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BIO-MONITORING AND OCCUPATIONAL RISK ASSESSMENT IN RANDOM HUMAN POPULATION IN NUCLEAR RESEARCH CENTRE USING EXFOLIATED BUCCAL EPITHELIAL CELLS AND PERIPHERAL BLOOD LYMPHOCYTES

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ABSTRACT

In present time a noninvasive method to estimate the radiation and mutagen exposure is the need of the day. For this, the buccal epithelial micronuclei assay was evaluated by monitoring the employees of our nuclear research centre, who come for annual medical checkup. Micronucleus assay is sensitive indicator and provides a measure of chromosome breakage, damage and chromosome loss. A study was undertaken for evaluating 50 random individuals working in nuclear research centre, to compare baseline micronuclei frequency in exfoliated buccal epithelial cells and lymphocyte micronuclei. Buccal micronucleus cytome assay (BMNcyt) and blood lymphocyte cytokinesis blocked micronuclei assay (CBMN) was performed on slides stained with Geimsa's stain. We report here the data on mean frequency of spontaneous micronuclei in buccal epithelial cells and blood lymphocytes. The mean Frequency of micronuclei (MN) observed in buccal epithelial cells of females 0.99 ± 0.21 ‰ (range 0.09 – 2.92) and of males 0.49 ± 0.10/10 ‰ (range 0.00 – 2.06). MN frequency in buccal epithelial cells was 0.66 ± 0.10 ‰ (range 0.00 – 2.92). The MN frequencies in human peripheral blood lymphocytes of same donors were found to be 8.61 ± 0.10 ‰ (range 3.00 – 17.00) in females and 9.15 ± 1.00 ‰ (range 3.00 – 22.00) in males. A total MN frequency in cytokinesis blocked lymphocytes was 8.96 ± 0.73 ‰ BN cells (Range: 3.00 – 22.00). The observed baseline frequency of spontaneous micronuclei in buccal epithelial cell and in human peripheral blood lymphocytes are correlating. Therefore buccal epithelial cell micronuclei are a good noninvasive bio-monitoring assay.

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INTRODUCTION

One of the cytogenetic techniques to determine the impact of environmental, genetic and life-style factors on genetic integrity in human population is micronucleus (MN) assay. Micronucleus (MN) is defined as microscopically visible, round or oval cytoplasmic chromatin masses next to the nucleus. MN originates from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division, or they are the results of aberrant mitosis and consist of acentric chromosome fragments, chromatid fragments, or aberrant chromosome. Thus, MN assay provides a measure of both structural and numerical chromosomal aberrations and it has been shown that this technique is sensitive indicator of chromosome damage. Cytogenetic endpoints such as chromosome aberration (CA), sister chromatid exchange (SCEs) and micronuclei assay in peripheral blood lymphocytes are time consuming to accurately read and interpret results. Thus micronucleated cell frequency have been proposed as sensitive parameters for assessing genotoxic effects of chemical or physical mutagens, for these reasons noninvasive investigation like application of the micronuclei test in exfoliated cells has been widely used as it is easy to collect the samples (Fenech 2002, 2007; Fenech *et al*. 1999; Duffaud *et al*. 1997; Nersesyan *et al*. 2002; Casartelli *et*

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al. 2000; Popescu 2003; Miller and Therman 2001; Timoshevsky and Nazarenko 2005; Yildirim *et al*. 2006). The buccal cell cytome assay was first proposed in 1983 and continues to gain popularity as a biomarker for genetic damage (Holland *et al*. 2008). Buccal mucosal cells are excellent for use in monitoring human exposure to occupational and environmental genotoxins because these cells are in the direct route of exposure to ingested pollutants. They are also capable of metabolizing proximate carcinogens to reactive chemicals. MN in exfoliated cells emerge during mitosis of the basal layers of the epithelium as extra chromosomal DNA particles when chromosome fragments or whole chromosomes lag behind and fail to be included in the main nuclei of the daughter cells. The formation of MN is therefore induced by substances that cause breakage of chromosomes (clastogens) as well as by agents, which affect the spindle apparatus (aneugens).

Buccal mucosal cells can be easily and rapidly collected for evaluating genomic damage and cell death in human aero-digestive tract (Holland *et al*. 2008; Torres-Bugarie *et al*. 2014; Salama *et al*. 1999; Majer *et al*. 2001; Ribeiro and Angelieri 2008). This assay was widely applied in biomonitoring studies to evaluate occupational or environmental exposures to genotoxins (Holland *et al*. 2008; Torres-Bugarie *et al*. 2014; Salama *et al*. 1999) medical procedures eg, radiation and chemicals, (Ribeiro and Angelieri 2008; Zamora-Perez *et al*. 2013; Carlin *et al*. 2010) micronutrient deficiencies eg, folate, (Thomas *et al*. 2011, 2015) lifestyle factors eg, alcohol, smoking, drugs, and stress (Chandirasekar *et al*. 2014; Alonso de Oliveira *et al*. 2014; Kausar *et al*. 2009) and genetic factors, such as inherited defects in DNA metabolism and/or repair (Rosin and Ochs 1986; Rosin *et al*. 1989; Rosin and German 1985; Tomanin *et al*. 1990). Increased micronucleus (MN) frequency in exfoliated buccal epithelial cells (BE cells) is associated with the risk of developing cancer and degenerative diseases (Bolognesi *et al*. 2015).

Therefore it is essential to have reliable and relevant minimally invasive biomarkers to improve the implementation of biomonitoring, diagnostics, and treatment of diseases caused by or associated with genetic damage. The study is aimed to investigate and compare the baseline frequency of the micronucleus in exfoliated buccal epithelial cells (BE cells) and peripheral blood lymphocytes of the same subjects in random human population of nuclear research centre.

MATERIALS AND METHODS

Ethical issues

The study was approved by Bhabha Atomic Research Centre Hospital Medical Scientific Committee (27th Meeting) & Bhabha Atomic Research Centre Hospital Medical Ethics Committee (Human Ethics Committee) (27th Meeting, BHMEC/51/2015). The Committee reviewed the project and approved the study. All methods were performed in accordance with the relevant guidelines and regulations. Before sample collection from random, healthy individuals, a written informed consent was obtained. Detailed information was obtained from each individual regarding age, gender, occupation, place of work, general health, lifestyle habits such as smoking, alcoholic, tobacco, paan, gutka, areca nut (betel-nut) chewing, drug intake and family history. Individuals were informed about the objectives of the study.

Subjects

The study involved 50 individuals (32 males and 18 females) with an age group of (range: 21-55 years) All the individuals were Bhabha Atomic Research Centre employees. They were selected randomly, during annual medical checkup at Occupational Health Centre (OHC), Bhabha Atomic Research Centre, Trombay, Mumbai. They volunteered for this project. Their general health records are maintained by OHC. All the samples collected analyzed in the study are from individuals who were nonsmokers, nonalcoholic and have no (tobacco, paan, gutka, areca nut) chewing habits. In addition, the individuals studied were healthy, without chronic illness.

Sample collection and preparation

Exfoliated epithelial cells

The participants were advised to rinse their mouths with water, with 8 to 10 ml of Listerine and again with water to remove unwanted debris prior to the sample collection. Subsequently, the cells were scraped with sterile wooden spatula from each cheek three to four times and the collected cells on wooden spatula were rinsed in plastic centrifuge tubes containing 10 ml of physiological saline solution (0.87%). After collection the tubes containing buccal cells were centrifuged. Buccal cell pellet was resuspended in Tris-HCl buffer solution three times [0.1 mol/L EDTA, 0.01 mol/L Tris-HCl, and 0.02 mol/L NaCl (pH 7.0)], and then fixed in chilled 3:1 methanol- acetic acid fixative. After two to three washes cells were smeared on clean chilled slides, air dried. Slides were stained with Geimsa's stain (Rosin 1992).

Peripheral blood lymphocytes

Heparinized peripheral blood samples (10ml) were obtained from same volunteers. 4 ml Hams F10 medium with 1% (200 mM) L-glutamine, 10% fetal bovine serum and 1% PHA (reconstituted medium) were inoculated with 0.3 ml whole blood. No antibiotics were added to the cultures at any stage. Cultures were incubated at 37°C. After 44h of culture stimulation with phytohemagglutinin, cytochalasin B was added, resulting in a final concentration of 6µg/ml to block cytokinesis. Cells were harvested at 72 h. Harvested cells were treated with 0.8% cold KCl solution for 5 min, followed by 3:1 methanol acetic acid treatment and then 2nd fixation with 3:1 methanol acetic acid including 1% formaldehyde. Fixation step was repeated twice and slides were prepared, air-dried and were stained with 1% Giemsa (Merck) in Sorensen's buffer, pH 6.8, for 20 min. (Fenech 1993; Sharma 2013; Krishnaja and Sharma 1998).

Cytological Analysis

A total number of 10000 buccal epithelial cells were analyzed for each individual from two slides (5000 each). Cells with intact nuclei (not clumped, smeared or overlapped) were included in the analysis. The criteria and the parameters used to assess micronuclei were as follows: 1) Rounded smooth perimeter suggestive of a membrane, 2) Less than a third the diameter of associated nucleus, exhibit similar focal plane 3) Staining intensity and stain similar to nucleus 4) Texture similar to nucleus 5) Same focal plane as nucleus 6) Absence

of overlap with or bridge to nucleus as described by Rosin and Tolbert (Rosin 1992; Tolbert *et al.* 1992,). Lymphocyte micronuclei were analyzed by Cytokinesis blocked Micronucleus assay (CBMN). 5000 binucleated lymphocytes from two slides (2500 each) were analyzed under bright field microscope (magnification 100X), in which the number of micronuclei was recorded according to standard recognition criteria. Briefly, micronuclei were (1) morphologically identical to the main nuclei but their diameters were from $1/16^{th}$ diameter of the main nuclei, (2) non-refractive and same intensity of stain like main nucleus, (3) not linked to the main nuclei (Fenech 1993).

RESULTS

A total number of 50 samples analyzed - 18 females and 32 males, age group 21 to 54 years. As shown in table 1, a total of 529148 buccal epithelial cells were analyzed out of which 0.043% buccal epithelial cells have shown micronuclei. Further the percentage of micronuclei was 0.067% in these cells. In females 0.057% buccal epithelial cells were with micronuclei, whereas in males the percentage was 0.034%. Only 4 buccal epithelial cells were observed which were positive for nuclear bud. The observed frequency of micronuclei (MN) in females buccal epithelial cells was 0.99 $\pm$ 0.21/1000 (range 0.09 – 2.92) and 0.49 $\pm$ 0.10/1000 (range 0.00 – 2.06) in males. The observed frequency of micronuclei in total buccal epithelial cells was 0.66 $\pm$ 0.10 /1000 (range 0.00 – 2.92).

Fig. 1 shows photomicrographs of (a) Buccal epithelial cells under low power (16x), (b) Buccal epithelial cells under oil immersion 100x, (c,d,e,f) Buccal epithelial cells with Micronuclei, (g,h,i) Buccal epithelial cells with nuclear bud.

Table 2, indicate total of 250000 Cytokinesis blocked peripheral blood lymphocytes cells analyzed for micronuclei. Binucleated cells with micronuclei were 0.89%. Cytokinesis blocked Binucleated cells with micronuclei in female were 0.86% and in male were 0.91%. The micronuclei (MN) frequencies in Cytokinesis blocked human peripheral blood lymphocytes (CBMN) of same donors were found to be 8.96 $\pm$ 0.73/1000 BN cells (Range: 3.00 – 22.00). The Micronuclei (MN) frequency in female 8.61 $\pm$ 0.10/1000 (range 3.00 – 17.00) and 9.15 $\pm$ 1.00/1000 (range 3.00 – 22.00) in males.
Fig. 2 shows photomicrographs of Blood Lymphocytes Micronucleus Cytokinesis Blocked Binucleated Cells with micronuclei.

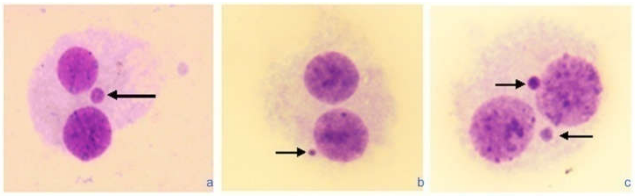

Fig. 2 Blood Lymphocytes Micronucleus,
Cytokinesis Blocked Binucleated Cells, Binucleated Cells with Micronuclei

DISCUSSION

The MN assay in exfoliated cells is a method in genotoxicity study, which holds assurance for the study of epithelial carcinogens (Tolbert *et al*. 1992). Various groups have found analysis of MN in buccal cells a sensitive method for monitoring genetic damage in human populations (Foiles *et al.* 1989; Sarto *et al*. 1990; Kayal *et al*. 1993).

Table 1 The Frequency of Micronuclei in Buccal Epithelial Cells.

Sample type	Number of Individuals	Age group Range	No. of BE Cells Scored	No. of BE Cells with MN	No. of MN in BE Cells	Frequency of BE cells with MN/ 1000 cells $\pm$ SE	Frequency of MN /1000 BE cells $\pm$ SE	MN Range / 1000 BE cells
Females	18	21 - 43	204341	117	197	0.58 $\pm$ 0.10	0.99 $\pm$ 0. 21	0.09 – 2.92
Males	32	29 - 58	324807	112	161	0.35 $\pm$ 0.07	0.49 $\pm$ 0.10	0.00 – 2.06
Total	50	21 - 58	529148	229	358	0.46 $\pm$ 0.06	0.66 $\pm$ 0.10	0.00 – 2.92

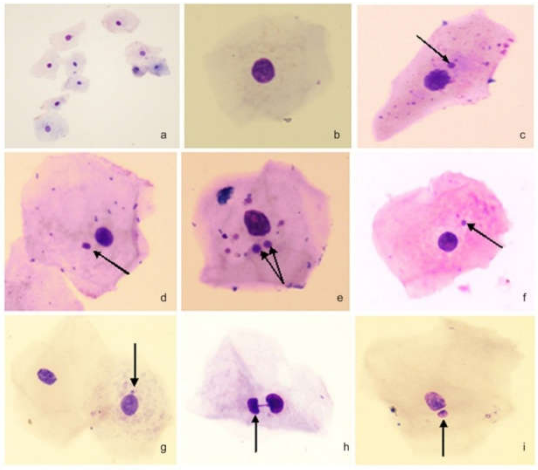

Fig. 1 (a) Buccal epithelial cells under low power (16x), (b) Buccal epithelial cells under oilimmersion 100x, (c,d,e,f) Buccal epithelial cells with Micronuclei, (g,h,i) Buccal epithelial cells with nuclear bud.

The analysis of MN incidence in the buccal mucosa cells is used in molecular epidemiological studies investigating the impact of lifestyle factors, and occupational exposure to potentially mutagenic, carcinogenic agents, diagnostic radiations and occupational radiation exposure at commercial nuclear power reactors and other facilities. Inhalation and ingestion of particles is considered as the primary route of exposure to potentially hazardous occupational and environmental genotoxic compounds. Buccal cells are the first barrier for the inhalation or ingestion of these residues and are considered as a suitable target site for monitoring human exposure to occupational and environmental genotoxins. Mucosa cells are capable of metabolizing chemical agents to reactive species. Micronuclei in buccal exfoliated cells are formed during mitosis of the basal layers of the epithelium, and their absolute frequency could reflect the real situation in target cells.The basal layer of buccal epithelial cells divides actively. They send the buccal exfoliated cells to surface within 5 to 14 days and which can exhibit nuclear damage. Stich *et al*. developed a protocol for MN assays with exfoliated buccal mucosa cells, which was widely used in occupational and lifestyle studies (Stich *et al*. 1990).

Table 2 The Frequency of Micronuclei in Blood Lymphocytes.

Sample type	Number of Individuals	Age group Range	No. of CB-BN Cells Scored	No. of BN Cells with MN	No. of MN in BN Cells	Frequency of BN Cells with MN/ 1000 cells ± SE	Frequency of MN /1000 BN cells ± SE	MN Range / 1000 BE cells
Females	18	21 - 43	90000	774	775	8.60 ± 0.10	8.61 ± 0.10	3.00 – 17.00
Males	32	29 - 58	160000	1452	1464	9.08 ± 0.98	9.15 ± 1.00	3.00 – 22.00
Total	50	21 - 58	250000	2226	2239	8.90 ± 0.71	8.96 ± 0.73	3.00 – 22.00

This noninvasive method is suitable for biomonitoring approach for the detection of increased cancer risk in humans because more than 90% of all human cancers are of epithelial origin (Burgaz *et al*. 1995; 1999).Oral epithelial cells are in immediate contact with genotoxic agents inhaled either with air or with food and water. The major advantage of the micronucleus test over other techniques is that it can be applied to interphase cells and does not require cell culture. Because of the low cost of the test it is suitable for the large-scale screening of population. Micronuclei are suitable internal dosimeters for revealing tissue specific genotoxic damage in individuals exposed to carcinogenic mixtures. Any different range of micronucleus number can be the result of chromosomal alternations. The micronuclei are the result of genomic damages to the cells. These structures can be used for individual biomonitoring. Exfoliated buccal mucosa cells are good sources for this biomonitoring. The micronuclei assay is a non-invasive and simple technique for evaluating the DNA damages (Holland *et al*. 2008).

We have undertaken a pilot study on buccal epithelial cells along with their peripheral blood lymphocyte samples obtained from workers in nuclear research centre for evaluation of health risk assessment. The frequency of MN in buccal epithelial cells and blood lymphocytes in our study is: in total buccal epithelial cells were 0.66 ± 0.10 /1000 (range 0.00 – 2.92), females 0.99 ± 0.21/1000 (range 0.09 – 2.92), males 0.49 ± 0.10/1000 (range 0.00 – 2.06). and 8.96 ± 0.73/1000 BN cells (range 3.00 – 22.00), females 8.61 ± 0.10/1000 (range 3.00 – 17.00), male 9.15 ± 1.00/1000 (range 3.00 – 22.00) respectively.

The frequency of MN in control subject reported by Fareed *et al*. (Fareed *et al*. 2011) was 0.75 ± 0.171/1500 cells /individual. Aurelio Flores-García1 *et al*. (Aurelio *et al*. 2014) investigated micronuclei and other nuclear anomalies in exfoliated buccal mucosa cells of Mexican women with breast cancer patients 1.79 ± 0.77/1000 BE cells and in matched control healthy women 0.85 ± 0.40 /1000 BE cells. The study carried out by Laura Aguiar Torres (Laura 2014) in buccal epithelial cells of individuals exposed (exposed group) and non-exposed (control group) to ionizing radiation reported 5.26 ± 0.653 (range 0-17) and 1.33 ± 0.215/ 2000 cells (range 0-6) as frequency of MN respectively. Halder *et al.* (Halder *et al*) studied 50 age and sex matched healthy controls compared with patients with oral lesions from the department of Faciomaxillary and ENT surgery, he reported oral mucosal MN frequency in the control population to be 0.35% (males 0.39%, females 0.32%). Our observations of Buccal epithelial cell micronuclei frequency in the population study is within the comparable limits of the above mentioned data. Although MN in buccal mucosa occurs at a lower frequency than in peripheral blood lymphocytes there is no reason that these should be excluded from study.

MN rates in peripheral blood lymphocytes (PBL) are moderately rare, typically occurring at levels of 5–25/1000 cells (Bonassi *et al*. 2011; Preethi *et al*. 2016) and at generally somewhat lower levels in the buccal mucosa, in the range 0.5–10/1000 cells (Fenech *et al*. 2011; Holland *et al*. 2008). It has been suggested that MN are suitable for biomonitoring genetic damage rates (Fenech *et al*. 2007, 1997; Fenech 2006) in particular damage arising from ionizing radiation exposure.

Several investigators have studied the frequency of MN in buccal epithelial cells of the individuals exposed to different genotoxic agents such as pesticides, heavy metals used in tanneries or individuals with different types of cancer (Idalia *et al*. 2016; Sellappa *et al*. 2011; Nersesyan *et al*. 2002). However, there are limited studies on MN frequency in buccal cells in individuals exposed to radiation. Laura Aguiar Torres (Laura 2014). has reported data of health professionals occupationally exposed to ionizing radiation, whereas Shitalkumar G Sagari *et al.* (Shitalkumar G Sagari *et al*). reported data based on nuclear power plant workers. As a result, there is a need to screen large population for buccal MN frequency and blood lymphocyte MN frequency that are occupationally exposed to radiation and general population living in high background area. The frequency of buccal MN seems to be a very sensitive assay for measuring exposure to genotoxic agents, the suitability and standardization of the buccal MN assay will allow a more reliable comparison of the data among human population. The study should be extended further for a large population to make particular the buccal mucosa, a marker of exposure.

CONCLUSION

Though our population was from nuclear research centre but findings did not show any variation compare to data reported by other groups. The test can be applied to large groups, it is quick and inexpensive. The frequency of micronuclei in exfoliated cells provides evidence of biological response to genotoxic agents in naturally dividing cells, cell culture is not required and they allow assessment of in vivo damage, such damage in exfoliated cells serves as an internal dosimeter.

The peripheral blood lymphocyte assay and exfoliated cell assay appears to be the most promising for future research. Our findings indicate the potential useful aspects of the BCMN assay. The impact of different confounding factors may influence the buccal cell MN assay such as gender, age and lifestyle habits and environmental factors have to be considered. Thus this assay can prove to be a good epidemiological tool. The need of the hour is to develop baseline values “range of normal values” for the BCMN assay. Genotoxic effect by carcinogen may be displayed in various ways like apoptosis or nuclear degeneration and therefore, only counting micronuclei in exfoliated epithelial cells as

biomarkers of exposure to lifestyle and occupation related genotoxic carcinogens warrants further evaluation.

Declarations

Ethics Approval and Consent to Participate

The study was approved by Bhabha Atomic Research Centre Hospital Medical Scientific Committee (27th Meeting) & Bhabha Atomic Research Centre Hospital Medical Ethics Committee (Human Ethics Committee) (27th Meeting, BHMEC/51/2015). The Committee reviewed the project and approved the study. Before sample collection, a written informed consent was obtained from individuals. Individuals were informed about the objectives of the study. We confirm that all methods were performed in accordance with the relevant guidelines and regulations.

Consent for Publication

Not applicable

Conflict of interest

No Conflict of interest.

Competing interests

The authors declare that they have no competing interests.

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