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Assessment of DNA Damage by Comet Assay in Buccal Epithelial Cells: Problems, Achievement, Perspectives


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Abstract

DNA damage risk assessment in comet assay by the use of buccal mucosa cells has great advantages in comparison with other cell type sample due to more safely, easier, cheaper, and non-invasive method for in vivo studies. According to the OECD Guidelines, the in vivo mammalian alkaline comet assay is well-established and validated method for measuring DNA strand breaks in single eukaryotic cells. Considering exposure to xenobiotics and endogenous damage inducers, buccal mucosa cells are the first to be in direct contact after exposure and this makes them an ideal biomatrices in evaluation of the level of individual genotoxicity to several compounds already mentioned. Their clinical diagnostic applicability confers a potential use in patients across time. However, the number of publications referring to the human buccal comet assay is low in the last two decades. This low growing interest may be explained by several factors, including its relative technical problems. Different procedures have been used in collecting and processing the samples. In order to have widespread acceptance and credibility in human population studies, the comet assay in buccal cells requires standardization of the protocol, of parameters analyzed, and a better knowledge of critical features affecting the assay outcomes, including the definition of the values of spontaneous DNA damage. There is a need for further collaborative work as in the HUMN (micronucleus assay on lymphocytes) and HUMNXL (micronucleus assay on buccal cells) collaborative projects. The creation of a network of laboratories will allow more focused validation studies, including the design of a classic, historic, prospective cohort study in order to explore the link between measures of genetic instability in the buccal mucosa and the risk of cancer and other chronic-degenerative diseases. One such network connection will start in 2016 as a COST project under the name “hCOMET—The comet assay as a human biomonitoring tool” launched by Prof. Andrew Collins.

Keywords: SCGE assay, buccal mucosa cells, genotoxic risk assays, DNA damage, comet assay

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1. Introduction

Human exposure to environmental chemical agents occurs as a result of contaminated air, water, soil, and food. Although many chemical agents are in use for more than two centuries, nowadays, it is known that a number of them can cause genetic damage. Chemicals that can cause this type of damage are specified and identified as mutagens, carcinogens, or teratogens based on the diverse type of investigations. It is estimated that chemicals play a predominant role in the etiology of a majority of human diseases. The possible genetic health hazards associated with chemicals are more difficult to evaluate in the human environment. There are tens of thousands of untested chemicals in the human environment, and some attempt must be made to identify the ones that are potentially hazardous to man. From 1972 when first UN Conference on the Human Environment was organized, World Health Organization and International Agency for Research on Cancer (IARC) have published many monographic editions categorizing dangerous chemicals based on collected in vitro and in vivo results of investigations [1,2]. Also, unique tools (methods) for assessing the potential effects of chemicals on human health, and the environment have been established under the name The OECD Guidelines for the Testing of Chemicals, methods, and guidelines internationally accepted as standard methods for safety testing [3] in which standardized and validated techniques are described that can estimate the level of DNA damage after the exposure.

During the past half century, the focus has been shifted from identification of these compounds in the environment to the risk assessment and minimization or prevention of unnecessary exposure in the first place. For this reason, along with an increasing understanding of mechanisms of action by which these chemicals can cause DNA or cell damage, and also cancer [4], a variety of hazard identification screening models have been developed and established. These models can serve in risk assessment studies. Risk is defined as the probability of a given toxicological hazard producing actual biological harm. This idea involves some form of mathematical relationship between exposure and toxicology. In the field of environmental toxicity assessment, the need for in-time risk management decisions requires setting up a battery of standardized and relatively easy to perform tests, allowing quick answers to pressing questions [5]. The use of diverse genotoxic bioassays is therefore unavoidable. Application of biomarkers in both qualitative and quantitative aspects of risk assessment has been eagerly anticipated for over a decade, since Hattis [6] first proposed their use in this process.

Numerous assays have been developed as screens for genotoxicity, beginning with the Salmonella mutagenicity assay. Genomic damage is probably the most important fundamental cause of developmental and degenerative disease. It is also well established that genomic damage is produced by environmental exposure to genotoxins, medical procedures, nutrient deficiency, lifestyle, and genetic factors [7]. It is essential to have reliable and relevant minimally invasive biomarkers to improve the implementation of biomonitoring, diagnostics, and treatment of diseases caused by, or associated with, genetic damage.

Since methods in molecular epidemiology have been improved with the use of reliable biomarkers of exposure in analysis, population biomonitoring has become an extremely
powerful approach to determine the effect of environmental mutagens on human populations [8]. On this way, early effects may be highlighted in all accessible cell types, such as blood cells, epithelial cells and exfoliated buccal or urothelial cells; thus, genetic biomonitoring allows detecting adverse effects of mutagenic chemicals in human somatic cells [9].

Among different types of cells and especially of epithelial cells, the collection of buccal cells is arguably the least invasive method available for measuring DNA damage in humans, especially in comparison with obtaining blood samples for lymphocyte and erythrocyte assays, or tissue biopsies [7]. Without the need for cell culture establishing (cells do not divide, but just differentiate from basal cells), buccal cells analyzed by other techniques, such as micronucleus assay, have shown good correlation with the level of damage observed on lymphocytes after 72-h cell culture with DNA damage cytogenetic test called cytochalasin B blocked micronucleus (MN) assay [10]. Buccal micronucleus cytome assay can measure frequency of MN (its origin is either from chromosome breakage/loss of entire chromosome), nuclear buds and/or broken egg, binucleated cells, and various forms of cell death phase measured as condensed chromatin, karyorrhectic, pyknotic, or karyolitic cells [11]. Chronic exposure leads to a steady-state elevated expression level of MN regardless of the cell division rate if the period of exposure exceeds the time frame for one nuclear division, that is, 20–30 h. Carcinogens delivered primarily through blood stream influence equally DNA damage measured in buccal cells and lymphocytes. Since collection of buccal cells and their processing is easy, fast and low cost, and they do not divide just differentiate, they have potential to replace the tests that need cell culture establishment in order to estimate DNA damage. HUMNxL group (The HUman MicroNucleus project on eXfoLiated buccal cells group) has collected data from 30 different laboratories on 5424 subjects in order to evaluate the impact of host factors, occupation, lifestyle, disease status, and protocol features on the occurrence of MN in exfoliated buccal cells [12]. The results of this survey have shown high correlation of micronucleus detection in buccal cells with exposure for occupational groups reporting exposure to solvents, polycyclic aromatic hydrocarbons (PAHs) and gasoline, arsenic, and antineoplastic drugs. Also, significant association of higher MN frequency was found for oro-pharyngeal and respiratory cancers, and for all the other cancers pooled together. Although micronucleus assay in buccal cells does not need cell culture, it requires at least 3000 cells examined under the microscope. Since this can also be time consuming, one of the other methods for measuring DNA damage is alkaline comet assay, one of the newest OECD guideline tests (from 2014) for chemical exposure in vivo (No. 489), an easy and low-cost assay that measures primary DNA damage on any type of single-cell suspension sample [13]. The use of comet assay on buccal cells would be a potential new and reliable combination for chemical exposure and DNA damage assessment. The comet assay in buccal cell was first reported in 1996 [14]. Like in HUMNxL project, it will be necessary to develop and implement the results of an international collaborative validation group established to identify and quantify the key variables affecting the damage evaluation in buccal mucosa cells using the comet assay. In addition, an inter-laboratory slide-scoring exercise could be undertaken to evaluate the intra- and inter-laboratory variability in the scoring of different parameters of comet assay in buccal cells, similar to the approach successfully used by the HUMN project for the MN assay in lymphocytes [15–17] and the HUMNxL project in buccal cells [7,12,17,18]. One such groups with prof. Andrew
Collins has started in 2016 a COST networking project under the name “hCOMET — The comet assay as a human biomonitoring tool”, in order to give response to the questions discussed in this review.

1.1. Comet assay

The comet assay is a cheap, easy, fast, reliable, and sensitive method for measuring the level of primary DNA damage in single-cell suspension of any type and requires a small sample material. For these reasons, the comet assay in its various modifications (alkaline, neutral, and with lesion-specific enzymes to detect specific types of DNA damage such as 8OHdG, formamidopyrimidine DNA glycosylase, endonuclease III, T4 endonuclease V.) has few serious competitors. The cells are embedded into agarose, and after lysis, denaturation, electrophoresis, and staining, the amount of DNA damage is measured either visually by dividing the damaged cells into five groups, or by the help of camera and software image program that analyses the image. Measured parameters are usually tail length (measured in micrometers), tail intensity or tail DNA percentage (when there is damage, DNA has a shape of a comet), and tail moment (combination of the first two parameters). It is recommended to use tail intensity parameter since the agents sometimes produce few small breaks that make comet tail long, but in fact, there is not a high percentage of DNA in the damaged part of the comet. When standardized and validated, the comet assay can provide valuable information in the areas of hazard identification and risk assessment of environmental and occupational exposure, diseases linked with oxidative stress (e.g., diabetes and cardiovascular disease), nutrition, monitoring the effectiveness of medical treatment, and investigating individual variation in response to DNA damage that may reflect genetic or environmental influences. The information obtained could lead to individual advice on lifestyle changes to promote health and especially on relative risks of genotoxic exposure to environmental pollution [19].

In human biomonitoring studies, the comet assay can provide crucial information on risk assessment of environmental, occupational, and lifestyle exposures. Earlier reviews have dealt with different aspects of the use of the comet assay in human biomonitoring studies [20–26], but without providing any specific, practical guidance for using the comet assay in human biomonitoring. Several general articles on biomonitoring are available [27–31] that can be helpful when designing biomonitoring studies using the comet assay. To avoid obtaining false-positive and false-negative results, certain basic principles should be respected and followed in study design and performing and these consider first of all matching of exposed and control group according to gender, age, alcohol, and smoking habits and their consumption, and also with other lifestyle and nutritional factors [19].

ComNet project group, established before last COST project that will make an effort in exposure type and DNA damage assessment, has made an effort to pool together data of all available comet assay biomonitoring studies, in order to establish baseline parameters of DNA damage, and to investigate associations between comet assay measurements and factors such as sex, age, smoking status, nutrition, lifestyle. Although this assay has been widely used in human biomonitoring for DNA damage measurement as a marker genotoxic agent’s exposure or for investigation of genoprotective effects, single research studies had usually small
numbers of subjects, with sub-optimal design also in other critical respects already mentioned, and also with the use of significantly different comet assay protocols. For these reasons, the ComNet project has recruited almost 100 research groups willing to share datasets. Collins et al. [32] provided a background of the ComNet project, and the history of the comet assay itself, and the most important, he has pointed out important practical issues that can critically affect its performance. The survey pointed out comet assays diverse applications in biomonitoring studies (environmental, occupational exposure to genotoxic agents), genoprotection studies that were controlled by dietary and other factors and DNA damage assessment studies associated with various diseases and intrinsic factors that affect DNA damage levels in humans. The survey also analyzed the quality of data from a random study selection, using epidemiological and statistical point of view. Most of the studies have been done on lymphocytes or whole blood, and they can show damage of DNA caused by long term exposure or also exposure in the past, since lymphocytes circulate through the body and can live for up to 3 years. A new step will be also to established basal levels of DNA damage in relation to different exposure, diseases, and cell types used, and to correlate them with long-term and short-term exposure. Considering the short term or recent exposure, buccal mucosa cell comet assay would be ideal since those cells among epithelial cells are short living cells with no division and DNA damage found in them can demonstrate recent exposure or direct contact exposure with oral mucosa, so the DNA damage measured by comet assay on buccal cells would be indication of recent exposure and severity of that exposure [33].

1.2. Exfoliated oral mucosa cells

Buccal cells form the first barrier for the inhalation or ingestion route and are capable of metabolizing proximate carcinogens to reactive products [34–37]. About 92% of human cancers are derived from the external and internal epithelium, that is, the skin, the bronchial epithelium, and the epithelia lining the alimentary canal [7,38]. Therefore, it could be argued that oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion [7,39].

In the early studies from the 1980s, exfoliated buccal mucosa cells were used with the MN assay to evaluate the genotoxic effects of multiple factors including environmental and occupational exposures, radiotherapy, chemoprevention, vitamin supplementation trials, lifestyle habits, cancer, and other diseases (see [7] for review), with possibility of cell degeneration in form of condensed/fragmented chromatin, pyknotic nuclei, loss of nuclear material in form of karyolytic or “ghost” cells [18,40,41]. In rare cases, some cells can also demonstrate other forms such as binucleated stage with two nucleus in the same cytoplasm, form of nuclear bud or “broken egg” or form small micronuclei (MN) near nuclei in the same cytoplasm. These biomarkers of genome damage (e.g., MN, nuclear buds) and cell death (e.g., apoptosis, karyolysis) can be observed in both the lymphocyte and buccal cell systems, and thus provide a more comprehensive assessment of genome damage then only MN in the context of cytotoxicity and cytostatic effects [7,39,41].
2. The comet assay in mucosa buccal cells

DNA damage assessment in exfoliated cells (buccal epithelium) may be an innovative promising tool for genotoxicity studies since sampling is easy. Some results indicate that alkaline single-cell gel electrophoresis, using buccal epithelial cells, could be a good biomarker of early effects, and can be utilized for human monitoring, since, in some cases, this kind of cell is the first to interact with xenobiotics [14]. Comet assay can detect DNA single-strand breaks and alkali labile sites at pH 13 (alkaline version) or double-strand breaks under neutral conditions (neutral version) [42–44]. The relevance of SCGE lies in its requirement for very small cell samples, and in its ability to evaluate DNA damage in proliferating or non-proliferating cells [45].

While biomonitoring studies employing cytogenetic techniques are mainly done in lymphocytes, the SCGE technique can be applied to any cell population. Over the last years, exfoliated cells have been used for biomonitoring studies utilizing several genotoxicity endpoints [40]; however, there are few studies which apply SCGE on epithelial cells [14].

Over 90% of cancers are epithelial in their origin [47] and since crucial mechanism in cancer development is the level and amount of DNA damage [48], DNA damage assessment in buccal epithelial cells may prove as a good biomarker of early damage. In their work, Rojas et al. [14] established for first time, the conditions for using the comet assay in buccal epithelial cells.

The use of surrogate cells, other than lymphocytes, such as exfoliated cells from epithelial tissues is of particular interest due to the ability to be collected with non-invasive methods, and the cells are explored with the aim to evaluate their suitability in biomonitoring studies [7,49]. Beside the minimally invasive sample collection from the inner wall of the cheek, the cells have advantage in exposure assessment to inhaled or ingested genotoxic agents, and this all makes them a good model for large biomonitoring studies, and also in pediatric researches.

The application of the comet assay test in uncultured buccal exfoliated cells (since the test does not need cell culture conditions), started in the 1996, when Rojas et al. [14] by comparing DNA damage level between smokers and non-smokers group in exfoliated buccal mucosa cells, found that DNA tail length significantly increased in the smoker group (89.30 + 16.18 μm) vs. non-smoker group (52.01 + 10.43 μm), indicating that the SCGE assay could be applied to human monitoring using exfoliated buccal epithelial cells.

In that moment, Rojas et al. [14] indicated that alkaline single-cell gel electrophoresis assay, using buccal epithelial cells could be a good biomarker of early effects, and can be utilized for human monitoring since; in some cases, this kind of cell is the first to interact with xenobiotics. However, 20 years later, <40 articles have been published with this bioassay. Table 1 represents the list of analyzed studies on buccal cells with comet assay with a point on sampling and preparation of slides for comet assay analysis. This table is extending the data collected in Rojas et al. [33] who only made observations in differences in preparing the slides, giving the highest impact on different lysis solution and enzyme digestion in preparation.
<table>
<thead>
<tr>
<th>Exfoliated Buccal Cells Sampling</th>
<th>Buccal comet assay technique</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rinsing</strong></td>
<td></td>
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<tr>
<td>With water</td>
<td></td>
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<tr>
<td>Small sterile spoon, kept in 1 mL of physiological solution at 37 °C</td>
<td>According with Singh et al. [106], Tice et al. [33], Sperti and Hartmann [107], with some modifications</td>
</tr>
<tr>
<td><strong>Collecting</strong></td>
<td></td>
</tr>
<tr>
<td>With a cytological brush, in a 20 circulaire expanding rotation, from the center of the cheek, both left and right cheek sampled with separate brushes, cells in 20 ml PBS, 4 °C until further process</td>
<td>Thomas et al. [111], Seto et al. [61]</td>
</tr>
<tr>
<td>Several times with distilled water</td>
<td></td>
</tr>
<tr>
<td>With water</td>
<td></td>
</tr>
<tr>
<td>Scrape the inner part of both cheeks 3 times with cytology brush, samples in sealed 1.5 ml Eppendorf tube with PBS, room temperature, no direct sunlight</td>
<td></td>
</tr>
<tr>
<td><strong>Followed protocol</strong></td>
<td></td>
</tr>
<tr>
<td>With water</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Centrifuged Slides preparation</strong></td>
<td></td>
</tr>
<tr>
<td>At 1000 rpm, 10 min</td>
<td></td>
</tr>
<tr>
<td>50 µL of cell pellet in 50 µL LMP agarose (5% in PBS), sample carefully stirred, dropped on a slide, covered with a coverslip precoated with NMP agarose (1% in PBS), and kept on ice during the polymerization of each gel layer</td>
<td>The coverslip removed, slides immersed in freshly made lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10), 10% DMSO, and 1% Triton X-100 for 24 h at 4 °C</td>
</tr>
<tr>
<td><strong>Enzyme treatment</strong></td>
<td></td>
</tr>
<tr>
<td>With water</td>
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<td></td>
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<tr>
<td><strong>Lysis</strong></td>
<td></td>
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<tr>
<td>With water</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td><strong>Pre-electrophoresis</strong></td>
<td></td>
</tr>
<tr>
<td>Horizontal electrophoresis chamber, fresh electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13), 20 min</td>
<td>Horizontal electrophoresis unit, with fresh alkaline solution (300 mM NaOH, 1 mM EDTA, pH 3.0) 20 min at 4 °C</td>
</tr>
<tr>
<td><strong>Electrophoresis</strong></td>
<td></td>
</tr>
<tr>
<td>Electrophoresis was 20 min at 25 V and 300 mA</td>
<td>Electrophoresis was 20 min at 25 V and 300 mA (0.90 V/cm)</td>
</tr>
<tr>
<td><strong>Neutralisation, fixation and staining</strong></td>
<td>3 times rinsing in fresh neutralisation buffer (0.4 M Tris, pH 7.5), fixed 5 min in absolute methanol, 75 µL Eth (20 µg/ml) 10 min</td>
</tr>
</tbody>
</table>

**Reference**

[82]
<table>
<thead>
<tr>
<th><strong>Exfoliated Buccal Cells Sampling</strong></th>
<th><strong>Buccal comet assay technique</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rinsing</strong></td>
<td><strong>Collecting</strong></td>
<td></td>
</tr>
<tr>
<td>Scraping the buccal mucosa with a wooden spatula, in a tube containing 1 mL of minimal essential media, wrapped in aluminum foil to protect them from light, stored in refrigerator at 4°C and processed next day</td>
<td>Following the method outlined by Singh et al. [100]</td>
<td>[72]</td>
</tr>
<tr>
<td>Water</td>
<td>Scarping the inner part of both cheeks with a cytology brush, cells kept in 0.9% NaCl and PBS in separate microcentrifuge tubes, brought to laboratory</td>
<td>Following the method outlined by Singh et al. [100]</td>
</tr>
</tbody>
</table>

**Exfoliated Buccal Cells Sampling**

**Collecting**

- Scarping the buccal mucosa with a wooden spatula, in a tube containing 1 mL of minimal essential media, wrapped in aluminum foil to protect them from light, stored in refrigerator at 4°C and processed next day

**Followed protocol**

- Ostling and Johanson [109], Sze et al. [61]

**Centrifuged Slides preparation**

- At 200 X g for 10 min, the cell pellet washed with 500 µL PBS and centrifuged

**Slides preparation**

- 10 µL of suspension mixed with 85 µL of prewarmed (48°C) LMP agarose 1% (w/v). Cells in LMP agarose were applied to a Trevigen comet slide and incubated at room temperature until the gel layer solidified

**Enzyme treatment**

- Layered with 50 µL trypsin solution (0.25% trypsin, 1 mM EDTA in Hanks balanced salt solution) and incubated for 30 min at 37°C. Slides washed with PBS.

**Lysis**

- Cell lysis with proteinase-K (1 mg/ml) for 60 min

**Pre-electrophoresis**

- Alkaline solution for 20 min at room temperature in the dark

**Electrophoresis**

- In electrophoresis buffer (0.01 M NaOH, 1 mM EDTA, pH 9.1), 0.9 V/cm, for 18-20 min

**Neutralisation, fixation and staining**

- Slides rinsed by dipping several times in distilled water. Fixation by immersing in 70% EtOH 5 min, then air dried. Etil stained (50 mg/mL)
<table>
<thead>
<tr>
<th>Exfoliated Buccal Cells Sampling</th>
<th>Buccal comet assay technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rinsing</strong></td>
<td><strong>Collecting</strong></td>
<td></td>
</tr>
<tr>
<td>Washing out the child’s mouth</td>
<td>Parents collected epithelial</td>
<td>Stained with Et-Br [86]</td>
</tr>
<tr>
<td>with tepid water to remove</td>
<td>mucosa cell samples by</td>
<td></td>
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<tr>
<td>exfoliated dead cells</td>
<td>gently brushing the</td>
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<td></td>
<td>inside of both cheeks</td>
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<td></td>
<td>with a cytology brush. The</td>
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<td></td>
<td>brush was then</td>
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<td></td>
<td>stirred in a PBS (pH 7.4)</td>
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<tr>
<td><strong>Water</strong></td>
<td>Scraping the inner part of</td>
<td>Stained with 50 ml of diluted SYBR Green [67]</td>
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<tr>
<td></td>
<td>the cheeks both sides with</td>
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<td></td>
<td>a cytology brush, cells kept</td>
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<td></td>
<td>in 0.9% NaCl and PBS in</td>
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<td></td>
<td>separate microcentrifuge</td>
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<td>tubes, brought to</td>
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<td></td>
<td>laboratory</td>
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<td><strong>Water</strong></td>
<td>The cells were collected by</td>
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<td>scraping the inner part of</td>
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<td>the cheeks both sides with</td>
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<tr>
<td></td>
<td>a cytology brush. Then, the</td>
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<td></td>
<td>cells were gently mixed with</td>
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<td></td>
<td>0.9% NaCl and PBS in separate</td>
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<td></td>
<td>microcentrifuge tubes and</td>
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<td></td>
<td>brought to the laboratory</td>
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<tr>
<td><strong>Followed protocol</strong></td>
<td>Cells were processed in</td>
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<td></td>
<td>alkali conditions and</td>
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<td>underwent</td>
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<td></td>
<td>electrophoresis assay</td>
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<td></td>
<td>[51, 110]</td>
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<tr>
<td><strong>Centrifuged</strong></td>
<td>Cell suspensions were</td>
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<td></td>
<td>washed twice with</td>
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<td></td>
<td>centrifugation at</td>
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<tr>
<td></td>
<td>room temperature</td>
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<tr>
<td><strong>Slides preparation</strong></td>
<td>Used the Treviglon Comet-</td>
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<tr>
<td></td>
<td>Assay7M kit protocol</td>
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<tr>
<td><strong>Enzyme treatment</strong></td>
<td>Cells + LMP agarose at 37°C</td>
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<tr>
<td></td>
<td>at the ratio of 1:10, and</td>
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<td></td>
<td>75 μl aliquots placed onto</td>
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<td></td>
<td>the slides and placed flat</td>
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<td></td>
<td>in a dark place at 4°C for</td>
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<td></td>
<td>10 min.</td>
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<tr>
<td><strong>Lysis</strong></td>
<td>The slides immersed in the</td>
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<tr>
<td></td>
<td>pre-chilled lysis solution</td>
<td></td>
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<tr>
<td></td>
<td>for 60 min.</td>
<td></td>
</tr>
<tr>
<td><strong>Pre-electrophoresis</strong></td>
<td>A freshly prepared alkaline</td>
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<tr>
<td></td>
<td>solution, pH=13, at room</td>
<td></td>
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<tr>
<td></td>
<td>temperature in the dark for</td>
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<tr>
<td></td>
<td>45 min.</td>
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<tr>
<td><strong>Electrophoresis</strong></td>
<td>The slides placed flat on a</td>
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<tr>
<td></td>
<td>gel tray, At 1 V/cm for 10</td>
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<td>min</td>
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<tr>
<td>Exfoliated Buccal Cells Sampling</td>
<td>Buccal comet assay technique</td>
<td>Reference</td>
</tr>
<tr>
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</tr>
<tr>
<td><strong>Rinsing</strong></td>
<td><strong>Collecting</strong></td>
<td><strong>Followed protocol</strong></td>
</tr>
<tr>
<td>Subjects rinsed their mouth thoroughly with saline solution to remove excess debris</td>
<td>Samples were obtained by scraping out from both cheeks with a moist wooden spatula. The spatula was then vigorously shaken in a dark plastic tube containing 10 ml of cold PBS, pH 7.4, and immediately refrigerated.</td>
<td>Within 1 h, exfoliated cells were processed by washing twice in PBS. After centrifugation at 800 x g for 3 min, the pellets were suspended in 40 μl PBS.</td>
</tr>
<tr>
<td><strong>Mouth washed with normal saline (0.9% NaCl) solution</strong></td>
<td>According to the alkaline single-cell gel electrophoresis method (100, 111)</td>
<td></td>
</tr>
<tr>
<td><strong>Water</strong></td>
<td>Brushing in the morning before taking any tobacco or tea. Collecting samples were taken in PBS</td>
<td>Using a standard protocol with some modifications [61]</td>
</tr>
<tr>
<td><strong>Scraping the inner part of both sides of the cheeks with a cytology brush</strong>. The cells were then gently mixed with 1.5 ml of 0.9% NaCl and PBS in a micro centrifuge tube, taken to the laboratory</td>
<td>Used the TempReigen Comet Assay™ kit protocol</td>
<td>The cells were combined with LMP agarose at 37°C at the ratio of 1:1.5, and 75 μl aliquots were immediately pipetted onto the slides. The slides were prepared in duplicate and placed flat in a dark place at 4°C for 10 min.</td>
</tr>
<tr>
<td>Exfoliated Buccal Cells Sampling</td>
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</tr>
<tr>
<td><strong>Rinsing</strong></td>
<td>Rinsing the mouth with temperate water to remove the exfoliated death cells.</td>
<td>[53]</td>
</tr>
<tr>
<td><strong>Collecting</strong></td>
<td>A soft interproximal toothbrush was used to collect buccal cells by gently scraping the inside cheek (right and left) of the mouth.</td>
<td></td>
</tr>
<tr>
<td><strong>Followed protocol</strong></td>
<td>The toothbrush was vigorously agitated in 5 ml of cold PBS in a 15 ml plastic tube and the resulting buccal cell suspension centrifuged at 1500 rpm and 15°C for 10 min.</td>
<td></td>
</tr>
<tr>
<td><strong>Centrifuged</strong></td>
<td>The toothbrush was vigorously agitated in 5 ml of cold PBS in a 15 ml plastic tube and the resulting buccal cell suspension centrifuged at 1500 rpm and 15°C for 10 min.</td>
<td></td>
</tr>
<tr>
<td><strong>Slides preparation</strong></td>
<td>Conventional microscope slides were treated with two layers of agarose. The bottom layer was prepared by dipping the slides into 10% of NMFP agarose, allowing the agarose to solidify at 4°C for a minimum of 5 min. Then, the top cell-containing layer consisted of 100 µl of a buccal cell suspension prepared in LMP agarose at 0.5%, (15 µl) of cell suspension and 85 µl agarose. After covering, the slide was kept at 4°C for 5 min.</td>
<td></td>
</tr>
<tr>
<td><strong>Enzyme treatment</strong></td>
<td>The cells were subjected to a lysing with 0.25% Trypsin in PBS (5 min, 37°C). Washed with 0.4 M Tris base solution and subsequently treated with proteinase K (1 mg/ml) for 30 min.</td>
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<tr>
<td><strong>Lysis</strong></td>
<td>The slides were rinsed, immersed in lysis solution (25 MNaCl, 0.1 M EDTA; 10 mM Tris Base; 7% Trixon X-100; and 10% DMSO; pH 10) for another hour at 4°C and washed again with 0.4 M Tris Base solution.</td>
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<tr>
<td><strong>Pre-electrophoresis</strong></td>
<td>Using an horizontal gel electrophoresis tank containing freshly prepared cold (8°C) electrophoresis buffer (1 mM Na2EDTA and 10 mM NaOH, pH 9) where the slides were submerged side by side in the gel tray and left for 20 min to produce single stranded DNA unwinding.</td>
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</tr>
<tr>
<td><strong>Electrophoresis</strong></td>
<td>Electrophoresis was run at 25 V and 300 mA for 20 min.</td>
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<tr>
<td><strong>Neutralisation, fixation and staining</strong></td>
<td>Rinsed with Tris solution. The cells were stained with 75 µl of a 20 µg/ml solution of BrdU.</td>
<td></td>
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</tbody>
</table>

**Before the start of the study, all subjects were instructed to continue brushing but not to use toothpastes and mouthwashes containing fluoride or chlorhexidine.**

**According to Tice and Vasquez [113]**

**The lysis step included an additional step of 100 ml of 1 mg/ml of proteinase K for 45 min to enhance the lysis step as recommended by Szeto et al. [61]**

**Unwinding for 40 minutes in electrophoresis buffer with the pH above 13**

**Slides were electrophoresed in the alkaline buffer at room temperature for 28 V for 40 minutes, level of the buffer was adjusted until 300 mA.**

**The slides were stained using 50 µl EthBr (20 µg/ml).**

**[54]**
<table>
<thead>
<tr>
<th>Exfoliated Buccal Cells Sampling</th>
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<tbody>
<tr>
<td>Rinsing Rinsing their mouth thoroughly by with water to remove unwanted debris</td>
<td></td>
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</tr>
<tr>
<td>Collecting Buccal mucosa cells were obtained by scraping the left inner cheek with a cervical brush</td>
<td>Slides preparation The cells were washed with PBS and centrifuged at 400 rpm for 10 min</td>
<td>20 min at 25 V (0.90 V/cm) and 300 mA</td>
</tr>
<tr>
<td>Followed protocol Then, 20 µL of the pellet was re-suspended in 80 µL of 0.75% LMP agarose</td>
<td>Enzyme treatment When the agarose solidified the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris pH 10.0-10.5) containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) DMSO for a minimum of 1 h and a maximum of 2 weeks</td>
<td>To allow DNA unwinding, slides were incubated in a freshly made alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA; pH &gt; 13) for 20 min in a horizontal electrophoresis tank</td>
</tr>
<tr>
<td>Centrifuged</td>
<td>Lysis</td>
<td>Neutralisation, fixation and staining Slides were washed three times in a neutralisation buffer (0.4 M Tris pH 7.5) for 5 min, rinsed 3 times in distilled water, and left to dry overnight at room temperature. Slides were stained with silver nitrate</td>
</tr>
<tr>
<td>The cells were washed with PBS and centrifuged at 400 rpm for 10 min</td>
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<tr>
<td>The buccal cell suspension was centrifuged</td>
<td>Electrophoresis</td>
<td>Stained with EtBr</td>
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<tr>
<td>The pellet obtained was mixed with 0.7% LMP agarose and placed on fully frosted roughened slides previously coated with 1% NMP agarose. To the solidified agarose, a third layer of 0.1% LMP was applied</td>
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<tr>
<td>Were immersed in freshly prepared ice cold lysis solution for 1 hour</td>
<td>[79]</td>
<td>[80]</td>
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<td><strong>Followed protocol</strong></td>
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<tr>
<td>Women rinse their mouth with saline water to remove extraneous materials</td>
<td>Exfoliated buccal epithelial cells (BECs) were collected by scraping the inside of both sides of the cheek with a soft bristle toothbrush. The toothbrush was then agitated in 30 ml cold PBS in a 50 ml plastic tube</td>
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<td></td>
<td>Buccal cell suspensions were centrifuged at 2000 rpm at 4°C for 10 min</td>
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<tr>
<td>Washed out the mouth three times with tepid water to remove dead exfoliated cells</td>
<td>Buccal swab taken by gentle brushing of the internal part of right and left cheek with a cup brush. The brushes were stirred in 5 ml of RPMI 1640, liqiuid (with L-glutamine, 25 mM HEPES), fetal bovine serum, and penicillin-streptomycin solution and transported within 30 min to the laboratory</td>
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<td>Collecting</td>
<td>Followed protocol</td>
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<tr>
<td>Water</td>
<td>The interior surfaces of right and left cheek were gently scraped with a toothbrush. The cells suspended in 25 ml of Titenko-Hollend buffer solution [114] and transferred within 2-4 hr, at 4°C and in the dark, to the laboratory.</td>
<td>According to Singh et al. [106]</td>
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</tbody>
</table>
Assessment of DNA Damage by Comet Assay in Buccal Epithelial Cells: Problems, Achievement, Perspectives

http://dx.doi.org/10.5772/62760

Exfoliated Buccal Cells Sampling

<table>
<thead>
<tr>
<th>Rinse</th>
<th>Collected</th>
<th>Followed protocol</th>
<th>Centrifuged</th>
<th>Slides preparation</th>
<th>Enzyme treatment</th>
<th>Lysis</th>
<th>Pre-electrophoresis</th>
<th>Electrophoresis</th>
<th>Neutralisation, fixation and staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal epithelial cells were collected by gently scraping the oral mucosa with a moist spatula. Suspended in phosphate buffered saline (PBS) and was processed for the comet assay.</td>
<td>According with Singh et al. [100]</td>
<td>The buccal epithelial cell samples were washed with PBS, centrifuged and recovered from the pellet.</td>
<td>On a clean, dry, plain slide 100 µl of 0.75% LMP agarose prepared in PBS was layered; these precoated slides were dried at 37°C. One layer of this layer, 30 µl of PBS, and buccal epithelial cells in PBS, mixed with 110 µl of 0.5% LMP agarose in PBS was layered. The third layer consisted of 100 µl of LMP agarose.</td>
<td>The slides were incubated in cold lysis buffer (2.3 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, 1% Triton X-100 and 10% DMSO added fresh) at 4°C overnight.</td>
<td>Immersing in freshly prepared alkaline electrophoresis buffer (1 mM Na2EDTA and 300 mM NaOH, pH 12) for 30 min.</td>
<td>30 min at 300 mA, 0.67 V/cm</td>
<td>Neutralising buffer (0.4 M Tris buffer, pH 7.5). The slides were then washed with distilled water and air dried. Silver staining.</td>
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</tbody>
</table>

The buccal cells were collected three times from each subject at 3 alternate days after the work shift. Workers rinsed the mouth with distilled water.

The buccal cells were collected using a toothbrush by scraping the inside cheek of the mouth. The toothbrush was agitated in 30 ml cold PBS buffer.

The buccal cells were collected by gentle brushing of the inside part of the lower lip with a cytological brush. The brushes were stirred in 50 ml plastic tubes containing 20 ml of PBS.

Washing out the mouth several times with tepid distilled water.

The alkaline version of the CA was employed in this study [51, 115]. Cells were washed twice, with centrifugation at 1500 rpm for 10 min at room temperature, and resuspended in PBS.

Briefly, 10 µl cell suspension was mixed with 75 µl LMP agarose (0.7%) and added to a slide precoated with 100 µl agarose (1%). Lysis was performed overnight at pH 10.

Cells were placed in a electrophoresis chamber, exposed to alkaloid pH13, for 25 min for 20 min at 25 V/cm. The slides were neutralized, fixed, and stained with silver nitrate [108].

[Reference] [64] [78] [52]
<table>
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<tr>
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</thead>
<tbody>
<tr>
<td><strong>Rinsing</strong></td>
<td></td>
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<tr>
<td>Prior to brushing</td>
<td>Buccal squamous cells were collected from subjects by oral brushing</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>Correct assay was performed under alkaline conditions by using a standard protocol [100] with some modifications</td>
<td></td>
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<tr>
<td></td>
<td>Cells were embedded in LMP agarose on glass slide precoated with 1% NMP agarose</td>
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<tr>
<td></td>
<td>After solidification, the slide was submerged into cool lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0), 1% L-55 buffered sarcosine sodium salt to which 10% DMSO, 1% Triton X-100 were freshly added and kept overnight at 4°C</td>
<td></td>
</tr>
</tbody>
</table>

<p>| <strong>Collecting</strong>                  |                               |           |
| Wash their mouth with water     | The interior surfaces of the right and left cheeks were gently scraped with a toothbrush. The cells were suspended in 25 mL of a buffer solution containing 0.01 M Tris-HCl, 0.1 M EDTA, and 0.02 M NaCl (pH 7.0), and immediately sent to the laboratory where the comet assay was performed. The exfoliated buccal cells were washed twice in PBS and then suspended in about 100 µL of the same buffer | [77]      |
|                                 | The procedure of Singh et al. [100] was used, with minor modifications |           |
|                                 | The exfoliated buccal cells were washed twice in PBS, then suspended in about 100 µL of the same buffer |           |
|                                 | 90 µL of 0.5% NMP agarose in PBS at 56°C were layered onto gel bond film, immediately covered with a coverslip, and allowed to solidify at 4°C for 5 min. The coverslip was then removed and about 80 µL of exfoliated cell suspension were mixed with 70 µL of 0.7% LMP agarose in PBS at 37°C and layered on top of the film. A coverslip was added and the film was left to solidify at 4°C for 5 min. After this, the coverslip was removed and a second layer of 0.7% LMP agarose was added and left to solidify |           |
|                                 | The coverslips were taken off and the films were layered onto glass slides and tauted in freshly prepared lysis solution (0.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, with 1% Triton X-100 and 10% DMSO added fresh) in the dark for 1 h at 4°C |           |
|                                 | Placed in a horizontal gel electrophoresis tank, filled with fresh alkaline buffer (1 mM Na2EDTA and 300 mM NaOH, pH 13) for 20 min at 4°C to allow denaturation and unwinding of the DNA, and the exposure of alkali-labile sites |           |
|                                 | At 25 V and 300 mA for 40 min |           |
|                                 | The slides were neutralized for 60 min in 0.4 M Tris/HCl, pH 7.5 on ice and stained in EtBr (25 µg/ml in distilled water) |           |
|                                 | The slides were washed three times for 5 min each with 0.4 M Tris-HCl. Slides were stained with 30 µl of 10 µg/ml EtBr |           |</p>
<table>
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<tbody>
<tr>
<td>Rinsing</td>
<td>Collecting</td>
<td>[116]</td>
</tr>
<tr>
<td>Rinsed their mouths with water</td>
<td>To collect exfoliated buccal cells, the right and left cheeks were gently scraped with a toothbrush. The cells were suspended in 25 ml of Tienko-Holland buffer solution [114]</td>
<td></td>
</tr>
<tr>
<td>Procedure of Collins et al. [116], with minor modifications</td>
<td>The exfoliated buccal cells were washed twice in PBS and then suspended in about 100 µl of the same buffer</td>
<td></td>
</tr>
<tr>
<td>Slides preparation</td>
<td>Centrifuged</td>
<td></td>
</tr>
<tr>
<td>Two gel bond films were prepared for each case (one to be treated with Fpg and the other left untreated) allowing the detection of oxidative DNA and direct DNA lesions (single-strand breaks and alkali-labile sites), respectively [116]. About 80 µl of exfoliated cell suspension were mixed with 70 µl of 0.7% LMP agarose in PBS at 37°C and layered on top of each film</td>
<td></td>
<td></td>
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<tr>
<td>Enzyme treatment</td>
<td>Lysis</td>
<td></td>
</tr>
<tr>
<td>Then they were bathed in lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Triton X-100 and 10% DMSO add- est新鲜 and kept in the dark for 1 h at 4°C</td>
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<tr>
<td>Pre-electrophoresis</td>
<td>Electrophoresis</td>
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<tr>
<td>The slides were washed 3 times in enzyme buffer (50 mM Na₂PO₄, 10 mM EDTA, 300 mM NaCl, pH 7.5), drained and incubated with 50 µl of either buffer or Fpg (1 µg/ml in enzyme buffer) in the dark for 30 min at 37°C. The slides placed in a horizontal gel electrophoresis tank filled with fresh alkaline buffer 1 mM Na₂EDTA and 300 mM NaOH, pH 13 for 40 min at 4°C</td>
<td></td>
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<tr>
<td>Electrophoresis</td>
<td>In the same buffer at 25 V and 300 mA for 30 min</td>
<td></td>
</tr>
<tr>
<td>Neutralisation, fixation and staining</td>
<td>The slides were then washed 3 times with Tris-HCl 0.4 M for 5 min and stained with 50 µl EtBr (10 µg/ml)</td>
<td>[75]</td>
</tr>
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<tr>
<td><strong>Rinsing</strong></td>
<td>Volunteers rinse their mouth thoroughly with filtered tap water</td>
<td>92</td>
</tr>
<tr>
<td><strong>Collecting</strong></td>
<td>Cell suspensions were obtained by scraping the inner cheek with a wooden stick or with a disposable brush moistened with PBS. The first scraping from each side of the cheek was discarded. The cells from each of the next four scrapings were rinsed into ice-cold PBS using individual coded centrifuge tubes, and were kept on ice until processed (within 30 min).</td>
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<tr>
<td><strong>Followed protocol</strong></td>
<td>According with Singh et al. [108] and Valverde et al. [83], with modifications</td>
<td></td>
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<tr>
<td><strong>Centrifuged</strong></td>
<td>The cells were centrifuged at 89 x g, for 5 min. 10 μL were used to perform the SCGE assay</td>
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<tr>
<td><strong>Slides preparation</strong></td>
<td>Briefly, aliquots of cell suspensions were suspended in 100 μL of molten 0.5% LMP agarose in PBS (cooled to 37°C). This mixture was layered onto a cooled slide, precoated with a thin layer of LMP agarose [117]. The agarose layer was covered with a cover slip and left for 5 min at 3°C to solidify</td>
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<tr>
<td><strong>Enzyme treatment</strong></td>
<td>Cell suspension was treated 1:1 with an enzyme “cocktail” (final concentration: 0.05 mg/ml DNase I, 0.15 mg/ml collagenase I, and 6.125 mg/ml trypsin in 0.01% EDTA, pH 7.4) for 30 min at 37°C (adapted from Ovle et al. [118]). After lysis, the slides were treated with PK for 1 hr at 37°C, by layering 100 μL of 1 mg/ml PK in PBS (pH 7.4) onto the slide and adding a coverslip to achieve an equal distribution of solution. Afterwards, the slides were immersed in 400 mM Tris-HCl (pH 7.5) for 5 min to remove excess salt</td>
<td></td>
</tr>
<tr>
<td><strong>Lysis</strong></td>
<td>Slides were immersed into either lysis solution I (2.5 M NaCl, 100 mM Na,EDTA, 10 mM Tris (pH 10), and 1% sodium sarcosine/CaCl₂, with 1% Triton X-100 and 10% DMSO added just before use) or Lysis Solution II (1% SDS and 30 mM Na,EDTA, pH 8) for at least 1 hr at 8°C</td>
<td></td>
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<tr>
<td><strong>Pre-electrophoresis</strong></td>
<td>Different alkaline unwinding times (0 - 40 min) and electrophoresis times (0.6A V/cm, 200 mA, for 5 - 30 min) were tested in the preliminary experiments. For the cross-sectional experiment, both pre- and postenrichment slides were randomized by location inside the electrophoresis box and by different runs.</td>
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<tr>
<td><strong>Electrophoresis</strong></td>
<td>Unwinding and electrophoresis were performed without an unwinding step and under neutral conditions (300 mM sodium acetate, 100 mM Tris, adjusted to pH 9 with glacial acetic acid [119]) for 1 hr at ~0.5 V/cm and 50 mA at 4°C so as to study the influence of alkali-labile sites on the migration of comets from cell samples</td>
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<tr>
<td><strong>Neutralisation, fixation and staining</strong></td>
<td>Slides were neutralized in Tris-HCl (pH 7.5) for 5 min, fixed with absolute ethanol, and stained. The slides were stained with 20 μg/ml EtBr</td>
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</tbody>
</table>
Assessment of DNA Damage by Comet Assay in Buccal Epithelial Cells: Problems, Achievement, Perspectives

Exfoliated Buccal Cells Sampling

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<th>Electrophoresis</th>
<th>Neutralisation, fixation and staining</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>Several</td>
<td>Buccal cell suspension was centrifuged at 2500 rpm 4°C for 30 min. Cell pellet was resuspended to 100 µL PBS</td>
<td>10 µL of tissue suspension was mixed with 85 µL of pre-warmed (60°C) 1% (w/v) LMP agarose in PBS, and immediately applied to a microscope slide which had been precoated with 0.5 µL of 1% (w/v) standard agarose in PBS. The slides were placed at room temperature until the gel layer solidified, and then put through the lysing procedure.</td>
<td>In some cases ly sis was followed by exposure of the cells to 6.1 mg/ml proteinase K in lysis solution at pH 7.5 without Triton X-100 for up to 5 h in a Coplin jar at 37°C. Also analyze the effects of trypsin treatment. The fixed (optimized) lysis protocol selected used cells pre-embedded in agarose on a microscope slide, with 50 µL trypsin solution layered onto the gel and left for 30 min at 37°C followed by aning proteinase K treatment.</td>
<td>Investigated the effect on buccal cells of immersion in standard lysis solution for up to 24 h at 4°C. Cell lysis analyze the effect of detergents.</td>
<td>Slides were then transferred to a Coplin jar containing electrophoresis solution at 4°C for 20 min (2-10 min). Electrophoresis was performed for 18 min at 12 V constant voltage.</td>
<td>Investigated the effect of electrophoresis at lower pH values. Electrophoresis was performed for 18 min at 12 V constant voltage.</td>
<td>Neutralized by immersing in three changes (3×5 min) of 0.4M Tris at pH 7.5. n were stained with EtBr</td>
<td>[61]</td>
</tr>
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<tr>
<td><strong>Rinsing</strong></td>
<td>Collect buccal epithelial cells directly from the inner cheek and sublingual region using a soft toothbrush. The cells collected on the toothbrush were transferred to a sterile PBS solution (pH 7). Cells were washed twice in PBS and then resuspended in RPMI-1640 medium.</td>
<td></td>
<td>[60]</td>
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<tr>
<td><strong>Collecting</strong></td>
<td>Collect buccal epithelial cells from the inner cheek and sublingual region using a soft toothbrush. The cells collected on the toothbrush were transferred to a sterile PBS solution (pH 7). Cells were washed twice in PBS and then resuspended in RPMI-1640 medium.</td>
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<td>Exfoliated Buccal Cells Sampling</td>
<td>Buccal comet assay technique</td>
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<td><strong>Rinsing</strong></td>
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<td><strong>Collecting</strong></td>
<td>Buccal epithelial cells were obtained by scraping the cheeks with a plastic spatula. The cells were added to 2 ml of RPMI-1640 medium</td>
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<td><strong>Followed by centrifuged at 600 rpm for 1 min</strong></td>
<td>Slides were prepared in duplicate, and 120 μl of 0.5% NP40 was layered on to pre-cleaned microscope slides, immediately covered with coverslips, and allowed to solidify. Then, cells were mixed with 75 μl of 0.5% LMP agarose in PBS at 37°C; the coverslips were removed, and the mixture was added to the slides. The coverslips were replaced and the slides were put on ice for 5 min. After solidification of the agarose, the coverslips were gently removed. A top layer of 75-μl LMP agarose was added, the coverslips were replaced, and the slides were again placed on ice</td>
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<td><strong>Centrifuged</strong></td>
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<td><strong>Enzyme treatment</strong></td>
<td>Slides were prepared in duplicate, and 120 μl of NP40 was layered on to pre-cleaned microscope slides, immediately covered with coverslips, and allowed to solidify. Then, cells were mixed with 75 μl of 0.5% LMP agarose in PBS at 37°C; the coverslips were removed, and the mixture was added to the slides. The coverslips were replaced and the slides were put on ice for 5 min. After solidification of the agarose, the coverslips were gently removed. A top layer of 75-μl LMP agarose was added, the coverslips were replaced, and the slides were again placed on ice</td>
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<td><strong>Lysis</strong></td>
<td>Slides were prepared in duplicate, and 120 μl of NP40 was layered on to pre-cleaned microscope slides, immediately covered with coverslips, and allowed to solidify. Then, cells were mixed with 75 μl of 0.5% LMP agarose in PBS at 37°C; the coverslips were removed, and the mixture was added to the slides. The coverslips were replaced and the slides were put on ice for 5 min. After solidification of the agarose, the coverslips were gently removed. A top layer of 75-μl LMP agarose was added, the coverslips were replaced, and the slides were again placed on ice</td>
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<td><strong>Pre-electrophoresis</strong></td>
<td>Slides were prepared in duplicate, and 120 μl of NP40 was layered on to pre-cleaned microscope slides, immediately covered with coverslips, and allowed to solidify. Then, cells were mixed with 75 μl of 0.5% LMP agarose in PBS at 37°C; the coverslips were removed, and the mixture was added to the slides. The coverslips were replaced and the slides were put on ice for 5 min. After solidification of the agarose, the coverslips were gently removed. A top layer of 75-μl LMP agarose was added, the coverslips were replaced, and the slides were again placed on ice</td>
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<td><strong>Electrophoresis</strong></td>
<td>Slides were prepared in duplicate, and 120 μl of NP40 was layered on to pre-cleaned microscope slides, immediately covered with coverslips, and allowed to solidify. Then, cells were mixed with 75 μl of 0.5% LMP agarose in PBS at 37°C; the coverslips were removed, and the mixture was added to the slides. The coverslips were replaced and the slides were put on ice for 5 min. After solidification of the agarose, the coverslips were gently removed. A top layer of 75-μl LMP agarose was added, the coverslips were replaced, and the slides were again placed on ice</td>
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<td><strong>Neutralisation, fixation and staining</strong></td>
<td>Slides were prepared in duplicate, and 120 μl of NP40 was layered on to pre-cleaned microscope slides, immediately covered with coverslips, and allowed to solidify. Then, cells were mixed with 75 μl of 0.5% LMP agarose in PBS at 37°C; the coverslips were removed, and the mixture was added to the slides. The coverslips were replaced and the slides were put on ice for 5 min. After solidification of the agarose, the coverslips were gently removed. A top layer of 75-μl LMP agarose was added, the coverslips were replaced, and the slides were again placed on ice</td>
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<td><strong>Rinsing</strong></td>
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<td>Buccal epithelial cells were obtained by scraping the internal part of the cheek with a wood stick and were added to 1 ml of cold RPMI-1640 medium.</td>
<td>Rojas et al. [14], with some modifications.</td>
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<td><strong>Followed protocol</strong></td>
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<td><strong>Slides preparation</strong></td>
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<td>Enzyme treatment</td>
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<td>After lysis (2.5 M NaCl, 100 mM NaN3EDA, 30 mM Tris-hydrochloride and 1% Naascorbinate, pH 10) at 4°C for 24 hr, the cells were treated with 100 μL protease K (10 mg/ml) at 37°C for 1 hr.</td>
<td>Slides were placed on a horizontal electrophoresis unit. The DNA was allowed to unwind for 20 min, in electrophoresis running buffer solution (30 mM NaOH and 1 mM NaN3EDA, pH 13).</td>
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<td>Neutralisation, fixation and staining</td>
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<td>20 min at 25 V and 300 mA. After 20 min, the slides were gently removed.</td>
<td>Neutralized with 0.4 M Tris, pH 7.5; dehydrated in 2 steps with absolute ethanol for 10 min each, staining with 75 ml EBBR (20 mg/ml) with coverslip.</td>
<td>[83]</td>
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<td><strong>Scraping the internal part of the cheek with a wood stick</strong></td>
<td>Tice et al. [120], with some modifications.</td>
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<td>The cells were added into 2 mL of RPMI-1640 medium and centrifuged at 600 rpm for 1 min, approx. 30000 cells resuspended in 75 μl LMP agarose.</td>
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<td>After lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% sodium sarcosinate, pH 10) at 4°C for 48 hr, the cells were dropped into a new lysis solution with 140 μl of protease K (10 mg/ml) at 37°C for 2 hr.</td>
<td>Slides were placed on a horizontal electrophoresis unit. The DNA was allowed to unwind for 20 min, in electrophoresis running buffer solution (300 mM NaOH and 1 mM NaN3EDA, pH 13).</td>
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<td>For 20 min at 25 V and 300 mA.</td>
<td>Neutralization with 0.4 M Tris-HCl, pH 7.5; staining with EBBR (75 μL of a 20 mg/mL solution) was added to each slide.</td>
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Table 1. List of the articles and detailed methodology for sampling, slide preparation, lysis step, enzymatic digestion, electrophoresis, neutralization, fixation and staining.
3. Use of comet assay in buccal cells

The comet assay in buccal cells has been used to evaluate DNA damage induced by different materials such as mouthrinses [50], metals released from orthodontic appliances [51–59], ionizing radiation [60], as well as assessment of DNA damage, and its modulation by lifestyle, dietary, genetic and healthy factors [61–74], occupational exposure [66–69,75–82], and environmental exposure [83–86]. Different procedures have been used in collecting and processing the samples that are presented and discussed in Rojas et al. [33]. The Table 2 represents classification according to the type of population study based on exposure and lifestyle factors with the results of comet assay.

3.1. Mouthrinses and metal released from orthodontic appliances

The genotoxic properties of mouthrinses and metals from orthodontic appliances are essential for determining the biological safety of those materials in patients. Current in vivo human studies are aimed at representing the real condition of the oral cavity by sampling buccal cells, which are directly exposed to the appliances [51,52].

Eren et al. [50] evaluated the stability of buccal epithelial cells for SCGE assay after the use of chlorhexidine digluconate (CHX), a mouthrinse used by dentists as a disinfecting agent for operation sites washing and for disinfection of root canals. A statistical increase was observed in the DNA damage after the CHX application. Considering orthodontic appliances, the first in vivo study was performed by Faccioni et al. [51], who conducted the alkaline comet assay in orthodontic patients. They reported genotoxic damage and found positive correlations between the concentrations of released cobalt and nickel and the number of comets as well as correlations between Co levels and comet tails. However, Westphalen et al. [52] did not find genetic damage after the placement of the orthodontic appliances.

According to Fernández-Miñano et al. [53], genotoxicity induced in buccal cells could be related to the composition of orthodontic appliances. Orthodontic apparatus made with titanium was not genotoxic for oral mucosa cells, whereas the stainless steel alloy and nickel-free alloy induced DNA damage in buccal mucosa cells. In contrast, Hafez et al. [54] observed that stainless steel brackets with stainless steel archwires produce the least damage, whereas titanium brackets with nickel–titanium archwires produced the highest amount of genotoxicity, assessed with the comet assay. Baričević et al. [55] assessed subjects with Co–Cr–Mo alloy and Ni–Cr alloy showed significantly higher comet assay parameters when compared with controls. Gonçalves et al. [59] showed the genotoxic effects of Hyrax auxiliary orthodontic appliances containing silver-soldered joints.

On the other hand, Hafez et al. [54] reported damage to the DNA in mucosa cells at 3 months of orthodontic treatment but not at 6 months. Thus, the difference in exposure period of prosthodontic and orthodontic appliances in oral cavity might explain discrepancies observed between results obtained by Faccioni et al. [51], and those of Westphalen et al. [52] and Baričević et al. [55].
Visalli et al. [56] found that both amalgams and resin-based composite fillings can induce genotoxic damage in human oral mucosa cells. They also report that lifestyle variables, including alcohol intake and smoking habits, did not affect the genotoxic response and did not act as confounding factors. Martín-Cameán et al. [57] observed induction of genotoxicity in buccal cells of subjects with orthodontic appliances and orthodontic appliances with micro-screws when compared with controls. In addition they found that damage was higher in women.

### 3.2. Radiation

Only one work that analyses and compares the DNA damage and repair following radiation challenge in buccal cells and lymphocytes using SCGE assay was found. The results suggested that baseline DNA damage in oral epithelial cells is greater than that in lymphocytes [60].

### 3.3. Life style, dietary, genetic and healthy factors

As mentioned above in the first work of this type, Rojas et al. [14] found a significantly increased tail length in a smoker group compared with a non-smoker group. Differences between genders either in the smoker or non-smoker group were not observed and were neither related to age or number of cigarettes smoked. Waterpipe smoking (a type of tobacco smoking) and its condensate have been examined for the genotoxic effects on buccal cells. The tail moment in buccal cells of smokers was found to be 186 ± 26, which is 371.9% higher than the tail moment in buccal cells of non-smokers. The other comet parameters such as tail length, % tail DNA, and fragmented DNA were 456 ± 71, 97.0 ± 19, and 32.0 ± 3.3, respectively, in buccal cells of smokers, whereas in control group (non-smokers), the values of tail length, % tail DNA, and fragmented DNA were extremely low [72].

Oral habits have also been associated with DNA damage. Khanna et al. [70] reported a case of a tobacco chawer in which the percentage of damaged cells was significantly higher than in the control. Also the effect of gutkha (a preparation of crushed areca nut, tobacco, catechu, paraffin wax, slaked lime, and sweet or savory flavorings) and pan masala (an herb, nut, and seed mixture that is commonly served in the Middle East countries) chewing along with and without smoking was studied in buccal epithelial cells using single-cell gel electrophoresis [71]. The increase in the mean comet tail length was observed as follows: non users < smokers < pan masala chewers < gutkha chewers < pan masala + smoking < gutkha + smoking. Like Rojas et al. [14], they conclude that these bioassay and biomarker are easier and safe methods to detect DNA damage among humans.

Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the comet assay has also been developed [87]. Pal et al. [62,63] analyzed the influence of regular black tea consumption on tobacco-associated DNA damage and human papilloma virus (HPV) prevalence in human oral mucosa. The increase in DNA damage was significantly associated with increase in age and tenure of tobacco habit. Reduced DNA damage was found to be significantly associated with increase in tea intake. In case of oral cancer patients, comparatively high frequency of DNA damage was observed. The frequency of DNA damage
and HPV infection was comparatively high in oral cancer patients than in the normal subjects. These studies indicated a chemopreventive role of black tea against reducing DNA damage risk of buccal cells due to tobacco exposure. Authors concluded that buccal cells could be used as cytological markers for detection of risk and risk reduction in normal population. Since, as mentioned above, more than 90% of human cancers arise from epithelial cells, it has been postulated that experiments with these cells may have particular relevance for the detection of cancer preventive effects [47].

On the other side, several polymorphisms in DNA repair genes have been reported to be associated with cancer risk [88]. The repair of DNA damage has a key role in protecting the genome from the insults of genotoxic agents. Tobacco-related compounds cause a variety of DNA damage, and DNA repair capacity plays an important role in agent-induced damage genotoxic. Several polymorphisms in genes that participate in different DNA repair pathways, such as XRCC1 399, hOGG1 326 [65], GSTP1 [66], CYP2E1 [67], CYP1A2 [68], and CYP1A1 [69], have been evaluated for their effects on different biomarkers [89], including comet tail length in buccal cells.

DNA damage effects of the used substances were confirmed in mechanical workshops workers, but with no confirmation of the influence of GSTP1 [66] or CYP1A1 [69] gene polymorphism on DNA damage, considering the comet assay performed on buccal cells. Conversely, workers with the wild genotype for CYP2E1 showed statistically significant higher comet tail length at the occupational exposure, while the mutated genotype did not have influence on this biomarker [67]. With CYP1A2 gene, the results showed that DNA damage in cells of workers carrying the mutated genotype was higher than workers carrying the wild genotype [68].

Sellappa et al. [65] found significant differences in the comet scores between smokeless tobacco users and control subjects when XRCC1399 and hOGG1326 polymorphisms and the frequencies of genetic damage among tobacco chewers were studied. These findings provided evidence for the view that polymorphisms in DNA repair genes may modify individual susceptibility to genotoxic agents and justify additional studies to investigate their potential role in development of genetic damage.

4. The use of the comet assay in buccal cells in biomonitoring the effect of pollution

4.1. Occupational exposure

Cavallo et al. [75] suggested the use of comet assay on exfoliated buccal cells to assess the occupational exposure to mixtures of inhalable pollutants at low doses since these cells represent the target tissue for this exposure and are obtained by non-invasive procedure. In their study, tail moment values from Fpg-enzyme-treated cells (TMenz) and from untreated cells (TM) were used as parameters of oxidative and direct DNA damage, respectively, and
found in the exposed group a higher value in respect to controls of mean TM and TMenz. An oxidative DNA damage was found, for exfoliated buccal cells in the 9.7% of exposed in respect to the absence in controls. On the other side, in healthcare workers in oncology hospital regularly handling antineoplastic drug mixtures, comet assay showed an increase on exfoliated buccal cells, also when it was not statistically significant, of mean TM with respect to controls in day hospital nurses (the group handling the highest amount of drugs during the administration process), while ward nurses and pharmacy technicians did not show the differences [77]. Increased levels of DNA damage were also found among jewellery workers occupationally exposed to nitric oxide using buccal cell comet assay, and also a synergistic effect of DNA damage with the cigarette smoking habit was found among the jewellery workers [78]. On the other hand, Cavallo et al. [76] evaluated two groups of workers, one exposed to antineoplastic drugs and the other exposed to PAHs, but the comet assay on exfoliated buccal cells did not show significant differences between exposed and control groups for comet percentages, whereas the TM value was higher in workers exposed to PAHs. Occupational risk assessment of paint industry workers with the comet assay in epithelial buccal cells showed that the damage index and damage frequency observed in the exposed group were significantly higher relative to the control group [79]. In other study on biomonitoring of genotoxic effects among shielded manual metal arc welders, Sudha et al. [80] showed a significantly larger mean comet tail length values. Among paddy farm workers exposed to mixtures of organophosphates was observed that the tail length formation showed significant increase of tail length differences between farmers compared with the matched control group [81]. Age, smoking status, duration of smoking, and secondhand smoker factors pointed out the significant intragroup variations, among the study population. Smokers and secondhand smokers generally showed higher levels of DNA damage, with increase connected with age and smoking duration increase. The last finding in this study leads again to the hypothesis that occupational risk factors contribute to the main effect on DNA damage. However, Carbajal-López et al. [82] did not find significant effect on genetic damage as a result of age, smoking, and alcohol consumption when genotoxic effect of pesticides in exfoliated buccal cells of workers occupationally exposed in Guerrero, Mexico was evaluated. The study revealed that the tail migration of DNA increased significantly in the exposed group.

4.2. Environmental exposure

After the first publication with comet assay in buccal cells by Rojas et al. [14], the same group [83] with this bioassay investigated differences in the level of DNA damage between young adults from the southern and northern areas of Mexico City and compared its effects with the damage induced in leukocytes and nasal epithelial cells. They found an increased DNA damage in leukocytes and nasal cells from individuals who lived in the northern part; however, no differences were observed for buccal epithelial cells, highlighting that it is important to study the genotoxic effects in other cells besides lymphocytes, as well as in cells of those tissues which are the first sites of contact with toxic pollutants. Although in their first work DNA damage in smokers was reported, in this work, they reported that smoking habit did not significantly increase DNA migration when compared with the non-smoker group.
A study of indoor air pollution from biomass burning was performed on Indian women engaged in biomass cooking (wood, dung, crop residues), and the group was compared with age-matched control women cooking with cleaner fuel liquefied petroleum gas. DNA damage was assessed on buccal epithelial cells (BEC) by comet assay and fast halo assay (FHA). Compared with control, BEC of biomass users showed higher comet tail % DNA, higher values for comet tail length, and olive tail moment, suggesting marked increase in DNA damage [84].

5. Clinical application of the comet assay in buccal cells

Significant stepwise increase in the DNA damage (basal/MNNG-treated/post-repair) was observed in buccal epithelial cells from control to pre-cancer patients and from pre-cancer to cancer patients. Considerable inter-individual and intercellular variability in DNA damage was observed, which also increased from control to pre-cancer patients and from pre-cancer to cancer patients [64]. Similar results were found in patients with oral squamous cell carcinoma (OSCC) and control group and suggested that comet assay may be used effectively to assess the prognosis of OSCC [73].

Among population studies regarding the health effects of air pollution, special attention should be given to children as a high-risk group, since some studies have shown significant correlation between early childhood exposure and development of chronic diseases in adulthood. Genotoxic biomarkers have been studied largely in adult population, but few studies so far have investigated children exposed to air pollution. Children are a high-risk group as regards the health effects of air pollution, and some studies suggest that early exposure during childhood can play an important role in the development of chronic diseases in adulthood. Genotoxic effects among farm children assessed with comet assay in buccal cells showed a significant increase in chromosome breakage and DNA strand breaks [85]. In other similar study, the exposure to pollutants was associated with markers of genotoxicity in exfoliated buccal cells of children living in a region with chipboard industries. The increase of outdoor formaldehyde was associated with a higher comet tail intensity and a higher tail moment [86].

6. Confounding factors in studies with the comet assay in buccal cells

A systematic and adequately powered investigation of key variables such as age, gender, genotype, season, diet, oral hygiene and dental health, life-style, smoking, alcohol, and other recreational drugs needs to be performed to identify the variables that have to be controlled [7].
<table>
<thead>
<tr>
<th>Exposed population</th>
<th>Control population</th>
<th>Interview</th>
<th>Results BCA</th>
<th>Parameters measured</th>
<th>Statistics</th>
<th>Other methods used</th>
<th>Results</th>
<th>Author</th>
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<td>detailed questionnaires regarding confounding factors for DNA damage such as smoking, viral diseases, recent vaccinations, and radiodiagnostic examinations.</td>
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<td>55 orthodontic patients with fixed appliances in both arches: nickel-titanium alloy, stainless steel or chromium-cobalt-nickel alloy</td>
<td>30, no dental restorations</td>
<td>Smoking, drinking</td>
<td>TL: 10.54 ± 2.41 vs 15.56 ± 6.78*</td>
<td>% DNA, tail length, TM</td>
<td>Wilcoxon's test</td>
<td>DNA damage in peripheral blood cells</td>
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<td>33 12-35</td>
<td>32 12-35</td>
<td>13 12-33</td>
<td>17 12-35</td>
<td>5.96 ± 0.21 vs 0.30 ± 0.49 vs 4.72 ± 1.51</td>
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<tr>
<td>14 16 + 2.5</td>
<td>6 16 + 2.5</td>
<td>14 16 + 2.5</td>
<td>6 16 + 2.5</td>
<td>Smoking or drinking or illnesses related to any genetic damage increase were not reported by any patient.</td>
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<td>2.5 ± 0.08 vs 1.5 ± 1.05</td>
<td>Damage was visually scored according to five classes, based on tail size (from undamaged - 0, to maximally damaged - 4). Damage index (DI) was thus assigned to each individual, according to Hartmann et al. [121]. The DI is a well-validated evaluation method as it is highly correlated with computer-based image analysis [122].</td>
<td>The one-tailed t-test with Welch’s correction was used</td>
<td>Micronucleus assay</td>
<td>MN frequency (p = 0.023)</td>
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<td>Exposed population</td>
<td>Control population</td>
<td>Interview</td>
<td>Results ICA</td>
<td>Parameters measured</td>
<td>Statistics</td>
<td>Other methods used</td>
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<td>Females (n), age</td>
<td>Males (n), age</td>
<td>15 12-16 the same patients before treatment</td>
<td>The inclusion criteria were: absence of systemic diseases, need of orthodontic treatments in both dental arches, absence of cavities or any repaired treatment in the oral cavity, with good oral health and absence of any disability to impede a correct oral hygiene, and that the treatment does not generate in the patients any psychological alteration or difficulties in their everyday relationships.</td>
<td>Exposure group vs control group</td>
<td>Stainless steel 69.35 ± 11.68; Nickel-free 68.41 ± 26.63; NiCr 71.10 ± 5.33 Titanium alloy and control/Office moment was similar</td>
<td>Olive moment</td>
<td>ANOVA, Tukey posthoc</td>
<td>[53]</td>
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<td>15 patients 12-16 after treatment with metal apparatus for orthodontic treatment: 4 tubes and 20 brackets for 30 days: 5 with stainless steel, 5 with titanium, 5 with nickel-free</td>
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<tr>
<td>22 20.2 ± 4.4</td>
<td>6 20.2 ± 4.4</td>
<td>10 21.5 ± 3.3</td>
<td>9 21.5 ± 3.3</td>
<td>DNA damage value, decreased from 125.5 ± 46.05 to 98.8 ± 33.70 at 6 months</td>
<td>Only nucleoids of the same size were chosen subjectively for scoring. A grade was given to each nucleoid according to DNA fragmentation in the comet tail. Also the damage frequency was calculated; this represents the number of comets per 100 examined nucleoids.</td>
<td>Normally distributed variables (compositional score and damage frequency) were tested with paired t tests.</td>
<td>[94]</td>
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<td>Exposed population</td>
<td>Control population</td>
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<td>Females (n), age</td>
<td>Males (n), age</td>
<td>Exhaustive medical history was documented for all subjects. A prestructured questionnaire on dietary and smoking habits, alcohol and drug intake, as well as on systemic diseases and verified allergy to known allergens and medications has been filled for each subject.</td>
<td>Significantly increased tail length and percentage DNA in the tail values in subjects wearing metal appliances</td>
<td>DNA damage was evaluated as percentage DNA in the tail (%DNA) and tail length</td>
<td>ANOVA vs Mann-Whitney U test t Test Newman–Keuls test</td>
<td>Evaluate influence of general characteristics of the subjects (age, gender, dietary habits, pH of saliva, alcohol and drug intake) on parameters of comet assay.</td>
<td>None of demographic or lifestyle factors tested as possible predictors have exhibited significant influence on values of comet assay parameters</td>
<td>[55]</td>
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<tr>
<td>30 69.56</td>
<td>25 71.68</td>
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<td>26 21.1 ± 0.30</td>
<td>17 21.1 ± 0.30</td>
<td>Collect information on age, gender, smoking, drinking, dietary habits, and previous drug intake. Moreover, chewing gum habits, toothbrushings per day, consumption of hot food and drinks, and bruxism behavior that could promote the release of restorative compounds</td>
<td>The DNA % was dose-dependent higher in subjects carrying dental fillings as compared with filling-free subjects. In subjects carrying at least two fillings</td>
<td>The results were expressed as percentage of DNA in the tail (TODNA %), measured by the automated image analysis system CASP (comet assay software project) (<a href="http://www.casperm.org">http://www.casperm.org</a>),</td>
<td>Mann–Whitney test and Poisson regression analyses</td>
<td>Morphological markers of cell death including pyknosis (condensed chromatin), karyorrhexis, and karyolysis, were evaluated at the microscopic analysis of the same slides used for the MN test.</td>
<td>MN frequency higher in subjects with restorative fillings than in filling-free subjects</td>
<td>[56]</td>
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<tr>
<td>20 persons as positive control (smokers)</td>
<td>20 patients with orthodontic treatment, 20 patients with orthodontic appliances and microscrews</td>
<td>20</td>
<td>% DNA in tail significantly different between all groups, females with orthodontic appliances</td>
<td>% DNA in tail</td>
<td>-</td>
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<td>[57]</td>
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<td>Exposed population</td>
<td>Control population</td>
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<td>Results RCA</td>
<td>Parameters measured vs control group</td>
<td>Statistics</td>
<td>Other methods used</td>
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<td>16 (7-14)</td>
<td>16 (7-14)</td>
<td>Each patient acted as his/her own control</td>
<td>Damage frequency 53.2% vs 35.94%</td>
<td>Wilcoxon's test</td>
<td>BMCT</td>
<td>No significant differences were observed</td>
<td>[59]</td>
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<td>Damage index 75.69% vs 50.31</td>
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<td>Radiation</td>
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<td>3 (34-45)</td>
<td>3 (34-45)</td>
<td>Participants were non-smokers, did not ever smoke and their weekly alcohol intake was less than 10 ml. Were healthy and had not received any medication for chronic/acute diseases were included in the study</td>
<td>DNA damage in oral epithelial cells is greater than in lymphocytes. There is no difference between the baseline DNA damage rate of buccal epithelial cells and sublingual cells</td>
<td>ANOVA Tukey's multiple comparison test Student’s t-test Pearson correlation factor between parameters</td>
<td>Damage in lymphocytes</td>
<td>There is no difference between the baseline DNA damage rate of C0 and C1 lymphocytes; For all cell types there is a significant difference in baseline DNA damage rate between individuals.</td>
<td>[60]</td>
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<td>Females (n), age</td>
<td>Males (n), age</td>
<td>Females (n), age</td>
<td>Males (n), age</td>
<td>Exposure group vs control group</td>
<td>DNA migration was measured with a scaled ocular as the total image length (including head and tail length).</td>
<td>Student’s t-test</td>
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<td>[14]</td>
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<td>6 (24–43)</td>
<td>5 (32–63)</td>
<td>6 (19–43)</td>
<td>3 (25–34)</td>
<td>89.30 ± 16.18 µm vs 82.01 ± 10.43 µm</td>
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<td>1 (28)</td>
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<td>59.16 ± 2.84 vs 14 ± 1.87</td>
<td>DNA damage was quantified by visual classification of cells into categories of comets corresponding to the DNA damage [123, 124]</td>
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<td>50 gutka chewers smokers (37.7 ± 1.30)/50 pan masala chewers smokers (32.2 ± 1.18)/50 gutka chewers (32.5 ± 1.60)/50 pan masala chewers (30.9 ± 1.42)/50 smokers (40.1 ± 1.71)</td>
<td>50 with no addition (29.7 ± 1.41)</td>
<td>36.9 ± 3.63/3.6 ± 3.59/3.6 ± 3.52/3.5 ± 3.41 ± 0.97 vs control (3.41 ± 0.41)</td>
<td>TL, using comet score 1.5 software</td>
<td></td>
<td>Chromosomal aberrations MN</td>
<td></td>
<td>CA2.4 ± 0.69 vs 1.2 ± 0.41 MN1,5 ± 0.5 % vs 0%</td>
<td>[71]</td>
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<td>20, 37, 59</td>
<td>20</td>
<td>Tail moment 100 ± 26 vs 0.00 ± 0.001 Tail length 456 ± 71 vs 9 ± 1.3 % tail DNA 97 ± 19 vs 1.2 ± 0.02 Fragmented DNA 32 ± 3.3 vs 3.4 ± 0.03</td>
<td>Analyzed for comet parameters using LTA Comet analysis system</td>
<td></td>
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<td>Cornet assay in human peripheral blood leukocytes</td>
<td>Juruk smoke Condensation Tail moment 12.41 ± 7.41 vs 0.01 Tail length 167.74 ± 47.66 vs 2.0 % tail DNA 22.36 ± 8.87 vs 0.3 % Fragmented DNA 5.09 ± 1.41 vs 2.8 Mousel smoke condensation Tail moment 21.66 ± 13.33 vs 0.01 Tail length 233.10 ± 75.22 vs 2.0 % tail DNA 29.03 ± 9.77 vs 0.3 % Fragmented DNA 5.23 ± 1.43 vs 2.7</td>
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<td>Exposed population</td>
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<td>Females (n), age</td>
<td>Males (n), age</td>
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<td>Males (n), age</td>
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<td>Cancer patients 6</td>
<td>Cancer patients 31</td>
<td>Tobacco users 2 No users 14</td>
<td>Tobacco users 84 No users 23</td>
<td>Prior to the study all subjects gave informed consent in project participation. Oral cancer patients who had medical treatment or radiotherapy were excluded. Studied subjects were interviewed using a questionnaire to survey possible confounding factors.</td>
<td>% DNA damage Oral cancer patients 19.1 ± 9.14 Tobacco Users 7.10 ± 3.66 Non-tobacco Users 4.56 ± 2.68</td>
<td>DNA damage is represented as percentage data</td>
<td>T-test of unequal variance, Chi-square test Multivariate analysis.</td>
<td>Confounding factors</td>
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<td>21</td>
<td>125</td>
<td>93</td>
<td>69</td>
<td>Were screened using a questionnaire to find out the possible factors (age, tobacco habit, tea habit) that could affect ROS generation and DNA damage</td>
<td>TDP% &lt;0.5 99.58 ± 4.18 =&lt;0.5 69.6 ± 4.64 Control &lt;0.5 40.4 ± 6.34 &gt;0.5 46.09 ± 3.8</td>
<td>Tail DNA percentage (TDP%) Of two tail moment (OTM) The Mean TDP% and OTM for each group were compared with mean values of control subjects of respective age groups.</td>
<td>Student's t-test One way ANOVA</td>
<td>Intracellular ROS levels Apoptosis rate</td>
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<tr>
<td>52 45.4 ± 10.2</td>
<td>164 45.4 ± 16.2</td>
<td>18 50.4 ± 8.7</td>
<td>52 50.4 ± 8.7</td>
<td>Questionnaires were completed to obtain detailed occupational, smoking, and medical histories.</td>
<td>Tail length (TL) and tail moment (TM) were evaluated, with Comet Assay II</td>
<td>Student's t-test MN chromosomal aberration assays</td>
<td>MN Tobacco smokers Male 2.2 ± 0.6 Female 2.0 ± 0.4 vs Control Male 0.8 ± 0.2 Female 2.2 ± 0.9</td>
<td>Total Chromosomal Aberrations (CA) Tobacco smokers 2.18 ± 1.31 vs Control 2.62 ± 0.9</td>
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<td>Females (n), age</td>
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<td>Exposure group</td>
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<tr>
<td>78 wild genotype 42 mutant genotypes</td>
<td>66 wild genotype 54 mutant genotypes</td>
<td>The subjects were interviewed to evaluate their health status and lifestyles. None of the occupationally exposed subjects wore gloves. None of the workshops had proper ventilation</td>
<td>Workers with the mutated genotype (Ile-Val) had a significantly greater comet tail length than controls. The same is true for workers with the wild genotype Ile-Ile.</td>
<td>Tail length. The cells were analysed using commercial TriTek Comet Score (version 1.3) software.</td>
<td>ANOVA Non-parametric Mann–Whitney U-test. Independent t-test. Chi-square test</td>
<td>MN PCR Restriction fragment length polymorphism (RFLP), Telomere length</td>
<td>Workers with the wild genotype (Ile-Val, Val-Val) had a significantly higher MN frequency, shorter telomere length than controls. The same is true for workers with the wild genotype Ile-Ile.</td>
<td>[66]</td>
</tr>
<tr>
<td>80 wild genotype 40 mutant genotypes</td>
<td>95 wild genotype 25 mutant genotypes</td>
<td>The subjects were interviewed to determine their health status and lifestyles.</td>
<td>c2c2 genotype 25.61 ± 9.35 vs 18.02 ± 8.49; c2c2 and c2c2 genotypes 24.09 ± 7.86 vs 15.42 ± 5.97; Mutated genotypes (c2c2 and c2c2) not influenced significantly by comet tail length</td>
<td>Tail length</td>
<td>Non-parametric Mann–Whitney U-test.</td>
<td>MN PCR RFLP Telomere length</td>
<td>Workers with the wild genotype showed statistically significant higher MN frequency, and shorter telomere length at the occupational exposure. The mutated genotype influenced significantly MN frequency in the workers, while the influence was not significant in relative telomere length</td>
<td>[67]</td>
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<tr>
<td>58 wild genotype 62 mutant genotypes</td>
<td>60 wild genotype 60 mutant genotypes</td>
<td>Subjects were interviewed about their health status, educational level, smoking habits, alcohol consumption, work history, duration of working at one occupation and other aspects relevant to the study.</td>
<td>WW genotype 23.79 ± 8.59 vs 17.14 ± 7.83; MW and MM genotypes 26.46 ± 9.01 vs 17.82 ± 8.21; No statistically significant effect was found in wild (WW) or mutated genotypes (MW, MM)</td>
<td>Tail length</td>
<td>Non-parametric Mann–Whitney U-test.</td>
<td>MNPCR-RFLP Telomere length</td>
<td>Difference in MN frequency between workers and controls was statistically significant in both wild and mutated genotypes. In addition, the results showed that the mutated genotype significantly affected the relative telomere length in workers.</td>
<td>[68]</td>
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<td>Exposed population</td>
<td>Control population</td>
<td>Interview</td>
<td>Results RCA Exposure group vs control group</td>
<td>Parameters measured</td>
<td>Statistics</td>
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<td>Females (n), age</td>
<td>Males (n), age</td>
<td>Subjects were interviewed about their health status, educational level, smoking habits, alcohol consumption, work history, duration of employment, and other aspects relevant to the study. In addition, duration of employment was assessed, and subjects were divided into 2 groups of more or less than 3 years of employment.</td>
<td>24.99 ± 9.14 vs 17.47 ± 8.40. Neither genotype showed any statistically significant effects</td>
<td>Tail length</td>
<td>Non-parametric Mann-Whitney U test</td>
<td>MNPCRFLP polymers length</td>
<td>The workers carrying wild or mutated genotypes showed a significantly higher MN frequency and shorter telomere length compared to controls</td>
<td>[69]</td>
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<td>56</td>
<td>64</td>
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<td>30 untreated patients with cancer 50.42 20 (20-72) 20 untreated patients at pre-cancer stage 29.55 (17-50)</td>
<td>79 untreated patients with cancer 50.42 (20-72) 118 untreated patients at pre-cancer stage 29.55 (17-50)</td>
<td>Case history and personal details were collected. Data included age and gender with similar smoking and tobacco use (chewing), dietary habits and socio-economic status.</td>
<td>Comet tail length Cancer 28.64 ± 4.97 Pre-cancer 20.91 ± 5.58 Control 11.5 ± 3.83</td>
<td>Tail length measured with an ocular micrometer fitted in the eyepiece</td>
<td>Students' t-test (paired and unpaired comparisons) and analysis of variance were carried out to evaluate various differences.</td>
<td>MN conducted on the buccal epithelial cells; Comet assay on peripheral blood leukocytes. The challenge comet assay on peripheral blood leukocytes.</td>
<td>% MN Cancer 0.48 ± 0.35 Pre-cancer 0.31 ± 0.24 Control 0.21 ± 0.18 There was a significant stepwise increase in comet tail length from control to patients with pre-cancer and then to cancer patients.</td>
<td>[64]</td>
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<td>30 patients with oral squamous cell carcinoma (OSCC)</td>
<td>30 without OSCC</td>
<td>Patients who were diagnosed as having OSCC formed the study group</td>
<td>OSCC 3.874 ± 2.5205 µm vs Normal subjects 0.86 ± 0.8142 µm</td>
<td>Total length and the diameter was measured.</td>
<td>Students' test. One way ANOVA &quot;F&quot;</td>
<td>To analyze DNA damage, patients having OSCC were divided into four stages, namely stage I, II, III, and IV</td>
<td>Stage I 2.312 ± 0.366 Stage II 3.171 ± 1.439 Stage III 3.490 ± 1.971 Stage IV 6.890 ± 3.710</td>
<td>[73]</td>
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<td>Females (n), age</td>
<td>Males (n), age</td>
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<td>Exposure group vs control group</td>
<td>arbitrary units, TL, TL, TM</td>
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<td>Occupational Exposure</td>
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<td>All subjects gave informed consent. Anaphoric, clinical, working information and lifestyle habits (smoking, dietary</td>
<td>31 43.35 ± 9.4</td>
<td>Tail moment from Fpg-treated cells (TMenx) and Fpg-un-</td>
<td>Student’s t-test</td>
<td>MN and Fpg-modified comet assay on lymphocytes and exfoliated buccal cells, and by chromosomal aberrations (CA) and sister chromatid exchange (SCE) analyses</td>
<td>The exposed group showed a higher mean value of SCE frequency in respect to controls (4.8 ± 3.8) and an increase (1.3-fold) of total structural CAs in particular breaks (up to 2.0-fold) and fragmenters (0.32% versus 0.00%), whereas there were no differences of MN frequency in both cellular types. Comet assay evidenced in the exposed group a higher value in respect to controls of mean TM and TMenx in lymphocytes (TM 43.01 vs 36.01); TMenx 55.06 versus 48.98.</td>
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<td>Pharmacy technicians 2 Day hospital nurses 37.6 ± 5.5 Ward nurses 11.32.7 ± 7.2</td>
<td>Pharmacy technicians 3 Day hospital nurses 35.8 ± 9.9 Day hospital nurses 2.37.6 ± 5.5 Ward nurses 2.32.7 ± 7.2</td>
<td>Data collection was by a questionnaire which included information on age, gender, life style, and habits (diet, smoking, alcohol consumption, chronic drug use), the types of antineoplastic drugs handled, and the number of mixtures prepared and administered</td>
<td>Pharmacy technicians 32.6 ± 182 Day hospital nurses 35.2 ± 38 Ward nurses 27.4 ± 13.9 Controls 28.6 ± 12.4</td>
<td>TMI</td>
<td>ANOVA Chi-square Student’s t test Kolinoglov-Smirnov non-parametric test Levene test</td>
<td>Comet assay in lymphocytes cells</td>
<td>Pharmacy technicians 20.8 ± 10.1 Day hospital nurses 15.5 ± 9 Ward nurses 14.7 ± 7.9 Controls 16.1 ± 8.1</td>
<td>[77]</td>
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</table>

Environmental Health Risk - Hazardous Factors to Living Species
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<tr>
<th>Exposed population</th>
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<td>Females (n), age</td>
<td>Males (n), age</td>
<td>A questionnare was used to collect the information on sex, age, duration of exposure, use of protective masks, general health status, smoking habits and exposure to drugs for each exposed and control subject.</td>
<td>The significant differences in the comet class between the control and jewellery workers show that the latter group has increased DNA damage who are occupationally exposed to nitrates.</td>
<td>Exposure group vs control group</td>
<td>The comets were analyzed by visual classification and the damage was assigned to 5 classes [125]. The percentage of tail DNA was calculated according to Zhao et al. [126]</td>
<td>Student's t-test</td>
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<td>57 (39.49 ± 9.11) 30 (35.17 ± 7.4) workers exposed to antineoplastic drugs and 57 workers exposed to PAHs included 41 airport workers (43 ± 8.3) and 16 paving workers (38.62 ± 10.0)</td>
<td>76 (39.72 ± 10.1)</td>
<td>Personal data, clinical and working information, and lifestyle habits (smoking, dietary habits, and alcohol consumption) were obtained from a questionnare administered by specialized medical personnel.</td>
<td>% Comets 13.74 ± 10.9 vs 13.78 ± 9.80 Tail moment 48.01 ± 30.1 vs 32.31 ± 12.79</td>
<td>% DNA in the tail, tail length, tail moment</td>
<td>Student's t-test, Mann-Whitney, U-test, ANOVA Kruskal-Wallis, and Bonferroni test</td>
<td>Comet and MN tests were performed on lymphocytes and exfoliated buccal cells.</td>
<td>The MN assay on lymphocytes did not show significant differences between exposed and controls, while the MN assay on exfoliated buccal cells showed higher values in workers exposed to antineoplastic drugs compared with controls (0.85 vs 0.48). The comet assay on lymphocytes showed a higher comet percentage value (18.11 vs 11.24 in controls) and mean tail moment (TM) value (21.84 vs 16.72 in controls) in individuals exposed to PAHs as compared with controls; no significant differences were found in exposed to antineoplastic drugs.</td>
<td>[78]</td>
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<td>Exposed population</td>
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<td>Females (n), age</td>
<td>Males (n), age</td>
<td>Males (n), age</td>
<td>Males (n), age</td>
<td>Exposed group vs control group</td>
<td>Fatal Frequency 22.38 ± 17.28 vs 13.56 ± 12.69</td>
<td>Damage index 32.42 ± 30.58 x 18.81 ± 18.93</td>
<td>Cells were scored visually into five classes according to tail size and shape (from undamaged – 0, to maximally damaged – 4, and a value (damage index (DI)) was assigned to each Comet according to its class (129). DI thus ranged from 0 (completely undamaged: 100 cells%) to 400 (with maximum damage: 100 cells%). The damage frequency (DF) (%) was calculated based on the percentage of damaged cells (0-100%).</td>
<td>Non-parametric Mann-Whitney U-test</td>
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<td>85 35 (20-42)</td>
<td>76 34 (21-41)</td>
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During personal interview, each participant was requested to furnish information about age, education, family size and income, habit, cooking time, yearly cooking, fuel and oven type, location and ventilation of kitchen, health problems in past 3 months and last one year.

Comet tail % DNA 32.21 ± 9.51 vs 12.41 ± 3.87 Comet tail length (μm) 37.81 ± 11.21 vs 14.22 ± 3.89 Olive tail moment in arbitrary unit 7.68 ± 2.11 vs 3.15 ± 1.97.

% Comets TL, TM Student’s t-test Mann-Whitney U-test Fast Halo Assay (FHA) Nuclear diffusion factor (NDF).

There was 5-fold increase in DNA damage in BEC of biomass users, implying greater DNA damage than that of control, NDF. | 114 |
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<th>Exposed population</th>
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<td>Females (n), age</td>
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<td>Interview</td>
<td>Exposure group vs control group</td>
<td>Tail length</td>
<td>Students 't' test.</td>
<td>Welders showed a significant increase in micronucleated cells compared to controls</td>
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<tr>
<td>66 37.3 ± 7.45</td>
<td>60 38.7 ± 8.21</td>
<td>The selection criteria for the subjects were based on a questionnaire according to the protocol published by the International Commission for Pro-tection against Environ-mental Mutagens and Carcinogens [27]</td>
<td>Welders showed a significant larger mean comet tail length compared to controls. In exposed group, a significant difference was observed between smokers and non-smokers and between alcohol drinkers and never drinkers in relation to DNA migration. DNA damage was further found to be significantly higher in subjects with a longer duration of work.</td>
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<td>160 40.13 ± 10.60</td>
<td>160 40.72 ± 9.79</td>
<td>Personal history, occupational, and residential information</td>
<td>Tail: 3.35 μm vs 12.8 μm</td>
<td>The cells were then analyzed by using the TriTek Comet Score (version 1.5) software. The tail length was measured (μm)</td>
<td>The effect of individual factors and levels of DNA damage by examining the significant differences in age, body mass index (BMI), smoker and secondhand smoker, smoking duration, and number of cigarette per day (smoking frequency) among the study population</td>
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Welders showed a significant increase in micronucleated cells compared to controls.
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<tr>
<td>Females (n), age</td>
<td>Males (n), age</td>
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<td>Exposure group vs control group</td>
<td>Comet tail length (DNA distance)</td>
<td>Kruskal-Wallis non-parametric test, ANOVA test, Tukey-Kramer multiple comparison test</td>
<td>MN assay and other nuclear anomalies such as nuclear buds, karyolysis, karyorhexis, and binucleate cells were also evaluated</td>
<td>Showed nuclear anomalies associated with cytotoxic or genotoxic effect. No significant effect on genetic damage was observed as a result of age, smoking, and alcohol consumption</td>
<td>[82]</td>
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<td>111 (45.75 ± 3.5)</td>
<td>60 (37.55 ± 0.2)</td>
<td>Complete a standardized questionnaire with personal data related to age, time of exposure, habits such as smoking and alcohol consumption, drugs, and diets; the type of work performed; and protective measures used. The questionnaire also included a history of recent illness and medical treatment, as well as of their knowledge about the pesticides used in those agricultural areas.</td>
<td>Tail migration of DNA increased significantly in the exposed group</td>
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<td>ENVIROMENTAL EXPOSURE</td>
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<td>South (32), 19</td>
<td>North (16), 19</td>
<td>Each student answered a self-applied questionnaire translated and validated from the American Thoracic Society (ATS) for respiratory tract symptoms</td>
<td>South 137.59 ± 55.88 vs North 121.96 ± 38.72</td>
<td>DNA migration (tail image length) Relative DNA damage index</td>
<td>U Mann-Whitney test</td>
<td>Leukocytes South 13.97 ± 9.32 vs North 8.76 ± 3.80, Neut South 40.07 ± 21.07 vs North 23.12 ± 10.36</td>
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<td>54 (10 ± 0.82)</td>
<td>41 (10.02 ± 0.80)</td>
<td>43 (10 ± 0.82)</td>
<td>42 (10.02 ± 0.80)</td>
<td>8.45 ± 3.89 vs 4.38 ± 1.66</td>
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<td>IS statistic</td>
<td>MN</td>
<td>5.05 ± 2.45 vs 2.92 ± 1.54</td>
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<td>Females (n), age</td>
<td>Males (n), age</td>
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<td>Exposure group vs control group</td>
<td>Tail intensity (%)</td>
<td>With Comet Assay II DNA damage was quantified as: Tail intensity Tail length Tail moment The median of each parameter was used as the representative value for each subject [130]</td>
<td>Analysis of variance for quantitative variables and Pearson’s chi-square test for categorical variables</td>
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<td>[86]</td>
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<td>231.9 ± 1.6</td>
<td>185.9 ± 1.6</td>
<td>The follow-up questionnaire is a short version of the baseline questionnaire on children’s health and risk factors [129], with some additional items on oral hygiene</td>
<td>Tail intensity (%) 3.25 ± 0.88 Tail length (µm) 11.60 ± 2.11 Tail moment 0.20 ± 0.05</td>
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<td>9</td>
<td>10</td>
<td>Samples were obtained from volunteers among the laboratory staff</td>
<td>Precrunch % Tail DNA 63.8 ± 70.2 vs 65.3 ± 13.9 Tail moment 25.8 ± 5.3 vs 23.1 ± 5.5 Postcrunch % Tail DNA 42.4 ± 20.4 vs 31.9 ± 10.5 Tail moment 15.4 ± 11.6 vs 9.1 ± 4.4</td>
<td>% Tail DNA Tail moment</td>
<td>Normality was tested by the Shapiro-Wilk test. Student’s t-test, paired and unpaired McNemar Chi² test</td>
<td>DNA damage in peripheral leukocytes</td>
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BCA: Buccal Comet Assay  
BMCy: Micronucleus Cytome Assays

Table 2. Information about exposure type, population studied, results, and statistics in observed articles with buccal comet assay.
None of demographic or lifestyle factors tested as possible confounding factors (age, gender, dietary habits, pH of saliva, alcohol, smoking habits, drug intake, and others) have exhibited significant influence on values of comet assay parameters in buccal cells [55,56,64,66,67,76,82,83,85]. On contrary, Pal et al. [62] in their evaluation of various confounding factors like age, tenure of tobacco habit, and tea habit showed significant associations with DNA damage. In the same line, Sudha et al. [80] showed that the combined exposure to cigarette smoke and Cr(VI) increased basal DNA damage in buccal epithelial cells of welders. How et al. [81] characterized potential risk factors that influence levels of DNA damage from exposure to mixtures of organophosphates, among all, age, smoking habit, smoking duration, number of cigarettes (per day); and secondhand smokers highlighted the significant differences between subjects and within groups. Martín-Cameán et al. [57] observed that DNA damage in buccal cells induced for orthodontic appliances was higher in women, and Jayakumar and Sasikala [78] found a synergistic effect of the habit of cigarette smoking among the jewellery workers.

7. Perspectives

The assessment of genotoxic risk in exfoliated buccal cells is a potentially useful and minimally invasive cytogenetic technique for measuring DNA damage in humans [7,12,17,18,46]. The comet assay is a widely used biomonitoring tool for DNA damage. The most commonly used cells in human studies are peripheral lymphocytes, harvested from venous or capillary blood. However, there is an urgent need to find an alternative target human cell that can be collected from normal subjects with minimal invasion [61].

Buccal cells are becoming an increasingly popular tissue source in human biomonitoring after exposure to occupational and environmental genotoxicants, particularly because they can be obtained non-invasively [50,61,90,91]. However, the number of publications referring to the human buccal comet assay is low in the last two decades. This low growing interest may be explained by several factors, including its relative technical problems.

A priority in this field should be to develop a protocol that could enable buccal cell lysis and DNA damage testing in the comet assay and to use the model to evaluate the potential of the buccal cells in human biomonitoring study [61].

Specialized cellular membranes, which make cell lysis difficult, contribute to making buccal mucosa cells a more complicated cell to SCGE assay [92]. As firstly mentioned in the review of Rojas et al. [33], there are studies that use proteinase K together with the lysis step in order to gain free nucleoids, and there are studies that do not use this enrichment, but only lysis solution, and it has been shown that results depend on this step. Szeto et al. [61] described the development of an improved protocol in which agarose embedded cells of epithelial origin from the mouth were digested with trypsin and proteinase K. Their early trials with buccal cells following the published protocol by Rojas et al. [14] were completely unsuccessful. They found that buccal cells sustained massive damage and disintegration at the high pH used, while at lower pH values, the cells were extremely resistant to lysis. According to these authors,
it is not possible to use earlier protocol developed as it leads to extremely high background levels. The adequate experimental design of SCGE trials in buccal cells is still a matter of debate, and the evaluation of the available data shows that there is an urgent need to develop guidelines [93].

Proper collection and storage of human (buccal) cells is essential step in order to preserve their integrity for later analysis by the comet assay [26,27]. After collection, more than 90% of the cells in a buccal sample are epithelial cells, a cell type with well-known low viability (10%) [91]. Although a prerequisite for using any cell type in the comet assay is that those cells must be viable [92,94], most of the reported studies did not consider this important factor. Failure in controlling of these confounding variables can lead to an over/under estimation of the DNA damage caused by exposure on work-place or in assessment of exposure to environmental genotoxicants [86]. Cell viability is expected to be low in epithelial tissue with terminally differentiated cell populations and a high renewal rate as buccal cells [95]. Dead or dying cells are extensively damaged (e.g., DNA fragmentation), and therefore, subjecting them to the alkaline conditions of the comet assay only increases DNA loss. Comet assay studies on epithelial buccal cell samples have reported high percentage of DNA “clouds” (>95%) [96]. Those clouds are excluded from the final quantitative analysis and that generally results in very low numbers of counted comets. Higher percentage of these atypical comets demonstrates that epithelial cells are not suitable for measuring DNA damage by the comet assay. Also enzymatic digestion such as proteinase K treatment is an essential step to enrich the number of epithelial viable cells, thus promoting necrotic cells destruction that are very numerous in the mucosa epithelium and have a very fast turnover. Enzymatic treatment with proteinase K caused degradation of leukocytes, mainly polymorphonuclear, which represent a great fraction of cells in the oral mucosa, due to migration from the blood through the gingival crevice [91].

Another problem in cell collection is that final cell suspension usually consists of mixture of epithelial cells and leukocytes with well-known fact that leukocyte fraction is more viable than epithelial cell fraction [91]. Pinhal et al. [92] investigated whether human buccal mucosa cells are suitable for use in the SCGE assay. After comparison of smoker/non-smoker group, there was no correlation of long-term smoking with the number of buccal cells that formed comets and represented damaged cells. They have also concluded that the cells that formed comets are probably leukocytes, and not buccal cells, and that the SCGE assay, used on a commonly performed way, without modifications, may not be useful for genotoxicity monitoring in human epithelial buccal mucosa cells. Similar conclusions were cited by Ribeiro [97].

In contrast, the uniform distribution of DNA within the heads of oral leukocytes and their greater viability indicates that this cell type is more suitable for assessing DNA damage in buccal samples [86]. Thus, recently McCauley et al. [98] and Kisby et al. [99] examined oral leukocytes of agricultural workers by the comet assay and demonstrated that DNA damage is greater in farmworkers who were exposed to pesticides.

As mentioned above, other alternative is to isolate lymphocytes from cells suspensions collected from the mouth and develop a technique for SCGE analyses, like it was followed by
Osswald et al. [91], and later, it was successfully implemented in an intervention trial with supplemented bread by Glei et al. [87].

The use of buccal epithelial cells to determine genotoxicity using the comet assay according to the procedure outlined by Singh et al. [100] was limited by the inability to obtain free nucleoids. In a recent review, Rojas et al. [33] showed that a broad variety of different protocols has been used in earlier investigations. No effort has been made so far to establish an international consortium which could develop and validate appropriate strategies for the use of SCGE assay in buccal cells. More information is required concerning the time and design of different phases, the duration of wash-out periods, the calibration of enzymes and other important factors which may influence the outcome of the experiments as has been proposed by Hoelzl et al. [93] for the use of SCGE assays for the detection of DNA-protective effects of dietary factors in humans.

8. Considerations

According to Rojas et al. [33], the use of alternative biomatrices to assess DNA damage in human populations has advantages and shortcomings focusing on the methodological characteristics of buccal mucosa cells and taking into consideration the sampling protocol, pre-processing, and post-sampling storage, as well as the possibilities of sample freezing and the need to adapt the classical alkaline comet assay protocol.

The use of buccal mucosa cells by comet assay in order to estimate DNA damage levels gives the possibility to obtain samples on cheap, safe, and non-invasive way in order to perform in vivo studies. Direct contact with xenobiotics and endogenous damage inductors makes this type of sample an attractive biomatrice for individual genotoxicity evaluation. Their applicability in clinical diagnostic confers a potential use in patients across time.

![Figure 1](image.png)

**Figure 1.** Picture of single buccal mucosa cells: (a) immediately stained after the solidification of agarose gel layer with sample cells, (b) the appearance of cells with cytoplasm after 1 h of classical lysis solution, (c) the appearance of the cells with cytoplasm after the combined treatment of lysis solution and proteinase K (1 mg/ml) for 1 h at 37°C, (d) the appearance of cells after 24 h of normal lysis, (e) the appearance of cells after 24 h of normal lysis and treatment with proteinase K 10 mg/ml for 1 h at 37°C, (f) 0.25% trypsin 30 min plus proteinase K 1 mg/ml 1 h, 37°C.
The comet assay in exfoliated buccal cells has been used since the 1990s to demonstrate cytogenetic effects of environmental and occupational exposures, lifestyle factors, dietary deficiencies, and different diseases.

The general guideline to perform comet assay in epithelial cells requires the correct sampling procedure, to follow the alkaline version proposed by Singh et al. [100]. In this sense, Rojas et al. [33] proposed protocols specific to sampling protocol and sample storage and comet assay sample preparation for buccal mucosa cells. We have also performed the protocols suggested by Rojas, but there have been some confusing factors. Rojas recommendation did not give free DNA neither in first case of lysis treatment for 1 h or lysis treatment with proteinase K for 1 h (pictures represented in Figure 1). We have also tried the protocols that Szeto et al. [61] have done in order to established the best one, but in our case, we have demonstrated that although cells are embedded on agarose gel, treatment with 0.25% trypsin and then proteinase K for 1 h is too aggressive and still gives cloudy free nuclei. For us, the best results were with lysis and proteinase K 10 mg/ml 1-h treatment on 37°C. It seems that also high pH of alkaline denaturation and electrophoresis makes massive DNA damage, as already mentioned in Szeto et al. [61]. As Szeto et al. [61] already mentioned, buccal cells as a type of stratified squamous epithelium do not divide but undergo a terminal differentiation from basal cells on order to form a protective barrier (cell envelope rich in a small prolinerich protein) that will protect the buccal cell from very harmful environment in the mouth and also will give resistance of buccal cells to lysis. On Figure 2, we have represented some pictures of the buccal cells after lysis and electrophoresis in alkaline conditions (pH > 13). Szeto el al. [61] suggested that denaturation and electrophoresis in neutral conditions would be more appropriate. According to our

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Pictures of buccal cells after different duration and type of lysis step, but all electrophoresis were at pH > 13: (a) treatment of lysis solution for 15 h 4°C, (b) lysis step for 20 h 4°C, (c) treatment with 0.25% trypsin for 30 min, and lysis for 30 min, both at 37°C, (d) 15 min of 0.25% trypsin a 37°C, 15 min of proteinase K 1 mg/ml, (e) 30 min of proteinase K 1 mg/ml at room temperature, 60 min of lysis at 4°C, (f) 24 h of lysis at 4°C, (g, h) 20 h of lysis at 4°C.
knowledge, alkaline conditions are also appropriate, but also this part needs further investigation.

A review of risk factors affecting background rates of parameters in the comet assay in cells of oral mucosa should be undertaken with a view to help in the interpretation of genotoxicity biomonitoring studies. Both endogenous factors and those due to methodological variation should be evaluated. Background variation of other indices of genotoxicity in buccal mucosa cells should be also considered as these data likely reflect overlapping causes of DNA damage and may provide some indicators for future research areas. A number of host risk factors, namely age, gender, smoking, vitamin status, alcohol consumption, disease conditions and infections, physical exercise, body mass index, and genotype should be identified, since there are evidences that they have an impact on background levels of genotoxicity biomarkers. Evaluation of these factors should be routinely included in genotoxicity biomonitoring studies [101].

However, important knowledge gaps remain about the methodologic procedures in laboratories around the world. To address these uncertainties, it will be necessary to develop similar projects as the HUMN and HUMNxL for validation of the lymphocytes and buccal cell MN assay, respectively [7,12,17,18]. Future research should explore sources of variability in the assay and resolve key technical issues, such as the method of buccal cell sample and sample storage, slide preparation, enzyme treatment, and optimal criteria for the classification of normal and degenerated cells. The harmonization and standardization of the buccal comet assay will allow more reliable comparison of the data among human populations and laboratories, evaluation of the assay’s performance, and consolidation of its worldwide use for biomonitoring of DNA damage.

In order that comet assay in buccal cells has widespread acceptance and credibility in human population studies, standardization of analyzed parameters and protocol is necessary and also a better knowledge of critical features affecting the assay outcomes, including the definition of the values of spontaneous DNA damage. Developing the network of laboratories using this technique and performing and international collaborative studies would be an ideal solution. Result of connecting would be the assembly of large databases which would allow a more detailed analysis of the assays performance and study of the biological/clinical events associated with this biomarker.

The need for a careful consideration of factors affecting the comet assay in cells of oral mucosa exists, which, in turn, should aid in the interpretation of studies of environmental and occupational chemical exposures and health risk. There is a need for further collaborative work as in the HUMN collaborative project which has reported data on ~7000 individuals [15,16,102–104]. If these measures are achieved, then it would be possible to use the data from biomonitoring studies in risk assessments to derive risk management measures [95]. Based on the experience of the HUMN project [96], the Conference on Environmental Mutagens in Human Populations [105,106], and the HUMNxL project, design of the studies could be similar to (i) identify technical variables that affect the measurement of DNA damage of buccal cells assessed with comet assay, (ii) identify lifestyle variables affecting this damage, (iii) identify protocol variables that affect the recovery of buccal cells and their scoring in comet assay, (iv)
design intra- and inter-laboratory validation studies based on the results of information collected for the method and scoring criteria, and (v) determine the role of buccal genomic damage monitoring and the prediction of cancer and other degenerative diseases.

The creation of a network of laboratories will allow more focused validation studies, including the design of a classic, historic, prospective cohort study, to explore the link between measures of genetic instability in the buccal mucosa and the risk of cancer and other chronic-degenerative diseases [12]. ComNet project and new COST project are a great step forward.

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