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RESEARCH ARTICLE

COMPARATIVE MICRONUCLEI FREQUENCY IN ORAL SUBMUCOUS FIBROSIS AND ORAL SQUAMOUS CELL CARCINOMA PATIENTS USING PAPANICOLAOU (PAP) AND MAY-GRUNWALD GIEMSA (MGG) STAIN

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ABSTRACT

Background: Presence of micronuclei in cells is a reflection of structural and chromosomal aberration in cells. Micronuclei frequency has been proved to be a reliable marker of genomic damage and can be applied to buccal cell cytology to determine the susceptibility. This method may help monitor and predict increased risk of cancer in humans. **Aim**: The present study aims to determine micronuclei frequency in control, oral submucous fibrosis (OSMF) and oral squamous cell carcinoma (OSCC) patients in oral cytology specimens and compare the efficiency of Papanicolaou (PAP) and May-Grunwald Giemsa (MGG) staining techniques for same. **Materials and methods**: Study groups consisted of 100 patients each of control, OSMF and OSCC. Two smears were obtained from each patient, each of which was stained with PAP and MGG stain. A total of 100 cells were evaluated for micronuclei in each slide using Tolbert's criteria and micronuclei frequency was calculated. **Results**: The mean micronuclei frequency was higher in the PAP stained smears in all the three groups than in the MGG stained smears and is the preferred staining method. Multiple comparisons between the three groups showed that MN frequency increased step-wise from control to OSMF to OSCC group of patients. **Conclusion**: Oral cytology is the least invasive and inexpensive method for measuring DNA damage and can be used as a field test using PAP stain. It can be used as a marker for epithelial precancer (OSMF) and cancer progression.

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INTRODUCTION

Oral squamous cell carcinoma is a multistage process from normal to dysplastic lesion and ultimately to squamous cell carcinoma. Development of oral cancer proceeds through discrete molecular genetic changes that are acquired from the loss of genomic integrity after continued exposure to environmental risk factors (Choi, 2008). Tobacco specific nitrosamines have been reported to be potent clastogenic and mutagenic agents which are responsible for the induction of chromatid/chromosomal aberrations. The genotoxic carcinogenic chemicals released from the areca nut, tobacco and also the calcium hydroxide content of lime present in the betel quid are thought to be responsible for promotion of reactive oxygen species which are responsible for DNA damage (Sellappa *et al*., 2009).

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Oral epithelium is maintained by continuous cell renewal whereby new cells produced by the basal layer migrate to the surface replacing those that are shed. The basal layer contains the stem cells that may express genetic damage (chromosome breakage or loss) as micronuclei (MN) during nuclear division. The daughter cells, which may or may not contain MN, eventually differentiate and exfoliate into the buccal cavity. Some of these cells may degenerate into cells with condensed chromatin, fragmented nuclei (karyorrhectic cells), pyknotic nuclei, or completely lose their nuclear material (karyolitic or ''ghost''cells). In rare instances, some cells may be blocked in a binucleated stage or may exhibit nuclear buds (also known as ''broken eggs'' in buccal cells), a biomarker of gene amplification. These biomarkers of genome damage (e.g. MN, nuclear buds) and cell death (e.g. apoptosis, karyolysis) can be observed in both the lymphocyte and buccal cell systems, and thus provide a more comprehensive assessment of genome damage in the context of cytotoxicity and cytostatic effects.

Oral exfoliative cytology can thus reveal various cellular alterations in squamous cell carcinoma (Holland, 2008). 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 (Yang *et al*., 2010). The buccal cell MN assay was first proposed in 1983 and continues to gain popularity as a biomarker of genetic damage in numerous applications. The usefulness of a micronucleus test to detect and quantitate the genotoxic action of carcinogens and mutagens has been well established (Hayashi, 1983). Its sensitivity is comparable to that of scoring chromatid breaks and exchanges. The MN assay in exfoliated buccal cells is potentially an excellent candidate to serve as such a biomarker. The purpose of the present study was to compare the micronuclei frequency in control, oral submucous fibrosis (OSMF) and oral squamous cell carcinoma (OSCC) patients using oral exfoliative cytology; and to compare the efficiency of Papanicolaou (PAP) and May-Grunwald Giemsa (MGG) stain on results of micronuclei in exfoliated cells. Our objective was to evaluate if micronuclei frequency test could be used as an early marker of genotoxicity for oral potentially malignant disorder and malignant lesions.

MATERIALS AND METHODS

Patient selection: A total of three hundred patients were included in the study and divided into three groups. Control group comprised of 100 individuals without any clinically observable lesions and without any tobacco (chewing and smoking) habits. Study group comprised of 100 clinically diagnosed OSMF patients and 100 clinically diagnosed and histopathologically confirmed OSCC patients. Patients suffering from any other systemic illness were excluded. Patients who had previously received any treatment in the form of radiotherapy or chemotherapy and recurrences were excluded from the study. Informed consent was obtained from all patients included in the study. Patient's charts were reviewed to obtain information such as age, sex, habits and details of the lesion. Ethical clearance was obtained by institutional ethics committee.

Sample collection: Before collection of sample all the patients were asked to thoroughly rinse mouth with water so that the surface for exfoliative cytology was prepared, such that it was free from all debris and necrotic tissue. One end of a wooden tongue blade was thoroughly moistened with water. The buccal mucosa was scrapped firmly with the tongue blade. The collected material was quickly spread on the precleaned slides and two smears were prepared, one for each stain. The slides were immediately fixed in 95% ethyl alcohol for at least 20 minutes. Two smears were obtained from each subject, since two staining techniques were used. One smear was stained with Rapid - PAP and second smear was stained with MGG for micronuclei evaluation.

Evaluation of micronuclei: Smears were evaluated for the presence of micronuclei in PAP and MGG stained slides. 100 cells were counted in PAP stained slides and 100 cells counted in the MGG stained slides. All the slides were observed under low power magnification x10 (×10 objective lens and ×10 ocular lens) for screening and high power magnification x40 (×40 objective lens and ×10 ocular lens) for counting micronuclei. Scoring criteria for Micronuclei was performed according to Tolbert *et al* (1992), which states that a micronucleus (MN) must be less than one-third the diameter of the main nucleus, be on the same plane of focus, have the same color, texture and refraction as the main nucleus, have a smooth, oval or round shape and be clearly separated from the main nucleus. MN are non-refractile and can therefore be readily distinguished from artifact such as staining particles; MN usually have the same staining intensity as the main nuclei but occasionally staining may be more intense. Only those structures fulfilling the above mentioned criteria were recorded as micronuclei. Micronuclei were counted out of 100 intact epithelial cells in each slide. All the stained smears were evaluated by two observers to eliminate inter-observer bias, and any disagreements were resolved with a pentahead microscope (Figure 1).

Statistical analysis: The statistical analysis was done by using statistical software STATA version 10.0. The total number of micronuclei observed in 100 intact epithelial cells was calculated as micronuclei frequency. Micronuclei frequency were presented as mean, standard deviation and minimum and maximum values (Range). Multiple groups were analyzed by Kruskal-Walli's (KW) ANOVA Test and a pairwise comparison was done by Dunn's Test. P-value of 0.05 or less was considered for statistical significance.

RESULTS

The present study has two factors influencing micronuclei viz. Groups and Stains. The Groups are three – Control group, OSMF and OSCC. The two stains are PAP and MGG (DNA non-specific stain). The mean age of patients in control, OSMF AND OSCC group was 36.51 yrs, 37.17 yrs and 52.86 yrs respectively. Table 1 summarizes mean micronuclei frequency using PAP stain in control, OSMF and OSCC groups. MN frequency gradually increased from control group through OSMF group and was highest in OSCC group. The difference in mean MN among the groups were found to be statistically significant ($P<0.05$). Post hoc multiple comparisons were done by Dunn's test in conjunction with ANOVA to determine which specific group pair(s) is statistically different from each other (Table 2). Table 3 shows mean MN frequency using MGG stain in control, OSMF and OSCC groups. The difference in mean micronuclei among the groups were found to be statistically significant ($P<0.05$). Post hoc multiple comparisons were done by Dunn's test in conjunction with ANOVA to determine which specific group pair(s) is statistically different from each other (Table 4). Table 5 shows comparison of mean micronuclei frequency in PAP and MGG Stains. As evident, between the two different stains, mean number of micronuclei was higher in the PAP stained smears in all the three groups than in MGG stained smears. The difference in mean MN between the two stains was found to be statistically significant ($P<0.001$).

DISCUSSION

The international collaborative project on micronucleus frequency on human population (HUMN) project established in 1997, is an program aimed at studying the MN frequency in human populations and standardize methodologies and characteristics and determine the extent to which the MN frequency is a valid biomarker of ageing and risk for diseases such as cancer. It initially focused on analysis of MN in peripheral lymphocytes from unexposed and exposed individuals and was later applies to exfoliated cells (Fenech *et al*., 1999).

Table 1: Comparison of mean micronuclei frequency using PAP stain in the groups by Kruskal Walli's test

PAP	Groups	Mean	Std. Dev.	Median	Range
	Control	7.6	6.41	6	0-21
	OSMF	9.11	4.97	9	0-23
	OSCC	11.46	5.69	10	2-30

One way ANOVA (KW= 30.55), P= 0.000 (HS= highly significant)

Table 2. Pairwise Comparison of mean micronuclei frequency in PAP stain three groups (Dunn's multiple comparison test)

Groups	Groups compared	Mean rank diff.	p-value
Control	Control vs OSMF	38.10	< 0.001, HS
OSMF	Control vs OSCC	67.33	< 0.000, HS
OSCC	OSMF vs OSCC	30.60	< 0.009,HS

(HS= highly significant)

Table 3. Comparison of mean micronuclei frequency using MGG stain in the three groups by Kruskal Walli's test

MGG	Groups	Mean	Std. Dev.	Median	Range
	Control	2.49	1.92	2	0-10
	OSMF	3.3	2.08	3	0-8
	OSCC	4.72	3.92	4	0-21

One way ANOVA (KW= 25.36); p=0.000; HS=Highly significant

Table 4. Pairwise Comparison of mean micronuclei frequency with MGG stain in three groups (Dunn's multiple comparison test)

Groups	Groups compared	Mean rank diff.	p value
Control	Control vs OSMF	35.69	< 0.000
OSMF	Control vs OSCC	32.84	< 0.009
OSCC	OSMF vs OSCC	29.86	< 0.013

(HS= highly significant)

Table 5. Comparison of mean micronuclei frequency in PAP and MGG stains using ANOVA test

Stain	Mean	Std. dev.	Min	Max
PAP	9.42	5.97	0	31
MGG	3.46	3.09	0	21

(P<0.001)

Buccal cells are the first barrier for the inhalation and ingestion route (tobacco smoke/ tobacco chemicals) and are capable of metabolizing proximate carcinogens to reactive products. Approximately 90% of human cancers originate from epithelial cells. Hence oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion (Vondracek, 2001; Spivack, 2004). The collection of buccal cells is a rapid, relatively simple, inexpensive and least invasive method available for measuring DNA damage in humans, especially in comparison to obtaining blood samples for lymphocyte and erythrocyte assays, or tissue biopsies. Buccal cells allow monitoring of genotoxic effects on cells including karyorrhexis, karyolysis, micronucleus formation, pyknosis, binucleation, broken egg nucleus (Nersesyan, 2006). Our findings show that the results of micronuclei assays in exfoliated oral mucosa cells of depend strongly on the staining methods. MN is easily seen in the clear cytoplasm in the PAP smears. Regarding the MGG smears, bacteria, salivary proteins and cell debris masked the effect of the MN as compared to the Pap smears, where the fixative destroyed the bacteria and clearly demarcated the cell boundaries. So PAP is a better stain as compared to MGG for counting micronuclei. In the present study the mean micronuclei count in the control group when stained with PAP was 7.60 ± 6.41 & our findings are in accordance with the study conducted by Sangeeta Palaskar, Chavi Jindal (2010)[12] in which the mean number of the micronuclei in the control group by using the PAP stain was 6.13 +/- 2.29.

However the study conducted by Devendra H Palve, Jagdish V Tupkari (2008) shows in contrast a very lower mean number of micronuclei i.e. 0.210 +/- 0.168. Many factors influence MN frequency including differences in cell collection, fixation, type of stain used, scoring criteria and number of cells counted, to name a few. In the present study, numbers of MN cells were significantly higher in OSMF (9.11 +/- 4.97) and OSCC (11.46 +/- 5.69) patients than those of controls. The increase of MN in these patients is likely due to a combination of factors which include the cumulative effect of acquired mutations in genes involved in DNA repair, chromosome segregation, and cell cycle checkpoint and numerical and structural aberrations in chromosomes caused by exposure to endogenous and exogenous genotoxins. Accumulation of such genetic alterations can lead to the development of premalignant lesions and subsequent invasive carcinoma (Ralhan, 2007). Also, ean micronuclei count was significantly higher in OSCC patients than OSMF patients. This may be due to the fact of increased genomic unstability and genomic damage associated with carcinogenesis than in dysplasia (Choi, 2008). Thus, micronuclei can be used as a reliable marker for early detection of cancer. Stich applied the micronuclei assay for studying the effects of tobacco/areca nut chewing habits on buccal mucosa and observed that saliva of Pan Bahar (a commercially available combination of ingredients like betel nut, catechu, lime, etc.) chewers was clastogenic to CHO (Chinese Hamster Ovary) cells and showed that role of micronuclei as internal dosimeters for revealing tissue-specific genotoxic damage (Stich, 1990).

Dave demonstrated dose dependent elevation in sister chromatid exchange and chromosomal aberrations, indicating genotoxicity of areca nut and its extracts (Dave *et al*., 1992). In recent years numerous studies have reported increased frequencies of micronucleated cells in exfoliated buccal mucosa and high incidence of genotoxic damage in peripheral lymphocytes in users of pan masala with and without tobacco in comparison with control individuals (Palaskar, 2010; Patel, 2007). In a similar study by Halder in oral precancerous (leukoplakia and erythroplakia) and cancer patients, MN frequency increased step wise from precancer to cancer patients (Halder, 2004). Anila *et al* reported elevated MN frequency in OSMF patients as compared to control group and further reported increase in MN frequency with increase in clinical staging (Anila, 2011).

In another study by Saran *et al* in precancerous and cancerous patients, a significant stepwise increase in the DNA damage was recorded in peripheral blood leucocytes and buccal epithelial cells (Saran, 2008). Hence, MN test for genotoxicity can be used to identify high risk individuals. Although Casartelli found similar results in precancer (leukoplakia) and cancer patients, he suggested use of micronuclei as a predictor of progression to malignancy with caution (Casartelli, 2000). Also, from various studies it can be concluded that tobacco, areca nut and their products are associated with increased micronuclei frequency (Gandhi, 2005; Fareed, 2011; Joshi, 2011). To conclude, ours is one of the few studies determining MN frequencies in OSMF and OSCC patients. Numerous studies have documented efficacy of buccal cells for evaluation of MN which parallels to evaluation of peripheral leucocytes for same. We report progressive increase in MN frequency from control to OSMF to OSCC patients and propose its use to identify genetic damage in OSMF patients and marker for progression to cancer. Also, PAP stain should be preferred stain for scoring MN in field studies.

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