaluate optimal doses of ozone and an optimal time exposure. Inconsistent data on the most adequate time of

janju primjene i primijenjenim koncentracijama u eliminaciji patoloških mikroorganizama u endodonciji, nisu dosljedni.

Zaključno, primjena ozona stvorenoga KP brizgalicom uređaja *Ozonytron* značajno je smanjila ukupan broj mikroorganizama te broj svake vrste bakterija. KP brizgalica statistički je značajno učinkovitija, u usporedbi s NaOCl-om kao pozitivnom kontrolnom skupinom, kad je riječ o *Staphyloccocus aureus* i *Staphyloccocus epidermidis*. Ukupan broj *Enterococcus faecalis* statistički je smanjen, no bez značajne statističke razlike između ispitivane i pozitivne kontrolne skupine. Između triju vrsta bakterija u suspenziji, KP brizgalica najslabije je djelovala na *Enterococcus faecalis*.

Sukob interesa

Nije ga bilo.

ozone application and concentration to use against endodontic pathogens have been proposed in the literature.

In conclusion, the application of ozone by KP syringe of Ozonytron unit significantly decreases the absolute count of microorganisms, as well as the count of each type of bacteria separately. KP probe was statistically more effective compared to NaOCl as positive control for *Staphylococcus aureus* and *Staphylococcus epidermidis*. The absolute count of *Enterococcus faecalis* was decreased without statistically significant difference among the tested groups and the positive control, respectively. Among the three types of bacteria in suspension, KP probe had the lowest effect on *Enterococcus faecalis*.

Conflict of interest

None declared.

Abstract

Aim: The aim of this study was to evaluate *in vitro* the antibacterial effect of ozone on suspension of three different bacteria inoculated in prepared canals of extracted human teeth. **Material and methods:** Ozone was produced by special KP syringe of high frequency ozone generator Ozonytron (Biozonix, München, Germany) from aspirated atmospheric air by dielectric barrier discharge and applied through the tip of the syringe to the prepared root canal. The microorganisms used were *Enterococcus faecalis*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. **Results:** However, none of the methods was 100% effective against the three bacterial types in suspension. Application of ozone significantly decreased the absolute count of microorganisms (89.3%), as well as the count of each type of bacteria separately (*Staphylococcus aureus* 94.0 %; *Staphylococcus epidermidis* 88.6% and *Enterococcus faecalis* 79.7%). Ozone generated by KP syringe was statistically more effective compared to NaOCl as positive control, for *Staphylococcus aureus* and *Staphylococcus epidermidis*. **Conclusion:** The absolute count of *Enterococcus faecalis* was statistically decreased without a statistically significant difference between the tested group and positive control, respectively. Among the three types of bacteria in suspension, KP probe had the lowest antimicrobial effect against *Enterococcus faecalis*.

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Key words
Dental Pulp Cavity; Root Canal Therapy; Anti-Bacterial Agents; Ozone; *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*

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