Testing of micronuclei and other nuclear alterations in oral mucosal cells as an alternative for early identification of oral cancer in smokers

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ABSTRACT
Micronuclei are structures made up of chromatid genetic material contained by a membrane, smaller than the main nucleus, and are usually formed around the karyotheca or scattered along the cytoplasm of cells. There are several advantages of the micronucleus test for the diagnosis of squamous cell carcinomas in chronic tobacco users in its various forms, one of the main ones being the possibility of early detection of genetic damage in the oral mucosa, even before the manifestation of any other clinical and histological signs that evidence carcinoma, in addition to being a non-invasive test; painless; low-cost; with high specificity and sensitivity and high predictive value

Keywords: Oral mucosal, Cancer, Smokers.

1 INTRODUCTION

Human beings are continuously being exposed daily to various cytotoxic and genotoxic substances, consciously or unconsciously. Among these substances, cigarette smoke is among the most harmful.

Cigarette smoke is a complex mixture of approximately 4,720 different toxic substances, of which 60 are considered potentially mutagenic. The main ones are: nicotine, benzopyrene, benzene, nickel, arsenic, radioactive (polonium 210 and carbon 14), heavy metals (lead and cadmium) and additives (preservatives, flavorings, enhancers, humectants and ammonium compounds). Not to mention pesticide residues.

It is already known in the scientific literature that the habit of chronic tobacco consumption is related to increased rates of development of oral carcinomas. In the process of cancer formation, cytotoxicity can lead to chronic injury followed by increased compensatory cell multiplication, hyperplasia, and then tumor development. Thus, there is a correlation between cytotoxicity and genetic

damage, and the accumulation of this damage in the genome can favor the progression of a normal cell into a malignant cell (CARVALHO et al., 2002).

According to Martins and Boschini Filho (2003), individuals genetically sensitive to cytotoxic and genotoxic agents, such as radiation, drugs and viruses, may present genomic DNA damage (genotoxicity), leading to the most diverse types of cellular alterations (cytotoxicity). Among these alterations, the formation of micronuclei stands out.

Since the classic studies by Stich et al. (1982) and Bugarin et al. (1998), who found that exfoliative epithelial cells of smokers always had a higher frequency of micronuclei and other nuclear abnormalities in relation to the control group, the micronucleus test has been used in the periodic evaluation of smokers, since the habit of smoking, Often, malignant neoplasms can result.

Micronuclei are structures made up of chromatid genetic material contained by a membrane, smaller than the main nucleus, and result from acentric chromosomal fragments that behave independently of the other chromosomes in the nucleus during cell division. Usually, they are formed around the karyotheca or scattered along the cytoplasm of the cells.

The micronucleus is formed as a response to genetic damage caused by the action of mutagens, and, for this reason, the evaluation of this bioindicator is widely used to indicate exposure to toxic agents contained in cigarettes (MENDES, 2018).

There are several advantages of the micronucleus test in cells of the exfoliative epithelium, among them the following: they reflect the cytogenetic changes that occurred in the population of cells in constant division in the basal layer; they monitored people at high risk of developing cancer such as smokers and alcoholics; it is a non-invasive method and simple preparation, which allows research at the epidemiological level; and enables results compatible with those obtained in more sophisticated techniques such as, e.g., *in vitro culture* of lymphocytes and satellite DNA.

Several studies have proven the efficacy of the micronucleus test as an indicator of cytogenetic damage in cells of the oral, bronchial and esophageal lining epithelium (CARVALHO et al., 2002; MUÑOZ et al; 1987; STICH et al; 1982; RAMIREZ et al; 1999 e LIPPMAN et al; 1990). This test is indicated as the main procedure, considering that it is fast, inexpensive, non-invasive, with the possibility of being repeated several times, for the prevention and monitoring of individuals under the recurrent use of tobacco and other mutagenic substances (CARVALHO et al., 2002) that can induce oral cancer.

Oral cancer can be defined as a set of malignant neoplasms that affect several anatomical sites in the head and neck region. In the international literature, there is no standardization of the primary sites included in the definitions of oral cavity cancer or oral cancer. In this context, malignant neoplasms of the lip, tongue, gums, floor of the mouth, hard palate, and other parts of the mouth, which correspond, respectively, to codes C00, C002 to C05.0, and C06 of the International Statistical

Classification of Diseases and Related Health Problems (ICD-10) will be considered oral cancer (BRASIL, 2022)

The epidemiological profile of individuals affected by oral cancer is well established in the literature. The disease is more prevalent in men, over 40 years of age, smokers, with low education and low income. The tongue is considered the most attacked organ, and squamous cell carcinoma (SCC) is the most frequent histopathological type (RUTKOWSKA et al., 2020).

The National Policy for Cancer Prevention and Control (PNPCC) states that cancer is a chronic disease and provides, in its guidelines, health promotion, primary prevention, early detection, diagnosis, treatment and palliative care actions. According to the regulation, the entire line of care in the health care network must be guaranteed, based on scientific evidence (BRASIL, 2013).

The fact that the neoplasms are painless, the delay in the recognition of the lesion by the dentist or physician, and the population's lack of access to health services hinder the early diagnosis of oral cancer and other premalignant lesions, such as leukoplakia, which can precede their appearance in the mouth and are therefore considered precursors of squamous cell carcinoma (SCC).

Considering the above-mentioned studies that demonstrate the importance of the micronucleus test in the biomonitoring of individuals exposed to mutagens, many of which are responsible for oral carcinogenesis, the present chapter aimed to present this alternative technique; non-invasive; painless; low-cost; with high specificity and sensitivity and high predictive value, for early identification of oral cancer in smokers, but little known among dentists and other health professionals.

2 DEVELOPMENT

2.1 EPIDEMIOLOGY OF ORAL CANCER

Oral cancer represents 3% of cancer cases worldwide, taking into account all types of cancer (SOARES, BASTOS NETO and SANTOS, 2019). According to the World Health Organization (WHO), for the year 2030 the estimated number of new cases is around 27 million, and Brazil has the highest incidence rate of oral cancer in South America, 3.6 cases per 100 thousand inhabitants, and the second highest mortality rate. of 1.5 deaths per 100 thousand inhabitants (BRASIL, 2022).

The etiology of oral cancer is multifactorial, with a greater predisposition to malignant neoplasms in the head and neck region. The main cases are related to epigenetic factors, in relation to individuals who make recurrent use of alcohol and tobacco, they have one.

The identification of risk factors that help in the early diagnosis of the disease is essential, however, in Brazil the identification of malignant lesions at an early stage corresponds to less than 10% of diagnoses (VOLKWEIS et al., 2014).

The increase in the prevalence, incidence and lethality of individuals affected by oral cancer in the Brazilian population point to the need for this pathology to be investigated through epidemiological research, not only for monitoring the

disease, but as a way to ensure the characterization of the profile of the population at risk and to outline public health policies for the entire population (SANTOS et al., 2012).

Although the oral cavity can be affected by different types of malignant neoplasms, such as salivary gland tumors (adenocystic, mucoepidermoid, acinar cell tumor, and adenocarcinomas), sarcomas, and melanomas, the most frequent histological type is squamous cell carcinoma (SCC), representing 90% of malignant tumors in this region (BRASIL, 2022).

The identification of risk factors and early diagnosis are of paramount importance for the development of public prevention policies.

2.2 RISK FACTORS FOR ORAL NEOPLASMS: TOBACCO CONSUMPTION.

Oral neoplasms, like others, are poorly reversible cellular alterations, resulting from mutations in genes involved in DNA repair mechanisms; in the control of cell proliferation and differentiation. In addition, alterations in genes of apoptosis pathways may contribute to the promotion of the disease, since cells that have damaged DNA escape death and may generate genetically altered offspring (HANAHAN; WEINBERG, 2000).

Risk factors for the development of neoplasms should, therefore, act to induce alterations in the genetic material, leading to the occurrence of both gene mutations and chromosomal alterations.

The consumption of industrialized tobacco is the most important independent risk factor for the development of oral and pharyngeal neoplasms, constituting the main preventable cause of premature death. It is estimated that 10 million people will die from tobacco-related diseases next year (2020) of which 70% will be in underdeveloped and developing countries, such as Brazil.

In addition to industrialized cigarettes, other forms of tobacco consumption, such as pipes, cigars, and chewing tobacco, are also associated with these neoplasms (WARNAKULASURIYA; SUTHERLAND; SCULLY, 2005).

Cigarette smoke is a complex mixture of approximately 4,720 different toxic substances, of which 60 are considered potentially mutagenic. The main ones are: nicotine, benzopyrene, benzene, nickel, arsenic, radioactive (polonium 210 and carbon 14), heavy metals (lead and cadmium) and additives (preservatives, flavorings, enhancers, humectants and ammonium compounds). Not to mention pesticide residues.

Of the 60 potentially mutagenic substances, aromatic hydrocarbons, benzopyrene and tobacco-specific nitrosamines (TSNs) stand out: nitroso-nor-nicotine (NNN), nitrosopyrrollidine (NPYR),

nitrosodimethylamine (NDMA), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which are soluble in water and present greater damage to cells (JOHNSON, 2001).

2.3 COLLECTION METHOD AND QUALITY OF ORAL MUCOSAL SMEAR

Cytopathology is a diagnostic method, based on the analysis of the morphological characteristics of a small set of cells that detach from the epithelial surfaces, resulting from the constant process of exfoliation and cell renewal.

The study of these cells can suggest a diagnosis according to the structural alterations found. There are reports of numerous methods of collecting these cells in the literature. Conventional exfoliative cytology and liquid-based exfoliative cytology are two of the most well-known among these methods. In the present project, we will focus only on considerations on conventional exfoliative cytology.

Cytological analysis is a minimally invasive technique, which constitutes less trauma, thus representing a diagnostic method of minimal morbidity for the patient. Thus, cytopathology is credited with a better psychological effect in relation to other diagnostic techniques, providing greater patient collaboration as well as treatment adherence. Another advantage is the larger sampling surface, since no continuity solution is created, since the examination does not compromise the integrity of the epithelium, and exfoliation can be performed in more than one site (LUCENA, et al. 2011).

As disadvantages, cytopathology is fundamentally characterized by the study of cells that have detached from a lining tissue by the friction of a collection instrument. Therefore, it is concluded that, by means of this diagnostic method, it is not possible to establish the normality of the intercellular relationship. The visualization of isolated cells, without the analysis of tissue architectural changes, has been considered one of the main limitations of the use of exfoliative cytology, as it increases the possibility of false-positives and false negatives (MERLIN, 2002).

In addition, the high degree of inconclusive results or with insufficient or inadequate material can be mentioned. Such results may be due to the non-standardization of the technique and/or the inexperience of the person responsible for the collection. Another negative point is the fact that the test is only used in surface lesions, i.e., lesions confined to the depth of tissues, such as the bone and the connective tissue itself, which cannot be diagnosed (FONTES, 2008).

The epithelium of the oral mucosa intended for the analysis of the micronucleus test has several advantages, such as the low cost and practicality of collection, which facilitates the development of biomonitoring research on cytotoxic and genotoxic substances.

The technique used in this test is scraping the epithelium of the buccal mucosa using a wooden or plastic spatula, as suggested by Tolbert et al. (1992) and Palaskara, Jindal, (2010).

When starting the collection, the first step is to ensure local asepsis by rinsing with water in order to prevent the presence of bacteria or other substances from interfering with the readings (SUHAS et al., 2004 and THOMAS, 2009). Next, the epithelium of the lateroinferior buccal mucosa of the patients is scraped with the wooden spatula. Finally, after collection, the samples will be sent to storage in 0.9% saline solution (MACHADO-SANTELLI, 1994 and BONASSI et al. 2009).

2.4 TYPES OF STAINING IN MICRONUCLEUS TESTS

The biggest differences in the way the micronucleus test is performed in scientific texts, that is, in their methodology, are in the type of staining used to identify the micronucleus. A low-cost and widely used staining method is the May-Grünwald/Giemsa (MGG) method. In this method, the slides with the buccal mucosal cell smear are air-dried and then stained with May-Grünwald for 5 minutes. After this phase, the slides are stained with Giemsa for 15 minutes and after this process they are washed in running water (PALASKARA; JINDAL, 2010). This staining method allows a better observation of the cell nucleus and is widely used in blood tissue cells, although it does not present specific DNA labeling (THOMAS et al., 2009; NERSESYAN et al., 2006).

According to Nersesyan et al. (2006), the major limitation of MGG is the lack of specificity for DNA labeling, which allows false-positive results to occur with some frequency. Fluorescent dyes can also be used to perform the micronucleus test, but this technique has a lower frequency of use (approximately 13%) when it comes to the laboratories that are part of the HUMN project (BONASSI et al., 2009). This evaluation method requires higher costs due to the use of antibodies and a fluorescence microscope.

The Feulgen technique is the most common for micronucleus studies (approximately 56%) in laboratories that perform this type of test (TOLBERT et al., 1992; THOMAS et al., 2009; BONASSI et al., 2009). The first to be done in this technique is the immersion of the slides, containing the cells of the oral mucosa, in HCl at 1 M at room temperature for 1 minute, then a new immersion of the slides is performed in HCl at 1 M at 60 °C for 10 minutes and, again, in HCl at 1 M at room temperature for 1 minute. The immersions performed in the HCl solution are responsible for the hydrolysis of the bases, being one of the most important phases for staining in the Feulgen technique. This hydrolysis has the purpose of separating the puric bases of the sugar from the deoxyribose, exposing the free aldehyde groups, leaving the DNA strand preserved and refined (CHIECO; DERENZINE, 1999). In the next step of hydrolysis, the slides are immersed in Schiff's reagent for 1 hour (Tolbert et al., 1992). At this stage, the specific regions of the cells with the free aldehyde groups in the apurin DNA molecule are associated with Pararosanilin, a non-methylated magenta dye dissolved in the Schiff reagent (CHIECO; DERENZINE, 1999). The bond between pararosaniline and the DNA molecule is considered specific, thus preventing the actions of non-specific interferents in the result. This staining

method is the most suitable when the objective is to quantify DNA due to the fact that it has a high degree of specificity for DNA molecule labeling (CHIECO; DERENZINE, 1999). In addition, in Feulgen/Fast Green staining, the cytoplasm is stained to help identify the micronucleus, which is a transparent and clear stain, which allows a more adequate identification of the micronucleus under a common optical microscope (HOLLAND et al., 2008; THOMAS et al., 2009).

2.5 CRITERIA FOR THE IDENTIFICATION OF THE MICRONUCLEUS IN BUCCAL MUCOSAL CELLS

For the identification of the micronuclei, the technique proposed by Tolbert will be used; Shy; Allen (1992), with modifications already made by Carvalho et al. (2202) and necessary for the success of the technique. The suggested patterns are:

a) Cell count: cells with normal and intact nuclei, with a smooth and distinct nuclear perimeter, should be included; that had intact cytoplasm stained by Fast Green, with the exception of those with small folds and little or no overlap with adjacent cells.

b) Micronucleus count: micronuclei should be considered to be those that present a surrounding halo suggestive of a membrane, less than 1/3 of the diameter of the associated nucleus, intensity of Schiff staining similar to the nucleus, and the same focal plane under microscopy. The analysis should be performed in 1,000 cells for each of the slides to be analyzed, from each individual, and then the number of micronuclei in regions A and B should be added and the median should be expressed as the number of micronuclei per 1,000 cells. The presence of micronucleated cells is a random event, which exhibits a Poisson distribution, and thus the comparison between the frequencies of micronuclei should be performed by non-parametric test with $p < 0.05$.

2.6 EVALUATION OF THE NUMBER OF CELLS

Following the suggestion of Tomas et al. (2009), for mucosal cells of the mouth, the quantitative evaluation of cells will be performed from the analysis of 1,000 to 2,000 cells, this number may vary due to the collection of the material and for better accuracy of the research. The initial research is individual and the material collected from each volunteer should be analyzed separately. The cells must be quantified and among them, those with the presence of micronuclei will also be counted, separating into groups.

2.7 HOW TO OBTAIN AND PREPARE MATERIAL FOR CYTOGENETIC STUDY: CHROMOSOMAL DAMAGE AND APOPTOSIS

The material intended for cytopathology analysis should be collected by gently scraping the oral mucosa with a tongue depressor spatula, and with it a smear should be performed on a clean glass

slide previously containing a drop of saline solution (0.9% NaCl.) The slides with the collected material should be air-dried at room temperature and then fixed in Carnoy's solution (ethane/acetic acid at a concentration of 3:1) for 24 hours.

The hydrolysis of the cells contained in the slides should be performed in hydrochloric acid (5N) for 15 minutes, followed by washing in distilled water three times.

The staining should be done using the Schiff reagent, and the counterstaining with 1% Fast Green. Permanent blades must be assembled with Entelan®.

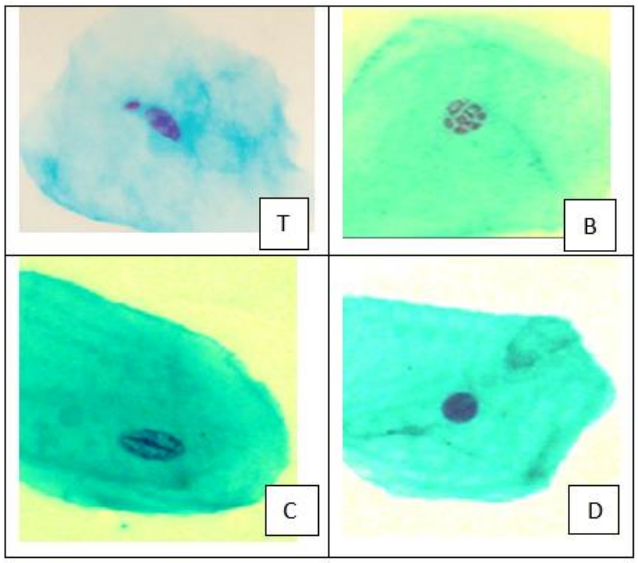
2.8 CAUTIONS FOR MICROSCOPIC ANALYSES OF MICRONUCLEUS TESTS

All cytopathology analysis should be performed under light microscopy (200x) and in a blind test on the collected samples.

At least 2,000 cells per individual should be analyzed for greater accuracy of the test, since random micronuclei and/or nuclear alterations, without statistical significance, can be identified in the oral mucosa. The criteria for identifying micronuclei should follow those described by Tolbert, Shy and Allen (1992), considering micronuclei to be rounded structures that are distinctly separated from the nucleus, with well-defined boundaries, measuring about 1/3 to 1/5 of the size of the nucleus and presenting a similar chromatine structure and coloration in relation to it, in addition to being visualized in the same plane (FIGURE 1a).

In the cytopathologies analyses, cells with degenerative nuclear alterations indicative of apoptosis should also be computed: karyorexis, condensed chromatin and pycnis (FIGURES 1b, c, d). Only cells with intact cytoplasm should be computed, following guidelines for general cytopathological patterns in benign and malignant conditions, according to the technical journal on cytopathology of the Ministry of Health (BRASIL, 2012).

FIGURE 1 - Photomicrographs indicative of the occurrence of Micronucleus (A), Karyorexis (B), Condensed Chromatin (C) and Pyknus (D).



2.9 SUGGESTIONS FOR STATISTICAL ANALYSIS PROCEDURES

The analysis of variance test (ANOVA) should be used to detect significant differences in micronucleus frequencies by the F test ($p < 0.05$) and the comparison of mean frequencies should be made by Tukey's test at the 95% probability level.

To confirm random effects on the analyzed parameters, the Poisson distribution should be verified to determine if the number of possible occurrences of discrete nuclear changes is much greater than the average number of occurrences in a given interval of time or space. In this way, the number of possible occurrences is often not known exactly. The results should occur randomly, i.e. totally by chance and the probability of occurrence should not be affected by whether or not the results occurred previously, so the occurrences are independent.

Other statistical tests should also be used, as suggested by Miranda (2006):

- ShapiroWilk test to verify whether the variables had a normal distribution;
- Spearman's correlation to verify the correlation between the groups;
- Wilcoxon Signpost Test to verify the existence of differences between dependent groups (intra-individual comparison of patients with oral cancer);
- Wilcoxon Rank Sums Test to check for the difference between independent groups;
- KrustalWallis test to compare independent groups;
- Fisher's Exact Test to verify association between variables;
- 'ROC' curves to determine cut-off points and variables with disease prediction power;
- McNemar's test to compare sensitivity and specificity between variables.

2.10 ETHICAL CONSIDERATIONS

As this is a study that involves the collection of biological samples and human beings, it must be registered in Plataforma Brasil and evaluated by a Research Ethics Committee (CEP) registered with the National Health Council.

The research subjects should be made aware of the objectives of the research and informed that ethical precepts should be ensured, including anonymity, and that a Free and Informed Consent Form should be signed, according to Resolution No. 466/12, which deals with research involving human beings (BRASIL, 2012).

3 FINAL THOUGHTS

From the above, it can be seen that the micronucleus test enables the early detection of genetic damage in the oral mucosa, even before the manifestation of any other clinical and histological signs that evidence cancer, making it a valuable tool in the prevention of squamous cell carcinomas that are

responsible for more than 90% of the malignant neoplasms that occur in the oral mucosa and have as their main risk factor the chronic use of tobacco in its various forms.

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