EVALUATION OF MICRONUCLEI IN SMOKELESS TOBACCO USERS FROM HALBA AND GOND TRIBES OF DURG DISTRICT OF CHHATTISGARH, INDIA

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In the present study genotoxic effect of smokeless tobacco on halba and gond tribes of Durg district of India was examined. From both tribes three set of experiments were designed comprising 20 population each for 1 year exposure, 5 years exposure and 10 years exposure of smokeless tobacco beside a parallel control group was maintained. To test genotoxicity, micronuclei test was performed from buccal epithelium and maximum micronuclei were found among population with 10 years exposure (6.25 ± 0.354 in Halba tribes and 5.80 ± 0.360 in Gond tribes) in comparison to control (0.40 ± 0.184 in Halba and 0.50± 0.136 in Gond tribes). The development of micronuclei in buccal epithelia explains damage of genetic material under influence of smokeless tobacco exposure which may leads to carcinoma like pathogenecity.

Consumption of tobacco is an old practice in India. It has been considered that tobacco was introduced in India by Portuguese about 400 years ago¹. Today a large number of tobacco products are available for consumption in India. Cigarette and Bidi are commonly used for tobacco which is used by burning tobacco and smoking. Smokeless tobacco (ST) or unburn tobacco is another form of tobacco which is used by more than 40% of population in India². Various forms of ST commonly available in India are Gutkha, Zarda, Khaini, Gudakhu, betel etc. All these forms of ST are blended with lime, areca nut, catechu and flavouring agent etc. to make it easily acceptable by the consumers. Nicotine the main constituent of tobacco is responsible for the addictive property of tobacco. ST also contains many carcinogenic compounds like 4-(methyl nitrosamino)-1-( pyridyl)-1- butanone (NNK), N-nitrosonornicotine (NNN) , N-nitrosoanatabin (NAT), N-nitrosoanabinobaspin, poly nuclear hydrocarbons, certain heavy metal like Polonium, Uranium etc. The extensive marketing of gutkha and other ST products leads to its widespread consumption among young school going children and make them addicted to it³. Many authors had reported the health effects of ST consumption which leads to various types of diseases. Gupta et al.⁴ determined the prevalence of major cardiovascular diseases in habitual tobacco users. ST consumption also affects the fertility of human population. A decrease in ejaculate volume, sperm density and total sperm count was observed in tobacco users by Dikshit et al.⁵. Said et al.⁶ also reported a decrease in sperm quality and azoospermia in men undergoing infertility evaluation is associated with habit of chewing tobacco in them. ST consumption among is also associated with still birth in early gestational period⁷. Mukerjee et al.⁸ reported the clastogenic effect of pan (betel) masala which affects the germinal cells of testes. ST consumption also affects the immune system⁹ and decrease in level of haemoglobin in pregnant women using ST was reported by Subromoney and Gupta¹⁰. Nair et al.¹¹ estimated that about 5 million young Indians are suffering from oral submucosa fibrosis, a precursor of oral cancer. Jyoti et al.¹² reviewed a large number of studies revealing genotoxicity of Pan Masala and gutkha.

According to World Health Organization (WHO) appraisal, tobacco causes 5.4 million death a year worldwide¹³-¹⁴. WHO also estimated that during 20th century, 100 million deaths occurred due to that is likely to increase to One billion in the 21st century if current trend continues. Several studies in various countries have identified the use of ST as a cause of oral cancer. Dikshit et al.¹⁵ conducted a study between 2001 - 2003 in India and concluded that tobacco related cancer is main cause of death due to cancer in India. A case control study in USA reported a high risk of oral cancer in ST users than non users a similar study in India reported two to 14 fold increase in the risk of oral cancer among chewers of tobacco (or tobacco plus lime) who are also non smokers¹⁶. A fivefold increase in risk of oesophageal cancer among chewers of tobacco in case control study from Assam, India had also reported¹⁷. The micronuclei are extranuclear cytoplasmic bodies associated with the chromosomal aberrations are induced in oral exfoliated cells. These are induced by variety of substances which are carcinogenic and genotoxic in nature and present in tobacco, areca nut, alcohol etc. The genotoxic effect of carcinogen and mutagens are determined by the...
induction of micronucleated cells\textsuperscript{18}. These are also observed in buccal exfoliated cells of people who are exposed to organic solvents, antineoplastic agents, diesel derivatives, polycyclic aromatic hydrocarbons, paints and solvents containing lead and drinking water contaminated with arsenic\textsuperscript{19}.

Micronuclei have been used as an indicator of exposure to genotoxic agent based on radiation studies conducted by Brenneke and Mather since 1937 as it is associated with chromosomal aberration\textsuperscript{20}. The assessment of micronuclei in exfoliated cells is a promising tool for the study of epithelial carcinogens and can be used to detect chromosome breakage and mitotic interference\textsuperscript{21}. The direct correlation between the micronuclei formation and genomic damage make the micronuclei assay an efficient alteration to the metaphase analysis\textsuperscript{18}. Based on above text present study is aimed to find out genotoxicity among smokeless tobacco users from Halba and gond tribes of Durg Chhattisgarh India. Our hypothesis is the target population is seriously ignorant about their health concern and frequently using smokeless tobacco, they are even hesitant to visit primary health centres.

**MATERIAL AND METHODS**

For this study we have selected tribes namely Gond and Halba from Durg district of Chhattisgarh, India to assess the genotoxic effect of ST on buccal epithelium. We have divided the two tribes into three groups each based on period of consumption of ST in any form viz. Up to 1 year of consumption, Up to 5 years of consumption and up to 10 years of consumption. Total 20 samples were collected from each group of individual belonging to age group between 15 to 40 years for each tribe. A healthy control in age match group was also maintained for both the tribes who are free from any form of addiction. Buccal epithelium cells were collected from oral cavity of each individual after rinsing their mouth thoroughly with tap water and scrapping of buccal epithelium stored in sterilized eppendorf's tube containing 1ml Phosphate buffer saline (1X PBS = NaCl, KCl, HCl, KH\textsubscript{2}PO\textsubscript{4}, Na\textsubscript{2}PO\textsubscript{4}, pH= 7.4) with the help of clean sterilised tooth picks. The cells were washed thoroughly with phosphate buffer by centrifugation; smear was prepared from cell suspension by smearing in clean sterilized slides and was fixed with methanol and air dried. The smears were than stained with Geimsa Stain and washed thoroughly with running water, air dried and observed under microscope (40x and 100x) to study the micronuclei formation in buccal epithelium. All the data were than statistically validated by 't' test using MINITAB 14.

**RESULTS AND DISCUSSION**

On observing the slide for formation of micronuclei in buccal epithelium of tobacco users maximum number of micronuclei per 100 cells was observed in individuals consuming smokeless tobacco up to 10 years for both the tribes. In case of Halba tribes individuals consuming ST for up to 10 years the mean value of micronuclei per 1000 cells were found 6.25± 0.354 followed by 3.05± 0.285 for up to 5 years of consumption and 0.55± 0.135 for up to one year of consumption and in case of control mean value was minimum i.e. 0.40 ± 0.184 (Table-1). Similarly in case of Gond tribes maximum number of micronucleated cells were found in consumers up to 10 years 5.80 ± 0.360 followed by up to 5 years and 1 years of consumption i.e. 2.45 ± 0.328 and 1.00 ± 0.178 respectively, and control set showed minimum number of micronuclei per 1000 cells i.e. 0.50± 0.136 (Table-2).

In Halba tribes the difference of micronuclei between control and 1 year user was found insignificant (t = 2.021>0.66 at 5% P) but among control to 5 year user (t = 2.021 <7.81 at 5%P), control to 10 years user (t = 2.021 <14.66 at 5%P) and 5 years user to 10 years user (t = 2.021 <7.03 at 5%P) were found significantly increased.

But Gond tribes the difference of micronuclei between control and 1 years user was found significant (t = 2.021 <2.24 at 5%P). Similarly in control to 5 year user (t = 2.021 <5.49 at 5%P), control to 10 years user (t = 2.021 <13.78 at 5%P) and 5 years user to 10 years user (t = 2.021 <6.88 at 5%P) had significantly increased. Comparatively the micronuclei per 1000 cells between Halba and Gond tribes was also found different. The difference in control set of both the tribe was reported 0.10 ± 0.05, between 1 year user 0.45 ±0.04, between 5 years user 0.06 ± 0.04 and between 10 years user 0.45 ±0.01. In halba tribes the micronuclei frequency among 5 years users and 10 years users were found higher in comparison to gond tribes.

Several authors have tried to analyse genotoxicity on the basis of micronuclei variation among tobacco chewers in different part of the world. Genotoxic effect of tobacco on buccal
epithelium was also studied by Biswas et al.\textsuperscript{22} and an increased rate of nuclear anomalies (condensed chromatin, Karyolysis and binucleation) in tobacco chewers and smokers as compared to healthy control was reported. Balachander et al.\textsuperscript{23} studied the cytogenic damage in khaini users of Tamil Nadu, India and performed micronuclei assay and chromosomal aberration in exfoliated buccal mucosa and showed a significant result in comparison to control. A similar result was observed by Sudha et al.\textsuperscript{24} in their study they collected 236 buccal cells and blood sample from 80 betel quid users and 76 with tobacco snuffing habits and observed a significant (p<0.01) increase in chromosomal aberration and micronuclei as compared to healthy individuals. Chadha and Yadav\textsuperscript{25} studied the genotoxic effect of gutkha in 50 gutkha consumers and compared the result with healthy control not consuming any type of tobacco products. They noted a significant increase in the value of three cytogenic markers viz. Chromosomal aberration (0.92 - 3.60), sister chromatid exchange (3.66 - 6.84) and micronucleated cell (0.09 - 0.98) as compared to control. The end point results were also found higher among high age group (> 31 years) compared with low age group individuals. A significant correlation was also established with duration of consumption and frequency of consumption per day.

Induction of micronuclei by smokeless tobacco on buccal mucosa cells of habitual users was studied by Ozkul et al.\textsuperscript{26} who observed a mean percentage of micro nuclei (1.86 ± 0.26) in smokeless tobacco users and 1.99 ± 0.30 in smokers as compared to non smokers/ non users of tobacco and concluded that genotoxic effect of ST should be considered in addition to other known hazards of tobacco. Bansal et al.\textsuperscript{27} also evaluated the micronuclei in tobacco users of Punjab, India and observed a significantly higher number of MN cells in ST users than smokers and controls and concluded that MN is a biomarker of genotoxicity. Genotoxic effect of tobacco dust exposure on

### Table 1: Micronucleated cells per 1000 buccal epithelium cells among Halba tribes (n = 20) for different period of Consumption.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Control N=20</th>
<th>Up to 1 year of consumption N=20</th>
<th>Up to 5 year of consumption N=20</th>
<th>Up to 10 years of consumption N=20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.4</td>
<td>0.55</td>
<td>3.05</td>
<td>6.25</td>
</tr>
<tr>
<td>SD</td>
<td>0.821</td>
<td>0.605</td>
<td>1.276</td>
<td>1.585</td>
</tr>
<tr>
<td>SE</td>
<td>0.184</td>
<td>0.135</td>
<td>0.285</td>
<td>0.354</td>
</tr>
</tbody>
</table>

### Table 2: Micronucleated cells per 1000 buccal epithelium cells among Gond tribes (n = 20) for different period of Consumption.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Control N=20</th>
<th>Up to 1 year of consumption N=20</th>
<th>Up to 5 year of consumption N=20</th>
<th>Up to 10 years of consumption N=20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.50</td>
<td>1.00</td>
<td>2.45</td>
<td>5.80</td>
</tr>
<tr>
<td>SD</td>
<td>0.607</td>
<td>0.792</td>
<td>1.468</td>
<td>1.609</td>
</tr>
<tr>
<td>SE</td>
<td>0.136</td>
<td>0.178</td>
<td>0.328</td>
<td>0.360</td>
</tr>
</tbody>
</table>
bidi rollers of Jabalpur, India was studied by Gautam et al. by evaluating Chromosomal aberration (CA), comet assay and urinary thioether estimation. Bidi rollers expressed a significantly increased CA % 3.0 ± 0.63 and 3.7 ± 0.39 for 30-35 years and 60-65 years age group respectively compared with age matched control (1.3 ± 0.32 and 1.8 ± 0.24). A significant increase in excretion of urinary thioether in exposed group and significant increase in comet and tail length was also reported as compared with control.

In tobacco major toxic components are nicotine, tar and polycyclic hydrocarbons which are carcinogenic, besides some other carcinogenic compounds are 4-(methylnitrosamino)-1-(pyridyl)-1-butanol (NNK), N-nitrosodimethylamine (NNN), N-nitrosoanatabin (NAT), N-nitrosoanabasine, certain heavy metal like Polonium, Uranium etc. It is also supposed that reactive oxygen species (ROS) in large amount are generated by tobacco which leads to DNA damage and other related toxicity.

In today’s scenario the micronuclei test is believed as perfect, reliable and simple sensitive test to detect genotoxicity. By the help of micronuclei test the incidence of genotoxicity between smokeless tobacco chewers and non chewers have been enumerated among insensitive tribal population (Halba and gond) of India and it was established that along with increase with duration of exposure micronuclei frequency in buccal epithelia of both tribes have been increased and comparatively in halba tribes was found much vulnerable in comparison to gond tribes. It is believed that several carcinogenic compounds present in tobacco specially nitrosamine is responsible for induction of micronuclei. These compounds are generally believed to be generated by bacterial enzymatic interference with nicotine and same incidence takes place in mouth under influence of saliva. The novelty of present finding is it is first time reporting on Halba and Gond tribes of Chhattisgarh, India which confirms the belief that tobacco is genotoxic and may leads to carcinoma.

REFERENCES


**Fig.-3 . Micronuclei in Buccal epithelium of tobacco user.**