The Use of the Micronucleus Test to Monitor Individuals at Risk of Oral Cancer

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1 Introduction

Oral squamous cell carcinoma is among the ten types of malignant neoplasia of highest incidence worldwide and is particularly frequent in developing countries (Marchione et al., 2007; Warnakulasuriya, 2009). In the United Kingdom, this neoplasia accounts for over 2% of all new cases of cancer in males and for more than 1% of all new cases in females (Cancer Research UK, 2012). In the United States between 2005 and 2009, 16.1 cases per 100,000 men and 6.2 per 100,000 women were diagnosed. In 2012, it is estimated that 28,540 men and 11,710 women will be diagnosed with cancer of the oral cavity and pharynx (U.S. National Institutes of Health, 2012). According to Jemal et al. (Jemal et al., 2011), the highest oral cavity cancer rates are generally found in Melanesia, South-Central Asia, and Central and Eastern Europe and the lowest in Africa, Central America and Eastern Asia, for both males and females.

Several risk factors have been shown to be associated with this neoplasia, but cigarette smoking is considered to be the most important factor for its development, particularly when in association with alcoholic beverages (Reibel, 2003; Rodriguez et al., 2004; Jemal et al., 2011). Smokeless tobacco products and HPV infection are also considered to be important risk factors for oral cavity cancer (Suhas et al., 2004; Syrjänen, 2005). Worldwide, considering deaths from cancers of both the oral cavity and the pharynx, smoking accounts for 42% and heavy alcohol consumption for 16% (Danaei et al., 2005).

Similarly to other types of malignant neoplasia, oral squamous cell carcinoma results from alterations (point mutations and chromosomal abnormalities) in genes that control the cell cycle, and/or in genes that are involved in DNA repair. In addition to the potential for metastasis, cancer is characterized by the loss of cells’ ability to evolve to death when genetic damage occurs (apoptosis) (Hanahan & Weinberg, 2000). As well as other types of malignant neoplasia, if oral squamous cell carcinoma is detected in its early stages, it can be treated successfully.

In this context, the use of biomarkers to identify genetic damage in individuals at higher risk of developing oral squamous cell carcinoma and to evaluate the malignant transformation potential of pre-cancerous lesions is considered to be an important tool for cancer prevention. The Micronucleus Test on exfoliated cells from oral epithelium has been widely used for these purposes.

2 The Micronucleus Test on Exfoliated Cells from Oral Epithelium

The use of the Micronucleus Test on exfoliated cells from oral epithelium with the aim of undertaking biomonitoring on human populations exposed to genotoxic agents was first proposed by Stich et al. (1982). The efficacy of this test for this purpose has been highlighted in many studies (Tolbert et al., 1992; Machado-Santelli et al., 1994; Salama et al., 1999; Cavallo et al., 2005). It is worth emphasizing that increased frequencies of micronuclei precede the clinical manifestations relating to the development of oral squamous cell carcinoma. According to Stich & Rosin (1983a) and Stich (1987), the frequency of micronuclei in human cells can be used as an “endogenous dosimeter” for tissues that are targets of these agents’ action.

According Salama et al. (1999), exfoliated oral cells are excellent for use in monitoring populations exposed to contaminants present in the environment, because these cells are in direct contact with pollutants that are ingested. Moreover, as highlighted by Zhang & Mock (1989) and Zhang (1994), they are capable of metabolizing carcinogens to reactive forms. Salama et al. (1999) also considered that when
the test is applied to these cells, it presents greater sensitivity in detecting the effects from exposure caused by the smoking habit, than when applied to lymphocytes.

Using the Micronucleus Test is also an important strategy for monitoring preneoplastic oral lesions, thereby guiding the therapeutic approach to be adopted (Casartelli, 2000; Halder et al., 2004; Kamboj & Mahajan, 2007; Saran et al., 2008; Chatterjee et al., 2009). Furthermore, according to Chatterjee et al. (2009), this test forms a very simple, practical, inexpensive and noninvasive screening technique for clinical prevention among individuals who are at risk of developing cancer.

2.1 Origin and Significance of Micronuclei

Micronuclei are formed by chromosomal fragments or whole chromosomes that fail to be included in the nuclei during the cell division process. They remain in the cytoplasm of interphasic cells as structures with a constitution and appearance similar to those of the nuclei (Cerqueira et al., 1998). Thus, micronuclei reflect both aneugenic and clastogenic events (Figure 1).

![Figure 1: Mechanisms of micronuclei formation](image)

2.2 Methodological issues

2.2.1 Choosing the sample

One important issue to be considered in the test is in relation to the number of individuals who should be included in the sample. Statistical software such as SPSS and Cytel Software can be used to calculate the sample size. Albertini et al. (2000) provided a practical description of how this calculation can be done. Segundo Au et al. (1991), in studies in which chromosome alterations are used as biomarkers, the minimum number of individuals to be analyzed should be 20, both for the exposed and for the control indi-
individuals. Ramirez (2000) considered that analysis on 30 individuals “ensures overall statistical representativeness for estimating sample parameters”.

In case-control studies, individuals in different groups should be matched according to gender and age. The criteria for including and excluding individuals should be rigorously consonant with the focus of the study, in order to minimize the interference of confounding factors, although statistical tools may get round the possible distortions introduced by such factors.

2.2.2 Collecting Cells and Obtaining Smears

The oral epithelium consists of stratified squamous cells, in four distinct layers: the stratum germinativum (or stratum basale) is the most internal layer, and this is successively overlain, going towards the surface of the oral cavity, by the spinosum, granulosum and corneum strata. Maintenance of this epithelium is done at the cost of intense proliferation of the basal cells of the stratum germinativum, which become differentiated as they migrate towards the upper layers. This process takes place over a period of 7 to 21 days (Squier & Kremer, 2001). Thus, the micronucleus test on exfoliated cells of the oral mucosa is a method indicated for evaluating recent genotoxic effects.

The material can be collected using different method, depending on the tissue to be studied, but according to Bonassi et al. (2011), the collection tool may influence the frequency of micronuclei. To collect cells from the oral cavity, the following have been used: toothbrush (Lucero et al., 2000; Pastor et al., 2001); wooden spatula (Özkul et al., 1997; Bloching et al., 2000); tongue depressors (Stich & Rosin, 1983b); or cervical cell collection brushes (Meireles, 2003; Cerqueira et al., 2004; Dórea, 2012). Cleansing of the oral cavity, by means of mouthwashes using drinking water, is recommended prior to sample collection.

The material collected can be directly transferred to a slide by means of cytocentrifugation or as a smear, or it can be dripped after centrifugation on saline solution (0.9% NaCl). Some authors have recommended that the material collected should be washed before dripping, using a buffer solution (0.01 M Tris-HCl, 0.1 M EDTA and 0.02 M NaCl at pH 7.0) (Titenko-Holland et al., 1994). From our experience, we consider that direct placement of a smear on a slide with the aid of two drops of physiological saline solution is not only extremely practical but also provides a greater number of cells and spreads them out well (Meireles, 2003; Cerqueira et al., 2004; Dórea et al., 2012). The transfer method does not interfere with the frequency of micronuclei (Bonassi et al., 2011). Additional references relating to collection and processing of epithelial cells from various tissues can be found in the extensive reviews by Majer et al. (2001) and Salama et al. (1999).

2.2.3 Fixing and Staining the Cells

The material is generally fixed using a 3:1 solution of methanol/acetic acid (Stich & Rosin, 1983b), but other fixing agents can also be used: 80%-85% ethanol (Stich et al., 1992), 3:1 ethanol/acetic acid (Burgaz et al., 1999) or 80% methanol (Lucero et al., 2000; Cavallo et al., 2005), which may or may not depend on the staining or methodology used.

The staining method most commonly used to show up the nuclear DNA and the micronuclei, in preparation for analysis under a conventional optical microscope, as proposed by Feulgen & Rossenbeck, back in 1924. Their preparations were counterstained using 1% fast green, but staining using Giemsa (Bloching et al., 2000) and aceto-orcein (Revazova, 2001) have also been used, even though non-DNA-specific staining agents lead to overestimates (Neresyan et al., 2006). Acridine orange or DAPI (4’, 6’-diamidino-2-phenylindole) is generally used for observations with fluorescence microscopy (Lucero et
According to Lucero et al. (2000), in preparations destined for observation using fluorescence microscopy, DAPI analysis (which is DNA-specific, thereby avoiding errors introduced through counting artifacts) is advantageous in relation to stains that are not DNA-specific. When the analysis is done using confocal microscopy (Cerqueira et al., 2004) or phase-contrast microscopy, only the nucleus should be stained (Tolbert et al., 1992).

The FISH technique (fluorescent in situ hybridization) for analyzing micronuclei is applied using a centromeric probe and propidium iodide for counterstaining (Moore et al., 1993; Titenko-Holland et al., 1994; Surrallés et al., 1997). This method was considered by Titenko-Holland et al. (1994) to be more effective than the Feulgen stain for detecting degenerative nuclear abnormalities, and makes it possible to precisely identify whether the micronucleus originated in an aneugenic event, or whether it resulted from chromosome breakage. Aneugenic or clastogenic origins for micronuclei can also be identified through using antibodies that recognized specific proteins of centromeres, but use of these antibodies on exfoliated cells runs into technical difficulties (Moore et al., 1993), or difficulties based on the size of the micronuclei (Sarto et al., 1987). Figure 2 illustrates the collection, processing and staining method used in our laboratory.

Figure 2: Methodology of the micronucleus test on exfoliated cells from oral epithelium
In 1985, Stich et al. (1985) proposed that cells presenting other nuclear alterations (karyorrhexis, multinucleated cells and cells presenting very large micronuclei) should be included in the count, based on their opinion that there could be some difficulty in correctly identifying micronucleated cells. Their proposal was also based on their view that the action of different carcinogens could preferentially induce different types of nuclear aberrations.

Two years later, Sarto et al. (1987) proposed a new analysis protocol that went against their initial proposal. They now proposed that the micronucleus count should only include cells with complete cytoplasm, with or without micronuclei, and that cells presenting degenerative nuclear phenomena such as karyorrhexis, karyolysis and pyknosis should be excluded from the analysis, along with cells presenting nuclear projections (nuclear buds and broken eggs). According to these authors, the nuclear alterations induced by these phenomena could be confounded with micronuclei (which we do not agree with, given that they are quite distinct). They also suggested that binucleated cells should also be excluded.

In 1991 and 1992, Tolbert et al. (1991, 1992) published two studies that suggested new criteria for cell counts in the Micronucleus Test. According to these authors, degenerative nuclear phenomena should be counted separately at the time of the micronucleus analysis, because greater occurrence of these phenomena, in itself, is indicative of apoptosis (karyorrhexis, condensed chromatin and pyknosis) and necrosis (karyolysis, karyorrhexis, condensed chromatin and pyknosis), which reveal the genotoxic and cytotoxic effects respectively of a given exposure.

Apoptosis is stimulated by exposure to mutagenic agents and acts as a protective mechanism against cancer by eliminating genetically damaged cells. High levels of occurrence of apoptosis may constitute evidence of genotoxic damage, and this would be related to the initiation of the process of malignant transformation (Tolbert et al., 1992).

Pyknosis, condensed chromatin and karyorrhexis accompany keratinization, which takes place as an adaptive response to cell damage in epithelium that usually is not keratinized (Pindborg et al., 1980). Karyolysis can occur in addition to these alterations, in cells undergoing necrosis, i.e. cell death, consequent to the action of exogenous agents on the cell environment (Wyllie, 1981; Galluzzi et al., 2012). Occurrences of keratinization and necrosis are indicative of cytotoxicity, and these may be associated with cancer promotion via stimulation of cell proliferation (Tolbert et al., 1992).

The criteria for micronucleus recognition are similar to what was described by Sarto et al. (1987). Micronuclei are distinctively individualized structures within the cytoplasm of interphasic cells measuring between one-fifth and one-third of the size of the main nucleus, observed in the same plane as the nucleus and presenting similar staining and chromatin distribution (Figure 4b). However, it has been suggested that a distinction should be made between micronuclei of high certainty (structures that meet all the identification criteria) and micronuclei of low certainty that are thus classified because the structure suggestive of a micronucleus does not fulfill all the requisites for inclusion (Figure 3). Nuclear projections that are described as broken eggs should also be included in the analysis. The cell count criteria proposed by these authors were ratified more recently by Thomas et al. (2009) in a paper in which they presented a detailed protocol for a test that they named the buccal micronucleus cytome test (BMCyt). In this protocol, the authors recommended that in addition, obviously, to making counts of cells without alterations, they should be made on the following: 1) cells with micronuclei and nuclear buds, to assess the DNA damage; 2) cells presenting karyorrhexis, karyolysis, pyknosis and condensed chromatin, to make inferences about occurrences of cell death; 3) basal cells, to assess the proliferative potential of the mucosa; and 4) binucleated cells, with information on defects in cytokinesis. The photomicrographs in...
Figure 4 illustrate cell without alteration (a), cell presenting micronuclei (b) and cells with degenerative nuclear phenomena (ctof).

Figure 3: Photomicrography of cell presenting micronuclei of low certainty

Figure 4: Photomicrographs of normal cell (a), cell presenting micronuclei (b) and cells with karyorrhexis (c), condensed chromatin (d), pyknosis (e) and karyolysis (f).

Attention needs to be drawn to the ambiguity relating to nuclear projections, with regard both to their significance in different cell types and to the nomenclature used to describe them, as also highlighted by Nersesyan (2005).

Nuclear projection structures known as broken eggs and buds have been analyzed in some studies (Tolbert et al., 1991; 1992; Torres-Bugárin et al., 1998; Meireles, 2003; Santos, 2003; Cerqueira et al., 2004), but the real significance of their occurrence, along with the mechanisms that originate them, are little known. In some studies, their occurrence has been counted, but there has been little discussion of their importance or significance consequent to exposure to genotoxic substances.
Broken eggs were described for the first time by Tolbert et al. (1991) and the first reference to buds appeared in the literature in 1998, in a report by Bhattathiri (1998) on alterations induced by radiation in oral epithelial cells.

With regard to the divergences relating to the use of the terms bud and broken eggs, Nersesyan (2005) analyzed studies in which these structures were assessed in lymphocytes and drew attention to the following: 1) both structures are presented in a photo published by Fenech & Crott (2002) under the same definition of “nuclear buds”; 2) Serrano-Garcia & Montero-Montoya (2001) described broken eggs as “buds attached to the nucleus by a stalk”, and nuclear buds as “stalk-less buds”; and 3) de Almeida et al. (2004) presented a photo of a “stalk-less bud” that they named a “broken egg”.

The term “nuclear blebbings” was used by Özkul et al. (1997) in reporting the exclusion of “structures similar to micronuclei, connected to the main nucleus by a bridge” from the micronucleus count, in exfoliated cells from the oral epithelium of users of Maras Powder.

From our experience with epithelial cells of the oral mucosa, we consider that the morphological distinction between nuclear buds and broken eggs is very clear (Figure 5). Both of them presented continuity with the nucleus, but we take the term “bud” to indicate nuclear projections that result from strangulation in a small and limited area of the nuclear surface from which a rounded protuberance (a bud) comes out, whereas a “broken egg” comprises a structure smaller than a nucleus that is connected to it by a fine Feulgen-positive filament. In broken eggs, attention is drawn to the fact that both in these and in the nucleus, there is a chromatin concentration close to the thread which under optical microscopy is sometimes difficult to see. In such cases, analysis using confocal microscopy always reveals the presence of the filament. Moreover, the nucleus and micronucleus are rounded, whereas in cells presenting broken eggs, it is sometimes seen that both in this and in the main nucleus, the circular outline is lost and an area of straight surface remains. Cells with these characteristics were presented by Ünal et al. (2005) as being cells with a micronucleus. A distinction with what Tolbert et al. (1991) named “micronucleus of low certainty” also needs to be established. In this, as shown well in Figure 4, the extranuclear structure has a well-defined outline and does not present any continuity solution with the nucleus (which is observed in the buds) but, rather, juxtaposition.

![Figure 5: Photomicrographs of cells presenting broken eggs (a) and a nuclear bud (b)](image-url)

It has been put forward that nuclear buds in lymphocytes may be the expression of the gene amplification process (Fenech & Crott, 2002; Fenech, 2002), and also that a micronucleus formation mecha-
nism that starts with formation of a bud in the S period of the interphase might mediate removal of double minutes: acentric autonomous chromatin replication structures that express the amplification of oncogenes in malignant tumors (Shimizu et al., 1998).

On the other hand, some studies in which occurrences of buds and broken eggs in lymphocytes were evaluated have correlated these structures as indicators of genotoxicity (Serrano-García & Montero-Montoya, 2001; Zeljejic & Garaj-Vrovak, 2004).

Although there is still no solidly substantiated evidence in the literature that would reveal the nature and significance of nuclear projections in lymphocytes, the results from some studies suggest that the yoriginate both in epigenetic events (Shimizu et al., 1998; Fenech & Crott, 2002; Fenech, 2002), and as a consequence of genotoxicity (Serrano-García & Montero-Montoya, 2001; Zeljejic & Garaj-Vrovak, 2004).

With regard to the real significance of nuclear buds and broken eggs in epithelial cells, the results from studies developed in the Toxicological Genetics Laboratory of Feira de Santana State University (UEFS), Brazil, strongly suggest that there is an association with the natural differentiation process of the oral epithelium. Cerqueira et al. (2004) observed that there was a significantly higher frequency of these structures before exposure to X-ray radiation than afterwards. In an analysis on oral mucosal cells from individuals exposed to chemical mutagens in the workplace, Meireles (2003) reported that there was a significantly higher frequency of broken eggs in the control group. Santos (2003) evaluated the genotoxic effects of exposure to pesticides and also observed that there was a significantly greater number of broken eggs in the control group than in the exposed group.

Concordant with these results, Torres-Bugarin et al. (1998) reported that the frequency of broken eggs was significantly higher in the negative controls than in individuals undergoing antineoplastic chemotherapy.

In a study conducted to assess the genotoxic effects of the habits of smoking and consuming alcoholic drinks, Bohrer et al. (2005) also described significantly higher frequencies of broken eggs among control individuals (abstemious in relation to alcohol and nonsmokers) than among either smokers or individuals who were both smokers and drinkers.

In some studies in which exfoliated cells from the oral epithelium were analyzed, no difference in the frequency of broken eggs was observed between exposed and control individuals. In evaluations on the effects of exposure to pesticides and cytostatic drugs, Gomez-Arroyo et al. (2000) and Torres-Bugarin et al. (1998; 2003) described similar frequencies of these structures between exposed and control individuals. However, in all these studies, significantly higher frequencies of micronuclei were detected in the exposed individuals. Results similar to these were obtained in a study developed in our laboratory (Cerqueira et al., 2008), in which the effects of the X-ray radiation used in producing panoramic radiographs were evaluated in gingival cells. If the frequency of broken eggs were indeed indicative of genotoxicity, it would be expected that in response to this exposure, higher frequencies of both micronuclei and broken eggs would be expected.

However, going against this logic, Montero et al. (2003) described a significantly higher frequency of “nuclear buds”, but not of micronuclei, in individuals in the central area of Mexico, a region that is recognized to be highly polluted.

Increased occurrence of broken eggs consequent to exposure to genotoxic substances was described by Montero et al. (2003) and Revazova et al. (2001), but these researchers merely presented data, without discussing the mechanisms that might lead to increased numbers of these structures consequent to the exposure.
Nersesyan et al. (2002a, b) evaluated occurrences of micronuclei and broken eggs in exfoliated cells from the uterine cervix of pre and postmenopausal women, in comparison with women who presented regular menstrual cycles. Significantly higher frequencies of broken eggs, but not of micronuclei, were observed among the pre and postmenopausal women. According to these authors, this suggested that occurrences of broken eggs were associated with the degenerative process in the cervical cells consequent to hormonal changes.

The hypothesis that broken eggs could be associated with degenerative processes receives backing, according to Nersesyan (2005), from a study conducted by Bindu et al. (2003). The latter study revealed that the frequency of micronuclei in cells of the oral epithelium in individuals subjected to radiation increased linearly with the dose (total dose of 38.5 Gy), but increased frequency of broken eggs was only observed close to the end of the radiotherapy treatment, which can be explained by degenerative changes in the tumor cells.

However, in a similar study, although Nersesyan et al. (1995) reported higher frequencies of micronuclei in oral epithelial cells from individuals who had been subjected to a radiation dose of 42 Gy, they did not observe any increase in the frequency of broken eggs. It has been proposed (Ramírez & Saldanha, 2002) that broken egg formation is an event that precedes micronucleus formation, but in our view there is a lack of experimental evidence to prove this supposition.

In summary, in the light of the contradictory results obtained from studies in which buds and broken eggs were analyzed in epithelial cells, we can conclude that the real significance of these abnormalities needs further investigation in order to establish this. The results from our laboratory and the data obtained by Bohrer et al. (2005) are concordant with the conclusion that Nersesyan (2005) reached after analyzing the literature, and strongly suggest that occurrences of these structures are not associated with genotoxicity.

### 2.2.5 Scoring Methods

With regard to the number of cells that should be counted, Albertini (2000) took the view that given the low frequencies of micronuclei in exfoliated epithelial cells, it was recommendable to count 3,000 to 5,000 cells per individual. Nonetheless, there is great variation in the numbers of cells considered in different studies. In the initial studies conducted, numbers of cells that today would be considered insufficient were counted, ranging from 300 to 500 cells (Stich & Rosin, 1983b; Stich et al., 1992). At the other extreme of this scale, Belién et al. (1995) took into account the low frequency of micronuclei and the intra and inter-individual variability that were detected in the results obtained from analyzing 1,000 cells from a single preparation of around 10,000 cells, and took the view that the analysis should include this total. Thus, in their view, this would require automation of the method. Since no automation methods have been established, most authors analyzed 2,000 to 3,000 or more cells (Sarto et al., 1987; Lucero et al., 2000; Pinto, 2000; Pastor et al., 2001; Santos, 2003; Cavallo et al., 2005), while others counted exactly 1,000 (Machado-Santelli et al., 1994; Burgaz et al., 1999; Bloching et al., 2000) or 1,500 cells (Surrallès et al., 1997) and yet others considered that 1,000 would be the minimum number acceptable but analyzed the maximum number of cells contained in the preparation (Meireles, 2003; Cerqueira et al., 2004). Thomas et al. (2009) suggested that initially, a minimum of 1,000 cells should be counted in order to determine the frequencies of the various cells, and that 2,000 differentiated cells should be counted in order to calculate the frequencies of the micronuclei and nuclear projections.

Microscopic analysis should be performed by a trained person, and preferably one who has not been involved in sample collection and slide preparation. In addition, the slides should be coded and ana-
alyzed in blind tests in relation to any information that might identify the subject of the sample (Thomas et al., 2009). These authors’ proposed protocol includes identification criteria for the different cell types, although broken eggs are described under the name of nuclear bud.

2.2.6 Statistical Analysis

The results obtained using the Micronucleus Test on exfoliated cells from oral epithelium should always be subjected to adequate statistical analysis, using parametric univariate analysis (Student’s t-test, ANOVA, Ancova or Pearson’s correlation) or nonparametric univariate analysis: Kruskal-Wallis test, Mann-Whitney U-test, Spearman’s correlation, chi-square or Wilcoxon (Thomas et al., 2009, Ceppi et al., 2010). Multivariate linear regression analyses may be necessary in order to determine the influence of confounding factors.

In our studies, we have often used a conditional test to compare proportions in situations of rare events (Bragança-Pereira, 1991). These are significance tests that form an alternative to the chi-square test along the lines of Fisher’s exact test and are appropriate for evaluating cytogenetic events when a large sample of cells is necessary for detecting occurrences of a specific chromosomal aberration.

2.2.7 Advantages and Limitations of the Test

The Micronucleus Test on exfoliated cells is considered to be a valuable tool for identifying genetic damage consequent to exposure to mutagens because of the various advantages that it presents. These include:

- It detects the action of both clastogenic and aneugenic agents, since micronuclei result from chromosome breakage or failure to join together during fusion;
- It methodology does away with culturing procedures, which makes it less expensive than the classical chromosome analysis procedures. It can thus be applied in biomonitoring programs that include large populations;
- Genetic damage expressed as micronuclei is easy to detect and can be observed directly in cells that form the target of the mutagenic and/or carcinogenic agent studied;
- Study material from some epithelial tissues (such as from the oral, cervical and nasal epithelia) is obtained through simple noninvasive procedures;
- When the test is applied to tissues that are chronically and repeatedly exposed to mutagens, the frequency of micronuclei reflects a set of different chromosomal alterations that occur in cells of the basal layer of the epithelium.

The intrinsic disadvantage of the test is that chromosome exchanges and other rearrangements are not detected, while there is also the possibility that some fragments will be included in the main nucleus, thus becoming imperceptible, which would lead the test to underestimate the real frequency with which chromosome damage occurs.
3 The Micronucleus as a Biomarker for Identifying Chromosome Damage in Individuals at Higher Risk of Developing Oral Squamous Cell Carcinoma: Tobacco and Alcoholic Beverage Users

The habit of smoking is the risk factor most consistently associated with development of premalignant and malignant lesions of the oral epithelium, particularly when done concomitantly with consumption of alcoholic beverages (Llewelyn et al., 2003; International Agency for Research on Cancer, 2004; Warnakulasuriya et al., 2005).

In the tobacco of manufactured cigarettes and in the smoke released from them, more than 4,000 substances can be identified (Husgafvel-Pursiainen, 2004). Among these, 200 are toxic to humans and more than 50 present known carcinogenic action. Prominent among the latter are polycyclic hydrocarbons and specific nitrosamines of tobacco that are found in tar (International Agency for Research on Cancer, 2004).

Certain enzymes may metabolize the hydrocarbons of tobacco and transform them into carcinogens that are more powerful, such as aryl hydrocarbon hydroxylase (AHH), which is effective in increasing the carcinogenic potential of benzopyrene. The risk of tumor development is thus greater among smokers who produce these enzymes at higher concentrations. In addition to the action of tobacco carcinogens, the heat released by tobacco combustion worsens the aggressive action on the mucosa of the oral cavity (DeMarini, 2004).

Forms of tobacco consumption other than manufactured cigarettes have also been correlated with cancer development. Pipe and cigar smoking multiply the risk of cancer of the lips, mouth, tongue and pharynx, depending on the quantity of consumption (Silverman & Shillitoe, 1990).

The habit of tobacco-chewing, in different preparations (“areca-nut”, “bidi” and “betel quid”), is considered to be a risk factor for development both of pre-malignant and malignant lesions of the oral epithelium. The use of snuff, especially when chewed, has also been correlated with development of oral squamous cell carcinoma (Lee et al., 2003; Suhas et al., 2004).

Evaluations on the chromosomal damage consequent to smoking, through micronucleus analysis on exfoliated cells from oral epithelium, have produced divergent results in the literature. Nonetheless, a significant number of studies have indicated that cigarette compounds are effective in inducing micronuclei.

Sarto et al. (1987) analyzed the aneugenic and clastogenic effects induced by tobacco on 25 individuals with such exposure (23 who smoked cigarettes and two who smoked cigars), and compared them with the same number of nonsmokers. They observed a significantly higher frequency of micronuclei consequent to chromosome breakage among the smokers.

On the other hand, using the same methodology, Stich & Rosin (1983b) did not detect any significant differences in the frequencies of micronuclei between 36 smokers (who were not alcoholic beverage consumers) and 15 nonsmokers (who were also non-drinkers).

In a study conducted in Orissa (India), Stich et al. (1992) analyzed occurrences of micronuclei among fishermen who habitually smoked with the lit end of the cigarette inside the mouth. Under such conditions, and given the way in which the smoke is expelled, the tongue and palate are more exposed than the cheeks. The mean frequencies of micronuclei observed in the tongue and palate were similar, but were significantly higher than those described for the cheeks.
Significantly higher frequencies of micronuclei in oral mucosal cells among smokers of manufactured cigarettes than among nonsmokers were described by Özkul et al. (1997) in a study that included 14 male smokers and 15 male nonsmokers.

Greater occurrence of chromosomal damage, as expressed by micronuclei, in cells of the oral mucosa, was also described by Konopacka (2003) in a study that included 50 smokers and 70 nonsmokers. The mean frequencies of micronuclei described per thousand cells were approximately three times greater among the smokers. Similar results were described by Martins & Boschini Filho (2003).

In a study that evaluated the effects of the quantity of cigarettes consumed per day with regard to induction of micronuclei, Wu et al. (2004) described higher frequencies of micronuclei only among individuals who smoked more than 20 cigarettes per day, in comparison with nonsmokers.

Bloching et al. (2000) observed significantly higher frequencies of micronuclei in cells of the oral epithelium of smokers, in comparison with a group of nonsmokers. Dose-response effects were also described. Evaluations in other studies, on occurrences of micronuclei consequent to exposure to tobacco consumed in forms other than manufactured cigarettes, have produced results that are even more consistent than those described for cigarette consumption.

In an investigation on the effects of using snuff, Tolbert et al. (1991) evaluated the frequency of micronuclei in exfoliated cells from the oral mucosa, among 38 women who used snuff orally and 15 women who did not use tobacco. The mean numbers of micronuclei of high and low certainty among the users was twice as high as what was observed in the control group. However, the frequencies of micronuclei did not differ significantly when only the micronuclei of high certainty were considered.

Among Indians, “khaini” tobacco is commonly used. This is placed in the lower gingival groove and is left there for long period of time. In an evaluation on occurrences of micronuclei in gingival and cheek cells from users of this tobacco, Stich et al. (1992) registered significantly greater frequency of these structures in exfoliated cells of the gingiva. Similar results had previously been described by Stich et al. (1982) among users in the city of Bihar (India). The effects from using toothpaste containing “gudakhu” tobacco with regard to induction of micronuclei were also evaluated by Stich et al. (1992). No differences were detected between users and controls.

Dave et al. (1992) evaluated the cytogenetic effects induced by consumption of “areca nut” among individuals without oral lesions and users presenting fibrosis of the oral submucosa or oral cancer. Comparisons with the control group (non-users presenting normal oral mucosa) showed that there were significantly higher frequencies of sister chromatid exchanges and chromosomal aberrations in peripheral lymphocytes in the three groups of users. A similar result was reported by these authors when they evaluated occurrences of micronuclei in exfoliated cells from the oral mucosa, in the same individuals.

In a study conducted in Turkey that included 25 men who were making oral use of a powder obtained from dehydrated tobacco originating from leaves of *Nicotiana rustica* ("Maras powder"), Özkul et al. (1997) reported that there was a significantly higher frequency of micronuclei in the oral mucosal cells of users than in the control group.

The effects from consuming tobacco in the form of “bidi” (tobacco rolled in leaves of *Diospyros melanoxylon* and tied together with cotton thread) were assessed by Suhas et al. (2004) among 25 individuals who were not consumers of alcoholic beverages and who solely consumed bidi, and compared them with 25 individuals without any history of exposure to any known genotoxic substance. The numbers of micronuclei were counted in cells of the oral mucosa, palate and tongue. Greater frequencies of these structures in the exposed group were recorded in cells from the oral mucosa and palate.
Genotoxic effects, expressed as greater occurrence of micronuclei in oral mucosal cells consequent to consumption of pan masala/gutkha, were described by Fareed et al. (2011). Similar results were obtained by El-Setouhy et al. (2008), among individuals who used a hookah for tobacco consumption.

However, Wu et al. (2004) did not detect higher frequencies of micronuclei in the oral mucosa of areca quid chewers. These authors also assessed the effects of this exposure concomitantly with consumption of manufactured cigarettes and did not observe any interaction. Thus, they suggested that the association described in the literature between oral carcinogenesis and use of areca quid might occur through routes other than genotoxicity.

An association between the habit of consuming alcoholic beverages and occurrences of oral cancer has been registered in the literature (Blot et al., 1988; International Agency for Research on Cancer, 1988; Silverman & Shillitoe, 1990; Longnecker, 1995; Seitz et al., 1998; Hindle et al., 2000; Salaspuro, 2003). It has been suggested that alcohol probably acts as a cofactor, thereby increasing the carcinogenic potential of tobacco components for inducing cancer (Rothman & Keller, 1972; La Vecchia et al., 1997; Du, 2000).

La Vecchia et al. (1997) suggested that alcohol may act as a solvent, thus facilitating the passage of carcinogens, especially those present in tobacco, through the cell membrane. Moreover, according to these authors, alcohol might present an association with carcinogenesis through increased metabolic activity of the liver, which could activate carcinogens, or through alterations induced directly in the metabolism of the target epithelial tissue.

With the aim of assessing the mutagenic effects of alcohol consumption, Reis et al. (2002) analyzed occurrences of micronuclei in exfoliated cells from the tongue and cheek mucosa of 40 nonsmokers who were chemically dependent on ethanol and 20 individuals who were abstemious in relation to alcohol and were nonsmokers. The number of micronuclei observed in the exfoliated cells from the tongue was significantly greater among the users of alcoholic beverages, but no significant difference was detected between the two groups in relation to cells from the cheek mucosa ($p > 0.05$).

Several studies evaluating the induction of chromosomal damage in lymphocytes or in exfoliated cells from the oral mucosa consequent to the habits of smoking and consuming alcoholic beverages have revealed that the alcohol present in these drinks does not, on its own, induce greater occurrence of this damage. However, in combination with the habit of smoking, additive and/or synergistic effects have been described (Stich & Rosin, 1983b; Xue et al., 1992; Castelli et al., 1999; Bloching et al., 2000).

In exfoliated cells from the oral mucosa, Stich & Rosin (1983b) evaluated the effects of these habits on the induction of micronuclei in four groups of individuals: Group I: nonsmokers and nonusers of alcoholic beverages; Group II: smokers and non-drinkers; Group III: nonsmokers but drinkers; and Group IV: smokers and drinkers. They did not detect any significant differences in occurrences of micronuclei between the individuals in groups I, II and III, but the frequency of micronuclei in the individuals in Group IV was significantly greater than what was observed in the other three groups. Thus, their study revealed synergistic effects from the habits of smoking and alcohol consumption, on micronucleus induction.

The synergistic effect from the habits of chewing Catha edulis leaves, smoking manufactured cigarettes and consuming alcoholic beverages, on micronucleus induction in oral epithelial cells, was investigated by Kassie et al. (2001). The sample analyzed was divided into three groups: Group I was formed by 25 individuals who chewed Catha edulis and were smokers and drinkers; Group II was formed by an equal number of individuals who smoked and drank; and Group III was formed by 25 individuals who did not chew these leaves and neither smoked nor drank. These authors observed that: 1) the number of
micronuclei among the individuals in Group I was twice what was observed among the individuals in Group II; 2) the incidence of micronuclei among the individuals in Group I was nine times greater than among the individuals in Group III; and 3) the incidence of micronuclei among the individuals in Group II was significantly greater than what was observed among the participants in Group III, thus revealing the additive effect of the habits of smoking and consuming alcoholic beverages on inducing chromosome damage.

In three studies conducted in our laboratory in which the effects of the habits of smoking and drinking were assessed, two of them did not reveal any significant differences between smokers and non-smokers (Santos, 2003; Freita et al., 2005) and in the other (Meireles, 2003), the number of micronuclei was significantly greater among the smokers ($p < 0.01$). The results described by Freita et al. (2005) and Santos (2003) are concordant with those obtained by Bohrer et al. (2005), who also did not observe any significant differences in micronucleus occurrence in a study in which epithelial cells from the oral mucosa of 21 individuals who were nonsmokers and abstemious regarding alcohol, 28 smokers and 19 cigarette and alcoholic beverage consumers.

The study by Freita et al. (2005) included 20 individuals (6 men and 14 women), among whom eight smoked and drank; eight only smoked and four only drank. The control group was formed of equal numbers of men and women who were nonsmokers, abstemious regarding alcohol and without any history of exposure to other genotoxic agents. No associations were found, and according to these authors, this may have been due to the low exposure of the individuals analyzed: the alcoholic beverage consumption consisted predominantly of beer (83%), at the frequency of once a week (67%), and only one of the smokers declared a consumption rate greater than 20 cigarettes/day, while the majority (75%) consumed fewer than 10 cigarettes/day.

The sample analyzed by Santos (2003) was formed by 60 individuals, of whom 14 were smokers and made use of alcoholic beverages, two only smoked, 28 were nonsmokers but made use of alcoholic beverages and 16 neither smoked nor drank. Occurrences of micronuclei were not shown to be associated with either of these two habits, either singly or together, and the author concluded that the lack of association with the smoking habit could be attributed to low exposure, given that 70% of the smokers declared that their consumption was less than or equal to 10 cigarettes/day. In relation to the habit of consuming alcoholic beverages, the majority (64%) of the users declared that their consumption was greater than five glasses on one or two days per week. The lack of association with higher levels of micronuclei corroborated results from other authors in which alcohol alone did not induce genotoxicity (Stich & Rosin, 1983b; Bloching et al., 2000).

Despite the divergent results obtained from studies in which occurrences of micronuclei in relation to the habits of smoking and drinking were investigated, the accumulated evidence indicates that the risk of this exposure is sufficient to discourage these habits and stimulate quitting them.

4 Use of the Micronucleus Test for Detecting Apoptosis in Individuals at Higher Risk of Developing Oral Squamous Cell Carcinoma: Tobacco and Alcoholic Beverage Users

Apoptosis is a genetically controlled process of cell death that occurs under normal conditions to eliminate cells that are no longer necessary to the organism or, as noted earlier, in response to genotoxic injury. Comprehension of the molecular events involved in the apoptotic process has advanced considerably
over recent years, and the nucleus is considered to be an important remodeling target during this process (Schulte-Hermann, et al., 2000; Miller et al., 2002).

Starting from the proposal put forward by Tolbert et al. (1991; 1992), for a protocol for analyzing micronuclei in exfoliated cells from the oral epithelium, in which not only micronuclei were counted but also other nuclear alterations indicative of apoptosis (karyorrhexis, pyknosis and condensed chromatin) and necrosis (karyorrhexis, pyknosis, condensed chromatin and karyolysis) would be included, several other studies using this protocol have been conducted (Ramirez & Saldanha, 2002; Santos, 2003; Çelik et al., 2003; Cerqueira et al., 2004; Freita, et al. 2005; Meireles et al., 2006).

In the studies that Tolbert et al. conducted in 1991 and 1992, to assess occurrences of micronuclei among snuff users, analyses on occurrences of pyknosis, karyorrhexis, condensed chromatin and karyolysis showed that these alterations were significantly more common among users, thus providing a good indication that this exposure produced genotoxic effects. Similar results were obtained by Freita et al. (2005) and Santos (2003), among individuals who were making use of cigarettes and/or alcoholic beverages. It should be emphasized that the individuals who formed the exposed group that these authors analyzed were considered to be moderate users. This highlights the sensitivity of this test for detected the effects of low exposure, when it is added to counting nuclear alterations other than micronuclei.

Induction of apoptosis in exfoliated cells from the oral mucosa of petrol/gasoline pump attendants in response to benzene exposure, and its interaction with the smoking habit, was also evaluated by Çelik, et al. (2003). The frequency of occurrence of karyorrhexis and karyolysis was significantly greater in the exposed group, and was also significantly greater among the smokers in this group and in the control group, in comparison with the nonsmokers in the respective groups. However, no interaction between benzene exposure and the smoking habit was observed.

In the study by Torres-Bugarín et al. (1998), in which occurrences of micronuclei in fire-eaters exposed to diesel were evaluated, several other nuclear alterations were counted, including those that Tolbert et al.(1991; 1992) considered to be indicative of apoptosis and necrosis. Like in other studies, the frequency of micronuclei was not significantly higher in the exposed group (n = 8) than among a group of 13 healthy individuals without any history of exposure to genotoxic agents, but the total count of other nuclear anomalies was significantly higher among the exposed individuals. Evaluation of the effects of the smoking habit between the exposed smokers (n = 5) and the exposed nonsmokers (n = 3) revealed that the smokers presented higher frequency of karyorrhexis. Comparison between the exposed smokers and a group of five smokers who had not been exposed to any other genotoxic agents showed that the exposed group presented a significantly greater number of cells with pyknosis, condensed chromatin and karyorrhexis, thus suggesting that there was some interaction between the smoking habit and exposure to diesel. Notwithstanding the small sample size evaluated by these authors, the results obtained indicated that the test had greater sensitivity for detecting genotoxicity when not only micronuclei but also other nuclear alterations were counted.

One important point to be emphasized in assessing occurrences of apoptosis in preparations for studying micronuclei is the need for correct interpretation of the nuclear alterations relating to apoptosis. Ünal et al. (2005), citing Çelik et al. (2003) and Tolbert et al. (1992), reported that karyolysis and karyorrhexis were evidence of apoptosis. In both studies by Tolbert et al. (1991; 1992) the affirmation that karyolysis is related to necrosis and not to apoptosis is very clear. In the study by Çelik et al. (2003), karyorrhexis and karyolysis were counted separately, but these authors reported that these alterations were considered to be indicative of apoptosis. Furthermore, although Ünal et al. (2005) correctly described the
morbidity of cells presenting karyorrhexis and karyolysis, the photographs corresponding to these were not representative of these events: two cells with karyorrhexis were indicated as presenting karyolysis.

5 The Micronucleus Test for Evaluating Malignant Transformation of Preneoplastic Lesions and for Biomonitoring on Individuals with Oral Squamous Cell Carcinoma

Cancer affecting the epithelium of the oral cavity is preceded by lesions that can be clinically detected, and among these, leukoplakia is the most frequently occurring type (Carnesolatas-Lázaro et al., 2007; Kamboj & Mahajan, 2007). Erythroplakia, leukoerythroplakia and lichenoid dysplasia are other types of lesion that have been indicated to have the potential to evolve to malignant transformation (Rodrigues et al., 2000; Kuffer & Lombardi, 2002; van der Meij et al., 2006). In addition to these lesions, oral lichen planus has been the focus of studies aimed towards evaluating its potential for malignant transformation, but the results from these studies have been a source of controversy (van der Meij et al., 2003; Lodi et al., 2005; González-Moles et al., 2006; van der Meij et al., 2006).

Use of biomarkers to indicate the potential of precursor lesions to evolve to the process of malignant transformation is a preventive measure that guides therapeutic management. Within this context, several authors have considered the Micronucleus Test to be a valuable tool for biological monitoring of premalignant lesions of the oral cavity (Bloching et al., 2000; Casartelli et al., 2000; Halder et al., 2004; Saran et al., 2008).

With the aim of evaluating whether the Micronucleus Test on exfoliated cells could be used as a biomarker indicative of greater risk of development of cancer of the upper digestive tract, Bloching et al. (2000) analyzed cells from regions of the oral mucosa without abnormalities, in 55 individuals with carcinomas of the upper digestive tract and 16 individuals with leukoplakia, and compared the results obtained with those of 99 individuals without lesions. The frequency of micronucleus occurrence was twice as high among the individuals with lesions. These authors took the view that the results obtained indicated that micronuclei used as a biomarker were effective in assessing the risk of cancer development and could thus be of assistance in establishing preventive measures aimed towards reducing the risk of developing this disease, although this biomarker was unable to provide information regarding when or if the malignant transformation would occur.

In the study by Casartelli et al. (2000), the frequencies of micronucleus occurrences of micronuclei in exfoliated cells from normal oral mucosa and from areas of leukoplakia and in situ carcinoma were registered. The frequencies of micronuclei were significantly greater in the premalignant and malignant lesions, than in cells from the normal mucosa. These authors concluded that micronuclei could be used as a biomarker for neoplastic progression in the oral epithelium.

Halder et al. (2004) reached the same conclusion after evaluating occurrences of micronuclei among 50 individuals without oral lesions, 32 with premalignant lesions, 10 with a diagnosis of oral cancer for which treatment had not yet begun and eight with the same diagnosis who had already undergone surgery to treat the lesion. The mean frequencies of micronuclei recorded for these groups were 0.35%, 0.63%, 1.36% and 0.44%, respectively. According to these authors, their results evidently corroborated those described by Casartelli et al. (2000) and suggested that micronuclei might be a marker for oral carcinogenesis, although further studies would be necessary.
Saran et al. (2007) evaluated occurrences of micronuclei in oral epithelial cells from 24 individuals with oral cancer, 29 with premalignant lesions and 60 without abnormalities of the mucosa. The results obtained revealed that there was a gradient of occurrences of these structures (from normal epithelium to cancer), thus indicating that the test was effective.

Ramirez and Saldanha (2002) compared micronucleus frequencies between individuals with oral and oropharyngeal carcinomas and individuals presenting oral mucosa without abnormalities. Exfoliated cells from three different regions of the oral cavity were analyzed: the region surrounding the lesion (B), the region contralateral to the lesion (A) and the region of the base of the upper gingival-labial sulcus. The results relating to comparisons within individual patients showed significant differences: micronuclei occurred most frequently in region B, followed by regions A and C. These authors emphasized the possibility that there might be a gradient of carcinogenic development (C → A → B). Comparison between individuals (patients and controls) indicated that there was a significantly higher frequency of micronucleus occurrence in the cells in the region of the lesion, in relation to the frequency of these structures in the individuals of the control group.

In a study developed in our laboratory (Dórea et al., 2012), which evaluated occurrences of micronuclei in exfoliated cells from the oral epithelium of 20 individuals with oral squamous cell carcinoma and forty individuals with normal oral mucosa, micronuclei were found significantly more frequently in cells collected from lesions than in cells from normal areas, independent of the presence/absence of cancer. Given that loss of cell capacity to evolve to death consequent to occurrence of genetic damage (apoptosis) is one of the most striking characteristics of the process of malignant transformation, these authors also evaluated occurrences of degenerative nuclear alterations that indicate this process. The results obtained showed that in cells obtained from the lesions and from areas of normal mucosa in individuals with cancer, the frequency of apoptosis was significantly lower than what was seen in individuals without oral squamous cell carcinoma. This suggested that impairment of the apoptosis process affects the epithelium in a generalized manner and forms an event that precedes and favors development of malignant transformation.

Table 1 summarizes results obtained in studies using the Micronucleus Test for evaluating malignant transformation of preneoplastic lesions and for biomonitoring on individuals with oral squamous cell carcinoma.

<table>
<thead>
<tr>
<th>Author</th>
<th>Kind of lesion</th>
<th>Endpoints analyzed</th>
</tr>
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<tbody>
<tr>
<td>Bloching et al., 2000</td>
<td>Oral cancer and leukoplakia</td>
<td>Micronucleus</td>
</tr>
<tr>
<td>Casartelli et al., 2000</td>
<td><em>In situ</em> carcinoma and leukoplakia</td>
<td>Micronucleus</td>
</tr>
<tr>
<td>Ramirez &amp; Saldanha, 2002</td>
<td>Oral and oropharyngeal carcinomas</td>
<td>Micronucleus</td>
</tr>
<tr>
<td>Halder et al., 2004</td>
<td>Oral cancer and premalignant lesions</td>
<td>Micronucleus</td>
</tr>
<tr>
<td>Saran et al., 2008</td>
<td>Oral cancer and premalignant lesions</td>
<td>Micronucleus</td>
</tr>
<tr>
<td>Dórea et al., 2012</td>
<td>Oral squamous cell carcinoma</td>
<td>Micronucleus and degenerative nuclear alterations</td>
</tr>
</tbody>
</table>

**Table 1:** Studies using the Micronucleus Test in exfoliated oral cells for evaluating chromosomal damage and/or apoptosis in premalignant and malignant lesions of the oral cavity
5 Conclusions

- The Micronucleus Test on exfoliated cells of the oral epithelium is a valuable tool for detecting genetic damage;
- Use of a differentiated protocol, as suggested by Tolbert et al. (1991, 1992) increases the sensitivity of the test;
- In oral epithelial cells, broken eggs may be not associated with occurrences of chromosome damage, and thus reflect alterations relating to the normal tissue differentiation process;
- The Micronucleus Test can be used in biomonitoring for premalignant oral lesions;
- The malignant transformation process takes place accompanied by increased frequency of chromosome damage;
- The apoptotic process is disparate, even in situations of low exposure to mutagens;
- Malignant transformation is accompanied by loss of cell capacity to evolve to death in situations of DNA damage.

References


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