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IZVORNI ZNANSTVENI RAD

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Effect of Different Bleaching Gels Thickeners on Cytotoxicity to Human Gingival Fibroblasts and Enamel Physical Properties: an *in Situ* Study

Citotoksičnost različitih zgušnjivača gelova za izbjeljivanje na fibroblaste ljudske gingive i učinak na fizikalna svojstva cakline: istraživanje in situ

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Abstract

Objective: This study evaluated *in vitro*, the effects of carbamide peroxide 10% (CP) associated with Carbopol® (CP-ct) and Aristoflex® (CP-at) thickeners on human gingival fibroblasts (HGF) cytotoxicity and assessed *in situ* their effects on dental enamel. **Material and methods:** The cytotoxicity was analyzed using MTT - Vybrant® proliferation test. For *in situ* stage, 144 bovine enamel/dentin blocks were randomized into seven groups (n=12). Samples were stained, fixed in intraoral palatal devices and bleached for 4 h, during 14 days, with: Carbopol thickener (ct), Aristoflex thickener (at), CP-ct, CP-at, CP without thickener (CP-wot), Commercial CP (CP-com). The samples had their microhardness (SMH), roughness (Ra) and color analyzed using a microdurometer, a rugosimeter and a spectrophotometer, respectively. The analyses were performed at baseline and 24-h after completion of tooth bleaching. **Results:** Different thickeners were similar regarding their cytotoxicity. The experimental gels with Carbopol exhibited lower SMH values, while the groups treated with CP exhibited higher Ra values. For the color change results, the groups treated with CP had values above the acceptability and perceptibility limits. **Conclusion:** CP-at was able to promote an effective bleaching with less alterations of the tooth surface compared to the CP-ct. Hence, Aristoflex stands as a promising thickener in conjunction with CP in order to preserve the physical properties of dental enamel after home bleaching.

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Introduction

Among bleaching gels, the gold standard treatment is carbamide peroxide 10 wt% (1). The bleaching treatment can be regarded as safe and minimally invasive (2). However, there are concerns remaining about its unfavorable effects on dental tissues such as alterations of the surface roughness or the tissue microhardness (3,4), apart from its possible cytotoxic effects on the gingiva (5).

The mechanism by which the tooth bleaching occurs is associated with hydrogen peroxide and its precursor, carbamide peroxide (CP), oxidizing capacity. These radicals are oxidizing agents which are able to break chromogenic molecules. Besides, they are able to remove these pigments by dif-

Uvod

Među gelovima za izbjeljivanje zuba zlatni standard je 10-postotni karbamid-peroksid (1). Postupak izbjeljivanja može se smatrati sigurnim i minimalno invazivnim (2). No postoji zabrinutost zbog nepovoljnih učinaka koje može generirati u zubnim tkivima, poput promjena u hrapavosti površine ili mikrotvrdoće tkiva (3, 4), osim njegovih mogućih citotoksičnih učinaka na gingivu (5).

Mehanizam prema kojemu se zubi izbjeljuju povezan je s oksidacijskim kapacitetom vodikova peroksida i njegova prekursora, karbamid-peroksida (CP). Ti su radikali oksidirajuća sredstva koja mogu razbiti kromogene molekule i također mogu ukloniti te pigmente difuzijom iz strukture cakline i

fusion out of the enamel and dentin structure. Consequently, this reduces the light absorption, thus making the tooth lighter (6–8).

CP-based bleaching gels formulations usually comprise Carbopol (*carboxypolymethylene*) as thickener which is an acidic and ionic polymer derived from carboxylic acid. Carbopol increases the bleaching gels viscosity (9), hence is able to promote better retention in tray and maintenance on the dental surface. In other studies, it has been proven that Carbopol could reduce the enamel microhardness due its capacity to bind to calcium (10,11), thus preventing the saliva minerals from being incorporated into the dental structure.

Considering the Carbopol limitations, this study intended to evaluate a bleaching gel that contained a thickener based on sulfonic acryloyldimethyltaurate acid copolymer and vinylpyrrolidone (Aristoflex), which is a pre-neutralized synthetic polymer that has been already assessed in an *in vitro* study (12). Aristoflex aids in the formulation of crystalline gels with an adequate consistency. This thickener has some important properties: It is stable in an acid pH has a cationic behavior, and it acts as an inert thickener within the formulations. In the pharmaceutical production it is already used as a thickener in personal care formulations. Moreover, it is also incorporated in oral hygiene products such as whitening toothpastes, mouth rinses, and gels (13), without posing long-term risks to human health.

As previously mentioned, this study evaluated the *in vitro* cytotoxicity and *in situ* physical alterations of dental enamel, such as color, roughness, and microhardness, after treatment with an experimental bleaching gel that contains carbamide peroxide 10 wt% and sulfonic acryloyldimethyltaurate acid copolymer and vinylpyrrolidone (Aristoflex). The study null hypotheses were: 1) Aristoflex would not be toxic to human gingival fibroblasts (HGF) cells; 2) 10 wt% carbamide peroxide gel containing Aristoflex would not interfere with the bleaching efficacy, enamel roughness, and microhardness.

Material and methods

Cytotoxicity Analysis

Based on initial cytotoxicity tests, dental bleaching gel solutions with different concentrations were formulated. First, the pH values of gels were measured in triplicate with a digital pH meter (Phs-3b, Phtek, Sao Paulo, SP, Brazil). The pH meter had a pH electrode with a temperature sensor connected to an ion analyzer. The electrode was put inside 3 g of each gel and the results were recorded (Table 1).

For the cytotoxicity analysis based on a previous study (14), human gingival fibroblasts (HGF) were cultivated in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with antibiotics (10000 U penicillin and 10 mg/mL streptomycin - Vitrocell Embriolife, Campinas, SP, Brazil) and 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and humidified atmosphere containing 5 % CO_2. Once an approximate 80 % confluence was reached, in order to detach them, the cells were washed with 0.25 % trypsin/EDTA (Gibco, Thermo Fisher Scientific, Waltham,

dentina. Posljedično se smanjuje apsorpcija svjetlosti, a zub postaje svjetliji (6 – 8).

Formulacije gelova za izbjeljivanje na bazi CP-a obično sadržavaju karbopol (karboksipoimetilen) kao zgušnjivač – to je kiseli i ionski polimer dobiven od karboksilne kiseline. Karbopol povećava viskoznost gelova za izbjeljivanje (9) i zato može pospješiti njihovo održavanje na površini zuba. U drugim istraživanjima dokazano je da može smanjiti mikrotvrdoću cakline za njezin kapacitet vezanja kalcija (10, 11), sprječavajući ugradnju minerala iz sline u strukturu zuba.

Uzimajući u obzir ograničenja karbopola, cilj ovog istraživanja bio je ispitati gel za izbjeljivanje koji sadržava zgušnjivač na bazi kopolimera sulfonske akriloildimetiltauratne kiseline i vinilpirolidona (Aristofleks), prethodno neutralizirani sintetski polimer koji je već ispitan u istraživanju *in vitro* (12). Aristoflex pomaže u formuliranju kristalnih gelova odgovarajuće konzistencije. Taj zgušnjivač ima važna svojstva: stabilan je u kiselome pH, ima kationsko ponašanje i djeluje kao inertni zgušnjivač unutar formulacija. U farmaceutskoj proizvodnji već se upotrebljava kao zgušnjivač u sredstvima za osobnu njegu. Štoviše, također se proučava njegova ugradnja u proizvode za oralnu higijenu kao što su zubne paste za izbjeljivanje, sredstva za ispiranje usta i gelovi (13), bez rizika za ljudsko zdravlje.

Prema već opisanome, u ovom istraživanju procjenjivala se citotoksičnost *in vitro* i fizikalne promjene zubne cakline *in situ*, kao što su boja, hrapavost i mikrotvrdoća nakon tretiranja eksperimentalnim gelom za izbjeljivanje koji sadržava 10-postotni karbamid-peroksid i kopolimer sulfonske akriloildimetiltauratne kiseline i vinilpirolidon (Aristoflex). Nulte hipoteze istraživanja bile su:

1) Aristoflex nije toksičan za stanice ljudskih gingivnih fibroblasta (HGF)

2) 10-postotni gel karbamid-peroksida koji sadržava Aristoflex ne utječe na učinkovitost izbjeljivanja, hrapavost cakline i mikrotvrdoću.

Materijal i metode

Analiza citotoksičnosti

Na temelju početnih testova citotoksičnosti formulirane su otopine gela za izbjeljivanje zuba različitih koncentracija. Najprije su pH vrijednosti gelova izmjerene tri puta digitalnim pH metrom (Phs-3b, Phtek, Sao Paulo, SP, Brazil). pH metar sastojao se od pH elektrode s temperaturnim senzorom spojenim na ionski analizator. Elektroda je stavljena u 3 g svakoga gela i rezultati su zabilježeni (tablica 1.).

Za analizu citotoksičnosti temeljenu na prethodnom istraživanju (14) ljudski gingivni fibroblasti (HGF) uzgajani su u Dulbeccovu modificiranom Eagleovu mediju (DMEM, Sigma-Aldrich, St. Louis, MO, SAD) s dodatkom antibiotika (10 000 i. j. penicilina i 10 mg/mL streptomicina – Vitrocell Embriolife, Campinas, SP, Brazil) i 10 % fetalnoga goveđeg seruma (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, SAD) na temperaturi od 37 °C i u vlažnoj atmosferi koja sadržava 5 % CO_2. Nakon što je postignuta konfluencija od približno 80 %, kako bi ih se odvojilo, stanice su isprane s 0,25 % tripsina/EDTA (Gibco, Thermo Fisher Scientific,

Table 1 Manufacturers, basic composition and pH of products
Tablica 1. Proizvođači, osnovni sastav i pH proizvoda

Product • Proizvod	Manufacturer • Proizvođač	Composition* • Sastav*	pH
Carbopol thickener (ct) • Zgušnjivač Carbopol (ct)	Drogal Manipulations (Piracicaba, Brazil)	Carbopol 940, nipagin, glycerin, amp-95 and deionized water • Carbopol 940, nipagin, glicerin, amp-95 i deionizirana voda	3.72
Aristoflex thickener (at) • Zgušnjivač Aristoflex (at)		Aristoflex AVC, nipagin, glycerin, amp-95 and deionized water • Aristoflex AVC, nipagin, glicerin, amp-95 i deionizirana voda	4.95
Carbamide peroxide 10 wt% + carbopol thickener (CP-ct) • 10-postotni karbamid- peroksid + zgušnjivač Carbopol (CP-ct)		Carbamide Peroxide 10 wt%, sodium fluoride, carbopol 940, nipagin, glycerin, amp-95 and deionized water • 10-postotni karbamid-peroksid, natrijev fluorid, karbopol 940, nipagin, glicerin, amp-95 i deionizirana voda	4.56
Carbamide peroxide 10 wt% + Aristoflex thickener (CP-at) • 10-postotni karbamid- peroksid + zgušnjivač Aristoflex (CP-at)		Carbamide Peroxide 10 wt%, sodium fluoride, Aristoflex AVC, nipagin, glycerin, amp-95 and deionized water • 10-postotni karbamid-peroksid, natrijev fluorid, aristofleks AVC, nipagin, glicerin, amp-95 i deionizirana voda	6.97
Carbamide peroxide 10 wt% (CP-wot) • 10-postotni karbamid- peroksid (CP-wot)		Carbamide Peroxide 10 wt% and deionized water • 10-postotni karbamid-peroksid i deionizirana voda	7.74
Whiteness Perfect or Commercial CP 10 wt% (CP-com) • Whiteness Perfect ili komercijalni 10-postotni CP (CP-com)	FGM (Sta Catarina, Brazil)	Carbamide Peroxide 10 wt%, potassium nitrate, carbopol neutralized, sodium fluoride and deionized water • 10-postotni karbamid-peroksid, kalijev nitrat, karbopol neutralizirani, natrijev fluorid i deionizirana voda	5.45

*MSDS data sheet • MSDS podatci

MA, USA). After detachment, the cells were centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was discarded and a new was added and mixed with the cells which were further seeded in 96-well culture plates (Corning Costar Corp., Cambridge, MA, USA).

Considering the final concentration in the culture medium, the cells were exposed to various gels concentrations as follows: 10 mg/mL; 5 mg/mL; 2.5 mg/mL; 1.25 mg/mL; 0.63 mg/mL; 0.31 mg/mL.

1) Carbamide Peroxide 10 wt%+ Carbopol (CP-ct); 2) Carbamide Peroxide 10 wt%+ Aristoflex (CP-at); 3) Carbamide Peroxide 10 wt% + Carbopol / commercial (CP-com); 4) Aristoflex gel (at); 5) Carbopol gel (ct); 6) Triton X-100 0.1 % (Sigma-Aldrich): negative control; 7) DMEM: positive control.

Culture plates (n = 7) were incubated at 37 °C for 4 h (bleaching treatment time). After the incubation had been completed, the cytotoxicity was determined by Vybrant® MTT cell proliferation assay (Thermo Fisher Scientific, Waltham, MA, USA). The MTT is an indirect method to determine the cell viability and proliferation according to the mitochondrial succinate dehydrogenase activity.

Within living cells, MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide], a yellow tetrazole salt, is reduced by succinate enzyme complex to Formazan [(E, Z)-5-(4, 5-dimethylthiazol-2-yl)-1, 3-diphenylformazan], that later precipitates as insoluble purple-gray crystals. After the crystal dissolution, the final solution color intensity (optical density), measured with the spectrophotometer, is the measurement of the cell viability.

For the cell counts, a Neubauer counting chamber was used. For this, the cells were seeded 24 h before the incubation with the gels extracts. For cytotoxicity assessment, 100 µL of solution (5 x 10^4 cells/mL) was added to each well of the culture plates. Then, the gel extracts were added to the wells. After

Waltham, MA, SAD). Nakon odvajanja stanice su centrifugirane na 3000 okretaja u minuti 5 minuta na 4 °C. Supernatant je odbačen i dodan je novi medij te pomiješan sa stanicama koje su dalje nasađene na ploče za kulture s 96 jažica (Corning Costar Corp., Cambridge, MA, SAD).

S obzirom na konačnu koncentraciju u mediju kulture, stanice su bile izložene različitim koncentracijama gela kako slijedi: 10 mg/mL; 5 mg/mL; 2,5 mg/mL; 1,25 mg/mL; 0,63 mg/mL; 0,31 mg/mL.

1. 10-postotni karbamid-peroksid + karbopol (CP-ct); 2. 10-postotni karbamid-peroksid + Aristoflex (CP-at); 3. 10-postotni karbamid-peroksid + Carbopol/komercijalni (CP-com); 4. Aristoflex gel (at); 5. Carbopol gel (ct); 6. Triton X-100 0,1 % (Sigma-Aldrich): negativna kontrola; 7. DMEM: pozitivna kontrola.

Ploče s kulturom (n = 7) inkubirane su 4 sata (vrijeme postupka izbjeljivanja) na temperaturi od 37 °C). Nakon što je inkubacija završena, citotoksičnost je određena Vybrant® MTT testom stanične proliferacije (Thermo Fisher Scientific, Waltham, MA, SAD). MTT je neizravna metoda za određivanje vitalnosti i proliferacije stanica prema aktivnosti mitohondrijske sukcinatne dehidrogenaze.

Unutar živih stanica, MTT [3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H-tetrazolij bromid], žuta sol tetrazola, reducira se sukcinatnim enzimskim kompleksom u formazan [(E,Z)-5-(4,5-dimetiltiazol-2-il)-1,3-difenilformazan] koji se poslije taloži kao netopljivi ljubičasto-sivi kristali. Nakon otapanja kristala, konačni intenzitet boje otopine (optička gustoća), izmjeren spektrofotometrom, mjera je vitalnosti stanica.

Za brojenje stanica korištena je Neubauerova komora za brojenje. Za to su stanice zasađene 24 sata prije inkubacije s ekstraktima gelova. Za procjenu citotoksičnosti 100 µL otopine (5 x 104 stanica/mL), u svaku jažicu dodana je ploča s kulturom. Zatim su u njih dodani ekstrakti gela. Na-

incubation had been finished, the liquids were aspirated and the wells were washed gently twice with phosphate buffered saline (PBS pH 7.4; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, 200 µL of 0.3 mg/mL of MTT solution in DMEM was placed into the wells. The plates were further incubated for 3 h with 5 % CO_2 at 37 °C. Finally, after having been emptied, the wells were filled with 200 µL of ethanol. The absorbance was read using a micro-spectrophotometer (Asys UVM340, Biochrom, Cambridge, England) at 570 nm to determine the optical density. The assays were made at two different times and all of them in quadruplicate. Based on da Silva et al. (15), the products cytotoxicity was scored.

In situ stage

This was a randomized double-bind *in situ* study. Freshly extracted bovine incisors, without fractures or enamel stains, were chosen and stored in 0.1 wt% thymol at 4 °C. Enamel/dentin blocks were prepared for color and roughness analysis (4 x 4 x 2.5 mm), and for microhardness analysis (4 x 4 x 2 mm). Decreasing-grit silicon carbide abrasive papers (#1200, #2500, and #p4000 - Isomet, Buehler, Lake Bluff, IL, USA) were employed to regularize the enamel surface and achieve an adequate height. To polish the samples, felts (Top-, Ram- and Supra-, Arotec, Cotia, São Paulo, SP, Brazil) with metallographic diamond pastes (6 µm - Top, 3 µm - Ram, 1 µm – Supra, Arotec) and lubricant (Arotec, Cotia, São Paulo, SP, Brazil) were used. In the course of each polishing step, in order to remove the polishing debris residuals, the samples were water-sonicated for 10 min.

After preparation, the samples were stained by putting them into a black tea solution (Leão Junior SA, Curitiba, PR, Brazil). The tea solution was renewed daily. After completing 6 days of staining, the samples were stored in artificial saliva, daily renewed, during 14 days in order to achieve the color stabilization (16). Subsequently, the samples were sterilized with ethylene oxide and were stored in water at 4 °C until the treatment.

After signing an informed consent form, twelve volunteers (six males and six females), aged 18-35 years, agreed to participate in this study (Ethics Committee register: 79720117.1.0000.5418). The inclusion criteria met by the volunteers were: normal salivary flow (checked by sialometry) and the absence of caries and/or periodontal disease- Patients who do not absorb any fluoride (by either fluoridated water intake, or use of fluoride toothpaste), wear orthodontic appliances, use medication that altered their salivary flow, smoke, or had fixed or removable prostheses were excluded.

A maxillary impression of each volunteer was obtained with alginate (Hydrogum, Zhermack, Badia Polesine, RO, Italy). Using the cast models, palatine acrylic-resin-based devices were created containing 6 cavities of 35 mm^2 wide and 3 mm deep. Inside the cavities, the teeth samples were inserted and stabilized in the palatal device employing sticky wax (ASFER - Chemical Industry Ltda, SP, Brazil). In each cavity, color/roughness and microhardness samples were placed from each group and exposed to saliva. The groups were in diverse places in each patient, considering the salivary ducts of the parotid gland location.

kon što je inkubacija završena, tekućine su aspirirane i jažice su nježno isprane dva puta fiziološkom otopinom puferiranom fosfatom (PBS pH 7,4; Gibco, Thermo Fisher Scientific, Waltham, MA, SAD). Zatim je u jažice stavljeno 200 µL 0,3 mg/mL MTT otopine u DMEM-u. Ploče su dalje inkubirane 3 sata s 5 % CO_2 na temperaturi od 37 °C. Na kraju, nakon pražnjenja, jažice su napunjene s 200 µL etanola. Apsorbancija je očitana mikrospektrofotometrom (Asys UVM340, Biochrom, Cambridge, Engleska) na 570 µm kako bi se odredila optička gustoća. Testovi su primijenjeni u dva različita vremena po četiri puta. Na temelju metode da Silve i suradnika (15) ocjenjivana je citotoksičnost proizvoda.

Istraživanje *in situ*

Provedeno je randomizirano, dvostruko slijepo istraživanje *in situ*. Svježe izvađeni goveđi sjekutići, bez fraktura ili obojenja cakline, odabrani su i pohranjeni u 0,1-postotnom timolu na 4 °C. Caklinski/dentinski blokovi pripremljeni su za analizu boje i hrapavosti (4 x 4 x 2,5 mm) te za analizu mikrotvrdoće (4 x 4 x 2 mm). Abrazivni papiri od silicijeva karbida padajuće granulacije (#1200, #2500 i #4000 – Isomet, Buehler, Lake Bluff, IL, SAD) korišteni su da bi se ujednačila površina cakline i postigla odgovarajuća visina uzoraka. Za poliranje uzoraka korišten je filc (Top-, Ram- i Supra-, Arotec, Cotia, São Paulo, SP, Brazil) s metalografskim dijamantnim pastama (6 µm – Top, 3 µm – Ram, 1 µm – Supra, Arotec) i sredstvom za podmazivanje (Arotec, Cotia, São Paulo, SP, Brazil). Poslije poliranja uzorci su čišćeni u ultrazvučnoj vodenoj kupelji 10 minuta.

Nakon pripreme uzorci su obojeni stavljanjem u otopinu crnoga čaja (Leão Junior SA, Curitiba, PR, Brazil) koja se svaki dan obnavljala. Nakon 6 dana odloženi su u umjetnu slinu, svakodnevno obnavljanu, tijekom 14 dana kako bi se postigla stabilizacija boje (16). Zatim su sterilizirani etilenoksidom i do tretmana čuvani u vodi na temperaturi od 4 °C.

Nakon potpisivanja informiranog pristanka dvanaest volontera (šest muškaraca i šest žena), u dobi od 18 do 35 godina, pristalo je sudjelovati u ovom istraživanju (registar Etičkoga povjerenstva: 79720117.1.0000.5418). Kriteriji za uključivanje bili su normalan protok sline (provjereno sijalometrijom) i zubi bez karijesa i/ili parodontne bolesti. Isključeni su pacijenti koji ne apsorbiraju fluor (bilo unosom fluoridirane vode, bilo upotrebom paste za zube s fluorom), koji se koriste ortodontskim aparatima, na terapiji su lijekovima koji mijenjaju protok sline, puše ili imaju fiksne ili mobilne proteze.

Otisak maksile svakoga dobrovoljca uzet je alginatom (Hydrogum, Zhermack, Badia Polesine, RO, Italija). S pomoću lijevanih modela izrađene su palatinalne naprave na bazi akrilne smole sa 6 kaviteta širine 35 mm^2 i dubine 3 mm. Unutar kaviteta umetnuti su uzorci zuba i stabilizirani ljepljivim voskom (ASFER - Chemical Industry Ltda, SP, Brazil). U svaki kavitet stavljen je uzorak boje/hrapavosti i mikrotvrdoće iz svake skupine i izložen slini. Skupine su bile na različitim mjestima kod svakog pacijenta, s obzirom na položaj parotidne žlijezde.

Prije početka tretmana palatinalne naprave ostale su u ustima volontera 24 sata kako bi se omogućio razvoj slinovne

Before the beginning of all treatments, the palatal devices stayed in a volunteer's mouth for 24 h to allow the salivary pellicle development. Volunteers were taught to remove the device only in order to consume food and drink. Meanwhile, the samples had to remain inside a water-filled device to avoid dehydration. One day after the device had been taken out of from the mouth and dried with absorbent paper and, the teeth samples were bleached. For each sample placed in the device, a single bleaching gel treatment was applied daily during 14 days. For this scope, the volunteers were trained to do the gel application over the samples' enamel surfaces, and let them undisturbed during 4 h at room temperature. Throughout the bleaching treatments, the device had to remain in a water-filled device. It is relevant to point out that the water did not get in contact with the area covered by the bleaching gel. After the treatment time was over, gels were rubbed out using cotton swabs, washed with running water, and dried with absorbent paper. Subsequently, the device was placed back to the patient's palate.

Microhardness Analysis (SMH)

An enamel surface microhardness (SMH) analysis was performed using a Knoop microhardness tester (HMV 2000, Shimadzu, Kyoto, Japan). At the samples central region, a Knoop diamond indenter applied a static load (50 gf/5 s). Analyses were taken initially - after staining (initial), and 24 h after the bleaching treatments finished (final) (Figure 1). For each sample, the average of five 100 µm equidistant indentations was used for statistical analyses.

Roughness Analysis (Ra)

For the purpose of surface roughness (Ra) assessment, a roughness tester (Surftest 211, Mitutoyo, Suzano, SP Brazil) was used. The Ra was also assessed at initial and final stages (Figure 1). On the sample surface, three equidistant measurements were made. The instrument was programed to have a cutoff point of 0.25 mm, reading extent of 1.25 mm, load of 5 N and speed of 0.1 mm/s. The average of three measurements of the same sample was considered in the statistical analysis.

ovojnice. Rečeno im je da napravu uklone samo tijekom konzumiranja jela i pića. U međuvremenu, radi vlaženja, uzorci bi trebali ostati unutar naprave napunjene vodom da bi se izbjegla dehidracija. Dan poslije toga naprava je izvađena iz usta i osušena upijajućim papirom, a uzorci zuba su izbijeljeni. Za svaki uzorak primjenjivan je tijekom 14 dana jedan tretman gelom za izbjeljivanje na dan. U tu svrhu volonterima je rečeno da nanesu gel na površinu cakline uzoraka i ostave ih nesmetano 4 sata na sobnoj temperaturi. Tijekom tretmana izbjeljivanja naprava je bila napunjena vodom. Bitno je istaknuti da voda nije došla u doticaj s područjem koje je prekrivao gel za izbjeljivanje. Kada je tretman završen, gelovi su istrljani vatom, isprani tekućom vodom i osušeni upijajućim papirom. Nakon toga naprava je vraćena na nepce pacijenta.

Analiza mikrotvrdoće (SMH)

Analiza mikrotvrdoće površine cakline (SMH) provedena je s pomoću Knoopove metode (HMV 2000, Shimadzu, Kyoto, Japan). Na središnjem području uzoraka Knoopovim dijamantnim utiskivačem primijenjeno je statičko opterećenje (50 gf/5 s). Analize su obavljene inicijalno – nakon bojenja (početno) i 24 sata poslije završenoga postupka izbjeljivanja (finalno) (slika 1.). Za svaki uzorak korišten je prosjek od pet ekvidistantnih udubljenja od 100 µm za statističke analize.

Analiza hrapavosti (Ra)

Za procjenu hrapavosti površine (Ra) korišten je tester hrapavosti (Surftest 211, Mitutoyo, Suzano, SP Brazil). Ra je također procijenjen na početku i na kraju (slika 1.). Na površini uzorka obavljena su tri ekvidistantna mjerenja. Instrument je programiran tako da ima graničnu točku od 0,25 mm, opseg očitanja od 1,25 mm, opterećenje od 5 N i brzinu od 0,1 mm/s. U statističkoj analizi uzet je u obzir prosjek triju mjerenja istoga uzorka.

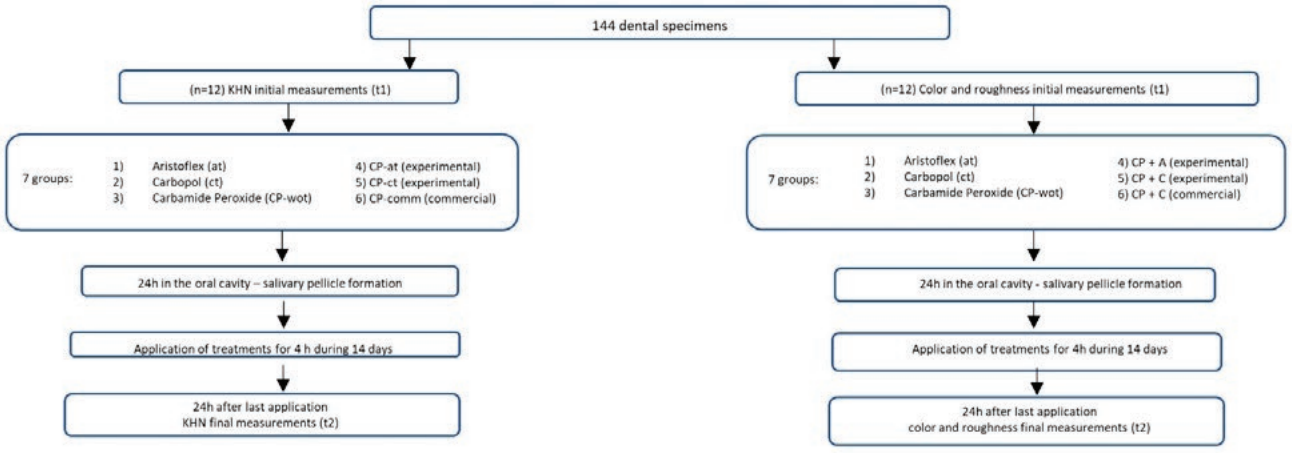

Figure 1 *In situ* phase experimental design summaries
Slika 1. Sažetak eksperimentalnog dizajna faze *in situ*

Color Analysis (ΔE^*_{ab}, ΔE_{00}, WI_D)

To analyze the color, a spectrophotometer was used (CM 700D, Minolta, Osaka, Japan) which was previously calibrated following the manufacturer's specifications. Samples were put in a polytetrafluoroethylene-based device in ambient light condition (MiniMatcher MM-1, GTI Graphic Technology, Newburgh, NY, USA) as to systematize the environment. The measurements were made after staining (initial) and 24 h after the treatment end (final). The ΔE^*_{ab}, ΔE_{00} and Whiteness Index for Dentistry (WI_D) values were obtained through the following formulas:

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

$$\Delta E_{00} = \sqrt{\left(\frac{\Delta L'}{K_L S_L}\right)^2 + \left(\frac{\Delta C'}{K_C S_C}\right)^2 + \left(\frac{\Delta H'}{K_H S_{LH}}\right)^2 + R_T\left(\frac{\Delta C'}{K_C S_C}\right)\left(\frac{\Delta H'}{K_H S_{LH}}\right)}$$

$$WI_D = 0{,}511L^* - 2{,}324a^* - 1{,}100b^*$$

where ΔL*, Δa* and Δb* are the differences between the assessment times (initial- final) (Figure 1) for the L*, a* and b*values, correspondingly. ΔL', ΔC' and ΔH' stand for the differences in light value (Light), chroma (Chroma) and hue (Hue), respectively, employing CIEDE2000 metric. S_L, S_C and S_H are factors to regulate the coordinate values as a function of variation in color difference. While K_L, K_C and K_H enable to do a correction concerning the experiment settings, and R_T is a parameter that takes in account the interaction between chroma and hue changes in blue region.

The following perceptibility and acceptability limits were taken as reference: (17, 18, 19)

- Delta Whiteness Index for Dentistry (ΔWI_D): 50%:50% perceptibility (0.72 ΔWI_D); 50%:50% acceptability (2.62 ΔWI_D)
- CIELAB and CIEDE2000: 50%:50% perceptibility (1.2 ΔE^*_{ab} and 0.8 ΔE_{00}) and 50%:50% acceptability (2.7 ΔE^*_{ab} and 1.8 ΔE_{00}).

Statistical Analyses

Statistical analyses were performed using GraphPad® Prism 6.0 software (GraphPad Software, San Diego, CA, USA). The normality and equality of variances were checked with the Shapiro-Wilk and Levene's tests. Cytotoxicity, ΔE^*_{ab} and ΔE_{00} data were analyzed by one-way analysis of variance (ANOVA) and Tukey test. For the microhardness and roughness data, mixed models for repeated measures and Tukey-Kramer test were used. ΔWI_D was analyzed by non-parametric Kruskal Wallis and Dunn tests. A 5% significance level was considered for all the analyses.

Results

Cytotoxicity

Cytotoxicity results are shown in Figure 2. Without CP, the Carbopol and Aristoflex gels had cytotoxicity values above the IC50 independently of their concentration. Still, above the 5 mg/mL concentration all groups that had CP in their formulation exhibited cytotoxicity values below IC50.

Analiza boje (ΔE^*_{ab}, ΔE_{00}, WI_D)

Za analizu boje korišten je spektrofotometar (CM 700D, Minolta, Osaka, Japan) koji je bio kalibriran prema specifikacijama proizvođača. Uzorci su stavljeni u uređaj na bazi politetrafluoretilena u uvjetima ambijentalnog svjetla (MiniMatcher MM-1, GTI Graphic Technology, Newburgh, NY, SAD) kako bi se standardiziralo okruženje. Mjerenja su obavljena nakon bojenja (početno) i 24 sata poslije završetka tretmana (finalno). Vrijednosti ΔE^*_{ab}, ΔE_{00} i indeksa bjeline za stomatologiju (WID) dobivene su s pomoću sljedećih formula:

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

$$\Delta E_{00} = \sqrt{\left(\frac{\Delta L'}{K_L S_L}\right)^2 + \left(\frac{\Delta C'}{K_C S_C}\right)^2 + \left(\frac{\Delta H'}{K_H S_{LH}}\right)^2 + R_T\left(\frac{\Delta C'}{K_C S_C}\right)\left(\frac{\Delta H'}{K_H S_{LH}}\right)}$$

$$WI_D = 0{,}511L^* - 2{,}324a^* - 1{,}100b^*$$

gdje su ΔL*, Δa* i Δb* razlike između vremena procjene (početno-konačno) (slika 1.) za L*, a* i b* vrijednosti. ΔL', ΔC' i ΔH' označavaju razlike u vrijednosti svjetline (Light), boje (Chroma) i nijanse (Hue), koristeći se metrikom CIEDE2000. SL, SC i SH faktori su za reguliranje koordinatnih vrijednosti kao funkcije varijacije razlike u boji. Dok KL, KC i KH omogućuju korekciju postavki eksperimenta, RT je parametar koji uzima u obzir interakciju između promjena boje i nijanse u plavom području.

Ti su pragovi vizualne percepcije i prihvatljivosti uzeti kao referencija (17, 18, 19):

- delta indeks bjeline za stomatologiju (ΔWI_D): 50 % : 50 % perceptibilnost (0,72 ΔWI_D); 50 % : 50 % prihvatljivost (2,62 ΔWI_D)
- CIELAB i CIEDE2000: 50 % : 50 % perceptibilnost (1,2 ΔE^*_{ab} i 0,8 ΔE_{00}) i 50 % : 50 % prihvatljivost (2,7 ΔE^*_{ab} i 1,8 ΔE_{00}).

Statistička analiza

Statističke analize provedene su u softveru GraphPad® Prism 6.0 (GraphPad Software, San Diego, CA, SAD). Normalnost i jednakost varijanci provjereni su Shapiro-Wilkovim i Leveneovim testovima. Podatci o citotoksičnosti, ΔE^*_{ab} i ΔE_{00} analizirani su jednosmjernom analizom varijance (ANOVA) i Tukeyjevim testom. Za podatke o mikrotvrdoći i hrapavosti korišteni su miješani modeli za ponovljena mjerenja i Tukey-Kramerov test. ΔWID je analiziran neparametrijskim Kruskal-Wallisovim i Dunnovim testovima. Razina značajnosti od 5 % uzeta je u obzir za sve analize.

Rezultati

Citotoksičnost

Rezultati citotoksičnosti nalaze se na slici 2. Bez CP-a, gelovi Carbopol i Aristoflex imali su vrijednosti citotoksičnosti iznad IC50, neovisno o njihovoj koncentraciji. Ipak, iznad koncentracije od 5 mg/mL sve skupine koje su imale CP u svojoj formulaciji pokazale su vrijednosti citotoksičnosti ispod IC50.

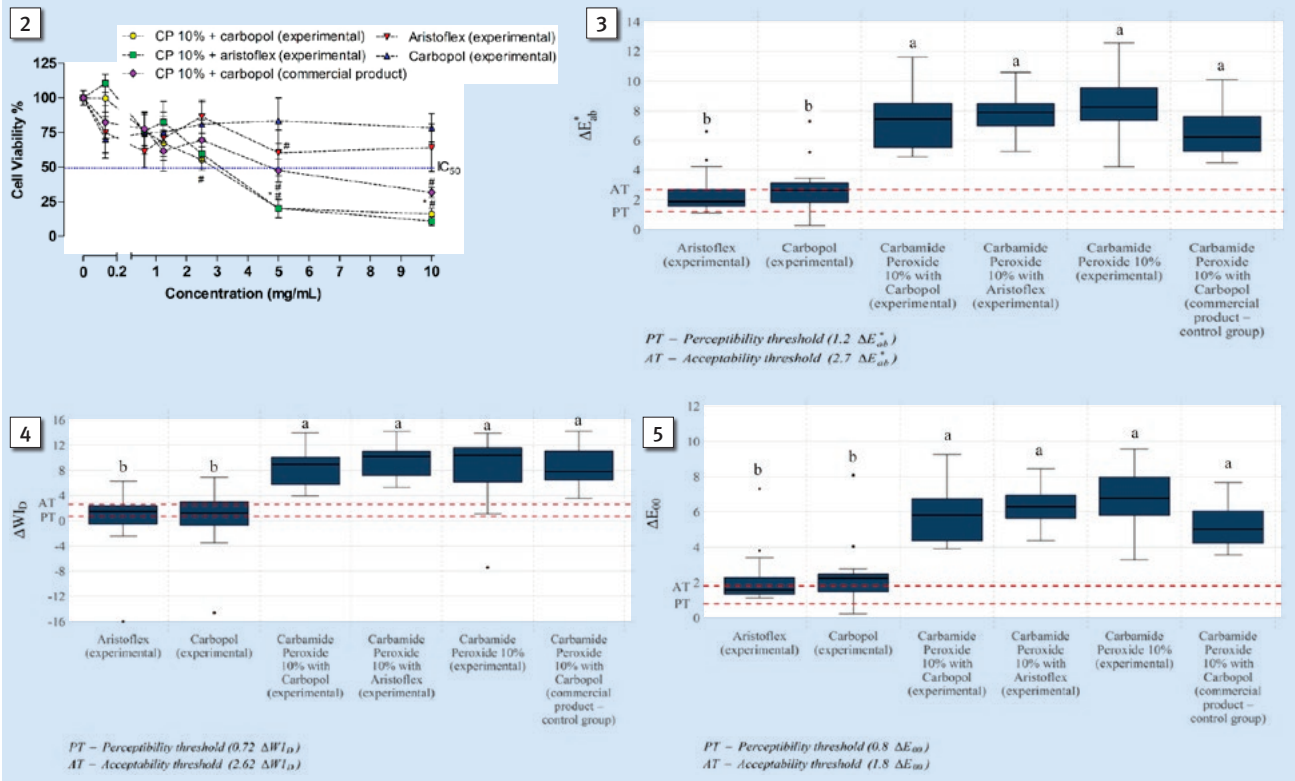

Figure 2 Cell viability (%) based on the employed gel concentration. #p < 0.05 compared to carbopol (ct) and *p < 0.05 compared CP 10% + carbopol (CP-com). $IC_{50:}$ concentration that after exposure causes 50% cell death or cell growth inhibition or other utilized cytotoxicity metric.

Slika 2. Vijabilnost stanica (%) na temelju upotrijebljene koncentracije gela. #p < 0,05 u usporedbi s karbopolom (ct) i *p < 0,05 u usporedbi s CP 10 % + karbopol (CP-com). IC_{50}: koncentracija koja poslije izlaganja uzrokuje 50 % stanične smrti ili inhibiciju rasta stanica ili druga korištena metrika citotoksičnosti

Figure 3 Boxplot of the ΔE * ab according to the group and time. Different letters denote significant differences (p≤0.05).

Slika 3. Okvirni prikaz ΔE * ab prema grupi i vremenu; različita slova označavaju značajnu razliku (p ≤ 0,05)

Figure 4 Boxplot of the delta whiteness index for dentistry (ΔWID) according to the group and time. Different letters denote significant differences (p≤0.05).

Slika 4. Prikaz delta indeksa bjeline za stomatologiju (ΔWI_D) prema skupini i vremenu; različita slova označavaju značajnu razliku (p ≤ 0,05)

Figure 5 Boxplot of ΔE_{00} according to the group and time. Different letters denote significant differences (p≤0.05).

Slika 5. Prikaz ΔE_{00} prema grupi i vremenu; različita slova označavaju značajnu razliku (p ≤ 0,05)

Microhardness (SMH)

For the SMH, the interaction among groups and times was significant (p < 0.05). At the initial time amid groups no statistical difference was found (p > 0.05). The groups treated with Carbopol: Carbopol thickener (ct) and Carbamide Peroxide 10 wt% + carbopol thickener (CP-ct) SMH decreased significantly (p < 0.05). At the final assessment (24 h after end of treatment) the ct group SMH values were lower than the rest of the groups, and the CP-ct group had significantly lower SMH compared to other groups, however the difference was not significant when compared to Carbamide Peroxide 10% with Aristoflex (CP-at) (p < 0.05), Table 2.

Roughness (Ra)

Regarding Ra (Table 3), the factors groups and times had a significant interaction (p < 0.05). At the initial time, the Ra between the groups was no significantly different (p > 0.05) and all of them showed significant Ra increase over time (p < 0.05). At the final assessment, the higher Ra was for the group treated with the Carbopol thickener (ct) (p < 0.05), but the difference was not significant when compared to Aristoflex thickener (at) (p > 0.05).

Mikrotvrdoća (SMH)

Za SMH je interakcija između skupina i vremena bila značajna (p < 0,05). U početnom vremenu između skupina nije pronađena statistički značajna razlika (p > 0,05). Skupine tretirane karbopolom: zgušnjivač Carbopol (ct) i 10-postotni karbamid-peroksid + zgušnjivač Carbopol (CP-ct) SMH se značajno smanjio (p < 0,05). Na konačnoj procjeni (24 sata poslije završetka tretmana) ct skupine SMH vrijednosti bile su niže od ostalih skupina, a CP-ct skupina imala je značajno niži SMH od većine ostalih, no razlika nije bila značajna u usporedbi s 10-postotnim karbamid-peroksidom s Aristoflexom (CP-at) (p < 0,05) (tablica 2.).

Hrapavost (Ra)

Što se tiče Ra (tablica 3.), faktori skupina i vrijeme imali su značajnu interakciju (p < 0,05). Na početku se Ra između skupina nije značajno razlikovao (p > 0,05), a sve su pokazale njegovo veliko povećanje tijekom vremena (p < 0,05). U konačnoj procjeni veći Ra bio je u skupini tretiranoj zgušnjivačem Carbopol (ct) (p < 0,05), ali razlika nije bila značajna u usporedbi sa zgušnjivačem Aristoflex (at) (p > 0,05).

Table 2 Mean (standard deviation) microhardness (KHN) of enamel before and after being submitted to different bleaching treatments.
Tablica 2. Srednja (standardna devijacija) mikrotvrdoća (KHN) cakline prije i poslije podvrgavanja različitim tretmanima izbjeljivanja

Group • Skupina	Time • Vrijeme	
	Initial • Inicijalno	Final • Završno
Aristoflex thickener – at • Zgušnjivač Aristoflex – at	293,87 (15,75) Aa	280,27 (25,53) Aa
Carbopol thickener – ct • Zgušnjivač Carbopol – ct	293,71 (16,43) Aa	151,00 (36,27) Bc
Carbamide peroxide 10 wt% with carbopol (experimental) – CP-ct • 10-postotni karbamid-peroksid s Carbopolom (eksperimentalno) – CP-ct	292,52 (16,56) Aa	250,28 (24,13) Bb
Carbamide peroxide 10 wt% with aristoflex (experimental) – CP-at • 10-postotni karbamid-peroksid s Aristoflexom (eksperimentalno) – CP-at	292,97 (15,41) Aa	275,09 (36,28) Aab
Carbamide peroxide 10 wt% (without thickener) – CP-wot • 10-postotni karbamid-peroksid (bez zgušnjivača) – CP-wot	292,66 (14,94) Aa	285,85 (9,32) Aa
Carbamide peroxide 10 wt% with carbopol (commercial product - control group) – CP-com • 10-postotni karbamid-peroksid s Carbopolom (komercijalni proizvod – kontrolna skupina) – CP-com	292,72 (15,21) Aa	280,60 (8,62) Aa

Means followed by distinct letter (uppercase in horizontal and lowercase in vertical comparison) differ from each other (p≤0.05); p (group) <0.0001; p(time)<0.0001; p(interaction)<0.0001 • Srednje vrijednosti iza kojih slijedi različito slovo (veliko slovo u vodoravnoj usporedbi i malo slovo u okomitoj usporedbi) uzajamno se razlikuju (p ≤ 0,05). p (skupina) < 0,0001; p (vrijeme) < 0,0001; p (interakcija) <0,0001

Table 3 Mean (standard deviation) roughness (Ra) of enamel before and after being submitted to different bleaching treatments.
Tablica 3. Srednja vrijednost (standardna devijacija) hrapavosti (Ra) cakline prije i poslije podvrgavanja različitim tretmanima izbjeljivanja

Group • Skupina	Time • Vrijeme	
	Initial • Inicijalno	Final • Završno
Aristoflex thickener – at • Zgušnjivač Aristoflex – at	0.12 (0.01) Ba	0.15 (0.02) Aab
Carbopol thickener – ct • Zgušnjivač Carbopol – ct	0.11 (0.01) Ba	0.16 (0.02) Aa
Carbamide peroxide 10 wt% with carbopol (experimental) – CP-ct • 10-postotni karbamid-peroksid s Carbopolom (eksperimentalno) – CP-ct	0.11 (0.01) Ba	0.14 (0.02) Ab
Carbamide peroxide 10 wt% with aristoflex (experimental) – CP-at • 10-postotni karbamid-peroksid s Aristoflexom (eksperimentalno) – CP-at	0.11 (0.01) Ba	0.14 (0.01) Ab
Carbamide peroxide 10 wt% (without thickener) – CP-wot • 10-postotni karbamid-peroksid (bez zgušnjivača) – CP-wot	0.12 (0.01) Ba	0.14 (0.01) Ab
Carbamide peroxide 10 wt% with carbopol (commercial product - control group) – CP-com • 10-postotni karbamid-peroksid s Carbopolom (komercijalni proizvod – kontrolna skupina) – CP-com	0.12 (0.01) Ba	0.14 (0.01) Ab

Means followed by distinct letter (uppercase in horizontal and lowercase in vertical comparison) differ from each other (p≤0.05); p (group) <0.0001; p(time)<0.0001; p(interaction)<0.0001 • Srednje vrijednosti iza kojih slijedi različito slovo (veliko slovo u vodoravnoj usporedbi i malo slovo u okomitoj usporedbi) uzajamno se razlikuju (p ≤ 0,05). p (skupina) < 0,0001; p (vrijeme) < 0,0001; p (interakcija) <0,0001

Color

The groups treated with gels that only contained thickeners (ct and at) displayed significantly lower ΔE*ab ($p < 0.05$) (Figure 3). Boxplot graphs also display the perceptibility and acceptability limits.

Similarly, the gels that contained only the thickeners (ct and at) ΔWI_D values were significantly lower than the rest of the groups ($p < 0.05$) (Figure 4). Color variations in the carbamide peroxide 10 wt% with Carbopol (CP-ct) carbamide peroxide 10 wt% with Aristoflex (CP-at), and carbamide peroxide 10 wt% with Carbopol commercial product (CP-com) groups were beyond the perceptibility and acceptability limits.

As for ΔE_{00} (Figure 5), the groups that contained only the thickeners (ct and at) had also lower values when compared to the rest ($p < 0.05$). All groups with 10% carbamide peroxide in their formulation displayed ΔE_{00} values above the perceptibility and acceptability limits.

Boja

Skupine tretirane gelovima koji su sadržavali samo zgušnjivače (ct i at) pokazale su znatno niže vrijednosti ΔE^*_{ab} ($p < 0,05$) (slika 3.). Kutijasti (boxplot) grafikoni također prikazuju granice vidljivosti i prihvatljivosti.

Slično tomu, za gelove koji su sadržavali samo zgušnjivače (ct i at), vrijednosti ΔWI_D bile su značajno niže od onih u ostalim skupinama ($p < 0,05$) (slika 4.). Varijacije boja u skupinama s 10-postotnim karbamid-peroksidom s karbopolom (CP-ct), 10-postotnim karbamid-peroksidom s Aristoflexom (CP-at) i 10-postotnim karbamid-peroksidom s karbopolnim komercijalnim proizvodom (CP-com) bile su iznad praga vizualne percepcije i prihvatljivosti.

Kada je riječ o ΔE_{00} (slika 5.), skupine koje su sadržavale samo zgušnjivače (ct i at) također su imale niže vrijednosti u odnosu prema ostalima ($p < 0,05$). Sve skupine s 10-postotnim karbamid-peroksidom, u svojoj formulaciji pokazale su vrijednosti ΔE_{00} iznad praga vizualne percepcije i prihvatljivosti.

Discussion

According to the obtained results, the first hypothesis, that the association of Aristoflex with carbamide peroxide 10 wt% (CP) would not be cytotoxic for human gingival fibroblasts (HGF) cells, was accepted. The usage of CP for the bleaching of vital teeth has been well-established and can be broadly implemented (20). Nonetheless, CP is placed into custom-made trays. Since these trays touch the gingival tissue, there exist the possibility of irritations and ulceration wounds caused by the CP and all its by-products as they have contact with oral cells. Besides, they have toxic effects on HGF (5,21).

Previous *in vitro* studies employed HGF to assess potential cytotoxic components within the bleaching gels (5,22). The current study demonstrated that the usage of CP-ct and CP-at for 4 h generated a comparable cytotoxicity curve. Nevertheless, the bleaching gel concentration augmentation induced the reduction of the fibroblast viability, particularly over 2 mg/mL. It is possible to assume that this effect is directly associated with the CP concentration, making this component liable for the product cytotoxic effect. Nevertheless, it is relevant to emphasize, that even at their highest concentrations, none of the thickeners (Carbopol and Aristoflex) without CP were cytotoxic.

Preceding studies have stated that mineral dissolution might occur throughout tooth bleaching procedure resulting in changes of the enamel surface (8,23,24). Based on the results of Basting et al., 2001, the bleaching gels acidic properties, extended application times, and the addition of Carbopol are considered to be likely the main responsible factors for dental structure surface alterations (25). Even if bovine enamel was evaluated in the current study, considering its physical-chemical similarity to human enamel (8), it is reasonable to deduce that this study obtained results that could be comparable to the results obtained with human tooth. Based on previous research (12,13), thickeners are polymers that, due to their bioadhesive capabilities, interrelate with dental structure. Bioadhesive capacity is associated with potential ionic bonds between polymers and the dental structure, generating a sort of "film" (meaning, a polymer coating that is located on the dental structure). This "film" has the capacity to generate a barrier that precludes the saliva-induced remineralization which is essential to reduce or hamper the hydrogen-peroxide-induced mineral loss generated during the bleaching treatment (11,24).

Of these thickeners, Carbopol is an anionic polymer with a high capacity to bind to the dental surface due to its high affinity with it. This closeness creates a "film" in a properly-adhered and thick way that would finally preclude the saliva-induced remineralization (12,13). Conversely, Aristoflex exhibits some properties that are different from Carbopol, starting by its cationic nature. Besides, it has low affinity for the dental structures, which results in the establishment of a not so closely-attached "film". Therefore, it may favor the saliva-induced remineralization (11,12).

Our results are in accordance with previous research (12,13). In the comparison of the initial and final times of our

Rasprava

Prema dobivenim rezultatima prihvaćena je prva hipoteza da veza Aristoflexa s 10-postotnim karbamid-peroksidom (CP) ne bi bila citotoksična za stanice humanih gingivnih fibroblasta (HGF). Primjena CP-a za izbjeljivanje vitalnih zuba dobro je uhodana i može se široko primijeniti (20). Unatoč tomu, CP se stavlja u individualne udlage, a s obzirom na to da one dodiruju gingivno tkivo postoji mogućnost iritacija i rana od ulceracija prouzročenih CP-om i svim njegovim nusproizvodima jer su u kontaktu s oralnim stanicama, osim njihovih toksičnih učinaka na HGF (5, 21).

U dosadašnjim istraživanjima *in vitro* primjenjivao se HGF za procjenu potencijalnih citotoksičnih komponenti unutar gelova za izbjeljivanje (5, 22). Ovo istraživanje pokazalo je da je uporaba CP-cta i CP-ata tijekom 4 sata stvorila usporedivu krivulju citotoksičnosti. Unatoč tomu, povećanje koncentracije gela za izbjeljivanje izazvalo je smanjenje vitalnosti fibroblasta, osobito iznad 2 mg/mL. Može se pretpostaviti da je taj učinak izravno povezan s koncentracijom CP-a, pretvarajući tu komponentu u odgovornu za citotoksični učinak proizvoda. Ipak, bitno je istaknuti da ni u najvišim koncentracijama nijedan od zgušnjivača (Carbopol i Aristoflex) bez CP-a nije bio citotoksičan.

Autori dosadašnjih istraživanja potvrđuju da se tijekom izbjeljivanja zuba može dogoditi otapanje minerala, a to se odražava u promjenama površine cakline (8, 23, 24). Na temelju rezultata Bastinga i suradnika (2001.) kisela svojstva gelova za izbjeljivanje, produženo vrijeme primjene i dodatak karbopola smatraju se vjerojatno glavnim čimbenicima odgovornima za promjene na površini zubne strukture (25). Čak i ako je u ovom istraživanju procijenjena goveđa caklina, s obzirom na njezinu fizikalno-kemijsku sličnost s ljudskom caklinom (8), razumno je zaključiti da bi se rezultati dobiveni u ovom istraživanju mogli usporediti s onima dobivenima na ljudskim zubima.

Prema dosadašnjim istraživanjima (12, 13) zgušnjivači su polimeri koji se zbog svojega bioadhezivnog svojstva povezuju sa strukturom zuba. Bioadhezivni kapacitet povezan je s potencijalnim ionskim vezama među polimerima i strukturom zuba, stvarajući neku vrstu „filma" (tj. polimerne presvlake koja se nalazi na površini zuba). Taj „film" može stvoriti barijeru koja onemogućuje remineralizaciju izazvanu slinom, a ključna je za smanjenje ili sprječavanje gubitka minerala izazvanoga vodikovim peroksidom koji nastaje tijekom postupka izbjeljivanja (11, 24).

Od ovih zgušnjivača, Carbopol je anionski polimer s velikom sposobnošću vezanja za površinu zuba zbog visokoga afiniteta. Time se stvara "film" koji je zalijepljen i gust, što u konačnici onemogućuje remineralizaciju izazvanu slinom (12, 13). Aristoflex, naprotiv, ima drukčija svojstva od Carbopola, počevši od svoje kationske prirode. To znači da ima nizak afinitet prema zubnim strukturama, a to rezultira stvaranjem ne tako tijesno pričvršćenoga „filma", stoga može pogodovati remineralizaciji prouzročenoj slinom (11, 12).

Naši rezultati u skladu su s dosadašnjim istraživanjima (12, 13). Usporedbom početka i završetka našeg istraživanja može se primijetiti smanjenje SMH-a u eksperimentalnim

study, a reduction in the SMH of the experimental groups that had Carbopol (ct and CP-ct) could be noticed. For the final assessment, the experimental groups that had Carbopol exhibited lower SMH values, while the experimental group that had carbamide peroxide 10 wt% with Aristoflex (CP-at) displayed more intermediary values than the rest of the groups.

Even if the commercial gel (CP-com) could have neutralized Carbopol thickener incorporated, which is able to decrease the occurrence of potential alterations on the enamel surface, the precise amount of this compound has remained unknown. Since the formulation details are undisclosed, it is challenging to determine how each particular component could have effects on the ultimate properties of the material. Nevertheless, it is possible to speculate that the Carbopol levels within the FGM gel are not sufficiently high to modify the enamel SMH, which happened with experimental gels that contained Carbopol.

The salivary fluid in the oral environment is overloaded with minerals such as calcium and phosphate that have the capacity to assist in the process of remineralization (24,26) or in the demineralization decrease (8). Along with the process of remineralization, the minerals from saliva could be deposited in an irregular manner on the enamel surface and this could increase the surface roughness (10) similarly as it occurs during the dissolution of the minerals. In the present research, all the groups presented an increase in Ra levels which could be related to the mineral restructuration that occurs over the surface of the enamel after the alternation between demineralization and remineralization processes. After completion of a 24-hour treatment, Carbopol gel (ct) displayed higher levels of Ra compared to the rest of the groups. It can be assumed that these results might be associated with acidic pH value (3.72) of this gel resulting in higher mineral loss. As a matter of fact, mineral loss in deeper levels (50, 75 and 100 µm), could not be restored by the saliva-induced remineralization methods (8,27). Therefore, it can be assumed that the measured microhardness values may tend to decrease as well as roughness increment.

To assess color alterations after bleaching in dental research, the ΔE and ΔE2000 formulas have been routinely employed, and are created according to the CIE L*, a*, b* system. Still, it is relevant to mention that while ΔE adopts the same value for all CIEL*, a*, b* coordinates, ΔE2000 takes into account parametric elements to evaluate CIEL*, a*, b* color alterations. Specifically, by using the CIEL*, a*, b* spectral dimension it is conceivable to assess bleaching alterations by means of changes in one or more of its coordinates (L*: luminosity; a*: red-green axis and b*: yellow-blue axis) (28, 29). The whitening index (white level), that is WID, has been used more recently in the dental research although it is also based on the CIELab system. When distinguished from the other indices, WID intends to achieve a higher correlation to visual perception (17,28). The effectiveness of the bleaching gels assessment is relevant, and the visual perception thresholds and color alteration acceptability act to check the quality control.

The samples used in the present study had their color standardized through a staining process with a black tea solution (7, 11). In the present study, all the samples present-

skupinama s karbopolom (ct i CP-ct). Za konačnu procjenu, eksperimentalne skupine koje su sadržavale karbopol pokazale su niže vrijednosti SMH, a eksperimentalna skupina koja je sadržavala 10-postotni karbamid-peroksid s aristofleksom (CP-at) pokazala je srednje vrijednosti u odnosu prema ostalim skupinama.

Čak ako komercijalni gel (CP-com) ima ugrađen neutralizirani zgušnjivač Carbopol koji bi mogao smanjiti pojavu potencijalnih promjena na površini cakline, točna količina toga spoja nije poznata. Budući da detalji o formulaciji nisu otkriveni, izazov je odrediti kako pojedinačna komponenta može utjecati na konačna svojstva materijala. Ipak, može se teoretizirati da razine karbopola unutar FGM gela nisu dovoljno velike da modificiraju caklinski SMH, kao što se dogodilo s eksperimentalnim gelovima koji sadržavaju karbopol.

Salivarna tekućina u oralnome okružju bogata je mineralima, poput kalcija i fosfata koji imaju svojstvo poticanja procesa remineralizacije (24, 26) ili smanjenja demineralizacije (8). Tijekom procesa remineralizacije, minerali iz sline mogli bi se nepravilno taložiti na površini cakline i to bi moglo povećati hrapavost površine (10), slično kao što se događa tijekom otapanja minerala. U ovom istraživanju u svim skupinama povećala se razina Ra, što bi se moglo povezati s mineralnom restrukturacijom koja se događa na površini cakline nakon izmjene procesa demineralizacije i remineralizacije. Dvadeset i četiri sata poslije završetka tretiranja Carbopol gel (ct) je prouzročio veći Ra u usporedbi s ostalim skupinama. Može se pretpostaviti da bi ti rezultati mogli biti povezani s kiselom pH vrijednošću gela (3, 72), što bi se moglo prevesti u veći gubitak minerala. Zapravo, gubitak minerala u dubljim razinama (50, 75 i 100 µm) nije se mogao obnoviti metodama remineralizacije izazvane slinom (8, 27). Stoga je vjerojatno da postoji smanjenje mikrotvrdoće i povećanje hrapavosti.

Za procjenu promjena boje poslije izbjeljivanja u stomatološkim istraživanjima rutinski se upotrebljavaju formule ΔE i ΔE2000, a izrađene su prema sustavu CIEL*, a*, b*. Ipak, važno je spomenuti da, dok ΔE usvaja istu vrijednost za sve CIEL*, a*, b* koordinate, ΔE2000 uzima u obzir parametarske elemente za procjenu CIEL*, a*, b* promjena boja. Konkretno, s pomoću spektralne dimenzije CIEL*, a*, b* mogu se procijeniti promjene poslije izbjeljivanja s pomoću promjena u jednoj ili više koordinata (L*: osvjetljenje; a*: crveno-zelena os i b*: žuto-plava os) (28, 29). Indeks izbjeljivanja (razina bijele boje) – WID – iako se također temelji na sustavu CIELab, odnedavno se upotrebljava u stomatološkim istraživanjima. Kada se razlikuje od ostalih indeksa, WI_D namjerava postići veću korelaciju s vizualnom percepcijom (17, 28). Učinkovitost procjene gelova za izbjeljivanje relevantna je, a pragovi vizualne percepcije i prihvatljivost promjene boje djeluju kao kontrola kvalitete.

Boja uzoraka korištenih u ovom istraživanju standardizirana je postupkom bojenja otopinom crnoga čaja (7, 11). U sklopu ovog istraživanja svi su uzorci pokazali slično ponašanje: skupine koje su sadržavale karbamid-peroksid (CP-ct, CP-at i CP-com) pokazale su vrijednosti iznad granica perceptibilnosti (PL) i prihvatljivosti (AL) sa svim formula-

ed similar behavior: the groups that had carbamide peroxide (CP-ct, CP-at, and CP-com) presented values over the perceptibility (PL) and acceptability (AL) limits with all the formulas - ΔE (PL: 1.2 / AL: 2.7), ΔWID (PL: 0.72 / AL: 2.62), and ΔE2000 (PL: 0.8 / AL: 1.8). This shows that the bleaching effectiveness of the CP-at is being comparable to the groups CP-ct and CP-com in terms of how noticeable and acceptable were the results of color changes. It means that the use of Aristoflex as a thickener did not hinder the bleaching effectiveness.

Nonetheless, Aristoflex (at) and Carbopol (ct) groups displayed color alterations above the PL with all the formulas. However, this color alteration was lower than the one observed in the carbamide-peroxide-based groups. Even if the thickeners cannot by themselves bleach the tooth, along with the bleaching treatment, these polymers have an important role to modulate the chemical reaction by liberating reactive oxygen species from hydrogen peroxide, which is actually responsible for bleaching (11). Additionally, the alteration of color could be associated with the acidic properties of the thickeners that are related to the demineralization of the surface and the enamel morphology alteration. This alteration could affect the samples surface reflectance patterns by inducing the color alteration.

Therefore, the second null hypothesis was partially accepted. Carbamide peroxide 10 wt% gel containing Aristoflex altered neither the effectiveness of bleaching gel nor the microhardness of the enamel between the initial and final times. Yet the roughness of the enamel had an increment. The current study design is restricted since it has not assessed some relevant aspects of the safety of experimental bleaching gels (e.g. trans-enamel-dentin cytotoxicity) or the bleaching effectiveness maintenance (e.g. after 14-days of bleaching). Further research is needed to consider the aforementioned features. In a similar manner, *in vivo* and clinical studies that can verify the current results are still required. Although the present study is corroborated by previous research, it points to the fact that Aristoflex is a favorable alternate thickener for at-home bleaching treatment.

Conclusion

In conclusion, the association of carbamide peroxide 10 wt% with Aristoflex is a promising alternative to the already commercially available Carbopol-containing bleaching products. It has exhibited significant improvements on whitening efficiency, microhardness and roughness of dental enamel without generating cytotoxicity to human gingival fibroblastic cells.

Conflict of interest

The authors declare they have no conflict of interest.

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ma – ΔE (PL: 1,2 / AL: 2,7), ΔWI_D (PL: 0,72 / AL: 2,62) i ΔE2000 (PL: 0,8 / AL: 1,8). To pokazuje učinkovitost izbjeljivanja CP-atom koja se može usporediti sa skupinama CP-ct i CP-c u smislu toga koliko su njihovi rezultati promjene boje zamjetni i prihvatljivi. Znači, korištenje aristofleksa kao zgušnjivača nije umanjilo učinkovitost izbjeljivanja.

Bez obzira na to, skupine Aristoflex (at) i Carbopol (ct) pokazale su promjene boje iznad PL-a sa svim formulama. No ta je promjena bila manja od one uočene u skupinama na bazi karbamid-peroksida. Čak ako zgušnjivači ne mogu sami izbijeliti zub, tijekom postupka izbjeljivanja ti polimeri imaju zadaću modulirati kemijsku reakciju oslobađanjem reaktivnih kisikovih vrsta iz vodikova peroksida, a ovaj posljednji je zapravo odgovoran za izbjeljivanje (11). Dodatno, promjena boje može biti povezana s kiselim svojstvima zgušnjivača, što uzrokuje demineralizaciju površine i promjenu morfologije cakline. Ta izmjena mogla bi promijeniti uzorak refleksije površine uzoraka, izazivajući promjenu boje.

Zato je druga nulta hipoteza djelomično prihvaćena. Naime, 10-postotni gel karbamid-peroksida koji sadržava aristofleks nije promijenio učinkovitost izbjeljivanja gela ni mikrotvrdoću cakline između početnoga i završnoga mjerenja. Ipak, hrapavost cakline se povećala. Primijenjeni studijski dizajn ograničen je na procijenjene relevantne aspekte sigurnosti eksperimentalnih gelova za izbjeljivanje (npr., transcaklinsko-dentinska citotoksičnost) ili održavanje učinkovitosti izbjeljivanja (npr., poslije 14 dana izbjeljivanja). Buduća istraživanja moraju uzeti u obzir te značajke. Na sličan način, i dalje su potrebna istraživanja *in vivo* i klinička istraživanja koja mogu potvrditi dobivene rezultate. Ipak, ovo istraživanje potvrđuje predodžbe utvrđene u dosadašnjim istraživanjima koja ističu Aristoflex kao povoljni alternativni zgušnjivač za postupak izbjeljivanja kod kuće.

Zaključak

U ovom istraživanju utvrđeno je da je kombinacija 10-postotnog karbamid-peroksida s aristofleksom obećavajuća alternativa već komercijalno dostupnim proizvodima za izbjeljivanje koji sadržavaju karbopol jer je pokazala usporedivu učinkovitost izbjeljivanja, mikrotvrdoću i hrapavost na zubnoj caklini, bez citotoksičnog učinka na fibroblastične stanice ljudske gingive.

Sukob interesa

Autori nisu bili u sukobu interesa.

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Sažetak

Svrha rada: U ovom istraživanju *in vitro* analizirani su učinci 10-postotnoga karbamid-peroksida (CP) povezanoga sa zgušnjivačima Carbopol® (CP-ct) i Aristoflex® (CP-at) na citotoksičnost ljudskih gingivnih fibroblasta (HGF) te su procijenjeni *in situ* njihovi učinci na zubnu caklinu. **Materijal i metode:** Citotoksičnost je analizirana s pomoću MTT - Vybrant® testa proliferacije. Za dio istraživanja *in situ* nasumično su, u sedam skupina (n = 12), podijeljena 144 bloka goveđe cakline/dentina. Uzorci su obojeni, fiksirani u intraoralne palatinalne naprave i izbjeljivani 4 sata tijekom 14 dana sljedećim sredstvima: zgušnjivačem Carbopol (ct), zgušnjivačem Aristoflex (at), CP-ctom, CP-atom, CP-om bez zgušnjivača (CP-wot) i komercijalnim CP-om (CP-com). Na uzorcima je analizirana mikrotvrdoća (SMH), hrapavost (Ra) i boja mikrodurometrom, rugosimetrom i spektrofotometrom. To je učinjeno na početku i 24 sata poslije završetka izbjeljivanja. **Rezultati:** Različiti zgušnjivači bili su slični kad je riječ o citotoksičnosti. Eksperimentalni gelovi s karbopolom pokazali su niže vrijednosti SMH-a. Skupine tretirane CP-om postignule su više vrijednosti Ra. Što se tiče rezultata u promjeni boje, skupine tretirane CP-om imale su vrijednosti iznad granica prihvatljivosti i vidljivosti. **Zaključak:** CP-at je uspio potaknuti učinkovito izbjeljivanje s manje promjena na površini zuba u usporedbi s CP-ctom. Stoga je zgušnjivač Aristoflex taj koji obećava u kombinaciji s CP-om da bi se očuvala fizikalna svojstva zubne cakline nakon kućnog izbjeljivanja.

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