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STUDY OF MICRONUCLEI OF PERIPHERAL LEUCOCYTES IN KHARRAH, GUTKHA, PANMASALA CHEWERS AND TOBACCO PRODUCT USER OF VIDARBHA REGION OF CENTRAL INDIA.

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ABSTRACT

Background: Tobacco and its product have accepted as important etiologic agents in oral cancer. Tobacco usage in any form is associated with etiology of many disease for many decades and any approach aimed at detection of population sub-group at increased risk should be considered a high priority task. The peripheral blood cells were known to exhibit changes in cancer of various organs. **Aim & Objectives**: To quantify the micronuclei in peripheral blood leukocytes of Gutkha Panmasala Chewers and Tobacco product user of Vidarbha region of central India. **Materials & Method**: Healthy tobacco chewers (n= 60) and healthy non-chewers as controls (n=60) with 30-50 years were selected. A thin blood smears were prepared. Slides were air-dried for 30 min followed by fixation using cold 3:1 methanol and acetic acid solution for 10-15 minutes, washed twice in PBS (phosphate buffer solution) and stained immediately in 5% Geimsa solution and dried on a hot plate for 5 minutes and observed under microscope. A minimum of 1000 cells from each individual was screened for calculating frequency of micronucleated cells (MNC). The identification of micronucleus was based on the criteria proposed by Sarto et al.[20] **Result**: Mean age of participants were 42.45± 7.47(SD). The leucocytes stained with Gimsa stain, and then the each slide was observed under Olympus® BX 21 microscope. The micronucleus frequency per 1000 observed cells in Addicted participants is found to be 38.13±5.59 SD whereas in 15.67±2.73 (SD) in Healthy Participants, The z-score is found to be 27.96 which is significant at $p \leq .0.001$. **Conclusion**: Micronuclei test is most primitive and simple indicator for genotoxicity damage than chromosomal aberration and statistically there is increased micronuclei frequency in the grossly normal appearing oral mucosa of high risk tobacco chewers subjects.

KEYWORDS: Gutkha Panmasala, genotoxicity, micronucleated.

INTRODUCTION

Micronuclei (MN), also known as Howell–Jolly bodies, were first identified at the end of nineteenth century in red cell precursors by William Howell, an American, and Justin Jolly[1] are extra-nuclear bodies that contain damaged chromosome fragments and/or whole chromosomes that were not incorporated into the nucleus after cell division. MN can be induced by defects in the cell repair machinery and accumulation of DNA damages and chromosomal aberrations.[2] Micronuclei are commonly seen in cancerous cells and may indicate genomic damage events that can increase the risk of developmental or degenerative disease.

Tobacco and its product have accepted as important etiologic agents in oral cancer. Tobacco along with various ingredients like areca nut catechu lime, cardamom, permitted spices unspecified flavouring agents have been reported to possess cytotoxic, mutagenic and genotoxic properties.[3] Carcinogens, such as polyaromatic hydrocarbons nitrosamines and aromatic amines present in tobacco, upon deactivation in the liver to electrophilic intermediates, react with DNA to form covalently bound adducts. The formation of specific DNA adducts and the resulting mutations can be viewed as being responsible for oncogene activation and inactivation of tumor suppressor genes, leading to cancer development.[4] Tobacco usage in any form is associated with etiology of many diseases for many decades and any approach aimed at expending the detection of population sub-group at increased risk should be considered a high priority task. It may be possible to use genotoxicity assays to identify tobacco users to the DNA damaging effect over base-line. The peripheral blood cells were known to exhibit changes in cancer of various organs. Even as early as 1959, Nieburgs[5] found abnormalities in the polymorphonuclear leukocytes

(PMNS), he named it as malignancy associated changes (MAC).

Very few studies of micronuclei in peripheral blood leukocytes of tobacco addicted individual have been done in Vidarbha region of central India. Prediction of cancer development by genotoxicity analysis is a major challange to identify tobacco users at greater risk. Therefore, present study aimed to analyse tobacco related genotoxic effects in Kharrah, Gutkha, Panmasala chewers and tobacco products user of Vidarbha region of central India.

MATERIALS AND METHOD

Subjects: Healthy tobacco chewers (n= 60) and healthy non-tobacco chewers as controls (n=60) were enrolled in this study. Age of subjects in both groups was ranging from 30-50 years. Chewers had habit of chewing kharrah, ghutkha and panmasala containig mixture of tobacco, areca nut and other ingredients like catechu, lime and unspecified flavoring agents. All subjects had habit of chewing kharrah, gutkha, gutkha, panmasala and tobacco products 4 to 10 times per day for 10 years.

Selection Criteria:

Inclusion criteria: Study group included subjects of age group 30 to 50 years having habit of chewing kharrah, ghutka and panmasala since minimum of 10 years with normal oral mucosa morphologically and they were not known cases of oral cancer or any other oral pathology.

Exclusion criteria: Study group subjects should not be below 30 years and above 50 years, subjects having abnormal oral mucosa**,** having habit of chewing tobacco and its products since less than 10 years and having blood cancer, lung cancer and other cancer were excluded.

Study ethics: The study design and subject consent to participate in the study was ethically approved by hospital based ethical committee of the institute.

Sample Collection: The peripheral blood samples (V=2ml) were collected under sterile conditions by venipuncture into heparinised tubes.

Micronucleus test in peripheral blood

Two ml of venous blood from each subject was taken in heparinised vacutainers. After the samples collection, they were coded to ensure an unbiased assay of the samples. The samples were sent to the genetic laboratory, department of Anatomy, NKP SIMS at room temperature. A thin blood smears were prepared on the same day of sample collection. The slides were air-dried for at least 30 minutes before fixing at room temperature. Once slides prepared, fixation were carried out using freshly prepared cold 3:1 methanol and acetic acid solution for 10-15 minutes. The slides were removed from the fixative and washed twice in PBS (phosphate buffer solution). Slides were then stained immediately in 5% Geimsa solution and dried on a hot plate for 5 minutes. Slides were stored in slide boxes before examining under a microscope.[6]

A minimum of 1000 cells from each individual was screened for calculating frequency of nucleated cells (MNC). The identification of micronucleus was based on the criteria proposed Sarto et al.[7] Extra chromosomal cytoplasmic DNA fragments scored as micronucleus were 2-4um in diameter and had the same texture and intensity as the nucleus. Fragments scored as micronuclei were in the same focal plane as the nucleus.

RESULT

The micronuclei levels in patients with tobacco habits were compared with that of the control group and results were found to be statistically significant. The mean micronuclei level in peripheral blood leukocytes between tobacco habituated patients with normal mucosa and oral cancer patients was found to be statistically significant. Micronuclei can differentiate higher tobacco exposure in chewers than chromosomal aberration.

Mean age of participants were 42.45± 7.47(SD). The leucocytes stained with Gimsa stain, then the each slide was observed under Olympus® BX 21 microscope. The micronucleus frequency per 1000 observed cells in Addicted participants is found to be 38.13±5.59 SD whereas in 15.67±2.73(SD) in Healthy Participants, The z score is found to be 27.96 which is significant at $p \leq .0.001$.

Table 1: Micronucleus frequency per 1000 observed cells in Addicted and Healthy Participants.

Sr. No	Healthy Participants	Addicted Participants	Sr. No	Healthy Participants	Addicted Participants
1	12	34	**31**	20	39
2	11	35	**32**	14	31
3	13	33	**33**	12	42
4	17	38	**34**	18	46
5	15	39	**35**	16	33
6	18	31	**36**	16	45
7	16	42	**37**	15	47
8	18	46	**38**	13	34
9	17	33	**39**	14	35
10	19	45	**40**	15	33
11	14	47	**41**	18	38
12	12	32	**42**	20	39
13	11	38	**43**	14	31
14	10	48	**44**	16	42
15	20	32	**45**	13	46
16	17	35	**46**	16	33
17	19	37	**47**	17	45
18	18	49	**48**	18	47
19	17	34	**49**	15	32
20	12	35	**50**	17	38
21	11	33	**51**	19	48
22	13	38	**52**	13	32
23	15	39	**53**	19	35
24	18	31	**54**	14	37
25	17	42	**55**	12	49
26	17	46	**56**	14	34
27	18	34	**57**	19	35
28	11	35	**58**	20	33
29	15	33	**59**	18	38
30	17	38	**60**	17	39
			Mean Total	**15.67 ±2.73 SD**	**38.13±5.59 SD**

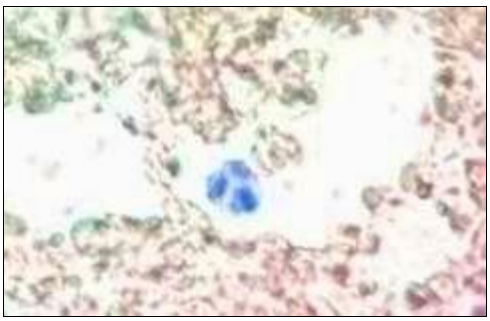

Fig 1: Micronucleus assay. Tertiary nucleus clearly visible.

Table 2: Statastical analysis of healthy participants & addicted participants with two-sided confidence interval 95%.

Result	*t* statistics	*df*	p-value[1]	Mean Difference	Lower Limit	Upper Limit
Equal variance	-27.966	118	<0.0000001	-22.5	-24.05	-20.87
Unequal variance	-27.966	86	<0.0000001	-22.5	-24.057	-20.863
		F statistics	*df*(numerator,denominator)		p-value[1]	
Test for equality of variance[2]		4.1927	59,59		0.000000135	

[1] *p-value (two-tailed)*

[2] *Hartley's f test for equality of variance*

DISCUSSION

Micronuclei(MN) assay is one of the most commonly used methods for measuring DNA damage in human populations because it is relatively easier to score than chromosomal aberrations.[8] If there is a marked increased in number of cells with micronuclei, it can be concluded that the chemical induces structural and / or numerical chromosomal damage. Micronucleus assay systems are very economical, require much less skill and much faster than these conventional tests. Since micronucleus assays reflect chromosomal aberrations reliably and rapidly, they are extremely useful for a quick assessment of chromosomal damage. DNA damage was found to be significant in epithelial dysplasia and carcinoma of the cervix using Single Cell Gel Electrophoresis (SCGE/Comet assay) technique.[9] Statistically significant result in DNA damage were found in the peripheral blood leukocytes with respect to the clinical staging and histopathological grading of oral squamous cell carcinoma.[10] It may possible to use genotoxicity assays to identify tobacco users to the DNA damaging effect over base line. Many of the substances contained in tobacco are genotoxic and therefore cytogenetic damage seems to be an excellent biomarker for determining effect of exposure to chromosome damaging agents in tobacco.[11]

There have been numerous attempts to establish or even develop tumour markers to determine the susceptibility of normal tissues to transform into cancer. Current predictive indication can be subdivided into morphologic and molecular. Among molecular predictive indicators, biomarkers of cytogenetic damage – chromosomal aberrations and micronuclei have been used as an important biological endpoint to study population at risk.[12] It has been hypothesized about direct association between the frequency of micronuclei in target tissues and cancer development, supported by different findings: like, increase in frequency of micronuclei in target tissues and lymphocytesin cancer patients.[13,14]

A correlation exists between carcinogenicity and genotoxicity for some agents who are able to increase micronuclei frequencies in humans and in animals, e.g., ionizing radiation,ethylene oxide, benzene, tobacco smoke.[15] These findings clearly suggest a causal link between micronuclei and cancer. The peripheral blood cells were known to exhibit changes in cancer of various organs. Even as early as 1959, Nieburg[5] found abnormities in the polymorphonulear leukocytes (PMNL) and he named it as malignancy associated changes. Chomet et al.[16] showed atypical nuclear changes in monocytes in routine peripheral blood smears of patients with malignant tumours.

Roias et al.[17] using the Comet assay, evaluated the DNA damage in exfoliated buccal mucosal cells of smokers and non-smokers. The extent of DNA damage was found to be significantly increased in the smoker group than in a non smoker group. It is quite probable that the leukocytes show a similar change in patients with tobacco habituation. To provide evidence to this assumption, the present study was carried out to evaluate DNA damage in peripheral blood leukocytes of patients with tobacco habituation, using the SCGE (Single cell gel electrophoresis) technique. Guttikoonda et. al.[10] noted statistically significant differences in DNA damage of peripheral blood leukocytes between tobacco users with normal oral mucosa and oral patients habituated to tobacco and higher mean values in cancer patients could be attributed to the continual insult of tobacco and its role in the progression to oral carcinoma.

The micronuclei test is a simple, practical, inexpensive, and non invasive screening technique for management of subjects under carcinogenic risk after exposure to genotoxic agents like tobacco and its products. Micronuclei test is better indicator for genotoxicity damage than chromosomal aberrations.[9] Stich and Stich[18] observed saliva of Pan Bahar (a commercially available combination flavour spices, fennel seeds, sugar, waxes, till seeds, colors, etc.) chewers was clastogenic to CHO cells. A very high frequency of micronuclei has been observed among tobacco users.[19,20] Similarly increased in frequency of micronuclei in “pan masala” consumers have also been reported by Gandhi and Kaur.[21] A correlation exists between carcinogenicity and genotoxicity for some agents who are to increase micronuclei frequencies in humans and in animals, e.g., ionizing radiation, ethylene oxide, benzene, tobacco smoke.[22]

The Comet assay was used to assess single-strand breaks in cervical carcinomas, oral neoplasm and potentially malignant lesions of cervix and oral cavity. Using the Comet assay, it has been shown that the patients with potentially malignant and cancerous lesions of cervix exhibited increased DNA damage levels in peripheral blood leukocytes (PBLs) when compared to normal individual.[9] According to a Pilot study conducted in PBLs of patients with oral squamous cell carcinoma by SCGS, a significant increase in DNA damage depending on the clinical staging and histopathological grading.[10] This result was attributed to SCGE assay, a sensitive technique to identify DNA damage in PBLs, even before morphological changes become apparent. As all the patients in the study group were tobacco users, the observed DNA damage could be attributed to tobacco habituation. These findings of various workers in recognizing changes in PBLs lend support to the concept of a systemic host response in malignancy.

CONCLUSION

Micronuclei test is most primitive, inexpensive, noninvasive screening technique and simple indicator for genotoxicity damage than chromosomal aberration in kharrah, ghutkha and panmasala chewers. There is statistically increased micronuclei frequency in the grossly normal appearing oral mucosa of kharrah, ghutka chewers subjects. Therefore the finding of increased

micronuclei in peripheral blood leukocytes in tobacco chewer subjects with a clinically normal oral mucosa, much before the alterations are evident, suggest that the micronuclei evalution in peripheral blood leukocytes is a sensitive indicator of tobacco insult.

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