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The Antibacterial and Antifungal Activity of Chlorhexidine Diacetate Incorporated into Acrylic Resins Used in Provisional Restorations

Antibakterijsko i antifungalno djelovanje klorheksidin-diacetata ugrađenog u akrilatne smole koje se upotrebljavaju za privremene nadomjestke

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Abstract

Objective: The surface of provisional restorations applied before conventional or implant- supported fixed restorations may cause bacterial or fungal biofilm formation. The aim of this study was to evaluate the antimicrobial activity of acrylic resins used in provisional restorations modified with chlorhexidine diacetate. **Methods:** 120 cylindrical, auto-polymerized resin samples modified with chlorhexidine diacetate were prepared at concentrations of 0 (control), 1, 3, 5 wt %. The antimicrobial activity was examined against *Streptococcus mutans*, *Enterococcus faecalis*, and *Candida albicans* using Crystal Violet quantification, MTT assay, and Scanning Electron Microscopy. Data were analyzed by ANOVA and paired sample t-tests (α=0.05). **Results:** The addition of chlorhexidine diacetate influenced the growth rate and metabolic activity of microorganisms. The antimicrobial effect against *C. albicans* and *S. mutans* statistically increased with the percentage of chlorhexidine diacetate. *E. faecalis* bacteria were less affected by chlorhexidine diacetate compared to other pathogens. **Conclusion:** It has been shown that the effectiveness of CHDA in inhibiting the proliferation of microorganisms correlated positively with increasing concentration levels. More research is needed to confirm the impact of different chlorhexidine concentrations on the mechanical properties, clinical efficacy, and antimicrobial properties of CDHA.

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Introduction

Provisional restorations, used in fixed prosthetic treatments are essential elements for the clinical success of dental treatments. Provisional restorations should have adequate physical, chemical, and thermal properties to ensure occlusal function, esthetics, and strength to prepared teeth (1). They can be used for correcting irregular occlusal planes in occlusal disorders. Also, they are used for adjusting the vertical dimensions and contouring the gingival tissue (2).

Various polymeric materials have been developed to increase the clinical success of provisional restorations (3,4).

Uvod

Privremeni nadomjestci koji se upotrebljavaju u fiksnoj protetičkoj terapiji ključni su elementi za njezin klinički uspjeh. Privremeni nadomjestci trebaju imati odgovarajuća fizikalna i kemijska svojstva da bi osigurali okluzijsku funkciju, estetiku i čvrstoću prepariranih zuba (1). Mogu se upotrijebiti za ispravljanje nepravilne okluzalne ravnine u slučaju okluzijskih poremećaja, podešavanja vertikalne dimenzije i konturiranja gingive (2).

Kako bi se povećao klinički uspjeh privremenih nadomjestaka, mogu se nabaviti različiti polimerni materijali (3, 4).

Auto-polymerized acrylic resin is one of the preferred materials for provisional restorations due to its low cost and easy handling features (5). However, its physical and chemical properties promote biofilm formation on the surface, which becomes a reservoir for pathogens, thus causing adverse reactions around tissues such as prosthetic stomatitis, secondary caries, and periodontal/peri-implant inflammation (6, 7). Provisional restorations are more subject to bacterial colonization than definitive restorations due to their higher surface roughness, especially when they have to be used for a long period of time. Research on antimicrobial effect of provisional restorations is limited (8).

Adhesion of bacteria to surfaces varies depending on bacterial species. A study (9) revealed that *Streptococcus mutans (S. mutans)* is the dominant species in the multispecies biofilm along with other bacterial species such as *Lactobacillus casei, Streptococcus salivarius, and S. sanguinis*. In addition, it has been reported that bacteria exposed to sucrose (or fermentable carbohydrates) can produce acids through fermentation and lower the pH of the dental biofilm, resulting in dental caries (9, 10). Furthermore, *Enterococcus faecalis (E. faecalis)*, a causative agent of various diseases, is a saprophytic commensal that lives in the oral cavity and gastrointestinal flora. *Enterococcus faecalis* is also commonly found bacterium after unsuccessful endodontic treatments (11). *Candida albicans (C. albicans)* is a pathogen that can cause inflammation in tissues, especially in immunocompromised patients (12, 13).

Antibacterial agents are effective in eliminating the undesirable impacts caused by bacteria (14). Chlorhexidine diacetate (CHDA) has a broad efficacy against microorganisms, and is successfully used as an oral antimicrobial and antiplaque agent (15-17). There are various studies on the effectiveness of chlorhexidine in dentistry (13, 15, 17). However, to the best of our knowledge, there are few studies evaluating CHDA incorporation into auto-polymerized acrylic resins used for provisional restorations. Therefore, this study aims to investigate the antibacterial and antifungal effect of CHDA incorporation into auto-polymerized acrylic resins. It was used the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (MTT), and the crystal violet (CV) assay for microbial viability evaluation. Null hypothesis was that CHDA addition with different concentrations into acrylic resins used for provisional restorations would have similar antibacterial and antifungal effect when compared to CHDA free provisional restorations.

Material and Methods

Test specimen preparation

To make the test specimens, resins were manipulated according to the manufacturers' recommendations in a ratio of 2 g polymer to 1 mL monomer to obtain 0.5%, 1.0%, and 2.0% samples by weight for each polymer material.

In total, 120 samples were prepared for this study (10 mm in diameter and 3 mm thick). Powder and liquid of the acrylic resins were prepared according to the manufacturer's recommendations in a ratio of 1 mL monomer to 2 g

Autopolimerizirajuća akrilatna smola jedan je od preferiranih materijala za privremene restauracije zbog niske cijene i jednostavne primjene (5). Međutim, njezina fizikalna i kemijska svojstva potiču stvaranje biofilma na površini koji postaje spremnik patogena i uzrokuje neželjene reakcije oko tkiva kao što su protetički stomatitis, sekundarni karijes i parodontitis/periimplantitis (6, 7). Privremeni nadomjestci podložniji su kolonizaciji bakterija nego trajni zbog veće površinske hrapavosti, osobito kada se moraju nositi dulje. Istraživanja o antimikrobnom učinku privremenih nadomjestaka su ograničena (8).

Prianjanje bakterija na površine varira ovisno o njihovoj vrsti. Tijekom istraživanja (9) otkriveno je da *Streptococcus mutans* (*S. mutans*) prevladava u biofilmovima u odnosu prema drugim bakterijskim vrstama kao što su *Lactobacillus casei*, *Streptococcus salivarius* i *Streptococcus sanguinis*. Uz to, objavljeno je da bakterije izložene saharozi (ili fermentirajućim ugljikohidratima) mogu proizvesti kiselinu tijekom fermentacije i sniziti pH zubnoga biofilma, što rezultira karijesom (9, 10). Uz to, *Enterococcus faecalis* (*E. faecalis*), uzročnik raznih bolesti, saprofitni je komenzal koji živi u usnoj šupljini i gastrointestinalnoj flori. Također se često nalazi poslije neuspješnoga endodontskog liječenja (11). *Candida albicans* (*C. albicans*) uzročnik je koji može izazvati upalu u tkivima, osobito ako je pacijent imunokompromitiran (12, 13).

Antibakterijski agensi učinkoviti su u otklanjanju nepoželjnih učinaka prouzročenih bakterijama (14). Klorheksidin-diacetat (CHDA) veoma je učinkovit kad je riječ o mikroorganizmima te se uspješno primjenjuje kao oralni antimikrobik i sredstvo protiv plaka (15 – 17). Postoje različita istraživanja o učinkovitosti klorheksidina u stomatologiji (13, 15, 17). No prema našim spoznajama u rijetkim se istraživanjima procjenjuje ugradnja CHDA-e u autopolimerizirane akrilatne smole koje se upotrebljavaju za privremene nadomjestke. Zato je cilj ovog istraživanja bio ispitati antibakterijski i antifungalni učinak ugradnje CHDA-e u autopolimerizirane akrilatne smole. Korišten je 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolij bromid test (MTT) i kristalna ljubičica (CV) za procjenu mikrobne vitalnosti. Nulta hipoteza bila je da će dodavanje CHDA-e različitih koncentracija u akrilatne smole koje se upotrebljavaju za privremene nadomjestke imati sličan antibakterijski i antifungalni učinak u usporedbi s privremenim nadomjescima bez CHDA-e.

Materijal i metode

Priprema uzoraka

Uzorci smole pripremljeni su prema preporukama proizvođača u omjeru 2 g polimera na 1 mL monomera da bi se dobili uzorci s 0,5 %, 1,0 % i 2,0 % po težini za svaki polimerni materijal.

Ukupno je za ovo istraživanje pripremljeno 120 uzoraka (promjera 10 mm i debljine 3 mm). Prah i tekućina akrilatnih smola pripremljeni su prema preporukama proizvođača u omjeru od 1 mL monomera prema 2 g polimera (Integra

polymer (Integra Cold-Cure Resin, Bg Dental, Turkey). The CHDA powder was separately weighed on a precision scale with (SF-400D, Tarez, Turkey, precision of ±0.01 g). Before the liquid addition, CHDA powder (< 200nm, Nanografi, METU Teknokent) was incorporated into the powder form of acrylic resin at a concentration of 0 (control), 1, 3, 5 wt % and mixed with a mixer (President Dental, Germany) in a 2900 rpm cycle for 30 seconds until a homogeneous mixture was achieved. The powder was dispensed into the liquid and mixed according to the manufacturer's recommendation. The proportions of powder/ monomer/ CHDA for each group were listed as follows: 0% CHDA, 5 g/2.5 mL/ 0 g; 1% CHDA, 5 g/2.5 mL/0.05 g; 3% CHDA, 5 g/2.5 mL/0.15 g; 5% CHDA, 5 g/2.5 mL/0.25 g. The mixed resin was put into the disk-shaped molds 30 samples were produced from each concentration (10 for *S. mutans, E. faecalis, and C. albicans*; total =120). The mold was covered with a glass slide and held under finger pressure for the time recommended by the manufacturer. The control group was prepared without addition of CHDA.

After the resin samples were retrieved from molds, they were polished with 1000, 2000, and 4000 grits respectively to obtain a standard surface (Waterproof silicon carbide paper, Atlas, Turkey). The mean surface roughness (Ra) of the samples was measured with a surface profilometer on three sites of each sample (Computerized Roughness Tester, Mitutoyo, Japan). Materials with roughness values below 0.2 µm were considered.

Culturing and inoculation of microorganisms:

S. mutans (ATCC 25175) strains were cultured in chemically defined medium (CDM) (10). CDM contained 2.0 g l-glutamic acid, 0.2 g l-cysteine, 0.02 g $MnCl_2 \cdot 4H_2O$, 0.9 g l-leucine, 1.0 g NH_4Cl, 1.2 g $MgSO_4 \cdot 7H_2O$, 2.5 g K_2HPO_4, 2.5 g KH_2PO_4 4.0 g $NaHCO_3$, 1.0 mg nicotinic acid, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.5 mg thiamine HCl, 0.6 g sodium pyruvate, 0.1 mg p-aminobenzoic acid, 1.0 mg riboflavin, 0.1 mg d-biotin per liter, 0.5 mg. It also contained Ca-pantothenate, 0.1 mg of folic acid and 1.0 mg of pyridoxal HCl per liter. The pH was adjusted to 7.0 with H_3PO_4. Inoculation was performed by adding 1000µl of CDM medium and 20µl of *S. mutans* (1.2×10^5) to the samples.

E. faecalis (ATCC 29212) was originally grown in the production of anaerobic blood agar plates (CDC, BioMerieux, Durham, NC, USA) (10). The bacterial cultures were then grown in Brain Heart Infusion (BHI) medium augmented with 5 g/l yeast extract (BHI-YE) in an anaerobic environment at 37 °C using gas generating bags. After *E. faecalis* was inoculated on the samples, the adherent bacteria were considered an initial biofilm and were further grown by the addition of 1000 µl of BHI-YE containing 0.5% glucose.

Sabouraud's dextrose agar (SDA) was used to grow *C. albicans* for 18 hours at 37 °C (18). Then, 100 mM glucose was added to yeast nitrogen-based (YNB) medium and a loopful of yeasts was added. Yeasts in the late exponential growth phase were harvested from overnight broth cultures. In order to prepare the yeast for adhesion and biofilm tests, they were washed twice with 5 ml of phosphate buffered saline

Cold-Cure Resin, Bg Dental, Turska). Prah CHDA-e odvojeno je izvagan na preciznoj vagi (SF-400D, Tarez, Turska, preciznost od ± 0,01 g). Prije dodavanja tekućine, prah CHDA-e (< 200 nm, Nanografi, METU Teknokent) usipan je u praškasti oblik akrilatne smole u koncentraciji od 0 (kontrola) te 1, 3 i 5 težinskih postotaka. i miješan mikserom (President Dental, Njemačka) u ciklusu od 2900 okretaja u minuti tijekom 30 sekunda dok se nije postigla homogena smjesa. Prašak je doziran u tekućinu i umiješan prema preporuci proizvođača. Omjeri praha/monomera/CHDA za svaku skupinu navedeni su kako slijedi: 0 % CHDA-e, 5 g/2,5 mL/0 g; 1 % CHDA-e, 5 g/2,5 mL/0,05 g; 3 % CHDA-e, 5 g/2,5 mL/0,15 g; 5% CHDA-e, 5 g/2,5 mL/0,25 g. Pomiješana smola stavljena je u kalupe u obliku diska. Iz svake koncentracije proizvedeno je 30 uzoraka (10 za *S. mutans*, *E. faecalis* i *C. albicans*; ukupno = 120). Kalup je prekriven predmetnim staklom i držan pod pritiskom prsta u trajanju koje je preporučio proizvođač. Kontrolna skupina pripremljena je bez dodatka CHDA-e.

Nakon što su uzorci smole izvađeni iz kalupa, polirani su s granulacijama od 1000, 2000 i 4000 kako bi se dobila standardna površina (vodootporni silicijev karbidni papir, Atlas, Turska). Srednja hrapavost površine (Ra) uzoraka izmjerena je površinskim profilometrom na trima mjestima na svakom uzorku (Computerized Roughness Tester, Mitutoyo, Japan). Razmatrani su materijali s vrijednostima hrapavosti nižima od 0,2 µm.

Uzgoj i inokulacija mikroorganizama

Sojevi *S. mutans* (ATCC 25175) uzgajani su u kemijski definiranom mediju (CDM) (10). CDM je sadržavao 2,0 g l-glutaminske kiseline, 0,2 g l-cisteina, 0,02 g $MnCl_2 \cdot 4H_2O$, 0,9 g l-leucina, 1,0 g NH_4Cl, 1,2 g $MgSO_4 \cdot 7H_2O$, 2,5 g K_2HPO_4, 2,5 g KH_2PO_4 4,0 g $NaHC0_3$, 1,0 mg nikotinske kiseline, 0,02 g $FeSO_4 \cdot 7H_2O$, 0,5 mg tiamin HCl-a, 0,6 g natrijeva piruvata, 0,1 mg p-aminobenzojeve kiseline, 1,0 mg riboflavina, i 0,1 mg d-biotina po litri, 0,5 mg. Također je po litri sadržavao Ca-pantotenat, 0,1 mg folne kiseline i 1,0 mg piridoksal HCl-a. Vrijednost pH podešena je na 7,0 s H_3P0_4. Inokulacija je provedena dodavanjem 1000 µl CDM medija i 20 µl *S. mutans* (1,2 x 105) u uzorke.

E. faecalis (ATCC 29212) izvorno je uzgajan u proizvodnji anaerobnih krvnih agar ploča (CDC, BioMerieux, Durham, NC, SAD) (10). Bakterijske kulture zatim su uzgajane u mediju Brain Heart Infusion (BHI) obogaćenom s 5 g/l ekstrakta kvasca (BHI-YE) u anaerobnom okruženju na 37 °C s pomoću vrećica za generiranje plina. Nakon što je *E. faecalis* inokuliran na uzorcima, prianjajuće bakterije smatrane su početnim biofilmom i dalje su uzgajane s dodatkom 1000 µl BHI-YE koji sadržava 0,5 % glukoze.

Sabouraudov dekstrozni agar (SDA) korišten je za uzgoj *C. albicans* tijekom 18 sati na 37 °C (18). Zatim je 100 mM glukoze dodano u kvaščev medij na bazi dušika (YNB) i dodana je petlja kvasca. Kvasci u fazi kasnoga eksponencijalnog rasta skupljeni su iz bujonskih kultura tijekom noći. Kako bi se kvasci pripremili za testiranje adhezije i biofilma, isprani su dva puta s 5 ml fosfatnog pufera (PBS; pH 7,2; bez Ca2+-

(PBS; pH 7.2; Ca2+- and Mg2+-free). After they have been washed, the cells were resuspended in growth medium (for adhesion experiments) or PBS (for biofilm assays) at a concentration of 10^7 cells/ml (optical density at 600 nm).

Biofilm formation assay

The formation of biofilm utilizing the microorganisms within the scope of the study was determined using a previously described method (18).

To evaluate biofilm formation of *S. mutans*, samples were prepared with 20 µl of a bacterial cell suspension (4.0 x 10^4 CFU) (or phosphate-buffered saline [PBS] as a control), 0.25 % sucrose in 24-well (flat bottom) plates, or 160 µl TSB supplemented with 3 % sucrose. The biofilm formation assay was conducted at 37°C for 10 or 16 hours in an aerobic atmosphere with 5% CO_2. After 10 or 16 hours of incubation at 37°C, the liquid medium was withdrawn from the plates and the biofilm-forming samples were rinsing twice with sterile distilled water (Elektromag, Etuv Incubator, Turkey). Afterwards, the samples were air-dried, and crystal staining and biofilm formation with MTT were assessed. For biofilm formation, *E. faecalis* (3×10^6 CFU/mL) was inoculated on samples (20µl (bacteria) +1000µl (media)) placed vertically in a 24-well plate. The culture was incubated at 37 °C for 24 hours in media containing BHI-YE + 0.5% glucose and biofilm formation was achieved. Colonies were cultured in BHI in aerobic incubation at 37°C. *C. albicans* (ATCC 10231) biofilm formation was achieved by making some modifications to the method proposed by Jin et al. (18) 100 µL of standard yeast cell suspensions (10^7 cells/mL) were transferred onto each sample and incubated on an orbital shaker (Shell Lab, Sheldon Manufacturing, USA) at 37°C at 120 rpm for 2 ho (adherence phase). After the adherence phase, the cell suspensions were gently removed. This was done carefully not to disturb the adherent cells, and each sample was washed twice with 1x PBS to remove any remaining planktonic cells. To increase the biofilm layer, 1000 µl of freshly prepared YEP (1% Bacto-Yeast Extract, 2% Bacto-Peptone) supplemented with 100 mM glucose was added on each sample.

Cristal Violet Assay

In an orbital shaker, samples were incubated for 72 hours at 120 rpm and 37°C. After 72 hours of shaking at 37°C at 120 rpm, the wells were rinsed twice with 1000 l of 10 mM potassium phosphate (pH 7.0) and stained for 30 minutes with 1000 µl of 0.1% CV. To remove surplus dye, 1000 l of demineralized water was used to wash the samples. The optical density at 585 nm was determined after dissolving the CV on the sample surfaces in 95% ethanol. Using a microtiter plate reader, the absorbance at 585 nm was measured. All experiments were performed three times using bacteria which were grown individually.

MTT Assay

Metabolic activities of living biofilm cells were assessed using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (19). Following incubation, the growth medium was removed from the specimens, which

i Mg2+). Nakon ispiranja stanice su resuspendirane u mediju za rast (za pokuse adhezije) ili PBS-u (za testove biofilma) u koncentraciji od 107 stanica/ml (optička gustoća od 600 nm).

Ispitivanje stvaranja biofilma

Formiranje biofilma mikroorganizama u sklopu istraživanja utvrđeno je prethodno opisanom metodom (18).

Za procjenu stvaranja biofilma *S. mutans*, uzorci su pripremljeni s 20 µl suspenzije bakterijskih stanica (4,0 x 104 CFU) [ili fiziološke otopine puferirane fosfatom (PBS) kao kontrola], 0,25 % saharoze u 24 jažice (ravno dno) ploče ili 160 µl TSB s dodatkom 3 % saharoze. Ispitivanje stvaranja biofilma obavljeno je na 37 °C tijekom 10 ili 16 sati u aerobnoj atmosferi s 5 % CO_2. Nakon 10 ili 16 sati inkubacije na 37 °C, tekući medij izvučen je iz ploča, a uzorci koji stvaraju biofilm dvaput su isprani sterilnom destiliranom vodom (Elektromag, Etuv Incubator, Turska). Nakon toga uzorci su osušeni na zraku i procijenjeno je bojenje kristala i stvaranje biofilma s MTT-om. Za stvaranje biofilma, *E. faecalis* (3 × 106 CFU/mL) inokuliran je na uzorke [20 µl (bakterije) +1000 µl (medij)] postavljene okomito u ploču s 24 jažice. Kultura je inkubirana na 37 °C 24 sata u mediju koji je sadržavao BHI-YE + 0,5 % glukoze i postignuto je stvaranje biofilma. Kolonije su uzgajane u BHI-ju u aerobnoj inkubaciji na 37 °C. Formiranje biofilma *C. albicans* (ATCC 10231) postignuto je uvođenjem nekih preinaka u metodu koju su predložili Jin i suradnici (18) 100 µL standardne suspenzije stanica kvasca (107 stanica/mL) preneseno je na svaki uzorak i inkubirano na orbitalnoj mućkalici (Shell Lab, Sheldon Manufacturing, SAD) na 37 °C pri 120 okretaja u minuti tijekom 2 sata (faza prianjanja). Nakon faze prianjanja stanične suspenzije oprezno su uklonjene kako se ne bi poremetile prianjajuće stanice, a svaki uzorak je dva puta ispran s 1x PBS-a da se uklone sve preostale planktonske stanice. Kako bi se povećao sloj biofilma, svakom uzorku dodano je 1000 µl svježe pripremljenog YEP-a (1 % bakto-kvasnog ekstrakta, 2 % bakto-peptona) dopunjenoga sa 100 mM glukoze.

Bojenje kristalnom ljubičicom (CV)

U orbitalnoj tresilici uzorci su inkubirani 72 sata pri 120 okretaja u minuti i 37 °C. Nakon 72 sata mućkanja na 37 °C pri 120 okretaja u minuti, jažice su isprane dva puta s 1000 10 mM kalijeva fosfata (pH 7,0) i obojene 30 minuta s 1000 ul 0,1 % CV. Da bi se uklonio višak boje, za pranje uzoraka utrošeno je 1000 ml demineralizirane vode. Optička gustoća na 585 nm određena je nakon otapanja CV-a na površini uzorka u 95-postotnom etanolu. S pomoću čitača mikrotitarskih ploča izmjerena je apsorbancija na 585 nm. Svi pokusi izvedeni su tri puta koristeći se bakterijama uzgojenima pojedinačno.

MTT test

Metaboličke aktivnosti živih stanica biofilma procijenjene su s pomoću MTT testa (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolijev bromid) (19). Nakon inkubacije medij za rast je uklonjen iz uzoraka koji su zatim tri puta isprani sa ste-

were then washed three times with sterile 1xPBS. Subsequently, 1000 µl of MTT solution (5 mg/mL) was added to the specimens and incubated in a dark place for 3 hours. After the incubation period, the supernatant was discarded, and 1000 µL of lysis solution (composed of 10% (v/v) sodium dodecyl sulfate and 50% (v/v) dimethylformamide in distilled water) was added to dissolve the biofilm. The specimens were incubated in the lysis solution for an additional 3 hours. Following the complete dissolution of the biofilm, the absorbance of the resulting solution was measured using a spectrophotometer or a microplate reader. This measurement provided an indication of the metabolic activity of the living biofilm cells, with higher absorbance values corresponding to greater metabolic activity.

Scanning Electron Microscopy

The samples were observed under scanning electron microscopy (SEM, Hitachi Regulus 8230 FE- SEM, Japan) at 10000X magnification.

Statistical analysis

Statistical package software (SPSS Version 24.0; SPSS Inc., Chicago, IL, ABD) was used for the statistical analyses of the results. The data obtained in the study displayed a statistically normal distribution. Since the data had normal distribution within the groups, the means and the variations among the CHDA groups were examined by using One-Way Variance Analysis (ANOVA) and the post-hoc Tukey test. The Pearson correlation was used to measure the strength of the relationship between growth rate/metabolic activity and CHDA percentage. A p-value of < 0.05 was considered statistically significant.

Results

2 different methodologies were used in this study: the MTT Assay and the Crystal Violet (CV) Assay. The mean metabolic activity and growth rate of the tested samples with different concentrations by MTT and CV Assays were listed in Table 1.

CHDA concentration increased (1% to 5%), and surface roughness increased. The antibacterial efficacy against microorganisms increased along with the concentration %

rilnim 1xPBS-om. Zatim je uzorcima dodano 1000 µl otopine MTT-a (5 mg/mL) i inkubirano na tamnome mjestu 3 sata. Nakon inkubacije supernatant je odbačen i dodano je 1000 µL otopine za lizu (sastavljene od 10 % (v/v) natrijeva dodecil sulfata i 50 % (v/v) dimetilformamida u destiliranoj vodi) da se otopi biofilm. Uzorci su inkubirani u otopini za lizu dodatna 3 sata. Nakon potpunog otapanja biofilma apsorpcija dobivene otopine izmjerena je spektrofotometrom ili čitačem mikropločica. Ovo mjerenje dalo je indikaciju metaboličke aktivnosti živih stanica biofilma s višim vrijednostima apsorbancije koje odgovaraju većoj metaboličkoj aktivnosti.

Skenirajuća elektronska mikroskopija

Uzorci su promatrani skenirajućom elektronskom mikroskopijom (SEM, Hitachi Regulus 8230 FE-SEM, Japan) pri povećanju od 10 000 x.

Statistička analiza

Za statističku analizu rezultata korišten je statistički paket softvera SPSS verzija 24.0 (SPSS Inc., Chicago, IL, ABD). Podatci dobiveni u istraživanju pokazali su statistički normalnu distribuciju. Budući da su imali normalnu distribuciju unutar skupina, srednje vrijednosti i varijacije među CHDA skupinama ispitane su korištenjem jednosmjerne analize varijance (ANOVA) i Tukeyeva post-hoc testa. Pearsonova korelacija korištena je za mjerenje snage odnosa između stope rasta/metaboličke aktivnosti i postotka CHDA-e. P-vrijednost $< 0,05$ smatrala se statistički značajnom.

Rezultati

U ovom istraživanju primijenjene su dvije različite metodologije: MTT analiza i bojenje kristalnom ljubičicom (Crystal Violet – CV). Prosječna metabolička aktivnost i brzina rasta testiranih uzoraka s različitim koncentracijama u MTT i CV testovima navedeni su u tablici 1.

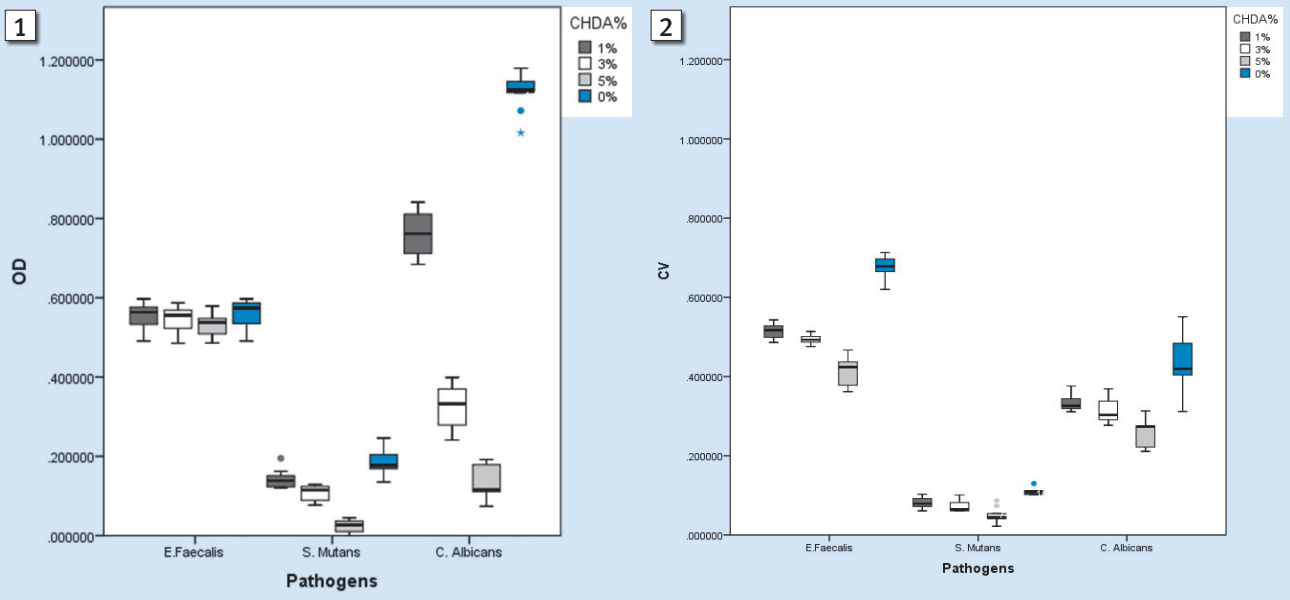
Povećavanjem koncentracije CHDA-e (1 % do 5 %) povećavala se hrapavost površine. Antibakterijska učinkovitost, kad je riječ o mikroorganizmima, povećavala se zajed-

Table 1 The mean metabolic activity and growth rate of the tested samples with different concentrations by MTT and CV Assays.
Tablica 1. Prosječna metabolička aktivnost i brzina rasta testiranih uzoraka s različitim koncentracijama s pomoću MTT i CV testova

	CHDA wt • tež. %	MTT	CV
E. faecalis	0%	0.56±0.01	0.67±0.01
	1%	0.55±0.01	0.52±0.01
	3%	0.54±0.01	0.49±0.01
	5%	0.53±0.01	0.42±0.01
S. mutans	0%	0.19±0.01	0.11±0.01
	1%	0.14±0.01	0.08±0.01
	3%	0.12±0.01	0.07±0.01
	5%	0.02±0.01	0.05±0.01
C. albicans	0%	1.12±0.01	0.44±0.02
	1%	0.76±0.02	0.33±0.01
	3%	0.33±0.02	0.31±0.01
	5%	0.13±0.01	0.26±0.01

of CHDA. For *S. mutans* pathogens, the MTT and CV Assay showed statistically significant antimicrobial effects on groups incorporated with 3%, 5% CHDA compared with the control group ($p<0.001$). For *C. albicans* group, 1%, 3%, 5% CHDA addition significantly affected the antimicrobial efficiency ($p<0.001$). On the other hand, *E. faecalis* group was mostly affected by 5 % CHDA added group, but it was not statistically different ($p>0.05$) (Figure 1 and Figure 2).

no s koncentracijom postotka CHDA-e. Za *S. mutans*, MTT i CV test pokazao je statistički značajne antimikrobne učinke na skupine koje su inkorporirane s 3 % i 5 % CHDA-e u usporedbi s kontrolnom skupinom ($p < 0,001$). Za skupinu *C. albicans*, dodavanje 1 %, 3 % i 5 % CHDA-e znatno je utjecalo na antimikrobnu učinkovitost ($p < 0,001$). S druge strane, u skupini *E. faecalis* najveći utjecaj imala je skupina s dodatkom 5 % CHDA-e, ali taj utjecaj nije bio statistički značajan ($p > 0,05$) (slike 1. i 2.).



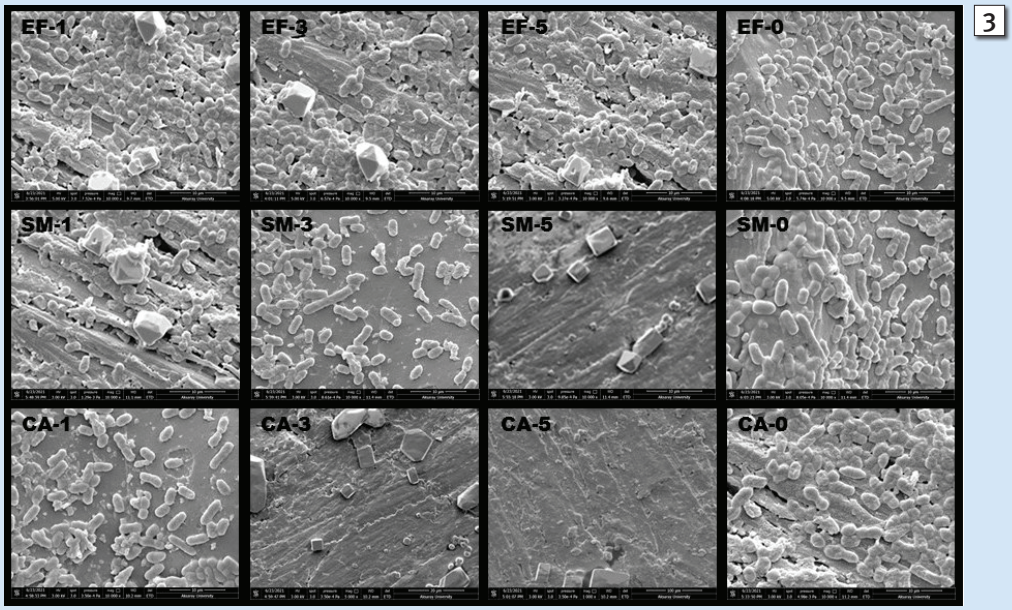


Figure 1 Box-plot diagram of the distribution of cell viability (%) data according to CV assay for *C. albicans, S. mutans, E. faecalis.*
Slika 1. Kutijasti dijagram (box-plot) raspodjele podataka o vitalnosti stanica (%) prema CV testu za *C. albicans, S. mutans* i *E. faecalis*
Figure 2 Box-plot diagram of the distribution of cell viability (%) data according to MTT assay for *C. albicans, S. mutans, E. faecalis.*
Slika 2. Kutijasti dijagram (box-plot) raspodjele podataka o vitalnosti stanica (%) prema MTT testu za *C. albicans, S. mutans* i *E. faecalis*
Figure 3 SEM images (X10K) showing the *C. albicans, S. mutans, E. faecalis* biofilms at different CHDA concentrations.(EF0*: E. faecalis* with 0%CHDA, EF1: *E. faecalis* with 1%CHDA, EF3: *E. faecalis* with 3%CHDA, EF5: *E. faecalis* with 5%CHDA; SM0: *S. mutans* with 0%CHDA, SM1: *S. mutans* with 1%CHDA, SM3: *S. mutans* with 3%CHDA, SM5: *S. mutans* with 5%CHDA; CA0: *C. albicans* with 0%CHDA, CA1: *C. albicans* with 1%CHDA, CA3: *C. albicans* with 3%CHDA CA5: *C. albicans* with 5%CHDA.
Slika 3. SEM slike (X10K) koje prikazuju biofilmove *C. albicans, S. mutans* i *E. faecalis* pri različitim koncentracijama CHDA-e (EF0: *E.faecalis* s 0 % CHDA-e, EF1: *E. faecalis* s 1 % CHDA-e, EF3*: E. faeealis* s 3 % CHDA-e, EF5: *E. faecalis* s 5 % CHDA-e; SM0: *S. mutans* s 0 % CHDA-e, SM1: *S. mutans* s 1 % CHDA-e, SM3: *S. mutans* s 3 % CHDA-e, SM5: *S. mutans* s 5 % CHDA-e; CA0: *C. albicans* s 0 % CHDA-e, CA1: *C. albicans* s 1 % CHDA-e, CA3: *C. albicans* s 3 % CHDA-e CA5: *C. albicans* s 5 % CHDA-e

There was an inverse correlation between the growth rate of *S. mutans, E. faecalis, C. albicans* and the percentage of CHDA due to the CV Assay [Very strong correlation for *S. mutans* (r = 0.82), and *E. faecalis* (r = 0.89), and strong correlation for *C. albicans* (r = 0.78)]. These correlations were statistically significant for all three microorganisms (p<0.001).

There was an inverse correlation between the metabolic viability *of S. mutans, E. faecalis, C. albicans* and the increase in the percentage of CHDA on the MTT Assay [Weak correlation for *E. faecalis* (r=0.31) and very strong correlation for *S. mutans* (r=0.91) and *C. albicans* (r=0.94)]. These correlations were statistically significant for all three microorganisms (p <0.05 for *E. faecalis*, p< 0.001 for *S. mutans, and C. albicans*).

The representative SEM images are shown in Figure 3. Few pathogen colonies can be seen in high concentration of CHDA groups.

Discussion

Provisional restorations are mostly prepared immediately after tooth preparation during the process of prosthetic restorations. The inclusion of antimicrobial agents in provisional restorations can prevent biofilm accumulation on the material as well as interference with the colonization of microorganisms around the tooth or implant surface. In the current study, the addition of CHDA inhibited the bacterial and fungal activity compared to the non-modified control group; therefore, the hypothesis was rejected.

Resin samples modified with CHDA showed significant antimicrobial properties in the current study. The results were almost consistent when compared to previous studies. Hiraishi et al. (17)-evaluated the antimicrobial activity against *S. mutans* and *E. faecalis* of chlorhexidine added acrylic resin cements and concluded that the concentration of CHDA was directly related to the antimicrobial activity. Kwon et al. (11) evaluated the antibacterial effects of resin containing chlorhexidine digluconate at concentrations from 0% (control) to 3% in agar diffusion test and they recommended that incorporation of 1%, 1.5% chlorhexidine digluconate could affect the antibacterial activity of resin. They also implied that more than 1% concentration inhibited the formation of biofilms except for *E. faecalis* (11). In the current study, the inhibition of *S. mutans* and *C. albicans* by CHDA incorporated groups was more noticeable than the inhibition of *E. faecalis*. *S. mutans* bacteria are known as the primary causative pathogens of human dental caries. This study suggests that dental caries can be prevented by adding CHDA to provisional restorations. On the other hand, the addition of CHDA to samples was not effective in reducing viable *E. faecalis* bacteria which exhibited higher resistance to CHDA. Gronroos et al. (20) reported in their study that CHDA did not affect the *E. faecalis* bacteria's growth rate compared to *S. mutans*. This is in parallel with our findings that the CHDA added to samples did not inhibit the growth rate of *E. faecalis* bacteria as much as other microorganisms. The reason for this difference may be that *E. faecalis* is able to survive in a wide range of pH environment and, is not only acid re-

Postojala je inverzna korelacija između stope rasta *S. mutans*, *E. faecalis*, *C. albicans* i postotka CHDA-e zbog CV testa [vrlo jaka korelacija za *S. mutans* (r = 0,82) i *E. faecalis* (r = 0,89) i jaka korelacija za *C. albicans* (r = 0,78)]. Ove korelacije bile su statistički značajne za sva tri mikroorganizma (p < 0,001).

Postojala je inverzna korelacija između metaboličke održivosti *S. mutans*, *E. faecalis*, *C. albicans* i povećanja postotka CHDA-e na MTT testu [slaba korelacija za *E. faecalis* (r = 0,31) i vrlo jaka korelacija za *S. mutans* (r = 0,91) i *C. albicans* (r = 0,94)]. Te su korelacije bile statistički značajne za sva tri mikroorganizma (p < 0,05 za *E. faecalis*, p <0,001 za *S. mutans* i *C. albicans*).

Reprezentativne SEM slike prikazane su na slici 3. Nekoliko kolonija patogena može se vidjeti u visokoj koncentraciji CHDA skupina.

Rasprava

Privremeni nadomjestci uglavnom se izrađuju neposredno poslije preparacije zuba tijekom procesa izrade protetičkih nadomjestaka. Uključivanje antimikrobnih sredstava u privremene nadomjestke može spriječiti nakupljanje biofilma na materijalu i ometati kolonizaciju mikroorganizama oko površine zuba ili implantata. U ovom istraživanju sadržaj CHDA-e inhibirao je aktivnost bakterija i gljivica u usporedbi s nemodificiranom kontrolnom skupinom. Zato je hipoteza odbačena.

Uzorci smole modificirani s CHDA-om pokazali su značajna antimikrobna svojstva u ovom istraživanju. Rezultati su pokazali slične nalaze u usporedbi s prethodnim istraživanjima. Hiraishi i suradnici (17) procijenili su antimikrobnu aktivnost cementa od akrilatne smole s dodatkom klorheksidina protiv *S. mutans* i *E. faecalis* te zaključili da je koncentracija CHDA-e izravno povezana s antimikrobnom aktivnošću. Kwon i suradnici (11) procijenili su antibakterijske učinke smole koja sadržava klorheksidin-diglukonat u koncentracijama od 0 (kontrola) do 3 % u testu difuzije agara i istaknuli su da ugradnja 1 % i 1,5 % klorheksidin-diglukonata utječe na antibakterijsko djelovanje smole. Također su implicirali da koncentracije više od 1 % inhibiraju stvaranje biofilmova, osim za *E. faecalis* (11). U ovom istraživanju inhibicija *S. mutans* i *C. albicans* u inkorporiranim skupinama CHDA-e bila je uočljivija od *E. faecalis*. Bakterije *S. mutans* poznate su kao primarni uzročnici zubnog karijesa kod ljudi. Ovo istraživanje sugerira da se zubni karijes može spriječiti dodavanjem CHDA-e u privremene nadomjestke. S druge strane, dodavanje CHDA-e uzorcima nije bilo učinkovito u smanjenju održivih bakterija *E. faecalis* koje su pokazale veću otpornost na CHDA-u. Gronroos i suradnici (20) izvijestili su u svojem istraživanju da CHDA nije utjecala na brzinu rasta bakterije *E. faecalis* u usporedbi sa *S. mutans*. To je u skladu s našim nalazima da CHDA dodana uzorcima nije inhibirala stopu rasta bakterije *E. faecalis* u tolikoj mjeri kao drugi mikroorganizmi. Razlog za razliku može biti to što *E. faecalis* može preživjeti u širokom rasponu pH okoline i nije otporan samo na kiseline kao *S. mutans*, nego je otporniji

sistant like *S. mutans*, but also more alkali resistant (21-23). In this study, *C. albicans* showed lower adherence in samples with 1% CHDA added. These findings are in line with those of the study performed by Regis et al. (24) who reported lower adherence of *C. albicans* on acrylic resin containing methacryloyloxyundecylpyridinium bromide. However, the effect was mostly seen in higher concentrations, suggesting that CHDA shows effective antifungal properties.

There are many factors affecting the surface roughness of resins, such as polymerization and polishing techniques, chemical structures, type, and size of incorporated materials (25-28). The presence of surface roughness in provisional restorations cannot only reduce the mechanical properties of the restorations, but can also cause periodontal inflammation around tooth or implant. Clinically acceptable surface roughness of acrylic resins should be below 0.2 µm (29). It was reported that plaque formation and microorganism colonization could increase above this level (29). In this study, the surface roughness values measured after polishing showed lower values than 0.2 µm. However, SEM examination showed that all surfaces were still rough, and the samples could provide sufficient adhesion conditions for microbial accumulation (30). Moreover, as CHDA concentration increased (from 1% to 5%), surface roughness increased. As the lower CHDA content group had lower Ra values, it is conceivable that there may be fewer salt particles on the surface of the samples and more irregular polymer chains in the resin.

MTT or CV assays can be used to detect cell viability (31). The MTT test has many advantages such as rapid results, ease of administration, and visualization of cell density in small cell cultures. However, it has been reported that the materials tested in the MTT test may yield non-consistent results about cell viability (31). The CV assay lacks the limitations undermining the accuracy of MTT. It is a non-enzymatic test for the rapid analysis of viable adherent cells and colonies. The test takes advantage of the proximity between the dye and the outer surface of the DNA double helix. The amount of dye absorbed depends on the total DNA material in the culture. Using a single assay may result in misinterpretation and yield incomplete or erroneous results. It was reported that the MTT assay is more reproducible than the CV tests, and it reduces the experimental error rate (32). In the current study, CV and MTT assays were used. Despite the differences between the two assays, the effects of microorganisms in our study were similar in both assays. *S. mutans* and *C. albicans* were more affected by CHDA added groups, while *E. faecalis* bacterium was less affected than CHDA groups. On the other hand, the fact that the antibiofilm effect was not evaluated by quantifying the colony forming units may have diminished the effectiveness of our study and impeded the interpretation of the results. As the MTT reduction assay does not always correlate with cell death and crystal violet can stain deceased cells, this may be one of the limitations of our study.

A primary limitation of this study is that the samples were not stored in saliva or subjected to thermal or physical treatments, as it occurs in clinical setting. It is necessary to evali na lužine (21 – 23). U ovom istraživanju je *C. albicans* pokazala slabiju adherenciju u uzorcima s dodatkom 1 % CHDA-e. Ovi su nalazi u skladu s drugim istraživanjem Regisa i suradnika (24) koji je izvijestio o nižem prianjanju *C. albicans* na akrilnu smolu koja sadržava metakriloiloksiundecilpiridinijev bromid. No učinak je uglavnom bio vidljiv u višim koncentracijama, što sugerira da CHDA pokazuje učinkovita antifungalna svojstva.

Mnogo je čimbenika koji utječu na hrapavost površine smola kao što su tehnike polimerizacije i poliranja, kemijska struktura, vrsta i veličina ugrađenih materijala (25 – 28). Pojava površinske hrapavosti u privremenim nadomjestcima ne samo da može negativno utjecati na mehanička svojstva nadomjestaka, nego može izazvati i upalu parodonta oko zuba ili tkiva oko implantata. Klinički prihvatljiva hrapavost površine akrilatnih smola trebala bi biti ispod 0,2 µm (29). Zabilježeno je da bi se stvaranje plaka i kolonizacija mikroorganizama mogli povećati iznad te razine (29). U ovom istraživanju su vrijednosti površinske hrapavosti izmjerene poslije poliranja bile niže od 0,2 µm. Međutim, SEM ispitivanje pokazalo je da su sve površine još uvijek hrapave, što znači da su uzorci imali uvjete za nakupljanje mikroba (30). Štoviše, kako se koncentracija CHDA-e povećavala (s 1 % na 5 %), tako se povećavala i hrapavost površine. Budući da je skupina s nižim sadržajem CHDA-e imala niže vrijednosti Ra, možda je bilo manje čestica soli na površini uzoraka i više nepravilnih polimernih lanaca u smoli.

MTT ili CV testovi mogu se upotrijebiti za otkrivanje viabilnosti stanica (31). MTT test ima mnoge prednosti kao što su brzi rezultati, jednostavnost primjene i vizualizacija gustoće stanica u kulturama malih stanica. No objavljeno je da materijali ispitani tim testom mogu dati nedosljedne rezultate o viabilnosti stanica (31). CV analizi nedostaju ograničenja koja potkopavaju točnost MTT-a. To je neenzimski test za brzu analizu održivih adherentnih stanica i kolonija. Test iskorištava blizinu boje i vanjske površine dvostruke spirale DNK. Količina apsorbirane boje ovisi o ukupnome DNK materijalu u kulturi. Korištenje jednog testa može rezultirati pogrešnim tumačenjem i dati nepotpune ili pogrešne rezultate. Zabilježeno je da je MTT test ponovljiviji od CV testova i smanjuje stopu eksperimentalne pogreške (32). U ovom istraživanju primijenjeni su CV i MTT testovi. Unatoč razlikama između tih dvaju testova, učinci mikroorganizama u našem istraživanju bili su slični u oba testa. *S. mutans* i *C. albicans* bili su više pogođeni u skupinama s dodanom CHDA-om, a bakterija *E. faecalis* bila je manje pogođena u CHDA skupinama. S druge strane, činjenica da učinak protiv biofilma nije procijenjen kvantificiranjem jedinica koje stvaraju kolonije možda je umanjila učinkovitost našeg istraživanja i spriječila tumačenje rezultata. Budući da analiza redukcije MTT-a nije uvijek u korelaciji sa staničnom smrću, a kristalno ljubičasto može obojiti mrtve stanice, to može biti jedno od ograničenja našeg istraživanja.

Primarno ograničenje ovog istraživanja jest to što uzorci nisu pohranjeni u slini ili podvrgnuti toplinskim ili fizičkim tretmanima kao u kliničkom okruženju. Potrebno je procijeniti druge varijable koje će podržati stvaranje biofilma. Ugradnja antimikrobnih sredstava u smolu sprječava na-

uate other variables that will support biofilm formation. Incorporation of antimicrobial agents into the resin prevents biofilm accumulation and it is also possible to form a material that interferes with colonization by microorganisms.

Conclusion

In conclusion, it has been demonstrated that the effectiveness of CHDA in preventing the growth of microorganisms positively correlates with increasing concentration percentages. It is necessary to determine the optimal chlorhexidine concentration in order to optimize the suppression of microbial proliferation while simultaneously preserving mechanical properties and therapeutic efficacy. Therefore, further research is needed to determine the effect of various chlorhexidine concentrations on mechanical properties, clinical efficacy, and antibacterial properties of CDHA.

Conflict of interest

The authors declare that they have no conflict of interest.

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kupljanje biofilma, a također je moguće stvoriti materijal koji ometa kolonizaciju mikroorganizama.

Zaključak

Zaključno, pokazano je da učinkovitost CHDA-e u sprječavanju rasta mikroorganizama pozitivno korelira s povećanjem postotaka koncentracije. Potrebno je odrediti optimalnu koncentraciju klorheksidina da bi se optimizirala supresija mikrobne proliferacije uz istodobno očuvanje mehaničkih svojstava i terapijske učinkovitosti. Zato su potrebna daljnja istraživanja kako bi se ustanovio učinak različitih koncentracija klorheksidina na mehanička svojstva, kliničku učinkovitost i antibakterijska svojstva CDHA-e.

Sukob interesa

Autori nisu bili u sukobu interesa.

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Doprinos autora: **S.O.A., C.A., E.M., E.E.** - Koncepti; Oblikovati; Analiza podataka; Priprema rukopisa; Redakcija rukopisa.

Sažetak

Svrha istraživanja: Površina privremenih nadomjestaka koji se koriste prije trajnih fiksnih nadomjestaka ili nadomjestaka postavljenih na implantate može prouzročiti stvaranje bakterijskoga ili gljivičnoga biofilma. Cilj ovog istraživanja bio je procijeniti antimikrobno djelovanje akrilatnih smola koje se upotrebljavaju u privremenim restauracijama modificiranima klorheksidin-diacetatom. **Materijal i metode:** Pripremljeno je 120 cilindričnih, autopolimeriziranih uzoraka smole modificirane klorheksidin-diacetatom u koncentracijama od 0 (kontrola), 1, 3, 5 težinskih postotaka. Antimikrobna aktivnost ispitivana je za mikroorganizme *Streptococcus mutans*, *Enterococcus faecalis* i *Candida albicans* kvantifikacijom Crystal Violeta, MTT testom i skenirajućom elektronskom mikroskopijom. Podatci su analizirani ANOVA-om i t-testom uparenih uzoraka ($\alpha = 0,05$). **Rezultati:** Dodatak klorheksidin-diacetata utjecao je na brzinu rasta i metaboličku aktivnost mikroorganizama. Antimikrobni učinak kad je riječ o *C. albicans* i *S. mutans* statistički se povećavao s postotkom klorheksidin-diacetata. Bakterija *E. faecalis* bila je manje pogođena klorheksidin-diacetatom u usporedbi s drugim patogenima. **Zaključak:** Pokazalo se da učinkovitost CHDA-e u inhibiciji proliferacije mikroorganizama pozitivno korelira s povećanjem razine koncentracije. Potrebna su dodatna istraživanja kako bi se utvrdio utjecaj različitih koncentracija klorheksidina na mehanička svojstva, kliničku učinkovitost i antimikrobna svojstva CDHA-e.

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