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IZVORNI ZNANSTVENI RAD

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Cytotoxicity and Genotoxicity of Fluoride Toothpastes in Buccal Cells

Citotoksičnost i genotoksičnost fluoridnih zubnih pasta u stanicama bukalne sluznice

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Abstract

Objectives: This study investigated whether toothpastes containing different fluoride compounds influence cytotoxic and genotoxic alterations in buccal mucosal cells, with particular attention to the type of fluoride, the presence of fluoride itself, and the duration of exposure. **Materials and Methods:** Eighty-eight participants were randomly assigned to four parallel groups: a control group using fluoride-free toothpaste and three intervention groups using formulations containing sodium fluoride, sodium monofluorophosphate, or amine fluoride. Buccal cell samples were obtained at baseline (T0), after 30 days (T1), and after 45 days (T2), and evaluated using the buccal micronucleus cytome assay to quantify nuclear abnormalities and cytotoxic markers. **Results:** All fluoride-containing toothpastes led to higher frequencies of micronuclei, nuclear buds, and "broken egg" cells at T1 and T2 compared with the control group ($P \leq 0.001$). Amine fluoride and sodium monofluorophosphate produced sustained increases in cytogenetic markers, including nuclear buds (AmF: $P = 0.013$; NaMFP: $P \leq 0.001$) and "broken egg" cells (AmF: $P \leq 0.001$; NaMFP: $P = 0.004$), while sodium fluoride demonstrated a slower, progressive increase in "broken egg" cells ($P = 0.036$). **Conclusion:** The findings suggest that fluoride-based toothpastes may modulate cytogenetic responses in buccal epithelial cells, and that these effects differ according to the fluoride compound and exposure duration. The findings should be interpreted with caution due to study limitations, and further research is needed to clarify the long-term biological consequences of repeated fluoride exposure in oral hygiene.

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Introduction

Toothpaste formulations typically include various chemical elements, and while these elements are generally safe when used as directed, it is essential to be aware of potential risks associated with certain components. To date, fluoride stands as the sole nonprescription toothpaste additive with demonstrated efficacy in preventing tooth decay. The introduction of fluoride toothpastes into daily oral hygiene has led to a significant reduction in the incidence of dental caries in many populations around the world. Fluoride has a remineralizing effect on tooth enamel, and it reduces tooth demineralization, thereby preventing the development of caries. Toothpastes

Uvod

Formulacije zubnih pasta obično sadržavaju različite kemijske komponente i, iako su u pravilu sigurne kada se koriste prema uputama, važno je biti svjestan mogućih rizika povezanih s određenim sastojcima. Do danas je fluorid jedini dodatak zubnim pastama bez recepta s dokazanom učinkovitošću u prevenciji karijesa. Uvođenje fluoridnih zubnih pasta u svakodnevnu oralnu higijenu značajno je smanjilo incidenciju zubnog karijesa u mnogim populacijama diljem svijeta. Fluorid remineralizacijski utječe na zubnu caklinu te smanjuje demineralizaciju, čime sprječava razvoj karijesa. Zubne paste s koncentracijom fluorida od 1000 do 1500 ppm doka-

containing 1000 to 1500 ppm fluoride have been proven effective in the prevention of dental caries and are internationally supported (1). The main fluoride compounds currently found in toothpastes are sodium fluoride and sodium monofluorophosphate, although stannous fluoride and amine fluoride are also used (2). Fluoride toothpastes differ not only in their total fluoride content but also in the chemical form and bioavailability of the fluoride compound. Sodium fluoride (NaF) dissociates rapidly, releasing free fluoride ions capable of interacting directly with the oral mucosa. Amine fluoride (AmF), an organic surfactant-bound fluoride compound, exhibits strong adhesion to soft and hard tissues, leading to prolonged retention in the oral cavity and enhanced substantivity (3). Sodium monofluorophosphate (NaMFP) requires enzymatic hydrolysis to liberate fluoride, resulting in a more gradual ion release and potentially different epithelial exposure kinetics (4). These pharmacochemical distinctions may influence the magnitude and pattern of cellular responses in the oral epithelium, particularly over repeated daily exposure.

The acknowledgment of potential toxicity of fluoride was largely overlooked due to its commendable reputation for caries prevention. Although the doses present in toothpastes are usually low, there are concerns regarding long-term daily exposure, especially in sensitive populations, such as children or individuals with weakened oral barriers. In recent years, there has been a resurgence of interest in investigating its adverse effects. This renewed focus stems from an increasing awareness that fluoride can interact with cellular systems, even at low doses. Numerous studies have shown that fluoride has the potential to induce oxidative stress (5), influence intracellular redox balance (6), promote lipid peroxidation (7), and induce alterations in gene expression, ultimately leading to apoptosis (8, 9). Fluoride has been tested in various *in vitro* and *in vivo* systems assessing mutagenicity and clastogenicity, including several studies on exposed humans. The results of *in vitro* cytogenetic studies are mixed, however, the majority of evidence indicates that sodium fluoride can induce chromosomal aberrations and sister chromatid exchanges in cultured mammalian cells (10, 11). Studies have shown that fluoride varnish exhibits a concentration dependent cytotoxic effect on human gingival fibroblasts, with cell viability decreasing as fluoride concentration increases (12, 13). A study by Kleinsasser using the Comet assay demonstrated a minor genotoxic impact on human oropharyngeal epithelial cells and peripheral lymphocytes when aminofluoride was applied (14). The frequent observation of positive results in tests assessing chromosomal aberrations and sister chromatid exchanges support the hypothesis that fluoride may influence the genome, potentially leading to DNA rearrangements (15). However, the analysis of human epidemiological data from over 30 studies fails to show a clear relationship between fluoride exposure and disease, particularly in studies examining the correlation between water fluoridation and cancer (16). The oral mucosa serves as the first point of contact when using toothpaste, making it essential to understand the effects of fluoride on this sensitive tissue. As a crucial protective barrier, any damage to the mucosa can lead to various health issues, including heightened vulnerability to infections, ulcerations, and other

zano su učinkovite u prevenciji karijesa te su međunarodno preporučene (1). Glavni fluoridni spojevi koji se danas nalaze u zubnim pastama jesu natrijev fluorid i natrijev monofluorofosfat, iako se koriste i kositrov fluorid te aminfluorid (2). Fluoridne zubne paste razlikuju se ne samo prema ukupnom sadržaju fluorida, nego i prema kemijskom obliku i bioraspoloživosti fluoridnoga spoja. Natrijev fluorid (NaF) brzo disocira, oslobađajući slobodne fluoridne ione koji mogu izravno djelovati na oralnu sluznicu. Aminfluorid (AmF), organski fluorid vezan uz surfaktant, pokazuje izraženo svojstvo vezanja za meka i tvrda tkiva, što rezultira produljenim zadržavanjem u usnoj šupljini i produljenim djelovanjem (3). Natrijev monofluorofosfat (NaMFP) zahtijeva enzimsku hidrolizu za oslobađanje fluorida, što rezultira postupnijim otpuštanjem iona i potencijalno drukčijom kinetikom izloženosti epitela (4). Te farmakokemijske razlike mogu utjecati na intenzitet i način staničnih odgovora u oralnom epitelu, osobito pri ponavljanoj svakodnevnoj izloženosti.

Potencijalna toksičnost fluorida dugo je bila zanemarivana zbog njegove dokazane učinkovitosti u prevenciji karijesa. Iako su doze u zubnim pastama obično niske, postoji zabrinutost vezana za dugotrajnu svakodnevnu izloženost, osobito u osjetljivim populacijama poput djece ili osoba s narušenom barijernom funkcijom oralne sluznice. Posljednjih godina ponovno raste zanimanje za istraživanje mogućih štetnih učinaka fluorida. Taj obnovljeni interes proizlazi iz sve većeg razumijevanja da fluoridi mogu stupati u interakcije sa staničnim sustavima i pri niskim koncentracijama. U mnogobrojnim istraživanjima autori su pokazali da fluoridi mogu inducirati oksidacijski stres (5), utjecati na intracelularnu redoks ravnotežu (6), poticati lipidnu peroksidaciju (7) te prouzročiti promjene u ekspresiji gena koje u konačnici mogu završiti apoptozom (8, 9). Fluoridi su ispitivani u mnogobrojnim sustavima *in vitro* i *in vivo* u procjeni mutagenosti i klastogenosti, uključujući i studije na ljudima. Rezultati citogenetskih istraživanja *in vitro* nisu ujednačeni, no većina dokaza upućuje na to da natrijev fluorid može inducirati kromosomske aberacije i izmjene sestrinskih kromatida u kultiviranim stanicama sisavaca (10, 11). Istraživanja su pokazala da fluoridni lakovi imaju koncentracijski ovisan citotoksični učinak na humane gingivne fibroblaste, pri čemu se vitalnost stanica smanjuje s porastom koncentracije fluorida (12, 13). Istraživanje Kleinsassera primjenom comet-testa (kometskoga testa) pokazalo je blagi genotoksični učinak na epitelne stanice orofarinksa i periferne limfocite nakon primjene aminfluorida (14). Česti pozitivni rezultati u testovima kromosomskih aberacija i izmjenama sestrinskih kromatida podupiru hipotezu da fluorid može utjecati na genom i potencijalno potaknuti strukturne promjene DNK (15). S druge strane, analiza epidemioloških podataka iz više od 30 studija ne pokazuje jasnu povezanost između izloženosti fluoridu i bolesti, osobito u istraživanjima u kojima se ispitivala povezanost fluoridacije vode i karcinoma (16). Oralna sluznica prvo je mjesto kontakta pri korištenju zubnih pasta, zbog čega je važno razumjeti učinke fluorida na to osjetljivo tkivo. Kao ključna zaštitna barijera, svako oštećenje sluznice može potaknuti različite zdravstvene probleme, uključujući povećanu sklonost prema infekcijama, ulceracijama i drugim

pathological changes. The oral mucosa is renewed through a continuous process in which cells generated by mitosis within the basal layer gradually migrate toward the surface, replacing exfoliated cells. This basal layer contains progenitor and stem cells, and any chromosomal damage that occurs during their division such as chromosome breakage or loss can manifest as micronuclei (MNi) in the resulting daughter cells (17). After a brief genotoxic insult, micronuclei typically become detectable in exfoliated buccal cells only after the time required for these newly formed cells to reach the epithelial surface, a process estimated to take approximately 5–7 days. However, it has been observed that the peak expression of micronuclei may be delayed for up to 21 days (18). The micronucleus test was introduced at the end of the 19th century by Howell and Jolly, and since then, it has become the most widely and most reliable assay to evaluate cytogenetic damage (19). Chromosomal abnormalities detected in human lymphocytes are a well-known predictor of future cancer risk, and similar associations have been demonstrated for micronuclei (MNi) in these cells (20). As an alternative to peripheral lymphocytes, gingival and buccal epithelial cells can also be used to assess genotoxic and cytotoxic effects. These epithelial cells offer several advantages: they can be collected quickly and non-invasively, do not require cell culture, and can be analyzed without mitogenic stimulation or metaphase preparation. For these reasons, the micronucleus assay applied to epithelial tissues is regarded as a highly sensitive approach for monitoring genetic damage in individuals exposed to various genotoxic agents (21).

The relationship between fluoride exposure and buccal cell genotoxicity and cytotoxicity remains a subject of ongoing research. To date, several studies have explored the cytotoxic and genotoxic effects of fluoridated toothpastes on buccal mucosal cells. An *in vivo* study by Tadin et al. (22), evaluating the toxicity of fluoride in toothpaste on buccal epithelial cells, concluded that sodium fluoride (NaF) does not exert cytotoxic or genotoxic effects on these cells. Similarly, another study found that the simultaneous daily use of NaF toothpaste and mouthwash over a 12-week period did not result in statistically significant fluoride dependent cytotoxic or genotoxic effects on exfoliated buccal mucosa cells for most endpoints in the buccal micronucleus cytome assay (23). However, none of these studies have systematically examined the effects of three distinct fluoride active substances and compared their impacts. This study aimed to evaluate the cytotoxic and genotoxic effects of toothpastes containing fluoride, incorporating different active substances; sodium fluoride, sodium monofluorophosphate, and amine fluoride.

The primary objective of this study was to compare the cytotoxic and genotoxic responses of buccal mucosal cells following the use of three different fluoride-containing toothpastes and fluoride-free control toothpaste. The study also aimed to examine whether exposure duration influenced the observed cytogenetic effects. We hypothesized that fluoride-containing toothpastes may differ in their ability to induce cytogenetic alterations compared with a fluoride-free formulation, and that these effects may vary across fluoride compound types and exposure durations.

patološkim promjenama. Regeneracija oralne sluznice kontinuirani je proces u kojemu stanice nastale mitozom u bazalnome sloju postupno migriraju prema površini zamjenjujući deskvamirane stanice. Bazalni sloj sadržava progenitorske i matične stanice, a svako kromosomsko oštećenje koje nastane tijekom njihove diobe, poput loma ili gubitka kromosoma, može se očitovati kao mikronukleusi (MNi) u novonastalim stanicama (17). Nakon kratkotrajnoga genotoksičnog podražaja, mikronukleusi se u eksfoliranim bukalnim stanicama mogu uočiti tek nakon što novonastale stanice dosegnu površinu epitela, za što je obično potrebno približno 5 do 7 dana. Međutim pokazalo se da maksimalna ekspresija mikronukleusa može kasniti i do 21 dan (18). Mikronukleusni test uveli su potkraj 19. stoljeća Howell i Jolly te je od tada jedna od najraširenijih i najpouzdanijih metoda za procjenu citogenetskih oštećenja (19). Kromosomske abnormalnosti u humanim limfocitima dobro su poznat prediktor budućega rizika za razvoj karcinoma, a slična povezanost opisana je i za mikronukleuse (MNi) u limfocitima (20). Kao alternativa perifernim limfocitima, epitelne stanice gingive i bukalne sluznice također se mogu koristiti za procjenu genotoksičnih i citotoksičnih učinaka. Te stanice imaju niz prednosti: mogu se prikupiti brzo i neinvazivno, ne zahtijevaju kultivaciju te se mogu analizirati bez mitogene stimulacije i pripreme metafaze. Zbog toga se mikronukleusni test, primijenjen na epitelna tkiva, smatra vrlo osjetljivom metodom za praćenje genetskog oštećenja kod osoba izloženih različitim genotoksičnim agensima (21).

Povezanost između izloženosti fluoridu i genotoksičnosti te citotoksičnosti u bukalnim stanicama i dalje je predmet istraživanja. Dosad su autori nekoliko studija ispitivali učinke fluoridnih zubnih pasta na stanice bukalne sluznice. U istraživanju *in vivo* Tadina i suradnika (22), u kojemu su procjenjivali toksičnost fluorida iz zubnih pasta na bukalne epitelne stanice, zaključeno je da natrijev fluorid nema ni citotoksične, ni genotoksične učinke na te stanice. Slično tomu, u jednom drugom istraživanju pokazano je da istodobna svakodnevna primjena zubne paste i tekućine za ispiranje usta s natrijevim fluoridom (NaF) tijekom 12 tjedana nije rezultirala statistički značajnim citotoksičnim ili genotoksičnim učincima povezanima s fluoridom u većini parametara mikronukleusnoga citom-testa bukalnih stanica (23). No ni u nijednom od tih istraživanja nisu sustavno ispitani učinci triju različitih fluoridnih aktivnih tvari i uzajamno uspoređeni.

Cilj ovog istraživanja bio je procijeniti citotoksične i genotoksične učinke zubnih pasta koje sadržavaju fluorid, uz primjenu različitih aktivnih tvari: natrijeva fluorida, natrijeva monofluorofosfata i aminfluorida. Primarni zadatak bio je usporediti citotoksične i genotoksične odgovore stanica bukalne sluznice nakon primjene triju fluoridnih zubnih pasta i kontrolne zubne paste bez fluorida. Također se ispitivalo utječe li trajanje izloženosti na uočene citogenetske učinke. Postavljena je hipoteza da se fluoridne zubne paste razlikuju u sposobnosti induciranja citogenetskih promjena u odnosu prema pasti bez fluorida te da ti učinci ovise o vrsti fluoridnoga spoja i trajanju izloženosti.

Materials and Methods

Study Design and Participants

The present study employed a prospective, randomized, triple-blind, parallel group clinical trial design to investigate the cytotoxic and genotoxic effects of toothpastes containing different fluoride-based active substances (sodium fluoride, sodium monofluorophosphate, amine fluoride), as well as to compare their effects. In this study, the investigators, participants, and the evaluator responsible for cytological scoring were all blinded to the type of toothpaste used. All statistical analyses were performed by an independent researcher who had access only to anonymized coded data and remained blinded to group allocation until completion of all analyses. A total of 100 participants aged 18 to 60 years were recruited from the Department of Restorative Dental Medicine and Endodontics at the School of Dental Medicine in Split, from students of the University of Split School of Medicine, and from adult volunteers who provided informed consent, as outlined in the recruitment flow presented in Figure 1. Inclusion criteria required participants to be in good general and oral health (ASA I) with a minimum of 20 teeth pres-

Materijali i metode

Dizajn istraživanja i ispitanici

Ovo istraživanje provedeno je kao prospektivno, randomizirano, trostruko slijepo i paralelno kliničko ispitivanje sa svrhom procjene citotoksičnih i genotoksičnih učinaka zubnih pasta koje sadržavaju različite fluoridne aktivne tvari (natrijev fluorid, natrijev monofluorofosfat i aminfluorid) te njihove uzajamne usporedbe. U istraživanju su istraživači, ispitanici i osoba odgovorna za citološku analizu bili zaslijepljeni u odnosu prema vrsti korištene zubne paste. Sve statističke analize obavio je neovisni istraživač koji je imao pristup isključivo anonimiziranim kodiranim podatcima te je ostao zaslijepljen za raspodjelu ispitanika po skupinama do završetka svih analiza. Ukupno je bilo uključeno 100 ispitanika u dobi od 18 do 60 godina, a odabrani su bili u Zavodu za restaurativnu dentalnu medicinu i endodonciju Stomatološkog fakulteta u Splitu, među studentima Medicinskog fakulteta Sveučilišta u Splitu te među odraslim dobrovoljcima koji su dali informirani pristanak, sukladno tijeku uključivanja ispitanika prikazanom na slici 1. Kriteriji za uključivanje obuhvaćali su ispitanike dobroga općeg i oralnog zdravlja

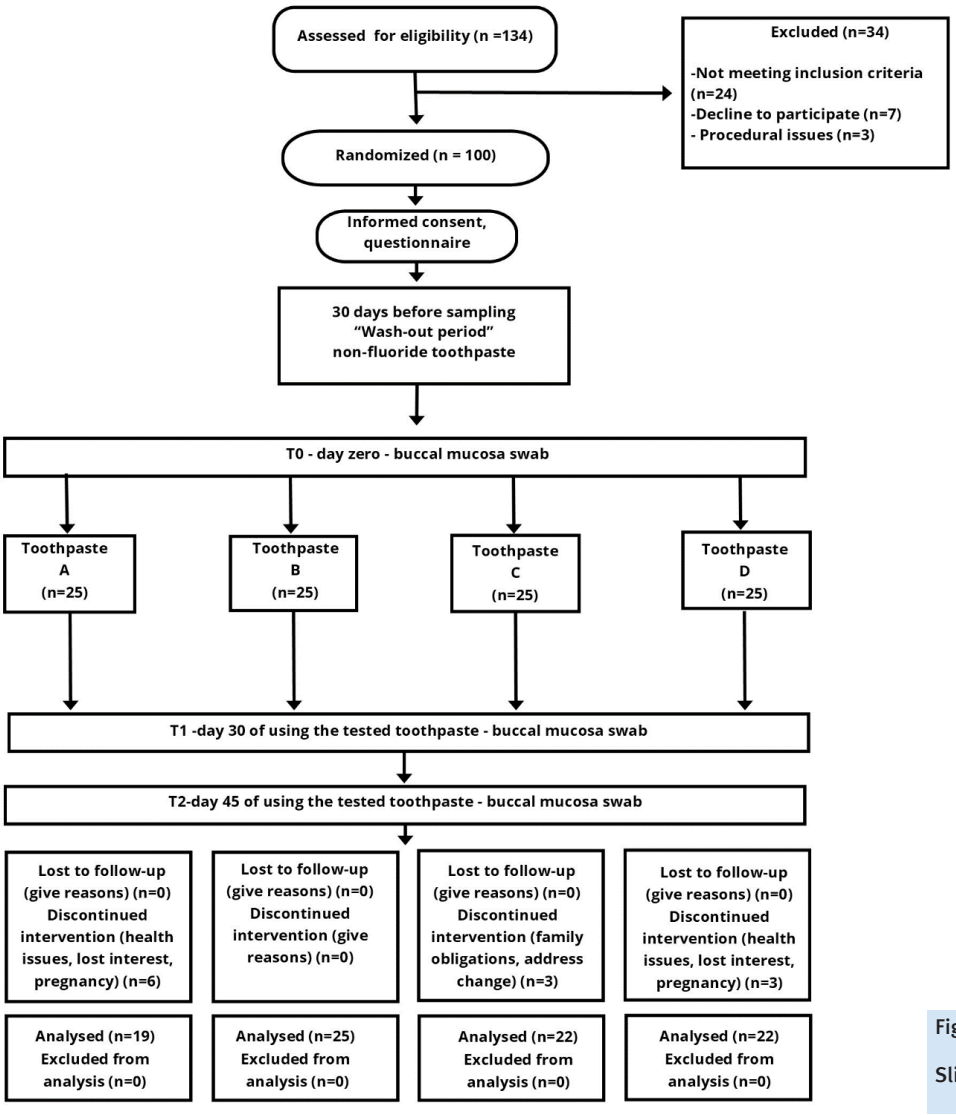

Figure 1 Flowchart of participant recruitment and follow-up.
Slika 1. Dijagram toka regrutiranja sudionika i praćenja

ent in both jaws. Exclusion criteria included individuals with infectious diseases, chronic inflammatory conditions, recent use (within the past six months) of antibiotics, corticosteroids, or anti-inflammatory drugs, as well as those with oral mucosal lesions, periodontal disease, fixed prosthetic restorations, or orthodontic appliances. Pregnant individuals, patients who had undergone radiation therapy in the head and neck region, and those with known allergies to ingredients in oral hygiene products were also excluded. Participants were also excluded if they had a history of chronic alcohol consumption and/or long-term smoking (more than 10 pack-years). Written informed consent was obtained from all participants after a thorough explanation of the study objectives. Basic demographic information (age and gender) was recorded for all participants. The sample size for the present trial was determined using the effect size reported in an earlier *in vivo* investigation by Tadin et al. (2019) (22), which examined fluoride and sodium lauryl sulfate related cytotoxic and genotoxic changes in buccal epithelial cells. In that study, the mean micronucleus frequency differed between users of fluoride-free toothpaste (0.55 ± 0.51) and those using a fluoride-containing formulation (1.15 ± 0.88), corresponding to a Cohen's d of 0.835. Using this effect size, together with a significance threshold of $\alpha = 0.05$ and a statistical power of 80%, the minimum number of participants needed for each study arm was calculated to be 19 (22). To account for potential attrition during follow-up, the minimal required sample size of 19 participants per group was exceeded by intentionally recruiting 25 participants per group at the time of randomization. The study was carried out at two collaborating institutions: the Department of Restorative Dental Medicine and Endodontics, School of Dental Medicine, University of Split, Split, Croatia, and the Institute for Medical Research and Occupational Health, Zagreb, Croatia. The study was conducted in accordance with the Declaration of Helsinki (1975, revised 2002) and approved by the Ethics Committee of the School of Dental Medicine, University of Zagreb (No: 05-PA-30-20-9/2023), and the Ethics Committee of the School of Medicine, University of Split (No: 2181-198-03-04-22-0003). Written informed consent was obtained from all participants prior to enrollment. All aspects of the trial adhered to CONSORT recommendations, and the study was prospectively registered on ClinicalTrials.gov under the identifier NCT05596149.

Materials, Clinical Procedure and Sample Collection

Before the study interventions were introduced, participants completed a 30-day washout period in which they exclusively used toothpaste without fluoride, a duration chosen to allow full turnover of the buccal epithelium and minimize any residual effects of previously used products. After baseline sampling, participants who met all eligibility criteria were randomly allocated into one of four study arms. The allocation sequence was generated using computer-based block randomization to ensure balanced group sizes throughout enrollment (24). An independent researcher, who was not involved in participant contact, sample collection, or microscopic evaluation, managed the alloca-

(ASA I) s najmanje 20 zuba u obje čeljusti. Iz istraživanja isključene su osobe s infektivnim bolestima, kroničnim upalnim stanjima, nedavnom primjenom (posljednjih šest mjeseci) antibiotika, kortikosteroida ili protuupalnih lijekova, te one s lezijama oralne sluznice, parodontnim bolestima, fiksnim protetičkim nadomjestcima ili ortodontskim aparatima. Također su isključene trudnice, ispitanici koji su bili podvrgnuti radioterapiji u području glave i vrata te osobe s poznatim alergijama na sastojke u proizvodima za oralnu higijenu. Dodatni kriteriji za isključivanje bili su anamneza kronične konzumacije alkohola i/ili dugotrajno pušenje (više od 10 paketa na godinu). Svi ispitanici potpisali su informirani pristanak nakon detaljnog objašnjenja ciljeva istraživanja. Za sve su također prikupljeni osnovni demografski podatci (dob i spol). Veličina uzorka za ovo ispitivanje određena je na temelju veličine učinka iz ranijeg istraživanja *in vivo* Tadina i suradnika (2019.) (22) koji su ispitivali citotoksične i genotoksične promjene u bukalnim epitelnim stanicama povezane s fluoridom i natrijevim lauril-sulfatom. U tom istraživanju srednja vrijednost učestalosti mikronukleusa razlikovala se između korisnika zubne paste bez fluorida (0,55 ± 0,51) i onih koji su se koristili fluoridnom formulacijom (1,15 ± 0,88), što odgovara Cohenovu d = 0,835. Na temelju te veličine učinka, uz razinu značajnosti $\alpha = 0,05$ i statističku snagu od 80 %, izračunato je da je minimalni broj ispitanika po skupini 19 (22). Kako bi se uzeli u obzir mogući gubitci tijekom praćenja, minimalni potreban broj ispitanika po skupini premašen je planiranim uključivanjem 25 ispitanika po skupini u trenutku randomizacije. Istraživanje je provedeno u dvjema suradničkim ustanovama: Zavodu za restaurativnu dentalnu medicinu i endodonciju Stomatološkog fakulteta Sveučilišta u Splitu te u Institutu za medicinska istraživanja i medicinu rada u Zagrebu. Istraživanje je provedeno u skladu s Helsinškom deklaracijom (1975., revidiranom 2002.) i odobrilo ga je Etičko povjerenstvo Stomatološkog fakulteta Sveučilišta u Zagrebu (br. 05-PA-30-20-9/2023) i Etičko povjerenstvo Medicinskog fakulteta Sveučilišta u Splitu (br. 2181-198-03-04-22-0003). Informirani pristanak potpisali su svi ispitanici prije uključivanja u istraživanje. Svi aspekti ispitivanja provedeni su u skladu sa smjernicama CONSORT, a istraživanje je prospektivno registrirano na ClinicalTrials.gov pod identifikacijskim brojem NCT05596149.

Materijali, klinički postupak i prikupljanje uzoraka

Trideset dana prije početka intervencije, tijekom pripremnog razdoblja (*Wash-out period*), svi su se ispitanici koristili zubnom pastom bez fluorida. To je razdoblje odabrano da bi se omogućila potpuna obnova bukalnoga epitela i smanjio mogući zaostali učinak prije korištenih proizvoda. Nakon uzimanja početnoga uzorka, ispitanici koji su zadovoljili sve kriterije za uključivanje randomizirano su raspoređeni u jednu od četiriju skupina. Raspored po skupinama generiran je računalnom blok-randomizacijom kako bi se tijekom uključivanja osigurala uravnotežena veličina skupina (24). Neovisni istraživač, koji nije sudjelovao u kontaktu s ispitanicima, prikupljanju uzoraka i mikroskopskoj

tion process and prepared the coded toothpaste tubes. All toothpastes were transferred into identical, opaque, precoded containers to maintain blinding. Coding was concealed from participants, the examiner responsible for buccal cell collection, and the examiner who performed microscopic scoring, thus ensuring full triple blinding. Baseline comparability among the four groups was confirmed using the Chi-square test for categorical variables and the Kruskal–Wallis test for age, with no significant differences detected (Table 2). Three of the groups used toothpastes containing fluoride, while the fourth control group continued to use fluoride-free toothpaste. The fluoride toothpastes tested contained either sodium fluoride (NaF), sodium monofluorophosphate (NaMFP), or amine fluoride (AmF). The composition of the toothpastes was the same, except for the active substances mentioned (Table 1). To maintain blinding, the toothpastes were labeled with letters (A, B, C, D) by pharmacy staff that were not familiar with the randomization process. All experimental dentifrices were prepared at a specialized pharma-

evaluaciji, vodio je postupak raspodjele i pripremao kodirane tube zubnih pasta. Da bi se očuvala zaslijepljenost, sve zubne paste prebačene su u identične, neprozirne, prije toga kodirane spremnike. Oznake su bile skrivene od ispitanika, ispitivača odgovornoga za uzimanje bukalnih stanica i ispitivača koji je provodio mikroskopsko bodovanje, čime je osigurana potpuna trostruka zaslijepljenost. Usporedivost četiriju skupina na početku istraživanja potvrđena je hi-kvadrat testom za kategorijske varijable i Kruskal-Wallisovim testom za dob, pri čemu nisu utvrđene statistički značajne razlike (tablica 2.). Tri skupine koristile su se zubnim pastama koje su sadržavale fluorid, a četvrta, kontrolna skupina nastavila je upotrebljavati zubnu pastu bez fluorida. Ispitivane fluoridne zubne paste sadržavale su natrijev fluorid (NaF), natrijev monofluorofosfat (NaMFP) ili aminfluorid (AmF). Sastav zubnih pasta bio je jednak, osim navedenih aktivnih tvari (tablica 1.). Radi očuvanja zaslijepljenosti, zubne paste označilo je slovima (A, B, C, D) ljekarničko osoblje koje nije bilo obaviješteno o postupku randomizacije. Sve eksperi-

Table 1 Composition of the experimental toothpastes used in the study.
Tablica 1. Sastav eksperimentalnih zubnih pasta korištenih u istraživanju

Toothpaste • Zubna pasta	Ingredients • Sastojci
Toothpaste A - 1000 ppm Amine fluoride • Zubna pasta A – 1000 ppm aminfluorida	Zeodent, Sodium lauryl sulfate, Sorbitol 70% solution, Sodium carboxymethylcellulose, Carbomer (Carbopol 980), Sodium hydroxide 18% solution, Sodium saccharin, Essential oil of eucalyptus, Purified water, Amine fluoride (1000 ppm) • Zeodent, natrijev lauril-sulfat, 70 %-tna otopina sorbitola, natrijeva karboksimetilceluloza, karbomer (Carbopol 980), 18 %-tna otopina natrijeva hidroksida, natrijev saharin, eterično ulje eukaliptusa, pročišćena voda, aminfluorid (1000 ppm)
Toothpaste B - 1000 ppm Sodium monofluorophosphate • Zubna pasta B – 1000 ppm natrijeva monofluorofosfata	Zeodent, Sodium lauryl sulfate, Sorbitol 70% solution, Sodium carboxymethylcellulose, Carbomer (Carbopol 980), Sodium hydroxide 18% solution, Sodium saccharin, Essential oil of eucalyptus, Purified water, Sodium monofluorophosphate (1000 ppm) • Zeodent, natrijev lauril-sulfat, 70 %-tna otopina sorbitola, natrijeva karboksimetilceluloza, karbomer (Carbopol 980), 18 %-tna otopina natrijeva hidroksida, natrijev saharin, eterično ulje eukaliptusa, pročišćena voda, natrijev monofluorofosfat (1000 ppm)
Toothpaste C - 1000 ppm Sodium fluoride • Zubna pasta C – 1000 ppm natrijeva fluorida	Zeodent, Sodium lauryl sulfate, Sorbitol 70% solution, Sodium carboxymethylcellulose, Carbomer (Carbopol 980), Sodium hydroxide 18% solution, Sodium saccharin, Essential oil of eucalyptus, Purified water, Sodium fluoride (1000 ppm) • Zeodent, natrijev lauril-sulfat, 70 %-tna otopina sorbitola, natrijeva karboksimetilceluloza, karbomer (Carbopol 980), 18 %-tna otopina natrijeva hidroksida, natrijev saharin, eterično ulje eukaliptusa, pročišćena voda, natrijev fluorid (1000 ppm)
Control - 0 ppm F • Kontrola – 0 ppm F	Zeodent, Sodium lauryl sulfate, Sorbitol 70% solution, Sodium carboxymethylcellulose, Carbomer (Carbopol 980), Sodium hydroxide 18% solution, Sodium saccharin, Essential oil of eucalyptus, Purified water • Zeodent, natrijev lauril-sulfat, 70 %-tna otopina sorbitola, natrijeva karboksimetilceluloza, karbomer (Carbopol 980), 18 %-tna otopina natrijeva hidroksida, natrijev saharin, eterično ulje eukaliptusa, pročišćena voda

Table 2 Demographic data of participants.
Tablica 2. Demografski podatci ispitanika

Characteristics • Karakteristike		Total • Ukupno (n=88)	Control • Kontrola (n = 22)	AmF (n = 19)	NaMFP (n = 25)	NaF (n = 22)	*P* - value • *P* - vrijednost
Age • Dob (X, SD)		36.0 (15.9)	43.5 (16.1)	32.6 (14.1)	32.5 (15.7)	35.36 (16.1)	0.092
Gender • Spol (n, %)	Male • Muškarci	31 (35.2)	9 (40.9)	5 (26.3)	9 (36.0)	8 (36.4)	0.912
	Female • Žene	57 (64.8)	13 (59.1)	14 (73.7)	16 (64.0)	14 (63.6)	

X – mean value; SD – standard deviation; n – number of participants; % – percentage of participants relative to the total number. *P*-value for age refers to between-group comparison of mean age. *P*-value for gender refers to the overall Chi-square test of sex distribution (male vs female). Statistical significance was set at $P < 0.05$ • X – srednja vrijednost; SD – standardna devijacija; n – broj ispitanika; % – postotak ispitanika u odnosu na ukupan broj. *P*-vrijednost za dob odnosi se na usporedbu srednje dobi između skupina. *P*-vrijednost za spol odnosi se na ukupni hi-kvadrat test raspodjele prema spolu (muškarci i žene). Statistička značajnost definirana je kao $P < 0,05$.

ceutical formulation laboratory in Zagreb. The toothpastes were prepared using standardized manufacturing procedures (controlled mixing, homogenization under reduced pressure) to ensure a uniform dispersion of ingredients and to maintain identical physicochemical characteristics across formulations. The base excipient composition was kept constant, with the fluoride active substance being the only variable. All participants received standardized written and verbal instructions regarding brushing frequency and technique. Participants were instructed to brush their teeth with the allocated toothpaste twice daily, once in the morning and once in the evening, using a pea-sized amount (approximately 0.5 cm) and following the modified Bass technique for a total of three minutes. Adherence was monitored through periodic follow-up contacts and short self-report checklists. They were explicitly advised not to use any additional toothpastes or oral hygiene products, including mouthrinses or topical fluoride preparations. Throughout the study period, all individuals received the same model of toothbrush (Swissdent Profi Soft, Swissdent Care AG, Zurich, Switzerland) to standardize brushing conditions. For cytological sampling, buccal epithelial cells were collected from both cheeks using a cytobrush (Cytobrush Plus, GmbH Dietramszell-Linden, Germany). The baseline sample (T0) was obtained immediately before participants began using their assigned toothpaste, following a 30-day washout phase with fluoride-free toothpaste. Follow-up samples were collected at two additional time points: T1, after 30 days of exposure to the assigned toothpaste, and T2, after 45 days. Participants in the control group used the fluoride-free toothpaste for the full 45-day duration, and the collection of buccal mucosa cell samples was carried out identically to the fluoride toothpaste groups at T0, T1, and T2. Before each sampling session, participants refrained from eating, drinking, and smoking for at least one hour, and were asked to rinse their mouths with water to remove superficial exfoliated cells. All buccal mucosa samples were collected by a single trained examiner using a standardized buccal exfoliation protocol to ensure methodological consistency. The same examiner performed all sampling at T0, T1, and T2 to minimize interexaminer variability. Using a cytological brush, the buccal mucosa was gently brushed on both sides, and the buccal cell swab was placed in Falcon tubes containing chilled saline solution (+4°C). The samples were centrifuged within 60 minutes of collection, and the cell suspension was applied to glass slides and fixed with a methanol and acetic acid solution (3:1) (School of Medicine, Split). Afterward, the preparations were sent to the Institute for Medical Research and Occupational Health in Zagreb, where they were stained with 1% Schiff reagent and 0.2% Light-Green dye (Merck, Darmstadt, Germany). The coded slides were independently evaluated by trained expert using a blind approach. A scoring scheme proposed by Thomas and Fenech in their protocol published in Nature was carefully applied (25). The analysis of the stained preparations was performed using a light microscope (Olympus CX 40, Tokyo, Japan) set to 600× magnification, which allowed precise visualization and reliable evaluation of the cellular features. In addition to anomalies in the organization of

mentalne zubne paste pripremljene su u specijaliziranom laboratoriju za farmaceutske formulacije u Zagrebu. Priprema zubnih pasta obavljena je standardiziranim proizvodnim postupcima (kontrolirano miješanje, homogenizacija pod sniženim tlakom) kako bi se osigurala jednolika raspodjela sastojaka i očuvala identična fizikalno-kemijska svojstva svih formulacija. Sastav pomoćnih tvari bio je stalan, a jedina varijabla bila je fluoridna aktivna tvar. Svi ispitanici dobili su standardizirane pisane i usmene upute o učestalosti i tehnici četkanja zuba. Također su upućeni da zube četkaju dodijeljenom zubnom pastom dva puta na dan, ujutro i navečer, koristeći se količinom veličine zrna graška (približno 0,5 cm) te modificiranom Bassovom tehnikom tijekom ukupno tri minute. Praćenje uputa provjeravalo se periodičnim kontrolnim kontaktima i kratkim upitnicima. Ispitanicima je izričito rečeno da se ne koriste dodatnim zubnim pastama, ni drugim proizvodima za oralnu higijenu, uključujući tekućine za ispiranje usta ili lokalne fluoridne pripravke. Tijekom cijelog istraživanja svi su se ispitanici koristili istim modelom četkice za zube (Swissdent Profi Soft, Swissdent Care AG, Zürich, Švicarska) zbog standardizacije uvjeta četkanja. Za citološku analizu epitelne stanice bukalne sluznice prikupljane su s obje unutarnje strane obraza citološkom četkicom (Cytobrush Plus, GmbH Dietramszell-Linden, Njemačka). Početni uzorak (T0) uzet je neposredno prije početka korištenja dodijeljene zubne paste, poslije 30-dnevne faze ispiranja zubnom pastom bez fluorida. Kontrolni uzorci prikupljeni su u još dvije vremenske točke: T1 poslije 30 dana izloženosti dodijeljenoj zubnoj pasti i T2 poslije 45 dana. Ispitanici u kontrolnoj skupini koristili su se zubnom pastom bez fluorida tijekom cijelog razdoblja od 45 dana, a uzorkovanje stanica bukalne sluznice provedeno je jednako kao i u skupinama koje su se koristile fluoridnim zubnim pastama, u vremenskim točkama T0, T1 i T2. Prije svakog uzorkovanja ispitanici su najmanje jedan sat apstinirali od hrane, pića i pušenja, nakon čega su ispirali usnu šupljinu vodom da bi se uklonile površinski eksfolirane stanice. Sve uzorke bukalne sluznice prikupljao je jedan educirani ispitivač prema standardiziranom protokolu eksfolijacije bukalne sluznice kako bi se osigurala metodološka dosljednost. Isti je ispitivač obavljao sva uzorkovanja u vremenskim točkama T0, T1 i T2, čime je smanjena međuispitivačka varijabilnost. Citološkom četkicom nježno je obrisana bukalna sluznica s obje strane, a uzorci bukalnih stanica pohranjeni su u epruvete Falcon koje su sadržavale ohlađenu fiziološku otopinu (+4 °C). Uzorci su centrifugirani unutar 60 minuta od prikupljanja, a stanična suspenzija nanesena je na predmetna stakalca i fiksirana otopinom metanola i octene kiseline u omjeru 3 : 1 (Medicinski fakultet u Splitu). Nakon toga preparati su poslani u Institut za medicinska istraživanja i medicinu rada u Zagrebu gdje su obojeni 1-postotnim Schiffovim reagensom i 0,2-postotnom svijetlo zelenom bojom (Merck, Darmstadt, Njemačka). Kodirana stakalca neovisno je ocjenjivao educirani stručnjak slijepim pristupom. Pažljivo je primijenjena shema bodovanja koju su predložili Thomas i Fenech u protokolu objavljenom u časopisu *Nature* (25). Analiza obojenih preparata provedena je svjetlosnim mikroskopom (Olympus CX 40, Tokio, Japan) pri povećanju od 600 puta, što je omo-

genetic material (micronuclei, nuclear buds, "broken eggs", nucleoplasmic bridges), the analysis of the slides included the identification and classification of various anomalies resulting from chromosomal instability and DNA damage, which pointed to specific forms of cell death. These anomalies were categorized according to established HUMNxl criteria, and 2,000 buccal exfoliated cells per participant were analyzed to ensure the reliability of the results (26). In the context of cytotoxicity parameters, cellular changes such as condensed chromatin (apoptosis), karyorrhexis (cell death characterized by nuclear fragmentation - apoptosis and necrosis), pyknosis (nuclear condensation - apoptosis), and karyolysis (nuclear dissolution - apoptosis and necrosis) were identified and categorized as indicators of early and late stages of apoptosis, pointing to cytotoxic effects. In addition to markers of cell death, the slides were analyzed for the presence of micronuclei, nucleoplasmic bridges, nuclear buds, and the "broken egg" phenomenon (specific membrane damage) as indicators of chromosomal and DNA damage (25). To ensure objective and unbiased scoring of cytogenetic and cytotoxic parameters, all slides were anonymized before evaluation. Each participant and sample timepoint (T0, T1, T2) was assigned a unique alphanumeric code generated by an independent researcher who was not involved in slide analysis. All cytological evaluations were performed by an experienced evaluator with postgraduate training in cytomorphology and prior calibration in the HUMNxl buccal micronucleus cytome assay scoring protocol. The evaluator was fully blinded to group allocation and to all sampling time points throughout the study. To ensure scoring consistency, repeated intraobserver calibration sessions were carried out on a randomly selected set of slides before a full analysis was initiated.

Statistical Analysis

Statistical analyses were performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics (mean and standard deviation) were calculated for all variables. The Shapiro–Wilk test demonstrated a non-normal distribution of cytogenetic and cytotoxic parameters; therefore, non-parametric tests were used throughout. Between-group comparisons of the four toothpaste groups at each time point (T0, T1, T2) were conducted using the Kruskal–Wallis test. Within-group comparisons across time points were performed using the Friedman test. Whenever the Friedman test indicated statistical significance, pairwise post-hoc analyses were carried out using the Wilcoxon signed-rank test with Bonferroni correction to control for Type I error. Superscript letters in Tables 3 and 4 denote statistically significant pairwise differences identified in these Bonferroni-adjusted post-hoc tests (adjusted $P < 0.05$). Comparisons of age among groups were assessed using the Kruskal–Wallis test, while gender distribution was analyzed using the Chi-square test. Statistical significance was set at $P < 0.05$.

gućilo preciznu vizualizaciju i pouzdanu procjenu staničnih obilježja. Uz anomalije u organizaciji genetskoga materijala (mikronukleusi, jezgreni pupovi, fenomen *broken egg*, nukleoplazmatski mostovi), analiza preparata obuhvaćala je i prepoznavanje te klasifikaciju različitih promjena nastalih zbog kromosomske nestabilnosti i oštećenja DNK koje upućuju na specifične oblike stanične smrti. Te su anomalije razvrstane prema utvrđenim kriterijima HUMNxl, a radi osiguravanja pouzdanosti rezultata analizirano je 2000 eksfoliranih bukalnih stanica po ispitaniku (26). U kontekstu parametara citotoksičnosti identificirane su i razvrstane stanične promjene poput kondenziranoga kromatina (apoptoza), karioreksije (stanična smrt obilježena fragmentacijom jezgre – apoptoza i nekroza), piknoze (kondenzacija jezgre – apoptoza) i kariolize (otapanje jezgre – apoptoza i nekroza) kao pokazatelji ranih i kasnih stadija apoptoze, odnosno citotoksičnih učinaka. Uz biljege stanične smrti, preparati su analizirani i na prisutnost mikronukleusa, nukleoplazmatskih mostova, jezgrenih pupova i fenomena *broken egg* (specifično oštećenje membrane) kao pokazatelja kromosomskih oštećenja i oštećenja DNK (25). Radi osiguravanja objektivnoga i nepristranoga bodovanja citogenetskih i citotoksičnih parametara, sva su stakalca prije evaluacije anonimizirana. Svakom ispitaniku i svakoj vremenskoj točki uzorkovanja (T0, T1, T2) dodijeljen je jedinstveni alfanumerički kod koji je generirao neovisni istraživač, a koji nije sudjelovao u analizi stakalaca. Sve citološke evaluacije proveo je iskusni ispitivač s poslijediplomskom edukacijom iz citomorfologije i prethodnom kalibracijom za bodovanje prema protokolu HUMNxl bukalnoga mikronukleusnoga citom-testa. Ispitivač je tijekom cijelog istraživanja bio potpuno zaslijepljen za raspodjelu po skupinama i za sve vremenske točke uzorkovanja. Kako bi se osigurala dosljednost bodovanja, prije početka cjelokupne analize provedene su ponovljene unutarispitivačke kalibracijske sesije na nasumično odabranom skupu preparata.

Statistička analiza

Statističke analize obavljene su u programu SPSS verzija 25.0 (SPSS Inc., Chicago, IL, SAD). Deskriptivna statistika (aritmetička sredina i standardna devijacija) izračunata je za sve varijable. Shapiro-Wilkov test pokazao je da citogenetski i citotoksični parametri ne slijede normalnu raspodjelu, zbog čega su u analizi korišteni neparametrijski testovi. Usporedbe između skupina (četiri skupine zubnih pasta) u pojedinim vremenskim točkama (T0, T1, T2) provedene su Kruskal-Wallisovim testom. Usporedbe unutar skupina, kad je riječ o različitim vremenskim točkama, obavljene su Friedmanovim testom. U slučajevima kada je Friedmanov test pokazao statistički značajnu razliku, primijenjene su parne post hoc analize Wilcoxonovim testom predznačenih rangova uz Bonferronijevu korekciju radi kontrole pogreške tipa I. Slova u tablicama 3. i 4. označavaju statistički značajne razlike između parova utvrđene u post hoc analizama uz Bonferronijevu korekciju ($P < 0,05$).

Usporedbe dobi među skupinama analizirane su Kruskal-Wallisovim testom, a raspodjela prema spolu hi-kvadrat testom. Statistička značajnost postavljena je na razinu $P < 0,05$.

Table 3 Overview of Genotoxic Parameters (number of cells with micronuclei, number of cells with nuclear buds, number of binucleated cells, nucleoplasmic bridges, number of cells with the "broken egg" phenomenon).
Tablica 3. Prikaz genotoksičnih parametara (broj stanica s mikronukleusima, broj stanica s jezgrenim pupovima, broj binuklearnih stanica, broj nukleoplazmatskih mostova, broj stanica s fenomenom *broken egg*)

Genotoxic parameters • Genotoksični parametri	Toothpastes used • Skupine zubnih pasta				*P* - value • *P* - vrijednost
	Control • Kontrola (n = 22)	AmF (n = 19)	NaMFP (n = 25)	NaF (n = 22)	
Micronucleus • Mikronukleusi					
T0	4.02 (1.45)	4.84 (3.13)	5.64 (2.38)^{A}	5.73 (2.55)	0.065
T1	4.06 (2.54)^{a,b,c}	6.53 (2.48)^{a}	7.44(2.43)^{A,b}	7.14 (3.11)^{c}	≤ 0.001
T2	3.95 (2.36)^{a,b,c}	6.79 (2.3)^{a}	6.28 (2.09)^{b}	6.14 (2.21)^{c}	≤ 0.001
P - value • *P* - vrijednost	0.923	0.104	0.007	0.074	
Nuclear buds • Jezgreni pupovi					
T0	5.32 (2.85)	5.37 (3.4)^{A,B}	5.47(2.52)^{A,B}	6.23 (2.74)^{A}	0.599
T1	4.27 (2.35)^{a,b,c}	8.05(4.03)^{A,a}	9.28(4.41)^{A,b}	10.05(4.18)^{A,c}	≤ 0.001
T2	4.32 (2.34)^{a,b,c}	7.79(4.49)^{B,a}	7.2 (4.15)^{B,b}	7.77 (3.41)^{c}	0.004
P - value • *P* - vrijednost	0.776	0.013	≤ 0.001	≤ 0.001	
Binucleated cells • Binuklearne stanice					
T0	7.91 (2.6)^{A,B}	7.16 (2.95)	8.76 (3.76)	9.27 (3.34)	0.323
T1	5.27 (2.45) ^{A,a,b}	6.79 (3.36)^{c}	9.2 6(3.74)^{a}	10.05 (3.8)^{b,c}	≤ 0.001
T2	5.59(4.44)^{B,a,b,c}	8.68 (3.6)^{a}	10.2 (4.46)^{b}	10.32 (4.9)^{c}	≤ 0.001
P - value • *P* - vrijednost	≤ 0.001	0.097	0.091	0.714	
Nucleoplasmic bridges • Nukleoplazmatski mostovi					
T0	0.27 (0.55)	0.84 (2.29)	0.16 (0.37)	0.23 (0.43)	0.603
T1	0.27 (0.46)	0.26 (0.56)	0.24 (0.52)	0.32 (0.57)	0.930
T2	0.36 (0.58)	0.26 (0.45)	0.28 (0.46)	0.32 (0.48)	0.960
P - value • *P* - vrijednost	0.819	0.558	0.535	0.850	
"Broken egg" • Fenomen *broken egg*					
T0	4.23 (2.18)	4.11(2.21)^{A,B}	5.44(2.62)^{A,B}	5.77 (2.14)	0.051
T1	4.86 (2.23)^{a}	6.05 (2.44)^{A}	8.2(3.89)^{A,a,b}	5.18 (4.01)^{A,b}	0.007
T2	4.41 (2.02)^{a,b,c}	7.89 (2.83)^{B,a}	9.04(5.17)^{B,b}	6.73 (3.99)^{A,c}	≤ 0.001
P - value • *P* - vrijednost	0.166	≤ 0.001	0.004	0.036	

The data are presented as mean and standard deviation values. Statistical analysis was performed using the Kruskal–Wallis test to compare differences between toothpaste groups at each time point. Within each toothpaste group, differences across T0, T1, and T2 were evaluated using the Friedman test. When the Friedman test was significant, post-hoc pairwise comparisons were performed using the Wilcoxon signed-rank test with Bonferroni correction. Uppercase superscript letters indicate statistically significant differences between time points within the same toothpaste group, while lowercase superscript letters indicate statistically significant differences between toothpaste types after Bonferroni correction (adjusted $P < 0.05$). Abbreviations: T0 – after 30 days of using fluoride-free toothpaste; T1 – 30 days after using the fluoride-containing toothpaste; T2 – 45 days after starting the use of the fluoride-containing toothpaste. • Podatci su prikazani kao srednja vrijednost i standardna devijacija. Statistička analiza provedena je Kruskal-Wallisovim testom kako bi se usporedila razlika između skupina zubnih pasta u svakoj vremenskoj točki. Unutar svake skupine zubne paste razlike između T0, T1 i T2 analizirane su Friedmanovim testom. Kada je Friedmanov test pokazao statistički značajnu razliku, provedene su post hoc parne usporedbe Wilcoxonovim testom predznačenih rangova uz Bonferronijevu korekciju. Velika slova u gornjem indeksu označavaju statistički značajne razlike između vremenskih točaka unutar iste skupine zubne paste, a mala slova u gornjem indeksu označavaju statistički značajne razlike između različitih zubnih pasta nakon Bonferronijeve korekcije (prilagođeni $P < 0,05$). Kratice: T0 – poslije 30 dana korištenja zubne paste bez fluorida; T1 – 30 dana poslije početka korištenja zubne paste s fluoridom; T2 – 45 dana poslije početka korištenja zubne paste s fluoridom.

Results

A total of 88 individuals were enrolled in the study, with a mean age of 36.03 ± 15.93 years. The key demographic variables for all four study groups are summarized in Table 2. No statistically significant differences were observed among the groups with respect to either age or sex distribution. The genotoxicity assessment across the four toothpaste groups—amine fluoride (AmF), sodium monofluorophosphate (NaMFP), sodium fluoride (NaF), and a fluoride-free control showed distinct temporal patterns in several cytogenetic markers, including micronuclei, nuclear buds, binucleated cells, nucleoplasmic bridges, and the "broken egg" phenomenon over the three sampling points (T0, T1, T2). As

Rezultati

Ukupno je u istraživanje bilo uključeno 88 ispitanika prosječne dobi 36,03 ± 15,93 godina. Ključne demografske značajke za sve četiri skupine prikazane su u tablici 2. Nisu ustanovljene statistički značajne razlike između skupina kad je riječ o dobi i raspodjeli prema spolu. Procjena genotoksičnosti između četiriju skupina zubnih pasta — aminfluorid (AmF), natrijev monofluorofosfat (NaMFP), natrijev fluorid (NaF) i kontrolna skupina bez fluorida – pokazala je različite vremenske obrasce u više citogenetskih parametara, uključujući mikronukleuse, jezgrene pupove, binuklearne stanice, nukleoplazmatske mostove i fenomen *broken egg* u svim trima vremenskim točkama (T0, T1, T2). Kako je prikazano u

Table 4 An overview of Cytotoxic Parameters (number of cells with karyolysis, number of cells with karyorrhexis, number of cells with pyknotic nucleus, number of cells with condensed chromatin).
Tablica 4. Prikaz citotoksičnih parametara (broj stanica s kariolizom, broj stanica s karioreksijom, broj stanica s piknozom, broj stanica s kondenziranim kromatinom)

Cytotoxic parameters • Citotoksični parametri	Toothpastes used • Skupine zubnih pasta				*P* - value • *P* - vrijednost
	Control • Kontrola (n = 22)	AmF (n = 19)	NaMFP (n = 25)	NaF (n = 22)	
Karyolysis • Karioliza					
T0	110.73 (27.26)	117.47 (22.77)	116.64 (24.12)	118.27 (22.93)	0.806
T1	130.68 (30.32)	126.21 (34.71)	130.56 (34.57)	128.5 (30.0)	0.939
T2	125.32 (31.34)	119.53 (33.81)	131.64 (34.17)	120.41 (23.73)	0.466
P - value • *P* - vrijednost	0.066	0.611	0.077	0.232	
Karyorrhexis • Karioreksija					
T0	15.23 (5.54)^{A}	19.74(5.19)^{A}	19.48 (7.53)^{A}	17.59 (7.75)^{A}	0.112
T1	16.73 (6.75)^{a,b,c}	22.95 (6.53)^{B,a}	24.48 (9.33)^{A,B,b}	24.27 (7.15)^{A,B,c}	0.002
T2	18.0 (4.31)^{A}	15.4(5.46)^{A,B}	18.4 (7.31)^{B}	17.41 (5.15)^{B}	0.470
P - value • *P* - vrijednost	0.016	≤ 0.001	≤ 0.001	≤ 0.001	
Pyknosis • Piknoza					
T0	11.95 (4.41)^{A}	10.68 (5.57)	14.4 (6.62)	14.09 (5.03)	0.144
T1	11.36(3.57) ^{a,b}	13.79(5.57)^{c}	18.44 (8.25)^{a,c}	16.95 (7.44)^{b}	0.001
T2	10.09 (4.36)^{A,a}	15.89 (10.44)	20.08 (10.26)^{a}	15.14 (8.95)	0.006
P - value • *P* - vrijednost	0.032	0.343	0.066	0.053	
Condensed chromatin • Kondenzirani kromatin					
T0	20.82 (3.83)	25.16 (7.89)	24.12 (5.68)^{A}	24.59 (8.65)^{A}	0.171
T1	18.09 (5.18) ^{a,b,c}	23.95 (8.46)^{a}	28.48 (10.06)^{B,b}	25.64 (6.94)^{B,c}	≤0.001
T2	19.5 (6.71)	21.89 (5.38)	19.16 (7.3)^{A,B}	18.73 (7.99)^{A,B}	0.447
P - value • *P* - vrijednost	0.644	0.670	≤ 0.001	0.004	

The data are presented as mean and standard deviation values. Statistical analysis was performed using the Kruskal–Wallis test to compare differences between toothpaste groups at each time point. Within each toothpaste group (comparisons across T0, T1, and T2), differences were evaluated using the Friedman test. Post-hoc pairwise comparisons following a significant Friedman test were performed using the Wilcoxon signed-rank test with Bonferroni correction. Uppercase superscript letters indicate statistically significant differences between evaluation periods within the same toothpaste group, while lowercase superscript letters indicate significant differences between toothpaste types based on Bonferroni-adjusted *P* values (adjusted *P* < 0.05). Abbreviations: T0 – after 30 days of using fluoride-free toothpaste; T1 – 30 days after using the fluoride-containing toothpaste; T2 – 45 days after starting the use of the fluoride- containing toothpaste. • Podatci su prikazani kao srednja vrijednost i standardna devijacija. Statistička analiza provedena je Kruskal-Wallisovim testom radi usporedbe razlika između skupina zubnih pasta u svakoj vremenskoj točki. Unutar svake skupine zubne paste razlike između T0, T1 i T2 analizirane su Friedmanovim testom. Kada je Friedmanov test pokazao statistički značajnu razliku, provedene su post hoc parne usporedbe Wilcoxonovim testom predznačenih rangova uz Bonferronijevu korekciju. Velika slova u gornjem indeksu označavaju statistički značajne razlike između vremenskih točaka unutar iste skupine zubne paste, a mala slova u gornjem indeksu označavaju statistički značajne razlike između različitih zubnih pasta nakon Bonferronijeve korekcije (prilagođeni *P* < 0,05). Kratice: T0 – poslije 30 dana korištenja zubne paste bez fluorida; T1 – 30 dana poslije početka korištenja zubne paste s fluoridom; T2 – 45 dana poslije početka korištenja zubne paste s fluoridom.

presented in Table 3, no significant differences in micronucleus frequency were observed at baseline (T0). However, by T1 and T2, all fluoride-containing toothpastes demonstrated significantly higher micronucleus counts than the control group ($P \leq 0.001$). Notably, NaMFP toothpaste showed a clear increase between T0 and T1 ($P = 0.007$). A similar pattern was observed for nuclear buds: all fluoride formulations displayed higher values than the control at T1 ($P \leq 0.001$) and T2 ($P = 0.004$). Significant within-group increases over time were observed for AmF toothpaste at both T1 and T2 compared to T0 ($P = 0.013$), and for NaMFP toothpaste, which displayed consistent rises from baseline ($P \leq 0.001$). NaF toothpaste demonstrated an increase from T0 to T1, with no further rise at T2 ($P \leq 0.001$). For binucleated cells,

tablici 3., na početku istraživanja (T0) nisu utvrđene značajne razlike u učestalosti mikronukleusa između skupina. No u vremenskim točkama T1 i T2 sve zubne paste koje su sadržavale fluorid pokazale su značajno veći broj mikronukleusa u usporedbi s kontrolnom skupinom ($P \leq 0,001$). Posebno se ističe zubna pasta s NaMFP-om, kod koje je zabilježen jasan porast između T0 i T1 ($P = 0,007$). Sličan model uočen je i za jezgrene pupove: sve fluoridne formulacije pokazale su više vrijednosti u odnosu prema kontroli u T1 ($P \leq 0,001$) i T2 ($P = 0,004$). Unutar skupina zabilježen je značajan porast tijekom vremena za AmF zubnu pastu u T1 i T2 u odnosu prema T0 ($P = 0,013$), te za NaMFP zubnu pastu koja je pokazala kontinuirani porast u odnosu prema početnim vrijednostima ($P \leq 0,001$). Zubna pasta s NaF-om poka-

NaMFP and NaF toothpastes exhibited significantly higher levels than the control at T1, with an additional difference observed between AmF and NaF ($P \leq 0.001$). By T2, all fluoride toothpastes maintained higher binucleated cell counts compared with the control ($P \leq 0.001$). The control group showed a progressive decline in binucleated cells over time, with significantly lower values at both T1 and T2 compared to T0 ($P \leq 0.001$). No statistically significant differences were detected in nucleoplasmic bridges across groups or time points, suggesting minimal sensitivity of this parameter to the tested formulations.

For the "broken egg" phenomenon, notable variations were observed. At T1, NaMFP toothpaste showed significantly higher counts compared with the control and NaF toothpaste ($P = 0.007$). By T2, all fluoride-containing toothpastes displayed markedly elevated counts relative to the control group ($P \leq 0.001$). Within-group analyses showed increases for AmF and NaMFP toothpastes at both T1 and T2 relative to baseline ($P \leq 0.001$ and $P = 0.004$, respectively), while NaF toothpaste demonstrated a rise between T1 and T2 ($P = 0.036$). Table 4 provides an overview of the cytotoxic parameters assessed across the study groups. For karyolysis, no statistically significant differences were observed between groups at any time point. All groups maintained comparable cell counts, with within-group analyses showing a trend of increased karyolysis from T0 to T1, although this did not reach statistical significance. In karyorrhexis, significant differences emerged between groups at T1. The fluoride toothpastes exhibited increased cell counts compared to the control ($P = 0.002$). Within-group comparisons showed that cell counts peaked at T1 across all fluoride groups. Notably, in the control group, T2 counts were significantly higher than T0 ($P = 0.016$), while AmF toothpaste showed a statistically lower count at T2 compared to both T0 and T1 ($P \leq 0.001$). NaMFP and NaF toothpastes similarly showed more karyorrhexis cells at T1 than at T0 and T2 ($P \leq 0.001$). The pyknotic nuclei parameter showed significant increases at T1 in NaMFP toothpaste compared to both the control and AmF toothpaste ($P \leq 0.001$). NaMFP also had a significantly higher count of pyknotic cells at T2 compared to the control ($P = 0.006$), although the increase within its own time points was not statistically significant. Additionally, NaF toothpaste displayed significantly more pyknotic cells than the control at T1 ($P \leq 0.001$). In the control group, T2 counts of pyknotic cells were significantly lower than T0 ($P = 0.032$). Lastly, condensed chromatin exhibited significant inter-group differences at T1, with all fluoride-containing toothpastes showing higher counts than the control ($P \leq 0.001$). Significant within-group reductions in condensed chromatin were noted over time for both NaMFP and NaF toothpastes, with notable decreases from T1 to T2 and from T0 to T2 ($P \leq 0.001$ and $P = 0.004$, respectively).

Discussion

This study investigated the cytotoxic and genotoxic effects of several fluoride-containing toothpastes on buccal mucosal cells, with the aim of determining whether cytozala je porast od T0 do T1, bez dodatnoga povećanja u T2 ($P \leq 0{,}001$). Za binuklearne stanice, zubne paste s NaMFP-om i NaF-om pokazale su značajno više vrijednosti u usporedbi s kontrolom u T1, uz dodatnu razliku između skupina AmF i NaF ($P \leq 0{,}001$). U T2 sve su fluoridne zubne paste zadržale više vrijednosti u odnosu prema kontrolnoj skupini ($P \leq 0{,}001$). U kontrolnoj skupini zabilježen je progresivan pad broja binuklearnih stanica tijekom vremena, uz značajno niže vrijednosti u T1 i T2 u odnosu prema T0 ($P \leq 0{,}001$). Za nukleoplazmatske mostove nisu utvrđene statistički značajne razlike između skupina, ni između vremenskih točaka, što upućuje na nisku osjetljivost toga parametra na ispitivane formulacije.

Za fenomen *broken egg* uočene su značajne razlike. U T1 pokazala je zubna pasta s NaMFP-om značajno veće vrijednosti u odnosu prema kontrolnoj skupini i NaF skupini ($P = 0{,}007$). U T2 su sve zubne paste koje su sadržavale fluorid pokazale značajno više vrijednosti u odnosu prema kontroli ($P \leq 0{,}001$). Unutar skupina zabilježen je porast za AmF i NaMFP zubne paste u T1 i T2 u odnosu prema početnim vrijednostima ($P \leq 0{,}001$ i $P = 0{,}004$), a zubna pasta s NaF-om pokazala je porast između T1 i T2 ($P = 0{,}036$). Tablica 4. prikazuje citotoksične parametre u svim skupinama. Za kariolizu nisu utvrđene statistički značajne razlike između skupina ni u jednoj vremenskoj točki. Sve skupine pokazivale su slične vrijednosti, uz trend porasta od T0 do T1 unutar skupina koji nije dosegnuo statističku značajnost. Kod karioreksije uočene su u T1 značajne razlike između skupina. Fluoridne zubne paste pokazale su više vrijednosti u odnosu prema kontrolnoj skupini ($P = 0{,}002$). Unutar skupina vrijednosti su dosegnule vrhunac u T1 za sve fluoridne skupine. U kontrolnoj skupini vrijednosti u T2 bile su značajno više nego u T0 ($P = 0{,}016$), a kod AmF zubne paste zabilježeno je značajno smanjenje u T2 u odnosu prema T0 i T1 ($P \leq 0{,}001$). Zubne paste s NaMFP-om i NaF-om također su pokazale veći broj stanica s karioreksijom u T1 u odnosu prema T0 i T2 ($P \leq 0{,}001$). Broj stanica s piknozom pokazao je značajan porast u T1 u skupini s NaMFP-om u odnosu prema kontroli i AmF skupini ($P \leq 0{,}001$). U T2 je zubna pasta s NaMFP-om također imala veće vrijednosti u odnosu prema kontroli ($P = 0{,}006$), iako unutargrupne razlike nisu bile statistički značajne. Zubna pasta s NaF-om također je pokazala veći broj piknotičnih stanica u odnosu prema kontroli u T1 ($P \leq 0{,}001$). U kontrolnoj skupini broj piknotičnih stanica bio je značajno niži u T2 u odnosu prema T0 ($P = 0{,}032$). Konačno, broj stanica s kondenziranim kromatinom pokazao je značajne razlike između skupina u T1, pri čemu su sve fluoridne zubne paste imale više vrijednosti u odnosu prema kontroli ($P \leq 0{,}001$). Unutar skupina zabilježeno je značajno smanjenje tijekom vremena za NaMFP i NaF zubne paste, s izraženim padom od T1 do T2 te od T0 do T2 ($P \leq 0{,}001$ i $P = 0{,}004$).

Rasprava

U ovom istraživanju ispitivani su citotoksični i genotoksični učinci zubnih pasta koje sadržavaju fluorid na stanice bukalne sluznice, sa svrhom utvrđivanja razlikuju li se cito-

genetic alterations differ according to fluoride compound, the presence of fluoride, and exposure duration. Fluoride-containing formulations showed distinct patterns compared with the non-fluoride control, and differences were also observed among individual fluoride compounds. Some markers decreased while others increased over time, indicating that cytotoxic and genotoxic responses fluctuate with continued exposure. Overall, these findings suggest that fluoride-containing toothpastes may induce modest but compound-specific variations in cytogenetic markers, thus supporting the possibility of a more complex cellular response to prolonged fluoride exposure (27). All fluoride-containing toothpastes showed higher frequencies of micronuclei and nuclear buds compared with the non-fluoride control, indicating a modest genotoxic response that varied among compounds. These findings are broadly consistent with previous research demonstrating that fluoride exposure can induce DNA damage and alter cell-cycle dynamics in oral epithelial cells (28, 29). The differing patterns observed among AmF, NaMFP, and NaF likely reflect their distinct fluoride-release mechanisms. NaMFP undergoes enzymatic hydrolysis, resulting in gradual ion release and prolonged low-level exposure (30), whereas AmF binds to salivary proteins and mucins, creating a sustained fluoride reservoir on oral surfaces (31). In contrast, NaF dissociates rapidly, producing higher immediate ion availability and potentially more acute cellular stress, consistent with reports of NaF-associated mitochondrial and metabolic disruption (32). Similar time-dependent cytotoxic effects have been observed in studies evaluating commercial toothpaste formulations (Ghapanchi et al.) (33), suggesting that prolonged or repeated fluoride exposure, together with formulation specific factors, may influence cytogenetic outcomes (34, 35). Fluctuations in binucleated cell counts and other genotoxic markers showed broadly similar trends across the fluoride-containing toothpastes, although the timing and magnitude of these responses differed among compounds. These compound-specific patterns likely reflect differences in fluoride release kinetics, as slower release formulations may produce more sustained but less acute cellular effects. In contrast, the non-fluoride control exhibited relatively stable genotoxic parameters, supporting the notion that the observed variations are attributable to fluoride exposure rather than natural temporal changes.

Nucleoplasmic bridges showed minimal variation across groups, suggesting that this endpoint may be less sensitive to the types or concentrations of fluoride tested. More distinctive patterns were observed in the "broken egg" phenotype, where fluoride-containing toothpastes consistently produced higher counts than the control. This aligns with evidence that fluoride retention in the oral cavity can prolong epithelial exposure and contribute cumulatively to nuclear abnormalities (36, 37). The stronger early response observed with NaMFP is consistent with its enzymatic hydrolysis and gradual ion release, whereas similar findings in NaF varnish studies (Escobar-García et al.) (38) support the broader concept that prolonged fluoride contact, regardless of formulation, may influence chromatin and nuclear morphology.

genetske promjene ovisno o vrsti fluoridnog spoja, prisutnosti fluorida i trajanju izloženosti. Formulacije koje su sadržavale fluorid pokazale su različite modele u odnosu prema kontrolnoj skupini bez fluorida, a razlike su uočene i među pojedinim fluoridnim spojevima. Neki su parametri tijekom vremena pokazivali porast, drugi su, pak, opadali, što upućuje na to da citotoksični i genotoksični odgovori variraju s produljenom izloženošću. Ukupno gledano, ti rezultati sugeriraju da zubne paste koje sadržavaju fluorid mogu prouzročiti umjerene promjene, ali ovisno o spoju u citogenetskim biljezima, što podupire pretpostavku o složenijem staničnom odgovoru na dugotrajnu izloženost fluoridu (27). Sve zubne paste koje su sadržavale fluorid pokazale su veću učestalost mikronukleusa i jezgrenih pupova u odnosu prema kontrolnoj skupini bez fluorida, što upućuje na umjeren genotoksični učinak koji se razlikovao među pojedinim spojevima. Ti su rezultati u skladu s dosadašnjim istraživanjima koja pokazuju da izloženost fluoridu može prouzročiti oštećenje DNK i promjene u dinamici staničnoga ciklusa u epitelnim stanicama usne šupljine (28, 29). Različiti obrasci opaženi između AmF-a, NaMFP-a i NAF-a vjerojatno odražavaju njihove različite mehanizme oslobađanja fluorida. NaMFP podliježe enzimskoj hidrolizi, što rezultira postupnim oslobađanjem iona i produljenom izloženošću niskim koncentracijama (30), a AmF se veže na salivarne proteine i mucine te stvara spremnik fluorida na oralnim površinama (31). Suprotno tomu, NaF brzo disocira, osiguravajući visoku trenutačnu dostupnost iona i potencijalno izraženiji akutni stanični stres, što je u skladu s izvješćima o poremećajima mitohondrijske funkcije i metabolizma povezanima s NaF-om (32). Slični vremenski ovisni citotoksični učinci opisani su i u istraživanjima u kojima su se ispitivale komercijalne formulacije zubnih pasta (Ghapanchi i sur.) (33), što upućuje na to da produljena ili ponavljana izloženost fluoridu, zajedno sa specifičnim značajkama formulacije, može utjecati na citogenetske ishode (34, 35). Promjene u broju binuklearnih stanica i drugim genotoksičnim parametrima pokazale su općenito slične trendove među zubnim pastama koje sadržavaju fluorid, iako su se vrijeme pojave i intenzitet tih odgovora razlikovali među spojevima. Ti obrasci, specifični za pojedini spoj, vjerojatno odražavaju razlike u kinetici oslobađanja fluorida, pri čemu sporije oslobađajuće formulacije mogu potaknuti produljene, ali manje izražene akutne učinke na stanice. Nasuprot tomu, kontrolna skupina bez fluorida pokazala je razmjerno stabilne genotoksične parametre, što podupire pretpostavku da su uočene promjene posljedica izloženosti fluoridu, a ne prirodnih vremenskih varijacija.

Nukleoplazmatski mostovi pokazali su minimalne varijacije između skupina, što upućuje na to da je taj parametar manje osjetljiv na vrstu ili koncentraciju ispitanih fluoridnih spojeva. Izraženiji obrasci uočeni su kod fenomena *broken egg*, pri čemu su zubne paste koje su sadržavale fluorid dosljedno pokazivale više vrijednosti u odnosu prema kontroli. To je u skladu s dokazima da zadržavanje fluorida u usnoj šupljini može produljiti izloženost epitela i kumulativno pridonijeti nastanku nuklearnih abnormalnosti (36, 37). Izraženiji rani odgovor uočen u skupini s NaMFP-om u skladu je s

However, the literature on this endpoint remains mixed. Ribeiro et al. reported no genotoxic effects at low NaF concentrations in rat oral mucosa, suggesting that dose, assay type, and exposure duration strongly influence outcomes (39). Their study used the comet assay, which detects strand breaks rather than chromosomal level anomalies; thus, the micronucleus cytome assay applied in our study captures complementary forms of genomic instability not detectable by comet analysis. The distinct temporal pattern observed with NaF, characterized by a more progressive increase, may reflect its rapid ion availability, which can impose acute oxidative or metabolic stress before repair mechanisms fully compensate. This interpretation is supported by evidence that NaF can impair mitochondrial function and cellular energy balance (40), raising the possibility of similar effects in buccal epithelial cells under prolonged or repeated exposure. Cytotoxicity-related markers provided additional insight into the cellular response to fluoride exposure. Karyolysis showed minimal variation across groups, suggesting that gross membrane disruption and advanced nuclear degeneration were not strongly affected by the fluoride formulations tested. This finding is consistent with the results of Tadin et al., who likewise reported no fluoride-dependent changes in karyolysis in buccal cells (22). In contrast, karyorrhexis exhibited more pronounced, though transient, changes. Fluoride-containing toothpastes generally showed an early increase in nuclear fragmentation, followed by a decline, a pattern compatible with an initial oxidative or metabolic stress response followed by partial cellular adaptation or repair. AmF tended to show a milder profile, which may relate to its protein binding properties and sustained but lower intensity fluoride release (31). NaMFP and NaF demonstrated patterns consistent with studies showing that fluoride induced oxidative stress can elevate cytotoxic markers before cellular homeostasis is restored (41). The potential contribution of formulation excipients should also be considered: Tadin et al. reported that sodium lauryl sulfate (SLS) can amplify fluoride related nuclear abnormalities by increasing membrane permeability (22). The findings of Vladislavić-Zorica et al. further support the notion of time-dependent fluctuations, as increases in karyorrhexis and condensed chromatin at 30–60 days were followed by a reduction at 90 days when using NaMFP and NaF-based toothpastes (42). Differences between their outcomes and ours likely reflect formulation variability, particularly the presence of whitening agents, which may impose additional oxidative burden and alter the cytotoxic trajectory compared with standard fluoride formulations. Pyknosis and condensed chromatin provided additional insight into the cytotoxic trajectory associated with prolonged exposure to different fluoride formulations. Fluoride-containing toothpastes generally produced early increases in pyknotic nuclei, which is consistent with an acute cellular stress response. NaMFP showed the most pronounced early effect, likely reflecting its enzymatic hydrolysis and gradual ion release pattern, while NaF's rapid ion availability aligns with reports of short-term oxidative stress induction. These observations are supported by Tadin et al., who found that formulations containing sodium lauryl sulfate (SLS) amplified pyknosis,

njegovom enzimskom hidrolizom i postupnim otpuštanjem iona, dok slični nalazi u istraživanjima fluoridnih lakova s NaF-om (Escobar-García i sur.) (38) podupiru širi koncept da produljeni kontakt s fluoridom, neovisno o formulaciji, može utjecati na kromatin i morfologiju jezgre.

Međutim, literatura vezana uz taj parametar i dalje je neujednačena. Ribeiro i suradnici nisu utvrdili genotoksične učinke pri niskim koncentracijama NaF-a u oralnoj sluznici štakora, što upućuje na to da doza, vrsta testa i trajanje izloženosti snažno utječu na ishode (39). U njihovu istraživanju korišten je comet-test koji detektira lomove DNK lanaca, ali ne i promjene na razini kromosoma; stoga mikronukleusni citom-test primijenjen u ovom istraživanju omogućuje detekciju komplementarnih oblika genomske nestabilnosti koji nisu vidljivi comet-analizom. Specifičan vremenski obrazac opažen kod NaF-a, obilježen postupnijim porastom, može odražavati njegovu brzu dostupnost iona koja može prouzročiti akutni oksidacijski ili metabolički stres prije potpune aktivacije mehanizama popravka. To tumačenje podupiru dokazi da NaF može narušiti mitohondrijsku funkciju i staničnu energijsku ravnotežu (40), što upućuje na mogućnost sličnih učinaka u bukalnim epitelnim stanicama tijekom produljene ili ponavljane izloženosti. Parametri povezani s citotoksičnošću omogućili su dodatni uvid u stanični odgovor na izloženost fluoridu. Karioliza je pokazala minimalne razlike između skupina, što sugerira da izražena oštećenja membrane i uznapredovala nuklearna degeneracija nisu značajno pogođeni ispitivanim formulacijama. Taj nalaz u skladu je s rezultatima Tadin i suradnika koji također nisu utvrdili promjene kariolize povezane s izloženošću fluoridu u bukalnim stanicama (22). Suprotno tomu, karioreksija je pokazala izraženije, iako prolazne promjene. Zubne paste koje su sadržavale fluorid općenito su pokazale rani porast fragmentacije jezgre, nakon čega je slijedio pad, što odgovara modelu početnoga oksidacijskoga ili metaboličkoga stresa praćenoga djelomičnom staničnom adaptacijom ili aktivacijom mehanizama popravka. AmF je pokazivao blaži model, što se može povezati s njegovim svojstvom vezanja na proteine i produljenim, ali manje intenzivnim otpuštanjem fluorida (31). NaMFP i NaF pokazali su obrasce u skladu s istraživanjima koja upućuju na to da fluoridom inducirani oksidacijski stres može privremeno povećati citotoksične parametre prije uspostave stanične homeostaze (41). Potencijalni doprinos pomoćnih tvari također treba uzeti u obzir. Tadin i suradnici pokazali su da natrijev lauril-sulfat (SLS) može pojačati fluoridom povezane nuklearne abnormalnosti povećanjem propusnosti stanične membrane (22). Rezultati Vladislavić-Zorice i suradnika dodatno podupiru koncept vremenski ovisnih promjena, pri čemu su porasti karioreksije i kondenziranog kromatina u razdoblju od 30 do 60 dana bili praćeni smanjenjem poslije 90 dana primjene zubnih pasta s NaMFP-om i NaF-om (42). Razlike između njihovih rezultata i nalaza u ovom istraživanju vjerojatno odražavaju razlike u formulacijama, osobito prisutnost izbjeljujućih sastojaka koji mogu dodatno povećati oksidacijski stres i promijeniti tijek citotoksičnih promjena u odnosu prema standardnim fluoridnim formulacijama. Piknoza i kondenzirani kromatin pružili su dodatni uvid u citotoksični tijek povezan s pro-

indicating that surfactants may potentiate fluoride-related membrane stress (22). Additional evidence from Tabatabaei et al. underscores the importance of concentration and exposure time, as NaF demonstrated a clear dose and time-dependent cytotoxic effect in gingival fibroblasts (41), although such responses may differ between gingival and buccal epithelial cells. Condensed chromatin followed a similar pattern, with fluoride formulations inducing a transient early increase suggestive of chromatin condensation during cellular stress, followed by a decline that may reflect partial adaptation or activation of repair mechanisms. The findings of Tadin et al. support the role of SLS in enhancing chromatin condensation (22), whereas Puizina et al. reported decreasing condensed chromatin levels over time (23), partially aligning with the later decline observed in our study. The differences between studies likely reflect variations in toothpaste composition, particularly the presence of whitening agents or surfactants, as well as differences in population characteristics and exposure conditions. This study provides valuable insights into the cellular effects of fluoride-containing toothpaste, but it also has some limitations. The observation period of the study is short (30 and 45 days), therefore it may not capture the full extent of cytotoxic and genotoxic effects associated with prolonged toothpaste use. It is challenging to control all variables within the oral cavity environment, where complex interactions between fluoride, saliva, and the oral microbiome can significantly influence cytotoxic and genotoxic effects. Additionally, the study relied solely on the micronucleus test for genotoxicity evaluation, which, while effective, does not detect all types of DNA damage, such as oxidative DNA lesions. Further research is required. It should extend observation periods to cover longer durations, potentially several months or years, to capture cumulative cellular effects of fluoride exposure. Incorporating additional genotoxicity assays, such as the comet assay, would offer a more thorough analysis by identifying single-strand and double-strand DNA breaks (43). A crossover study design could allow participants to act as their own controls, helping to control for individual oral hygiene habits. Although participants were instructed not to use additional fluoride sources (e.g., mouthrinses), residual variability in dietary fluoride intake or drinking water fluoride content cannot be completely excluded. Moreover, assessing saliva pH and oral microbial composition would provide valuable context for understanding how these environmental factors influence fluoride's cytotoxic and genotoxic effects, ultimately giving a more nuanced view of fluoride's impact in the oral cavity.

duljenom izloženošću različitim fluoridnim formulacijama. Zubne paste koje su sadržavale fluorid općenito su uzrokovale rani porast stanica s piknozom, što odgovara akutnom staničnom stresnom odgovoru. U skupini s NaMFP-om zabilježen je najizraženiji rani učinak, vjerojatno zbog enzimske hidrolize i postupnog otpuštanja iona, a brza dostupnost iona kod NaF-a odgovara nalazima o kratkotrajnoj indukciji oksidacijskoga stresa. Te nalaze podupiru Tadin i suradnici koji su pokazali da formulacije koje sadržavaju natrijev lauril-sulfat (SLS) pojačavaju piknozu, što upućuje na to da surfaktanti mogu potencirati fluoridom prouzročeni membranski stres (22). Dodatni dokazi iz istraživanja Tabatabaeija i suradnika ističu važnost koncentracije i trajanja izloženosti, pri čemu je NaF pokazao jasan citotoksični učinak ovisan o dozi i vremenu u gingivnim fibroblastima (41), iako se takvi odgovori mogu razlikovati između gingivnih i bukalnih epitelnih stanica.

Kondenzirani kromatin pokazao je sličan model, s početnim porastom u ranoj fazi koji upućuje na kondenzaciju kromatina tijekom staničnoga stresa, nakon čega slijedi smanjenje koje može odražavati djelomičnu adaptaciju ili aktivaciju mehanizama popravka. Nalazi Tadin i suradnika podupiru ulogu SLS-a u pojačavanju kondenzacije kromatina (22), a Puizina i suradnici navode smanjenje kondenziranog kromatina tijekom vremena (23), što je djelomično u skladu s kasnijim padom opaženim u ovom istraživanju. Razlike između studija vjerojatno proizlaze zbog varijacija u sastavu zubnih pasta, osobito prisutnosti izbjeljujućih sredstava ili surfaktanata, te razlika u populaciji i uvjetima izloženosti. Ovo istraživanje pruža vrijedan uvid u stanične učinke zubnih pasta koje sadržavaju fluorid, ali ima i određena ograničenja. Razmjerno kratko razdoblje praćenja (30 i 45 dana) možda ne odražava u cijelosti citotoksične i genotoksične učinke povezane s dugotrajnom uporabom zubnih pasta. Također je teško kontrolirati sve varijable u oralnom okolišu u kojem složene interakcije između fluorida, sline i oralnoga mikrobioma mogu značajno utjecati na citotoksične i genotoksične učinke. Istraživanje se također oslanjalo isključivo na mikronukleusni test koji, iako pouzdan, ne detektira sve oblike oštećenja DNK, poput oksidacijskih lezija. Buduća istraživanja trebala bi uključivati dulje razdoblje praćenja, potencijalno tijekom više mjeseci ili godina, kako bi se obuhvatili kumulativni učinci izloženosti fluoridu. Uključivanje dodatnih metoda procjene genotoksičnosti, poput comet-testa, omogućilo bi sveobuhvatniju analizu detekcijom jednostrukih i dvostrukih lomova DNK (43). Ukrižen (*crossover*) oblik istraživanja omogućio bi da ispitanici budu vlastite kontrole, čime bi se smanjio utjecaj individualnih navika oralne higijene. Iako su ispitanici bili upućeni da se ne koriste dodatnim izvorima fluorida (npr., tekućinama za ispiranje usta), preostala varijabilnost u prehrambenom unosu fluorida ili koncentraciji fluorida u vodi za piće ne može se potpuno isključiti. Također, procjena pH vrijednosti sline i sastava oralnoga mikrobioma pružila bi dodatni kontekst za razumijevanje utjecaja okolišnih čimbenika na citotoksične i genotoksične učinke fluorida, čime bi se omogućio precizniji uvid u njegov učinak u usnoj šupljini.

Conclusion

This study examined potential cytotoxic and genotoxic effects of fluoride-containing toothpastes on buccal mucosal cells, assessing the influence of fluoride compound type, fluoride presence, and exposure duration. Statistically significant differences were observed between fluoridated and non-fluoridated toothpastes, as well as among different fluoride compounds, suggesting that fluoride exposure may be associated with cytogenetic changes in oral mucosal cells. Observed variations among different fluoride compounds may reflect differences in their release profiles, potentially influencing cellular responses over time. While some markers suggested possible cellular adaptation or stabilization, others raised questions regarding cumulative effects that warrant further investigation. Given the methodological limitations of the buccal micronucleus cytome assay, these findings should be interpreted with caution. The study underscores the complex interactions between fluoride and oral epithelial tissues and highlights the need for further research using complementary genotoxicity assays to better characterize early and progressive cellular effects. Complementing these investigations, longitudinal studies are needed to better understand the long-term biological implications of fluoride use in oral hygiene products, particularly in the context of prolonged exposure and varying fluoride release mechanisms.

Conflict of interest: The authors declare no conflicts of interest.

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Zaključak

U ovom istraživanju ispitivani su mogući citotoksični i genotoksični učinci zubnih pasta koje sadržavaju fluorid na stanice bukalne sluznice, uz procjenu utjecaja vrste fluoridnoga spoja, prisutnosti fluorida i trajanja izloženosti. Utvrđene su statistički značajne razlike između fluoridnih i nefluoridnih zubnih pasta te među različitim fluoridnim spojevima, što upućuje na to da izloženost fluoridu može biti povezana s citogenetskim promjenama u stanicama oralne sluznice. Uočene razlike među pojedinim fluoridnim spojevima mogu odražavati razlike u njihovoj kinetici oslobađanja, što može utjecati na stanične odgovore tijekom vremena. Iako su neki parametri upućivali na moguću staničnu adaptaciju ili stabilizaciju, drugi su otvorili pitanje mogućih kumulativnih učinaka koji zahtijevaju dodatna istraživanja. S obzirom na metodološka ograničenja bukalnoga mikronukleusnog citom-testa, ove rezultate treba tumačiti s oprezom. U ovom istraživanju dodatno se ističu složene interakcije između fluorida i oralnoga epitela te potreba za daljnjim istraživanjima uz primjenu komplementarnih metoda procjene genotoksičnosti radi preciznijeg određivanja ranih i progresivnih staničnih učinaka. Uz to, potrebna su longitudinalna istraživanja da bi se bolje razumjele dugoročne biološke implikacije primjene fluorida u proizvodima za oralnu higijenu, osobito u kontekstu produljene izloženosti i različitih mehanizama oslobađanja fluorida.

Izjava o sukobu interesa: Autori nisu bili u sukobu interesa.

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Doprinos autora: **A. T.** – konceptualizacija; **A. T., D. Ž., I. Š. i K. P.** – metodologija; **A. T. i J. V** – softver; **A. T., J. V. i D. Ž** – validacija; **J. V. i A. T.** – formalna analiza; **A. T. i J. V.** – istraživanje; **A. T., D. Ž., I. Š., K. B. i K. P.** – resursi; **A. T. i J. V** – obrada podataka; **J. V. i N. Z. V.** – pisanje – izvorni nacrt; **A. T., I. Š., K. B. i K. P.** – pregled i uređivanje teksta; **J. V. i N. Z. V.** – vizualizacija; **A. T., D. Ž., I. Š. i K. P.** supervizija; **A. T., J. V. i K. P.** – administracija projekta; **A. T., D. Ž. i K. P.** – osiguravanje financiranja. Svi autori sudjelovali su u pisanju i reviziji konačne verzije teksta. **K. P.** i **J. V.** dopisni su autori. **A. T.** i **J. V.** jednako su pridonijeli ovom radu i dijele prvo autorstvo.

Sažetak

Cilj rada: U ovom istraživanju ispitivan je utjecaj zubnih pasta koje sadržavaju različite fluoridne spojeve na citotoksične i genotoksične promjene u stanicama bukalne sluznice, s posebnim naglaskom na vrstu fluorida, prisutnost fluorida te trajanje izloženosti. **Materijali i metode:** U istraživanje je bilo uključeno 88 ispitanika koji su randomizirano raspoređeni u četiri paralelne skupine: kontrolnu skupinu koja se koristila zubnom pastom bez fluorida te tri intervencijske koje su upotrebljavale formulacije s natrijevim fluoridom, natrijevim monofluorofosfatom ili aminfluoridom. Uzorci stanica bukalne sluznice prikupljeni su na početku istraživanja (T0), poslije 30 dana (T1) i poslije 45 dana (T2) te su analizirani primjenom bukalnoga mikronukleusnog citom-testa radi kvantifikacije nuklearnih abnormalnosti i citotoksičnih markera. **Rezultati:** Sve zubne paste koje su sadržavale fluorid potaknule su veću učestalost mikronukleusa, jezgrenih pupova i stanica s tzv. fenomenom *broken egg* u vremenskim točkama T1 i T2 u usporedbi s kontrolnom skupinom ($P \leq 0{,}001$). Aminfluorid i natrijev monofluorofosfat prouzročili su trajno povećanje citogenetskih markera, uključujući jezgrene pupove (AmF: $P = 0{,}013$; NaMFP: $P \leq 0{,}001$) i stanice s fenomenom *broken egg* (AmF: $P \leq 0{,}001$; NaMFP: $P = 0{,}004$), a natrijev fluorid pokazao je sporiji, progresivni porast stanica s tim fenomenom ($P = 0{,}036$). **Zaključak:** Rezultati upućuju na to da zubne paste na bazi fluorida mogu utjecati na citogenetske odgovore u epitelnim stanicama bukalne sluznice te da se ti učinci razlikuju ovisno o vrsti fluoridnoga spoja i trajanju izloženosti. Zbog ograničenja istraživanja, rezultate treba tumačiti s oprezom, te su potrebna daljnja istraživanja kako bi se razjasnile dugoročne biološke posljedice ponavljane izloženosti fluoridu u oralnoj higijeni.

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