

Evaluation of Genotoxicity and Efficacy of At-home Bleaching in Smokers: A Single-blind Controlled Clinical Trial

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Clinical Relevance

The results of this study indicate that 10% carbamide peroxide gel did not induce DNA damage in gingival tissue during the evaluated period. The bleaching procedure was effective in smokers.

SUMMARY

Objective: This single-blind controlled study evaluated the genotoxicity and efficacy of at-home bleaching in smokers and nonsmokers.

Methods: We selected 60 patients with central incisors A2 or darker: 30 smokers (experimental group) and 30 nonsmokers (control group). The bleaching was carried out with 10% carbamide peroxide for three hours a day for three weeks. The color was evaluated using a shade guide,

Vita Bleachedguide 3D-Master, at baseline, during bleaching (first, second, and third weeks), and one week and one month after bleaching. Smears were obtained with a moistened wooden spatula from marginal gingiva. All the cytologic smears were stained with Giemsa solution. From each slide, 1000 cells were examined under 40× magnification and where micronuclei (MN) were located, they were examined under 100× magnification. The change in shade guide units at the different assessment periods and the frequency of MN were subjected to a two-way repeated measures analysis of variance and Tukey test ($\alpha=0.05$).

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Results: In both groups we detected a whitening of approximately 4 to 5 shade guide units, without color rebound after one month ($p>0.05$). The frequency of MN was significantly higher in the experimental group than in the control group, regardless of the bleaching treatment ($p>0.001$).

Conclusion: The efficacy of bleaching does not appear to be affected by the smoking habit. Additionally, at-home bleaching did not induce DNA damage to the gingival tissue during the bleaching period.

INTRODUCTION

The demand for dental esthetics has increased the number of dental bleaching procedures among dental professionals.¹ As dental bleaching is a relatively simple procedure, dentists usually offer it to their patients² to solve discoloration problems in the permanent dentition. This procedure became more popular after the introduction of at-home bleaching in 1989.³

Although the effectiveness of this procedure is well reported in the literature,⁴⁻⁶ the clinical trials on this technique are usually performed on patients with sound teeth and who are nonsmokers. Smoking is usually included as an exclusion criterion in most clinical trials of at-home^{4,7-12} and in-office bleaching.^{1,13,14}

There are at least two reasons for this exclusion. First, cigarette smoke contains water, air, carbon monoxide, carbon dioxide, and tar. During cigarette burning, components such as tar, sugar, and cocoa are transferred to the smoke hue,¹⁵ which probably stain teeth because of their dark hue and ability to adhere to the dental surface.¹⁶

Another concern is that there are around 1.2 billion smokers in the world, and it is estimated that this habit causes more than 1 million cancer deaths per year;¹⁷ this is probably the rationale behind excluding smokers from studies of dental bleaching procedures. The prevalence of self-assessed tooth discoloration in smokers is almost twice that reported by nonsmokers,¹⁸ and therefore, they are probably the main candidates for bleaching procedures in a daily practice.

DNA damage in the cells of the oral mucosa of smokers usually signals the genotoxicity potential of the smoking habit.^{19,20} This can be indirectly observed by the increase in the frequency of micronuclei (MN) in exfoliated epithelial cells.^{21,22} During the division of the cells from the basal layer

of the mucosa, the damage to the DNA molecule leads to the formation of MN, which consist of acentric chromosomes, chromatid fragments, or whole chromosomes that failed to be incorporated in the daughter nuclei during mitosis. The formation of MN is, therefore, induced by substances that cause breakage of chromosomes (clastogens) and by agents that affect the spindle apparatus (aneugens). This usually occurs days or weeks after contact with a carcinogenic agent.^{21,22} Evaluation of the frequency of MN is a viable method for detecting risk of cancer in humans, because most tumors possess epithelial origin.²³ In regard to smoking, a positive correlation was already reported between a higher frequency of MN and tobacco users.^{20,24,25}

Although the effects of bleaching agents on hard tissues have already been extensively investigated,²⁶ the response of the soft tissue to these agents remains largely unknown in humans.²⁷ Although it was demonstrated that hydrogen peroxide can induce pathological alterations in soft tissues in animal models^{28,29} and in cell research,³⁰⁻³⁶ few studies have been conducted on humans.^{37,38} This is especially important when it comes to smokers as it is well established that they have an increased risk of developing oral cancer or other forms of epithelial cancer.³⁹⁻⁴¹ Therefore, the aim of this single-blind controlled clinical trial was to evaluate the efficacy and genotoxicity of at-home bleaching in smokers and nonsmokers.

METHODS AND MATERIALS

This controlled single-blind nonrandomized clinical trial recruited patients by printed advertising at the local universities. During the screening, dental prophylaxis was performed for dental screening and color evaluation at baseline, which usually occurred two weeks before starting the bleaching protocol.

Inclusion and Exclusion Criteria

Participants included in this clinical trial were between 18 and 40 years old and had good general and oral health. Each subject had at least one central incisor with shade 1M2 or darker, assessed by comparison with a value-oriented shade guide (Vita Bleachedguide 3D-Master, Vita Zahnfabrik, Bad Säckingen, Germany). Participants who underwent previous dental bleaching procedures during orthodontic treatment, pregnant or lactating women, and participants with bruxism habits were not included in the trial.

Additionally, participants with restorations on the labial surfaces of anterior teeth and noncarious cervical lesions, teeth veneers or full crowns, gingival recession, spontaneous tooth pain, severe internal tooth discoloration, and teeth with endodontic treatment or stains classified as 3 or higher according to the Thylstrup-Fejerskov Index⁴² were also excluded from this trial. A total of 60 volunteers signed the consent form and were enrolled in this study.

Sample Size Calculation

The sample size calculation was based on the frequency of MN per 1000 cells in nonsmokers. In the pilot study it was observed that the normal frequency of MN in nonsmokers is about 1 ± 1.1 .^{24,43-45} In order for the bleaching procedure to be considered safe, it was expected that we would find a mean difference of not more than 1.0. Thus, we needed a minimum sample size of 27 participants for a study with a predictive power of 90% and an alpha of 5%.

Experimental Groups

Participants who met the inclusion criteria were asked about their daily smoking habits. Those who did not smoke were part of the group of nonsmokers and those who smoked at least 10 cigarettes a day belonged to the group of smokers. Thirty participants were included in each group.

Bleaching Procedure

Alginate impressions were made of each subject's maxillary and mandibular arch, and these were filled with dental stone. To produce study models, no block-out material was applied to the labial surfaces of teeth.⁴⁶ A 1-mm soft vinyl material provided by the manufacturer (FGM Dental Products, Joinville, Brazil) was used to fabricate the custom-fitted tray that would hold the bleaching gel. The excess material from the labial and lingual surfaces was trimmed 1 mm from the gingival junction. The tray and 10% carbamide peroxide gel (Whiteness Perfect, FGM) were delivered to each subject, with verbal instructions for use. All subjects were instructed to wear the tray containing the bleaching agent for at least three hours a day for a period of three weeks. After the daily three-hour period, they were instructed to remove the tray, wash it with water, and brush their teeth as usual. With regard to oral hygiene, all participants were instructed to brush their teeth regularly and were asked to not use whitening toothpastes and mouthwashes containing peroxides.

Shade Evaluation

The shade evaluation was performed with a value-oriented shade guide (Vita Bleachedguide 3D-Master). Two calibrated evaluators with a previous agreement of at least 85%, as determined by weighted kappa statistics, recorded the shade of the upper central right incisor at different time assessments: at baseline, during treatment (after the first, second, and third weeks of bleaching), and one week and one month after the end of the bleaching protocol. As evaluators could guess which group the participants were from, usually by the smoking smell, this procedure could not be blinded.

The area of interest for measuring tooth color matching was the middle third of the facial surface of the anterior central incisor, according to the American Dental Association guidelines.⁴⁷ Shade changes were calculated from the beginning of the active phase to the individual recall times by calculating the change in the number of shade guide units (Δ SGUs), which occurred toward the lighter end of the value-oriented list of shade tabs. In the event of disagreements between the examiners during shade evaluation, a consensus was reached.

Tooth Sensitivity (TS) Evaluation

Subjects were instructed to keep a daily record of whether they experienced TS, using a visual analog scale.^{1,7,11,13} They were asked to place a line perpendicular to a 10-mm long line with zero at one end indicating "no TS" and the 10-mm end indicating "unbearable TS."

Sample Collection for MN in Exfoliated Epithelial Cells

Exfoliated oral mucosa was collected before and immediately after the third week of at-home bleaching. Before cell collection, the participants rinsed their mouths with tap water for one minute. Subsequently, the cells were scraped with wooden spatulas from the marginal gingiva.^{24,25,44,48} The scraped cells were placed on clean glass slides, and smears were prepared. The smear was dried with a jet of air from a triple syringe for one minute at a distance of approximately 30 cm, avoiding excessive dehydration of the cells.⁴⁸

Staining Procedures

The staining protocol was prepared immediately after the smear collection. Five to six drops of Giemsa stock solution (Cinética Química, São Paulo, Brazil) was applied directly over the slide

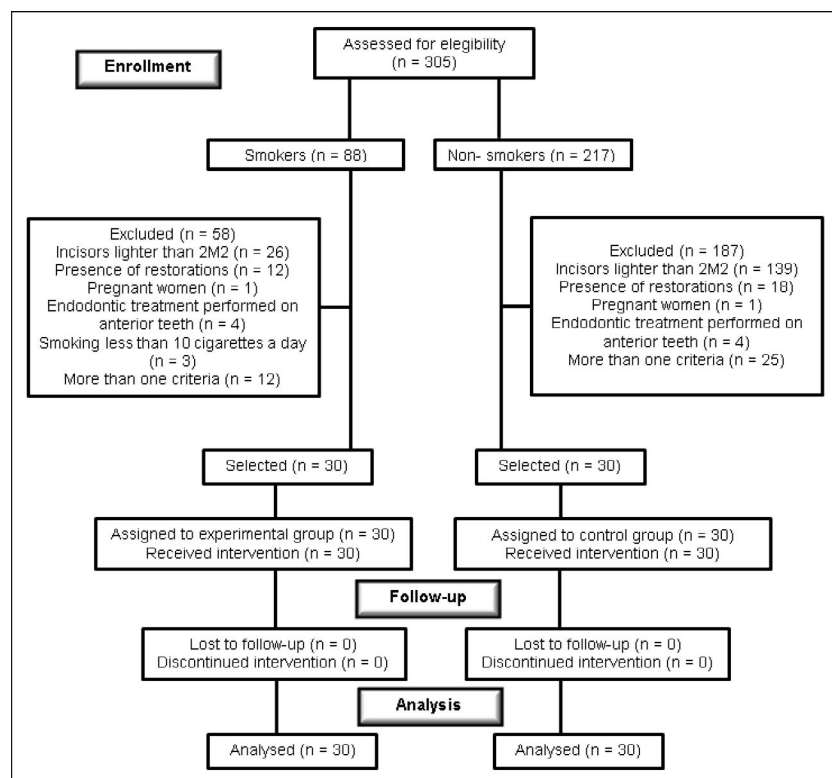


Figure 1. Study Design

for two minutes; then the slides were washed in a container with tap water (container 1 = three to four washes, container 2 = two to three washes). The differentiation of the cells was performed in a third container (1200 mL of tap water and one drop of glacial acetic acid, Vetec Quimica Fina Ltda., Rio de Janeiro, Brazil). After this process, the slide was dried for one minute in the same manner described before. Then, three drops of the adhesive Entellan (Merck KGaA, Darmstadt, Germany) were applied on the visibly dry slide for cover glass positioning.⁴⁸

Evaluation of the Slides

Two blinded examiners were trained and calibrated for the evaluation of the slides by one expert in stomatology. From each participant, at least 1000 cells were evaluated at each period with the staining procedure. Cell counting was performed under an optical microscope with 100× magnification, and when MN were found, the magnification was increased to 400× (Nikon E800, Tokyo, Japan). Criteria for inclusion in the total cell count were the following: 1) cytoplasm intact and lying relatively flat; 2) little or no overlap with adjacent cells; 3) little or no debris; and 4) nucleus normal and intact, nuclear perimeter smooth and distinct.⁴⁹

The parameters for identifying micronuclei were as follows: 1) rounded smooth perimeter suggestive of a membrane, 2) less than a third of the diameter of associated nucleus but large enough to discern shape and color, 3) staining intensity similar to nucleus, 4) texture similar to nucleus, 5) same focal plane as nucleus, and 6) absence of overlap with or bridge to nucleus.⁴⁹ Dead or degenerating cells (karyolysis, karyorrhexis, nuclear fragmentation) were excluded from evaluation. Nuclear blebbings (micronucleus-like structure connected with the main nucleus by a bridge) were also not considered.

Statistical Analysis

The ΔSGU at the different assessment periods and the data frequency of MN were tabulated using the software SigmaPlot 5.0 for Windows (Systat Software Inc, San Jose, CA, USA) and subjected to a two-way analysis of variance (ANOVA) (treatment group vs time; $\alpha=0.05$) for repeated measures (time). The Tukey test was performed for contrast of means ($\alpha=0.05$). The percentage of participants who experienced TS at least once during the bleaching therapy was determined to be the absolute risk of TS. The absolute risk and intensity of TS of both groups was compared with the χ^2 and Mann-Whitney tests ($\alpha=0.05$), respectively.

Table 1: Demographic Characteristics of the Participants

| | Nonsmokers | Smokers |
|---|----------------|----------------|
| Baseline color (SGU; mean \pm SD ^a) | 8.2 \pm 1.3 | 7.8 \pm 1.1 |
| Age (years; mean \pm SD) | 24.1 \pm 6.8 | 26.3 \pm 6.5 |
| Sex (male; %) | 53.3 | 63.3 |
| Cigarettes/day (mean \pm SD) | -- | 13.2 \pm 4.0 |
| Average smoking years (mean \pm SD) | -- | 8.0 \pm 5.9 |

^a Abbreviations: SD, standard deviation; SGU, shade guide unit.

RESULTS

A total of 305 participants in the range of 18 to 40 years old were screened to select 60 participants who met the inclusion criteria (Figure 1). The mean age and baseline tooth color of the participants were similar between groups. Most of the participants were men (Table 1). All participants attended the recall visits during the bleaching protocol. None of the patients continued bleaching as they were satisfied with the outcome reached after three weeks of treatment.

Shade Evaluation

Two-way ANOVA revealed that the interaction of treatment group vs time ($p=0.372$) and the main factor treatment group ($p=0.098$) were not significant. Only the main factor time was statistically significant (Table 2; $p<0.001$). A significant color change of approximately 4.5 to 5.0 SGUs was observed after bleaching for both groups, which was stable one month after the procedure (Table 2).

Tooth Sensitivity Evaluation

Table 3 presents data on the prevalence of dental sensitivity for the sample investigated. The risk of TS was similar between the two study groups (χ^2 , $p=1.0$),

Table 2: Means and Standard Deviations Between Assessment Points for the Characteristics of Nonsmokers and Smokers in Change in Shade Guide Units (Δ SGUs) Assessed by Means of Subjective Using Vita Bleachedguide 3D-Master^a

| Assessment Time Intervals | Δ SGU | |
|--------------------------------|-----------------|-----------------|
| | Nonsmokers | Smokers |
| Baseline vs 1 wk | 2.1 \pm 1.1 C | 1.9 \pm 1.0 C |
| Baseline vs 2 wk | 3.9 \pm 1.3 B | 3.4 \pm 1.2 B |
| Baseline vs 3 wk | 4.9 \pm 1.4 A | 4.4 \pm 1.0 A |
| Baseline vs 1 wk postbleaching | 5.0 \pm 1.4 A | 4.4 \pm 1.0 A |
| Baseline vs 1 mo follow-up | 4.7 \pm 1.4 A | 4.1 \pm 1.1 A |

^a Two-way analysis of variance and Tukey test ($p<0.001$).

Table 3: Comparison of the Number of Patients Who Experienced Tooth Sensitivity (TS) at Least Once During the Bleaching Regimen in Both Groups and Intensity of TS for Both Groups Under the Pain Scale

| Treatment | Number of participants with TS | | Absolute Risk (95% Confidence Interval) ^a | Visual Analog Scale ^b |
|------------|--------------------------------|----|--|----------------------------------|
| | Yes | No | | |
| Nonsmokers | 14 | 16 | 47 (30–64) | 0.5 \pm 0.9 |
| Smokers | 15 | 15 | 50 (33–67) | 0.7 \pm 1.2 |

^a χ^2 test ($p=1.0$).

^b Mean \pm standard deviation; Mann-Whitney ($p=0.83$).

and approximately 50% of patients had at some point in the procedure, tooth sensitivity. Similarly, the TS intensity (Mann-Whitney test, $p=0.83$) was not significantly different between groups. None of the patients from this trial gave up the treatment.

Assessment of Genotoxicity by MN frequency

The two-way ANOVA revealed that the interaction of treatment group vs time ($p=0.067$) and the main factor time ($p=0.248$) were not statistically significant (Table 4). Only the main factor treatment group was statistically significant ($p<0.001$), which means the bleaching procedure did not increase the frequency of MN. The amount of MN was significantly higher in smokers than in nonsmokers, regardless of the bleaching procedure ($p<0.001$).

DISCUSSION

Although smokers also require dental bleaching in daily practice, the literature lacks information about the efficacy and safety of the procedure in such patients. Most of the studies in dental bleaching use shade guides for color evaluation.^{4-6,12,13} Although these shade guides were primarily designed for shade matching with composite resins, their use is supported in the literature for evaluating bleaching effectiveness.^{4-6,12,13} Compared with the spectropho-

Table 4: Means and Standard Deviations of the Micronuclei Frequency per 1000 Exfoliated Buccal Mucosa Cells of Nonsmokers and Smokers.^a

| Assessment Periods | MN Frequency | |
|--------------------|-----------------|-----------------|
| | Nonsmokers | Smokers |
| Before bleaching | 1.4 \pm 2.2 A | 3.7 \pm 2.0 B |
| After bleaching | 0.5 \pm 0.7 A | 3.9 \pm 1.8 B |

^a Two-way analysis of variance and Tukey test ($p<0.001$). Same letters are statistically similar.

tometer, the shade guides show better visual correlation and have the potential to allow for more accurate monitoring, and consistent and reliable color of teeth.⁴⁹

Vita Classical (Vita Zahnfabrik)^{4,5,11,13} and Trubyte Bioform (Dentsply Intl, York, PA, USA)⁵⁰⁻⁵³ are the most frequently used shade guides in dental bleaching studies. However, they have a nonlinear color arrangement, as they were not primarily designed to evaluate dental bleaching. This is why we used the shade guide Vita Bleachedguide 3D-Master. This new shade guide is already organized from lowest to highest value; it contains lighter shade tabs with subtle color gradation and more uniform color distribution compared to Vita Classical and Trubyte Bioform scales.⁵⁰ Additionally, Vita Bleachedguide was scored as the easiest to rearrange and the most preferred for dental bleaching monitoring and other dental procedures that require shade matching.⁵⁴

An effective bleaching was observed after three weeks of treatment, which remained stable one month after bleaching. An overall bleaching of 4 to 5 SGUs herein reported is in agreement with earlier at-home studies that used 10% carbamide peroxide gel.^{4,5,9,12,55} Surprisingly, no significant difference was observed between groups. As reported in the introduction, some components of cigarette smoke are responsible for tooth discoloration in smokers; however, it appears to be superficial and easily removed with professional cleaning⁵⁶ and dental bleaching.¹⁶ Perhaps, color rebound may occur earlier in smokers than nonsmokers because of the continuous deposition of cigarette smoke components on the enamel surface. This hypothesis was not confirmed with the results of the present study because the bleaching results after one month did not show any effect in terms of color rebound. However, this is a short-term follow-up, and only long-term clinical evaluations can assess this hypothesis.

Several *in situ*³⁰⁻³⁶ and *in vivo* studies in animals^{28,29} observed different types of DNA damage by various concentrations of bleaching agents. Different *in vivo* studies in animals showed no risk involved in the bleaching procedure.⁵⁷⁻⁶⁰ However, these results cannot be directly extrapolated to humans, as they do not resemble the clinical scenario.

In light of these considerations, methods that assess the genotoxicity potential of bleaching agents under a realistic condition are essential. An increased frequency of chromosome breaks has been recently demonstrated to be an initial event in

carcinogenesis, suggesting that these alterations may play a significant role in assessing oncogenic risk.^{61,62} Among biomarkers that can be used for this purpose, the measurement of MN appears to be one of the most suitable. An increased frequency of MN in exfoliated cells from oral mucosa has served traditionally as an index for evaluating the genotoxicity of exposure to various carcinogens.^{63,64} MN originates from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. They reflect chromosome damage and may thus provide a marker of early-stage carcinogenesis.

In the present study, we observed a statistically significant difference between the frequency of MN in smokers and nonsmokers, which has been shown previously in most recent studies.^{20,24,25,46} The frequency of MN in normal oral mucosa is between 0.5 and 2.0/1000 cells,^{24,44,45} which is within the range we detected for nonsmokers.

On the other hand, an average of 3.8/1000 cells was detected in smokers in this study, which is also within the range reported by some studies.^{65,67} This is probably due to the high number of carcinogens in the tobacco smoke that produces related DNA damage.⁶⁸

Although many studies have already assessed the frequency of MN in smokers,^{20,24,25,44,45,62,65,66} only one used this method to assess the genotoxicity of in-office bleaching agents.³⁸ Fortunately, we demonstrated that the frequency of MN was not increased after bleaching with 10% carbamide peroxide in both study groups, suggesting that the low-concentration carbamide peroxide gel did not seem to induce DNA damage to the gingival tissue when used for three hours daily over a three-week period.

These findings, however, are not in agreement with those of Klaric and others,³⁸ who observed a higher MN frequency 72 hours after in-office bleaching. A more concentrated bleaching gel (35% hydrogen peroxide) was used in the aforementioned study; additionally, the authors isolated the gingival tissue from the participants with a light-curing gingival barrier, which may also have played a role in the results obtained.

Another *in vivo* study in humans,³⁷ which assessed the effects of bleaching agents on the gingival tissue by biopsy, observed a proliferative activity of the gingival epithelium after bleaching with 10% carbamide peroxide gel (eight hours daily for a five-week period). However, this study should be interpreted with caution as an increase in the prolifera-

tive activity of epithelial cells does not necessarily mean that the agent has a genotoxic potential.

One should also mention the current study's limitations. First, during color evaluation the examiners could not be blinded. Although patients were asked to rinse their mouth with mouth rinses, the smoking smell was impregnated in the participant's clothes, hair, hands, and breath. Second, the frequency of MN was only evaluated soon after the end of the bleaching procedure. It is known that it takes approximately 10-12 days for the regeneration of the cells from the gingival tissue,⁶⁹ which is a little bit shorter than the period of the bleaching protocol. The study of DNA damage in exfoliated cells collected from the oral cavity holds great promise as a minimally invasive method for monitoring exposure to genotoxic agents; according to Thomas and others,⁷⁰ as the buccal cells turn over every 7 to 21 days, thereafter it is theoretically possible to observe the genotoxic effects of acute exposure within this period. Last, care should be taken during the extrapolation of the study results to women, as most of the participants in the smokers group were men.

Future studies should be conducted with other bleaching agents and protocols. Additionally, samples from the gingival tissue should be collected for longer periods after the end of the bleaching protocol to definitely ensure the safety of this cosmetic procedure in dentistry.

CONCLUSION

It can be concluded that the effectiveness of dental bleaching does not seem to be affected by smoking and at-home bleaching does not induce DNA damage to the gingival tissue.

Acknowledgment

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Human Subjects Statement

This study was conducted in accordance with all the provisions of the local human subject oversight committee guidelines and policies. The approval code for this study was 208.355 under protocol number 16457/2012. This study was conducted at State University of Ponta Grossa, Paraná, Brazil.

Conflict of Interest

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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