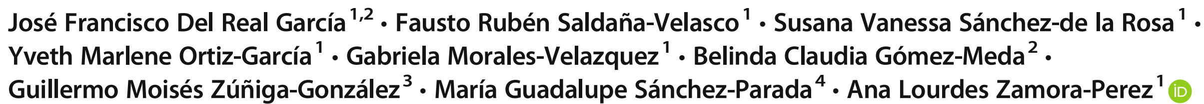
Clinical Oral Investigations (2019) 23:3033–3046
https://doi.org/10.1007/s00784-018-2716-3

ORIGINAL ARTICLE

In vivo evaluation of the genotoxicity and oxidative damage in individuals exposed to 10% hydrogen peroxide whitening strips



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Received: 18 June 2018 / Accepted: 17 October 2018 / Published online: 14 November 2018
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Abstract

Objective This study assessed the impact of 10% hydrogen peroxide whitening strip exposure on the genotoxicity and oxidative damage by means of the buccal micronucleus cytome assay by counting nuclear abnormalities (NAs) in buccal mucosa and attached gingiva cells and by analyzing in whole saliva the molecule 8-hydroxy-2′-deoxyguanosine (8-OHdG).

Materials and methods The study was conducted on 113 subjects divided into two groups: group 1 or control ($n = 53$), non-whitening strip exposed, and group 2 ($n = 60$), whitening strip exposed (Crest® 3D Whitestrips® premium plus, 10% hydrogen peroxide). Oral epithelial cells and whole saliva samples were taken at the beginning and 30 days later for group 1 and immediately before bleaching and 15 and 30 days after the end of the bleaching for group 2.

Results An increased frequency of NAs ($p < 0.05$) and higher levels of 8-OHdG ($p < 0.05$) were observed after bleaching exposure. Also, a positive correlation exists between oxidative stress produced by hydrogen peroxide and micronuclei was found.

Conclusion Individuals exposed to 10% hydrogen peroxide whitening strips exhibit NAs increased in oral epithelial cells and 8-OHdG in saliva, which is directed related to nuclear and oxidative DNA damage, respectively.

Clinical significance Hydrogen peroxide is the active agent of tooth whitening and this compound induced DNA damage. Individuals exposed to whitening strips with 10% hydrogen peroxide exhibit increased genotoxic and oxidative damage. Therefore, self-application of bleaching agents should be handled carefully since it could be a risk to human health.

Keywords DNA damage · Genotoxicity · Cytotoxicity · Nuclear abnormalities · Dental bleaching · Oxidative stress · Buccal mucosa cells · Attached gingiva cells · Saliva

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Introduction

Dental whitening (bleaching) is one of the simplest procedures for the restoration of vital discolored teeth and has reached popularity due to esthetic demand [1, 2]. Different self-applied tooth bleaching products are offered on the market, such as whitening strips, which contain a 10% hydrogen peroxide gel distributed uniformly across the strip surface [3]. Recently, there are some investigations about the genotoxic potential of dental bleaching agents. For example, some investigation analyzes the issue of oral mucosal damage and carcinogenicity relating to the use of hydrogen peroxide in the mouth for tooth whitening [4, 5]. In vitro studies have demonstrated that commercial dental bleaching agents were genotoxic [6–8], as well as in vivo studies in human oral mucosa cells exposed to whitening products, the micronucleus number increased [9, 10] and also it has observed tissue

damage, from direct contact with the oral epithelial mucosa and the whitening product [11]. Hydrogen peroxide is the active agent of tooth whitening and it is known that this compound presents genotoxic effects due to free radical formation [12]. Hydrogen peroxide can interact with DNA by means of the increase of reactive oxygen species and free radical concentration leading to oxidative DNA damage [12, 13]. Oxidative DNA damage includes the oxidation of nucleosides, which could cause DNA strand breaks (Fig. 1) [14, 15], failures in the DNA repair system, and apoptosis, and this type of damage could have carcinogenic consequences [16]. Due to its genotoxic potential, hydrogen peroxide has been shown to induce an increased frequency of nuclear abnormalities (NAs) in buccal mucosa cells [10, 17]. Buccal micronucleus cytome (BMCyT) assay is an alternative in vivo method for evaluating the genotoxic and cytotoxic effects in epithelial cells and is used as a biomarker of exposure to genotoxic agents in human population [17–20]. In this assay, the size, density, and distribution of chromatin experiment change, which lead to DNA damage. NAs in exfoliated cells include micronuclei (MN) caused by a loss of genetic material, binucleated cells (BN) initiated by a failure in cytokinesis, nuclear buds (NBs) triggered by gene amplification or excess of DNA that has been expelled from the main nucleus, karyolysis (KL) defined as loss of integrity of the nucleus, karyorrhexis (KR) known as nucleus fragmentation, abnormally condensed chromatin (CC) defined as a nucleus with a striated pattern, and pyknosis (PYK) or small nucleus. MN, BN, and NBs are considered genotoxic markers and KL, KR, CC, and PYK are associated with cytotoxicity events [21, 22].

On the other hand, a high concentration of reactive oxygen species that exceeds the antioxidant capacity of the organism results in oxidative stress, considered an imbalance between oxidants and antioxidants species in the organism [23–25] which has been associated as an indicator of health status, or exposure to external stressors [26].

An important biomarker of oxidative stress is 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is formed through oxidation of guanine from damaged DNA [24, 25] and increased levels have been reported in body fluids and tissues in diseases, including diabetes mellitus [27], cancer [27, 28], rheumatoid arthritis [29, 30], and periodontitis [19].

The aim of this work was to evaluate the genetic damage caused by the exposure of 10% hydrogen peroxide whitening strips. The BMCyt assay was used for detecting the possible genotoxic and cytotoxic effect of the whitening strips by counting NAs in oral epithelial cells. Oxidative damage was assessed by 8-OHdG quantification in whole saliva. The null hypotheses of this in vivo study were that in individuals exposed to 10% hydrogen peroxide whitening strips:

1. The number of NAs increases significantly in buccal mucosa and attached gingiva cells.
2. The levels of 8-OHdG increase significantly in saliva.

Materials and methods

Study population

The study was conducted on 113 unrelated, healthy adults who voluntarily participated in the study on the basis of informed consent. The selected participants (males and females) aged between 19 and 26 years old (22.80 ± 2.75) had a similar socioeconomic background. All participants were in good health, with good oral hygiene and no periodontal disease or gingival irritation. Pregnant women or nursing, alcohol consumers, smokers, and individuals with stained teeth by tetracycline or fluorosis, with previous tooth bleaching treatments and individuals with any systemic disease, or those who were on long-term medications that could be confounding factors were excluded from the study. Exclusion criteria also included subjects with rheumatic diseases, diabetes, chronic liver diseases, and cancer and those who had recently undergone radiological procedures (< 2 months). All participants used the same toothpaste for oral hygiene during the study.

This study was approved by the Medical Ethics Review Committee at the Universidad de Guadalajara, Guadalajara, Jalisco, Mexico. All participants provided their written

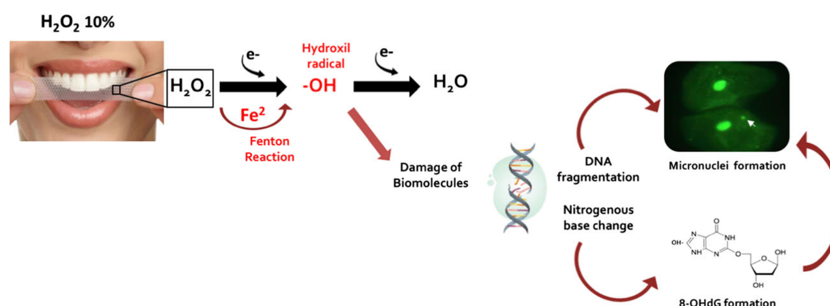

Fig. 1 Genotoxicity and oxidative damage caused by hydroxyl radicals. Hydrogen peroxide (H_2O_2) due to the Fenton reaction generates the release of hydroxyl radicals (–OH), which damage biomolecules such as DNA, resulting in fragmentation of DNA or change in nitrogenous bases originating micronuclei and 8-OHdG formation

approval before participating, according to the Mexican General Health Law Official Mexican Standard (NOM-008-SSA2–1993), Nutrition, Growth and Development Surveillance for Children and Adolescents.

Participants were divided randomly into two groups: the control group, $n = 53$, non-whitening strip-exposed group (0% hydrogen peroxide gel on flexible strips; 30 min twice daily over a 10-day period) and the exposed group, $n = 60$, whitening strip-exposed group, where subjects used the 10% hydrogen peroxide whitening strips (Crest® 3D Whitestrips® premium plus) following the manufacturer's instructions, which specified wore strips for 30 min twice daily over a 10-day period.

Samples from buccal mucosa and attached gingiva cells and saliva were taken from all participants. In the non-whitening strip-exposed group, samples were collected at the beginning of the experiment and 30 days later and in the whitening strip-exposed group, samples were taken immediately before bleaching and 15 and 30 days after the end of the bleaching.

Buccal micronuclei cytome assay

Cells samples were collected using a pre-coded slide from each participant and from two locations: buccal mucosa and attached gingiva. Subjects were asked to rinse their mouths with water, before taking the samples. Cells from the buccal mucosa were collected by scraping both cheeks with a slide with a ground edge. The obtained cells were spread directly on separate precoded slides in duplicated [22, 31].

Cells from attached gingiva were collected with a cytological brush that was rubbed gently but firmly on the surface of the attached gingiva from left to right moving across the surface gingiva, in the anterior dental area. The head of the cytological brush was stored in a sterile Eppendorf tube containing 500 μL of the physiological buffer by making circular movements to release the collected material. The procedure was repeated to obtain a duplicate. Subsequently, samples were centrifuged at 7826×g in a centrifuge Legend Micro 21R Thermo Scientific, for 10 min at 37 °C; once the centrifugation was completed, a whitish button with a foamy appearance was formed at the bottom of the tube. With a manual micropipette of adjustable volume, 450 μL of the supernatant was withdrawn to isolate the button in the remaining 50 μL. Once the button was isolated, it was resuspended to obtain a cloudy solution again and 50 μL of the tube was taken and spread on a clean and uniformly precoded slide in duplicate [32].

Cell smear samples from buccal mucosa and attached gingiva were air-dried and fixed with 80% methanol for 48 h and then stained with acridine orange (CAS no. 10127023; Sigma-Aldrich, St. Louis, MO, USA) [33]. Pre-coded slides were examined by one reader, who blindly counted NAs including MN, BN, NBs, KL, KR, CC, and PYK (Fig. 2). The criteria used for scoring NAs were according to those described by Thomas et al. [22], and the number of cells with NAs was evaluated among 2000 cells using an Olympus CX31 microscope equipped with epifluorescence and oil immersion objectives (60× and 100×; Olympus, Tokyo, Japan). The results are presented as the number of cells with NAs per 1000 cells. NAs 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 [22, 31].

Salivary 8-hydroxy-2-deoxyguanosine determination

Whole, non-stimulated saliva samples were collected in the morning under resting conditions in a quiet room. The saliva collection was performed at least 30 min after food or liquid ingestion. Participants mouths were required to rinse their mouths with water and 2 mL of whole saliva was collected in disposable tubes with a collection time of approximately 5 min. Samples were stored at − 80 °C a single freezing process until analysis. A competitive ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA) was used for determination of 8-OHdG biomarker in the saliva samples. Saliva samples were centrifuged at 10,000g for 10 min, and determination of 8-OHdG levels was conducted following the manufacturer's protocol. The DNA damage ELISA kit uses an 8-OHdG monoclonal antibody to competitively bind 8-OHdG in the sample, standard, or 8-OHdG pre-bound to the wells of a 96-well immunoassay plate. Anti-8-OHdG bound to 8-OHdG in the sample or standard were washed away, whereas those captured by the immobilized 8-OHdG were detected using a horseradish peroxidase-conjugated secondary antibody. The assay was developed with a tetramethylbenzidine substrate, and the absorbance was measured in a microplate reader at 450 nm. The intensity of the yellow color is inversely proportional to the concentration of 8-OHdG.

Statistical analysis

Power analysis indicated that with 40 participants in each group, the study would have 95% power and a 95% significance level. As this work is a pre- and post-treatment study, and in case of any dropout, 50 participants at least per group were examined for inclusion in the study. The results are expressed as means ± SD. All data were tested for normality using the Kolmogorov–Smirnov test. Differences in NAs and 8-OHdG values were evaluated using the Wilcoxon test for dependent samples and intragroup comparison. Spearman's correlation was performed to test the relationship between MN and 8-OHdG. All tests were performed using the Statistical Program for Social Sciences (SPSS v11.0) for

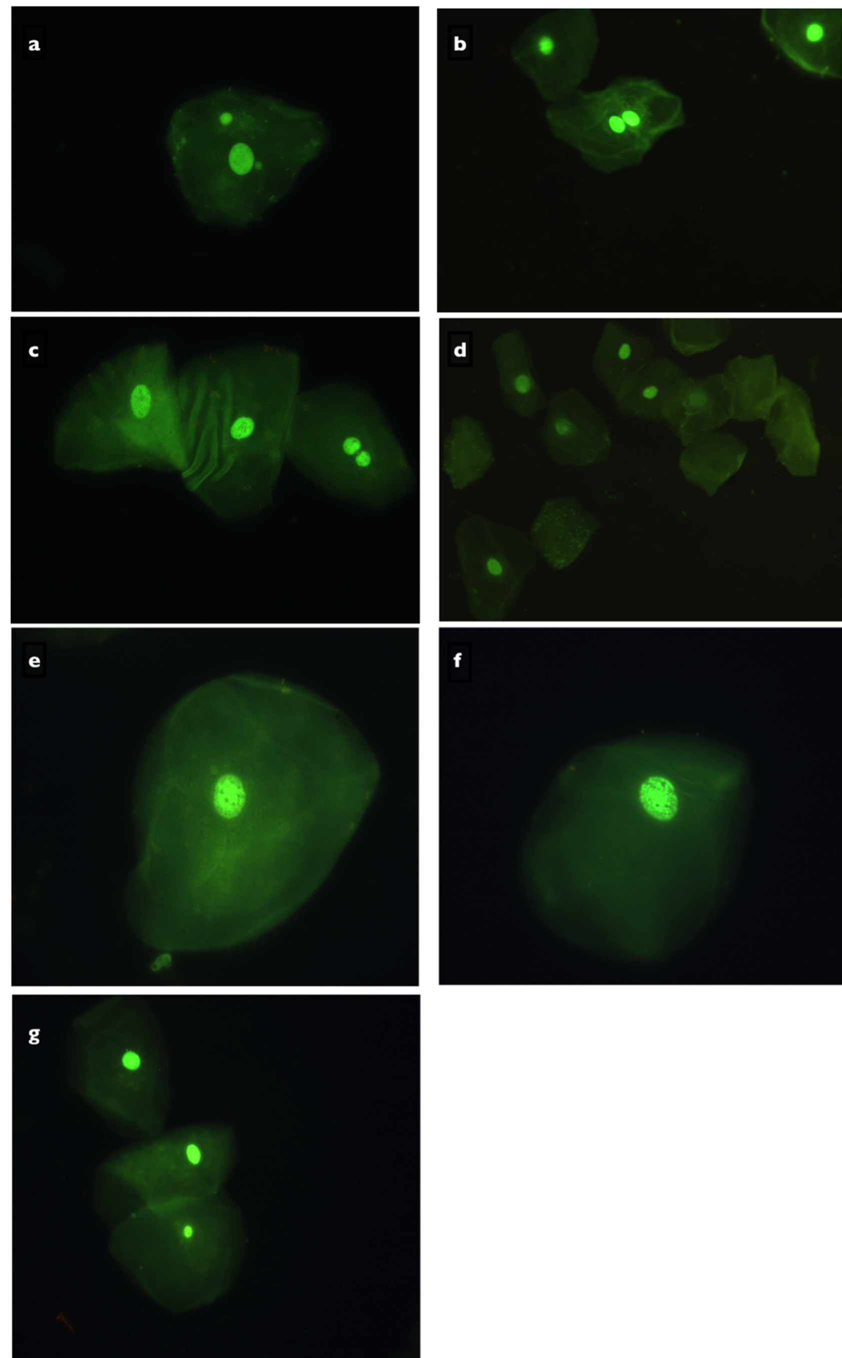

Fig. 2 Genotoxicity and cytotoxic markers from epithelial oral cells: **a** micronuclei, **b** binucleated cells, **c** nuclear bud, **d** karyolitic, **e** karyorrhectic, **f** condensed chromatin, and **g** pyknotic. Oil immersion objective 60×, acridine orange stain

Windows (SPSS, Inc., Chicago, IL, USA) where a $p < 0.05$ was considered statistically significant.

Results

Participants were classified into two groups: non-whitening strip exposed and whitening strip exposed. The demographic characteristic (age and gender) of both groups are shown in Table 1. The mean age of participants was similar between non-exposed and exposed groups. Neither age nor gender was used as a stratification variable because there were no significant differences among groups.

Genotoxicity analysis

The BMCyT assay was used to measure the genotoxicity effect in buccal mucosa and attached gingiva cells in vivo (Fig. 2). The mean frequencies of cells with NAs—MN, BN, and NBs (genotoxicity markers) and KL, KR, CC, and PYK

Table 1 General profile from participants per group

Variables	Groups	
	1: non-whitening strip exposed (%)	2: whitening strip exposed (%)
n	53 (100%)	60 (100%)
Male	30 (56.7%)	30 (50%)
Female	23 (43.3%)	30 (50%)
Age, year	21.16 ± 2.37	22.80 ± 2.75

Age is expressed as mean ± standard deviation; *n* refers to the sample size

(cytotoxicity/cell death markers), measured at the different sampling times in both groups—are shown in Figs. 3 and 4.

According to the results of the intragroup comparisons, in individuals who participated in the control or non-whitening strip-exposed group, there were no statistically significant increases observed in NAs (MN, BN, NBs, KL, KR, CC, PYK) in buccal mucosa and attached gingival cells at any sampling time (Fig. 3).

On the other hand, in the whitening strip-exposed group, the mean values of NAs obtained in buccal mucosa and gingival epithelial cells during sampling times are shown in Fig. 4. Whitening led to an increase of most genotoxic and cytotoxic markers observed in both buccal mucosa and attached gingival cells.

According to our results of the Wilcoxon test for dependent samples, intragroup comparisons, in buccal mucosa cells, the number of MN recorded 15 days after bleaching with whitening strips was on average 11.00 times higher (0.60 ± 0.43) compared to the basal value (0.05 ± 0.15; $p = 0.001$) and 10.06 times higher compared to the MN number obtained 30 days after the bleaching process (0.58 ± 0.48; $p = 0.001$). In addition, no significant bleaching effect was observed on day 30 after bleaching treatment compared with day 15 after bleaching (Fig. 4). BN cells increased significantly, 0.75-fold, after 15 and 30 days of bleaching (4.15 ± 2.21) compared with the basal values (2.36 ± 1.17; $p = 0.005$) and decreased 0.31-fold after 30 days (2.83 ± 1.39) of whitening treatment compared with values obtained after 15 days of whitening treatment ($p = 0.002$) (Fig. 4).

Furthermore, NBs increased 1.98- and 0.33-fold higher after 15 days (3.61 ± 2.21) and 30 days (1.61 ± 1.36) of whitening treatment compared to basal values (1.21 ± 1.09; $p = 0.001$ and $p = 0.003$ respectively) and decreased 0.55-fold after 30 days compared with values obtained 15 days after whitening treatment ($p = 0.002$) (Fig. 4).

Also, bleaching with whitening strips significantly increased the frequency of cytotoxic markers. The number of KL significantly increased 5.54-fold and 3.72-fold higher after 15 days (11.00 ± 4.55) and 30 days (7.93 ± 3.58) after whitening treatment compared with basal values (1.68 ± 1.10; $p = 0.001$ and $p = 0.001$ respectively). The number of cells with KR also tended to increase significantly 15 days (11.16 ± 5.73) and 30 days (7.38 ± 2.51) of whitening treatment (2.94-fold higher, $p = 0.010$ and 1.60-fold higher, $p = 0.03$ respectively) compared with basal values (2.83 ± 2.08) and the number of CC increased significantly 15 days (2.35 ± 1.06) and 30 days (1.90 ± 1.21) after bleaching (1.16-fold higher, $p = 0.03$ and 0.63-fold higher, $p = 0.04$ respectively) compared with basal value (1.16 ± 1.31) (Fig. 4). However, the number of cells with PYK tends to increase 15 days (0.70 ± 0.55) after whitening treatment compared with the basal value (0.43 ± 0.40) (0.62-fold higher, $p = 0.01$), but the increased was not high (Fig. 4).

Moreover, in attached gingiva cell samples, most of the genotoxic and cytotoxic markers tend to increase after the whitening process. In the case of genotoxic markers, the number of cells with MN, BN, and NBs increased significantly at 15 and 30 days after whitening (MN: 0.93 ± 0.46, 4.16-fold higher, $p = 0.010$ and 0.63 ± 0.43, 2.50-fold higher, $p = 0.001$; BN 3.40 ± 1.24, 0.41-fold higher, $p = 0.001$, 2.53 ± 1.25, 0.79-fold higher, $p = 0.001$; NBs: 3.03 ± 1.19, 1.46-fold higher, $p = 0.001$ and 2.00 ± 1.06, 0.62-fold higher, $p = 0.001$) compared with the baseline values (MN, 0.18 ± 0.27; BN, 1.41 ± 0.95; and NBs, 1.23 ± 0.52) (Fig. 4). However, in all genotoxic biomarkers, a non-significant bleaching effect was observed 30 days after the bleaching treatment compared to 15 days after bleaching.

Also, in the case of the cytotoxic biomarker, the number of cells with KL, KR increases significantly 15 and 30 days after whitening (KL: 106.98 ± 30.52, 0.78-fold higher, $p = 0.001$ and 88.48 ± 25.79, 0.47-fold higher, $p = 0.001$; KR: 2.55 ± 1.16, 1.29-fold higher, $p = 0.001$ and 2.33 ± 1.46, 1.09-fold higher, $p = 0.001$) compared with the baseline values (KL, 59.90 ± 12.20; KR, 1.11 ± 1.16) (Fig. 4). The number of CC observed 30 days (0.55 ± 0.67) after bleaching was 0.57 times higher compared to basal values (0.35 ± 0.69, $p = 0.05$) and the number of PYK cells observed 15 days (0.50 ± 0.36) after bleaching increased 1.17-fold compared with baseline values (0.23 ± 0.15, $p = 0.01$). Furthermore, no significant bleaching effect was observed in the number of KL and PYK 30 days after the bleaching treatment compared with measured made 15 days after bleaching in the number of KL and PYK.

Moreover, there were no significant differences between groups when we performed the comparisons of NAs basal values in buccal mucosa and attached gingiva cells (Figs. 5 and 6). On the other hand, most of NA values obtained 30 days later from the beginning of the experiment were significantly higher in the whitening strip-exposed group as compared with the control group (Figs. 5 and 6). In buccal mucosa cells, the highly significant NA values were MN (control, 0.33 ± 0.46; exposed, 0.68 ± 0.48, $p = 0.025$), BN (control, 1.1 ± 1.69; exposed, 2.83 ± 1.39, $p = 0.001$), NBs (control, 1.25 ± 1.62; exposed, 2.61 ± 1.36, $p = 0.001$), KL (control, 1.06 ± 0.16; exposed, 7.93 ± 3.58, $p = 0.001$), KR (control, 1.37 ± 0.76; exposed, 7.38 ± 2.51, $p = 0.001$), and CC (control, 1.07 ± 1.25; exposed, 2.9 ± 1.21, $p = 0.01$) (Fig. 5). Also, in attached gingiva

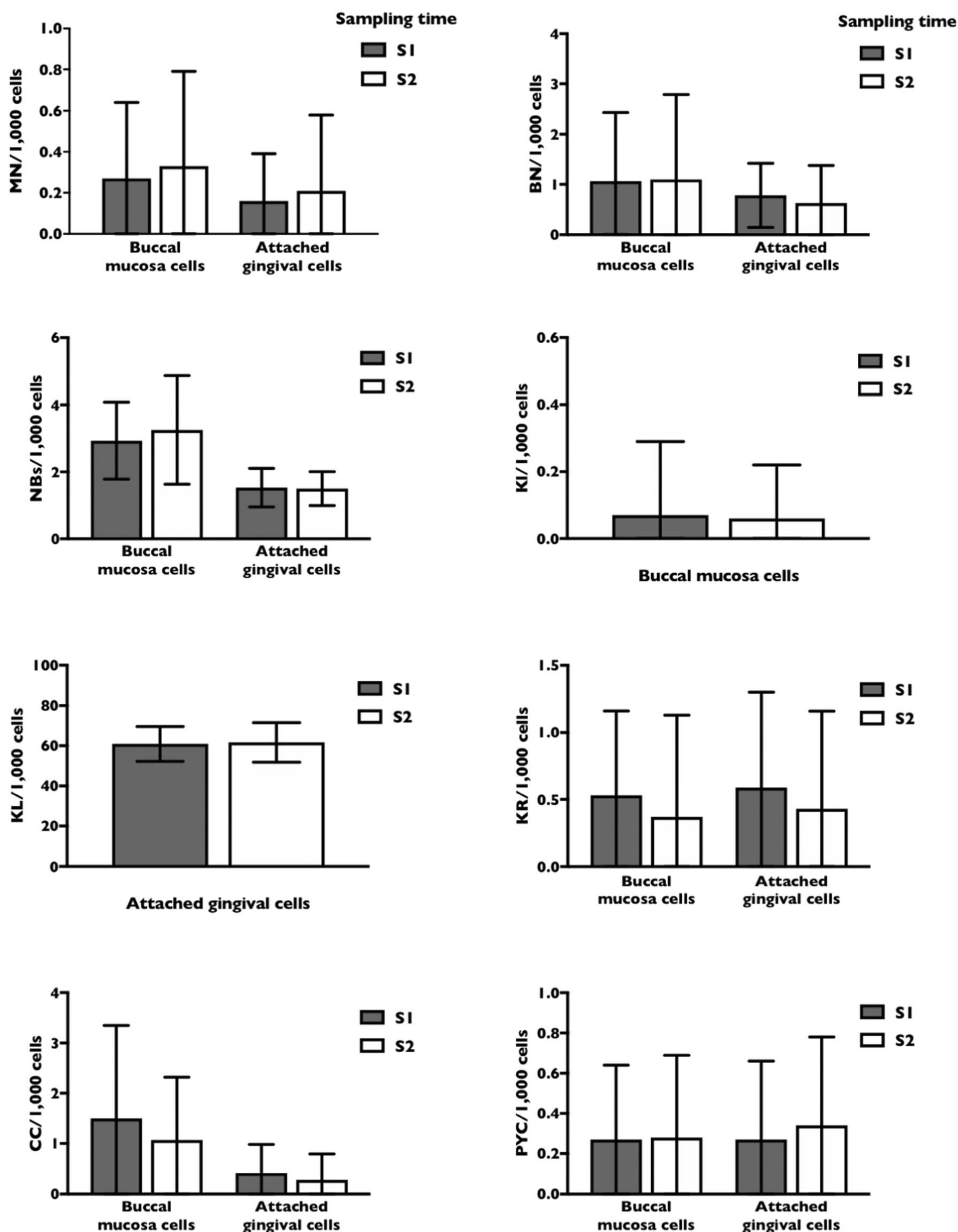

Fig. 3 Frequency of NAs in buccal mucosa and attachment gingival cells from the non-whitening strip-exposed group at the different sampling time. Participants used 0% hydrogen peroxide gel on a flexible strips 30 min twice daily over a 10-day period. Mean values are expressed as columns and error bars represent standard deviation. Sampling time: S1, basal sample (basal value); S2, sample taken 30 days after S1. MN, micronuclei; BN, binucleated cells; NBs, nuclear buds; KL, karyolysis; KR, karyorrhexis; CC, abnormally condensed chromatin; PYK, pyknotic nucleus. Intragroup comparisons were made between NAs recorded at S1 vs S2. No statistically significant increases were observed in NAs in none of the tissues sampled at any sampling time

cells, the highly significant NA values were MN (control, 0.21 ± 0.37; exposed, 0.63 ± 0.43, $p = 0.001$), BN (control, 0.63 ± 0.75; exposed, 2.53 ± 1.25, $p = 0.001$), NBs (control, 1.5 ± 0.51; exposed, 2.61 ± 1.36, $p = 0.04$), KL (control, 61.62 ± 9.86; exposed,

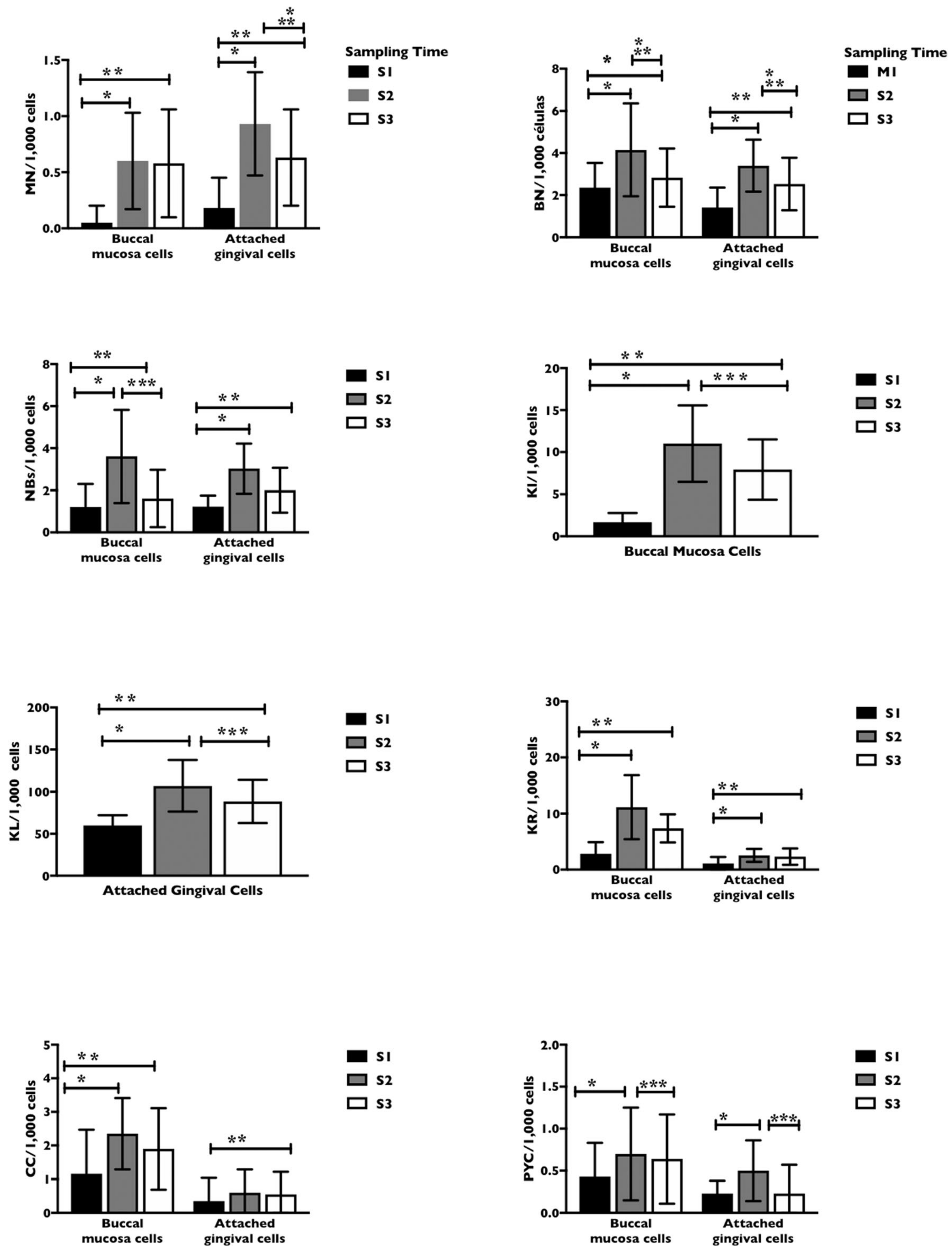

Fig. 4 Whitening strips effect on frequency of NAs in buccal mucosa and attachment gingival cells at the different sampling time. Participants used 10% hydrogen peroxide whitening strips 30 min twice daily over a 10-day period. Mean values are expressed as columns and error bars represent standard deviation. Results are presented as number of cells with NAs per 1000 cells. Sampling time: S1, basal sample before whitening process (basal value); S2, sample taken 15 days after whitening process; S3, sample taken 30 days after the whitening process. Intragroup comparisons were made *S2 vs S1, **S3 vs S1, and ***S3 vs S2. Statistical significance was considered with a p value < 0.05. *Statistically significant differences. MN, micronuclei; BN, binucleated cells; NBs, nuclear buds; KL, karyolysis; KR, karyorrhexis; CC, abnormally condensed chromatin; PYK, pyknotic nucleus

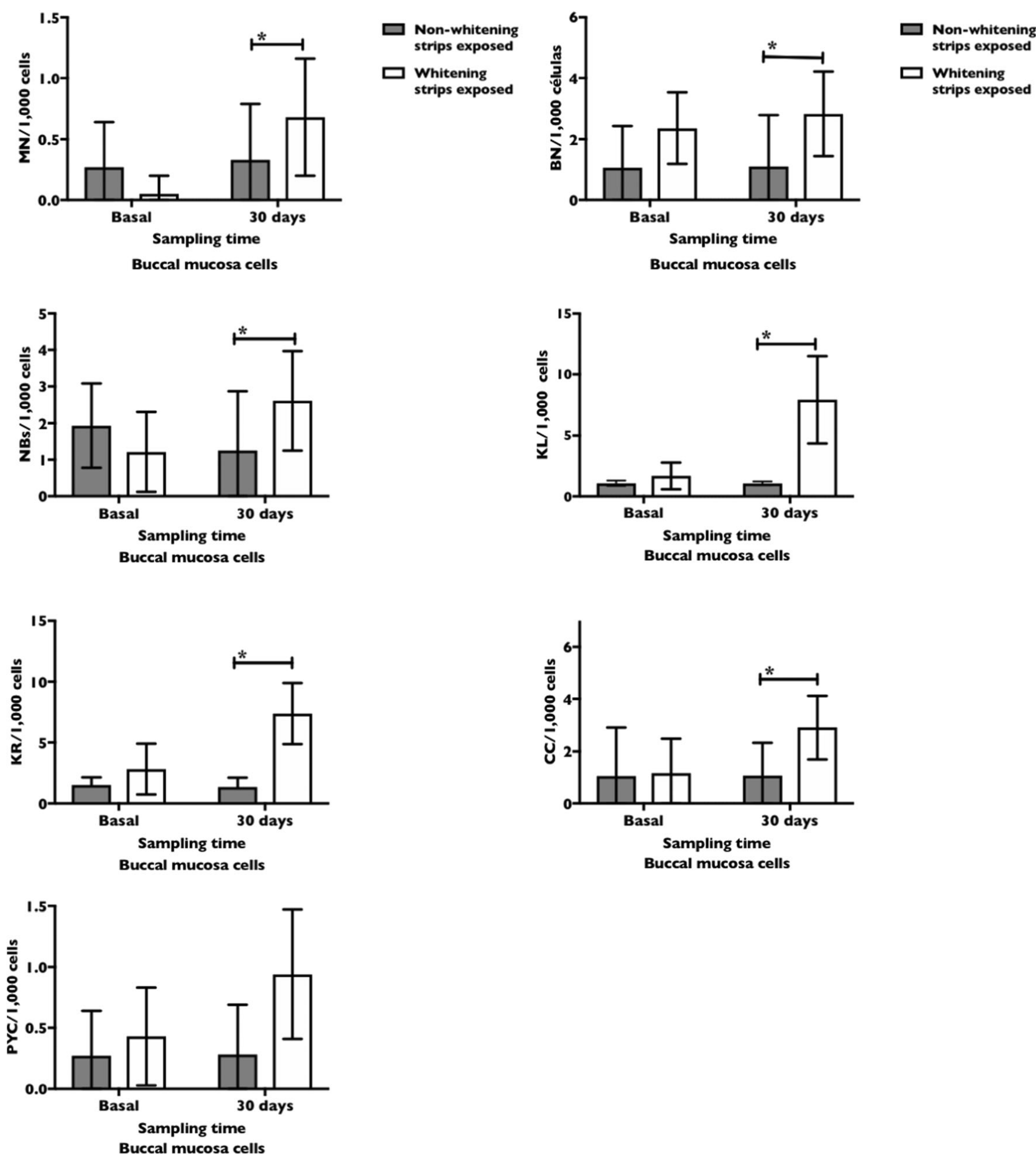

Fig. 5 Intergroup comparisons of NAs in buccal mucosa cells between groups. Mean values are expressed as columns and error bars represent standard deviation. Results are presented as number of cells with NAs per 1000 cells. Differences in NA values were evaluated using Mann–Whitney's *U* test for intergroup comparison (non-whitening strip exposed vs. whitening strip exposed). Statistical significance was considered with a p value < 0.05. *Statistically significant differences. MN, micronuclei; BN, binucleated cells; NBs, nuclear buds; KL, karyolysis; KR, karyorrhexis; CC, abnormally condensed chromatin; PYK, pyknotic nucleus

106.98 ± 30.52, $p = 0.001$), and KR (control, 0.43 ± 0.73; exposed, 2.33 ± 1.46, $p = 0.001$) (Fig. 6).

Oxidative stress

Oxidative DNA damage was determined by quantifying the 8-OHdG enzyme in whole saliva samples from all participants. Intragroup comparisons showed that 8-OHdG levels did not increase significantly in the non-whitening strip-exposed group at any sampling time (Fig. 7). Meanwhile, in the whitening strip-exposed group, 8-OHdG levels increased significantly 1.85 times higher 15 days (3.97 ± 0.79 ng/mL) after whitening and 1.50 times higher 30 days (3.48 ± 1.00 ng/mL) after whitening compared with baseline levels ($p = 0.001$) and 1.50 times higher 30 days (3.48 ± 1.00 ng/mL) after whitening compared with baseline levels (1.39 ± 0.47 ng/mL; $p = 0.001$). Also, non-significant effect of bleaching on 8-OHdG levels

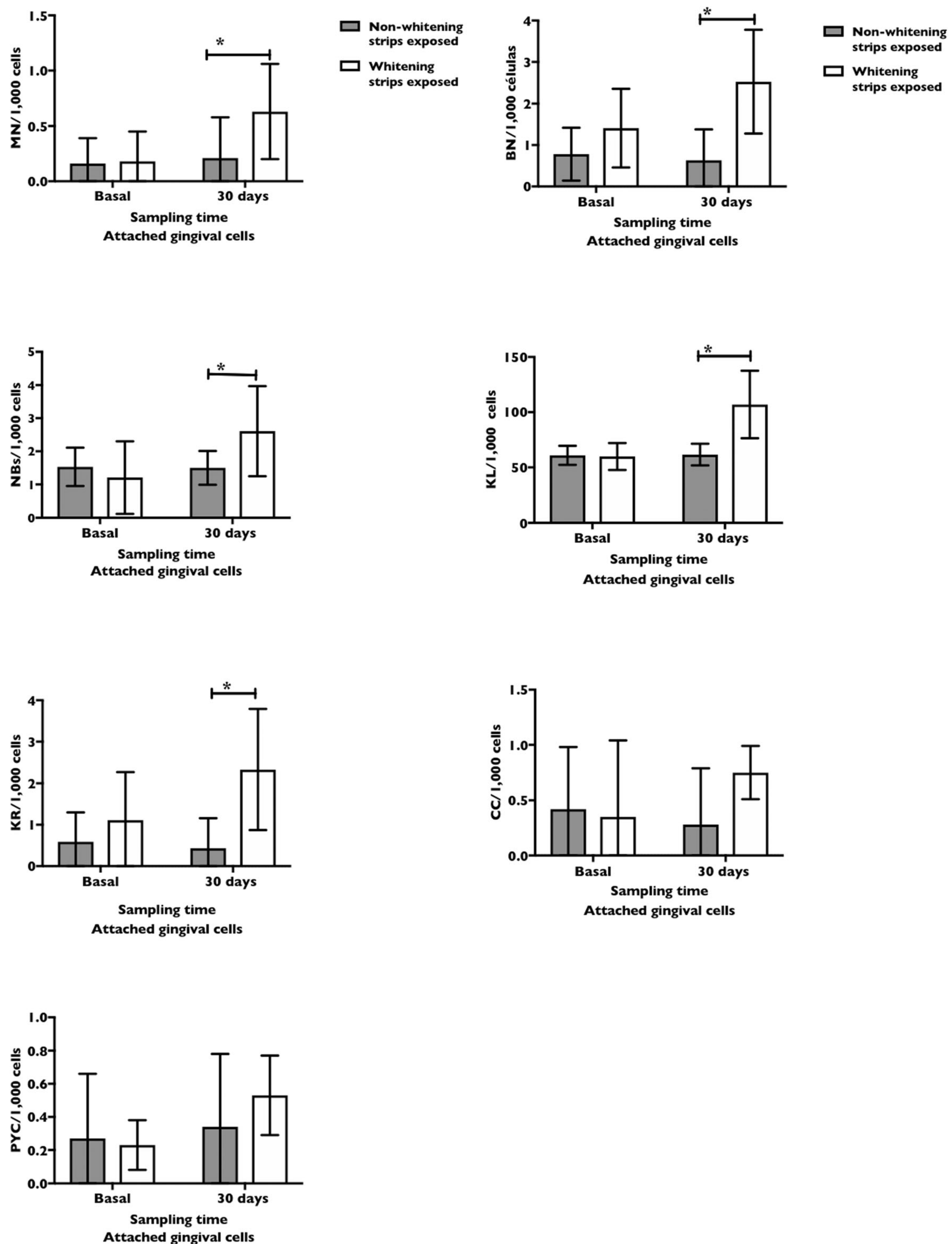

Fig. 6 Intergroup comparisons of NAs in attached gingival cells between groups. Mean values are expressed as columns and error bars represent standard deviation. Results are presented as number of cells with NAs per 1000 cells. Differences in NA values were evaluated using Mann–Whitney's *U* test for intergroup comparison (non-whitening strip exposed vs. whitening strip exposed). Statistical significance was considered with a p value < 0.05. *Statistically significant differences. MN, micronuclei; BN, binucleated cells; NBs, nuclear buds; KL, karyolysis; KR, karyorrhexis; CC, abnormally condensed chromatin; PYK, pyknotic nucleus

was observed 30 days after bleaching treatment compared to 15 days after bleaching (Fig. 7).

Non-significant differences were found between groups for the 8-OHdG basal levels (Fig. 9). By contrast, a significant

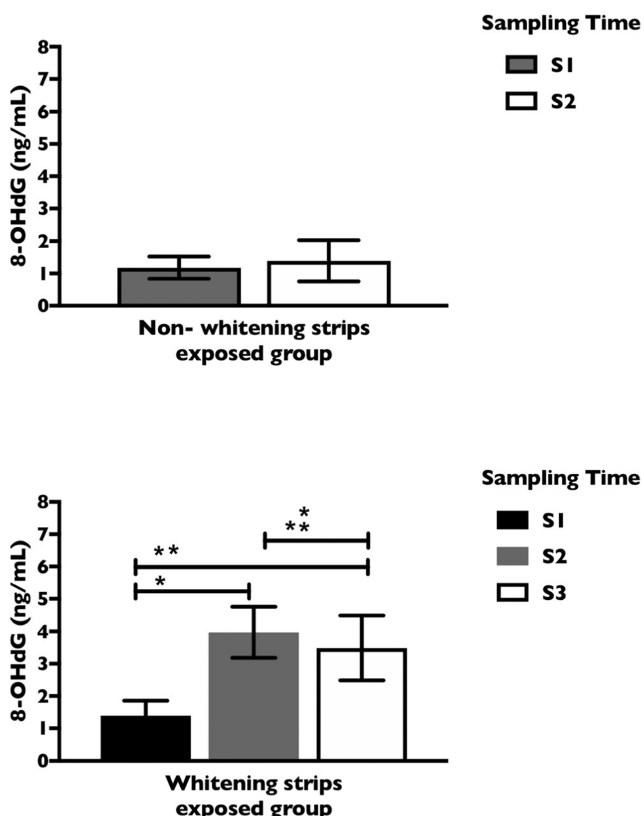

Fig. 7 Salivary levels of 8-hydroxy-2-deoxyguanosine in the study groups. Mean values are expressed as columns and error bars represent standard deviation. Results are presented as levels of 8-OHdG in ng/mL. Sampling time: non-whitening strip-exposed group, S1, basal sample (basal value); S2, sample taken 30 days after basal sample; whitening strip-exposed group, S1, basal sample before whitening process (basal value); S2, sample taken 15 days after whitening process; S3, sample taken 30 days after the whitening process. Intragroup comparisons were made *S2 vs S1, **S3 vs S1, and ***S3 vs S2. Statistical significance was considered with a p value < 0.05. *Statistically significant differences. 8-OHdG, 8-hydroxy-20-deoxyguanosine; ng, nanogram; mL, milliliters

difference between groups was observed for 8-OHdG concentration obtained 30 days later from the beginning of the experiment (control, 1.39 ± 0.65; exposed, 3.48 ± 1.01, $p = 0.001$) (Fig. 8).

We searched for a correlation between MN and 8-OHdG enzyme levels in subjects exposed to whitening strips. Statistically significant positive correlations ($p = 0.001$) were observed between MN and 8-OHdG enzyme levels with a confidence level of 95%. The fitted model explains 28.72% of the variability in MN and 8-OHdG, with a correlation coefficient of 0.53 (Fig. 9).

Discussion

Genotoxicity is the property of an agent that damages the genetic material within a cell that may cause mutations and lead to cancer. This means that in order for a chemical,

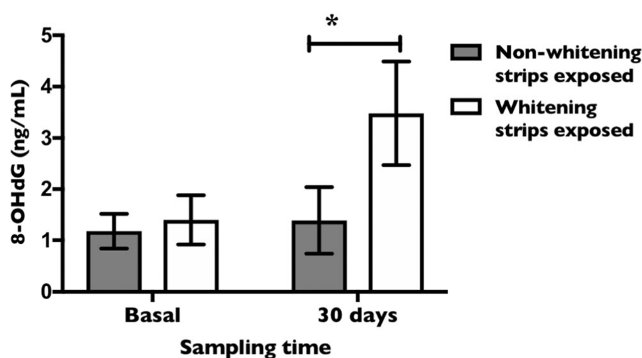

Fig. 8 Intergroup comparisons of salivary levels of 8-hydroxy-2-deoxyguanosine. Mean values are expressed as columns and error bars represent standard deviation. Results are presented as levels of 8-OHdG in ng/mL. Differences in NA values were evaluated using Mann–Whitney's U test for intergroup comparison (non-whitening strip exposed vs. whitening strip exposed). Statistical significance was considered with a p value < 0.05. *Statistically significant differences. 8-OHdG, 8-hydroxy-20-deoxyguanosine; ng, nanogram; mL, milliliters

physical, or biological agent to be considered as genotoxic, it needs to interact directly or indirectly with the DNA sequence and structure [34]. Taking in to account that DNA damage is the first mechanism that occurs during carcinogenesis, genotoxic information about dental materials is necessary for clarifying the health risk to patients and professionals [35].

This work was designed to evaluate DNA damage by assessing the increase in NAs in buccal mucosa and attached gingiva cells and oxidative damage by quantifying the enzyme 8-OHdG in whole saliva from individuals exposed to 10% hydrogen peroxide whitening strips or from a control group. The first null hypothesis was accepted because the number of NAs in buccal mucosa and attached gingiva cells was statistically increased in individuals exposed to 10% hydrogen peroxide whitening strips.

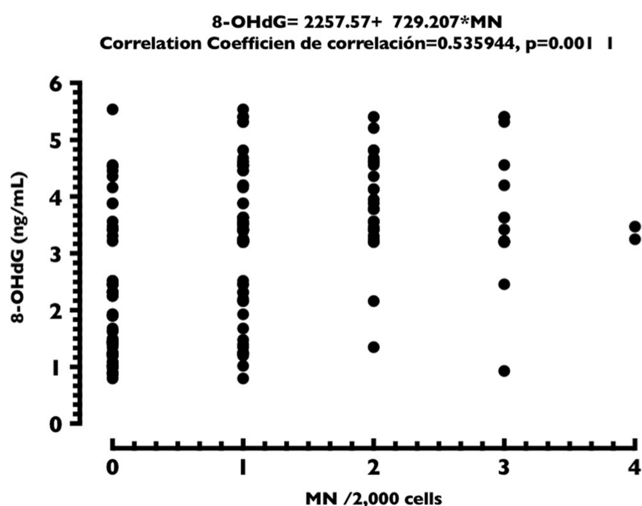

Fig. 9 Spearman's correlation coefficient between 8-OHdG and MN in the whitening strip-exposed group. The scatter plot of the correlation between 8-OHdG and MN indicates the relationships between oxidative and nuclear DNA damage

The results of the present work showed a statistically significant increase of genotoxic (MN, BN, and NBs) and cytotoxic (KL, KR, CC, and PYK) biomarkers in buccal mucosa and attached gingiva cells from individuals exposed to whitening strips (Fig. 4). Data thus indicate that exposure of 10% hydrogen peroxide whitening strip leads to chromosomal breakage or loss, gene amplification, and failure of cytokinesis as might be evidenced by the observed increased in MN, NB, and BN cells respectively. This study also showed other mechanism of damage to DNA in addition to genotoxic markers (MN, BN, and NBs), such as necrosis (KL) and apoptosis (KR, CC and PYC). Since MN, BN, and NBs may originate from different mechanisms associated to genotoxicity [31], the observed increased rate of these biomarkers in the group exposed to the dental bleaching agent indicates the harmful effect of whitening strip compounds. In this work, the increased frequency of KL, KR, CC, and PYC in the exposed group was observed, thus evidencing the cytotoxic potential of the bleaching agent.

Our results are in accordance with those reported by Klaric et al. [11] who evaluated in vivo the genotoxic effect of two bleaching agents on gingival and upper lip mucosa cells from 22 individuals. Their results showed a significant increase in MN, BN, NB, and KL frequencies where the researchers conclude that both preparations demonstrated potential genotoxic effects [10]. Unlike the present work, Klaric et al. [11] used an in-office bleaching treatment and the concentration of hydrogen peroxide bleaching agent was higher (25% and 38%). Also, one of the bleaching agents required light activation and the other chemical activation. Similarly, de Almedida et al. [36] evaluated the genotoxic effect by means of MN assay, after the exposure of two concentrations (10% and 16%) of carbamide peroxide for 21 days in gingival marginal cells from 37 participants. The authors observed an increased in MN frequency for both carbamide peroxide concentration, compared with the basal values and 15 days after the exposure [36]. However, Rezende et al. [11] evaluated in 30 volunteers the genotoxicity of in-office bleaching with 35% hydrogen peroxide in epithelial cells from the gingival and lip tissue and their results indicated that MN frequency was not increased after bleaching. The authors discussed that the absence of DNA damage after a high concentration of hydrogen peroxide exposure is due to the controlled in-office bleaching, since during home bleaching, accidental contact can occur with the highly concentrated bleaching material such as oxygen free radicals from peroxides and the buccal epithelial tissue, and this contact could increase MN frequency in the oral mucosa [11]. However, Rezende et al. [12] report that the absence of increased MN cells might be justified by considering the small number of participants compared with our work and the number cells examined from each slide and the non-specific cell stained. Another concern might be that the hydrogen peroxide concentration used was toxic, avoiding cell division and thus no MN increase was observed. Therefore, analyzing all ranges of the cytological NAs is essential in addition to MN in order to show another mechanism of genetic material damage, known as the genotoxic effect, such as necrosis, apoptosis, gene amplification, and cytokinesis defects.

On the other hand, there are some in vitro studies investigating the possible genotoxic effect of dental bleaching agent. Ribeiro et al. [8, 9] demonstrated that six commercial dental bleaching products show genotoxic effects in mouse lymphoma cells or Chinese ovary hamster cells. Also, Camargo et al. [10] evaluated the cytotoxicity and genotoxicity of whitening and common toothpaste using the MTT assay and a genotoxic effect in human gingival cells was also observed.

In the present work, three samples of oral epithelial cells (buccal mucosa and attached gingiva cells) were taken: the first before bleaching treatment (used as a control), the second 15 days after bleaching, and the third 30 days after bleaching. This sampling scheme allows to observe the potential genotoxic effect as an increase of NAs since oral epithelial cells turn over every 15 days approximately [21, 30]. Then, the genotoxic and cytotoxic effect of dental bleaching exposure was observed in buccal mucosa and attached gingival cells in samples taken after bleaching, but the most significant increase of NAs was observed in the samples taken 15 days after bleaching. This increase could be due to the exposure time to the whitening strip.

As shown in Figs. 3 and 4, the frequencies of NAs are similar in buccal mucosa and attached gingiva cells. However, a marked high number of KL was noted in attached gingiva cells even in the basal sample and also after bleaching compared with buccal mucosa cells. This could be because unlike the buccal mucosa that is a non-keratinized epithelium, the attached gingiva is a keratinized epithelium whose last layer, the stratum corneum, is composed mostly of corneocytes, without a nucleus, whereby this value is higher in gingiva tissues [37].

The buccal mucosa is a barrier for xenobiotic exposure, mostly because of its flattened surface cell layers and intracellular material [21]. Therefore, the detection of increased NA number in oral epithelial cells requires that the genotoxic agents overcome the permeability barrier and induce genetic damage that are observed as NAs in the external surface of the tissue.

On the other hand, oxidative stress contributes in many pathological conditions and diseases, including cancer and it can be measured in vivo in various sample types including saliva [28, 37]. Saliva offers an advantageous alternative because of its low cost and easy and noninvasive collection [25, 28]. There are several biomarkers for oxidative stress such as salivary 8-OHdG which is an indicator of DNA damage due to the hydroxyl radical attack at the C8 position of the nucleobase guanine or its nucleoside guanosine and this

damage, if left unrepaired, it may contribute to a mutagenicity and carcinogenesis process [24]. Also, several studies show that salivary 8-OHdG is a good biomarker for the risk assessment of various cancers, degenerative diseases, and oral pathologies [24, 25, 28]. In the present work, the results obtained for oxidative DNA damage by means of salivary 8-OHdG quantification from non-exposed and exposed individuals to whitening strip show that individuals exposed to the bleaching product present higher levels of this enzyme at different sampling times compared with 8-OHdG basal levels. Therefore, the second null hypothesis can be accepted. Furthermore, a correlation between genotoxicity and oxidative damage was performed to determine whether free radicals were the cause of DNA damage. We observed a significant positive correlation between MN and 8-OHdG in the group exposed to whitening strips, showing that these two biomarkers are related. It is well-known that 8-OHdG level increases in the mitochondria because of its high susceptibility to oxidative damage and its limited DNA repair compared to nuclear DNA, which makes it more vulnerable to damage and mutations [38–40]. On the other hand, Nomoto et al. [41] concluded that the cytoplasmic expression of 8-OHdG reflects mitochondrial DNA damage in liver disease and that nuclear DNA damage needs stronger oxidative cellular damage than mitochondrial damage. Their results indicated that mitochondrial DNA suffers oxidative stress earlier than nuclear DNA [41].

The significant positive correlation observed between MN frequency (biomarker associated to nuclear damage) and 8-OHdG levels (biomarker associated with oxidative DNA damage) are related to a more severe genotoxicity and oxidative damage in individuals exposed to 10% hydrogen peroxide whitening strips. Therefore, DNA oxidative damage is the results of the interaction of DNA with radical oxygen species specifically the radical $OH^{\cdot}$. So that, this oxidative damage produces the increased of MN frequency due to DNA strand breaks and the highest levels of 8-OHdG, which are products of DNA oxidation (Fig. 1) [27].

According to our results, an increase of NA frequency in oral epithelia cells and 8-OHdG levels in whole saliva from individuals exposed to 10% hydrogen peroxide whitening strips was observed. These results suggest that the genotoxic and oxidative effect exhibited by whitening strips could be a result of a component that may induce oxidative stress. Hydrogen peroxide that contains the bleaching strip is a reactive oxygen species and its production can be followed by the liberation of highly reactive oxygen species in the body via enzymatic and spontaneous redox reactions [7, 13]. Hydrogen peroxide is a component of living cells [6, 42] but, when exogenous hydrogen peroxide levels overwhelm cellular protective mechanisms, this molecule shows health risk effects [6, 42]. Evidence suggests that an increase in the cellular levels of hydrogen peroxide plays an important role in cancer development because this molecule is related to DNA damage, mutations, and genetic instability [5, 13].

Conclusions

Based on our results, it can be concluded that individuals exposed to whitening strips with 10% hydrogen peroxide exhibit increased in NAs in oral epithelial cells and 8-OHdG levels in saliva, which is directly related with nuclear and oxidative DNA damage respectively. Therefore, self-application of bleaching agents should be handled carefully since it could be a risk to human health.

Funding The work was supported by the Dental Institute Research, Department of Clinical Odontalgics, University of Guadalajara, Jalisco, Mexico.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the medical ethics review committee at the Universidad de Guadalajara, Guadalajara, Jalisco, Mexico.

Informed consent Informed consent was obtained from all participants included in the study.

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