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RESEARCH

Cytotoxicity and genotoxicity of whitening toothpastes in buccal mucosal cells: a randomized controlled trial

Nada Zorica Vladislavic[1] · Jasen Vladislavic[2] · Ivana Franic[3] · Antonija Tadin[4,5]

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Abstract

Objectives To assess genotoxic and cytotoxic effect of commercially available toothpastes with the different whitening ingredients.

Materials and methods In vivo assessment of cytotoxicity and genotoxicity of whitening toothpastes with different ingredients using a buccal micronucleus cytome assay (BMCyt assay) comprised 199 participants randomly divided into ten groups based on used whitening or control/conventional toothpaste. The exfoliated buccal mucosal cells were collected, stained, and microscopically evaluated at baseline (T0), 30 days (T1), and 60 days (T2) after the beginning of treatment and 30 days after completing treatment (T3). Statistical evaluation was performed by repeated-measures analysis of variance (two-way ANOVA), Tukey's test, and multiple regression analysis.

Results The genotoxic parameters showed no biologically significant changes in any of the observed period for the tested toothpastes, while cytotoxic parameters (number of cells with karyorrhexis and condensed chromatin) showed statistically significant difference ($P < 0.05$) among evaluation periods for the three peroxide-containing toothpastes.

Conclusions Peroxide-containing whitening toothpastes exhibit an increase in certain cytotoxic parameters only during the application period, which return to control values after the cessation of application.

Clinical significance Whitening toothpastes show no genotoxic effect, while peroxide-containing whitening toothpastes may present significant increase of cytotoxicity (measured by the number of karyorrhexis and condensed chromatin) during the application period. However, these changes observed in clinical conditions cannot be considered significant.

Trial registration ClinicalTrials.gov: NCT04460755.

Keywords Micronucleus test · Toothpastes · Buccal mucosa · DNA damage · Peroxides

Introduction

In the last decade, there has been an increasing trend in the use of oral hygiene products that contain bleaching substances. Over-the-counter (OTC) products present secondary products that achieve a whiter effect on the teeth. Unlike other treatments in esthetic dentistry, they are widely available at an affordable price. Application of whitening dentifrices, rinses, whitening dental floss and toothbrushes, chewing gum, paint-on gels, OTC tray with gel activated by light, and whitening strips containing substances may have potential harmful results, especially for the young patients [1]. Whitening toothpastes are often used in the oral hygiene with the aim of removing and controlling the surface staining of the teeth. Considering that during the act of brushing, the teeth are in direct contact with the oral mucosa, it is important to know the potentially negative effect of chemical agents in these toothpastes and determine their effect on the mucosa through cyto- and genotoxicity parameters. In general, oral hygiene products, including toothpastes and mouthwashes, are classified as cosmetic products and as

Nada Zorica Vladislavic, Jasen Vladislavic, Ivana Franic and Antonija Tadin contributed equally to the work.

Highlights
- All tested whitening toothpastes showed no genotoxic effect.
- Peroxide-containing toothpastes revealed increased cytotoxicity (number of cells with karyorrhexis and condensed chromatin) for the application period only.
- In clinical conditions, the obtained changes cannot be considered significant.
- Generally, whitening toothpastes containing abrasives, peroxides, and/or enzymes and charcoal are safe for usage.

Extended author information available on the last page of the article

such do not have to undergo the same rigorous risk assessment tests as medicines do [2].

In previous research, the toxicity of various everyday oral hygiene products and dental materials was evaluated in in vivo and in vitro conditions [2–10]. These researches emphasized that some components in toothpastes can be potentially toxic—sodium lauryl sulfate, sodium tripolyphosphate, hydrated silica, aluminum oxide, hydroxides, sodium monofluorophosphate, silicon dioxide, sodium benzoate, preservatives, colors, flavors, and buffering agents. The research by Barbier et al. [11] highlights that the reason for cytotoxicity of toothpastes might be due to fluoride which can induce oxidative stress and decrease intracellular homeostasis and lipid peroxidation, altering gene expression and consequently apoptosis. Although there is no direct correlation between the occurrence of oral cavity cancers and any of the active ingredients in toothpastes, these ingredients cause many controversies as the research results show conflicting conclusions. Some in vitro and in vivo studies showed that commercial tooth whitening agents show genotoxicity [3, 12–15]; some did not show signs of genotoxicity in in vivo and in vitro conditions [10, 16], while others claim the limited, biologically insignificant genotoxic effect [4]. A detailed knowledge of cytotoxic and genotoxic effect of whitening agents in commercially available whitening toothpastes can contribute to selection and recommendation of whitening toothpastes available on the market.

The cells of the buccal mucosa represent the first barrier during the ingestion and inhalation of carcinogens that enter the body through the oral and nasal routes. Therefore, the cells of the oral epithelium are considered a desirable target site for early genotoxic detection of changes caused by carcinogenic agents. It is considered that 90% of human cancers originate in epithelial cells; therefore, the buccal mucosa could be used to monitor early genotoxic events [17]. Although not routinely performed, oral cytopathology can detect early genetic damage and it can be recommended as a screening model for early damage detection in alcohol and tobacco users who do not have visible oral lesions yet [18]. In addition, the buccal mucosa is an easily accessible tissue that can be used for cell sampling in a minimally invasive way. It was used in research to assess the rate of division of proliferating basal cells, their genetic stability, and the tendency to death [19]. It should be noted that the buccal mucosa is well supplied with vascular and lymphatic drainage, so permeability of the buccal mucosa is 4–4000 times higher than the permeability of the skin [20]. Hence, the elimination of potentially harmful substances occurs faster.

Today, the dental market offers a wide selection of whitening toothpastes with different ingredients. Therefore, it is important to analyze and understand their toxicity and potential harm to humans. This analysis should be conducted not only using laboratory methods in vitro but also clinically, by applying objective methods. Consequently, the purpose of this study was to evaluate the cytotoxic and genotoxic effects of toothpastes containing various combinations of whitening ingredients commonly found in commercially available whitening toothpastes. The null hypothesis of the study stated that the use of commercially available whitening toothpastes would not result in an increase in cytogenetic damage in exfoliated buccal cells.

Materials and methods

Study design, materials, and participants

This prospective, parallel randomized controlled clinical trial evaluated cytotoxic and genotoxic effects of nine different whitening toothpastes and one conventional non-whitening toothpaste, for 4-month monitoring (first month, usage of control non-whitening toothpaste; second and third month, usage of whitening toothpastes; fourth month, usage of control non-whitening toothpaste). The study was conducted at the Department of Restorative Dental Medicine and Endodontics, Study of Dental Medicine, School of Medicine, the University of Split, and the protocol was reviewed and approved by the Ethics Committee of School of Dental Medicine, University of Zagreb (No. 05-PA-30–9/2018), and Ethics Committee of School of the Medicine, University of Split (No. 2181–198-03–04-17–0063; No. 2181–198-03–04-20–0067). The research was in full accordance with World Medical Declaration of Helsinki (version 2013). The study is registered at clinical trials (ClinicalTrials.gov, Study ID number: NCT04460755) and was performed by the Consolidated Standards of Reporting Trials guidelines (CONSORT) [21].

Participation was voluntary, anonymous, and with no compensation. All respondents signed the informed consent before inclusion in the research and were acquainted with the purpose of the investigation.

Inclusion criteria for the participants were non-smokers aged 18 years or older with good oral and general health (ASA I physical status). In contrast, the exclusion criteria were pregnant and breastfeeding women, individuals with tooth sensitivity, gingival recession, oral mucosa disorders, prosthetic, orthodontic and implant-supported rehabilitation, and history of allergy to any dental hygiene product. Individuals who had previously undergone any tooth whitening treatment were excluded, too. Medical and dental anamnesis was taken in written form for each participant individually and each patient was introduced in detail in the aim and background of the study. The patients were also given a structured questionnaire, prepared for this research, where they provided answers related to demographic factors (age, gender), personal factors (amalgam and composite fillings), their eating habits

(meat, fruit, and vegetable consumption), smoking habits, and alcohol consumption. Eligibility criteria were assessed by the principal investigator who was blinded to the whitening toothpaste brand.

The minimum sample size was calculated according to the results from the study of Tadin et al. in 2018 about the cytotoxic and genotoxic effects of conventional and whitening toothpastes on the oral mucosa [4]. A power analysis was conducted, with the effect of Cohen's size $d = 1.21$ of the mentioned study, 80% power, and 95% confidence interval, at least 10 participants per group. However, as the study was conducted as part of the larger research that examined also the whitening effect of the tested toothpastes, a sample size of 21 was chosen based on the calculation from the previous study [22].

One month prior to the start of the study, all subjects participated in a wash-out period where they used the same toothpaste (Kalodont Multi Repair, Saponia, Osijek, Croatia). This wash-out period was aimed at establishing a standardized baseline before the introduction of the tested whitening toothpastes. During this preparation phase, baseline buccal mucosa samples were collected from all participants. These samples were collected to assess any potential variations in the number of cytogenetic impairments based on demographic and social factors.

Following the baseline sampling, eligible participants were randomly assigned to ten groups with a sample size of 21 participants each. The randomization process was carried out using computer software and followed a block randomization procedure [23]. This method was employed to ensure an equal distribution of participants across the groups. The randomization process was performed by an independent research member who was not involved in the evaluation procedures.

Nine tested groups used a different brand of whitening toothpaste with various whitening ingredients: Colgate Max Expert White (CMEW, Colgate-Palmolive Company, New York City, USA), Signal Daily White (SDW, Unilever House, London, UK), Himalaya Sparkly White Herbalis (HSWH, The Himalaya Drug Company, Makali, India), Signal White System (SWS, Unilever House, London, UK), Rembrandt Deeply White + Peroxide (RDWP, Rembrandt Trust Proprietary Limited, Johannesburg, South Africa), Splat Extreme White (SEW, Splat-Cosmetica, Moscow, Russia), Splat White Plus (SWP, Splat-Cosmetica, Moscow, Russia), Deep White (BDW, Biobaza, Sveta Nedelja, Croatia), and Dontodent Black Shine (DBS, DM Drogerie, Karlsruhe, Germany). At the same time, the control group used toothpaste classified as conventional/regular toothpaste, Kalodont Multi Repair (Saponia, Osijek, Croatia). The toothpaste brands and their ingredients are presented in Table 1.

The participants were instructed to use the tested whitening toothpastes for a duration of 2 months. Detailed instructions were provided in written form, directing them to apply the toothpaste twice a day: once in the morning and once in the evening. They were instructed to brush their teeth for 3 min using a modified Bass brushing technique, applying approximately 1 g of toothpaste (equivalent to approximately 2 cm in length). All respondents used the same type of toothbrush during the research (Splat Professional Complete Medium, Splat-Cosmetica, Moscow, Russia). It is important to note that during the research period, the participants did not use any toothpaste, other than the one being tested. Additionally, they refrained from using any other oral hygiene agents such as mouthwash, topical fluoridation, or whitening agents.

Clinical procedure and sample collection

Cell sampling was performed for four times at different time intervals: T0, before the treatment (baseline–after usage of conventional non-whitening control toothpaste); T1, 30 days after the beginning of using whitening toothpaste; T2, 60 days after the beginning of using whitening toothpaste; and T3, 90 days after the beginning of the study (30 days after completing the treatment and usage of conventional non-whitening toothpaste). Before taking the samples, all participants were asked to rinse the oral cavity twice with tap water for 1 min. It was done in order to remove exfoliated dead cells. All participants were asked to abstain from eating and drinking alcoholic beverages for 1 h before sampling. Using a cytological brush (Cytobrush Plus, GmbH Dietramszell-Linden, Germany) by gently brushing the buccal mucosa on both sides for 30 s, a smear of buccal cells was taken. The samples were transferred into 15-ml plastic tubes with chilled saline (5 ml) and centrifuged. After centrifugation, the cells were suspended in a small volume of fixation solution, methanol/acetic acid, in a ratio of 3:1 and five drops of dimethyl sulfoxide. After that, the cell suspension was applied to a pre-cleaned glass slide and left to dry for 24 h at room temperature. All cytologic preparations were made in duplicate. The slides were stained using the Feulgen/Fast green method, which is considered the standard protocol for staining buccal cells [24]. The protocol involved immersing the slides in 5 mol/l HCl, followed by rinsing with distilled water and drying. After being immersed in Schiff's reagent, the slides were washed with distilled water, and then, Fast green at a concentration of 1.0% (w/v) was applied to them. All chemicals, materials, and reagents used were from Biognost (Biognost d.o.o, Zagreb, Croatia) and Merck (Merck KGaA, Darmstadt, Germany).

The samples were coded by an independent coordinator who was not involved in the research and were stored in boxes for microscope slides at room temperature until the moment of microscopy. For each subject, 2000 cells were analyzed with an Olympus CX40 light microscope (Olympus, Tokyo, Japan) under × 400 magnification according to

Table 1 Toothpaste ingredients based on the manufacture's information

Toothpaste	Manufacturer	Tooth whitening agents	Ingredients
Kalodont Multi Repair (not whitening/conventional)	Saponia, Osijek, Croatia	Abrasives	Glycerin, PEG-8, hydrated silica, potassium nitrate, aqua, silica, dicalcium phosphate, sodium lauryl sulfate, aroma, tetrapotassium, pyrophosphate, sodium saccharin, DMDM hydration, xanthan gum, benzyl alcohol, limonene, linalool, CI 77891, sodium monofluorophosphate (1000 ppm F)
Colgate Max Expert White (whitening)	Colgate-Palmolive Company, New York City, USA	Hydrogen peroxide, abrasives	Glycerin, propylene glycol, PEG/PPG-116/66 copolymer, PEG-12, PVP, sodium lauryl sulfate, silica, aroma, sodium saccharin, phosphoric acid, BHT, CI 74160, calcium pyrophosphate, tetrasodium pyrophosphate, sodium monofluorophosphate (1450 ppm F)
Signal Daily White (whitening)	Unilever House, London, UK	Abrasives, sodium bicarbonate	Aqua, sorbitol, calcium carbonate, hydrated silica, sodium lauryl sulfate, aroma, sodium phosphate, cellulose gum, benzyl alcohol, sodium saccharin, propylene glycol, glycerin, CI 74160, CI 74260, sodium monofluorophosphate (1450 ppm F)
Himalaya Sparkly White Herbalis (whitening)	The Himalaya Drug Company, Makali, Bengaluru	Bromelain, papain, abrasives	Sorbitol, aqua, glycerin, silica, sodium lauryl sulfate, xanthan gum, titanium dioxide, aroma, sodium saccharin, sodium benzoate, potassium sorbate, menthol, *Salvadora persica* extract, *Prunus amygdalus dulcis* shell extract, *Cinnamomum zeylanicum* bark oil, *Eugenia caryophyllus* bud oil, thymol, citric acid, eugenol
Signal White System (whitening)	Unilever House, London, UK	Abrasives	Aqua, hydrogenated starch hydrolysate, calcium carbonate, hydrated silica, sodium lauryl sulfate, aroma, cellulose gum, benzyl alcohol, trisodium phosphate, sodium saccharin, glycerin, CI74160, sodium monofluorophosphate (1450 ppm F)
Rembrandt Deeply White + Peroxide (whitening)	Rembrandt Trust Proprietary Limited, Johannesburg, South Africa	Urea peroxide, papain, sodium citrate, abrasives	Glycerin, modified food starch, flavor, silica, propylene glycol, cocamidopropyl betaine, sodium lauryl sulfate, carbomer, sodium saccharin, calcium disodium EDTA, hydrated silica, aluminum hydroxide, sodium monofluorophosphate (0.884%)
Splat Extreme White (whitening)	Splat-Cosmetica, Moscow, Russia	Urea peroxide, papain, abrasives	Aqua, hydrogenated starch hydrolysate, PEG-8, sodium lauryl sulfate, hydrogenated palm oil, hydrated silica, aroma, PVP, xanthan gum, PEG-200, sodium methylparaben, sodium saccharin, sodium fluoride (0.05%), titanium dioxide, lecithin, CI 73360, CI 16255, limonene

Table 1 (continued)

Toothpaste	Manufacturer	Tooth whitening agents	Ingredients
Splat White Plus (Whitening)	Splat-Cosmetica, Moscow, Russia	Papain, abrasives	Aqua, hydrogenated starch hydrolysate, potassium nitrate, hydrated silica, calcium pyrophosphate, dicalcium phosphate dihydrate, PEG-8, pentasodium triphosphate, aroma, sodium coco-sulfate, cellulose gum, titanium dioxide, sodium lauroyl sarcosinate, PVP, sodium methylparaben, sodium saccharin, menthol, limonene, sodium monofluorophosphate (1000 ppm F)
Dontodent Black Shine (whitening)	DM-drogerie, Karlsruhe, Germany	Abrasives, charcoal	Aqua, sorbitol, hydrated silica, propylene glycol, pentasodium triphosphate, tetrapotassium pyrophosphate, sodium C14-16 olefin sulfonate, disodium pyrophosphate, aroma, xanthan gum, charcoal powder, sodium fluoride, sodium saccharin, limonene
Biobaza Deep White (whitening)	Biobaza, Sveta Nedelja, Croatia	Abrasives, charcoal	Hydrated silica, aqua, charcoal powder, citric acid, glycerin, lauryl glucoside, Mentha piperita leaf extract, menthol, potassium sorbate, silica, sorbitol, titanium dioxide, xanthan gum

EDTA ethylenediaminetetraacetic acid, *PEG* polyethylene glycol, *PPG* polypropylene glycol, *PVP* polyvinylpyrrolidone

the Thomas and Fenech protocol [25]. The rules according to the HUMNxl project criteria were followed when the samples were analyzed [26]. Protocol mandates the examination of minimum 1000 cells, within which the number of anomalies associated with cell death and nuclear anomalies indicating chromosomal instability or DNA damage is determined. Accordingly, 2000 differentiated cells were analyzed per slide. As parameters of genotoxicity, the number of binuclear cells that indicate a cytokinesis defect, i.e., the second phase of cell division, and cells with micronuclei and nuclear buds representing appropriate measures of chromosomal and DNA damage was evaluated. Cells with condensed chromatin, karyorrhexis (cell death with significant nuclear disintegration; disintegration into smaller parts), pyknosis (thickening), and karyolysis (decaying) are classified as markers of early to late stages of apoptosis and cell death [27] and show a cytotoxic effect [17, 28].

Statistical analysis

Statistical analysis was done using SPSS software version 25.0 (SPSS Inc., Chicago, IL, USA) and Excel MS office (Microsoft, Redmond, Washington, SAD). The normality of the data (distribution of variables) was tested by the Kolmogorov–Smirnov test.

The primary statistical parameters (mean, median, standard deviation, minimum, maximum, and interquartile range values) were determined by descriptive statistical analysis. The differences in the number of cells with micronuclei and other nuclear anomalies between different sampling times for tested groups and between groups at the same sampling time were evaluated by repeated-measures analysis of variance (two-way ANOVA) and Tukey's test. A general regression model from linear/nonlinear modeling method was used for the assessment of the influence of predictor variables (age, gender, and eating habits) on dependent variables (number of micronuclei, nuclear buds, number of binucleated cells, pyknosis, condensed chromatin, karyolysis, and karyorrhexis). Using Pareto diagram, the results of general regression model were demonstrated (*t*-values). Statistical significance was set up to $P < 0.05$.

Results

The study included 199 participants, students and employees of the School of the University of Split, Croatia. There was a total of 79 (39.7%) males and 120 (60.3%) females, aged between 21 and 55 (mean age 29.41 ± 7.81, min 21, max 55). Participant's demographic data are presented in Table 2.

The results of buccal micronucleus assay (BMCyt assay) are presented as genotoxic parameters (chromosomal and DNA damage markers) in numbers of cells with

Table 2 Demographic data divided by treatment groups

Treatment groups	*N*	Gender		Age
		Male (%)	Female (%)	
Control	20	7 (35.0%)	13 (65.0%)	29.25 (6.48)
CMEW	20	10 (50.0%)	10 (50.0%)	27.40 (9.20)
SDW	20	9 (45.0%)	11 (55.0%)	32.60 (8.59)
HSWH	20	8 (40.0%)	12 (60.0%)	26.80 (4.70)
SWS	20	9 (45.0%)	11 (55.0%)	29.45 (5.82)
RDWP	20	7 (35.0%)	13 (65.0%)	31.55 (8.40)
SEW	20	8 (40%)	12 (60%)	27.00 (7.69)
SWP	21	8 (38.1%)	13 (61.9%)	26.76 (5.53)
DBS	20	4 (20.0%)	16 (80.0%)	27.10 (6.00)
BDW	18	9 (50%)	9 (50%)	37.06 (9.415)

Data are presented as whole numbers and percentages or mean (SD)

Control Kalodont Multi Repair, *CMEW* Colgate Max Expert White, *SEW* Splat Extreme White, *RDWP* Rembrandt Deeply White + Peroxide, *SWP* Splat White Plus, Himalaya *HSWH* Sparkly White Herbalis, *SDW* Signal Daily White, *SWS* Signal White System, *DBS* Dontodent Black Shine, *BDW* Biobaza Deep White, *SD* standard deviation

micronuclei, number of binucleated cells, and number of cells with nuclear buds. Cytotoxic parameters (indicators of cell death and apoptosis) are presented as number of cells with condensed chromatin, number of cells with karyorrhexis, number of cells with pyknosis, and number of cells with karyolysis. The CONSORT diagram of the study (i.e., enrolment, intervention allocation, follow-up, and data analysis) is presented in Fig. 1.

Genotoxic parameters showed no biologically significant changes in any of the observed period for the tested toothpastes, while the cytotoxic parameters (number of cells with karyorrhexis and condensed chromatin) showed statistically significant increase ($P < 0.05$) at T1 and T2 compared to baseline for three peroxide-containing toothpastes—Colgate Max Expert White, Rembrandt Deeply White + Peroxide, and Splat Extreme White.

The analysis of ANOVA indicates statistically significant difference neither for genotoxic parameters nor for cytotoxic parameters between groups for the same sampling time. The same applies for the genotoxic parameters for all the ten groups between sampling times. The statistically significant difference ($P < 0.05$) was observed only for cytotoxic parameters condensed chromatin and karyorrhexis between sampling times for the following whitening toothpastes: CMEW-karyorrhexis (increase: T1 vs. T0; T2 vs. T0), CMEW-condensed chromatin (increase: T1 vs. T0; T2 vs. T0; decrease: T3 vs. T1; T3 vs. T2), RDWP-karyorrhexis (increase: T1 vs. T0; T2 vs.T0; decrease: T3 vs. T1; T3 vs. T2), RDWP-condensed chromatin (increase: T1 vs. T0; T2 vs. T0; T2 vs. T1; T3 vs. T0; decrease: T3 vs. T1; T3 vs. T2), SEW-karyorrhexis (increase: T1 vs. T0; T2 vs.T0; decrease: T3 vs. T1; T3 vs. T2), and SEW-condensed chromatin (increase: T1 vs. T0; T2

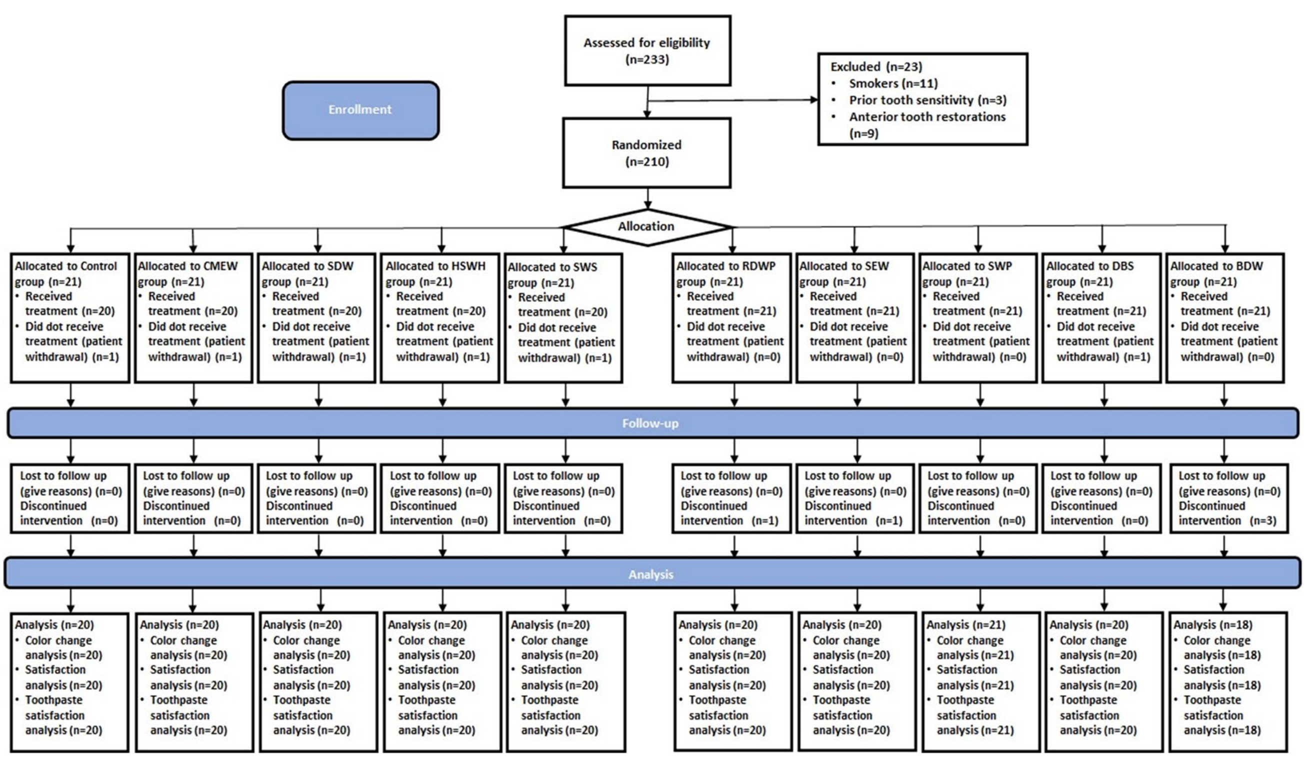

Fig. 1 Flowchart of participant's recruitment and follow-up

vs. T0; T2 vs. T1; T3 vs. T0; decrease: T3 vs. T1; T3 vs. T2). Mean (standard deviation) of DNA damage parameter (micronuclei, nuclear buds, and binucleated cells) in 2000 buccal epithelial cells of tested group participants for each time point of measurement is presented in Table 3. Mean (standard deviation) of cytotoxic parameters (karyolysis, karyorrhexis, pyknosis, and condensed chromatin) in 2000 buccal epithelial cells of participants for each time point of measurement is presented in Table 4.

Relation of the cytotoxic damage score to demographic and lifestyle factors as possible predictors was determined by multiple regression analysis and presented in the form of Pareto diagrams and *t*-values (Fig. 2). The number of cells with condensed chromatin was statistically significantly related to age ($\beta = 0.116$, SE = 0.05, $P \leq 0.001$, alcohol consumption ($\beta = -0.075$, SE = 0.571, $P = 0.018$), and number of amalgam filling ($\beta = 0.064$, SE = 0.28, $P = 0.033$). The number of karyorrhexis was statistically significantly related to gender ($\beta = -0.101$, SE = 0.469, $P = 0.002$), coffee consumption ($\beta = 0.086$, SE = 0.136, $P = 0.005$), and number of composite filling ($\beta = -0.089$, SE = 0.075, $P = 0.006$). Relation of genotoxic parameters to demographic and lifestyle factors as possible predictors was also determined by multiple regression analysis and presented in the form of Pareto diagrams and *t*-values (Fig. 3). The number of cells with micronuclei was statistically significantly related to gender ($\beta = 0.121$, SE = 0.067, $P \leq 0.001$), number of amalgam filling ($\beta = 0.059$, SE = 0.022, $P = 0.049$), number of composite filling ($\beta = -0.077$, SE = 0.011, $P = 0.016$), and fruit consumption ($\beta = -0.148$, SE = 0.061, $P \leq 0.001$). The number of cells with nuclear buds was statistically significantly related to gender ($\beta = -0.080$, SE = 0.054, $P = 0.011$), age ($\beta = -0.131$, SE = 0.003, $P \leq 0.001$), meat consumption ($\beta = -0.133$, SE = 0.042, $P \leq 0.001$), coffee consumption ($\beta = 0.129$, SE = 0.016, $P \leq 0.001$), and number of composite filling ($\beta = -0.089$, SE = 0.009, $P = 0.005$). The number of cells with binucleated cells was statistically significantly related to gender ($\beta = -0.064$, SE = 0.212, $P = 0.038$), meat consumption ($\beta = -0.099$, SE = 0.227, $P = 0.001$), fruit consumption ($\beta = -0.111$, SE = 0.223, $P = 0.001$), alcohol consumption ($\beta = -0.164$, SE = 0.197, $P \leq 0.001$), and number of composite filling ($\beta = -0.173$, SE = 0.046, $P \leq 0.001$).

Discussion

The results in this research showed that the toothpastes with different whitening ingredients have no genotoxic effect (statistically significant differences in number of micronuclei, binucleated cells, or nuclear buds were found neither between evaluation periods within the same group nor between the groups for the same evaluation periods), while peroxide-containing toothpastes—Colgate Max Expert (CMEW), Rembrandt Daily White + Peroxide (RDWP), and Splat Extreme White (SEW)—showed cytotoxic effect in terms of statistically significant difference in the number of karyorrhexis and condensed chromatin that was identified. Based on this fact, the null hypothesis was partially rejected.

Statistically significant increase in the number of karyorrhexis and condensed chromatin cells was detected after 30 days (T1) and 60 days (T2) of using the toothpaste compared to baseline sampling at T0. Furthermore, the results point to statistically significant increase in the number condensed chromatin cells even at T2 compared to T1 for RDWP and SEW. Both cytotoxic parameters were decreased in the follow-up period (T3) for the three whitening toothpastes. Interestingly, no statistically significant difference for the three whitening toothpastes was observed against control non-whitening toothpaste (Kalodont Multi Repair) as for no other tested whitening toothpaste. Based on these results, it can be judged that a slight cytotoxic effect may be present for all tested whitening toothpastes, while only for some of them (CMEW, RDWP, SEW), this effect is statistically significant. The differences between the evaluation periods of the three whitening toothpastes further indicate direct correlation of toothpaste application period with the observed alterations. Finally, it is important to highlight that all the three whitening toothpastes revealing statistically significant difference for the two cytotoxic parameters (karyorrhexis and condensed chromatin) contain peroxides in its composition (hydrogen peroxide and urea peroxide) as an active whitening ingredient. This implies peroxides to dominantly influence the revealed cytotoxic effect of the three whitening toothpastes.

Due to a limited number of studies [3, 4, 10, 29–31] in the available literature that analyze the cytotoxicity and genotoxicity performance of toothpastes classified as whitening toothpastes, this study was compared to studies that evaluated similar effects of other over the counter products and bleaching agents for professional usage.

Complementary to the results of this study, an in vitro study of Rode et al. [10] applying the micronucleus test (MNT) suggests no genotoxic potential of the whitening toothpastes while the elements of cytotoxic potential exist. The cytotoxic potential was presented by fluoride whitening toothpastes for which they showed that cytotoxicity in gingival fibroblasts was related to concentration of the toothpaste (higher concentration caused cytotoxicity). Based on their research, they claim that the cytotoxicity of the toothpastes is mainly caused due to fluoride in its composition. Opposed to this, results of this study conclude that the increased cytotoxicity alterations (in terms of karyorrhexis and condensed chromatin) are dominantly caused by peroxide in the whitening toothpaste composition, despite the concentration of peroxides in toothpaste which is usually low—1% hydrogen peroxide or 0.5–0.7%

Table 3 Mean (standard deviation) of DNA damage parameters in buccal mucosal cells of tested group participants at different time points

Cytogenetic parameters	Treatment groups									
	Control	CMEW	SDW	HSWH	SWS	RDWP	SEW	SWP	DBS	BDW
Number of cells with micronuclei										
T0	1.51 (1.22)^{Aa}	1.69 (0.85)^{Aa}	1.77 (0.73)^{Aa}	1.64 (1.13)^{Aa}	1.87 (0.82)^{Aa}	1.87 (1.14)^{Aa}	1.79 (1.15)^{Aa}	1.84 (0.96)^{Aa}	1.75 (0.87)^{Aa}	1.60 (1.32)^{Aa}
T1	1.48 (1.17)^{Aa}	1.56 (0.83)^{Aa}	1.77 (0.82)^{Aa}	1.52 (1.08)^{Aa}	1.75 (0.83)^{Aa}	1.73 (1.09)^{Aa}	1.68 (1.20)^{Aa}	1.78 (1.00)^{Aa}	1.60 (0.87)^{Aa}	1.46 (1.26)^{Aa}
T2	1.50 (1.23)^{Aa}	1.55 (0.87)^{Aa}	1.69 (0.79)^{Aa}	1.50 (1.10)^{Aa}	1.84 (0.81)^{Aa}	1.74 (1.11)^{Aa}	1.66 (1.18)^{Aa}	1.83 (0.96)^{Aa}	1.62 (0.88)^{Aa}	1.46 (1.26)^{Aa}
T3	1.47 (1.17)^{Aa}	1.60 (0.88)^{Aa}	1.73 (0.80)^{Aa}	1.54 (1.06)^{Aa}	1.84 (0.80)^{Aa}	1.78 (1.13)^{Aa}	1.71 (1.16)^{Aa}	1.86 (0.98)^{Aa}	1.65 (0.94)^{Aa}	1.51 (1.27)^{Aa}
Number of cells with nuclear buds										
T0	2.52 (0.85)^{Aa}	2.58 (0.97)^{Aa}	2.64 (0.79)^{Aa}	2.75 (1.05)^{Aa}	2.51 (0.84)^{Aa}	2.78 (1.01)^{Aa}	2.50 (0.65)^{Aa}	2.63 (0.49)^{Aa}	2.48 (0.86)^{Aa}	2.64 (0.68)^{Aa}
T1	2.59 (1.27)^{Aa}	2.40 (1.18)^{Aa}	2.48 (1.08)^{Aa}	2.70 (1.17)^{Aa}	2.43 (0.99)^{Aa}	2.46 (1.24)^{Aa}	2.31 (1.04)^{Aa}	2.66 (1.19)^{Aa}	2.69 (0.74)^{Aa}	2.51 (0.88)^{Aa}
T2	2.62 (1.18)^{Aa}	2.43 (1.36)^{Aa}	2.52 (0.93)^{Aa}	2.72 (1.13)^{Aa}	2.46 (1.33)^{Aa}	2.64 (1.17)^{Aa}	2.33 (1.26)^{Aa}	2.60 (1.15)^{Aa}	2.79 (0.85)^{Aa}	2.61 (1.22)^{Aa}
T3	2.48 (1.07)^{Aa}	2.47 (1.20)^{Aa}	2.45 (1.16)^{Aa}	2.58 (1.07)^{Aa}	2.45 (1.17)^{Aa}	2.48 (1.30)^{Aa}	2.41 (1.10)^{Aa}	2.39 (1.04)^{Aa}	2.70 (0.74)^{Aa}	2.74 (1.19)^{Aa}
Number of binucleated cells										
T0	6.85 (4.69)^{Aa}	6.91 (5.74)^{Aa}	6.44 (5.08)^{Aa}	7.03 (5.46)^{Aa}	6.28 (2.79)^{Aa}	7.13 (4.82)^{Aa}	6.85 (3.32)^{Aa}	6.90 (3.50)^{Aa}	6.36 (4.68)^{Aa}	6.39 (4.68)^{Aa}
T1	6.93 (4.73) ^{Aa}	6.92 (5.73)^{Aa}	6.53 (5.19)^{Aa}	7.14 (5.44)^{Aa}	6.36 (2.86)^{Aa}	7.18 (4.87)^{Aa}	6.91 (3.28)^{Aa}	7.01 (3.50)^{Aa}	6.44 (4.72)^{Aa}	6.46 (4.64)^{Aa}
T2	6.87 (5.43) ^{Aa}	6.98 (5.72)^{Aa}	6.52 (5.18)^{Aa}	7.13 (5.43)^{Aa}	6.33 (2.84)^{Aa}	7.21 (4.91)^{Aa}	6.95 (3.26)^{Aa}	7.04 (3.49)^{Aa}	6.47 (4.73)^{Aa}	6.53 (4.63)^{Aa}
T3	6.97 (5.29) ^{Aa}	6.89 (5.62)^{Aa}	6.39 (5.07)^{Aa}	7.10 (5.45)^{Aa}	6.26 (2.80)^{Aa}	7.10 (4.90)^{Aa}	6.88 (3.35)^{Aa}	6.93 (3.52)^{Aa}	6.32 (4.73)^{Aa}	6.34 (4.74)^{Aa}

Different capital letters indicate a significant difference among the evaluation periods, and different lowercase letters indicate a significant difference among the treatment groups ($P < 0.05$)

Control Kalodont Multi Repair, *CMEW* Colgate Max Expert White, *SEW* Splat Extreme White, *RDWP* Rembrandt Deeply White + Peroxide, *SWP* Splat White Plus, *HSWH* Himalaya Sparkly White Herbalis, *SDW* Signal Daily White, *SWS* Signal White System, *DBS* Dontodent Black Shine, *BDW* Biobaza Deep White, *T0* baseline, *T1* 30 days of treatment beginning, *T2* 60 days of treatment beginning, *T3* 90 days of treatment beginning (30 days after treatment–follow-up)

Table 4 Mean (standard deviation) of cytotoxic parameters in buccal mucosal cells of tested group participants at different time points

Cytogenetic parameters	Treatment groups									
	Control	CMEW	SDW	HSWH	SWS	RDWP	SEW	SWP	DBS	BDW
Number of cells with karyolysis										
T0	103.21 (38.36)^{Aa}	104.53 (36.82)^{Aa}	104.81 (38.52)^{Aa}	111.25 (37.69)^{Aa}	111.58 (37.63)^{Aa}	110.49 (35.58)^{Aa}	106.18 (34.21)^{Aa}	111.37 (35.21)^{Aa}	109.39 (38.69)^{Aa}	110.50 (26.40)^{Aa}
T1	103.13 (38.34)^{Aa}	107.04 (36.18)^{Aa}	107.31 (38.07)^{Aa}	114.74 (29.53)^{Aa}	113.76 (37.52)^{Aa}	113.96 (31.51)^{Aa}	108.89 (34.82)^{Aa}	112.48 (35.03)^{Aa}	112.90 (38.95)^{Aa}	113.94 (28.80)^{Aa}
T2	103.90 (39.68)^{Aa}	107.40 (36.37)^{Aa}	107.38 (38.28)^{Aa}	114.33 (28.69)^{Aa}	113.21 (37.65)^{Aa}	112.15 (30.71)^{Aa}	108.36 (34.38)^{Aa}	111.87 (35.07)^{Aa}	112.34 (38.40)^{Aa}	113.36 (26.89)^{Aa}
T3	103.44 (38.79)^{Aa}	105.45 (36.57)^{Aa}	106.91 (38.39)^{Aa}	112.84 (30.83)^{Aa}	112.64 (37.62)^{Aa}	111.12 (33.29)^{Aa}	106.98 (33.44)^{Aa}	110.78 (35.41)^{Aa}	110.27 (39.03)^{Aa}	111.42 (25.32)^{Aa}
Number of cells with karyorrhexis										
T0	15.41 (7.43)^{Aa}	15.07 (7.30)^{Aa}	15.14 (7.20)^{Aa}	15.88 (6.50)^{Aa}	15.12 (7.58)^{Aa}	14.86 (6.95)^{Aa}	15.50 (7.63)^{Aa}	15.26 (7.49)^{Aa}	15.78 (6.39)^{Aa}	15.23 (7.59)^{Aa}
T1	15.63 (7.48)^{Aa}	16.84 (9.43)^{Ba}	16.14 (7.23)^{Aa}	16.88 (8.72)^{Aa}	16.08 (9.17)^{Aa}	16.77 (8.17)^{Ba}	17.40 (7.42)^{Ba}	15.48 (7.30)^{Aa}	16.62 (6.34)^{Aa}	15.46 (7.35)^{Aa}
T2	15.81 (7.44)^{Aa}	16.60 (9.26)^{Ba}	15.59 (7.29)^{Aa}	16.84 (8.18)^{Aa}	16.11 (8.88)^{Aa}	16.94 (8.05)^{Ba}	17.50 (7.53)^{Ba}	15.97 (7.31)^{Aa}	16.88 (5.62)^{Aa}	15.73 (7.34)^{Aa}
T3	15.50 (7.24)^{Aa}	16.00 (7.74)^{ABa}	15.25 (7.79)^{Aa}	16.01 (8.11)^{Aa}	15.98 (8.99)^{Aa}	15.76 (7.73)^{Aa}	16.00 (7.03)^{Aa}	15.25 (7.20)^{Aa}	16.34 (6.10)^{Aa}	15.64 (7.51)^{Aa}
Number of cells with pyknosis										
T0	3.76 (1.54)^{Aa}	3.43 (1.76)^{Aa}	3.77 (1.69)^{Aa}	3.23 (1.63)^{Aa}	3.89 (1.29)^{Aa}	3.61 (2.32)^{Aa}	3.53 (1.71)^{Aa}	3.74 (2.20)^{Aa}	3.42 (1.23)^{Aa}	3.60 (2.10)^{Aa}
T1	3.83 (1.42)^{Aa}	3.83 (2.12)^{Aa}	3.84 (1.55)^{Aa}	3.68 (2.19)^{Aa}	3.88 (2.31)^{Aa}	3.96 (1.55)^{Aa}	3.83 (1.98)^{Aa}	3.94 (2.18)^{Aa}	3.65 (1.30)^{Aa}	4.08 (2.14)^{Aa}
T2	3.90 (1.31)^{Aa}	3.83 (2.15)^{Aa}	3.82 (1.77)^{Aa}	3.71 (2.21)^{Aa}	3.97 (2.32)^{Aa}	3.95 (2.18)^{Aa}	4.01 (2.14)^{Aa}	3.90 (2.15)^{Aa}	3.73 (1.55)^{Aa}	4.06 (2.33)^{Aa}
T3	3.81 (1.87)^{Aa}	3.67 (1.46)^{Aa}	3.61 (2.49)^{Aa}	3.46 (1.90)^{Aa}	3.79 (1.88)^{Aa}	3.73 (2.19)^{Aa}	3.83 (2.16)^{Aa}	3.89 (2.25)^{Aa}	3.82 (1.66)^{Aa}	3.72 (2.29)^{Aa}
Number of cells with condensed chromatin										
T0	41.12 (10.80)^{Aa}	40.25 (14.91)^{Aa}	39.89 (11.22)^{Aa}	40.43 (12.99)^{Aa}	40.93 (13.21)^{Aa}	38.78 (12.61)^{Aa}	39.21 (15.55)^{Aa}	40.52 (11.46)^{Aa}	40.28 (10.53)^{Aa}	40.62 (14.81)^{Aa}
T1	41.62 (11.83)^{Aa}	41.60 (15.45)^{Ba}	40.25 (11.33)^{Aa}	40.93 (13.18)^{Aa}	41.36 (13.41)^{Aa}	41.82 (12.19)^{Ba}	43.75 (15.11)^{Ba}	40.98 (11.32)^{Aa}	40.85 (10.67)^{Aa}	41.24 (14.71)^{Aa}
T2	41.33 (11.68)^{Aa}	41.44 (14.99)^{Ba}	40.39 (11.46)^{Aa}	40.86 (13.23)^{Aa}	41.44 (13.44)^{Aa}	42.51 (12.17)^{Ca}	44.32 (15.09)^{Ca}	41.10 (11.61)^{Aa}	40.96 (10.84)^{Aa}	41.35 (14.83)^{Aa}
T3	41.15 (11.28)^{Aa}	40.43 (15.49)^{Aa}	40.21 (11.13)^{Aa}	40.64 (12.95)^{Aa}	41.41 (13.34)^{Aa}	40.17 (12.35)^{Da}	42.71 (14.87)^{Da}	41.05 (11.38)^{Aa}	40.84 (10.48)^{Aa}	40.70 (14.72)^{Aa}

Different capital letters indicate a significant difference among the evaluation periods, and different lowercase letters indicate a significant difference among the treatment groups ($P < 0.05$)

Control Kalodont Multi Repair, *CMEW* Colgate Max Expert White, *SEW* Splat Extreme White, *RDWP* Rembrandt Deeply White + Peroxide, *SWP* Splat White Plus, *HSWH* Himalaya Sparkly White Herbalis, *SDW* Signal Daily White, *SWS* Signal White System, *DBS* Dontodent Black Shine, *BDW* Biobaza Deep White, *T0* baseline, *T1* 30 days of treatment beginning, *T2* 60 days of treatment beginning, *T3* 90 days of treatment beginning (30 days after treatment–follow-up)

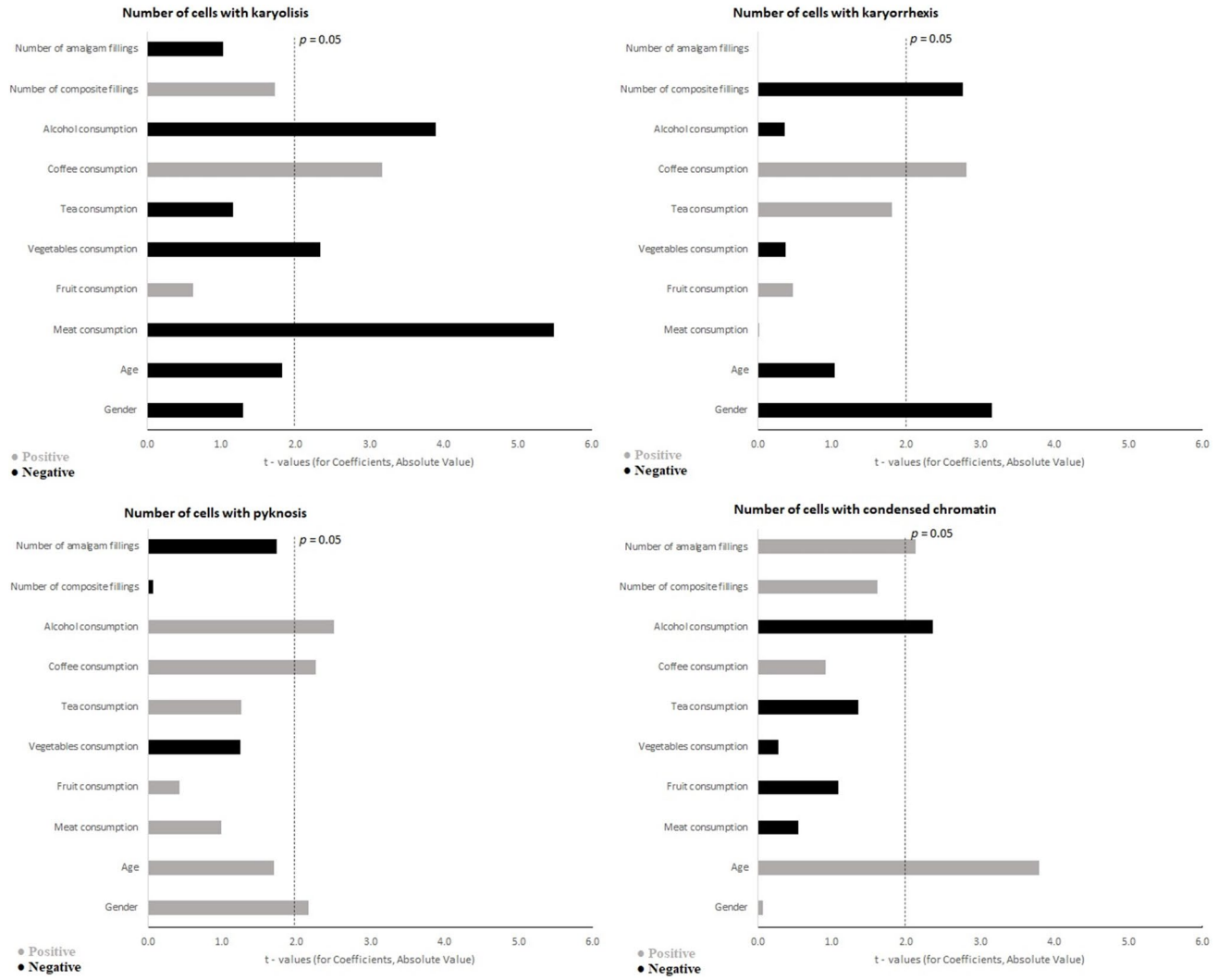

Fig. 2 Multiple regression analysis results. Relation of cytotoxic parameters in buccal mucosal cells (number of cells with condensed chromatin, karyolysis, karyorrhexis, and pyknosis) with participant's demographic variables and lifestyle factors as possible predictors

calcium peroxide [32, 33]. However, it is important to highlight here that all tests in this study were conducted with constant same concentration of the toothpaste throughout all sampling time. Next, based on the results in this study solely, the influence of other ingredients, such as fluoride, cannot be entirely neglected. Similar results for cytotoxic effect of toothpastes to those of Rode et al. [10] were shown by Camargo et al. [3] that were using in vitro MNT test on Chinese hamster fibroblasts and reported higher concentration of the toothpaste caused cytotoxicity in V79 cells. In their work, cytotoxicity of whitening toothpastes was presented through cell survival. The most cytotoxic toothpaste showed to be Colgate Whitening, which had viable cells lower than 5%. Based on this fact, they concluded that the whitening toothpastes promote the highest cytotoxicity among toothpastes. Unlike the work of Rode et al. [10] and this study, Camargo et al. [3] report also genotoxic effect of the whitening toothpastes. In their in vitro study, using the methyl tetrazolium test (MTT) on human gingival fibroblasts (HGF-1), they reported Oral-B whitening toothpaste, having fluoride and abrasives as the main whitening ingredients, as the most genotoxic one. Although in this study no test was conducted on Oral-B whitening toothpaste, other fluoride- and abrasive-based toothpastes were examined. Irrespective of the whitening ingredients of the toothpastes, the results of this study point to a conclusion of no genotoxic effect of the whitening toothpastes. In their study, Bruno et al. [30] used the MTT assay in in vitro conditions and reported that all tested toothpastes, including whitening toothpaste, Colgate Luminous White, were classified as highly cytotoxic—cell viabilities were lower than 50%. They emphasize that decrease in cell viability, implying cytotoxicity, can be attributed to the various components in toothpastes,

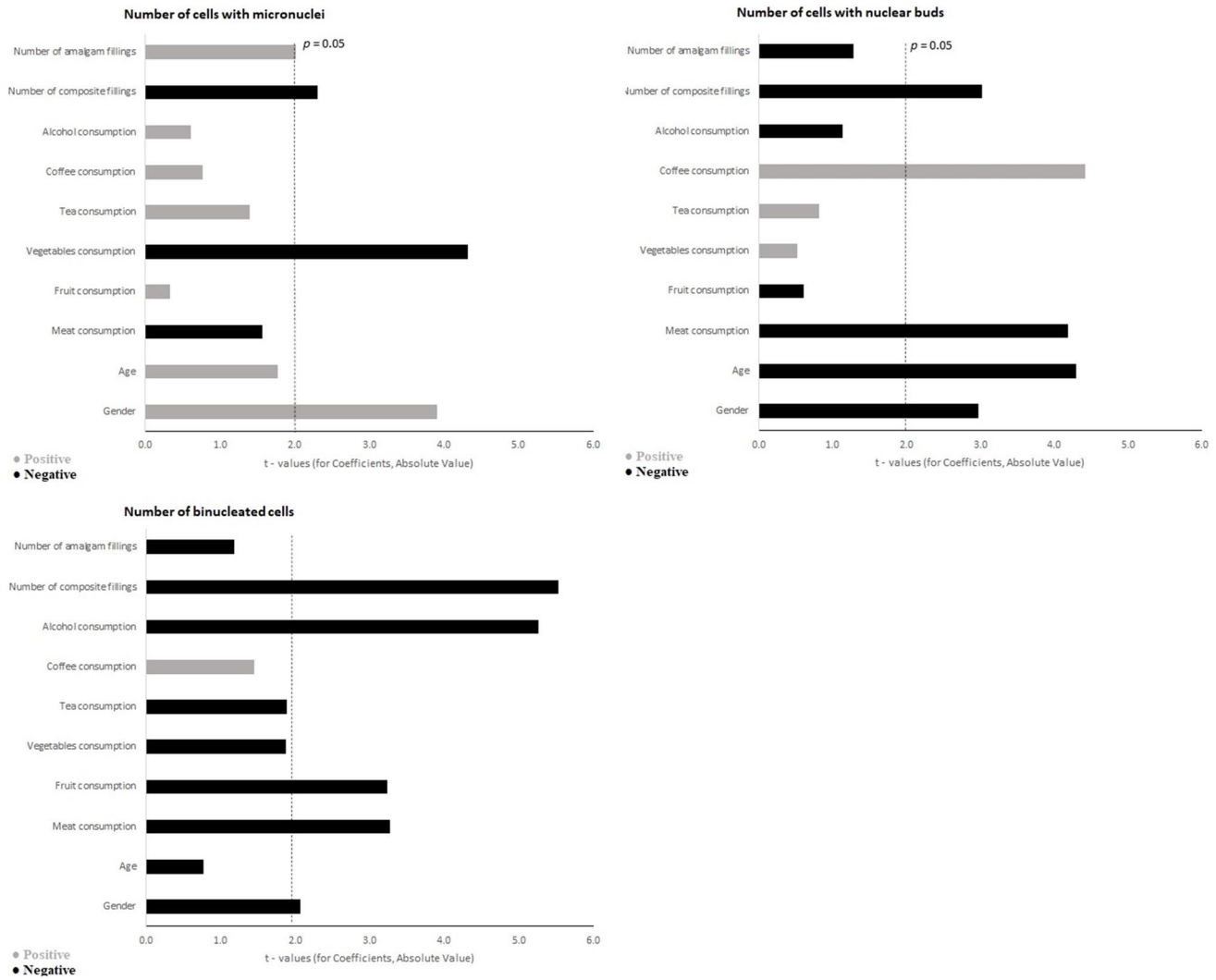

Fig. 3 Multiple regression analysis results. Relation of genotoxic parameters in buccal mucosal cells (number of binucleated cells, number of cells with micronuclei, and number of cells with nuclear buds) with participant's demographic variables and lifestyle factors as possible predictors

either alone or combined. This study points to nearly the same conclusion yet implying peroxides to dominantly attribute to cytotoxicity of the whitening toothpastes. In their research, Tadin et al. [4] compared the toxicity of whitening toothpastes and non-whitening toothpastes, and only one of these tested whitening toothpastes showed significant increase in number of micronucleated cells after 60 days of usage of Colgate whitening toothpaste. They reported that whitening toothpastes can express genotoxic effects on buccal epithelial cells, but the obtained results were classified as limited and biologically insignificant. The results of this research suggest no genotoxic potential of the observed whitening toothpastes at all.

In in vitro study on mouse fibroblasts cells L929, reported by Torrado et al. [29], the authors highlighted (based on MTT assay) that the commercially available Crest Extra Whitening toothpaste caused an inhibition percentage not greater than 50% and they concluded that cytotoxicity was not increased with the duration of process. Based on the results of this study, the cytotoxicity of the three tested whitening toothpastes (CMEW, RDWP, and SEW) was directly related to the time of usage. Ghapanchi et al. [31] tested cytotoxicity in in vitro study of 16 commercial toothpaste in primary epithelial cells of the oral cavity and HeLa cell line for exposure from 1 to 5 min, and cytotoxic effects on buccal mucosa cells were evaluated for different duration; thus, an increase in cytotoxicity was positively correlated with duration of exposure. All tested toothpastes showed cytotoxic potential, but to a different extent. Nearly the same results, but in various concentrations of hydrogen peroxide,

were reported by Furukawa et al. [34] for different duration exposure on cultured human gingival fibroblasts (HGFs). They results showed that the low concentration of hydrogen peroxide (0.0015%) had no effect on the survival of the cells after 30 min of exposure, but a reduced survival rate was obtained after 60 min. Lower concentration of hydrogen peroxide (0.00015%) did not affect cell survivor even after 60 min. Concentration of ≥ 0.15% hydrogen peroxide affected cell survival after 90 s of exposure. Both studies [31, 34] showed correlation between the level of cytotoxicity and the time of exposure. Moreover, some chemical ingredients such as hydrogen peroxide, significantly increased cytotoxicity even in a short exposure time. Although the exact concentrations of peroxides and other active ingredients in tested whitening toothpastes were not compared in this study, the results suggest nearly same conclusion to those in [31, 34], but for different time intervals of exposure (1–5 min vs. 2 months of usage). It is important to note that in this study, the toothpaste was always in contact with the buccal mucosa for the same duration (3 min for each application of the toothpaste) during the experiment, so the magnitude of daily application was always the same during the 2-month treatment.

Concentrations of hydrogen peroxides in other bleaching agents for professional and non-professional usage are various (3–38%). The toxic potential of various bleaching substances has been evaluated in the literature. Del Real Garcia et al. [35] in their study assessed the impact of 10% hydrogen peroxide whitening strip exposure on the genotoxicity and oxidative damage. They concluded that strips with 10% hydrogen peroxide exhibit increased in NAs in oral epithelial cells and 8-OHdG levels in saliva which produce oxidative DNA damage. According to these results, they recommend careful and rational handling of self-application bleaching agents. Contrary to these results, Monteiro et al. [16] evaluated the genotoxic potential of 10% hydrogen peroxide at-home bleaching gels and concluded that there was no sign of genotoxic effect during the application of this bleaching agent for 30 min/day for 14 days. Klaric et al. [36] investigated the genotoxic effect of two hydrogen peroxide bleaching agents on oral mucosal cells and both demonstrated potentially genotoxic effect. It is important to mention that the concentration of hydrogen peroxide bleaching agent which they used was higher than in whitening toothpastes (25% and 38%). High concentration of peroxide is only allowed for professional usage, which is not the case with products with significantly lower concentrations of peroxide such as tooth whitening pastes. However, it is important to know that the hydrogen peroxide can interact with DNA and increase the concentration of reactive oxygen species and free radicals leading to consequent oxidative DNA damage [37, 38]. The EU Scientific Committee on Consumer Safety (SCCP 2007) concluded that products with 0.1% hydrogen peroxide or the release up to 0.1% hydrogen peroxide are safe for human usage [39]. In our study, none of the tested whitening toothpastes containing chemical agents (peroxides and/or enzymes; CMEW, HSWH, RDWP, SEW, and SWP), abrasives (SDW and SWS), and charcoal (BDW and DBS) showed statistically significant increase of genotoxic parameters. Comparing our results to aforementioned studies for the bleaching agents, we can conclude that the concentration of peroxides in the whitening toothpastes is low enough not to cause any genotoxic effects.

Generally, the side effects on the cells of the oral mucosa can be directly related to the presence of fluoride and fluoride concentration in fluoride containing products for oral hygiene, SLS, triclosan, sodium monofluorophosphate, silicon dioxide, sodium benzoate, preservatives, colors, and flavors [2, 8, 31, 40]. Also, lifestyle, dental status, and habits (factors such as alcohol and tobacco consumption, subgingival plaque) may be of genotoxic relevance [41]. During the cytotoxic and genotoxic analysis of whitening toothpastes, it is important to note that components such as environmental, biological, and demographic factors, as well as professional exposure to some toxins, can be predilection factors for the appearance of increased toxic parameters in in vivo conditions. In our research, the results of multiple regression showed significant dependence of genotoxicity parameters with demographic and lifestyle factors as possible predictors (numbers of cells with micronuclei with gender, alcohol consumption, and number of amalgam and composite fillings; binucleated cells with alcohol consumption and number of composite fillings; nuclear buds with gender, coffee consumption; and number of composite filling). Also, the cytotoxic parameters showed the significant dependence with demographic and lifestyle factors as possible predictors (number of cells with condensed chromatin with age, alcohol consumption, and number of amalgam fillings; number with karyorrhexis with gender, coffee consumption, and number of composite fillings; karyolysis with meat, coffee, and alcohol consumption; pyknosis with gender and alcohol and coffee consumption).

The results in this study indicate a connection between the increase of cytotoxicity in tested toothpastes and the peroxide content in them. Namely, whitening toothpastes, that showed an increase in cytotoxicity, contained peroxide (urea or hydrogen peroxide) in their composition. As expected, with the cessation of the use of toothpastes with peroxides, a decrease in cytotoxic parameters was recorded. Our results suggest that the cytotoxic behavior of toothpastes might be due to peroxides in their compositions, although the influence of other components/ingredients in whitening toothpastes cannot be ruled out, especially in combination with peroxides in their composition. In clinical condition, the obtained results of tested whitening toothpastes indicate

a limited, biologically insignificant cytotoxic effect on buccal mucosal cells.

Upon reviewing the available literature, it becomes evident that only a limited number of studies have explored the cytotoxic and genotoxic effects of whitening toothpastes containing various ingredients that are currently available on the market [3, 4, 10, 29–31]. This work contributes to new knowledge about commercially available whitening toothpastes with different ingredients in respect of the safety when using them. Nevertheless, this study had some limitations. It did not test individual components/ingredients of toothpastes and their exact concentrations in them, so it is suggested for the future studies to consider testing individual components of whitening toothpastes and test the influence on cell survivor in in vitro conditions, using, for example, MTT on human gingival fibroblasts (HGF). In addition, it would be desirable to analyze the cells of the buccal mucosa and samples from the gingiva or oropharynx to get a better insight into the toxicity of whitening toothpastes. It would be desirable to test the toxicity of different concentrations of individual components during the various exposure times—for example, for a duration of 1 to 5 min. Other possible limitations are the small sample size per group and the limited representation of age and gender among the participants. It would have been advantageous to have larger test groups that were more closely matched in terms of demographic characteristics. However, since each participant served as their own control, it is less likely that these differences had a substantial impact on the study's results. Despite these limitations, the study yields significant findings concerning the toxicity of commercially available whitening toothpastes. It is noteworthy as the first in vivo study conducted on a substantial number of subjects, investigating various whitening toothpastes with different whitening agents. Further research, encompassing both in vitro and in vivo studies employing diverse methods, is essential to explore the biocompatibility of individual ingredients found in whitening toothpastes. Therefore, more in vitro/in vivo studies are required to investigate biocompatibility of individual ingredients of whitening toothpastes. Ultimately, for consumers, it is important to pay attention to the ingredients in whitening toothpastes when choosing toothpastes.

Conclusion

To the best of our knowledge, this study represents the first comprehensive evaluation of the cytotoxic and genotoxic effects of whitening toothpastes with different ingredients available on the market. The findings of this study indicate that none of the ten tested toothpastes, including one regular and nine whitening toothpastes, demonstrated any genotoxic effects. However, it was observed that whitening toothpastes containing peroxide, such as Colgate Max Expert White, Rembrandt Deeply White + Peroxide, and Splat Extreme White, showed an increase in cytotoxicity as evidenced by an increase in the number of karyorrhexis and condensed chromatin. Importantly, this increase in cytotoxicity was observed only during the period of toothpaste application, and we believe that it is not considered significant in clinical conditions. Overall, the study suggests that whitening toothpastes containing abrasives, peroxides, enzymes, or charcoal can be considered safe for usage.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Nada Zorica Vladislavic, Jasen Vladislavic, Ivana Franic, and Antonija Tadin. The first draft of the manuscript was written by Nada Zorica Vladislavic and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate All participants were introduced to the background and the study's aim and gave their informed consent in writing before inclusion in the investigation. The study protocol was approved by the Ethics Committee of School of Dental Medicine, University of Zagreb (No. 05-PA-30–9/2018), and Ethics Committee of School of the Medicine, University of Split (No. 2181–198-03–04-17–0063; No. 2181–198-03–04-20–0067), who also confirmed that the study was in full accordance with ethical principles including the World Medical Association Declaration of Helsinki (version 2013).

Consent for publication Consent to submit has been received from all authors.

Conflict of interest The authors declare no competing interests.

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Authors and Affiliations

Nada Zorica Vladislavic[1] · Jasen Vladislavic[2] · Ivana Franic[3] · Antonija Tadin[4,5]

✉ Nada Zorica Vladislavic
nada_zorica@yahoo.com

[1] Dental Polyclinic Split, A.G. Matosa 2, 21000 Split, Croatia

[2] Department of Pulmonology, Clinical Hospital Centre Split, 21000 Split, Croatia

[3] 5Th Gymnasium "Vladimir Nazor" Split, 21000 Split, Croatia

[4] Department of Restorative Dental Medicine and Endodontics, Study of Dental Medicine, School of Medicine, University of Split, 21000 Split, Croatia

[5] Department of Maxillofacial Surgery, Clinical Hospital Centre Split, 21000 Split, Croatia