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LYMPHOCYTES DNA DAMAGE FROM PANMASALA, GUTKHA, KHARRAH, CHEWING TOBACCO USERS AND CHAIN SMOKERS OF CENTRAL INDIA, BY USING SINGLE-CELL GEL ELECTROPHORESIS ASSAY

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ABSTRACT

Background: Oral submucous fibrosis (OSMF) is a chronic, complex potential potent pre-cancerous condition characterized by juxta-epithelial inflammatory reaction and progressive fibrosis of the lamina propria and deeper connective tissues. As the disease progresses, the jaws become rigid to the point that the sufferer is unable to open his mouth. These events are further influenced by exposures to carcinogenic agents including panmasala, gutkha, kharrah and tobacco consumption. Single-cell gel electrophoresis assay or comet assay is a sensitive and rapid method for DNA strand breaks; it further provides information on amount of damage among individual cells. **Aim and Objective:** In this study, we aimed to analyse the lymphocyte DNA damage from panmasala, gutkha, kharrah and chewing tobacco users of central India, by using Single-cell gel electrophoresis assay. **Materials & Methods:** The peripheral blood samples from 60 addicted participants of age group 30-70yr were collected under sterile conditions in heparinised tubes used for Leukocytes culture and 30 healthy non-cancerous participants of same age group ware taken as control. The informed consent was obtained. The comet assay conducted using three well OxiSelect™ Comet Assay Kit and stained with vista green dye, the slides were analysed by using Olympus® BX 51 fluorescence microscope. The results were statistically analysed. **Result:** Mean age of addicted participants were 45.31± 16.24 (SD) and Mean age of control participants were 40.55± 11.15 (SD). Obtained comets were analysed by the CometScore 1.5 Software. The Comet score analysis shows that the mean % TDNA (Tail DNA) of comet in Leukocytes of addicted participants is found to be 32.61%±3.38(SD) than 7.16%±3.18(SD) mean % TDNA of control participants. **Conclusion:** It can be conclude that addiction to panmasala, gutkha, kharrah and chewing tobacco can damage DNA of peripheral blood leukocytes.

KEYWORDS: juxta-epithelial, CometScore, panmasala, gutkha.

INTRODUCTION

Oral submucous fibrosis (OSMF) is an oral precancerous condition characterized by inflammation and progressive fibrosis of the submucosal tissues resulting in marked rigidity and trismus.[1] In 1966, Pindborg[1] defined OSMF as "an insidious chronic disease affecting any part of the oral cavity and sometimes pharynx. It is associated with juxta-epithelial inflammatory reaction followed by fibroelastic changes in the lamina propria layer, along with epithelial atrophy which leads to rigidity of the oral mucosa proceeding to trismus and difficulty in mouth opening." Other terms used to describe this condition are juxta-epithelial fibrosis, idiopathic scleroderma of the mouth, idiopathic palatal fibrosis, submucous fibrosis of the palate and pillars, sclerosing stomatitis, and diffuse OSMF.[2]

As the disease progresses, the jaws become rigid to the point that the sufferer is unable to open his mouth. These events are further influenced by exposures to carcinogenic agents including panmasala, gutkha, kharrah, tobacco consumption and smoking.[3-4] Single-cell gel electrophoresis assay or comet assay is a sensitive and rapid method for DNA strand breaks; it further provides information on amount of damage among individual cells.

The condition is well recognized for its malignant potential and is particularly associated with areca nut chewing, the main component of betel quid. Betel quid chewing is a habit practiced predominately in Southeast Asia and India that dates back for thousands of years. It is similar to tobacco chewing in westernized societies.[5]

Various researchers have studied the adverse effects of carcinogenic agents using various research tools although the increased knowledge of these mechanisms unknown. In our study we used the advance assay method like Single-cell gel electrophoresis assay its sensitivity and rapidity for DNA strand breaks study in blood leukocytes provides information on amount of damage among individual cells. The analysis of the comet using software tools further provide more precise statistics to affirm result.

This study is carry out with an aimed to perform the comet assay on peripheral blood leukocytes culture of patients with addiction and without addiction, to give significant relation between Leukocytes DNA damage and addiction of carcinogenic agents, if any.

AIMS AND OBJECTIVES

In this study, we aimed to analyse the lymphocyte DNA damage from panmasala, gutkha, kharrah and chewing tobacco users of central India, by using Single-cell gel electrophoresis assay.

MATERIAL AND METHOD

The present study was carried out during the period of Dec. 2014 to Oct. 2016.in the Department of Anatomy, of NKP Salve Institute of Medical Sciences and research Centre, Nagpur India.

Patient Selection

This study comprise participant of all age, addicted to panmasala, gutkha, kharrah and chewing tobacco users of central India The informed consent was obtained from the participants. The 30 addicted participants from OPD of our institute. The peripheral blood samples were taken for study. The cases with regular panmasala, gutkha, kharrah and chewing tobacco users for more than six month will be included for the study and the all types of oral pathological diseases, cancer patients will be excluded from the study. The study was approved by the Institutional Ethics Committee of our college.

Samples Collection

The peripheral blood samples (V = 2 ml) was collected under sterile conditions by venipuncture into heparinized tubes for comet assay from addicted and non addicted participants from Hospital OPD.

Tissue culture of human blood leukocytes cells

Human peripheral blood leukocytes culture

Cell culture was carried out as per standard protocol to acclimatize the leukocytes cells, 200ul blood was cultured in 1 ml of RPMI Medium containing 10 foetal calf serum in a single well of a 6 well plate and was incubated for 1hr 30 min on appropriate CO2 pressure at 37°C.

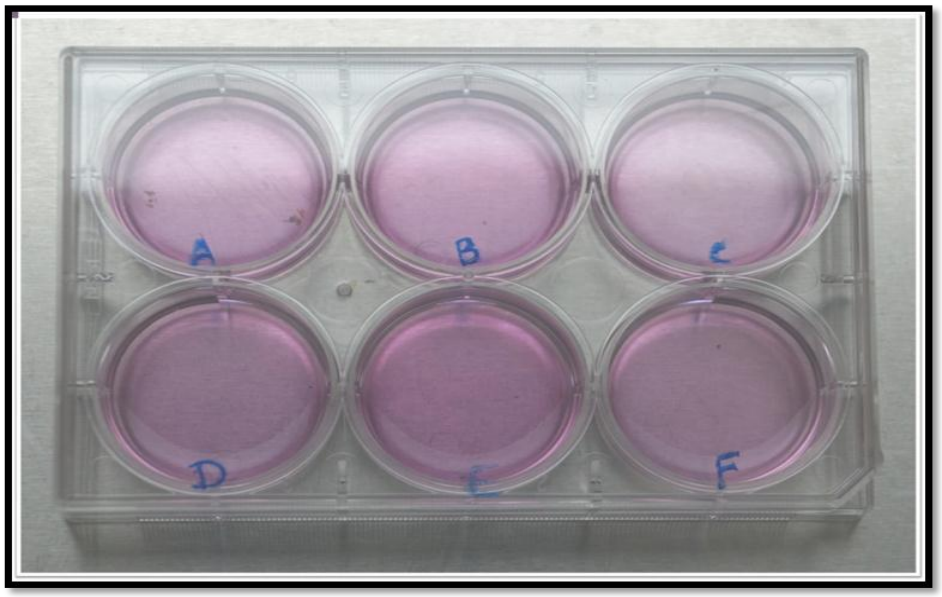


Fig. 1: Wells NEST® Cell Culture plate.

The Comet Assay

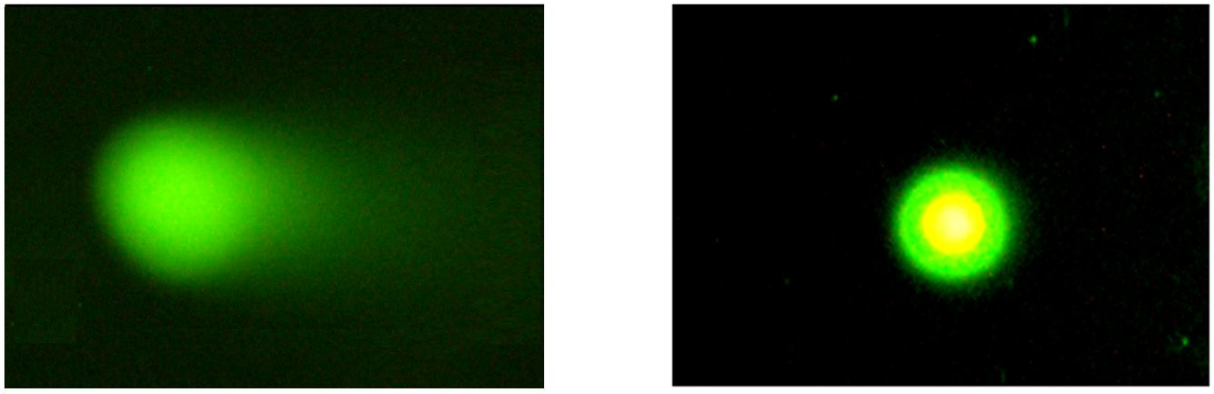
The comet assay conducted using three well OxiSelect™ Comet Assay Kit as per the standard protocol and stained with vista green dye, then the each slide was analyzed by using Olympus® BX51 fluorescence microscope. DNA damage was further quantified by visual classification of cells into categories of 'comets' corresponding to the amount of DNA in the tail by using Comet Score 1.5 Software.

Statistical Analysis

Statistical analysis was carried out using the Epi Info® (version 6.0). The cell viability was calculated as subject means with standard deviation. Statistical significance was defined at $P \leq 0.05$.

OBSERVATIONS AND RESULTS

Mean age of addicted participants were 45.31± 16.24 (SD) and Mean age of control participants were 40.55± 11.15 (SD). The comet stained with vista green dye, then the each slide was observed under Olympus® BX 51 fluorescence microscope.



a b

Fig. 2: Comet assay (a) Participants addicted to carcinogenic agents (b) Healthy non-addictive medical students.

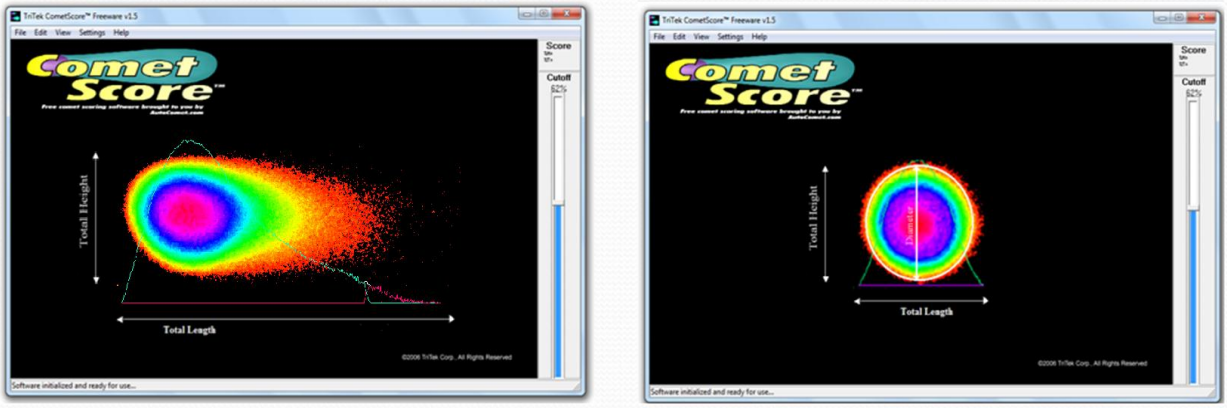


Fig. 3: Comet assay analysis by comet score (a) Participants addicted to carcinogenic agents (b) Healthy non-addictive medical students.

The comets were analysed by the CometScore 1.5 Software. The Comet score analysis shows that the mean % TDNA (Tail DNA) of comet in Leukocytes of addicted participants is found to be 32.61%±3.38(SD) than 7.16%±3.18(SD) mean % TDNA of control participants.

Table 1: TDNA% after comet assay analysis by CometScore

(a) Participants addicted to carcinogenic agents

Sr. No	Mean TDNA%(px)
1	25.93
2	30.27
3	33.69
4	32.55
5	28.24
6	31.46
7	28.58
8	29.20
9	32.43
10	32.73
11	28.07
12	33.64
13	35.69
14	35.51
15	34.42
16	35.95
17	35.34
18	36.14
19	35.82
20	30.39
21	26.03
22	27.92
23	34.74
24	29.54
25	35.51
26	34.50
27	34.41
28	35.85
29	35.66
30	37.99
Mean TDNA%	**32.61%±3.38SD**

(b) Healthy non-addictive medical students of institute

Sr. No.	Mean TDNA%(px)
1	5.65
2	2.64
3	9.54
4	14.25
5	6.27
6	3.47
7	6.74
8	10.41
9	7.55
10	4.66
11	1.44
12	11.02
13	7.26
14	4.63
15	7.22
16	8.22
17	7
18	8.02
19	7.36
20	0.89
21	7.33
22	12.89
23	4.28
24	6.71
25	7.64
26	7.6
27	9.97
28	11.4
29	9.47
30	3.4
Mean TDNA%	**7.16%±3.18SD**

Mean % TDNA of comet in Leukocytes of addicted participants = ***32.61%±3.38 (SD).*** Mean % TDNA of comet in Leukocytes of control non-addicted participants. ***=7.16±3.18 (SD)***

DISCUSSION

According to Joti et al[6] the addiction is directly related to the tail length. The highest DNA migration was found among gutkha chewers with smoking habit. SCGE or Comet assay in buccal epithelial cells is easier and a safe method to detect DNA damage among humans.[7] When the amount of ROS generated in cells increases from the capacity of the normal detoxification system then oxidative stress leads to cellular damage, along with the DNA damage.[8] DNA damage can occur as single-stranded (ss) breaks or doublestranded (ds) breaks.[9] The main objective of the study was to evaluate the extent of the DNA damage due to various addictions. In the present study the higher values in comet tail length were observed among gutkha chewers along with smoking. The tobacco present in cigarette/beedi induces DNA adducts and oxidative DNA damage in human tissues. The formation of carcinogens may lead to DNA mutation and by disturbing the protein function may lead to cancer.[10,11] The tobacco-specific nitrosamines can induce miscoding in the DNA that could result in the tumourigenic process in the oral cavity.[12] Pan masala or smokeless tobacco causes genotoxicity that affects DNA repair pathways.[6] In smokers, comet tail length was found to be more as compared to the non-users (control) group which may be due to oxidative stress in smokers. This causes an imbalance between the formation of reactive oxygen species (ROS) and the ability to neutralize ROS.[13] The formation of the DNA adduct is the initiating step in the process of carcinogenesis. Pan masala and gutkha also contain various irritating substances that make the skin lose its elasticity.[14] The main carcinogens in gutkha are derived from their ingredients (arecanut, catechu, and tobacco). A high level of nitrite and nitrate reductase activity has been reported in the saliva of gutkha chewers.[15,16] There are reports for the generation of ROS by the aqueous extract of arecanut and catechu leading to the genotoxic damage in buccal epithelial cells.[17] The occurrence of oral cancer has been well documented independently in association with oral habits such as smoking, betel quid chewing and tobacco chewing.[18,19] These oral habits have also been associated with DNA damage. Comet assay is used for the biomonitoring study and the tail length has been the most commonly used parameter for DNA damage measurement.[20] In our present study the highest DNA damage was observed among the gutkha + smoking group. Gutkha is a mixture of arecanut, catechu, lime, cardamom, unspecified flavouring agents, and tobacco. Arecanut is the main component of gutkha responsible for the oral submucosis fibrosis (OSMF).[21] In our earlier studies the high frequency of micronucleus was found among gutkha users.[22] The high frequency of micronucleus was also found among OSMF patients (gutkha chewers).[23] However, earlier studies have shown that the ROS produced by arecanut is responsible for the initiation of OSMF.[24] The aqueous extract of *N*-nitroso compounds related to arecanut, that is, 3-(methylnitrosamino) proprionitrile is highly cytotoxic and genotoxic in cultured human buccal epithelial cells, responsible for the induction of tumours among betel quid chewers.[25]

Speit G et al[26] performed a comprehensive investigation on blood samples from smokers and non-smokers. Because tobacco smoke is a well-documented source of a variety of potentially mutagenic and carcinogenic compounds, smokers should be a suitable study group with relevant mutagen exposure. Here, we report our results for the first sample of 20 healthy male smokers and 20 healthy male non-smokers. Baseline and benzo(a)pyrene diolepoxide (BPDE)-induced effects were analysed by two investigators using two image analysis systems. The study was repeated within 4 months. Furthermore, the influence of a repair inhibitor (aphidicolin, APC) on baseline and BPDE-induced DNA damage was comparatively analysed. In all experiments, a reference standard (untreated V79 cells) was included to correct for assay variability. None of these approaches

revealed significant differences between smokers and non-smokers. Although more data is needed for a final conclusion, this study indicates some limitations of the comet assay with regard to the detection of DNA damage induced by environmental mutagens in peripheral blood cells.

Gichner et al[27] measured the DNA repair as the reduction of the tail moment values as a function of time after the mutagen treatment ended. DNA damage in leaf nuclei of EMS-or ENU-treated tobacco plants persisted over a 72h recovery period. However, a reduction of the SCGE tail moment values in nuclei isolated from leaves was observed over a 4-week period of recovery. Newly emerged leaves expressed a lower level of DNA damage due to more efficient repair and/or dilution of initial DNA lesions during cell division. After 24h recovery, leaf nuclei from cells exposed to 20 or 40Gy of gamma-radiation expressed complete DNA repair. These data indicate that DNA lesions induced by alkylating agents are not readily repaired and persist beyond 4 weeks. Enzymes necessary to repair gamma-induced DNA lesions are fully functional in non-replicating leaf cells and single and double strand breaks are rapidly repaired.

In the comet assay Roy et al[28] found percent of tail DNA gradually increases among the groups and has statistical significance. Spearman correlation revealed strong positive correlation between the arsenic exposed peoples and the binucleated cells ($r=0.4763$; $P<0.001$). Amount of chewing tobacco had significant positive correlation with micronucleus frequency ($r=0.268$; $P<0.05$) and karyolitic cells ($r=0.217$; $P<0.05$) and also in the percentage of tail DNA ($r=0.5532$, $P<0.001$). A statistically significant increase in glucose content and decrease in hemoglobin content as well as acetylcholine esterase in the blood of exposed individuals was observed. Our preliminary study indicate that population exposed to arsenic through drinking water may become more susceptible towards chewing tobacco induced nuclear damage as evaluated by buccal cytome assay and comet assay.

CONCLUSION

From this research it can be conclude that the panmasala, gutkha, kharrah and chewing tobacco may damage DNA of peripheral blood leukocytes though these carcinogenic agents contact only on oral cavity.

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