ejpmr, 2019,6(5), 521-525

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

COMPARATIVE CYTOTOXICITY ANALYSIS OF EXFOLIATED MUCOSAL EPITHELIAL CELLS IN MAVA, TOBACCO AND PANMASALA CHEWERS

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Article Received on 03/03/2019 **Article Revised on 24/03/2019** **Article Accepted on 16/04/2019**

ABSTRACT

The rationale of the present study was to evaluate cytological alterations in panmasala chewers and compare it with other chewing stuff's such as tobacco and mava chewers. The buccal cytome assay was used to determine nuclear anomalies. The assay was applied to exfoliated buccal mucosal cells of 100 subjects (nonchewers-50 and different groups of chewers-50) having different habits, such as tobacco, mava, panmasala and arecanut chewing. 1000 cells per individual were examined microscopically. The results showed significant difference in cytotoxicity of all the products consumed by people. The mava chewers showed significantly higher anomalies in buccal cells followed by tobacco and panmasala. This might be due to physical damage caused by arecanut and chemical exothermic reaction due to presence of lime; these two processes goes hand in hand which could be potent reason.

KEYWORDS: Mava, panmasala, tobacco, micronucleus, binucleus, karyohexis, karyolysis, pyknotic, fragmented cells.

INTRODUCTION

Oral cancer is one of the most common cancers in South and Southeast Asian countries.[1] The chewing habit associated with the use of arecanut and tobacco is more prevalent in South Asian countries and is spreading to Western countries among settled Asian migrant communities.[2,3] Different forms of smokeless tobacco (SLT) such as mava, gutkha, panmasala etc are very popular and practice of chewing these is a leading cause for increasing health problem, especially in continents like Asia and Africa. Mava is a mixture of tobacco, arecanut and lime water where as panmasala contains powdered or crushed form of ingredients like catechu, lime, arecanut, cardamom and other flavouring agents. Mava, tobacco and panmasala chewing is very dangerous for health. Inspite of this known fact their consumption is becoming very popular. However, there are around 100 million users of SLT products in developing countries like India.[4] Among all the cancers occurring worldwide oral cancer ranks eighth position in cancer incidence.[5] With the adoption of this cancer causing habits such as chewing mava, gutkha, khaini and panmasala the global burden of cancer continues to increase in economically developing countries. Epidemiological data have shown a correlation between the use of tobacco and arecanut products, premalignant lesions of the oral cavity, and incidence of oral cancer.[5] Annual mortality ascribed to tobacco use in India, has been estimated to be 1 million.[6] Oral cancer is one of the top ten most common cancers with 575000 new cases and 320000 deaths per year worldwide.[7] The prevalence of oral cancer in the world is correlated with the pattern and level of consumption of tobacco products.[8] Tobacco and betel nut chewing along with other ingredients like catechu, lime, permitted spices and unspecified flavouring agents have been reported to have cytotoxic, mutagenic and genotoxic properties.[9,10] yet its consumption has continued to increased among all age groups. In spite of awareness among the people about these products they find it helpless to quit.

Oral cancer arises through an accumulation of genetic alterations, including chromosomal alterations, DNA changes and/or epigenetic alterations. Exfoliative cytology, which is a simple, non-invasive diagnostic technique, could increase the chances of earlier detection of premalignant and malignant lesions.[11,12] One of the best techniques for studying the effects of environmental factors on genetic stability in human cells is the micronucleus (MN) test.[13] MN may be products of early events in carcinogenesis, especially in the oralcavity, which is directly exposed to cigarette smoke.[14] Apart from this there are many various anomalies that depicts the genomic instability.

The objective of the present study was to detect various nuclear anomalies like binucleus, micronuclei, karyohexis, keryolysis, picknosis from exfoliated buccal mucosal cells of the chewers.

MATERIALS AND METHODS

Age matched individuals were taken into consideration. Prior to sample collection volunteers were made to explain the purpose of study and an informed consent form was filled. Inclusion criteria of the study are (1) those subjects consuming arecanut, tobacco, mava and panmasala, (2) healthy individuals with age group less than 65 years, and (3) those who were willing to participate in the study. Exclusion criteria includes (1) Old age (65+ years) people having chewing habit, (2) smokers, (3) individuals consuming Alcohol, (4) those having any kind of pre-cancerous lesion, and (5) those who were not willing to participate

For collecting the buccal cell the subjects were asked to gargle their mouth thoroughly with listrin so that bacteria and their debris don't hinder in scanning. A small headed soft brush (cytome brush) was used to collect the cell from the inner wall of both the cheeks and fixed in fixative containing 1:3 aceto- methanol. The slides were prepared from cell suspension stained with Giemsa for 2-3 min dried and observed under microscope. For scoring 1000 cells were scanned per sample. Nuclear anomalies were counted.[13,15]

Data were subjected to statistical analysis using graphpad prism (version 0.5). One-way analysis of variance (ANOVA) followed by Tukey's test was performed to compare MN, BN, KR, KL, RUP and PK between different groups.

RESULTS

The representative photographs of micro nucleated cell, nuclear buds and binucleated cell in buccal epithelial cells have been shown in Fig. 1. Table 1 shows different types of anomalies observed in buccal cells of subjects having different type of chewing habit. The results shows that binucleus and micronucleus was found more than the other kind of anomalies, of which panmasala chewers exhibited highest number of binucleus (9.54±1.19) while micronucleus was observed highest in tobacco chewers (15.07±1.53). The extreme stages such as karyohexis, karyolysis and pykotic cells were observed significantly higher in mava and panmasala consuming group. Mava showed significantly higher amount of keryohexix (11.80±1.64), followed by tobacco (11.80±1.64). karyolysis was also observed highest in buccal cell of mava chewers (12.32±1.20) whereas cells with pyknotic nucleus were observed highest in tobacco chewers (9.94±1.29) than in mava chewers (9.37±0.20). Ruptured and fragmented nucleus was also found highest in mava chewers.

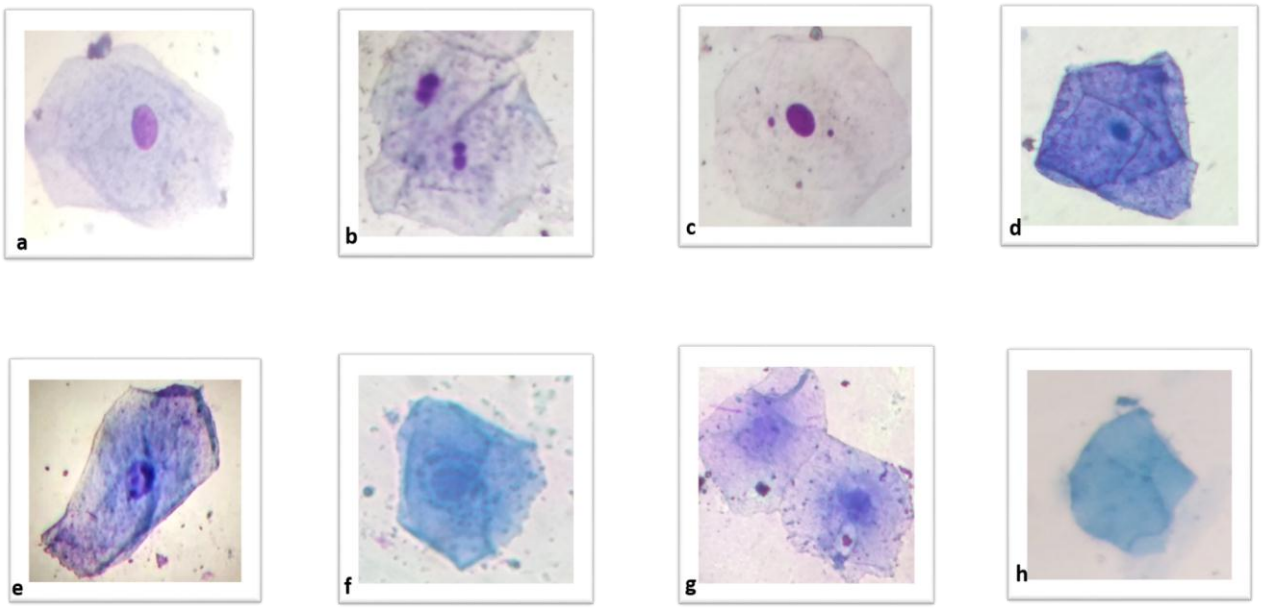
On analysis of frequency of chewing in all three groups (mava, panmasala and tobacco) was found to same. Similarly there was no significant difference found in number of years of chewing; as well as in duration of chewing material (tobacco, panmasala and mava) was on an average the same.

Table 1: **Different types of anomalies found in buccal cell of subjects having different type of chewing habit**

Groups	Binucleus	Micronucleus	Karyohexis	Keryolysis	Pyknotic	Ruptured	Fragmentation
Control	1.35 ±0.11^{b,c,d}	0.92 ±0.23^{b,c,d,e}	0.00 ±0.00^{b,c,d,}	0.03 ±0.02^{b,c,d,e}	0.35 ±0.08^{b,c,d,e}	0.06 ±0.04^{b,c,d}	0.06 ±0.05^{b,c,d}
Mava	8.78 ±0.87	15.66 ±1.12	13.48 ±1.28	12.36 ±1.58	9.72 ±0.83	7.03 ±0.48	5.84 ±0.68^{a}
Tobacco	10.90 ±0.90	16.00 ±2.14	11.80 ±1.97	6.70 ±1.63^{b}	10.30 ±1.35	6.22 ±1.20	5.20 ±1.13^{b}
Panmasala	10.00 ±1.84	15.10 ±0.982	8.50 ±1.40^{a,b}	3.50 ±1.09^{b}	5.20 ±1.45^{b,c}	4.10 ±0.93^{b}	3.28 ±0.44^{a}
Arecanut	6.20 ±0.58	10.60 ±1.69	0.00 ±0.00^{b,c,d}	0.00 ±0.00^{b}	0.20 ±0.20^{a,b,c,}	0.00 ±0.00^{b,c,d}	0.00 ±0.00^{b,c,d}

The mean difference is significant at the 0.05 level

a: as compared to control; b:as compared to mava; c: as compared to tobacco; d: as compared to panmasala; e: as compared to arecanut



a: Normal cell; b: Binucleates; c: Micronucleus; d: pyknotic cell; e: Ruptured Nucleus; f: Fragmented Nucleus; g: karyohexis; h: karyolysis

Fig 1: Buccal cell anomalies observed in chewers.

DISCUSSION

MN is studied from exfoliated buccal mucosa of tobacco chewers. MNs are fragments or whole chromosomes, which did not reach spindle poles during mitosis and remained encapsulated at telophase in a separate nucleus, they are extent of chromosomal break in early cell division i.e is they are separated from the nucleus and is formed by condensation of acrocentric chromosome fragments or whole chromosome.[16] Besides MN presence, described other NA as phenomena that can occur not only during physiologic cellular differentiation but also during death cell[17] with DNA damage.

Occurrence of increased frequencies of KL anomaly has significance as these occur in the pre-keratinization process.[18] This anomaly represents cytotoxicity, which is also evident in necrotic cells.[19] A study carried out among firefighters indicated that in addition to elevated MN frequency, raised prevalence of several other nuclear anomalies like N Buds, binuclei, karyorrhexis and karyolysis were also exhibited.[20] Karyolysis is associated with cytotoxicity, and karyorrhexis accompany apoptosis.[21] Furthermore, these biomarkers offer additional endpoints for possible evaluation of chromosomal instability and gene amplification (via nuclear buds), cytokinesis arrest due to aneuploidy (by means of binucleated cells), and different cell death events (e.g., karyorrhectic and pyknotic cells).[22] The mechanisms through which each of these abnormalities is produced or its biological meaning is still unknown. MN detection can also be used to describe beneficial effects against genotoxicity produced by changes in lifestyle and/or as a consequence of supplements intake.[23] It has been described that the choice and the amount of foods and supplements intake have influence on cellular concentrations of micronutrients that are required either as substrates (e.g.,5,10-methylene tetrahydrofolate) or cofactors (e.g., Zn and Mg) in DNA synthesis and repair.[24]

Accurately MN presence in cells is observed in epithelial tissue exfoliated cells, derived from cell division that takes place in the basal layer and then they migrate towards the surface within 5 to 14 days. In this manner, the epithelial tissue can reflect the damage occurred at this time. Oral mucosa cells are useful for determining exposure to compounds not only because they are the first line of encounter with several environmental factors like tobacco and alcohol, but also since several systemic conditions and treatments limit the proliferation rate of epithelial cells.[25] Furthermore, it is important to outline the fact that nearly 60% of the oral mucosa surface is stratified non-keratinized epithelia, which allows cells in the most superficial layer to maintain their nuclei well defined and almost intact, a characteristic that favors colorant absorption and eases observation and proper identification of nuclei cells morphologic characteristics with the use of a microscope. Moreover, keratinocytes are big cells with abundant cytoplasm[26] and they can be studied without the need of a cell culture, which makes this test both simple and cheap.

In the present study, we observed a low number of MN cells in the oral mucosa of the control groups (non-chewers). However, the mean number of MN in chewers was similar to findings reported previously in oral cancer samples.[27] In the current study, we found that MN and BI was observed almost in all the groups where as KH, KL, PK and FRAG in chewers were the most frequent anomalies as compared to that of normal. Many

investigators have similarly shown higher frequency of PK and KL in chewers.[28,29] The extreme stages such as karyohexis, karyolysis and pykotic cells were observed significantly higher in mava and panmasala consuming group. Mava showed significantly high amount of karyohexix (11.80±1.64), followed by tobacco (11.80±1.64). keryolysis was also observed highest in buccal cell of mava chewers (12.32±1.20) and in cells with pyknotic nucleus were observed highest in tobacco chewers (9.94±1.29) than in mava chewers (9.37±0.20). Ruptured and fragmented nucleus was also found highest in mava chewers. Likewise, in the study by Sharma et al which the same anomalies as ours were studied, KL, PK and MN in chewers and smokers were the most frequent anomalies.[30,31] MN formation has been observed in precancerous lesions of the oral cavity of chewers.[32] Since the prolonged use of the chewing items such as arecanut, pan masala/gutkha can generate a risk of developing nuclear anomalies and ultimately into different types of oral cancer, it becomes necessary to screen the population for its possible risk.

Results of the present study affirms that any mixture containing areca nut and tobacco have genotoxic and cytotoxic potential that induces the nuclear anomalies in the buccal mucosa cells indicating higher oral diseases among chewers.

Mava chewers showed highest anomalies in buccal cells. This might be due to physical damage caused by arecanut and chemical exothermic reaction due to presence of lime, these two process goes hand in hand which could be potent reason.

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