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Buccal Micronucleus Cytome Assay- A Biomarker of Genotoxicity

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Abstract

The buccal micronucleus cytome assay in exfoliated buccal cells is utilized as biomarkers for DNA damage, cell death and basal cell frequency. It offers great opportunity to evaluate genotoxicity by the way of quantifying mean frequencies of micronuclei, binucleated cell, broken egg, karyolysis, karyorrhexis, pycknosis and condensed chromatin. This assay is sensitive, minimally invasive, simple, cheap, easy and fast. It has precision and statistical power obtained from scoring large number of cells. Micronucleus assay has been extensively used to assess genetic damage due to lifestyle characteristics, occupational exposure, diseases and environmental risk. It also has applications in human biomonitoring, ecotoxicology, cancer risk assessment, pharmaceutical drug testing and the impacts of dietary micronutrients and micronutrient combinations on DNA damage. Fluorescence in situ hybridization is a valuable addition to micronucleus assay as combination of both enables us to characterize the genetic contents of the micronuclei. The present article reviews and updates on usefulness of buccal micronucleus cytome assay as a biomarker. It gives a detailed description of the methodology of buccal micronucleus test and analysis of the results. We also discussed the criteria for identification and classification of nuclear anomalies. We have also proposed the future directions namely high-throughput automation for further enhancing the reliability of micronucleus assay to be applicable on large scale experimental and epidemiological studies. It would help in overcoming many of the problems caused by inter-observer variability in evaluation of slides.

Keywords: Biomarkers; Buccal micronucleus cytome assay; Exfoliated cells; Genetic damage; Genotoxicity; Micronuclei

Introduction

The continuous discovery and development of new chemical, biological and physical agents necessitates the utilization of rapid and reliable test methods and biomarkers for the screening of genotoxicity [1]. The National Institutes of Health Biomarkers Definitions Working Group [2] defined a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other health care interventions. Biomarkers are as indicators of molecular and cellular events in biological system that may illuminate relationships between hazards and human health and disease processes [3]. Biomarkers are potentially useful tool for determining unintended environmental exposure, such as to chemicals or nutrients. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction [4].

The frequency of MN in exfoliated cells is extensively used in molecular epidemiology and cytogenetics as a biomarker to evaluate the presence and the extent of chromosomal damage in human populations exposed to genotoxic agents or bearing a susceptible genetic profile and genomic stability in human populations [5-7]. The micronucleus (MN) assay in exfoliated buccal cells is a useful and minimally invasive method for monitoring genetic damage in humans [8]. The MN assay involves examination of cells to determine the prevalence of cells with micronuclei (MNi), extranuclear bodies composed of whole chromosomes or chromosomal fragments that failed to be incorporated into daughter nuclei during mitosis [9].

Kirsch-Volders et al. [10] used florescence in situ hybridization with human pan-centromeric probes for discriminating the negatively labeled MN containing acentric chromosome fragments and positively labeled MN containing one or several whole chromosomes. Literature showed that it is a cytological approach based on not only scoring MN frequency but also other genomic damage markers (nuclear buds or broken egg, karyolysis, karyorrhexis), dead or degenerated cells and assures a comprehensive measure of cytotoxic and genotoxic effects and provide important mechanistic insights [11]. MN assay has greater accuracy and statistical power as thousands of cells can be scored as compared to a few hundred in the *in vitro* chromosomal aberration test.

Besides, the minimal invasiveness of cell collection, low cost, ease of storage and slide preparation make the MN assay with buccal epithelial cells the ideal choice for molecular epidemiological studies [12]. The buccal cell MN assay first proposed by Stich et al. [13] still continues to gain popularity as a biomarker of genetic damage in numerous applications. High reliability and low cost of MN technique has contributes to the worldwide success of genome damage and can be used for the early detection of carcinogenic effects in the cell exposed to various carcinogenic agents [14]. It has been widely used in occupational and lifestyle studies [15-17]. MN assay has also been successfully applied to identify dietary factors that have a significant impact on genome stability [18].

Any tissue possessing dividing cells such as cervix epithelia [19], oesophagus, bladder, nasal, bronchial and buccal mucosa [20] can be used for evaluation of MN. However, buccal mucosa cells are preferred as they are the first line of contact with many hazardous compounds. It is well understood that several systemic conditions and treatments decrease the proliferative rate of epithelial cells. About 60% of mucosa surface are stratified non keratinized epithelia (big cells with well

defined, intact nuclei and abundant cytoplasm) making it suitable for analysis [21].

Epithelial tissue exfoliated cells are derived from actively dividing basal layer. These cells migrate towards the surface within 5 to 14 days and can exhibit nuclear damage happen at this time. Basal layer also provides the first barrier against potential carcinogens. Thus, it is more likely to suffer damage by these agents before reflecting a systemic condition. According to Holland et al. [8], about 90% of all cancers are derived from epithelial cells.

Since more than 90% of all human cancers are of epithelial origin, MN assay with buccal epithelial cells is the most suitable biomonitoring approach for the detection of increased cancer risk in humans. Buccal cells have limited DNA repair capacity relative to peripheral blood lymphocytes, and therefore, may more accurately reflect age-related genomic instability event in epithelial tissue [22]. Being in immediate contact with inhaled and ingested genotoxic agents, and metabolites of the chemicals, epithelial tissues are the first to express the genotoxic effects of these agents [23]. Micronuclei in exfoliated buccal cells reflect genotoxic events that occurred in the dividing basal layer 1-3 weeks earlier [24,25]. The frequency of occurance of MNi is a measure of chromosome breakage in early cell divisions, and the number of micronuclei is known to increase with carcinogenic stimuli, long before the development of clinical symptoms [25]. The presence of MNi and other nuclear anomalies within these cells has been shown to be associated with genetic defects in genome maintenance, accelerated ageing, exposure to genotoxic agents, oral cancer risk and neurodegenerative diseases and was also useful in chemo-preventive studies [8].

Methodology for Detection of MN and other Nuclear Anomalies in Exfoliated Buccal Epithelial Cells

Presampling procedure

Prior to sampling, an ethical clearance should be obtained in accordance to Helsinki's Declaration, World Medical Association (2013) and governmental as well as institutional regulations. Epidemiological survey regarding the individual's age, gender, occupation, weight, height, general health, lifestyle habits, drug intake and family history if any must be completed during face to face interview. Written informed consent must be obtained from each subject before the sampling.

Collection of exfoliated buccal epithelial cells

Prior to buccal cells collection, it is appreciated that each volunteer had mouth wash thoroughly with water in order to remove any unwanted debris that may interfere with the analysis. Exfoliated buccal epithelial cells could be collected from inner side of both the cheeks using a small headed toothbrush [26], or wooden spatula [27], or wooden tongue- depressor, or metal spatula [19,28], or toothpick [20], or toothbrushes [29]. Small headed brushes or cytobrushes seemed to be most commonly used and most effective tool for collection of buccal epithelial cells. The method used for the sample collection should be constant i.e. application of same strength and skill for collection of sample.

Slide preparation

Usually, after collection of buccal cell sample with wooden or metal spatula leads to preparation of buccal smears by spreading the cells on clean slide. In number of studies, the toothpicks and cytobrushes used to collect buccal cells was transferred into a tube containing Tris/EDTA washing buffer (pH=7) to release the cells and centrifuged [26,20]. The buffer aids in inactivating the endogenous DNAase and helps in removing bacteria that interferes with scoring. Cell suspensions are fixed and transferred to slides and air dried at room temperature [19]. Fixatives that are commonly used include 80% methanol, or 80% ethanol, or methanol-ethanol mixture (3:1) or methanol- glacial acetic acid mixture (3:1). It is preferred to make two slides per individual. Coding of the slides should be done simultaneously in a manner which avoids the identification of the volunteer.

Staining

Several staining methods have been used, although stains with high affinity to DNA are suitable for differentiating nuclear anomalies in exfoliated buccal epithelial cells. Modified Feulgen (Feulgen- Schiff's-Fast green) staining is preferred by many researchers as it minimizes the incidence of false positives. With this staining method, DNA material such as nuclei and MN could be observed under fluorescent microscope [30]. Several investigators have used a number of DNA specific fluorescent dyes such as DAPI [31], acridine orange [32], Hoechst [33] and propidium iodide [34] in different biomonitoring studies using MN assay in human buccal cells. Giemsa (May-Grunwald- Giemsa) (2-10%) has been used in different laboratories worldwide [21,35]. But it is suggested to avoid using Giemsa stain since it enhances the chances of false positive scoring leading to overestimation of micronuclei by scoring of non- nuclear bodies, bacteria, keratohyalin bodies in cytoplasm that resemble micronuclei. In certain studies, higher MN frequencies were found to be associated with Giemsa or Aceto-orcein stain in contrast to DNA specific stains [33,36].

Slide analysis

During 1980s, few cells (approximately 500 per person) were evaluated [13,24]. Later, Tolbert et al. [37] suggested scoring at least 1000 cells per person. They also recommended to score 2000-3000 cells if less than 5 micronucleated cells were observed after counting 1000 cells. Ceppi et al. [38] suggested evaluating 3000-4000 cells. Most of the studies published have scored between 1000-3000 cells [19,39-41]. The analysis should be done by the same observer to eliminate inter-observer variability in the results. The criteria of Tolbert et al. [42] for identification and classification of the nuclear anomalies is most widely used. The various nuclear anomalies in comparison to normal cell nucleus (Figure 1A) that may be encountered include micronuclei (Figure 1B and 1C), binucleated cell (Figure 1D), broken egg (Figure 2E), karyorrhexis (Figure 2F), karyolysis (Figure 2G) and pyknosis (Figure 2H).

Figure 1: Exfoliated buccal epithelial cells showing various nuclear anomalies at 1000X (A) Normal cell; (B) Micronuclei; (C) five micronuclei; (D) Binucleated cell.

Figure 2: Exfoliated buccal epithelial cells showing various nuclear anomalies at 1000X (E) Broken egg; (F) Karorrhexis; (G) Karyolysis; (H) Pyknosis.

Classification Criteria for Micronuclei (MNi) and other Nuclear Anomalies

Micronucleated (MN) cell

MN is a small extranuclear DNA particle formed when chromosome fragment or acentric chromosomes lag behind and fails to be included in the main nuclei of daughter cells [43]. This may arise from unrepaired dsDNA breaks, dysfunctional error-free homologous recombinational DNA repair pathway, defective repair enzymes in non-homologous end joining pathway, simultaneous occurrence of excision repair mechanisms in proximity and on complementary strands, in which improper gap filling step leads to double strand breaks formation and ultimately MN formation. Malsegregation of whole chromosomes at anaphase may also cause MN formation as a result of hypomethylation of cytosine in centromeric and pericentromeric repeat sequences, reduction in heterochromatin integrity, defects in mitotic spindle assembly, alteration in functioning of mitotic checkpoint genes, abnormal centromere amplification and telomere end fusions [44]. MN could be inherited from mother cells. During mitosis, nuclear fragmentation of cytoplasmic bridges occur which may lead to MN. Nuclear budding has also been proposed as one of the important mechanisms for formation of MN, detected by live cell imaging [45]. In order for the cell to be considered micronucleated, it is required to meet the following criteria:

- Cell has one or more micronuclei along with main nucleus.
- MNi have circular or oval shape with smooth perimeter suggestive of membrane.
- MNi have less than 1/3rd the diameter of the main nucleus but large enough to discern shape and color.
- MNi have same texture, focal plane and staining intensity as main nucleus.
- MNi are Fuelgen- positive bodies.

Binucleated (BN) cell

Formation of BN cell seems to be related to cytokinesis failure either due to defects in microfilament ring formation or cell cycle arrest due to aneuploidy or telomere dysjunction [28]. Shi and King [46] reported that chances of occurrence of non-disjunction are higher in cells that fail to complete cytokinesis than those which have completed cytokinesis resulting in formation of two normal mononucleated cells. In order for the cell to be considered binucleated, it is required to meet the following criteria:

- Presence of two nuclei within a cell.
- Two nuclei are of similar size and staining intensity
- Both the nuclei may be either in close proximity or touching each other.

Nuclear Buds (NBUDs) or Broken Eggs (BE)

NBUDs represent amplification of DNA. The most likely mechanism for the formation of NBUDs is the elimination of amplified DNA, DNA repair complexes and possibly excess chromosomes from aneuploid cells. Nucleoplasmic bridge formation is suggestive of presence of dicentric chromosomes due to misrepair of DNA breaks and telomere end fusions. Defective separation of sister chromatid at anaphase due to failure of decatenation also results in NBUDs [44]. The cell with NBUDs or BE has following characteristics:
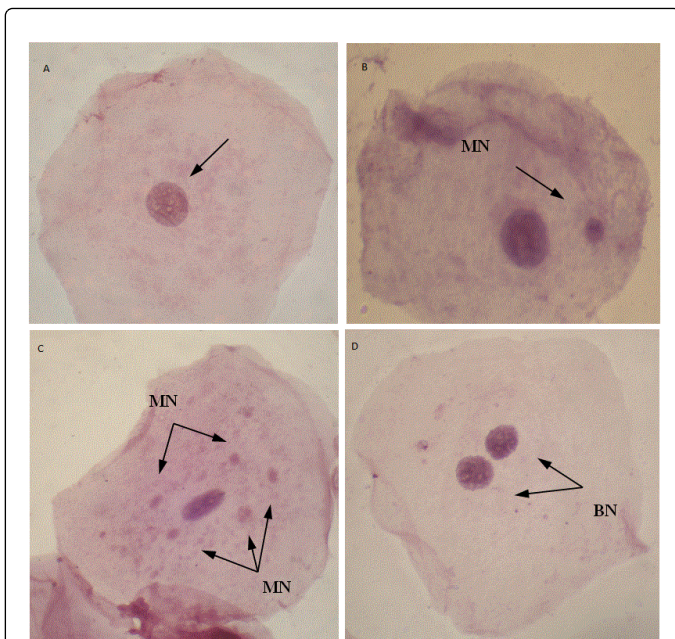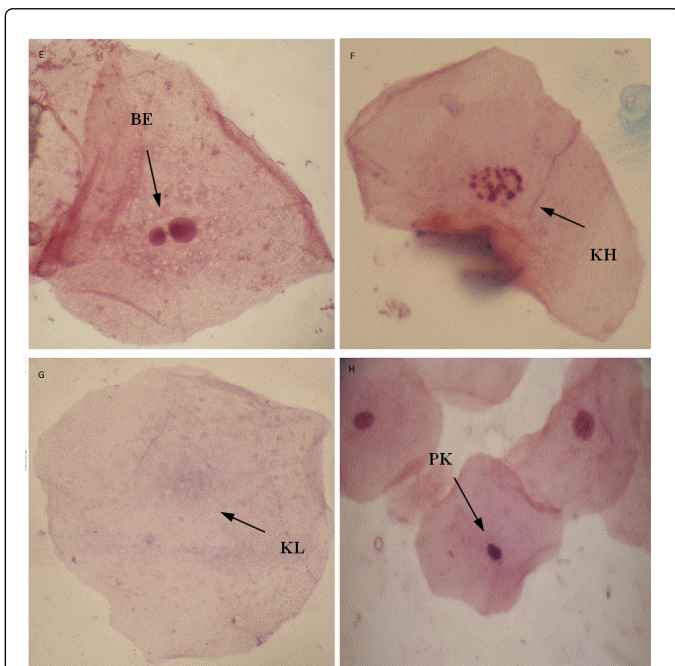
The main nucleus presents a sharp constriction in one extreme forming a bud of nuclear material.

- NBUDs have same texture, focal plane and staining intensity as the main nucleus.
- NBUDs are connected to the main nuclei with narrow or wide nucleoplasmic band.
- NBUDs have diameter that is 1/3-1/16th of main nucleus but in certain rare cases could be greater and almost upto same size as the main nucleus.

Karyolytic (KL) cell

Karyolysis represent a advanced stage of necrosis and apoptosis [47]. In order for the cell to be considered karyolytic, it is required to meet the following criteria:

- They are angular and flat in shape with cytoplasmic area that is the size of terminally differentiated cell.
- Cell with nuclear dissolution, in which aceto-orcein negative, ghost-like image of nucleus remains.

Karyorrhectic (KH) cell

Karyorrhexis is a typical later stage of apoptosis [48]. In order for the cell to be considered karyorrhectic, it is required to meet the following criteria:

- Cell with nuclear disintegration involving loss of integrity of the nucleus.
- Nucleus constitutes more densely aggregated chromatin than that of condensed chromatin cells.

Pyknotic (PK) cell

They are the cells that are in process of dying as the frequency of PK cells has been reported to be positively correlated with frequency of condensed chromatin and karyorrhectic cells [49]. In order for the cell to be considered pyknotic, it is required to meet the following criteria:

- Cell with small and shrunken nucleus with diameter approximately 1/3rd of normal nucleus.
- Nucleus is uniformly and highly stained.

Condensed chromatin (CC)

Condensed chromatin represents the stages of apoptosis which occurs due to rapid proteolysis of nuclear matrix proteins [50]. In order for the cell to be considered condensed chromatin, it is required to meet the following criteria:

- Cells with intensively stained nucleus in distinct areas of chromatin condensation.
- Nuclei are characterized by striated pattern of parallel tracts of aggregated chromatin.

Applications

MN assay is used by the academics, industry and contract laboratory organizations for internal hazards identification and compound prioritization as an alternative/ replacement of the *in vitro* chromosome abberation test as it offers significant advantages over the chromosome abberation test [5]. The frequency of MN is extensively used as a biomarker of genomic instability, genotoxic exposure and early biological effect in human biomonitoring studies [51-53]. The test allows the detection of both clastogens and aneugens and it can simultaneously detect mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction [54]. In a meta-analysis of 63 human population studies with the exfoliated buccal MN assay, Ceppi et al. [55] reported a statistically strong correlation between the increase in frequency of micronucleated cells in peripheral blood lymphocytes and buccal cells in genotoxic exposed groups. Thus, supporting the use of buccal MN assay as a biomarker of DNA damage in epithelial cells.

Induction of MN is regarded as an effective biomarker of diseases and processes associated with induction of DNA damage [23]. Shimizu [56] observed a strong correlation between binucleated cells rate and diseases like, cancer and neurodegenerative diseases, which is relevant to the cumulative damage mechanism of MN formation. Significant increase in number of binucleated cells in Down syndrome patients was observed by Thomas et al. [49]. A significant increase in the frequencies of MN was observed in exfoliated buccal cells of polycystic ovarian syndrome patients [57]. This test when used on exfoliated cells helps to identify the genotoxic damage in human tissues, which are targeted by carcinogens and from which carcinogens develop [24]. The proportion of exfoliated buccal mucosal cells with MNi gives the opportunity to assess sensitivity to genotoxic compounds and monitor the effectiveness of cancer intervention studies [58]. Yadav and Sharma [19] reported an increase in the frequency of MNi in the subjects exposed to mobile phone radiations as compared to control subjects. MN assay in exfoliated cells of buccal mucosa is suitable for human biomonitoring [59]. Under the HUMNXL, Bonassi et al. [28] compiled and analyzed a database of 5424 subjects with buccal MN values obtained from 30 laboratories worldwide to investigate the influence of several conditions affecting MN frequency. They reported that frequency of MNi increased in heavy smoking (40 cigarettes/ day) and decreased with daily fruit consumption. Ceppi et al. [55] reported a strong correlation of MN frequency in buccal exfoliated cells with MN frequency in lymphocytes. This correlation suggests that systemic genotoxic effects within the blood stream may also impact on and be detectable in buccal cells. The genetic factors and exposure affecting MN frequency in lymphocytes may possibly also apply to some degree to buccal cells, including the association of MN with cancer risk [28].

The potential of MN assay can be enhanced by the combination with fluorescence in situ hybridization (FISH) technique. MN assay when combined with FISH is able to assess the frequency of occurrence of different chromosomes to form MN. It could also evaluate the potential chromosomal targets of mutagenic substances [60,61] and the mechanism of aneuploidy with the help of chromosome specific centromeric probes [62]. Chromosome locus specific FISH probes play an important role in studying the nature of genome instability in tumour cells [63]. In a further advancement of MN assay, Huang et al. [45] used DNA- binding fluorescent dyes to visualize MNi in live cells under fluorescence illumination. This technique allows for the real-time study of the mechanism of formation of MN and other anomalies.

Future perspectives

MN assays is successfully used to study DNA damage in humans, there are still several challenges to be met. MN assay can have high inter-observer variability, even under optimized laboratory conditions. Therefore, it is suggested to score 10,000 cells to generate statistically significant data, 50% increase in MN frequency [64]. Although, MN

and other nuclear anomalies could be easily visualized but scoring large cell numbers is a time consuming process. As MN assay is an excellent marker for human biomonitoring and genotoxicity studies, there is an urgent need for automation of MN and nuclear anomalies analysis for quicker and relatively more reliable detection of anomalies, allowing the applicability on large scale.

Acknowledgement

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