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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 inductors, 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

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, micronutrient 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 karyolytic 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 assay 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 ($89.30 \pm 16.18 \mu\text{m}$) vs. non-smoker group ($52.01 \pm 10.43 \mu\text{m}$), 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.

Buccal comet assay technique										
Exfoliated Buccal Cells Sampling	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	Reference
With water	Small sterile spoon, kept in 1 mL of physiological solution at 37 °C	According with Singh et al. [100], Tice et al. [23], Speit and Hartmann [107], with some modifications	At 1000 rpm 10 min	50 µL of cell pellet in 50 µL LMP agarose (1% 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		The coverslip removed, slides immersed in a 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	Horizontal electrophoresis chamber. Fresh buffer (300 mM NaOH and 1 mM Na ₂ EDTA, pH 13); 20 min	Electrophoresis was 20 min at 25 V and 300 mA	3 times rinsing in fresh neutralization buffer (0.4 M Tris, pH 7.5), fixed 5 min in absolute methanol, 75 µL EtBr (20 µg/mL) 10 min	[82]
Several times with distilled water	With a cytological brush, in a 20 circular expansion, 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	Thomas et al. [11], Szeto et al. [61]	At 1500 rpm 10 min, resuspended in 1 mL PBS in Eppendorf tube centrifuged (1000 rpm, 5 min)	10 µL cell suspension mixed with 0.75% LMP agarose, 75 µL immediately spread onto a glass microscope slide pre-coated with a layer of 1% NMP agarose. The LMP layer was allowed to set at 4 °C for 5 min	0.25% trypsin solution was added, 30 min, 37 °C, after, cells were treated with proteinase K (1 mg/mL) 10 min.	Ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4 °C for 1 week. This procedure removes cell proteins and leaves the DNA as 'nucleoids'	Horizontal electrophoresis unit, with fresh alkaline solution (300 mM NaOH, 1 mM EDTA, pH 13.0) 20 min at 4 °C	20 min at 25 V and 300 mA (0.90 V/cm)	Slides were then neutralized (0.4 M Tris, pH 7.5), washed in distilled water, AgNO ₃ staining protocol as described by Nadin et al. [108]	[59]
With water	Scraping 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		At 2500 rpm 1 min	Mixing 500 µL of molten LMA with 50 µL of cells specimen at 37 °C and immediately pipetting 75 µL of 1:10 (v/v) aliquot onto a comet slide, placed flat at 4 °C in the dark 10 min		The slide was immersed in freshly chilled lysis solution for 60 minutes	Followed by immersion in freshly prepared alkaline solution, pH >13, for 45 minutes at room temperature in the dark	1 V/cm, 1 h	Slides rinsed by dipping several times into deionized water, fixation in 70% EtOH 5 min, 50 µL of dilute SYBR green	[81]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique							Reference	
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
-	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	Odling and Johanson [109], Szeto et al. [61]	At 200 X g for 10 min, the cell pellet washed with 500 µL PBS and centrifuged	10 µL of suspension mixed with 85 µL of pre-warmed (40°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	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.	Cell lysis with proteinase-K (1 mg/ml) for 60 min	Alkaline solution for 20 min at room temperature in the dark	In electrophoresis buffer (0.01 M NaOH, 1 mM EDTA, pH 9.1), 0.9 V/cm, for 18-20 min	Slides rinsed by dipping several times in distilled water. Fixation by immersing in 70% EtOH 5 min, then air dried. EtBr staining (50 mg/ml)	[72]
						Were lysed by detergents and salts at high concentrations		For 15 min under high pH, at 20 V and 400 mA	Stained with EtBr	[73]
Water	Scraping the inner part of both cheeks with a cytology brush, cells kept in 0.9% NaCl and PBS in separate micro-centrifuge tubes, brought to laboratory	Following the method outlined by Singh et al. [100]								[69]
	Exfoliated buccal mucosa cells were collected by gently scraping the mucosa of the inner lining of one or both cheeks	Rojas et al. [14], and modified based on standard procedures from comet assay kit								[85]

Buccal comet assay technique										
Exfoliated Buccal Cells Sampling	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	Reference
Rinsing										
	Parents collected epithelial mucosa cell samples by gently brushing the inside of both cheeks with a cytology brush. The brush was then stirred in a PBS (pH 7.4).	Cells were processed in alkali conditions and underwent submarine electrophoresis accioni [51, 110]	Cell suspensions were washed twice with centrifugation at room temperature						Stained with Et-Br	[86]
Water	Scraping the inner part of the cheeks both sides with a cytology brush, cells kept in 0.9% NaCl and PBS in separate microcentrifuge tubes, brought to laboratory	Used the Trevigen Comet-Assay™ kit protocol		Cells + LMP agarose at 37°C at the ratio of 1:10, and 75 µL aliquots pipetted onto the slides and placed flat in a dark place at 4°C for 10 min.		The slides immersed in the pre-chilled lysis solution for 60 min	A freshly prepared alkaline solution, pH>13, at room temperature in the dark for 45 min	The slides placed flat on a gel tray. At 1 V/cm for 10 min	Stained with 50 ml of diluted SYBR Green	[67]
Water	The cells were collected by scraping the inner part of the cheeks both sides with a cytology brush. Then, the cells were gently mixed with 0.9% NaCl and PBS in separate microcentrifuge tubes and brought to the laboratory	Protocols described in a previous paper [66]								[68]

Exfoliated Buccal Cells Sampling			Buccal comet assay technique					Reference		
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	Reference
Subjects rinsed their mouth thoroughly with saline solution to remove excess debris	Samples were obtained by scraping cells 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		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.	Samples were assayed in duplicate using 5 µL of the cell suspension for each spot	Under dim, indirect light, 8 µL of a 10 mg/ml, proteinase K solution was added to 20 µL of cell suspensions and kept in PBS for 15 min at 40 °C. Microtubes were centrifuged 800 x g for 1 min. The pellets were washed in PBS and, after centrifugation at 6,000 x g for 2 min, suspended in 10 µL of PBS.	After slide preparation, lysis was performed overnight at 4°C in a cold solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 10), where 10 % DMSO and 1 % Triton X-100 were added just before use	DNA unwinding and electrophoresis were performed in an alkaline solution (0.3 M NaOH, 1 mM EDTA, pH 13), for 20 min and 40 min, in a horizontal electrophoresis tank filled with an ice-cold alkaline solution.	At 300 mA and 25 V (0.86 V/cm)	After neutralization step, the slides were dehydrated with absolute ethanol and stored in dry air up to the end of sampling, slides were stained with EtBr (2 µg/mL in H ₂ O)	[56]
-	-	According to the alkaline single-cell gel electrophoresis method [100, 111]							Silver staining method [108]	[70]
Mouth washed with normal saline (0.9% NaCl) solution	Brushing in the morning before taking any tobacco or tea. Collected samples were taken in PBS	Using a standard protocol with some modifications [61]		Cell suspension mixed with 1% LMP agarose in 1:2 ratio and spread on a microscopic slide pre-coated with 1% NPM agarose.	Trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA) for 30 min followed by proteinase K (1 mg/ml) digestion for 1 h at 37°C		Slides were equilibrated in electrophoresis buffer (10 mM NaOH, 1 mM EDTA, pH 9.1) for 10 min	At 30 V for 20 min	Neutralization for 15 min in 400 mM Tris/HCl, pH 7.4 staining with EtBr (20 µg/ml)	[63]
Water	Scraping the inner part of both sides of the cheeks with a cytology brush. The cells were then gently mixed with 1.5 mL of 0.9 % NaCl and PBS in a micro-centrifuge tubes, taken to the laboratory	Used the Trevigen Comet Assay™ kit protocol		The cells were combined with LMP agarose at 37°C at the ratio of 1:10, 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		Precultured lysis solution for 60 min	Freshly prepared alkaline solution, pH>13, room temperature in the dark for 45 min. After that, the slides were placed flat on a gel tray	1 V/cm (measured from electrode) and applied for 10 min	Slides stained with 50 µL of diluted SYBR Green	[66]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique						Reference	
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining
Rinsing the mouth with temperate water to remove the exfoliated death cells.	A soft interproximal toothbrush was used to collect buccal cells by gently scraping the inside cheek (right and left) of the mouth		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.	Conventional microscope slides were treated with two layers of agarose. The bottom layer was prepared by dipping the slides into 1.0% of NMP agarose, allowing the agarose to solidify at 4°C for a minimum of 5 min. Then, the top or 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	The cells were subjected to a lysis with 0.25% Trypsin in PBS (15 min, 37°C). Washed with 0.4 M Tris Base solution and subsequently treated with proteinase K (1 mg/ml) for 30 min.	The slides were rinsed, immersed in lysis solution (2.5 M NaCl; 0.1 M EDTA; 10 mM Tris Base; 1% Triton X-100; and 10% DMSO; pH 10) for another hour at 4°C and washed again with 0.4 M Tris Base solution.	Using an horizontal gel electrophoresis tank containing freshly prepared cold (4°C) electrophoresis buffer (1 mM Na ₂ EDTA 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).	Electrophoresis was run at 25 V and 300 mA for 20 min	Rinsed with Tris solution. The cells were stained with 75 µL of a 20 µg/ml solution of BrEt
Before the start of the study, all subjects were instructed to continue brushing but not to use toothpastes and mouthwashes containing fluoride or chlorhexidine	The cells harvested, according to Besaratinia et al. [112] by gentle scraping of the internal part of the right and left cheeks with a wooden tongue depressor. Each tongue depressor was stirred in a 2 mL tube prefilled with 1.5 mL of ice-cold PBS pH 7.4	According with 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 alkali buffer at room temperature at 20 V for 40 minutes, level of the buffer was adjusted until 300 mA	The slides were stained using 50 µL EtBr (20 µg/mL)

Exfoliated Buccal Cells Sampling		Buccal comet assay technique						Reference		
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis		Electrophoresis	Neutralisation, fixation and staining
-	Buccal mucosa cells were obtained by scraping the left inner cheek with a cervical brush		The cells were washed with PBS and centrifuged at 800 rpm for 10 min	Then, 20 µL of the pellet was resuspended in 80 µL of 0.75% LMP agarose		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	To allow DNA unwinding, slides were incubated in a freshly made alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA; pH > 13) for 20 min in a horizontal electrophoresis tank	20 min at 25 V (0.90 V/cm) and 300 mA	Slides were washed three times in a neutralization 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	[79]
Rinse their mouth thoroughly with water to remove unwanted debris	Gently rubbing the inside of both cheeks with an extra soft toothbrush for 1 min each. The participant then rinsed the mouth with 20 ml of 0.9% saline and expectorated into a 50 ml conical-based tube. The toothbrush rinsed in the tube and 30 ml saline was added before the cells were pelleted. The cells washed with PBS (pH 7.4)		The buccal cell suspension was centrifuged	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% LMA was applied		Were immersed in freshly prepared ice cold lysis solution for 1 hour			Stained with EtBr	[80]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique							Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	
Women rinse their mouth with saline water to remove extraneous materials	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	Szeto et al. [61]	Buccal cell suspensions were centrifuged at 2500 rpm at 4°C for 10 min	The supernatant was discarded and the cell pellet was resuspended in 100 µl of PBS. 10 µl cell suspension was mixed with 85 µl of pre-warmed (at 40°C) 1% (w/v) LMP agarose in PBS, and immediately applied to a microscopic slide already precoated with 85 µl of 1% (w/v) standard and agarose in PBS. The slides were placed at room temperature until the gel layer solidified	50 µl trypsin solution (0.25% trypsin and 1 mM EDTA in Hanks balanced salt solution) was layered onto the gel and left for 30 min at 37°C. The slides were washed with PBS. 50 µl of proteinase K solution (1 mg/ml of PBS) applied to each slide 1 h at 4°C	Slides were then immersed in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, and 1% Triton X-100, pH 10) for 1 h at 4°C	Then the slides were transferred to a Coplin jar containing electrophoresis solution (0.01 M NaOH and 1 mM EDTA, pH 9.1) and left for 20 min (2 × 10 min) at 4°C	18 min at 12 V constant voltage, after which slides were removed and neutralized by immersing in three changes (3×5 min) with 0.4 M Tris at pH 7.5	[64]
Washed out the mouth three times with tepid water to remove dead exfoliated cells	Buccal swab taken by gentle brushing of the internal part of right and left cheek with a cytobrush. The brushes were stirred in 5 ml of RPMI 1640, liquid (with L-glutamine, 25 mM HEPES), fetal bovine serum, and penicillin-streptomycin solution and transported within 30 min to the laboratory	According with Singh et al. [100]	3 min/3,200 rpm; resuspended in PBS (pH 7.4)	8 µl of cell suspension was mixed with 100 µl of LMP agarose and added to a microscope slide pre-coated with 1.0% of NMP agarose.	Cells were lysed (2.5 M NaCl, 100 mM Na ₂ EDTA, 10 mM Tris-HCl, 1% Na-lauroyl-sarcosinate, 1% Triton X-100, and 10% DMSO, pH 10) for 72 h at 4°C and denatured (300 mM NaOH, 1 mM Na ₂ EDTA, pH 13.0) for 10 min	Cells were lysed (2.5 M NaCl, 100 mM Na ₂ EDTA, 10 mM Tris-HCl, 1% Na-lauroyl-sarcosinate, 1% Triton X-100, and 10% DMSO, pH 10) for 72 h at 4°C and denatured (300 mM NaOH, 1 mM Na ₂ EDTA, pH 13.0) for 10 min	Electrophoresis was performed at 0.66 V/cm, 300 mA for 16 min	Neutralization (0.4 M Tris-HCl, pH 7.5) staining with EtBr (20 µg/mL) was performed	[55]

Exfoliated Buccal Cells Sampling			Buccal comet assay technique					Reference		
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	Reference
-	-	-	The buccal cell suspension was centrifuged	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% LMA was applied and were immersed in freshly prepared ice cold lysis solution for 1 hour			Stained with EtBr	[65]
Water	The interior surfaces of right and left cheeks gently scraped with a toothbrush. The cells suspended in 25 ml of Tienko-Holland buffer solution [114] and transferred within 2-8 hr, at 4°C and in the dark, to the laboratory	According with Singh et al. [100]	Cells were washed twice in the buffer solution and then suspended in about 100 µL of the same buffer, immediately before performing the comet assay	90 µl of 0.5% NMP agarose in PBS at 50°C layered onto bonded gel film, immediately covered with a coverslip, and allowed to solidify at 4°C for 5 min. The coverslip was then removed, and about 40 µL of lymphocytes suspension or 80 µL of exfoliated cells 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. The coverslip was then removed, and a second layer of 0.7% LMA was added and left to solidify		The coverslips were taken off, the films were layered onto glass slides, and bathed in freshly prepared lysis solution (2.5 M NaCl, 100 mM Na ₂ EDTA, 10 mM Tris with 1% Triton X-100, and 10% DMSO added fresh) in the dark for 1 hr at 4°C	The slides were removed from the lysis solution, placed in a horizontal gel electrophoresis tank filled with fresh alkaline buffer (1 mM Na ₂ EDTA and 300 mM NaOH, pH 13) for 20 min at 4°C	Alkaline buffer (1 mM Na ₂ EDTA and 300 mM NaOH, pH 13) at 20 V and 300 mA for 20 min	Slides were then washed three times with 0.4 M Tris HCl for 5 min, and stained with 50 µL EtBr (10 µg/mL)	[76]

Exfoliated Buccal Cells Sampling	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	Reference	
Rinsing	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	According with Singh et al. [100]	The buccal epithelial cell samples were washed with PBS, centrifuged and resuspended from the pellet	On a clean, dry, plain slide 100 µL of 0.75% NMP agarose prepared in PBS was layered. These pre-coated slides were dried at 37°C. On top 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.		The slides were incubated in cold lysis buffer (2.5 M NaCl, 100 mM Na ₂ EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate; 1% Triton X-100 and 10% DMSO added fresh) at 4°C overnight	Immersed in freshly prepared alkaline electrophoretic buffer (1 mM Na ₂ EDTA and 300 mM NaOH, pH 13) for 30 min	30 min at 300 mA, 0.67 V/cm	Neutralizing buffer (0.4 M Tris buffer, pH 7.5). The slides were then washed with distilled water and air dried. Silver staining	[64]
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	Comet assay was performed as described by Szeto et al. [61]	The suspension was centrifuged at 2500 rpm at 4°C for 10 min. The cell pellet was resuspended in 100 µL PBS buffer	10 µL of the buccal cell suspension was mixed with 85 µL of pre-warmed (40°C) 1% (w/v) LMP agarose in PBS (0.137 M NaCl, 2.68 mM KCl, 6.4 mM Na ₂ HPO ₄ ·7 H ₂ O, 1.47 mM KH ₂ PO ₄ , pH 7.4), and immediately applied on a clean microscopic slide precoated with 85 µL of 1% (w/v) NMP agarose in PBS buffer. The slides were allowed to solidify in room temperature	A 50 µL of trypsin solution was layered onto the gel and left for 30 min at 37°C followed by washing with PBS buffer, proteinase K (1 mg/ml) treatment for 1 h at 37°C	Immersion in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h at 4°C	The slides were kept in electrophoresis tank filled with electrophoretic buffer (0.01 M NaOH and 1 mM EDTA, pH 9.1) for 20 min	At 12 V for 18 min	Neutralized 3 times with 0.4 M Tris at pH 7.5. The slides were then stained with EtBr (50 µL of 20 µg/ml)	[78]	
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 alkali, pH 13, for 25 min	For 20 min at 25 V (0.86 V/cm) and 300 mA, at room temperature	The slides were neutralized, fixed, and stained with silver nitrate [108]	[52]	

Exfoliated Buccal Cells Sampling		Buccal comet assay technique						Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	
Prior to brushing subjects wash their mouth with 0.9% NaCl solution	Buccal squamous cells were collected from subjects by oral brushing	Comet assay was performed under alkaline conditions by using a standard protocol [100] with some modifications		Cells were embedded in LMP agarose on glass slide precoated with 1% NMP agarose		After solidification of gel the slide was submerged into cooling solution [2.5 M NaCl, 10 mM EDTA, 10 mM Tris (pH 10.0), 1% LSS lauryl sarcosine sodium salt to which 10% DMSO, 1% Triton X-100 were freshly added] and kept overnight at 4°C	The slides were then placed on the horizontal electrophoresis unit filled with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 30 min	At 25 V and 300 mA for 40 min
Neutralisation and staining	The slides were neutralized for ~60 min in 0.4 M Tris/HCl, pH 7.5 on ice and staining in EtBr (25 µg/ml in distilled water)							[62]
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	The procedure of Singh et al. [100] was used, with minor modifications	The exfoliated buccal cells were washed twice in PBS and then suspended in about 100 µL of the same buffer	90 µL of 0.5% NMP agarose in PBS at 50°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 bathed in freshly prepared lysis solution (2.5 M NaCl, 10 mM Na ₂ EDTA, 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 Na ₂ EDTA and 300 mM NaOH, pH 13) for 20 min at 4°C to allow denaturing and unwinding of the DNA, and the expression of alkali-labile sites	20 V and 300 mA for 20 min
							The slides were washed three times for 5 min each with 0.4 M Tris-HCl. Slides were stained with 50 µL of 10 µg/mL EtBr	[77]

Buccal comet assay technique										
Exfoliated Buccal Cells Sampling	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	Reference
Rinsed their mouths with water	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]	Procedure of Collins et al. [116], with minor modifications	The exfoliated buccal cells were washed twice in PBS and then suspended in about 100 μ L of the same buffer	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 μ L of exfoliated cell suspension were mixed with 70 μ L of 0.7% LMP agarose in PBS at 37°C and layered on top of each film		Then they were bathed in lysis solution (2.5 M NaCl, 100 mM Na ₂ EDTA, 10 mM Tris with 1% Triton X-100 and 10% DMSO added fresh) and kept in the dark for 1 h at 4°C	The slides were washed 3 times in enzyme buffer (50 mM Na ₂ PO ₄ , 10 mM EDTA, 100 mM NaCl, pH 7.5), drained and incubated with 50 μ L of either buffer or Fpg (1 μ g/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 Na ₂ EDTA and 300 mM NaOH, pH 13) for 40 min at 4°C	In the same buffer at 25 V and 300 mA for 30 min	The slides were then washed 3 times with Tris-HCl 0.4 M for 5 min and stained with 50 μ L EtBr (10 μ g/mL)	[75]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique					Reference			
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Volunteers rinse their mouth thoroughly with filtered tap water	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).	According with Singh et al. [100] and Valverde et al. [83], with modifications	The cells were centrifuged at 89 x g, for 5 min. 10 µL were used to perform the SCGE assay	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 coded slide, precoated with a thin layer of NMP agarose [117]. The agarose layer was covered with a coverslip and left for 5 min at 4°C to solidify	Cell suspension was diluted into 150–300 µL PBS, and treated 1:1 with an enzyme "cocktail" (final concentration: 0.05 mg/ml DNase I, 0.15 mg/ml collagenase I, and 0.125 mg/ml trypsin in 0.01% EDTA, pH 7.4) for 30 min at 37° (adapted from Olive 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	Slides were immersed into either lysis solution 1 (2.5 M NaCl, 100 mM Na ₂ EDTA, 10 mM Tris (pH 10), and 1% sodium sarcosine°C at 37°C) or lysis solution 2 (1% SDS, 10 mM Na ₂ EDTA, pH 8) for at least 1 hr at 4°C	Different alkaline (pH > 13) unwinding times (5–40 min) and electrophoresis times (0.66 V/cm, 300 mA, for 5–40 min) were tested in the preliminary experiments. For the cross-sectional experiment, both pre- and post-enrichment slides were randomized by location inside the electrophoresis box and by different runs.	Unwinding and electrophoresis for 20 and 10 min, respectively. In some experiments, the electrophoresis was 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 alkaline labile sites on the migration of comets from cell samples	Slides were neutralized in Tris-HCl (pH 7.5) for 5 min, fixed with absolute ethanol, and stored. The slides were stained with 20 µg/ml EtBr	[92]

[92]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique						Reference		
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis		Electrophoresis	Neutralisation, fixation and staining
Distilled water	A soft bristle toothbrush was used to collect buccal cells by scraping the inside cheek of the mouth gently. The toothbrush was then agitated in 30 ml cold PBS in a 50 ml plastic tube	Several	Buccal cell suspension was centrifuged at 2500 rpm 4°C 10 min. cell pellet was resuspended in 100 µL PBS	10 µL of this suspension were mixed with 85 µL of pre-warmed (40°C) 1% (w/v) LMP agarose in PBS, and immediately applied to a microscope slide which had been precoated with 85 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	In some cases lysis was followed by exposure of the cells to 0.1 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 final (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 ashing-proteinase K treatment.	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	Slides were then transferred to a Coplin jar containing electrophoresis solution at 4°C for 20 min (2×10 min). Electrophoresis solution comprised 1mM EDTA with various concentrations of NaOH (0.0003-0.3 M), with pH ranging from 5.9 to >13	Investigated the effect of electrophoresis at lower pH values. Electrophoresis was performed for 18 min at 12 V constant voltage.	Neutralized by immersing in three changes (3×5 min) of 0.4M Tris at pH 7.5. s were stained with EtBr	[61]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique					Reference		
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining
Volunteers were required first to rinse their mouths with water, then to discard the water used in the mouthwash	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			Slides were prepared in duplicate as follows: 120 μ L NMP agarose (1% in PBS) were layered on to pre-cleaned frosted microscope slides, immediately covered with a cover slip and allowed to solidify. Then, 20 μ L of cell suspension was mixed with 75 μ L 0.5% LMP agarose in PBS at 37°C, the cover slips were removed and the mixture was added to the slides. The cover slips were replaced and the slides were put on ice for 5 min. After solidification of the agarose, the cover slips were gently removed. Another top layer of 75 μ L LMP agarose was added, the cover slips were replaced and the slides were again placed on ice	The slides with buccal epithelial and sublingual cells were treated at 4°C for 25 min in the lysis solution containing 10 μ L proteinase K (10 mg/ml) in 100 μ L lysis solution	Once the top layer had solidified, the cover slips were removed and the slides gently immersed in cold lysing solution (2.5M NaCl, 100 mM Na ₂ EDTA, 1% N lauryl sarcosine, 10 mM tris-HCl [pH 10], to which 1% Triton X-100 and 10% DMSO had been freshly added)	2 washings with distilled water for 10 min, in a horizontal gel electrophoresis unit containing fresh buffer (300 mM NaOH, 1 mM Na ₂ EDTA, pH 13) to a level of 0.25 cm above the slides for 20 min.	20 min, 25 V, 300 mA (0.8 V/cm)	The slides were stained with 50 μ L of propidium iodide (200 μ M/mL in 50 ml PBS) for 10 min
									[60]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique							Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	
Rinse their mouths with tap water	Buccal epithelial cells were obtained by scraping the cheeks with a plastic spatula. The cells were added to 2 ml of RPMI-1640 medium		Buccal cells were washed with RPMI-1640 medium and centrifuged at 6,000 rpm for 1 min	Slides were prepared in duplicate, and 120 µl of NMP agarose 0.5%, in PBS 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	After lysis, buccal cells were treated with 140 µl of proteinase K (10 mg/ml) at 37°C for 2 h	Once the top layer had solidified, the coverslips were removed and the slides gently immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 1% N lauryl sarcosine, 10 mM tris-HCl [pH 10], to which 1% Triton X-100 and 10% DMSO had been freshly added). The slides were left at 4°C for at least 1 h	The slides placed close together in a horizontal gel electrophoresis tank near the anode. This was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) to a level of 0.25 cm above the slides, which were then left to soak for 40 min in the alkali	For 40 min at 19 V, 300 mA	[50]
								Neutralising buffer (0.4 M tris-HCl, pH 7.5) was added dropwise to the slides 3 times, 5 min each time. Slides stained with 50 µl EtBr (40 mg/ml)	

Exfoliated Buccal Cells Sampling										Refer- ence
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre- electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
-	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.	Rojas et al. [14], with some modifications			After lysis (2.5 M NaCl, 100 mM Na ₂ EDTA, 10 mM Tris-hydrochloride and 1% Nasarcosinate, pH 10) at 4°C for 24 hr, the cells were treated with 100 µL proteinase K (10 mg/ml) at 37°C for 1h		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 Na ₂ EDTA, pH 13).	20 min at 25 V and 300 mA. After, the slides gently removed	Neutralized with 0.4 M Tris, pH 7.5; dehydrated in 2 steps with absolute ethanol for 10 min each, staining with 75 ml EtBr (20 mg/ml) with coverglass	[83]
-	Scrapping the internal part of the cheek with a wood stick	Tice et al. [120] with some modifications	The cells were added into 2 mL of RPMI-1640 medium and centrifuged at 6000 rpm for 1 min, approx. 5000 cells resuspended in 75 µL LMP agarose		After lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% sodium sarcosinate, pH 10) at 4°C for 48 h, the cells were dropped into a new lysis solution with 140 µL of proteinase K (10 mg/ml) at 37°C for 2 h		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 Na ₂ EDTA, pH 13).	For 20 min at 25 V and 300 mA	Neutralization with 0.4 M Tris-HCl, pH 7.5. EtBr (75 µL of a 20 mg/ml solution) was added to each slide	[14]

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 life-style, 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 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 chewer 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].

Exposed population		Control population		Interview	Results BCA	Parameters measured arbitrary units, TL, TL, TM	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age							
MOUTHPIECES AND METAL RELEASED FROM ORTHODONTIC APPLIANCES										
9	4	9	4	Subjects filled in detailed questionnaires regarding confounding factors for DNA damage such as smoking, viral diseases, recent vaccinations, and radiodiagnostic examinations.	Before CHX treatment Undamaged nuclei 91.54 ± 6.75 Intermediate nuclei 6.00 ± 4.85 Tailed nuclei 2.46 ± 3.73 After CHX treatment Undamaged nuclei 15.77 ± 4.6 Intermediate nuclei 71.15 ± 7.12 Tailed nuclei 13.08 ± 4.94	Determine the degree of damage by grading the cells as undamaged, intermediate, and tailed.	Wilcoxon's test	DNA damage in peripheral blood cells	Before CHX treatment Undamaged nuclei 93.77 ± 7.65 Intermediate nuclei 0.62 ± 1.50 After CHX Treatment Undamaged nuclei 82.62 ± 8.35 Intermediate nuclei 11.31 ± 7.93 Tailed nuclei 6.08 ± 4.44	[50]
55 orthodontic patients with fixed appliances in both arches: nickel-titanium alloy, stainless steel or chromium-cobalt-nickel alloy				Smoking, drinking	TL: 10.54 ± 2.41 vs 15.56 ± 6.78 TM: 0.46 ± 0.21 vs 0.30 ± 0.09 TL: 5.44 ± 1.89 vs 4.72 ± 1.51	% DNA, tail length, TM	Mann-Whitney U test	Apoptosis, viability	Apoptosis: 3.15 ± 4.93 vs 1.00 ± 2.26, Viability: 50.40 ± 13.55 vs 73.43 ± 12.29,	[51]
33 12-35	32 12-35	13 12-33	17 12-35							
14 16 + 2.5	6 16 + 2.5	14 16 + 2.5	6 16 + 2.5	Smoking or drinking or illnesses related to any genetic damage increase were not reported by any patient.	2.5 ± 3.08 vs 1.5 ± 1.05	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]	The one-tailed t-test with Welch's correction was used	Micronucleus assay	MIN frequency (p = 0.0213)	[52]

Exposed population	Control population		Interview	Results BCA Exposure group vs control group	Parameters measured		Statistics	Other methods used	Results	Author
	Females (n), age	Males (n), age			arbitrary units, TL, TL, TM	TL, TM				
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	15 12-16 the same patients before treatment		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.	Stainless steel 69.35 ± 11.68; Nickel-free 68.41 ± 26.63; H ₂ O ₂ 71.10 ± 5.15 Titanium alloy and controls Olive moment was similar	Olive moment		ANOVA, Tukey posthoc			[53]
22 20.2 ± 4.4	6 20.2 ± 4.4	8 21.5 ± 3.3	Subjects were initially screened with a questionnaire to check whether they fit the criteria of the study. The eligibility criteria for subject selection included nonsmokers; no oral diseases, systemic diseases, oral restorations or prosthetics; clinically healthy oral mucosa; no previous orthodontic treatment; no occupational exposure to metals; not receiving any medications or supplements; no radiographic examination during the previous 6 months; and no known allergy to costume jewelry, watches, or sources of nickel and chromium.	DNA damage value, decreased from 125.6 ± 46.05 to 98.8 ± 33.70 at 6 months	Only nucleosids 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.		Normally distributed variables (composite score and damage frequency) were tested with paired t tests.			[54]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age							
30 69.56		25 72.68		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.	Significantly increased tail length and percentage DNA in the tail values in subjects wearing metal appliances	DNA damage was evaluated as percentage DNA in the tail (% DNA) and tail length	ANOVA vMann-Whitney U test t Test Newman-Kuels test	Evaluate influence of general characteristics of the subjects (age, gender, dietary habits, pH of saliva, alcohol and drug intake) on parameters of comet assay.	None of demographic or lifestyle factors tested as possible predictors have exhibited significant influence on values of comet assay parameters	[55]
26 21.1 ± 0.30	17 21.1 ± 0.30	12 20.0 ± 0.55	8 20.0 ± 0.55	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	The DNA % was dose-dependently higher in subjects carrying dental fillings as compared with filling-free subjects. In subjects carrying at least two fillings	The results were expressed as percentage of DNA in the tail (TDNA %), measured by the automated image analysis system CASP (comet assay software project) (http://isp.sourceforge.net).	Mann-Whitney test and Poisson regression analysis	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.	MN frequency higher in subjects with restorative fillings than in filling-free subjects	[56]
20 persons as positive control (smokers)20 patients with orthodontic treatment,20 patients with orthodontic appliances and micro screws		20			% DNA in tail significantly different between all four groups, females with orthodontic appliances	% DNA in tail	-	-	-	[57]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age							
16 (7-14)		16 (7-14). Each patient acted as his/her own control			Exposure group vs control group <i>Damage frequency</i> 53.25 % vs 35.94 % <i>Damage indice</i> 75.69 vs 50.31	Cells were scored visually according to tail length into five classes: class 0: undamaged, without a tail; class 1: with a tail shorter than the diameter of the head (nucleus); class 2: with a tail 1 to 2X the diameter of the head; class 3: with a tail longer than 2X the diameter of the head and class 4: comets with no heads.	Wilcoxon's test	BM/Cyt	No significant differences were observed	[59]
RADIATION										
3 (34-45)	3 (34-45)	3 (34-45)	3 (34-45)	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	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;	A slide's visual score was converted to an arbitrary DNA damage score. The nuclei showing comets were categorized as follows: 1° (>80% DNA in comet head), 2° (50-79% DNA in comet head), 3° (20-49% DNA in comet head) and 4° (<20% DNA in comet head). Nuclei without DNA damage were listed in category 0	ANOVA Tukey's multiple comparison test Student's t-test Pearson correlation factor between parameters	Damage in lymphocytes	There is no difference between the baseline DNA damage rate of G0 and G1 lymphocytes; For all cell types there is a significant difference in baseline DNA damage rate between individuals.	[60]

Exposed population		Control population		Interview	Results BCA	Parameters measured arbitrary units, TL, TL, TM	Statistics	Other methods used	Results	Author	
Females (n), age	Males (n), age	Females (n), age	Males (n), age								
LIFE STYLE, DIETARY, GENETIC AND HEALTHY FACTORS											
6 (24-43)	5 (32-63)	6 (19-43)	3 (25-34)		89.30 ± 16.18 µm vs 52.01 ± 10.43 µm	DNA migration was measured with a scaled ocular as the total image length (including head and tail length).	Student's t-test			[14]	
	1 (28)			Chewing tobacco since the last 17 years	59.16 ± 2.84 vs 14 ± 1.87	DNA damage was quantified by visual classification of cells into categories of comets corresponding to the DNA damage [123, 124]		Chromosomal aberrations MN	CA 2.4 ± 0.69 vs 1.2 ± 0.41 MN 1.5 ± 0.5 % vs 0.05%	[70]	
50 gutkha chewers smokers (37.7 ± 1.50)/50 pan masala chewers smokers (32.2 ± 1.18)/50 gutkha chewers (32.5 ± 1.63)/50 pan masala chewers (30.0 ± 1.42)/50 smokers (40.1 ± 1.71)		50 with no addiction (29.7 ± 1.41)		Age, smoking habit, consumption of gutkha and pan masala	36.9 ± 3.60/33.6 ± 3.59/31.6 ± 3.52/29.3 ± 3.41/14.9 ± 0.79 vs control/3.41 ± 0.41	TL, using comet score 1.5 software	Student's 't' test	-	-	[71]	
20/37.55		20			Tail moment 186 ± 26 vs 0.05 ± 0.001 Tail length 456 ± 71 vs 9 ± 1.3 % tail DNA 97 ± 19 vs 1.12 ± 0.02 Fragmented DNA 32 ± 3.3 vs 3.4 ± 0.03	Analyzed for comet parameters using LAI Comet analysis system		Comet assay in human peripheral blood leukocytes	Jurak smoke Condensate Tail moment 12.61 ± 7.41 vs 0.01 Tail length 160.74 ± 47.66 vs 2.0 % tail DNA 22.36 ± 8.87 vs 0.31 Fragmented DNA 5.09 ± 1.41 vs 2.8 Massed smoke condensate Tail moment 21.86 ± 13.33 vs 0.01 Tail length 213.10 ± 75.22 vs 2.0 % tail DNA 29.03 ± 9.77 vs 0.31 Fragmented DNA 5.23 ± 1.43 vs 2.78	[72]	

Exposed population		Control population		Interview	Results BCA	Parameters measured arbitrary units, TL, TL, TM	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age							
Cancer patients 6	Cancer patients 31	Tobacco users 2 No users 14	Tobacco users 84 No users 31	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.	% DNA damage Oral cancer patients 19.1 ± 9.14 Tobacco Users 7.10 Users 4.56 ± 2.68	DNA damage is represented as percentage data	T-test of unequal variance, Chi-square test Multivariate analysis.	Confounding factors	The evaluation of various confounding factors like age, tenure of tobacco habit and tea habit showed significant associations with DNA damage age	[62]
21	125	93	69	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% <65 59.58 ± 4.18 >65 61.86 ± 4.64 Control <65 40.46 ± 6.34 >65 46.09 ± 3.8	Tail DNA percentage (TD%) Olive tail moment (OTM) The Mean TD% and OTM for each group were compared with mean values of control subjects of respective age groups.	Student or to the study all t-test One way ANOVA	Intracellular ROS levels Apoptosis rate	In the <65 y age group percentage of apoptotic cells was low in the control subjects as well as in the subjects with/without tobacco and/or tea habit (7-9%). In case of >65 y age group, percentage of apoptotic cells was comparatively higher in the control subjects (17 ± 2.8%) and slight increase in apoptosis was observed in rest of the subgroups.	[63]
52 45.4 ± 10.2	104 45.4 ± 10.2	18 50.4 ± 8.7	52 50.4 ± 8.7	Questionnaires were completed to obtain detailed occupational, smoking, and medical histories.	Tail Length <45 years 34.3 ± 1.12 vs 32.1 ± 1.14 >45 years 34.3 ± 1.21 vs 32.5 1.01	Tail length (TL) and tail moment (TM) were evaluated, with Comet Assay II	Student's t-test	MIN chromosomal aberration assays	MIN Tobacco drivers Male 2.2 ± 0.67 Female 2.0 ± 0.47 vs Control Male 0.86 ± 0.52 Female 1.2 ± 0.91 Total Chromosomal Aberrations (CA) Tobacco drivers 2.18 ± 1.31 vs Control 1.21 ± 0.91	[65]

Exposed population		Control population		Interview	Results BCA		Parameters measured arbitrary units, TL, TL, TM	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group						
78 wild genotype 42 mutant genotypes		66 wild genotype 54 mutant genotypes		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	Workers with the mutated genotype (Ile-Val, Val-Val) had a significantly greater comet tail length than controls. The same is true for workers with the wild genotype Ile-Ile,		Tail length. The cells were analysed using commercial TriTek Comet Score (version 1.5) software.	ANOVA Non-parametric Mann-Whitney U-test. Independent t-test, Chi-square test	MN PCR Restriction fragment length polymorphism (RFLP). Telomere length	Workers with the mutated 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,	[66]
80 wild genotype 40 mutant genotypes		95 wild genotype 25 mutant genotypes		The subjects were interviewed to determine their health status and lifestyles.	c1c1 genotype 25.64 ± 9.35 vs 18.02 ± 8.40 c1c2 and c2c2 genotypes 24.09 ± 7.86 Men 15.42 ± 5.97 Mutated genotype (c1c2 and c2c2) not influenced significantly by comet tail length		Tail length	Non-parametric Mann-Whitney U-test.	MN PCR RFLP Telomere length	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	[67]
58 wild genotype 62 mutant genotypes		60 wild genotype 60 mutant genotypes		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	WW genotype 23.70 ± 8.59 vs 17.14 ± 7.81 MW and MM genotypes 26.46 ± 9.01 vs 17.82 ± 8.24 No statistically significant effect was found in wild (WW) or mutated genotypes (MW, MM)		Tail length	Non parametric Mann-Whitney U-test.	MN PCR RFLP Telomere length	Difference in MN frequency between workers and controls was statistically significant in both wild and mutant genotypes. In addition, the results showed that the mutated genotype significantly affected the relative telomere length in workers.	[68]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age							
56	64	52	68	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 5 years of employment.	24.99 ± 9.14 vs 17.47 ± 8.40 Neither genotype showed any statistically significant effects	Tail length	Non parametric Mann-Whitney U test.	MN/PCR/ELF/PTelomere length	The workers carrying wild or mutated genotypes showed a significantly higher MN frequency and shorter telomere length compared to controls	[69]
50 untreated patients with cancer 50.42 (20-72) 20 untreated patients at pre-cancer stage. 29.55 (17-50)	79 untreated patients with cancer 50.42 (20-72) 118 untreated patients at pre-cancer stage. 29.55 (17-50)	35 healthy 30.80 (16-67)	141 healthy 30.80 (16-67)	Case history and personal details were collected. Data included age and gender with similar smoking and tobacco use (chewing), dietary habits and socioeconomic status.	Comet tail length Cancer 28.64 ± 4.97 Pre-cancer 20.93 ± 5.58 Controls 9.15 ± 3.83	Tail length was measured with an ocular micrometer fitted in the eyepiece	Student's t-test (paired and unpaired comparisons) and analysis of variance were carried out to evaluate various differences.	MN conducted on the buccal epithelial cells; Comet assay on peripheral blood leukocytes; The challenge comet assay on peripheral blood leukocytes.	% MN Cancer 0.48 ± 0.33 Pre-cancer 0.31 ± 0.24 Controls 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.	[64]
30 patients with oral squamous cell carcinoma (OSCC)	30 without OSCC			Patients who were diagnosed as having OSCC formed the study group	OSCC 3.874 ± 2.5205 µm vs Normal subjects 0.8616 ± 0.8142 µm	Total length and the diameter was measured.	Students' test, One way ANOVA "F"	To analyze DNA damage, patients having OSCC were divided into four stages, namely stage I, II, III, and IV	Stage I 2.312 ± 0.366 Stage II 3.171 ± 1.439 Stage III 3.490 ± 1.971 Stage IV 6.890 ± 3.710	[73]

Exposed population				Control population		Interview	Results BCA Exposure group vs control group	Parameters measured arbitrary units, TL, TL, TM	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age									
OCCUPATIONAL EXPOSURE												
41 43.0 ± 8.3		31 43.35 ± 9.4		All subjects gave informed consent. Anagraphic, clinical, working information and lifestyle habits (smoking, dietary habit, alcohol consumption) were obtained from a questionnaire administered by specialized medical personnel	TM 118.87 vs 68.20 TMenz 146.11 vs 78.32	Tail moment from Fpg-treated cells (TMenz) and Fpg-untreated cells (TM). Values TMenz and from TM were used as parameters of oxidative and direct DNA damage, respectively. TMenz/TM ratio higher than 2.0 was used to indicate the presence of oxidative damage.	Student's t-test	MN and Fpg-modified comet assay on lymphocytes and exfoliated buccal cells, and by chromosomal aberrations (CA) and sister chromatid exchange (SCE) analyses	The exposed group showed a higher mean value of SCE frequency in respect to controls (4.6 versus 3.8) and an increase (1.3-fold) of total structural CA in particular breaks (up to 2.0-fold) and fragments (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 TMenz in lymphocytes (TM 43.01 versus 36.01; TMenz 55.86 versus 43.98).	[75]		
Pharmacy technicians 2 35.8 ± 9.9 Day hospital nurses 10 37.6 ± 5.5 Ward nurses 11 32.7 ± 7.7		25 34.9 ± 8.5			5 34.9 ± 8.5	Pharmacy technicians 32.6 ± 18.2 Day hospital nurses 43.2 ± 36 Ward nurses 27.4 ± 13.9 Controls 28.6 ± 12.4	TM	ANOVA Chi square Student t test Kolmogorov-Smirnov non-parametric test Levene test	Comet assay in lymphocytes cells	Pharmacy technicians 20.8 ± 10.1 Day hospital nurses 15.5 ± 9 Ward nurses 14.7 ± 7.9 Controls 16.1 ± 8.1	[77]	

Exposed population		Control population		Interview	Results BCA	Parameters measured		Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age			arbitrary units, TL, TI, TM	TL, TM				
				A questionnaire 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.	The significant differences in the comet class between the controls and jewellery workers shows that the later group has increased DNA damage who are occupationally exposed to nitric oxide.	The comets were analyzed by visual classification and the damage was assigned to 5 classes [125]. The DNA was calculated according with Zhao et al. [126]		Student's t-test		[78]	
87 (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 ± 10.6).		76 (39.72 ± 10.1)		Personal data, clinical and working information, and lifestyle habits (smoking, dietary habit, and alcohol consumption) were obtained from a questionnaire administered by specialized medical personnel.	% Comets 13.74 ± 10.9 vs 13.78 ± 9.80 Tail moment 48.01 ± 30.1 vs 32.31 ± 12.79	The percentage of comets on total cells was calculated. Measurements of comet parameters were: % DNA in the tail, tail length, tail moment		Students' t-test, Mann-Whitney U-test, ANOVA, Kruskal-Wallis, and Bonferroni test	Comet and MN tests were performed on lymphocytes and exfoliated buccal cells.	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 as 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 workers exposed to antineoplastic drugs.	[76]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age							
85.35 (20–42)	58.29.03 ± 9.98		30.28.24 ± 10.99	According to the protocol published by the International Commission for Protection against Environmental Mutagens and Carcinogens [127] and participate in a face-to-face questionnaire which included standard demographic data (age, gender,) as well as questions relating to medical issues (exposure to X-rays, vaccinations, medications), life style (smoking, coffee, alcohol, diet,) and their occupation (number of hours worked per day, time exposed to organic solvents, use of protective measures).	Damage Frequency 22.38 ± 17.28 vs 13.56 ± 12.69 Damage index 33.43 ± 30.18 vs 18.81 ± 18.93	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 [128]. DI thus ranged from 0 (completely undamaged: 100 cells=0) to 400 (with maximum damage: 100 cells=4, [22]. The damage frequency (DF) (%) was calculated based on the percentage of damaged cells (0–100%).	Non-parametric Mann–Whitney U-test	In peripheral blood lymphocytes and oral mucosa cells of paint industry workers	No significant difference was detected between the control and paint industry workers. Comet assay data in peripheral blood leukocytes showed that both analysis parameters (DI and DF) were significantly greater than that for the control group	[79]
		76.34 (21–41)		During personal interview, each participant was requested to furnish information about age, education, family size and income, habit, cooking time per day, years of cooking, fuel and oven type, location and ventilation of kitchen, health problems in past 3 months and last one year.	Comet tail % DNA 32.23 ± 8.51 vs 12.41 ± 3.87 Comet tail length (µm) 37.81 ± 11.21 vs 14.22 ± 3.89 Olive tail moment in arbitrary unit 7.08 ± 2.11 vs 3.15 ± 0.97	% 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 diffusion in BEC of biomass users, implying greater DNA damage than that of control. NDF	[84]

Exposed population		Control population		Interview	Results BCA	Parameters measured arbitrary units, TL, TL, TM	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age							
66 37.3 ± 7.45		60 38.7 ± 8.21		The selection criteria for the subjects were based on a questionnaire according to the protocol published by the International Commission for Protection against Environmental Mutagens and Carcinogens [127]	Exposure group vs control group 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	Tail length	Students' 't' test.	MN	Welders showed a significant increase in micronucleated cells compared to controls	[80]
160 40.13 ± 10.60		160 40.22 ± 9.79		Personal lifestyle, occupational, and residential information	TL 24.35 µm vs 12.8 µm	The cells were then analyzed by using the TriTek Comet Score (version 1.5) software. The tail length was measured (µm)		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	Age, smoker, smoking duration, and secondhand smoker highlighted the significant difference within groups among the study population. Overall, smokers and secondhand smokers reported with higher levels of DNA damage, and this impairment increased with age and smoking duration.	[81]

Exposed population		Control population		Interview	Results IBCA Exposure group vs control group	Parameters measured arbitrary units, TL, TL, TM	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age							
111 45.75 ± 3.5		60 37.55 ± 0.2		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 these agricultural areas.	Tail migration of DNA increased significantly in the exposed group	Comet tail length (DNA distance)	Kruskal-Wallis non-parametric test, ANOVA test, Tukey-Kramer multiple comparison test.	MN assay and other nuclear anomalies such as nuclear buds, karyolysis, karyorrhexis, and binucleate cells were also evaluated	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	[82]
ENVIRONMENTAL EXPOSURE										
South 32 19		North 16 19		Each student answered a self-applied questionnaire translated and validated from the American Thoracic Society (ATS) for respiratory tract symptoms	South 137.59 ± 55.88 vs North 121.96 ± 58.72	DNA migration (tail image length) Relative DNA damage index	U Mann-Whitney test	Alkaline SCG assay using leukocytes and nasal cells	Leukocytes South 13.97 ± 9.32 vs North 8.76 ± 3.80 Nasal South 40.07 ± 21.07 vs North 23.12 ± 10.36	[83]
54 10 ± 0.82	41 10.02 ± 0.80	43 10 ± 0.82	42 10.02 ± 0.80		8.45 ± 3.89 vs 4.38 ± 1.66	The level of DNA damage was measured using comet assay following the method described previously [14] and modified based on standard procedures from the comet assay kit	t statistic	MN	5.05 ± 2.45 vs 2.92 ± 1.54	[85]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age							
231 9.4 ± 1.6	182 9.5 ± 1.6			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	Exposure group vs control group <i>Tail intensity (%)</i> 3.25 ± 0.88 <i>Tail length (µm)</i> 11.69 ± 2.11 <i>Tail moment</i> 0.20 ± 0.05	arbitrary units, TL, TM 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]	Analysis of variance for quantitative variables and Pearson's chi-square test for categorical variables			[86]
9		10		Samples were obtained from volunteers among the laboratory staff	<i>Preirradiation %</i> Tail DNA 63.8 ± 70.2 vs 65.3 ± 13.9 Tail moment 25.8 ± 5.3 vs 23.1 ± 5.5 <i>Postirradiation %</i> Tail DNA 42.4 ± 20.4 vs 31.9 ± 10.5 Tail moment 15.4 ± 11.6 vs 9.1 ± 4.4	% Tail DNA Tail moment	Normality was tested by the Shapiro-Wilks W test. Student's t-test, paired and unpaired McNemar Chi ² test	DNA damage in peripheral leukocytes	PK digestion increased the DNA migration and head diameter of leukocytes, regardless if they were untreated or treated with MMS. Therefore, PK digestion did not affect the ability of the assay to detect MMS-induced DNA damage	[92]

BCA: Buccal Comet Assay
BM-Cyt: 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 Gleib 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 inducers 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.

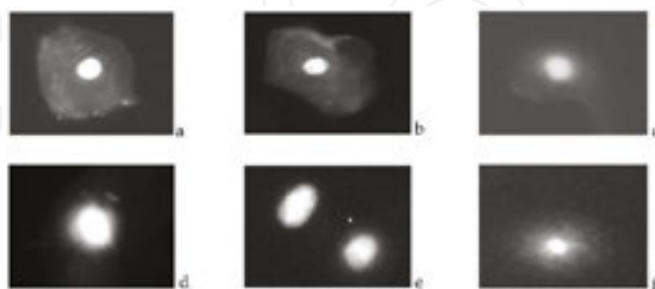


Figure 1. Picture of single buccal mucosa cells: (a) immediately stained after the solidification of agarose gel layer with sample cells, (b) the appearance of cells with cytoplasm after 1 h of classical lysis solution, (c) the appearance of the cells with cytoplasm after the combined treatment of lysis 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 et al. [61] suggested that denaturation and electrophoresis in neutral conditions would be more appropriate. According to our

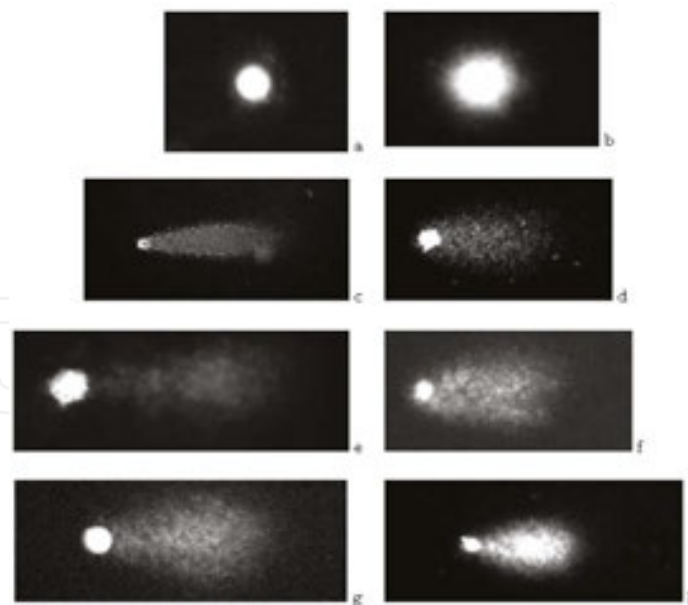


Figure 2. Pictures of buccal cells after different duration and type of lysis step, but all electrophoresis were at pH > 13: (a) treatment of lysis solution for 15 h 4°C, (b) lysis step for 20 h 4°C, (c) treatment with 0.25% trypsin for 30 min, and lysis for 30 min, both at 37°C, (d) 15 min of 0.25% 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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