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ORIGINAL ARTICLE

Cytogenetic damage induced by mouthrinses formulations in vivo and in vitro

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Abstract The aim of the present study was to comparatively evaluate DNA damage and cellular death in cells exposed to various commercially available mouthrinses: Listerine® Cepacol®, Plax alcohol free®, Periogard®, and Plax Whitening®. A total of 75 volunteers were included in the search distributed into five groups containing 15 people each for in vivo study. Exfoliated buccal mucosa cells were collected immediately before mouthrinse exposure and after 2 weeks. Furthermore, blood samples were obtained from three healthy donors for in vitro study. The micronucleus test was used to evaluate mutagenicity and cytotoxicity in vivo. The single-cell gel (comet) assay was used to determine DNA damage in vitro. After 2 weeks exposure, Periogard® showed 1.8% of micronucleated cells with significant statistical differences ($p<0.05$) compared to before exposure (0.27%). Plax Whitening® presented high tail moment value (4.5) when compared to negative control (0.6). The addition of all mouthrinses to cells incubated with methyl methanesulfonate did not alter the number of strand breaks in the genetic material. Listerine® was able to reduce genetic damage induced by hydrogen peroxide because a decrease of tail moment was noticed. The results of the present study suggest that Periogard® and Plax Whitening® can induce genetic damage, whereas Listerine® is an antioxidant agent. Since DNA damage is considered to be prime mechanism during chemical carcinogenesis, these data may be relevant in risk assessment for protecting human health and preventing carcinogenesis.

Keywords Buccal mucosa cells · Mouthrinses · Genetic damage · Cellular death · Micronucleus test · Single-cell gel (comet) assay

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Introduction

Mouthrinses are used as adjuncts to mechanical oral hygiene [1]. The use of mechanical control alone to reduce recalcitrant biofilms in the oral cavity has been challenged because it is considered to be a rather time-consuming and, most importantly, insufficient activity for effective oral hygiene [2]. In this regard, numerous mouthwashes are available for use as part of a daily oral hygiene routine. The formulations contain actives that may inhibit microbial growth and enzymatic reactions or may react directly with volatile sulfur compounds to reduce their levels in the mouth [3]. However, to the best of our knowledge, little information is available in the literature whether mouthrinses can induce noxious activities on eukaryotic cells, particularly genetic damage and/or cellular death so far.

Therefore, it would be useful to know whether, and to what extent, mouthrinses have direct effects on the genetic apparatus and/or on cellular machinery after continuous or even following acute exposure.

For many years, biomarkers have been used in medicine and toxicology to assist in diagnosing, staging disease, as well as to evaluate risk assessment. They should allow statements concerning chemical exposure and give further information on the status of susceptibility. Biomarkers are divided into three groups: the first to define the exposure to harmful agents; the second to show biological effects on the target tissue; and the third to give information about the individual susceptibility [4]. To date, a variety of assays have been proposed as potential biomarkers in biomonitoring studies, including those assessing metaphase chromosomal aberrations, sister chromatid exchanges, and host cell reactivation. Yet, such methods are typically laborious and time consuming or even require highly trained technicians to accurately read and interpret slides. For this purpose, a great deal of enthusiasm was raised by the application of the micronucleus test to uncultured exfoliated cells [5]. Micronucleus arises from acentric fragments or whole chromosomes which are not included the main nuclei of the daughter cells. The formation of micronuclei can be induced by substances that cause chromosome breakage (clastogens) as well as by agents affecting the spindle apparatus (aneugens) [6]. Recently, we have successfully applied such a methodology to individuals exposed to dental X-ray [7] or xenobiotics [8].

A useful method for quantifying DNA damage is the alkaline comet assay (single-cell gel electrophoresis) [9]. The alkaline version of the single-cell gel (comet) assay used is sensitive for a wide variety of DNA lesions. Among them, there are single- and double-strand breaks, alkali-labile sites including abasic and incomplete repair sites, and DNA–DNA/DNA–protein/DNA–drug cross-linking in any eukaryotic cell [9]. Due to its simplicity and sensitivity, the comet assay has gained fast acceptance as a genotoxicity assay. Our group has consistently demonstrated that the single-cell gel (comet) assay is a useful tool for detecting DNA breaks in multiple organs and under different paradigms [10–12].

Nowadays, it is well established that either exogenous or endogenous agents can modify the cellular DNA along with other cellular components [13]. Hydrogen peroxide, a potent endogenous oxygen reactive species, is able to interact both directly with DNA and through highly reactive oxygen and radical species to cause extensive oxidative DNA damage [14]. Oxidative DNA damage has been recognized as a major cause of cell death and mutations in all aerobic organisms. In humans, oxidative DNA damage is also considered an important promoter of cancer [15]. Alkylating agents have been classified for many years as a DNA-damaging agent to induce mutagenesis [16]. DNA damage caused by alkylating agents is predominantly repaired by the base excision repair pathway and DNA alkyltransferases [17]. Some authors support the idea that these compounds are the most potent and abundant chemical DNA damagers found in our environment [18].

As a result, and because of inappropriate evidence, the aim of this study was to investigate the frequencies of micronucleated cells and pyknosis, karyolysis, and karyorrhexis in the oral mucosa from individuals exposed to various commercially available mouthrinses as a predictor of mutagenic and cytotoxic effects, respectively. In order to monitor genotoxicity, the single-cell gel (comet) assay was also evaluated in such a setting.

Material and methods

In vivo study

The subjects of this study comprised a total of 75 healthy adults (45 men and 30 women) with a mean age of 37.3+ 11.1 years. Subjects' individual characteristics were collected and included, such as gender, age, habits, and exposure to genotoxic agents such as alcohol consumption or smoking habits. Each person was interviewed concerning possible confounding factors and was excluded from this study when there was lesion on the oral mucosa visible at clinical examination, history of cancer, previous radio- or chemotherapy, use of therapeutic drugs, exposure to diagnostic X-rays in the last 6 months, exposure to diagnostic X-rays during the preceding 6 months, intensive sports activities during the preceding week, a history of smoking, cancer, high alcohol consumption, or use of therapeutic drugs, lesion on the buccal mucosa visible at clinical examination, or previous radio- or chemotherapy.

The study was approved by the Ethics Committee of UNIFESP, Federal University of Sao Paulo. Informed consent was obtained from the individuals included in the study.

Micronucleus test in buccal mucosa cells

Individuals were distributed into five groups containing 15 people each. The following groups were defined according to the mouthrinse under investigation. The following mouthrinses were tested: Listerine® (Johnson & Johnson®, SP, Brazil), Cepacol® (Avantis-Pharma®, SP, Brazil), Plax alcohol free® (Colgate-Palmolive®, SP, Brazil), Periogard® (Colgate-Palmolive®, SP, Brazil), and Plax Whitening® (Colgate-Palmolive®, SP, Brazil). Test materials, manufacturers, and ingredients are listed in Table 1. All volunteers were requested to rinse their mouths with 15 ml of mouthrinse twice a day for 2 weeks as described elsewhere [19]. The rinsing procedures took approximately 30 s each time. No attempt was made to change the oral hygiene.

Table 1 Mouthrinses tested

Mouthrinse tested	Manufacturer	Country	Composition
Listerine	Johnson & Johnson	Brazil	Thymol, 0.064%; eucalyptol, 0.092%; methyl saliciate, 0.06%; menthol, 0.042%; sorbitol; alcohol, 21.6%; benzoic acid
Cepacol	Avantis Pharma	Brazil	Sodium fluoride, 225 ppm; cethylperidine chloride, 0.05%; alcohol, 17.6%
Plax alcohol free	Colgate-Palmolive	Brazil	Sodium fluoride, 225 ppm; cethylperidine chloride, 0.05%
Plax Whitening	Colgate-Palmolive	Brazil	Sodium fluoride, 225 ppm; cethylperidine chloride, 0.05%; acohol, 17.6%; hydrogen peroxide 1.5%
Periogard	Colgate-Palmolive	Brazil	Chroxhexidine digluconate, 0.12%; alcohol, glicerol

Exfoliated buccal mucosa cells were collected immediately before the mouthrinse exposure and after 2 weeks. After rinsing the mouth with tap water, cells were obtained by scraping the right/left cheek mucosa with a moist wooden spatula. Cells were transferred to a tube containing saline solution, centrifuged (800 rpm) for 5 min, fixed in 3:1 methanol/acetic acid, and dropped onto pre-cleaned slides. Later, the air-dried slides were stained with the Feulgen/Fast Green method, and examined under a light microscope at ×400 magnification to determine the frequency of micronucleated cells [6]. Two thousand cells were scored from each person for each sampling time (before and after mouthrinse exposure).

Data analysis

Micronuclei were scored according to the criteria described by Sarto et al. [20] as a parameter of DNA damage (mutagenicity). For cytotoxicity, the following nuclear alterations were considered: pyknosis, karyolysis, and karyorrhexis. Results were expressed in percentage (%). Such analysis was established in a previous study conducted by our research group [21].

In vitro study

A total of three healthy donors were used in this study. All donors were female and 21, 23, and 24 years of age. Each person was interviewed about possible confounding factors and was excluded from this study when there was a history of smoking or cancer, previous radio- or chemotherapy, use of therapeutic drugs, exposure to diagnostic radiographs during the preceding 6 months, intensive sports activities during the preceding week, or high alcohol consumption; lesion on the buccal mucosa visible at clinical examination, or previous radio- or chemotherapy.

Heparanized blood samples were obtained by venous puncture. A total of 2 mL of peripheral blood was collected from each donor. The same tested materials were used to the in vivo study (Table 1). Ten microliters of the tested mouthrinse was then added to human peripheral blood cells for 1 h at 37°C. After that, all treatments were incubated with either of two known genotoxins: methyl methanesulfonate (MMS), an alkylating agent (Sigma Aldrich®, St. Louis, MO) at 10 μmol/L concentration in phosphate-buffered solution (PBS), pH 7.4, for 15 min at 37°C or/and H_2O_2, an oxidizing agent, (Merck®, St. Louis, MO) at 100 μmol/L concentration in distilled water for 5 min on ice [22]. The negative control cells were treated with PBS for 1 h at 37°C. Each individual exposure was tested in at least three separate experiments in triplicate for each individual treatment. After incubating, the cells were centrifuged at 1,000 rpm for 5 min, washed twice with fresh medium, and resuspended with fresh medium.

Single-cell gel (comet) assay

The protocol used for single-cell gel (comet) assay followed the guidelines proposed by Tice et al. [9]. Slides were prepared in duplicate per treatment. Thus, a volume of 10 μL of treated or control cells (~1×10^4 cells) was added to 120 μL of 0.5% low-melting-point agarose at 37°C, layered onto a precoated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and slides immersed in lysis solution (2.5 M NaCl; 100 mM EDTA [Merck®, St. Louis, MO]; 10 mM Tris–HCl buffer pH=10 (Sigma Aldrich®); 1% sodium sarcosinate (Sigma Aldrich®); with 1% Triton X-100 [Sigma Aldrich®]; and 10% DMSO [Merck®, St. Louis, MO]) for about 1 h. Before electrophoresis, the slides were left in alkaline buffer (0.3 mM NaOH [Merck®] and 1 mM EDTA [Merck®]; pH>13) for 20 min and electrophoresed for another 20 min, at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris–HCl (pH=7.5) for 15 min, fixed in absolute ethanol, and stored at room temperature until analysis. All of the steps described above were conducted in the dark to prevent additional DNA damage.

Throughout this study, diluted and treated aliquots were tested for viability by trypan blue exclusion, and constantly more than 75% of cells excluded trypan [23].

Comet capture and analysis

A total of 50 randomly captured comets per treatment (25 cells from each slide) [24] were examined blindly by one expert observer at ×400 magnification using a fluorescence microscope (Olympus Optical Co., Tokyo, Japan) connected through a black and white camera to an image analysis system (Comet Assay II®, Perceptive Instruments, Suffolk, Haverhill, UK). This software was previously calibrated according to the manufacturer's instructions. The computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and then evaluates the range of derived parameters. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. To quantify the DNA damage, tail moment was evaluated. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. The comet tail moment is positively correlated with the level of DNA breakage in a cell. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in such a sample.

Statistical methods

The Mann–Whitney non-parametric test was used to compare the frequencies of micronuclei and other cellular alterations among the samples between exposed versus control groups using SigmaStat® software, version 1.0 (Jadel Scientific, USA). Parameter from the comet assay (tail moment) was assessed by the Friedman test, using SigmaStat® software, version 1.0 (Jadel Scientific, Rafael, CA). The level of statistical significance was set at 5%.

Results

In vivo study

Table 2 shows the frequencies of micronucleated cells in individuals exposed to various commercially available mouthrinses formulations. Before exposure Listerine®, Plax alcohol free®, and Cepacol® showed 0.07%, 0.04%, and 0.02% frequencies of micronucleated cells, respectively. No statistically significant differences ($p>0.05$) in the micronucleus frequencies before and 2 weeks after exposure were noticed to all groups evaluated. Plax Whitening® did not induce any signs of mutagenicity either since similar values of micronucleated cells were observed before and after exposure (0.02% and 0.04%, respectively). Nevertheless, Periogard® showed 1.8% of micronucleated cells with significant statistically differences ($p<0.05$) compared to before exposure (0.27%).

Additionally, there was no observed increase of other nuclear alterations closely related to cytotoxicity as depicted by frequencies of karyorrhexis, pyknosis, and karyolysis in volunteers exposed to all mouthrinses evaluated in this study. Such findings are displayed in Table 2. Figure 1 shows a micronucleated cell. Figure 2 displays karyorrhexis, pyknosis, and karyolysis.

Table 2 Frequency of micronucleated cells (MNC) and other nuclear alterations (karyorrhexis, pyknosis, and karyolysis) in individuals exposed to various commercial mouthrinses

Groups	MNC (%)		Other nuclear alterations[a] (%)	
	Sampled size	Mean±SD	Sampled size	Mean±SD
Listerine				
Before exposure	15	0.07±0.02	15	7.1±1.0
After exposure	15	0.04±0.08	15	9.4±2.1
Periogard				
Before exposure	15	0.27±0.23	15	28.3±8.4
After exposure	15	1.8±0.2*	15	26.4±11.3
Plax alcohol free				
Before exposure	15	0.04±0.08	15	13±3.6
After exposure	15	0.16±0.01	15	13.2±4
Plax Whitening				
Before exposure	15	0.02±0.04	15	19.6±3.5
After exposure	15	0.04±0.05	15	20.1±4.3
Cepacol				
Before exposure	15	0.02±0.04	15	19.2±5
After exposure	15	0.02±0.04	15	18.6±4.7

*$p<0.05$ when compared to before exposure

[a] Karyorrhexis, pyknosis, and karyolysis

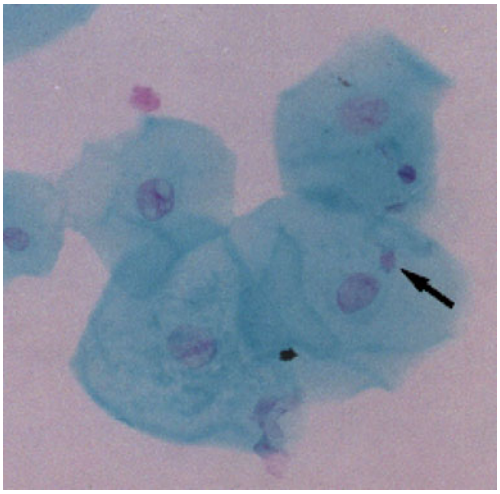

Fig. 1 Micronucleated cell (*arrow*), ×100 magnification, Feulgen/Fast Green stain

In vitro study

The single-cell gel (comet) assay was used to measure DNA damage in peripheral blood cells in vitro. DNA strand breaks were represented by the mean tail moment for 50 comets/sample. As seen in Table 3, the single treatment with Cepacol®, Periogard®, Plax alcohol free®, and Listerine® did not induce strand breaks in DNA with tail moment values of 0.5, 0.6, 0.8, and 0.8, respectively. By contrast, Plax Whitening® induced extensive genetic damage in peripheral blood cells after in vitro exposure as demonstrated by high tail moment, i.e., 4.5.

In order to detect potential interactions with known genotoxins, samples incubated with the mouthrinses were further assayed together with MMS or H_2O_2. The addition of Periogard®, Cepacol®, Plax alcohol free®, or Listerine® to cells incubated with MMS did not alter the number of strand breaks in the genetic material since no significant statistical differences were noticed to tail moment values. In the same way, this picture was also observed, with respect to H_2O_2, for Periogard®, Cepacol®, or Plax alcohol free®. Interestingly, Listerine® was able to reduce genetic damage induced by hydrogen peroxide because a decrease of tail moment was noticed. Such data are summarized in Table 3. It is important to stress that DNA damage was unscorable regarding Plax Whitening® for H_2O_2 or MMS because comets pointed out all DNA in the tail. Therefore, this mouthrinse was not included in the experimental design.

Figure 3 contains representative comet images of a blood cell from a negative control (a), a cell exposed to Periogard® (b), and an MMS-treated cell (positive control; c).

Discussion

The aim of this study was to evaluate, both in vivo and in vitro, genetic damage and cellular death induced by various mouthrinses. To the best of our knowledge, the approach has not been demonstrated so far.

Micronucleus assay in exfoliated buccal mucosa cells has been systematically used in genetic biomonitoring of populations exposed to several genotoxic chemicals, such as tobacco products, pesticides, and alcohol consumption [25]. The key advantage of the micronucleus is the relative ease of scoring, the limited costs and required person-time, and the precision obtained from scoring larger numbers of cells.

Micronucleated cell indexes may reflect genomic instability [26]. The detection of an elevated frequency of micronuclei in a given population indicates an increased risk of cancer [27]. Our results have demonstrated that Periogard® containing chlorhexidine at 0.12% was able to induce cytogenetic damage as depicted by the increase of micronucleus frequency after continuous exposure. By comparison, a level of DNA damage in peripheral blood lymphocytes and buccal mucosa cells was evidenced in individuals exposed to chlorhexidine digluconate at 0.12% for 2 weeks by single-cell gel (comet) assay [19]. In particular, a previous study conducted by our research groups has consistently demonstrated that Periogard® induced genetic damage in peripheral lymphocytes and buccal mucosa cells of rats [28]. No remarkable significant differences were noticed as to Cepacol®, Listerine®, and Plax alcohol free®, between groups. Cepacol® and Plax® contain high concentration of sodium fluoride. Previous

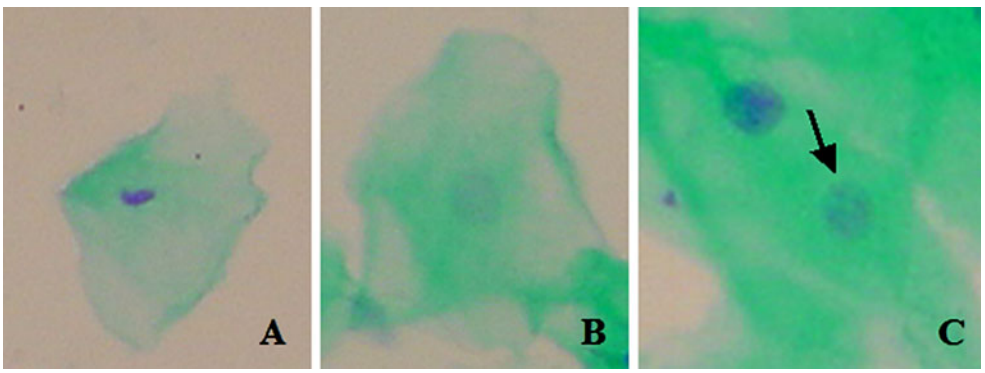

Fig. 2 Cytotoxicity parameters evaluated in this study: **a** pyknosis, **b** karyolysis, and **c** karrhyorexis (*arrow*; ×100 magnification, Feulgen/Fast Green stain)

Table 3 Mean±SD of DNA damage (depicted by the mean tail moment) in human peripheral blood cells exposed to methyl methanesulfonate (MMS) or hydrogen peroxide (H_2O_2) in the presence or absence of the mouthrinses

Groups	DNA damage (tail moment)		
	Basal damage	With MMS[a]	With H_2O_2[b]
Negative control	0.6±0.4	2.5±1.2	3.4±1.2
Listerine	0.8±0.5	2.5±0.7	1.6±0.5*
Plax alcohol free	0.8±0.5	3.2±1.6	4.2±1.8
Plax Whitening	4.5±0.8*	–	–
Cepacol	0.5±0.6	3.5±1.5	4±1.3
Periogard	0.6±0.4	2±1.3	3.9±1.8

*$p<0.05$ when compared to negative control within columns

[a] Methyl methanesulfonate at 10 μmol/L

[b] Hydrogen peroxide at 100 μmol/L

studies conducted by our group have demonstrated that sodium fluoride did not cause genetic damage either in vitro or in vivo [29, 30]. Regardless of the biological phenomenon involved in this process, we assumed that Periogard® is a mutagenic agent as a result of chromosomal breakage or loss in buccal mucosa cells.

To monitor cytotoxic effects, the frequencies of karyorrhexis, karyolysis, and pyknosis were evaluated in this experimental design. Our results have demonstrated that all tested mouthrinses did not induce cellular death in vivo. Some authors have postulated that Listerine® produces significant morphological changes that may be associated with cell death [31]. In the same way, others have revealed that chlorhexidine-induced cytotoxicity in vitro [32, 33]. The variation of cytotoxicity indicates relative large differences between the groups in this study. Therefore, the results should be interpreted cautiously.

The single-cell gel (comet) assay was also applied in this in vitro study. The cells most frequently used for in vitro studies when screening for the potential genotoxicity of chemicals are Chinese hamster ovary cells or L5178Y mouse lymphoma cells. However, there are some drawbacks associated with cell lines. For example, the chromosomal material has usually undergone extensive rearrangement in which some key genes involved in affecting the viability of cells that sustain DNA damage, such as TP53, may be mutated [34]. Human peripheral blood cells have the advantage of being karyotypically normal human cells as well as good indicators of the systemic burden caused by exposure factors [35]. For this reason, we chose to use the human peripheral blood cells in the current study.

Our results have demonstrated that Plax Whitening® was able to induce genetic damage in human blood in vitro. Plax Whitening® is composed by hydrogen peroxide. Hydrogen peroxide is a molecule that easily goes through the cell membrane and is transformed in hydroxyl radicals by a non-enzymatic process occurring in the cytoplasm and in the presence of metal ions (Fe^{2+} or Cu^{2+}), known as the Haber–Weiss or Fenton reaction. Hydroxyl radicals can induce single-strand breaks, double-strand breaks, alkali-labile sites, and various species of oxidized purines and pyrimidines [36]. Other free oxygen radicals/oxygen free radicals derived from H_2O_2 can also interact with DNA to induce a broad spectrum of DNA lesions. In fact, it has been widely reported that oxygen reactive species derived from H_2O_2 are the major endogenous source of DNA damage and induce carcinogenesis [37]. Nevertheless, the micronucleus test did not demonstrate a positive response in vivo as for buccal mucosa cells. This requires further study.

To further elucidate the possible interactions of mouthrinses in combination with DNA-damaging agents, we performed additional experiments with the single-cell gel (comet) assay. Methyl methanesulfonate, an alkylating agent, modifies both guanine (to 7-methylguanine) and adenine (to 3-methlyladenine/methyladenine) to cause base mispairing and replication blocks, respectively. In this context, clear genotoxic effects were obtained for MMS-induced DNA damage in human peripheral blood cells. Nevertheless, MMS-induced DNA damage was not altered by the simultaneous treatment with all mouthrinses evaluated in this scenario. Moreover, we obtained extensive DNA migration after incubation of the cells with H_2O_2. Interestingly, the combination of H_2O_2 and Listerine® produced a reduction of DNA damaging in human peripheral blood cells. Some authors have postulated that methylsalicylate exerts potent antioxidant activity because

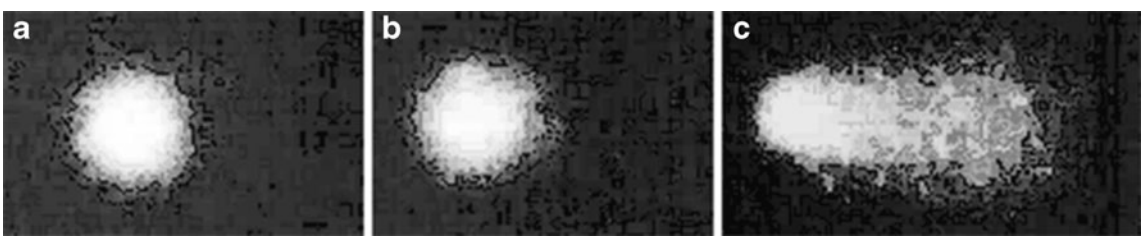

Fig. 3 Representative comet images of a blood cell from a negative control (**a**), a cell exposed to Periogard (**b**), and an MMS-treated cell (positive control, **c**). DNA was stained with ethidium bromide. ×40 magnification

salicylic acid and its derivatives readily react with OH^- [38]. Periogard®, Cepacol®, and Plax alcohol free® do not exert any positive response. This was confirmed by others [39]. Taken as a whole, it seems that Listerine® is an antioxidant agent capable of preventing oxidative DNA damage in human peripheral blood cells.

In conclusion, the results of the present study suggest that Periogard® and Plax Whitening® can induce genetic damage in vivo and in vitro, respectively, whereas Listerine® is an antioxidant agent.

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Conflict of Interest Statement None declared.

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