

# Genotoxic Study of Chewing Leaf Tobacco in Swiss Albino Mice

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**ABSTRACT-** The tobacco plant *Nicotiana* has probably been responsible for more deaths than any other herb as it is market driven commodity of economic benefit. While the majority will likely be killed by use of cigarettes, tobacco use in other forms also contributes to worldwide morbidity and mortality. Chewing leaf tobacco is less used now as the ban is imposed on it. We tested the genotoxic potential of chewing leaf tobacco using *in vivo* cytogenetic tests- peripheral blood micronucleus test, and sperm abnormality assay. Three doses of tobacco viz., 3%, 5%, and 10% were given for 14 days. Cyclophosphamide, an indirect acting clastogen was used as positive control agent and it was injected intra peritoneally to the animals only once. Double distilled water was used as negative control. The frequency of micronucleated normochromatic erythrocytes (MNNCE) was increased in tobacco treated mice with the maximum MN being induced in NCEs at 10% dose. All three tobacco doses used in this study, induced significant abnormal sperms compared to controls ( $P < 0.05$ ). The chewing leaf tobacco at a certain concentration is genotoxic.

**Key-words-** Chewing Leaf tobacco, Peripheral blood micronucleus, Swiss albino mice, Howell-Jolly bodies

## INTRODUCTION

The tobacco plant has conquered the world as a powerful drug in the form of cigarettes, cigars, and pipes<sup>[1,2]</sup> *Nicotiana* varieties originate mainly from South America. The tobacco plant, *Nicotiana*, has probably been responsible for more deaths than any other herb. At present, tobacco smoking is causing over 3 million deaths a year worldwide, and if current smoking trends continue the annual mortality will exceed 10 million by around 2030. Undoubtedly, tobacco is the most important avoidable cause of premature death and disease in the world<sup>[3]</sup>.

Smokeless tobacco is consumed without burning the product and can be used orally and through nasal route. Oral smokeless tobacco products are placed in the mouth, cheek or lip and sucked (dipped) or chewed. In India, smokeless tobacco is famous in the form of Gudakhu, Mishri, snuff, chewing tobacco, Masher, Mawa, Sadagura etc. Maybe tobacco is the only one plant now known the world over for the overall health burden it causes to public health. And probably this is the only one plant whose references are made in almost every life science journals at least once.

The use of smokeless tobacco among women is increasing in India<sup>[4]</sup>. Tobacco use is projected to kill one billion people during the 21<sup>st</sup> century. While the majority will likely be killed by use of cigarettes, tobacco use in other forms also contributes to worldwide morbidity and mortality<sup>[5]</sup>.

There is evidence that some tobacco and nicotine products may pose less health hazard than cigarette smoking and so could reduce morbidity and mortality due to smoking<sup>[6]</sup>. There is general agreement in the scientific community that the health hazards from low nitrosamine smokeless tobacco are lower than those of cigarette smoking on the individual level. With respect to population impact, there is some concern about attracting new users with reduced risk products into the overall pool of tobacco users, whose acquired disease risk would then offset the reduced disease burden among smokers<sup>[7]</sup>.

Micronuclei (MN), also known as Howell-Jolly bodies, were first identified at the end of the nineteenth century in red cell precursors by William Howell, an American, and Justin Jolly, a Frenchman<sup>[8]</sup>. They found small inclusions in the blood taken from cats and rats. The small inclusions were also observed in the erythrocytes of peripheral blood from severe anemia patients<sup>[9]</sup>. MN test is widely used for screening of different agents for their genotoxic potential. It is a recommended bioassay in the test battery for genotoxicity evaluation and has become increasingly popular for regulatory acceptance. Micronucleus in peripheral blood is used as an index for genotoxic potential. The mouse erythrocyte micronucleus assay has been traditionally carried out using one or two exposures to the test agent, followed by sampling at two

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or three post-exposure times to obtain a sample near the time of the transient peak of micronucleated polychromatic erythrocytes (PCEs)<sup>[10]</sup>.

In animals, exposure of males to toxic chemicals and radiation<sup>[11,12]</sup> can result in wide variety and combination of reproductive dysfunction such as changes in the sexual behavior, spermatogenic killing, diminished sperm quantity and quality, chromosomal defects in germ cells, reduced fertility etc<sup>[13,14]</sup>. The mouse sperm morphology test also has potential in identifying chemicals that induce spermatogenic dysfunction and perhaps heritable mutations<sup>[15]</sup>.

To the present date, more than 4700 different chemicals have been identified in tobacco smoke, ranging from heavy metals to polycyclic aromatic hydrocarbons to mutagenic chemicals. "Sadagura" a smokeless tobacco of Assam has shown genotoxicity through micronuclei induction and sperm head abnormality<sup>[16]</sup>. Compared with nonusers of tobacco, chewing tobacco users in India and snuff users in Sweden have lower sperm count, concentration, motility, and normal morphology among men seeking infertility treatment<sup>[17]</sup>. Thus we employed Sperm abnormality assay for the investigation of genotoxic effect of chewing leaf tobacco in Swiss albino mice.

## MATERIALS AND METHODS

In the present investigation we used Swiss albino mice (*Mus musculus*), bred and maintained in the institutional animal house of Department of Applied Zoology, Mangalore University, Mangaluru, Karnataka, India. The animal studies were conducted after obtaining the approval from the Institutional Animal Ethical Committee (IAEC) of Mangalore University. Care of the animals and experimental procedures were conducted as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experimentation on Animals) India. Animals were housed in polypropylene shoe box cages bedded with clean, dry paddy husk and kept in air-conditioned room at a temperature of 22± 2°C and relative humidity 50±15%. They were fed with a standard pelleted diet and water ad libitum. 8-10 weeks old animals of both the sexes with average body weight, 25±2g were used for the experiments. In each experimental and control groups, five animals were maintained. Cyclophosphamide (CP, CAS No.6055-19-2; Batch No. GL3045, Asta Medica AG Germany), marketed as Endoxan by German Remedies Ltd, Ponda, India was used as the positive control. All other chemicals were obtained from MERCK, SRL or HI media, India.

For the present study, three doses of tobacco viz., 3%, 5%, and 10% were chosen. Different doses were prepared by homogenizing in mortar and pestle using suitable amount of distilled water. These doses were administered orally in 0.2 ml quantity for 14 consecutive days. Cyclophosphamide, an indirect acting clastogen<sup>[18]</sup> (CP, 50 mg/kg b w) was used as positive control agent and it was injected intraperitoneally to the animals only once. Double distilled water was used as negative control.

The peripheral blood MN assay was done by using the method of Schlegel and MacGregor<sup>[19]</sup>. The blood was drawn from the tail vein and thin smears were prepared on clean grease free slides. They were fixed in absolute methanol for 10 minutes. The slides were then stained with buffered 10 % Giemsa (pH-6.8) taken in vertical couplin jars. Giemsa staining technique has been shown to give the best results for micronuclei and other biomarkers. It should be mentioned here that we filtered the buffered Giemsa using Whatman no 1 filter paper before staining the slides. A thorough washing under the running tap water was preferably carried out soon after removing from the stain to remove any stain particles adhering on the blood cells, which would otherwise impede the scoring of the micronuclei. About 2000 NCE per animal were scanned for the presence of MN. The number of PCE corresponding to 2000 NCE was also determined<sup>[20]</sup>.

The animals used for sperm abnormality assay was those mentioned above, and they were sacrificed after 35 days of last tobacco treatment. Animals were killed, and the testes were dissected out and weighed. Both the cauda epididymis were removed and minced thoroughly in a watch glass containing 1 ml phosphate buffered saline (pH=7.2), stained with 2% aqueous eosin for about 45 min. Thin smears were made. Two thousand sperm/animal were scored from each group for the presence of sperm shape abnormalities following the criteria of Wyrobek and Bruce<sup>[15]</sup>. For sperm count, an aliquot from the sperm suspension was diluted (1:40) with PBS and mixed thoroughly and counted using the Neubaur counting chamber and the total sperm count in 8 squares of 1 mm<sup>2</sup> was determined and multiplied by 5×10<sup>4</sup> to calculate the number of sperms per epididymis.

**Statistical Analysis-** The results of the experiments were expressed as the mean %±SEM. The significance of differences among the groups was assessed using one-way analysis of variance (ANOVA) test. The mean between two groups was analyzed by Students t-test. For all analyses p<0.05 was set as the critical level of significance.

## RESULTS AND DISCUSSION

Measuring numbers of micronuclei in mammalian red blood cells is relatively straight forward because normoblasts (erythrocyte precursor cells that reside in the bone marrow) expel their nuclei during red blood cell development, leaving behind any micronuclei that have formed. It therefore, makes an ideal test for the detection of genome instability *in vivo* that is minimally invasive and does not require the death of the animal, meaning that it can easily be incorporated into a phenotyping screen<sup>[21]</sup>. Polychromatic erythrocytes in mouse bone marrow are abundant, easily recognizable, and have a lifetime of ~2 days before maturing into normochromatic erythrocytes<sup>[22]</sup>.

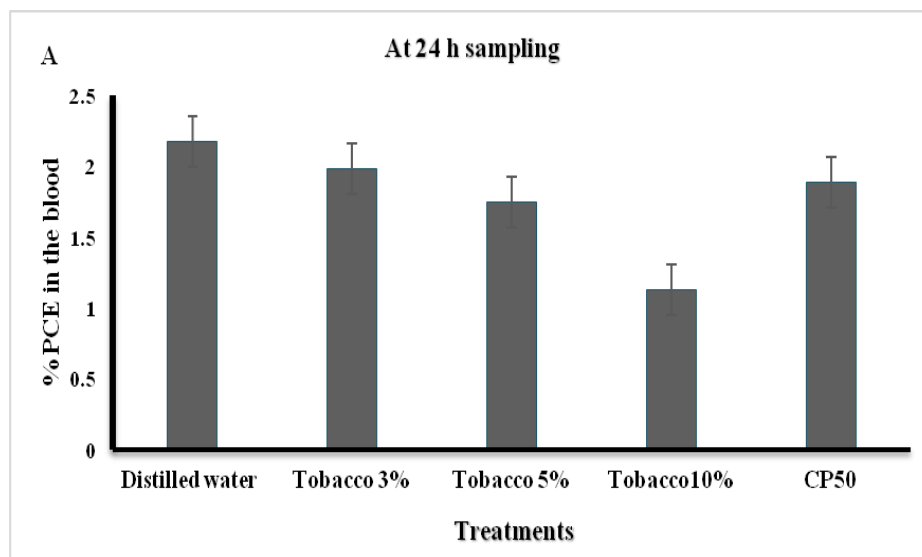
**Table 1:** Peripheral blood MN test in Swiss albino mice treated for 14 days with different doses of chewing leaf Tobacco at different sampling timings

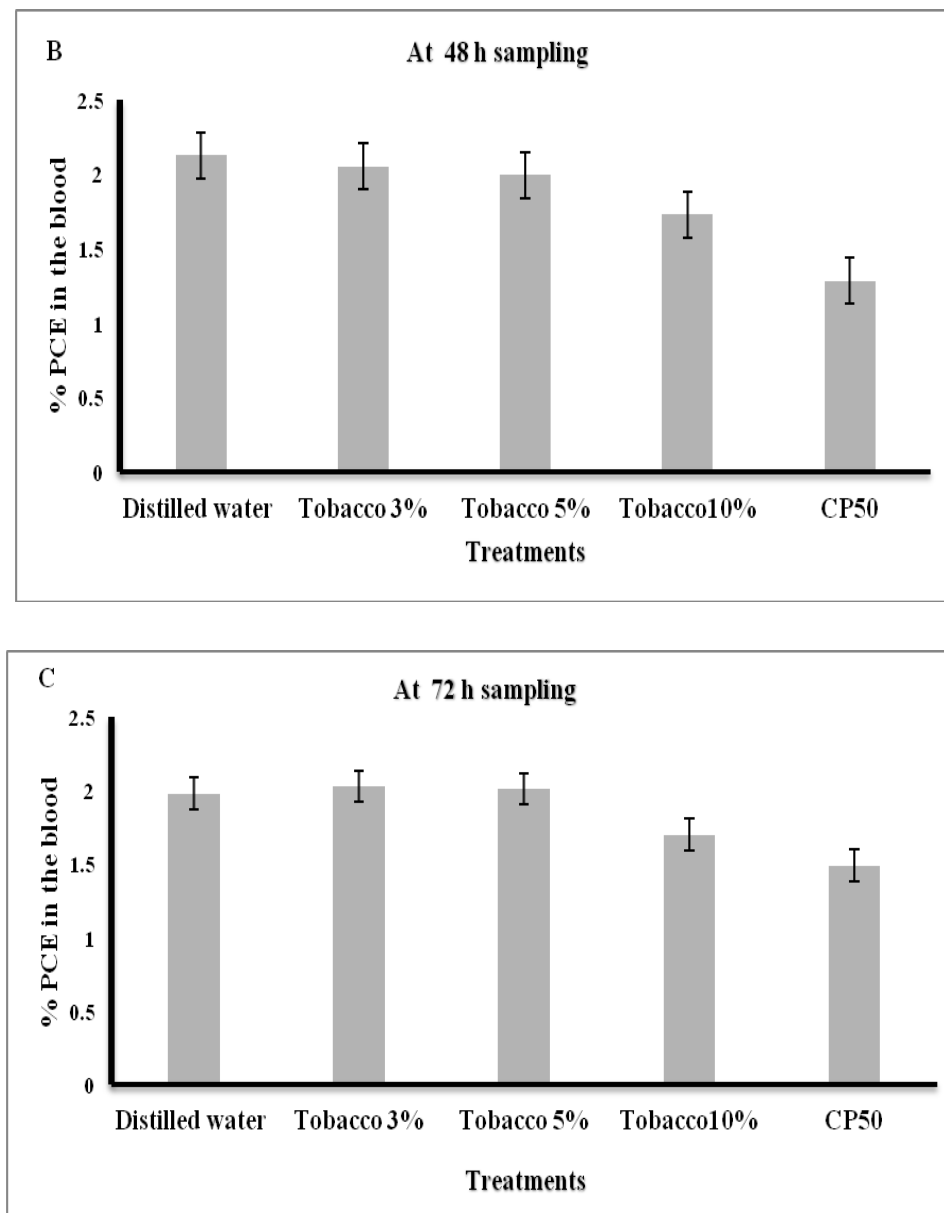
Treatment/dose (mg/kg bw)	Sample time	Mean%NCE ± SEM	MN-NCE/1000NCE (Individual data)	Mean MN in NCE±SEM
Dist. Water	24 h	97.82±0.11	1.5,0.5,0.5,1,1	0.80±0.25
	48 h	97.87±0.13	1,2,1.5,2,1	1.50±0.22
	72 h	98.02±0.08	1.5,1,2,1.5,2	1.60±0.18
3% Tobacco	24 h	98.02±0.06	2,1.5,3.5,2.5,2	2.50±0.35 <sup>a</sup>
	48 h	97.95±0.08	1,2.5,1.5,3.5,2	2.10±0.43
	72 h	97.97±0.12	1.5,2,2,0.5,2.5	1.70±0.33
5% Tobacco	24 h	98.25±0.04	2,2.5,1,3,2	0.21±0.33 <sup>a</sup>
	48 h	98.01±0.09	4,2.5,2,3,2	2.70±0.37
	72 h	97.99±0.09	1.5,2,1.5,2.5,2	1.90±0.18
10 % Tobacco	24 h	98.87±0.06	4.5,2.5,5,6,3	4.20±0.64 <sup>a</sup>
	48 h	99.27±0.11	4.5,3,5,5,5,6	4.80±0.40 <sup>a</sup>
	72 h	99.68±0.07	3.5,2.5,3,2,2	2.60±0.29
Positive control (CP 50)	24 h	98.11±0.10	1.5,0.5,1.5,1.5,2	1.40±0.24
	48 h	98.72±0.06	4,4.5,2.5,5,3.5	3.90±0.29 <sup>a</sup>
	72 h	98.51±0.06	3,4.5,5,5,6,5	4.80±0.51 <sup>a</sup>

NCE- Normochromatic erythrocytes; MN-NCE- Micronucleated normochromatic erythrocytes; CP50- Cyclophosphamide 50mg/kg bw; SEM- Standard error of the mean ANOVA test: t- test; <sup>a</sup>P<0.05

The frequency of MN NCE (Table 1 & Fig. 2) was increased in tobacco treated mice with the maximum MN being induced in NCEs at 10% dose. The MN-NCE in the positive control was significant only at 48h and 72h. This

may be because the positive control was treated for only once through intraperitoneal route. The highest dose, 10% tobacco showed MN-NCE at statistically significant levels from the distilled water control group (Table 1).





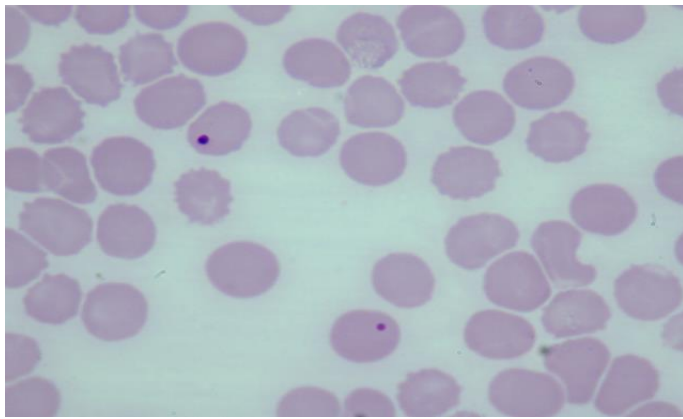
**Fig. 1 (A-C):** Effect of dosing of chewing leaf tobacco for 14 days on % PCE in peripheral blood in Swiss albino mice at different sampling time

A dose-dependent decrease in the percentage frequency of PCE was found at all sampling times (Fig. 1 and Fig. 3). The polychromatic erythrocytes are not comparable to that found in the bone marrow. In the bone marrow, at early developmental stages, the ratio of PCE to NCE is more than one. As it matures, ratio decreases slowly. The ratio of PCE to NCE decreases when an animal gets exposed to toxic agent or genotoxic compounds either through environmental contamination or through the emergency medications. In our study, this effect was more pronounced in 10% tobacco treated group. This indicated the suppressive effect of higher dose of chewing leaf tobacco on bone marrow proliferation.

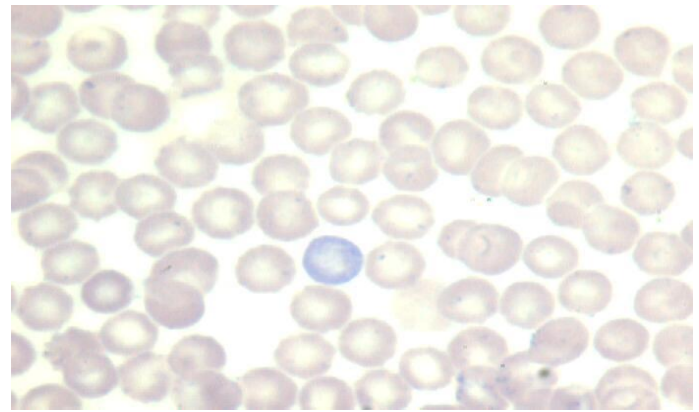
Background Micronuclei (MN) in mammalian cells serves as a reliable biomarker of genomic instability and genotoxic exposure [23]. Elevation of MN is commonly observed in cells bearing intrinsic genomic instability and

in normal cells exposed to genotoxic agents. Tobacco can cause and increase the rate of nuclear anomalies in both smoking and smokeless forms compared to healthy

controls. Earlier works demonstrated significant increase in the number of micronucleated polychromatic erythrocytes (MN-PCE) and decrease in the number of polychromatic erythrocytes (PCE) in bone marrow of nicotine-treated animals using micronucleus assay [24]. Others have investigated the *in vivo* micronucleus assay as part of a rat cigarette inhalation study and have also demonstrated that the level of micronuclei were not increased following cigarette smoke exposure in peripheral blood or bone marrow samples [25]. The micronuclei inductions were not significantly increased following 6 weeks of cigarette smoke exposure [26].



**Fig. 2:** Micronucleus in NCE



**Fig. 3:** One PCE among NCE

Erythrocyte MN represented the consequence of chromosomal aberrations induced during the preceding mitotic divisions in the erythroblasts [27]. Swiss albino mice are suitable for *in-vivo* micronucleus assay using peripheral blood because the spleen does not remove the micronucleated erythrocytes from the circulation, whereas rats are not suitable. The frequency of micronucleated erythrocytes in the peripheral blood of splenectomized rats can be used as an index of both acute and cumulative chromosomal damage, while in normal rats the use of peripheral blood for cytogenetic monitoring is restricted by the selective removal of these micronucleated cells [28].

For sperm abnormality assay parameters selected are hook less, banana shaped, amorphous, double tailed, doubled head and folded (Table 2 & Fig. 4). The data shows that percent abnormality in control group was  $1.91 \pm 0.11$  and in CP treated group  $6.47 \pm 0.10$  i.e., CP induced significant frequency of abnormal sperms. The number of amorphous sperms were very less in controls, whereas, in treated individuals, they were in significant frequencies. Statistically, all three tobacco doses used in this study, induced significant abnormal sperms compared to controls ( $P < 0.05$ ).

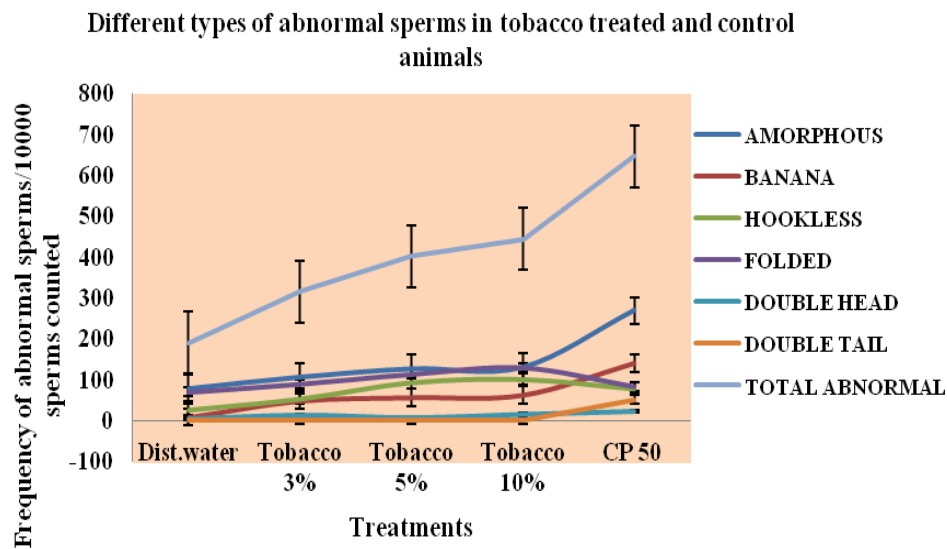
**Table 2:** Effect of chewing leaf tobacco on epididymal spermatozoa in Swiss albino mice after sub-acute treatment

Drug / Dose	Hookless	Banana shaped	Amorphous	Folded	Double Tails	Double Head	Total	Percent Abnormal sperms (Mean $\pm$ SEM)
Control	5.40 $\pm$ 0.60	1.80 $\pm$ 0.37	16.00 $\pm$ 1.48	14.00 $\pm$ 1.81	0.00 $\pm$ 0.00	1.00 $\pm$ 0.44	38.20 $\pm$ 2.22	1.91 $\pm$ 0.11
3% tobacco	10.80 $\pm$ 0.86	9.80 $\pm$ 0.86 <sup>a</sup>	21.60 $\pm$ 1.74	18.00 $\pm$ 1.48	0.20 $\pm$ 0.20	2.80 $\pm$ 0.73	63.20 $\pm$ 1.85 <sup>a</sup>	3.16 $\pm$ 0.09 <sup>a</sup>
5% tobacco	18.80 $\pm$ 1.42 <sup>a</sup>	11.40 $\pm$ 0.67 <sup>a</sup>	25.60 $\pm$ 1.77 <sup>a</sup>	22.80 $\pm$ 1.65	0.40 $\pm$ 0.24 <sup>a</sup>	1.60 $\pm$ 0.50	80.60 $\pm$ 2.15 <sup>a</sup>	4.03 $\pm$ 0.10 <sup>a</sup>
10% tobacco	20.40 $\pm$ 2.29 <sup>a</sup>	12.60 $\pm$ 1.20 <sup>a</sup>	26.40 $\pm$ 2.27 <sup>a</sup>	26.00 $\pm$ 1.51	0.40 $\pm$ 0.24 <sup>a</sup>	3.20 $\pm$ 0.37	89.00 $\pm$ 4.70 <sup>a</sup>	4.45 $\pm$ 0.23 <sup>a</sup>
CP 50	15.80 $\pm$ 1.49 <sup>a</sup>	28.00 $\pm$ 1.22 <sup>a</sup>	53.80 $\pm$ 2.05 <sup>a</sup>	16.80 $\pm$ 1.39	10.4 $\pm$ 1.07 <sup>a</sup>	4.60 $\pm$ 0.81 <sup>a</sup>	129.40 $\pm$ 2.15 <sup>a</sup>	6.47 $\pm$ 0.10 <sup>a</sup>

Five animals in each group; 2000 sperms/animal; ANOVA test; <sup>a</sup>p < 0.05

Daily smoking has shown a steady rise in defective sperm morphology [29]. Cigarette smoking is associated with lowered sperm density. Vine *et al.* [30] calculated that smokers had a 13%–17% reduction in sperm concentration compared with nonsmokers [30]. Smokeless or non-combusted oral tobacco use as a substitute for cigarette smoking has been gaining greater interest and attention by the public health community and the tobacco industry. A study by Kumari *et al.* [31] demonstrated that chronic exposure of mice for six months to 3% pan

masala with tobacco (a South Asian product similar to gutkha) in the diet produced adverse reproductive outcomes including reductions in spermatid count, mature sperm count, and sperm production [31]. Abnormal spermatozoa have also been reported in tobacco chewing sub-fertile males. The adverse impact of tobacco chewing on semen parameters was evident even with mild chewers, but with the intensive chewing practice, phenotypes of sperms, mainly defect in the head and cytoplasmic residue was severely affected [32].



**Fig. 4:** Graph showing different types of sperm shape abnormalities induced by chewing leaf tobacco in Swiss albino mice. Each group contained five animals

## CONCLUSIONS

The parameters selected to confirm the genotoxicity of chewing leaf tobacco proved that chewing leaf tobacco at certain concentration is genotoxic. Future research is required in unraveling the therapeutic uses of chewing leaf tobacco in the field of chemotherapy because every chemotherapeutic agents are highly genotoxic. They also produce cancers. This chewing leaf tobacco, though a culprit in the society, clinically may be useful, if the pharmacological properties are investigated and incorporated. These Bioengineered Tobacco Plants may grow pharmaceuticals of the future. Plant derived medicines have been around forever, but until the development of a process called biopharming, they've been restricted to whatever naturally occurring medicines the plants themselves could produce.

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