Genotoxic Effect of Two Bleaching Agents on Oral Mucosa

EVA KLARIC1, MATEJ PAR2, IVONA PROFETA2, NEVENKA KOPJAR3, RUZICA ROZGAJ3, VILENA KASUBA3, DAVOR ZELJEZIC3 and ZRINKA TARLE1

1Department of Endodontics and Restorative Dentistry, School of Dental Medicine, University of Zagreb, Zagreb, Croatia; 2Private Dental Office, Zagreb, Croatia; 3Institute for Medical Research and Occupational Health, Zagreb, Croatia

Abstract. Aim: To analyze the genotoxic effect of two hydrogen peroxide-containing bleaching products on oral mucosal cells. Materials and Methods: The research was conducted on 22 individuals divided into two groups. Group 1 used ZOOM2 and group 2 the Opalescence BOOST bleaching agent. Specimens of the gingival and the upper lip mucosa were obtained before, immediately after, and 72 h after the bleaching procedure and were analyzed using a micronucleus test. Results: Seventy-two hours after bleaching treatment with BOOST, samples collected from the oral mucosa exhibited a statistically significant increase of all genotoxicity markers, with large effect sizes (Cohen’s d>0.8) observed in the total number of micronuclei (MN), number of cells with 3+ MN, karyolysis and bi-nuclear cells. ZOOM2 treatment showed a significant increase, with medium-to-large effect sizes, in the number of cells with 1 MN, karyolysis, nuclear buds and bi-nuclear cells. Conclusion: Both preparations demonstrated potential genotoxic effects.

An active agent of tooth bleaching preparations is hydrogen peroxide (1, 2). Genotoxic effects of hydrogen peroxide are the result of the formation of free radicals which can damage a number of intracellular structures (3, 4). Previous studies of hydrogen peroxide toxicity were conducted in vitro on cell cultures and in vivo in animal models. Genotoxicity of hydrogen peroxide has been demonstrated in bacterial cell cultures (5-7), as well as in eukaryotic cell cultures (8, 9). However, the addition of catalase prevented genotoxic effects in cell cultures (10). Genotoxic effects of hydrogen peroxide in vivo are questionable – the lack of genotoxic, carcinogenic or promoting activity has been demonstrated in several studies (11-13). However, some studies reported an increased incidence of duodenal hyperplasia and its malignant transformation after long-term oral ingestion of hydrogen peroxide (4-16).

Differences in the results of in vivo and in vitro studies are probably due to different experimental conditions under which cell cultures were exposed (5-16). Cells in vitro are directly exposed to high concentrations of hydrogen peroxide and lack protective mechanisms such as catalase and antioxidants. Moreover, the DNA of bacterial cells is located in the cytoplasm and is hence more susceptible to damage caused by free radicals than is eukaryotic DNA, which is protected by a nuclear membrane and whose integrity is protected by effective repair mechanisms. Furthermore, cells under physiological conditions are not exposed to such high concentrations of hydrogen peroxide and also have a higher activity of enzymes that neutralize free radicals (4). For all these reasons, cells in their natural in vivo environment are much more capable of resisting the genotoxic effect of free radicals than are cells in culture.

Previous clinical research of the side-effects of dental bleaching has mostly focused on postoperative sensitivity and gum irritation, while data on genotoxicity are lacking (17). Although gum irritation occurs frequently, it is not considered a risk factor for development of oral cancer (17). There is also no evidence of any cancer-promoting activity of hydrogen peroxide in smokers and people who consume large amounts of alcohol (18).

To date, although scarce, in vivo studies have not clearly revealed any genotoxic effect of hydrogen peroxide. Clinical studies of hydrogen peroxide contained in tooth bleaching products are not found in the available literature. Regarding the available data on genotoxicity and carcinogenicity of hydrogen peroxide, the International Agency for Research on Cancer (IARC) 1999 made the following conclusions: there is inadequate evidence in humans for the carcinogenicity of hydrogen peroxide, and there is limited evidence in experimental animals for the carcinogenicity of hydrogen peroxide. Accordingly, the IARC classifies hydrogen peroxide as not classifiable as to its carcinogenicity to humans (18).
The micronucleus test on human oral epithelial cells is a minimally-invasive and relatively simple cytogenetic technique that can detect damage to the genome in primarily exposed cells of living organisms in vivo. In recent years, the formation of micronuclei (MN) in oral epithelial cells is increasingly being used as a biomarker of exposure to various genotoxic agents and a significant correlation with the risk of cancer has been demonstrated (19-24).

The aim of this study was to investigate the possible genotoxic effect of two bleaching products. The null hypothesis tested was that there is no genotoxic effect on oral mucosa cells caused by using two different in-office dental bleaching treatments.

Materials and Methods

The study was approved by the Ethics Committee of the School of Dental Medicine, University of Zagreb (study approval no. 565-119). The study was conducted on 22 individuals who volunteered in the procedure of tooth bleaching on the basis of informed consent. The selected individuals were younger people, both males and females, between 18 and 25 years of age (mean=22.8 and SD=1.3 years), without any contraindication for tooth bleaching. They had good oral hygiene, were free of periodontal disease and gingival irritation, had healthy teeth without major restoration and were free of cervical lesions. All patients were non-smokers. Patients were excluded from the study if they were pregnant or nursing, had severely-stained teeth (tetracycline stains, fluorosis, endodontic treatment), and had previously undergone tooth bleaching procedures. Participants were randomly assigned equally to two groups according to the bleaching agent used. In the first group, bleaching was carried out using ZOOM2 (Discus Dental, Culver City, CA, USA) which is based on 25% hydrogen peroxide and is supported by light activation. In the second group, Opalescence BOOST (Ultradent Products, South Jordan, UT, USA) based on chemically activated 38% hydrogen peroxide was used. Cell samples were collected using the swab technique from each participant and from two locations: from the gingival area (the first sample) and from the upper lip lining (the second sample). Each of these samples was collected thrice: immediately before, immediately after and 72 h after the bleaching. One participant dropped out before the end of the experiment and was excluded from the analysis. In total, the analysis included 66 measurements for BOOST and 60 measurements (due to drop-out) for ZOOM2 treatment for each of the genotoxicity markers analyzed.

Prior to collecting the first cell sample, the participants rinsed their mouth with water. The surface layer of dead cells from gingiva in the frontal region was removed using sterile gauze and the sample was collected using a cytological brush. The sample was then stored in a sterile eppendorf tube containing a buffer solution (0.1 M Na2EDTA, 0.02 M NaCl, 0.01 M Tris-HCl, pH 7.0) and refrigerated to +4°C. Cell samples from the upper lip lining were collected in the same manner. Teeth were cleaned using Proxyl prophylactic paste (RDA 7) (Ivoclar Vivadent, Schaan, Lichtenstein) and the mouth was thoroughly rinsed with water. In the first group, the retractor was set in place, the teeth were dried by air stream and the gums were isolated by protective Liquidam gel (Discus Dental) which was illuminated by the polymerization unit (Bluephase; Ivoclar-Vivadent). A 1 to 2 mm-thick layer of bleaching ZOOM2 gel was applied to the labial surfaces of teeth 14-24 and 34-44 using the brush from the original package. Teeth were illuminated by the light source for 15 min. The application of the gel was repeated three times, each application lasting for 15 min. Upon completion of bleaching, the protective gel and retractor were removed and the mouth was rinsed with water. Cell samples from gums and the upper lip lining were collected once again. Participants were scheduled for an appointment in three days, during which the final cell samples from gums and upper lip lining were collected.

Samples were obtained in the same manner for the second group treated with Opalescence BOOST gel, except that the gums were isolated by protective Opalad gel (Ultradent Products) and the gel was chemically-, not light-activated. Treatments were performed in the morning at the Department of Endodontics and Restorative Dentistry School of Dental Medicine from February until May.

On the day of collection, samples were submitted to the Institute for Medical Research and Occupational Health, Zagreb, Croatia. Upon delivery, samples were centrifuged at 2000 x g for 4 min. The supernatant was removed and the cell precipitate was resuspended in fresh buffer solution (0.1 M Na2EDTA, 0.02 M NaCl, 0.01 M Tris-HCl, pH 7.0). The centrifuging and rinsing procedure was repeated twice. Resuspended cell sediment was applied to a microscopic glass slide warmed to 37°C. Preparations were dried at 37°C for 15 min and then fixed in methanol (80% v/v) at 4°C for 20 min. Fixed preparations were dried at room temperature and stained in a 5% Giemsa solution for 10 minutes. Stained preparations were analyzed by light microscope at ×1000 magnification. The total number of micronuclei (MN) determined in cell samples was counted, as well as the number of cells with one, two, and more than three MN. The inclusion criteria were: MN was entirely separated from the nucleus or was touching the nucleus while the nuclear edge was clearly visible, and the diameter of the MN was less than one-third of the nuclear diameter and of the same color as the nucleus (21). A total of 2,000 cells were analyzed in each sample. Damaged cells and cells with morphological changes of the nuclei were not included in the analysis. Such cells were recorded separately.

Statistical analysis. Due to the high heteroscedasticity between measurements and non-normality, data were transformed prior to analysis. Positively-skewed data and Box-Cox test indicated that logarithmic transformation was appropriate. A repeated measures ANOVA was applied to the transformed data to assess differences among measurements observed at the baseline, immediately after bleaching and 72 h after bleaching. Deviation of residuals from normality was assessed by the Shapiro-Wilk test and normal probability plot. Huynh-Feldt adjustment was used for univariate effects when the assumption of sphericity had been violated. Mean differences in log-transformed data were transformed back to the original scale for reporting so that the difference between groups is expressed as a ratio of geometric means. If ANOVA residuals significantly deviated from normality, Friedman and Wilcoxon signed rank tests were used instead. Furthermore, comparison of gum and lip measurements was performed by Wilcoxon rank sum test.

The size of the population for sample selection was substantially constrained by the study requirements. Nevertheless, the main aim of the study was to address potentially large differences in values of genotoxicity markers observed in the oral mucosa, as small changes were not considered clinically relevant. The selected sample
size was therefore large enough to capture the large effects according to Cohen’s effect size conventions (25), i.e. Cohen’s $f=0.40$, with a satisfactory statistical power of 80%.

As this was an exploratory study, data were analyzed without multiplicity adjustment (26, 27). The significance level was set to 0.05. Analysis was carried out with SAS System 8.2 (SAS Institute Inc., Cary, NC, USA) and G*Power (Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany).

**Results**

The average values of genotoxicity markers measured before, immediately after and 72 h after bleaching with BOOST and ZOOM2 are presented in Figure 1. Bleaching led to somewhat higher values of most genotoxicity markers observed. The smallest change was observed in the number of cells with nucleoplasmatic bridges and nuclear buds.

According to the results of repeated measures ANOVA, based on the ratio of geometric means, the total number of MN recorded 72 h after bleaching with BOOST was on average 1.93-times higher compared to baseline ($p<0.001$) and 1.57-times higher compared to the number of MN measured immediately after the bleaching process ($p<0.001$) (Table I). Similar effects, but of a smaller sizes, were also obtained for the number of cells with one MN, two MN and 3+ MN, when bleaching with BOOST led to significant increase in the number of cells after 72 h. The number of cells with one MN was on average 1.45-times higher 72 h after bleaching compared to the baseline ($p=0.012$) and 1.28-times higher compared to the measurements made immediately after bleaching ($p=0.036$). Furthermore, bleaching with BOOST, on average, led to a 1.54- and 1.73-fold increase in the number of cells with two MN and 3+ MN measured 72 h after bleaching compared to baseline ($p=0.007$ and $p<0.001$, respectively) and to a 1.84- and 1.44-fold increase compared to the measurements made immediately after bleaching ($p<0.001$ and $p=0.007$). On the other hand, a significant increase in the number of cells with karyolysis was obtained immediately after bleaching ($p=0.005$) and was 2.22-times higher 72 h after bleaching compared to baseline ($p<0.001$). The number of cells with nucleoplasmatic bridges and nuclear buds also tended to be higher immediately after ($p=0.008$ and $p=0.007$) and 72 h after bleaching ($p=0.043$ and $p=0.003$) compared to baseline measurements, but the observed effects of bleaching were not large in size. Furthermore, treatment with BOOST on average led to 2.12-fold higher number of binuclear cells 72 h after bleaching compared to baseline ($p<0.001$) and was 1.67-times higher compared to values recorded immediately after bleaching ($p=0.002$).

Bleaching with ZOOM2 also significantly affected the values of most genotoxicity markers. The total number of MN was on average 1.42-times higher in measurements recorded 72 h after the bleaching process than immediately after ($p=0.016$) (Table I). A significant effect of bleaching with ZOOM2 was also detected in the number of cells with one MN which was 1.45 times higher 72 h after bleaching compared to baseline ($p=0.007$) and measurements recorded immediately after bleaching ($p=0.013$). Results indicated no statistically significant effect of bleaching with ZOOM2 on
the number of cells with two MN and 3+ MN. On the other hand, the number of cells with karyolysis significantly increased 72 hours after bleaching compared to the baseline (1.80-fold increase, $p=0.018$) and measurements made immediately after bleaching (1.68-fold increase, $p<0.001$).

Bleaching with ZOOM2 did not induce a significant increase in the number of cells with nucleoplasmatic bridges. However, the number of cells with nuclear buds tended to be higher 72 h after bleaching compared to the baseline measurements ($p=0.016$), but the change was not large. The same pattern was observed for the number of binuclear cells. Bleaching with ZOOM2 did not induce a significant increase in the number of cells with nucleoplasmatic bridges. However, the number of cells with nuclear buds tended to be higher 72 h after bleaching compared to the baseline measurements ($p=0.016$), but the change was not large. The same pattern was observed for the number of binuclear cells. Bleaching with ZOOM2 did not induce a significant increase in the number of cells with nucleoplasmatic bridges. However, the number of cells with nuclear buds tended to be higher 72 h after bleaching compared to the baseline measurements ($p=0.016$), but the change was not large. The same pattern was observed for the number of binuclear cells.

Comparison of gum and lip measurements revealed a significant difference in the change of number of cells with one MN from baseline to 72 h after bleaching with BOOST (Wilcoxon signed rank test; $p=0.049$). The median change in the number of cells was 1.0 for gum and 8.0 for lip measurements. Lip measurements also revealed a significantly greater change in the number of cells with two MN recorded immediately after bleaching with BOOST (median=0.9) compared to gum measurements (median=3.0) (Wilcoxon rank sum test; $p=0.022$). On the other hand, ZOOM2 treatment significantly increased the number of cells with karyolysis between gum and lip measurements. The increase from baseline to 72 h after bleaching was greater for gum (median=8.5) than lip measurements (median=1.0) (Wilcoxon rank sum test; $p=0.030$), respectively.

**Discussion**

Both bleaching treatments demonstrated a potential genotoxic effect. The null hypothesis that there is no genotoxic effect on oral mucosa cells caused by using two different in-office bleaching treatments was therefore rejected. Nevertheless, the statistically significant increase in the values of genotoxicity markers was relatively small and clinically almost negligible.

Following the bleaching, two samples of cells were collected: the first immediately after bleaching and the second 72 h after bleaching. This allows time for cells to undergo mitosis and the possible genotoxic effect to manifest as an increase in the number of MN. For the formation of MN as a morphological manifestation of genomic damage, a cell must undergo at least one cell cycle. The sample collected immediately after bleaching was used as a control since it was taken about an hour after application of bleaching.

**Table I. The statistical analysis of the effects of two bleaching treatments on genotoxicity markers.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Agent</th>
<th>Overall treatment effect</th>
<th>Pairwise comparisons of measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$p$-Value</td>
<td>Cohen’s $f$</td>
<td>Geometric mean</td>
</tr>
<tr>
<td>Total no. MN</td>
<td>BOOST</td>
<td>$&lt;0.001$</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>ZOOM2</td>
<td>0.045</td>
<td>0.44</td>
</tr>
<tr>
<td>Cells with 1 MN</td>
<td>BOOST</td>
<td>0.021</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>ZOOM2</td>
<td>0.009</td>
<td>0.53</td>
</tr>
<tr>
<td>Cells with &gt;3 MN</td>
<td>BOOST</td>
<td>$&lt;0.001$</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>ZOOM2</td>
<td>0.173</td>
<td>-</td>
</tr>
<tr>
<td>Karyolysis</td>
<td>BOOST</td>
<td>$&lt;0.001$</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>ZOOM2</td>
<td>0.017</td>
<td>0.55</td>
</tr>
<tr>
<td>Nucleoplasmatic bridges b</td>
<td>BOOST</td>
<td>0.017</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ZOOM2</td>
<td>0.562</td>
<td>-</td>
</tr>
<tr>
<td>Nuclear buds b</td>
<td>BOOST</td>
<td>0.005</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ZOOM2</td>
<td>0.046</td>
<td>-</td>
</tr>
<tr>
<td>B-inuclear cells</td>
<td>BOOST</td>
<td>$&lt;0.001$</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>ZOOM2</td>
<td>0.045</td>
<td>0.42</td>
</tr>
</tbody>
</table>

MN: Micronuclei; M1, baseline; M2, immediately after bleaching; M3, 72 h after bleaching. aReferring to the differences in the number of cells among three repeated measurements. bFriedman test with Wilcoxon signed rank test as a post hoc test applied due to the deviations from normality. cHodges-Lehmann estimate of median treatment difference with corresponding 95% confidence intervals.
preparations. This time is not sufficient for genomic damage to become manifested as the emergence of MN. Accordingly, an eventual increase of MN was expected to be found in the sample taken 72 h after bleaching, whereas in the sample taken immediately after bleaching, such an increase was not expected. Morphological changes in cell nuclei and karyolysis indicate the initiation of molecular mechanisms of cell death. Karyolysis is the morphological manifestation of necrosis. It should be noted that necrosis can be partly induced by mechanical damage to oral cells due to the swabbing technique used. Furthermore, oxidative stress can lead to genomic damage. An increased number of MN suggests that oxidative stress and damage to the genome occurred after bleaching. The number of bi-nuclear cells is an indicator of toxic effects on a cell’s protein structures, particularly of the cytoskeleton. Damage to the cytoskeleton affects cytokinesis and normal chromosome migration. As a manifestation of cytokinesis disturbance, cells with two nuclei appear (28). An increased number of binuclear cells implies that damage also affects microtubules of the mitotic spindle, along with other intracellular protein formations. Nucleoplasmatic bridges most commonly occur by fusion of chromosomes damaged in telomeric regions. The appearance of nucleoplasmatic bridges represents a significant disturbance of the integrity of the genome. Nuclear buds are morphological manifestation of the separation of amplified or heavily damaged sections of the genome from the nucleus (19, 29). As statistically significant increase in the number of nuclear buds was detected during BOOST treatment. The observed genotoxic effect on gingival and lip cells may be a result of the direct action of bleaching preparations due to inadequately set gingival protection, or of hydrogen peroxide and its degradation products released from hard dental tissues after completion of the bleaching. The latter is more likely, since no leakage of gingival protection was noticed throughout bleaching treatment.

Furthermore, it is possible that the application of gingival protection gel contributed to the observed increase in the number of MN. Light-curing resin that is used in gingival protection gels contains methacrylate monomers with a potential genotoxic effect (30). As a light-curing resin is never completely polymerized (31), a certain amount of monomer remains in contact with the gingiva for the entire duration of bleaching. The cytotoxic effect of resin composites was demonstrated on several different cells and tissues (32-34).

This study has a number of limitations. High variability present in all three samples (immediately before and immediately after and 72 h after bleaching) was inevitable, since significant differences in the number of MN among the participants were already present in basal conditions. As with many clinical studies, daily food and drink intake, as well as hygienic habits of the individual, were not fully-controlled, relying only on the subjective assessment of respondents. The observed variability is probably a result of exposure to various genotoxic factors in daily life, and reflects the pronounced sensitivity of the MN test manifested as an increase in genotoxicity markers. Fernández et al. evaluated the cytotoxicity and genotoxicity of hydrogen peroxide, carbamide peroxide, sodium percarbonate and sodium perborate on mouse fibroblasts and found that all agents exhibited a dose-dependent cytotoxicity. Hydrogen peroxide had the highest cytotoxic and genotoxic effect (35). Ceppi et al. analyzed 63 studies which used the MN test to determine the genotoxic effect of various chemicals in occupational or accidental exposure. They concluded that the MN test is a very good minimally-invasive method for monitoring genetic damage in human populations (21). Ionizing radiation plays an important role in the treatment of many neoplasias and several studies evaluated MN in buccal cells and peripheral lymphocytes of patients undergoing radiotherapy in the head and neck. An increase in MN frequency shortly after the initiation of radiotherapy was found (36-38). Lifestyle factors, which include smoking, alcohol consumption, and different types of diet, can also cause an increase in MN frequency (39-41). Today, MN in buccal mucosa cells is used to study pre-neoplastic effects by collecting the cells directly from the affected tissues. MN in buccal mucosa may predict for cancer risk for the upper aerodigestive tract, including pre-malignant stages, such as oral leukoplakia and MN can also be found in patients with diabetes mellitus (42).

It is known that some dental materials increase the frequency of MN (43) and this could also affect the results of the study. In this study the participants were chosen carefully, and five of them had only minor restorations. Therefore, subject selection was one of the most important parts of this study. Goldberg et al. reported that in clinical conditions, the daily low-level doses of bleaching product used to produce tooth whitening never generate general acute and sub-acute toxic effects. Genotoxicity and carcinogenicity only occur at concentrations that are never reached during dental treatments (44).

Since no studies evaluating genotoxicity of bleaching preparations in clinical setting by micronucleus test were found in available literature, our results cannot be compared to those of others. As this research was exploratory in nature, the findings should be considered indicative and the corresponding hypothesis should be tested in further confirmatory studies. Our study showed that the bleaching preparations caused certain damage to the genome of the oral mucosal cells, but at this point it is not possible to estimate their actual genotoxic potential. The observed increases of genotoxicity markers, although statistically significant, were relatively small in magnitude, considering high individual variability which exists under basal conditions (21). Since cells of the oral mucosa have a short lifespan, a single exposure to such mild genotoxic noxa probably has
negligible carcinogenic potential. Further research is needed to evaluate the possible genotoxicity and carcinogenicity of bleaching products in clinical setting. Since it is virtually impossible to reduce the variability of genotoxicity markers among respondents in a clinical setting, an increased number of participants is required for a more accurate and detailed assessment, as the results indicate that significant differences between surfaces do exist. Nevertheless, separate analysis of gum and lip was not performed due to the lack of sensitivity, as the study was designed to enable assessment of the influence of bleaching on oral mucosa in general. Separate assessments of gum and lip remain for future study.

**Conclusion**

Both bleaching preparations demonstrated potential genotoxic effects on oral mucosal cells. The evidence obtained suggests that bleaching treatment affects the genome of mucosal cells to a certain extent, but it is difficult to assess the clinical significance of these findings. Due to the lack of similar studies, it is not possible to discuss the ultimate genotoxic potential of bleaching preparations, although it is probably negligible compared with daily exposure to other genotoxic factors. The patterns presented here provide a framework for ongoing further research, aimed at quantifying the observed effects and their long-term consequences on cells of the oral mucosa.

**Authors Contribution**

The Authors do not have any financial interest in the companies whose materials are included in this article. The work has not been published before, is not being considered for publication elsewhere and has been read and approved by all Authors. Commercial affiliations as well as consultancies, stock or equity interests, and patent-licensing arrangements which could be considered a conflict of interest are also excluded.

**Acknowledgements**

This study was supported by the Ministry of Science, Education and Sport, Republic of Croatia (project no. 065-0352851-0410) and Croatian Science Foundation.

**References**


Received August 16, 2013
Revised September 29, 2013
Accepted September 30, 2013