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IZVORNI ZNANSTVENI RAD
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Antimikrobni kapacitet kazein fosfopeptida/amorfnoga kalcijeva fosfata i enzima u stakloionomernom cementu pri sanaciji karijesnih lezija

Antimicrobial Capacity of Casein Phosphopeptide/Amorphous Calcium Phosphate and Enzymes in Glass Ionomer Cement in Dentin Carious Lesions

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Sažetak

Svrha: Željela se procijeniti moguća inhibicija rasta bakterije *S. mutans* na karioznom modelu koristeći se stakloionomernim cementom (GIC) pomiješanim s kazein fosfopeptidom/amorfnim kalcijevim fosfatom (CPP/ACP) te lizozimom, laktoferinom i laktoperoksidazom (LLL). **Materijali i metode:** Odabrano je osamdeset trajnih trećih kutnjaka (molara). Dentin tih zuba ogoljen je i zagladen. Osim koronalnoga dijela dentina, ostatak zuba hermetički je zabrtvljen, steriliziran i izvrgnut karijesnom utjecaju *S. mutans*. Karijesne lezije zatvorene su kako slijedi: grupa 1 (n = 20): GIC bez dodataka; grupa 2 (n = 20): GIC + CPP/ACP; grupa 3 (n= 20): GIC + LLL; grupa 4 (n = 20): GIC + CPP/ACP + LLL. Broj bakterija *S. mutans* određivao se prije nego što su karijesni zubi zatvoreni (n = 5), nakon 24 sata (n = 5), nakon mjesec dana (n = 5) i nakon šest mjeseci (n = 5). Rezultati su analizirani opisnom statističkom analizom i Kruskal-Wallisovim testom (Student-Newman-Keulsov test). **Rezultati:** Mjesec dana nakon zatvaranja kombinacija GIC + LLL uzrokovala je značajnu redukciju rasta *S. mutans* ($p < 0,01$), no unatoč tomu zabilježen je značajan rast *S. mutans* šest mjeseci nakon zatvaranja. Uzorci GIC-a, GIC + CPP/ACP i GIC + CPP/ACP + LLL imali su značajnu redukciju *S. mutans* nakon 24 sata ($p < 0,05$), ali i porast broja bakterija nakon mjesec dana i šest mjeseci. **Zaključak:** Dodavanje LLL-a u GIC snažnije antimikrobno djeluje na *S. mutans*. To rezultira jednomjesečnom kontrolom bakterijskoga biofilma, što na kraju zaustavlja karijesnu leziju.

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Ključne riječi
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Uvod

Minimalno invazivna stomatologija predlaže uklanjanje inficiranog dentina te očuvanje dentina zahvaćenog karijesom. Za inficirani dentin svojstvena su omekšana i degenerirana vlakna kolagena, a karijesom zahvaćeni dentin mekši je i sadržava vlakna kolagena koja se mnogu obnoviti (1, 2). U karijesnim lezijama možemo prepoznati šest slojeva:

1. vanjski ireverzibilno demineralizirani,
2. translucentni,
3. subtransparentni,
4. sklerotični,
5. zdravi dentin,
6. predentin.

Drugi, treći i četvrti sloj mogu se oporaviti (3).

Prehrana mikroba ograničena je zatvaranjem pulpodentinskoga kompleksa, pa se tako zaustavlja progresija karije-

Introduction

Minimally invasive dentistry recommends the removal of the infected dentin and the preservation of the caries-affected dentin. The infected dentin is characterized by severe softening and degeneration of its collagen fibers, whereas the caries-affected dentin is characterized by moderate softening with repairable collagen fibers (1, 2). Six different layers have been observed in carious lesions: 1- outer irreversibly demineralized layer; 2- translucent layer; 3- subtransparent layer; 4- sclerotic layer; 5- healthy dentin; 6- predentin. Layers 2, 3, and 4 are the repairable caries-affected tissues (3).

The microbial nutrition is restricted by the sealing of the dentin-pulp complex, thus stopping the progression of carious lesions (1-4). Demineralization and remineralization of the dentin are dynamic processes involved in the onset, progression, and reversal of the carious lesion. The balance

sne lezije (1 – 4). Demineralizacija i remineralizacija dentina dinamični su procesi koji uključuju nastanak, progresiju i nazadovanje karijesne lezije. Ravnoteža između tih procesa ključna je u prevenciji i tretmanu te dentalne lezije (5, 6).

Postoje različite terapijske metode u procesu remineralizacije, uključujući i upotrebu kazein fosfopeptida/amorfnoga kalcijeva fosfata (CPP/ACP). CPP/ACP bioaktivni je spoj koji stimulira reparaturu strukture zuba otpuštanjem iona kalcija i fosfata (7, 8) na površinu zuba te tako povećava gradijent koncentracije ispod površine cakline i potiče remineralizaciju *in situ* (5, 9). CPP/APP komercijalno je dostupan kao zubna pasta koja inducira mineralizaciju intra- i interfibrilarnog dentina na površini kolagena tako da elektrostatski privlači ione kalcija (10). Mjesto nukleacije formira se kako bi potaknulo remineralizaciju potrebnu za rast kristala hidroksiapatita (10). Nakon što je caklina remineralizirana CPP/ACP-om, mnogo je otpornija na kiseline u usporedbi s normalnom caklinom (11). Ovaj spoj je osteokonduktivan, biorazgradiv, bioaktivan, potiče adheriranje stanica, nije citotoksičan i kinetički je stabilan (7). U literaturi se mogu pronaći podatci o korištenju CPP/ACP-a kao aktivne komponente u mnogim proizvodima i materijalima, posebice u vodicama za ispiranje usta, zubnim pastama, gelovima za izbjeljivanje, abrazivnim pastama, lakovima i stakloionomernim cementima (GIC) (5, 7).

Dodatak antimikrobnih supstrata stakloionomernom cementu znatno povećava antimikrobni potencijal toga materijala (12 – 14). U literaturi se ističe da se u GIC najčešće kao antimikrobni agensi dodaju antibiotici (12), propolis (15), klorheksidin (13, 16, 17), triklosan (14, 18), cetrimid (19) i ketilpirimidov klorid (19). Rezultati istraživanja pokazuju da dodatak tih antimikrobnih agensa smanjuje broj vitalnih bakterija, pa tako mogu pridonijeti uspješnoj sanaciji karijesa uz minimalnu intervenciju. Prema stajalištu Tüzünea, Ulusa, Mazzaoua i ostalih (20, 21), dodavanjem antimikrobnih agensa ne utječe se na fizička i mehanička svojstva GIC-a.

Kao dodatna terapijska mogućnost nameće se uporaba lizozima, laktoferina i laktoperoksidaze (LLL) (22). Spomenuti enzimi nalaze se u slini i antimikrobno djeluju na bakterijske, virusne i gljivične patogene (23, 24). Interakcija bakterija sa salivarnim komponentama koje formiraju biofilm na površini cakline povezana je sa selektivnom adherencijom *S. mutans* na površinu cakline. Salivarne komponente koje djeluju sa *S. mutans* su mucini, lizozimi, laktoperoksidaze, aglutinin, prolin i sekretorni imunoglobulin. Goodman i suradnici (25) dokazali su da interakcija lizozima i ostalih sastojaka sline pozitivno utječe na lizu mikroorganizama. Laktoferin je glikoprotein zaslužan za vezanje željeza i nalazi se u slini. Proizvode ga neutrofili i stanice žljezdanoga epitela (26). Taj isti enzim je multifunkcinalan i bioaktivan i njegova je molekula važna u fiziologiji organizma (26). Salivarna laktoperoksidaza katalizira konverziju tiocijanata u hipotiocijanat, a taj proces koji kontrolira nastanak karijesa (27).

Svrha ovog istraživanja bila je procijeniti mogućnost CPP/ACP-a i LLL-a dodanih u GIC da inhibiraju rast *S. mutans* u karioznom modelu.

between these processes is essential for the prevention and treatment of this dental disease (5, 6).

Different treatment options to promote the remineralization process have been evaluated, including the use of casein phosphopeptide/amorphous calcium phosphate (CPP/ACP). CPP/ACP is a bioactive compound that stimulates the repair of tooth structure by first releasing calcium and phosphate ions (7, 8) and then attracting them to the tooth surface, thus increasing the concentration gradient on the enamel subsurface and promoting *in situ* remineralization (5, 9). CPP/ACP is commercially available as toothpaste and induces the mineralization of the intrafibrillar and interfibrillar dentin on the collagen surface by attracting calcium ions through electrostatic force (10). A nucleation site is formed to trigger the remineralization needed for the growth of hydroxyapatite crystals (10). After enamel is remineralized using CPP/ACP, it becomes more resistant to acid changes when compared with normal carbonated enamel (11). This compound has osteoconductivity, biodegradability, bioactivity, high cell adherence, no cytotoxicity, and kinetic stabilization (7). There are reports in the literature on the use of CPP/ACP as an active component of many products and materials, especially mouthwashes, toothpastes, some dental bleaching, abrasive pastes, varnishes, and glass ionomer cement (GIC) (5, 7).

The addition of antibacterial substances to GIC has been shown to increase the antimicrobial potential of this material (12-14). According to the literature, the most commonly used antimicrobials added to GIC are antibiotics (12), propolis (15), chlorhexidine (13, 16, 17), triclosan (14,18), cetrimide (19), and cetylpyridinium chloride (19). The results reported in these studies show that the addition of these antimicrobials is able to reduce the number of viable bacteria and may contribute to the successful treatment of caries using minimal intervention. Moreover, according to Tüzüner & Ulusu and Mazzaoui et al. (20, 21), the addition of antimicrobial agents does not affect the physical and mechanical properties of GIC.

Another therapeutic possibility is the use of enzymes such as lysozyme, lactoferrin, and lactoperoxidase (LLL) (22). These enzymes are present in saliva and exert an antimicrobial effect against bacterial, viral, and fungal pathogens (23, 24). The interaction of bacteria with the salivary components that form the biofilm on the enamel surface is associated with the selective adherence of *S. mutans* to the surface. The salivary components that interact with *S. mutans* are mucin, lysozyme, lactoperoxidase, agglutinin, proline, and secretory immunoglobulin. Goodman et al. (25) demonstrated that, when lysozyme and other substances found in saliva interact, they may be effective in the lysis of microorganisms. Lactoferrin, an iron-binding glycoprotein present in saliva, is produced by neutrophils and glandular epithelial cells (26). This enzyme is a multifunctional bioactive molecule playing an important role in the physiological system (26). Salivary lactoperoxidase catalyzes the conversion of thiocyanate into hypothiocyanite, which regulates the incidence of caries (27).

The objective of the present study was to evaluate the ability of CPP/ACP and LLL added to GIC to inhibit the growth of *S. mutans* in a caries model.

Materijali

GIC korišten u ovom istraživanju bio je Ketac™ Cem Easymix (3M ESPE, Seefeld, Njemačka) koji se komercijalno može nabaviti kao prašak s priloženom tekućinom za miješanje. Razlog da je odabran za ovaj niz pokusa jest njegov potencijal da služi kao podloga te se, ako dodavanjem LLL-a i CPP/ACP-a oslabe mehanička svojstva GIC-a, kavitet i dalje može zaštititi drugim ispunom. Korišteni su sljedeći enzimi: lizozim (Sigma, Sao Paulo, SP, Brazil), laktoferin (Sigma) i laktoperoksidaza (Sigma). Nakon što su izmjereni na preciznoj vagi (Uni Bloc, Shimadzu Auy220, Kjoto, Japan), jedan posto svih enzima dodan je u prah GIC-a i zatim je sve izmiješano u mužaru. Kako bismo pripremili CPP/ACP, upotrijebljen je 1 g GC Tooth Mousse Plus paste (GC Corporation, Tokio, Japan) nakon što je izvagan na preciznoj vagi (Uni Bloc) te razrijeđen u 4 ml destilirane vode kako bi se smanjile moguće promjene u kemizmu vezanja GIC-a. Nakon toga je 3 posto te otopine dodano u tekućinu GIC-a i pomiješano dok nije dobivena homogena otopina. Materijali korišteni u ovom istraživanju te ime proizvođača i serijski broj nalaze se u tablici 1.

Materials

The GIC used throughout this study was the Ketac™ Cem Easymix (3M ESPE, Seefeld, Germany), which comprises powder/liquid components and is commercially available for manual mixing. This GIC was chosen for this series of experiments because it has the potential for serving as a cavity liner; thus, in case the procedure of adding LLL and CPP/ACP weakened the mechanical strength of GIC, the cavity would be protected by a filling layer. The following enzymes were used: lysozyme (Sigma, Sao Paulo, SP, Brazil), lactoferrin (Sigma), and lactoperoxidase (Sigma). Using a precision scale (Uni Bloc, Shimadzu Auy220, Kyoto, Japan), 1% of each of the enzymes was added to the powder of GIC and mixed in a sterile porcelain mortar and pestle set. To prepare CPP/ACP, 1 g of GC Tooth Mousse Plus paste (GC Corporation, Tokyo, Japan) was weighed on a precision scale (Uni Bloc) and diluted in 4 ml of distilled water to minimize possible changes in the setting chemistry of GIC. Then, 3% of this solution was added to the liquid of GIC and mixed until a homogeneous solution was obtained. The materials used in the study along with the manufacturer's name and batch number are described in Table 1.

Tablica 1. Materijali, proizvođači i serijski brojevi
Table 1 Materials, manufacturers, and batch numbers

Materijal • Product	Proizvođač • Manufacturer	Serijski broj • Lot
Glass Ionomer Cement (Ketac Cem Easymix)	3M ESPE. Germany	494562
Lysozyme	SIGMA – Aldrich Brasil Ltda. São Paulo, Brazil	SLBF1341V
Lactoferrin	SIGMA – Aldrich Brasil Ltda. São Paulo, Brazil	SLBB2642V
Lactoperoxidase	SIGMA– Aldrich Brasil Ltda. São Paulo, Brazil	SLBB5243V
Tooth Mousse Plus paste	GC Corporation. Tokyo, Japan	120816S

Metode

Ovo istraživanje odobrilo je sveučilišno Etičko povjerenstvo (Protokol: 0345/11).

Odabir uzoraka

Odabrano je osamdeset trajnih trećih kutnjaka. Svi pacijenti potpisali su informirani pristanak.

Kriterij za uključivanje u istraživanje

- Trajni treći kutnjaci
- Zubi bez frakturnih pukotina pregledani pod povećanjem od 10 puta (povećalo Stemi DV4 Carl Zeiss, São Paulo, Brazil)

Postupak

Zubi su pohranjeni u 0,9-postotni natrijev klorid s dodatkom 0,02 posto natrijeva azida (LabCenter, São Paulo, Brazil) na temperaturi od 4 °C (28). Okluzalna trećina zuba uklonjena je dijamantnim diskom (KG Sorensen Indústria e

Methods

This study was approved by the Research Ethics Committee of PUC-Campinas (Protocol: 0345/11).

Selection of samples

Eighty permanent third molars were selected at the Dental Clinic of PUC-Campinas. All donor patients signed an Informed Consent Form.

The inclusion criteria were:

- Permanent third molars;
- No fissures or fractures detected by a 10x magnifying glass (Stemi DV4 Carl Zeiss, São Paulo, Brazil).

Procedures

The teeth were stored in sodium chloride 0.9% containing sodium azide (0.02%) (LabCenter, São Paulo, Brazil) at 4°C (28). The occlusal third was removed from the specimens using double-sided diamond disc (KG Sorensen In-

Comercio LTDA, São Paulo, Brazil) na niskoj brzini s vodenim hlađenjem. Površina dentina ispolirana je mokrim silikonsko-karbidnim brusnim papirom gradacije P 600 (Água T223 advance, Norton, Indústria Brasileira, São Paulo, Brazil). Svi uzorci zabrtvljeni su epoksidnom smolom (Araldite, São Paulo, Brazil) i lakom za nokte (Colorama, Sao Paulo, Brazil), osim koronalne trećine uzorka u ventilacijskoj komori (Veco, Campinas, SP, Brazil). Nakon toga sterilizirani su 20 minuta u autoklavu (Sercon, São Paulo, Brazil) na temperaturi od 121 °C i pod pritiskom od 1 atm (29).

Kako bi simulirali karijesom zahvaćeni dentin, uzorci su stavljeni u sterilne epruvete koje su sadržavale medij BHI (LabCenter, São Paulo, Brazil), 0,5 posto ekstrakta plijesni (LabCenter, São Paulo, Brazil), 1 posto glukoze (LabCenter, São Paulo, Brazil) i 1 posto sukroze (LabCenter, São Paulo, Brazil). U to je zatim dodan soj *S. mutans* (ATCC 25175) (Fundação André Tosello, Campinas – SP, Brazil) klasificiran na 0,5 prema MsFarlandovu standardu. Uzorci su mjesec dana inkubirani na 37 °C u anaerobnim staklenkama koje su sadržavale omotnice s plinovima i atmosferu od 85 posto dušika (N_2), 10 posto ugljičnog dioksida (CO_2) i 5 posto hidrogena (H_2). Nakon toga svaki je zub pohranjen u bakteriološki inkubator (Fanem Ltda, São Paulo, SP, Brazil). Tijekom inkubacije BHI medij mijenjan je svaka dva dana (prema de Carvalhu i suradnicima)(30). Nakon toga uzorci su nasumce podijeljeni u četiri grupe (n = 20 po grupi):

Grupa 1: GIC bez aditiva

Grupa 2: 3 posto CPP/ACP-a dodano u tekući dio GIC-a

Grupa 3: 1 posto lizozima, 1 posto laktoferina i 1 posto laktoperoksidaza dodano u prah GIC-a

Grupa 4: 3 posto CPP/ACP-a dodano u *tekuću* komponentu GIC-a, uz 1 posto lizozima, 1 posto laktoferina i 1 posto laktoperoksidaze dodanih u *prah* GIC-a

U svakoj grupi (n = 20) određen je broj *S. mutans* prije zatvaranja karijesne lezije (n = 5), nakon 24 sata (n = 5), nakon mjesec dana (n = 5) i nakon šest mjeseci (n = 5). GIC je uklonjen s uzorka velikom brzinom sterilnim sferičnim dijamantnim svrdlom (KG Sorensen, São Paulo, Brazil) i pritom je hlađen fiziološkom otopinom. Uzorci karioznog dentina skupljeni su sterilnim ekskavatorom #20 (SSWhite Duflex, Rio de Janeiro, Brazil). Odmah su stavljeni u BHI medij (LabCenter, São Paulo, Brazil). Svi su obrađeni u roku od 24 sata i homogenizirani 3 minute (Phoenix, Araraquara, SP, Brazil). Odmah nakon toga pripremljeno je 5 decimalnih razrjeđenja. Tri alikvota od 25 uL ovih razrjeđenja stavljeno je na površinu *mitis salivarius* bacitracin medija (MBS). Inkubacija u posudama (Oxoid Ltd., Basingstoke, Hampshire, England) trajala je 5 dana na temperaturi od 37°C i u atmosferi od 85 posto dušika (N_2), 10 posto ugljičnog dioksida (CO_2) i 5 posto hidrogena (H_2). Takva atmosfera dobivena je s pomoću anaerobnih staklenki koje sadržavaju omotnice plinova i anaerobne indikatore (Oxoid Ltd., Basingstoke, Hampshire, England). Nakon inkubacije prebrojene su vitalne bakterije (31).

dústria e Comercio LTDA, São Paulo, Brazil) at low speed under water-cooling for dentin exposure. The dentin surfaces were polished with wet silicon carbide sandpaper sheets, P600 grit (Água T223 advance, Norton, Indústria Brasileira, São Paulo, Brazil). The specimens were sealed using epoxy resin (Araldite, São Paulo, Brazil) and nail polish (Colorama, Sao Paulo, Brazil), except for the coronal dentin in the laminar flow (Veco, Campinas, SP, Brazil). After sealing the teeth, the specimens were sterilized in an autoclave (Sercon, São Paulo, Brazil) for 20 minutes at 121°C and 1 atm (29) and submitted to cariogenic challenge.

With the purpose of simulating caries-affected dentin, the teeth were placed in sterile test tubes with brain heart infusion (BHI) broth (LabCenter, São Paulo, Brazil) plus 0.5% yeast extract (LabCenter, Sao Paulo , Brazil), 1% glucose (LabCenter, São Paulo, Brazil), and 1% sucrose (LabCenter, São Paulo, Brazil). The standard strain of *S. mutans* (ATCC 25175) (Fundação André Tosello, Campinas - SP, Brazil) standardized to 0.5 McFarland standard was added to the BHI (LabCenter, São Paulo, Brazil). The specimens were incubated at 37°C for 1 month in anaerobic jars with gas-generating envelopes (LabCenter, São Paulo, Brazil) in an atmosphere containing 85% nitrogen (N_2), 10% carbon dioxide (CO_2), and 5% hydrogen (H_2). After that, the teeth were stored in a bacteriological incubator (Fanem Ltda, São Paulo, SP, Brazil). During this period, the BHI broth (LabCenter, São Paulo, Brazil) was replaced every 2 days (adapted from de Carvalho et al.) (30). Then, the teeth were randomly assigned to one of four groups of materials for sealing the carious lesion (n=20 per group):

Group 1: GIC (powder/liquid components) without any additives.

Group 2: 3% of CPP/ACP added to the liquid of GIC.

Group 3: 1% of lysozyme, 1% of lactoferrin, and 1% of lactoperoxidase added to the powder of GIC .

Group 4: 3% of CPP/ACP added to the liquid of GIC + 1% of lysozyme, 1% of lactoferrin, and 1% of lactoperoxidase added to the powder of GIC.

In each group (n=20), *S. mutans* counts were performed before sealing the carious tissue (n=5), after 24 hours (n=5), 1 month (n=5), and 6 months (n=5). The GIC restorations were removed using sterile spherical diamond burs (KG Sorensen, São Paulo, Brazil) at high speed under saline cooling. The collections of caries-affected dentin were performed using a sterile #20 spoon excavator (SSWhite Duflex, Rio de Janeiro, Brazil). The specimens were immediately immersed in BHI broth (LabCenter, São Paulo, Brazil). This material was manipulated within a period of 24 hours and homogenized for 3 minutes in a tube shaker (Phoenix, Araraquara, SP, Brazil). Immediately after, 5 decimal dilutions were performed. Three aliquots of 25 uL of these dilutions were seeded in the surface of the *mitis salivarius* bacitracin (MSB) medium. All plates were incubated in jars (Oxoid Ltd., Basingstoke, Hampshire, England) for 5 days at 37°C in 85% nitrogen (N_2), 10% carbon dioxide (CO_2), and 5% hydrogen (H_2). Such atmosphere was obtained using anaerobic jars with gas-generating envelopes and anaerobic indicators (Oxoid Ltd., Basingstoke, Hampshire, England). After incubation, we counted the total number of viable bacteria (31).

Statistička analiza

Usporedili smo ukupan broj vitalnih bakterija *S. mutans* prije zatvaranja karijesnih lezija, nakon 24 sata, jednog mjeseca i šest mjeseci, a koristili smo se Bioestatom 4,0. Rezultati su analizirani opisnom statističkom analizom te Kruskal-Wallisovim i Student-Newman-Keulsovim testom. Razina statističke značajnosti određena je na 5 posto ($p < 0,05$).

Rezultati

Uspoređujući rezultate *S.mutans* u karijesnim lezijama prije zatvaranja, nakon 24 sata, jednog mjeseca i 6 mjeseci, vidjeli smo da GIC bez dodataka, GIC s dodatkom CPP/ACP-a i GIC s dodatkom CPP/ACP-a i LLL-a pokazuju slične rezultate – kod svih je značajno reduciran broj *S. mutans* nakon 24 sata ($p < 0,05$) ali je značajno porastao nakon mjesec dana i nakon šest mjeseci. GIC s dodatkom LLL-a značajno je reducirao *S. mutans* nakon mjesec dana ($p < 0,01$). Unatoč tomu, nakon 6 mjeseci lezije zatvorene GIC-om s dodatkom LLL-a imale su značajan porast vrijednosti *S. mutans* (tablica 2., slika 1.).

Statistical Analysis

We compared the total amount of viable *S. mutans* before sealing the carious tissue, after 24 hours, 1 month, and 6 months using Bioestat 4.0. The results were analyzed using descriptive statistical analysis and the Kruskal-Wallis test followed by Student-Newman-Keuls test. The level of significance was set at 5% ($p<0.05$).

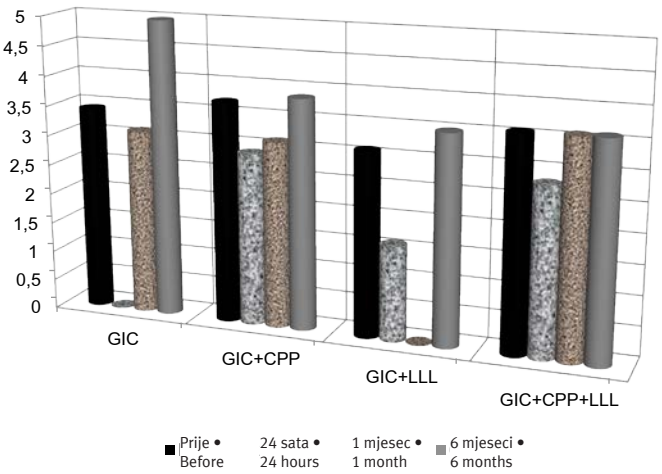
Results

After comparing the counts of *S. mutans* in the carious lesions before sealing and after 24 hours, 1 month, and 6 months, we found that GIC without additives, GIC with the addition of CPP/ACP, and GIC with the addition of CPP/ACP and LLL showed similar behavior with significant reduction of *S. mutans* after 24 hours ($p<0.05$) and increased count after 1 and 6 months. GIC with the addition of LLL resulted in a significant reduction of *S. mutans* after 1 month ($p<0.01$). However, at 6 months, carious lesions sealed with GIC with the addition of LLL showed a significant growth of *S. mutans* (Table 2 and Figure 1).

Tablica 2. Usporedba vrijednosti *S. mutans* u karijesnim lezijama prije i poslije zatvaranja različitim stakloionomernim cementima (GIC)
Table 2 Comparison of *S. mutans* counts in carious lesions before and after sealing with different glass ionomer cement (GIC)-based materials

Sealing material	Prije • Before	Poslije 24 sata • 24 hours	poslije 1 mjeseca • 1 month	poslije 6 mjeseci • 6 months	p*
GIC	3.44 (0.20)[a]	0.00 (0.00)[b]	3.11 (4.77)[a]	4.98 (0.17)[a]	0.0085
GIC+CPP/ACP	3.71 (0.94)[a]	2.90 (0.06)[b]	3.12 (0.30)[a]	3.86 (0.11)[a]	0.0052
GIC+LLL	3.10 (0.30)[a]	1.60 (0.00)[a]	0.00 (0.00)[b]	3.50 (0.36)[a]	0.0004
GIC+CPP/ACP+LLL	3.58 (0.50)[a]	2.82 (0.39)[b]	3.60 (0.26)[a]	3.61 (0.04)[a]	0.0186

GIC: GIC bez dodataka • GIC without additives
GIC + CPP/ACP: GIC s dodacima kazein fosfopeptida/amorfnoga kalcijeva fosfata • GIC with addition of casein phosphopeptide/amorphous calcium phosphate
GIC + LLL: GIC s dodatkom lizozima, laktoferina i laktoperoksidaze • GIC with addition of lysozyme, lactoferrin, and lactoperoxidase
GIC + CPP/ACP + LLL: GIC s dodatkom kazein fosfopeptida/amorfnoga kalcijeva fosfata i dodatkom lizozima, laktoferina i laktoperoksidaze • GIC with addition of casein phosphopeptide/amorphous calcium phosphate and addition of lysozyme, lactoferrin, and lactoperoxidase
Vrijednosti su izražene kao srednja vrijednost (interkvartilna devijacija); različita slova u istom redu označuju statističku značajnost ($p < 0,05$) • Values are expressed as median (interquartile deviation). Different letters in the same row indicate significant difference ($p<0.05$)
* Kruskal-Wallisov test i Student-Newman-Keulsov test • Kruskal-Wallis test followed by Student-Newman-Keuls test



Slika 1. Medijani broja S. mutans prije i nakon 24 sata te 6 mjeseci nakon zatvaranja različitim staklenoionomernim cementima (GIC)
Figure 1 Medians of *S. mutans* counts before and 24 hours, 1 month, and 6 months after sealing of caries with different glass ionomer cement (GIC)-based materials

Rasprava

Naše istraživanje razlikuje se od ostalih u literaturi jer nismo stavljali uzorke u destiliranu vodu (16, 17, 20) i fiziološku otopinu (32). Pohranjivanjem uzoraka u BHI medij uspjeli smo sačuvati vitalitet mikroba tijekom eksperimenta, ako su postojale marginalne pukotine između GIC-a i zuba.

Prema našim rezultatima možemo zaključiti da konvencionalni GIC značajno reducira *S. mutans* u prva 24 sata nakon zatvaranja lezije. Unatoč tomu nakon mjesec dana i nakon šest mjeseci porastao je broj tih bakterija u usporedbi s vrijednostima nakon 24 sata. Rezultati su u skladu s ishodima prijašnjih istraživanja (13, 17, 21) zato što postoje saznanja o tome da je antimikrobno djelovanje ovog materijala najjače prvih nekoliko sati nakon zatvaranja lezije. To se najvjerojatnije događa zbog pojačanog otpuštanja florida (17) dok se GIC veže. Proces vezanja istiskuje ione (uključujući i floride) tijekom rane gel-faze. Još jedno moguće objašnjenje jest da učinkovito zatvaranje dentinsko-pulpnog kompleksa, zahvaljujući kemijskom vezanju i interakciji karboksilne grupe i polialkatične kiseline GIC-a s ionima kalcija i hidroksiapatita, ograničava i onemogućuje prehranu mikroba te tako zaustavlja progresiju karijesne lezije (1 – 4).

Dodatak LLL-a GIC-u u ovom istraživanju rezultirao je redukcijom *S. mutans* nakon 24 sata i mjesec dana nakon zatvaranja. Unatoč tomu, nakon šest mjeseci zabilježen je porast broja te bakterije. Ovaj rezultat u skladu je s rezultatima Jyotia i suradnika (33) zato što ti autori zagovaraju upotrebu laktooperoksidaze u proizvodima za oralnu higijenu. LLL bakteriostatski i baktericidno djeluje na oralne patogene, uključujući i *S. mutans.* Lizozim uzrokuje lizu bakterija tako što kida veze između N-acetil muramične kiseline i N-acetil glukozaminske kiseline. To uzrokuje propadanje peptidoglikana bakterijskoga staničnog zida posredstvom aktivnosti muramidaze te pokazuje njezinu neaktivnost uzrokovanu rupturom membrane (34). Lizozim pokazuje baktericidna svojstva i nakon inaktivacije toplinom, vjerojatno zbog određenih kationskih svojstava (35). Laktoferin ima bakteriostatska antimikrobna svojstva jer reducira količinu željeza potrebnog za bakterijski vitalitet (36). Ljudska slina ima antibakterijski sustav laktoperoksidaze, vodikov peroksid te ione tiocijanata. Vodikov peroksid u oralnoj šupljini proizvod je mikrobakterija. Laktoperoksidaza katalizira oksidaciju tiocijanata s pomoću vodikova peroksida te proizvodi hipotiocijanoznu kiselinu ili hipotiocijanatni anion. Kiselina i anion antibakterijski su aktivni. Utječući na bakterijski metabolizam hipotiocijanat djeluje kao inhibitor rasta (27). Djelovanje aniona na inhibiciju metabolizma glukoze *S. mutans* pospješuje se kada je reduciran pH medij (37).

Dodavanje CPP/ACP-a i CPP/ACP + LLL-a u GIC pokazalo je značajnu redukciju *S.mutans* u roku od 24 sata nakon zatvaranja karijesne lezije, u usporedbi s brojem te bakterije prije zatvaranja. Unatoč tomu, nakon mjesec dana i nakon 6 mjeseci porastao je broj *S. mutans*. Stoga možemo zaključiti da se dodavanjem enzima LLL-a u GIC-u poboljšavaju antibakterijska svojstva materijala. S dodatkom CPP/ACP-a, materijal se ponaša kao čisti konvencionalni GIC, najvjerojatnije zato što je CPP/ACP inhibirao antimikrobni

Discussion

Our study is different from other studies from the literature that stored the specimens in distilled water (16, 17, 20) and saline (32). By storing our specimens in BHI broth, we could preserve microbial viability during the experimental period if there were marginal fissures in the interface between GIC and the tooth.

Based on our results, we found that conventional GIC showed a significant reduction of *S. mutans* in the first 24 hours after sealing; however, after 1 and 6 months there was bacterial growth when compared with the count performed after 24 hours. This finding is in agreement with the literature (13, 17, 21), since there are reports that the antibacterial action of this material is more prevalent in the first hours after sealing. This may be explained by the higher fluoride release that occurs (17) during GIC setting. Such release displaces the ionically active elements (including fluoride) in the early stages of gelation. In addition, the effective sealing of the dentin-pulp complex is another possible explanation for the antibacterial action, because it promotes chemical adherence through the interaction between the carboxyl groups of the polyalkenoic acid of GIC and the calcium ions of hydroxyapatite, thus restricting microbial nutrition and stopping the progression of carious lesions (1-4).

The addition of LLL to GIC in this study resulted in reduction of *S. mutans* 24 hours and 1 month after sealing. However, after just 6 months of sealing, there was growth of *S. mutans.* This is in agreement with Jyoti et al. (33) because these authors considered that the action and addition of lactoperoxidase in oral hygiene products were effective. LLL have bacteriostatic and bactericidal effect against oral cavity pathogens, including *S. mutans.* Lysozyme causes bacterial lysis by breaking the binding between the N-acetyl muramic acid and the N-acetyl-glucosamine acid. Therefore, it degrades the peptidoglycan of the bacterial cell wall by muramidase activity and shows non-muramidase activity caused by the rupture of the membrane (34). Lysozyme has bactericidal activity even after heat inactivation, probably because of its cationic characteristic (35). Lactoferrin has bacteriostatic antimicrobial properties because it reduces the amount of viable iron provided to the bacteria (36). Human saliva also includes the antibacterial system of lactoperoxidase, hydrogen peroxide, and thiocyanate ion. The hydrogen peroxide present in the oral cavity is produced by microorganisms. Lactoperoxidase catalyzes the oxidation of thiocyanate ions by means of hydrogen peroxide, thus generating hypothiocianous acid or hypothiocyanite anion, both with antibacterial action. Hypothiocyanite acts as bacterial inhibitor interfering in cell metabolism (27). The action of hypothiocyanite inhibiting glucose metabolism of *S. mutans* becomes more effective as the pH of the medium is reduced (37).

The addition of CPP/ACP and CPP/ACP + LLL to GIC showed a significant reduction of *S. mutans* 24 hours after sealing of the caries lesions when compared with the *S. mutans* count before sealing. However, after 1 and 6 months, there was growth of *S. mutans.* Thus, we found that the addition of LLL enzymes to GIC improves the antibacterial

učinak enzima zbog antagonističke kemijske reakcije. To se može objasniti činjenicom da CPP/ACP, osim što potiče remineralizaciju, povećava i pH vrijednosti medija (38, 39) tako što raste koncentracija iona kalcija i fosfata (5, 7, 8). To je razlog da karijes u zahvaćenom dentinu postaje više bazičan. Zato su u ovom istraživanju promjene u vrijednostima pH mogle utjecati na aktivnost LLL enzima kojima je optimalan raspon pH između 5 i 6 (26, 37, 40, 41).

Rast *S. mutans* u svim grupama nakon šest mjeseci objašnjen je oštećenjem ili gubitkom pečatnoga materijala tijekom pohrane. Czarnecka i suradnici (32) pronašli su marginalne pukotine u GIC-u na zubima koji su imali karijesne lezije i to 21 dan nakon pohrane uzoraka u fiziološkoj otopini. Tijekom manipulacije GIC-om u materijalu nastaju mjehurići zraka te uzrokuju poroznost koja je eksponirana tijekom abrazije ili erozije kiselinom, što pridonosi hrapavosti površine. Hrapavost GIC-a može umanjiti otpornost na trošenje te pridonijeti većoj sklonosti površine prema odlaganju bakterijskoga biofilma, a posljedica je degradacija površine na rubno propuštanje (42).

Na kraju možemo reći da dodatak lizozima, laktoferina i laktoperoksidaze u testiranom GIC-u poboljšava kratkotrajna antimikrobna svojstva i može se koristiti za mikrobnu redukciju *S. mutans* u dentinu zahvaćenom karijesom. Dodatkom CPP/ACP-a nisu poboljšana antimikrobna svojstva GIC-a. Nakon šest mjeseci u svim grupama uzoraka uočena je vitalnost bakterija i povećanje broja *S. mutans. To* može biti povezano sa starenjem ispuna i marginalnim propuštanjem, što pridonosi metabolizmu preostalih bakterija.

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Sukob interesa

Nije ga bilo.

properties of the material. Nevertheless, when CPP/ACP is added, the material begins to have the same behavior as pure conventional GIC, thus demonstrating that CPP/ACP probably inhibited the antimicrobial effect of the enzymes because of antagonist chemical action. This may be explained by the fact that CPP/ACP, in addition to promoting remineralization, increases the pH of the medium (38, 39) by increasing the concentration of calcium and phosphate ions (5, 7, 8), thus the medium becomes more alkaline in caries-affected dentin. Therefore, in the present study, changes in pH may have inhibited the activity of LLL, which have an antimicrobial effect between pH 5 and 6 (26, 37, 40, 41).

The growth of *S. mutans* in all groups after 6 months is explained by the loss and/or wear of the seal during storage. Czarnecka et al. (32) found marginal fissures in GIC in teeth with preexisting carious lesions after 21 days of storage of specimens in saline. GIC incorporates air bubbles during handling, which cause porosities that are exposed during the acid erosion or abrasion, thus contributing to increased surface roughness. GIC roughness may decrease the wear resistance and make this surface more prone to increased deposition of bacterial biofilm with consequent surface degradation and marginal leakage (42).

In conclusion, the addition of lysozyme, lactoferrin, and lactoperoxidase to the tested GIC enhanced its short-term antimicrobial properties and it may be used for microbial reduction of *S. mutans* in caries-affected dentin. The addition of CPP/ACP did not add antimicrobial properties to GIC. After 6 months, all groups showed bacterial viability and increase of *S. mutans*. This may be related to the aging process of the restoration and the presence of marginal fissures, thus enabling the nutrition of the remaining bacteria.

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Conflict of interest

None declared.

Abstract

Objective: To evaluate the ability of casein phosphopeptide/amorphous calcium phosphate (CPP/ACP) and lysozyme, lactoferrin, and lactoperoxidase (LLL) added to glass ionomer cement (GIC) to inhibit the growth of *S. mutans* in a caries model. **Material and methods:** Eighty permanent third molars were selected. The dentin of these teeth was exposed and flattened. Except for the coronal dentin, the specimens were waterproofed, autoclaved, and submitted to cariogenic challenge with standard strain of *S. mutans*. The carious lesions were sealed as follows: group 1 (n=20): GIC without additives; group 2 (n=20): GIC + CPP/ACP; group 3 (n=20): GIC + LLL; group 4 (n=20): GIC + CPP/ACP + LLL. *S. mutans* counts were performed before the caries were sealed (n=5), after 24 hours (n=5), at 1 month (n=5), and at 6 months (n=5). The results were analyzed using descriptive statistical analysis and the Kruskal-Wallis test (Student-Newman-Keuls test). **Results:** GIC + LLL caused a significant reduction of *S. mutans* 1 month after sealing ($p<0.01$); however, there was a significant growth of *S. mutans* 6 months after sealing. GIC, GIC + CPP/ACP, and GIC + CPP/ACP + LLL showed similar behavior with significant reduction of *S. mutans* after 24 hours ($p<0.05$) and increase after 1 and 6 months. **Conclusion:** The addition of LLL to GIC increases the antimicrobial action of GIC on *S. mutans*. This leads to control of bacterial biofilm for 1 month, thus stopping the progression of carious lesions.

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Key words
Caries; Streptococcus mutans; casein phosphopeptide-amorphous calcium phosphate nanocomplex; Glass Ionomer Cements; Anti-Bacterial Agents; Muramidase; Lactoferrin; Lactoperoxidase

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