ORIGINAL ARTICLE

Influence of smoking on oral cells genotoxicity after at-home bleaching using 22% carbamide peroxide: a cohort study

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Abstract
Objective This study evaluated the influence of smoking on the oral cells genotoxicity before and after at-home bleaching using 22% carbamide peroxide (CP).
Materials and methods This is a prospective observational analytics cohort study which evaluated nonsmokers (NS; $n = 24$) and smokers (S; $n = 16$) patients. At-home bleaching was performed using 22% CP gel in individual trays for 1 h per day for 14 days in both groups. Scrapped cells from marginal gums were collected before the bleaching treatment (D0-*baseline*) and 1 day (D1), 15 days (D15), and 1 month (D30) after its finishing. Cells were stained with Giemsa 10%, and the micronucleus (MN) and metanuclear alterations (MA) were counted by a trained operator in 1000 cells per patient. The collections and data analysis occurred blindly. Data was analyzed by Kruskal–Wallis, Dunn, and Mann–Whitney test ($\alpha = 0.05$).
Results MN frequency was not influenced by smoking or bleaching. An increase of MA was observed between D0 and D30 for both groups ($p < 0.001$); however, no statistical difference was found between NS and S ($p > 0.05$) in the evaluation times.
Conclusion Smoking associated with 22% carbamide peroxide gel for at-home bleaching does not show genotoxic potential analyzed by the MN counts. However, a significant increase of MA was found for smokers and nonsmokers.
Clinical relevance Despite of the increase in MA, smoking associated with 22% CP peroxide at-home bleaching showed no important genotoxic potential (MN) for oral cells. Therefore, at-home bleaching treatment is safe for nonsmokers and smokers even with a high carbamide peroxide concentration of 22%.

Keywords Tooth bleaching · Carbamide peroxide · Genotoxicity · Smokers

Introduction

At-home bleaching is reported as an efficient, lasting, and conservative procedure to manage the esthetics problems related to darkened teeth [1, 2]. The most frequently used agent is the carbamide peroxide (CP) gel with concentrations varying from 10 to 22% used in individual trays for a period that varies with the gel concentration and patients' characteristics [3, 4]. In contact with the enamel surface, the CP breaks down into urea and carbon dioxide and further on ammonia, water, and oxygen [5, 6]. The generated reactive oxygen species and some free radicals break the double bonds of the organic and inorganic particles inside the dental structure [7].

Despite the desirable bleaching effect, a genotoxic potential of the reactive oxygen has been reported in the literature [3, 5, 8, 9]. In contact with human cells, -OH radical can induce lipid peroxidation and DNA alteration followed by cell lysis and death [5]. Previous in vitro study [10] showed irreversible cytotoxic effects in cells exposed to 20% CP for 1 h. However, although no genotoxicity effect of lower concentration of CP was used [6, 11–13], a closer view of clinical studies that evaluated higher concentrated bleaching materials for at-home technique showed controversial results in terms of micronucleus frequency [11, 13–15].

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Genotoxicity is the ability of a substance to damage the human cells genetic content [16]. The micronucleus test consists in micronuclei (MN) formation count in cells and is frequently used to analyze the cytogenetic damage (such as the genotoxic effect of substances) on human target tissues such as oral mucosa, tongue, alveolar bone, gum, palate, and floor of the mouth [11, 12, 16].

Smokers are commonly discouraged to perform bleaching treatments because of potentially lower efficiency of the bleaching treatment or teeth staining in these patients and the potential genotoxic effect (increase on MN counts) of the bleaching agent and cigarettes association [2, 12, 17–19]. In fact, cigarettes can present up to 3800 components and some of them with well-known genotoxic effect [17, 20]. A previous study analyzed the potentialized cytotoxic effect of the association of 10% CP bleaching and smoking [13]. This study showed an increase on the metanuclear alterations (MA), related to cells irritability, as a transitory effect (only during treatment) and no genotoxic effect (MN counts). With higher concentrations of CP, this cytotoxic potential of smoking can be significantly increased, highlighting the importance of more studies on the area.

Given that both CP at higher concentrations and smoking have cytotoxic effects, the objective of this study is to analyze the influence of smoking on the oral cells genotoxicity after at-home bleaching using 22% carbamide peroxide (CP). The hypothesis 1 is that MA is not affected by the bleaching when compared smokers and nonsmokers. The hypothesis 2 is that MN is not affected by the bleaching when compared smokers and nonsmokers.

Materials and methods

Ethical considerations.

This study was approved by the University of Passo Fundo Ethics Committee (n. 3.095.118/2018) and registered at REBEC (Brazilian Register of Clinical trials, n. RBR-2×2czx, available at http://www.ensaiosclinicos.gov.br/rg/RBR-2x2czx/). The report followed the recommendation of the STROBE (Strengthening the Reporting of Observational studies in Epidemiology) for this study.

Study design

This is a prospective observational cohort study. The project included the collection of marginal gingiva cells to access the frequency of micronuclei (DNA damage) and metanuclear alterations (binucleate cells, nuclear buds, nuclear alterations: karyorrhexis, picnose, karyolysis, and "broken-egg"). Thus, the main target of the present investigation is to evaluate the potential genotoxic effect of smoking and 22% CP.

Sample selection

Inclusion criteria

The study included nonsmokers and smokers of at least 5 cigarettes per day for more than 1 year which have not performed bleaching treatment for at least 6 years [21]. Volunteers aged between 18 and 54 years, presenting healthy anterior–superior teeth A2 (Vita Classical Shade Guide) or darker, were included [19].

Exclusion criteria

The study excluded the volunteers using orthodontic appliances (brackets), presenting caries, restorations, loss of pulp vitality, or any alterations (endodontic treatment, tetracycline stain, imperfect amelogenesis, fluorosis) that could result in color change of the upper and lower anterior teeth. Volunteers affected by periodontitis, bruxism, cervical lesions (erosion, abrasion, or attrition), or dentin sensitivity were also excluded [22]. Additionally, systemic conditions were also part of exclusion criteria: the presence of oral neoplasm or cancerous lesions, pregnancy or breastfeeding, decompensated diabetes, heart problems, and high blood pressure. Data from volunteers that were unable to attend any of assessments periods and collections with insufficient histopathological material for analysis were excluded.

Sample size

The sample size calculation was based on the frequency of MN per 1000 cells in adults. In the pilot study, it was observed that the normal frequency of MN was about 1 ± 1.1 [15, 19, 21]. Therefore, 32 patients (16 for each group) are required to have an 80% chance of detecting, as significant at the 5% level, an increase in the primary outcome measure from 1 in the control group to 2 in the experimental group. A 75–100% increase in sample size was considered for potential losses, refusals, and difficult of collections cells.

Bleaching procedure

For at-home bleaching, alginate (Avagel™; Dentsply, Milford, DE, USA) impressions were taken to obtain upper and lower arch stone models (Asfer™; São Caetano do Sul, SP, Brazil). The customized bleaching trays (without reservoirs [23, 24]) were made pressing a soft 1-mm-thick vinyl material (FGM™, Joinville, SC, Brazil) over the stone models using a vacuum plasticizer (Bio Art™; São Carlos, SP, Brazil). The excess of material was trimmed from the tray leaving a 1 mm marginal gum covered. In a clinical appointment,

the trays fit to the arcs was checked, and the patients received two syringes of the bleaching agent gel (Whiteness Perfect 22%, FGM Joinville, SC, Brazil) containing 22% carbamide peroxide, neutralized carbopol, potassium nitrate, sodium fluoride, humectant (glycol), and deionized water. Oral and written instructions on the bleaching protocol were given to the patients: application of a small amount (rice-size) for each tooth of the bleaching gel inside the tray, in the area corresponding on the buccal face of anterior teeth; wear during 1 h for 14 days.

Training and examinations

One examiner was trained by a clinical stomatologist to collect oral cells, and other one was trained by a cell biology specialist to perform cell analysis (MA and MN counting) in a pilot study. After training, a Kappa test was performed to assess the calibration between trainers and examiners ($k = 0.90$). Cells collections, histochemical laboratorial assessments, and statistical analysis occurred blindly. For cells collection, a special mask (N95, PFF2, 3 M, St. Paul, MN, USA) was used to prevent the detection of typical cigarette smell.

Cell analysis

Before the cells collection, all volunteers performed a mouthwash with water for 1 min to eliminate debris. Scrapped cells from the upper and lower marginal gums (area with the highest contact with the bleaching agent) were collected using a wooden spatula. Cells were collected before bleaching (D0-baseline) and 1 day (D1), 15 days (D15), and 30 days (D30) after the end of the treatment.

The collected material was transferred to a falcon tube, fixed using a 3:1 (volume) solution of methanol and acetic acid (Merck KGaA™, Darmstadt, Germany). The material was centrifuged at 1000 rpm for 10 min, and the supernatant was removed, remaining only the white pallet. The fixator was applied for the second time to rinse the cells, obtaining a colorless and clean (no debris) material. The material was dripped over histologic slides, left to dry at room temperature (23 °C), and stained using 10% Giemsa (Renylab Química, Barbacena, MG, Brazil).

Micronucleus and metanuclear alterations evaluation

The criteria used for scoring micronucleus (MN) and metanuclear alterations (MA) were according to those described by Thomas et al. [25]. For each patient and each collection, 1000 cells were examined using an Olympus BX 50™ microscope (Corporation of the Americas, Center Walley, PA, USA) in a $100 \times$ magnification. The abnormalities were evaluated by assessing the staining intensity, texture, and focal plane of the nucleus. Normal cells were identified as follows: intact and relative homogeneous cytoplasm, little or no contact with adjacent cells, and an intact homogenous nucleus with a smooth and distinct nuclear perimeter [25, 26]. The results are presented as the number of cells with MN and MA per 1000 cells.

The amount of observed MA, including binucleate cells, nuclear buds, nuclear alterations type 1 (karyorrhexis), 2 (picnose), 3 (karyolysis), and 4 ("broken-egg"), were recorded [14]. MN and MA were count following the criteria [25, 26]: *Micronucleated cells* were characterized by the presence of the main nucleus and a smaller one, called micronucleus, resulting from a chromosomal fragmentation by genotoxicity. The MN cells were characterized according to the following criteria: (a) regular contour, round or elliptical, and inside the cell cytoplasm; (b) similar color to the main nucleus; (c) less than one-third of the diameter of the nucleus; and (d) completely separated from the nucleus, allowing clear identification between the nucleus and MN limits. *Karyorrhexis cells* were characterized by more extensive chromatid aggregation indicating fragmentation and nuclear disintegration in the advanced stage of cell death by apoptosis. *Pycnotic cells* were characterized by a small nucleus with condensed chromatin and intense staining. Nuclear diameter is 1/3 to 2/3 smaller than that of the differentiated cells and is related to an advanced stage of cell death by necrosis. *Karyolytic cells* have a lightly stained chromatin, related to a more advanced stage of cell death process due to necrosis. "Broken egg" cells showed the main nucleus and the nearby accessory core connected by fine chromatin filaments. The accessory core has the same morphological and coloring characteristics as the main core, but a diameter less than 25% of the core. It is believed that this type of morphology originates from the presence of dicentric chromosomes with abnormal anaphasic behavior during segregation.

Statistical analysis

A descriptive analysis was performed. Differences on age between the S and NS groups were performed using chi-square test. Shapiro–Wilk test was used to analyze data distribution. Data failed normality and equal variances test; thus, MN and MA counts of the groups were compared using Kruskal–Wallis and Dunn post-hoc tests at the evaluation times. Finally, Mann–Whitney test was used to compare the MN and MA frequency between S and NS within the same evaluation time. Data analysis was performed using STATA 14™ (Statistics/Data Analysis, College Station, Texas, USA) software with an $\alpha = 0.05$.

Results

From the initially examined 96 volunteers (and 34 S and 62 NS), 6 S and 30 NS were excluded from the study because they did not meet the eligibility criteria. Thus, the initial sample was composed of 60 participants (28 S and 32 NS). During the follow-up times, 20 volunteers were lost (Fig. 1) due to the lack of time to attend the appointments, no show, or abandonment (no answer in contact attempts). In addition, data from 4 S and 1 NS were excluded because collections had less than 1000 cells, which is insufficient for the analysis.

Demographic characteristics of the participants are shown in Table 1. No significant age differences were found between S and NS. Most of the volunteers were

Table 1 Volunteers' social-demographic characteristics

Characteristics	Groups	
	Smokers	Nonsmokers
Female (%)	9 (56%)	16 (67%)
Male (%)	7 (44%)	8 (33%)
Age (years; mean ± standard deviation)	31.6 (± 10.6)	23.3 (± 5.9)
Cigarettes/day (mean ± standard deviation)	7.6 (± 5.1)	-
Smoking years (mean ± standard deviation)	11.3 (± 10.8)	-

Fig. 1 Flow diagram of the observational prospective study, including information of the excluded volunteers
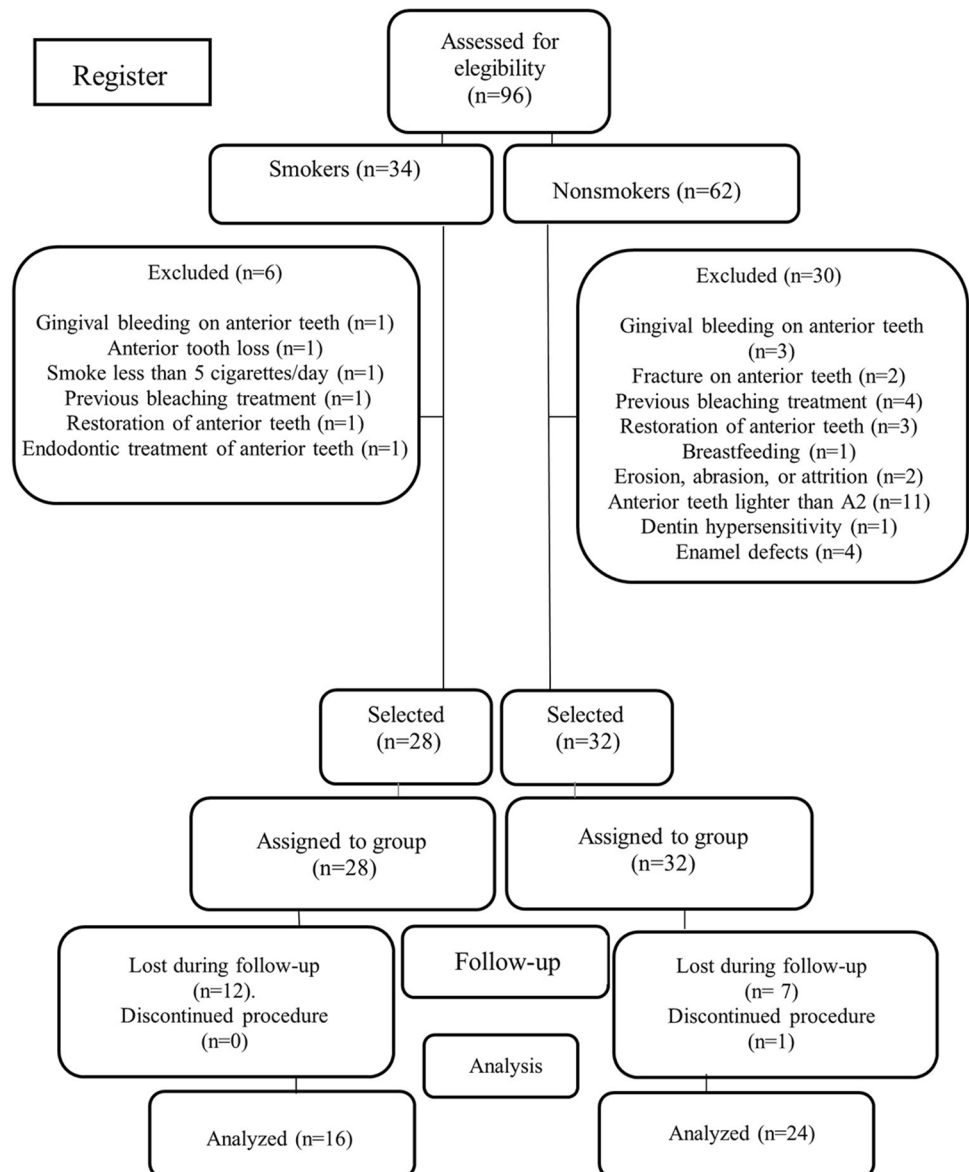
female. Smokers consumed 7.6 cigarettes per day on average.

The MA counts increased significantly at D30, compared to D0 ($p < 0.0001$) for both groups (Fig. 2), while MN counts remained the same ($p > 0.05$).

Within the same evaluation times, no differences on MA and MN were found between S and NS ($p > 0.05$) (Table 2).

Discussion

The present study showed no differences on MN and MA counts between smokers and nonsmokers neither before the bleaching treatment nor after its finishing, which lead us to not refuse the two null hypotheses. The potential genotoxic effect of the smoking and bleaching combination was probably neutralized by the eukaryotic cells [16]. As similar results were presented in previous studies [11, 27, 28] which

used carbamide peroxide, bleaching treatment is being demonstrated as safe for smokers as it is for nonsmokers.

The genotoxic potential of bleaching agents was mostly investigated by in vitro studies (cell cultures) [8] or animal models [29]. These studies use controlled peroxide concentrations (ranging from 0.03 to 30 mM) [30] resulting on a dose-dependent DNA damage increase [29, 30]. Due to the possibility of strict control of the concentration of bleaching agent in in vitro studies, most of the time, bleaching agents can be considered genotoxic in vitro but not in vivo [8]. In the clinical environment, the oral mucosa counts with many protective mechanisms, such as saliva, gingival crevicular fluid, tooth brushing, and cells exfoliation [7, 31]. In addition, oral mucosa cells present important antioxidant systems such as catalases, glutathione peroxidases (GSH-Px), and the superoxide dismutases (SODs), which are activated under oxidative stress and eliminate free radicals originated by the bleaching gel [7]. These mechanisms can contribute to reduce the genotoxicity of CP on smokers, highlighting

Fig. 2 Boxplots of metanuclear alterations (MA) counts in smokers and nonsmokers comparing the evaluated times. Different uppercase letters show statistical differences on MA counts between the evaluation times in the same group
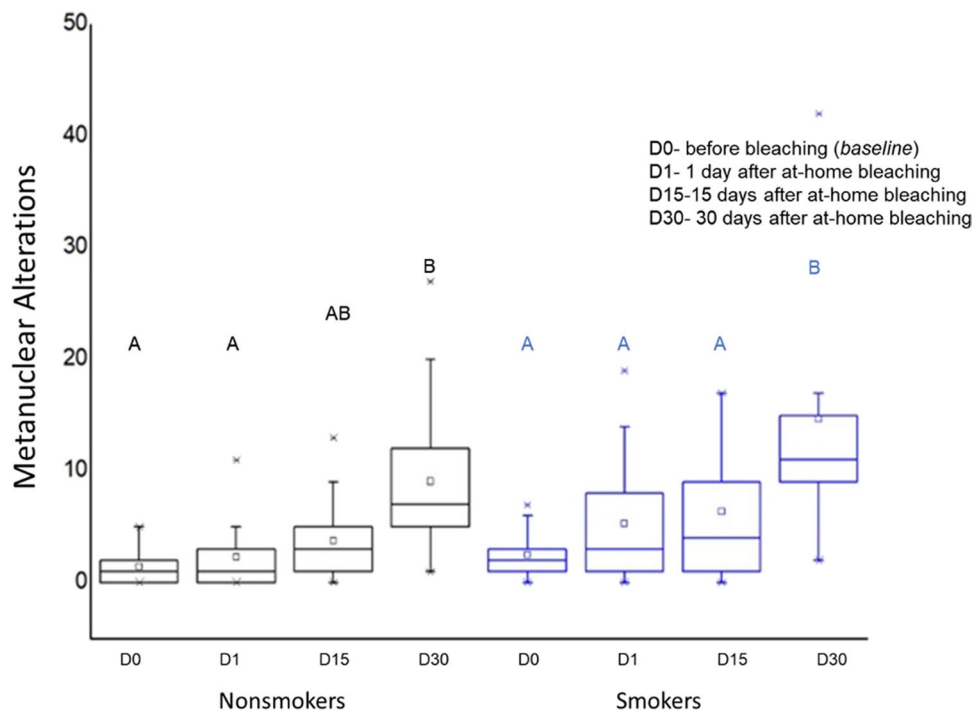
Table 2 Comparison of metanuclear alterations (MA) and micronucleus (MN) median counts at determined evaluation times between smokers and nonsmokers (*)

	Metanuclear alterations (MA)*		p-value	Micronucleus (MN)*		p-value
	Smokers	Nonsmokers		Smokers	Nonsmokers	
D1-D0	2.0 (−1.5; 6.5)	5.0 (−1.0; 3.0)	0.45	0 (0; 0)	0 (0; 0)	0.75
D15-D0	2.5 (0; 8.0)	1.0 (−1.0; 5.0)	0.41	0 (0; 0)	0 (0; 0)	0.23
D30-D0	8.5 (3.0; 14.0)	6.0 (4.0; 10.0)	0.32	0 (0; 1)	0 (0; 1)	0.82
D15-D1	−1.0 (−3.5; 5.5)	2.0 (3.0; 10.0)	0.25	0 (0; 0)	0 (0; 0)	0.63
D30-D1	6.0 (1.0; 13.0)	6.0 (3.0; 10.0)	0.25	0 (0; 1)	0 (0; 1)	0.90

*Expressed by the median of counts (first and second quartiles).

the importance of clinical studies, which considers the complexity of the oral environment.

Despite the most concerning side effect of bleaching substances and smoking is unquestionably its genotoxic potential, the presence of gingival irritation [6, 11], tooth sensitivity [24, 28, 32], and enamel hardness decrease is frequently reported in the literature [33, 34]. Soft tissue irritation can occur due to an inadvertent gel extrusion from the bleaching tray [11, 35], which is made of a flexible silicon material and is extended in up to 1 mm over the gingival margin [23]. This unintentional contact of CP with the oral cells can result in injuries and ulcerations responsible for DNA interaction and oxidative damage changing the genetic content repair ability [6, 11, 12, 36].

The MN test was previously being used to evaluate the genotoxicity of CP and smoking [12, 15, 17, 18, 20], showing a potential genotoxic effect when these factors are evaluated separately. Both agents have the potential to cause genetic damage that could lead to cell cycle impairment, cell death, and malign neoplasms [16, 35]. It seems quite reasonable that the safety of the combination of both substances (CP and smoke) needed to be investigated. Previous study reported that in normal oral cell, up to 2.0 MN/1000 cells (1.7%) can normally be present [37]. In the present study, both evaluation groups showed MN counts lower than this normal value, suggesting no genotoxicity of bleaching with 22% CP and smoking on the tested population and agreeing with data from the literature which report average MN counts/1000 cells between 0.4 and 2 [11, 15].

Comparing MN and MA frequency in smokers and nonsmokers, the present study showed that smoking, associated or not to 22% CP, did not show genotoxic effect on oral mucosa cells, differently from previously published report [12]. Distinct results obtained by the studies could be attributed to variations in the daily amount of cigarettes consumed, which is associated to MN increase in exfoliated mucosa oral cells comparing smokers to nonsmokers [37]. The volunteers of the present study used to smoke approximately a half of the cigarettes (7.6) of the previous study volunteers (13.2) [12], which could have reduced the genotoxic effect of tobacco and other substances since this is a dose-dependent response [29, 30].

The present study demonstrated no genotoxic effect (MN counts) of smoking and bleaching, but a significant increase of metanuclear alterations (MA) was observed from the treatment start to 30 days after bleaching. MA counts represents less serious changes in oral cells (death by necrosis or apoptosis) than MN (loss of genetic material and atypical mitosis) for cancerization but can represent an irritation of oral cells [15]. Previous in vitro studies have investigated cells proliferative response (normal epithelium response) to the oxidative stress potentially caused by the whitening products. Such approaches demonstrated that a similar cell

irritation response occurs after carbamide and hydrogen peroxides gel exposure, characterized by morphological alterations of the oral mucosa tissues, increased cells proliferation and apoptotic cells (cells death) in multiple layers of the epithelium (including basal and suprabasal layers) after exposure to whitening products [35, 38]. In addition, no differences on cells irritation were found between smokers and nonsmokers [38], agreeing with the present study findings.

Most of the studies on CP genotoxicity observed inexpressive MN counts during and after bleaching (no genotoxic effect) [6, 11, 12, 27]. A previous study of our group [15] evaluated MA counts during and after at-home bleaching using 22% CP. The frequency MA significantly increased during the treatment and reduced to baseline values 30 days after the bleaching conclusion. In the present study, however, MA frequency showed no reduction and continued to increase in S group by 30 days after finishing the bleaching treatment ($p < 0.0001$), highlighting the importance of extended periods of evaluation. Based on these data and the incipient literature reporting the genotoxic effect of the combination of 22% CP and smoking, further studies should be conducted with longer follow-up times to reach the effect of cells exfoliating and turnover times [39]. While morphologic alterations, cells proliferation, and induction to apoptosis were seen in the oral mucosa cells exposed to whitening products, it is important to notice that previous studies have demonstrated that the tissues maintained their viability [15, 35, 38]. Oral mucosa cells continually proliferate and induce cells death to maintain their barrier function in response to injury [35, 38]. Therefore, it may be speculated that the metanuclear alterations and micronucleus incidence would fall to reference level after stop of the at-home bleaching. Our first study design included larger follow-up times (2, 3, and 6 months after bleaching); however data collection was interrupted due to the COVID-19 pandemic, and only data from evaluation times up to 30 days after bleaching were included in the analysis.

Conclusion

The smoking habit associated with 22% carbamide peroxide gel for at-home bleaching does not show genotoxic potential when MN was evaluated. However, a significant increase proportional to evaluating times of MA was found for smokers and nonsmokers subjects.

Author contribution Conceptualization: Paula Benetti, João Paulo De Carli, Kauê Collares.

Study design/methodology: Paula Benetti, João Paulo De Carli, Kauê Collares.

Data collection: Rodrigo Rohenkohl Silva, Júlia Vanini, Júlia Stephenie Presotto, José Eduardo Vargas.

Formal analysis and investigation: Kauê Collares; Rodrigo Rohenkohl Silva; Alessandro Dourado Loguercio.

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All the mentioned authors had an important intellectual to the conception and design of the work and to the execution of the protocols of the research, data acquisition and analysis, interpretation of the results, and writing and critically revising the manuscript. The authors agreed in all aspects of the work, ensuring that questions related to the work were appropriately investigated and resolved. In addition, the authors approve the presented version of the manuscript.

Declarations

Ethical approval This study was approved by the University of Passo Fundo Ethics Committee (n. 3.095.118/2018) and registered at REBEC (Brazilian Register of Clinical trials, n. RBR- 2×2czx, available at http://www.ensaiosclinicos.gov.br/rg/RBR-2x2czx/).

Informed consent All the volunteers who met inclusion and exclusion criteria signed an informed consent form.

Conflict of interest The authors declare no competing interests.

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