

MICRONUCLEI ASSAY OF EXFOLIATED ORAL MUCOSAL CELLS: A REVIEW

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Abstract

The micronuclei (MN) assay is potentially an excellent biomarker to detect chromosome loss or malfunction of mitotic spindle which is caused by aneugenic mechanisms. This assay is a suitable internal dosimeter that allows to evaluate the biomarkers of DNA damage (micronuclei & nuclear bud), cytokinetic effects (binucleated cells), proliferative potential (frequency of basal cells) and/or cell death (condensed chromatin, karyorrhexis, pyknotic & karyolytic cells) which are associated with ageing, neurovegetative disorders, high risk of cancer and the carcinogenic process with increased genetic instability. In this brief review, we summarize the cytogenetic effects of environmental and occupational exposures, dietary deficiencies, lifestyle factors, and different diseases along with the characteristics of micronuclei and other nuclear abnormalities.

Keywords: Aneugenic, Exfoliative, micronuclei, nuclear

Introduction

Genomic damage is considered to be the most important cause of developmental and degenerative diseases. It is also well known that genetic damage is produced by genotoxins, various medical procedures that includes radiation & chemicals, micronutrient deficiency, lifestyle factors and genetic factors such as inherited defects in DNA metabolism or repair.¹

To evaluate the genotoxic risks, DNA damage can be assessed by cytogenetic markers like chromosomal aberrations, sister chromatid exchanges and micronuclei. Out of all these, micronucleus test is preferable as it does not require tedious procedures like cell culture and metaphase preparation. To further add, as it is applicable on interphase cells only, it is the best indicator of mitotic interference and chromosomal mutations or breakages. It is a non-invasive and very economical procedure.²

Micronuclei (MN) and other nuclear abnormalities are biomarkers of genotoxic events and chromosomal instability and are collectively measured in micronucleus cytome assay. The molecular mechanisms behind these events have been investigated using molecular probes and genetically engineered cells. Presence of these nuclear anomalies could increase the risk of developmental and degenerative diseases.¹

People who are exposed to organic solvents, antineoplastic drugs, polycyclic aromatic hydrocarbons, drinking water contaminated with arsenic and paints with lead content shows significantly higher frequencies of MNs in exfoliated mucosal cells.³ The lifestyle factors like smoking, alcohol consumption and diet, especially vitamin deficiencies and supplementation are also associated with the genetic damage.⁴

Epidemiological studies reveal a positive correlation between micronutrient deficiencies and development of cancer. Thus the measurement of frequency of micronuclei becomes a valuable tool to study the link between nutrition and DNA damage. This in turn will assist in stepping up

implementation of public health strategies to reduce diseases of ageing and cancer.⁵

The aim of this article is to provide an overview of the current status of the MN assay in exfoliated oral mucosal cells and to highlight the strengths and limitations for collection, staining, and scoring of oral exfoliated mucosal cells in micronucleus assay.⁶

Background

In the early 1970s, the term micronucleus was first time suggested by Boller and Schmidt and Heddle who showed that this is a simple method to detect genotoxic potential of mutagens after in vivo exposure of animals using bone marrow erythrocytes.⁶

About 25 years ago, Stich and co-workers developed a protocol for micronucleus assay with exfoliated human epithelial cells which has been widely used in occupational & lifestyle studies.⁷ The buccal cell micronucleus assay was proposed in 1983, thereafter it gained popularity as a biomarker of genetic damage in numerous applications.⁴ A few years later, Countryman & Heddle showed that peripheral blood lymphocytes could be used for micronucleus approach and recommended to use micronuclei as a biomarker in testing schemes.⁶

In 1997, Human micronucleus (HUMN) project was established to standardize the micronucleus assay in peripheral blood lymphocytes and to assess the effects of protocol and scoring criteria on the values obtained.⁶

Origin of Micronuclei and Other Nuclear Abnormalities

In mitotic cells, MN could arise from the chromosome breakage and the dysfunction of the mitotic apparatus.[8] It is now well established that micronuclei mainly originate from the acentric chromosome fragment, acentric chromatid fragments or whole chromosome that fail to be included in the daughter nuclei at the completion of telophase during mitosis because they did not attach properly with the spindle during segregation process in anaphase.¹ Bigger micronuclei results from the damage to the spindle

apparatus of the cell resulting in the exclusion of whole chromosome (aneugenic effect) whereas smaller MN results from structural aberrations causing fragments of chromosomes (clastogenic effect).⁹ It is evident that multiple molecular mechanisms can lead to the formation of micronuclei and other nuclear abnormalities (Table 1).¹

Assay Biomarker	Molecular Events Associated With Biomarker
MN	Lagging acentric chromosome or chromatid fragment anaphase <ul style="list-style-type: none"> • Misrepair of DNA breaks • Unrepaired DNA breaks Lagging whole chromosomes at anaphase <ul style="list-style-type: none"> • Hypomethylation of repeat sequences in centrometric and pericentrometric DNA • Defects in kinetochore proteins or assembly • Dysfunctional spindle • Defective anaphase checkpoint genes • Unresolved replication stress intermediates
Nuclear Buds	Active process of elimination of nuclear material from nucleus <ul style="list-style-type: none"> • Elimination of amplified DNA possibly generated via BFB cycles • Elimination of DNA repair DNA protein complexes • Elimination of excess chromosomes – May occur in polyploidy cells to facilitate aneuploidy rescue Shrinkage of the remnants of a broken NPB between two nuclei can result in a temporary NBUD on one or both nuclei.

Table 1: - Molecular events associated with micronuclei and nuclear buds

Exfoliated cell micronucleus assay also demonstrates certain background prevalence of nuclear anomalies (Figure 2).

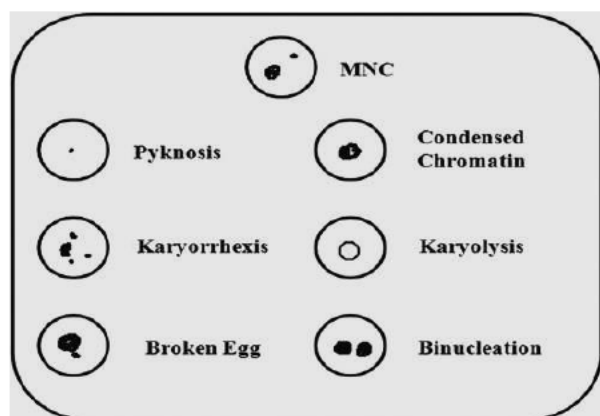


Figure 1: - Nuclear anomalies seen along with micronuclei in exfoliated cells

Basal cells: These cells have a uniformly stained nucleus and are smaller in size and more oval in shape when compared to the more angular and flat differentiated buccal cells.

Binucleated cells: Presence of two nuclei within the cell which is indicative of failed cytokinesis following the last nuclear division in the basal cell layer. It has recently been shown that a higher frequency of chromosomal dysjunction occurs in those binucleated cells that fail to complete cytokinesis than those cells with completed cytokinesis. This mechanism is thought to be a cytokinesis checkpoint for aneuploid binucleated cells.¹⁰

Broken eggs or cells with nuclear buds: They contain nuclei with a sharp constriction at one end of the nucleus which is suggestive of a budding process. The nuclear bud and the nucleus are usually in very close proximity and appear to be attached to one another. The morphology and staining properties of the nuclear bud are same as that of the nucleus; however its diameter may range from a half to a quarter of that of the main nucleus.¹¹

Cells with condensed chromatin: shows a roughly striated nuclear pattern in which the aggregated chromatin is intensely stained. In these cells it is apparent that the chromatin is aggregating in some regions of the nucleus whilst being lost in the other areas. When chromatin aggregation is extensive the nucleus may appear to be fragmenting.¹¹

Pyknotic cells: Cells characterized by a small shrunken nucleus which contains a high density of nuclear material that is uniformly but intensely stained. They may represent as an alternative mechanism of nuclear disintegration that is distinct from the process leading to the condensed chromatin and karyorrhectic cell death stages.¹⁰

Karyorrhectic cells: Cells with nuclear disintegration involving the loss of integrity of the nucleus.¹⁰ They have nuclei that are characterized by more extensive nuclear chromatin aggregation relative to condensed chromatin cells. They have densely speckled nuclear pattern indicative of nuclear fragmentation leading to the eventual disintegration of the nucleus.¹¹

Karyolytic cells: Cells in which the nucleus is completely depleted of DNA and is apparent as a ghost like image.¹¹

Cell Sampling and Preparation

There are several factors that affect the MN in exfoliated oral mucosal cells like differences in timing and implements used in cell collection, fixation, staining techniques, number of cells counted, scoring criteria, and other nuclear abnormalities in normal or degenerated cells.¹² Oral exfoliative cytology has been shown to have a sensitivity of 94%, specificity of 100%, and an accuracy of 95% and is particularly valuable for mass screening purpose.⁶

To obtain a smear from the oral cavity wooden spatula, metal spatula, toothpicks or toothbrush, or a moistened

cytobrush can be used. It is also advised to rinse the oral cavity before collection of the samples to remove the food debris and necrotic slough, if any, which could alter the quality of the smear.⁸ Once the exfoliated cells are collected, they are smeared immediately on pre-cleaned microscopic slides followed by fixation in 80% methanol or absolute ethanol or a methanol-glacial acetic acid mixture.⁶

There are numerous staining methods out of which DNA specific stains are ideal for staining. Among them, the most widely used are Feulgen reaction followed by counterstaining with fast green to delineate cell cytoplasm. Acridine orange can also be applied with MN fluorescing bright green.¹⁰ Other stains include diamidino-2-phenylindole DAPI, propidium iodide,¹³ papanicolou stain, Hoescht, May Grunwald Geimsa stain and orcein.

Scoring Criteria

The turnover rate for the appearance of MN in exfoliated buccal cells in an otherwise normal cell after exposure to an acute genotoxic event is estimated to be a minimum of 5–7 days.¹⁴ As the intralaboratory and interlaboratory variations exist in studies for micronuclei in exfoliated cells, efforts were made to standardize the assay. Since the first publications of Stich and Rosin for the basic criteria for identification of MN,¹⁴ a number of studies have been done by different authors to standardize the MN identification criteria.

In 1992 Tolbert *et al* developed the criteria for choosing the cells which consists of the following parameters.⁴

- A. Parameters for cell inclusion in the cells to be scored:
 - i) Intact cytoplasm and relatively flat cell position on the slide.
 - ii) Little or no overlap with adjacent cells.
 - iii) Little or no debris.
 - iv) Nucleus normal and intact, nuclear perimeter smooth and distinct.
- B. Parameters for identifying micronucleus:
 - i) Rounded smooth perimeter suggestive of a membrane.
 - ii) Less than a third the diameter of associated nucleus, but large enough to discern shape and color.
 - iii) Staining intensity similar to nucleus.
 - iv) Texture similar to nucleus.
 - v) Same focal plane as nucleus.
 - vi) Absence of overlap with bridge to nucleus.

Tolbert *et al*, also recommended the scoring of at least 1000 cells, which can be increased to 2000-3000 if less than 5 micronucleated cells are observed after counting 1000 cells.⁶

Association of Micronuclei Assay with other Factors & Disease States

Since the frequencies of micronuclei are increasingly being used as a biomarker, it is important to know that what factors influence their frequency in health and diseased states. Epidemiological studies reveal a strong association between micronuclei frequency and various factors that

include environmental and occupational exposures, radiotherapy, chemoprevention, lifestyle, habits, vitamin supplement trials, cancer and other diseases.

Occupational & environmental exposures: The assessment of the frequency of micronuclei has become an important biosurveillance tool for quantifying genomic damage associated with occupational and environmental exposures.¹⁵ Human populations are exposed to a variety of mutagenic and carcinogenic agents, such as antineoplastic drugs, arsenic in drinking water, fertilizers, pesticide mixtures, chemicals and all forms of tobacco.^{3,6} Many studies report that exposed individuals shows a significantly elevated levels of MN than controls, although the effects are relatively small, ranging between 1.1-4 fold.

Lifestyle factors: Lifestyle factors include smoking, alcohol consumption and diet which are related to high risk of oral cancer. A number of studies have been performed for MN in the subjects having specific lifestyle habits like chewers of betel quid, reverse smokers, snuff dippers, khaini tobacco, and other similar practices.⁶ And the results of the impact of tobacco on micronuclei frequencies have led to varied conclusions.

Researchers have consistently found an increase in micronuclei frequencies with the consumption of alcohol in individuals who have genetic variants in the alcohol metabolising enzyme, alcohol dehydrogenase.¹⁵ Teo and Fenech suggested an interaction between folic acid levels and alcohol use. According to them, increased folic acid values provides a protective influence against potential DNA damage which is caused by increased ethanol ingestion.¹⁶ Studies proves that micronutrients, including beta-carotene and other vitamins significantly decreases MN levels (1.4–4-fold) in both the healthy tobacco users and individuals with precancerous lesions.¹⁴

Naderi *et al* through their study found that micronuclei count in buccal mucosal cells of non-smokers was significantly lower than that of the smokers. They also found that increasing the smoking duration can heighten the frequency of micronuclei; however the difference was not that significant.¹⁷ Bansalet *al*, also concluded a positive correlation between increased micronuclei frequency and tobacco using habits with the frequency of MN significantly higher in smokeless tobacco users than in smokers and controls.³

There are many studies analysing the frequency of micronuclei in individuals using cell phones over a period of time. Significantly elevated micro nucleated buccal cells were observed in these studies.

Radiation: Ionizing radiation plays an important role in the treatment of many neoplasms, but at the same time it also produces genetic damage. For the evaluation of micronuclei frequency in mucosal cells of patients undergoing radiotherapy in the head and neck region several studies have been conducted and it was observed that radiation sensitive tumours have higher MN levels in exfoliated cells

after radiation therapy than the more radiation resistant ones. Thus the assay can be used as a predictor of tumor radio sensitivity.

Oral cancer: Oral cancer, one of the 10 most common cancers as stated by World Health Organization, is a complex disease with abnormal growth, altered gene expression and disruption of normal function of cells caused by genotoxic agents. It results in genomic instability at an early stage of cancer, which is often reflected as leukoplakia, erythroplakia, lichen planus and oral submucous fibrosis.²

Micronucleus assay is useful for screening the populations under the risk of mutagenic agents that may cause oral neoplasms, and also for the identification of pre-clinical steps of carcinogenic process.¹⁸ Casartelliet *al* observed that there is a gradual increase in MN counts from normal to precancerous lesions to carcinoma, and suggested a link of this biomarker with neoplastic progression.³

Conclusion

Micronuclei assay is a sensitive, non-invasive and low cost technique that offers a very simple method for obtaining information on status of the epithelial cells, particularly DNA damage, proliferative potential of basal cells and cell death. Although many studies have shown a statistically significant increased MN frequency in the buccal cells of populations exposed to occupational and environmental insults, various lifestyle factors, radiation and oral cancer; but the magnitude of changes is usually relatively small. Simplicity, accuracy, multipotentiality and large tissue applicability of the MN technology made it attractive in the past and will ensure a key role in the evaluation of mutagenicity and primary prevention in the future.

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