P: ISSN NO.: 2321-290X RNI : UPBIL/2013/55327 VOL-6 * ISSUE-6 * (Part-1) February- 2019
E: ISSN NO.: 2349-980X **Shrinkhla Ek Shodhparak Vaicharik Patrika**

Effect of Aqueous Absorbent of Tobacco Smoke on The Peripheral Blood Erythrocytes of Common Toad, *Bufo melanostictus (Duttaphrynus melanostictus)*

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Abstract

Several testing protocols for detecting the mutagenic potentials of odd agents are in use, of which Micronucleus test (MNT) has been claimed to be an acceptable short term screening method.MN are small chromatin containing bodies arising from chromosome fragments or whole chromosomes that were not incorporated into daughter nuclei following mitosis. MN form only in dividing cells in the case of erythropoiesis in erythroblasts.The newly formed erythrocytes which are formed by extrusion of nuclei from erythrocytes still contain rRNA and are called polychromatic erythrocytes (PCEs). The stain differently can be distinguished from the normochromatic erythrocytes (NCEs) which lack ribosomes. Only the PCEs will gave micronuclei induced by a recent treatment, within 2-3 days of sampling. However, this is the case in mammalian erythropoiesis.

In India large number of people has the habit of “Hooka” smoking or Pipe smoking. It is widely used by the village folk of Assam, Orissa, West Bengal and other neighbouring states. In usual smoking the tobacco smoke directly enters into the lungs,in Hooka smoking, the smoke first passes through water and then goes to our lung. The Genotoxic effect of aqueous absorbend of tobacco smoke (Hooka water) on the blood erythrocytes of a common toad, *Bofo melanostictus (Duttaphrynus melanostictus)* have been studied. Aqueous absorbend of tobacco smoke were injected to the toads at the rate of 4cc/100gm and sacrificed after 1,3,7 and 15 days. Control groups received only equal amount of water. For each sacrifice in treated series 4 animals were used and 4000 cells per individual were scored from the coded slides. The chemical induced higher frequency of micronuclei over the control in treated series. Significance and relevance of micronucleus assay in mutagenicity study from cytological view point has been discussed. It is revealed that Hooka smoking seems to be less injurious to health than direct smoking.

Keywords: Micronucleus, Hooka Water, Tobacco Smoking, Nicotine, Genotoxic, Cytotoxic.

Introduction

Several mutagenic agents are used for micro-nucleus test. Tobacco is one of the most dreadful mutagenic chemical. Tobacco smoke contains many mutagenic and carcinogenic chemicals both whole tobaccos smoke and extracts induce tumours in experimental animals. Among the 3800 chemicals that have been identified in tobacco smoke a large number of biologically active compounds are induced (Viner and Caporase, 1995).The most important chemical families of carcinogens are polycyclic aromatic hydrocarbons, aromatic amines and nitroso compounds. Some of these chemicals, in particular, aromatic amine and nitrous amines are potent carcinogens in animal system where they induce tumours in several organs. Also exposure of animals (Rats, Hamsters, Mice and Rabbits) to whole smoke or condensate induce tumours of the respiratory tract or of the skin. Globally, large number of people dies every year due to tobacco smoking but if current smoking patterns persist, this number will increase markedly over the next thirty to forty years. There is also growing tendency

among young women for smoking and these young women who are currently smoking reach, one in four of them can be expected to die as a result of smoking tobacco. Both men and women who smoke, face increased risks of developing one or more of the some two dozen diseases which are caused by smoking. Most of these diseases are incurable and fatal, for example various cancers of lung and circulatory diseases. The preliminary data suggest that there may be inverse associations of smoking with uterine fibroids and endometriosis and protective effects on hypertensive disorders and vomiting of pregnancy are likely. Smoking has consistently been found to be inversely relared to the risk of endometrial cancer, but cancer of the breast and colon seem unrelated to smoking. Inverse associations with venous thrombosis and fatality after myocardial infection are probably not casual, but indications of benefits with regard to current aphthous ulcers, ulcerative colitis and control of body weight may well reflect a genuine benefit. Evidence is growing that cigarette smoking and nicotine may prevent or ameliorate Parkinson's disease and could do so in Alzheimer's dementia. A variety of mechanisms for potentially beneficial effects of smoking have been proposed but three predominate – the anti-estrogenic effect of smoking alterations in prostaglandin production; and stimulation of nicotinic cholinergic receptors in the central nervous system. The ingredients of cigarette smoke is highly cytotoxic, genotoxic and carcinogenic in various test systems (De Marine,1983, Curval et.al 1984, IARC, 1986). The use of smokeless tobacco has been suggested to be associated with cancer in human oral cavity and pharynx (Winn et.al, 1981). In India large number of people has the habit of smoking direct and indirect. Since long, pipe smoking or "Hooka" smoking has been the practice of Rajas, Maharajas and also village folk. Pipe (Hooka) smoking facilitates filtration of smoke in water. It is of the common experience that this "Hooka" water quickly turns to brown colour and highly toxic in nature. In a way this water absorbs many harmful ingredients of smoke especially in nicotine. The assessment of the genotoxicity of this "Hooka" water would highlight the beneficial effects of such type of smoking over direct (Cigarette or Bidi) smoking.

Cytogenetic assay is one of the established parameters for the evaluation of the environmental pollutants for health hazards. Micronucleus test system has been accepted to be a term dependable cytogenetic test. Micronucleus test is one of the most surest bio-assays for screening the metagenicity of genobiotics (Schmid,1976). From the point of health risk the widespread use of different tobacco preparation and smoking warrants extensive evaluation for their potential genotoxic effects. Considering all these, the present work "Effect of aqueous extract of tobacco smoke on the peripheral Blood Erythrocyte of common toad, *Bufo melanostictus (Duttaphrynus melanostictus)* was undertaken.

Objective of the Study

The bone marrow cells of some mutagen treated animal reveal micronucleus after completion of the last mitosis when on expulsion of the main nucleus from the erythroblasts, the micronucleus if present would disappear as a minute chromatin structure in the free cytoplasm which was formed generally from anaphase legards, asymmetrical exchange etc (Schmid, 1976). The MN could be detected for long time until it was made to disappear by the cytoplasmic enzymes, nucleases and proteases.

Review of Literature

The first serious attempt to use micronuclei as a monitor of cytogenetic damage reported by Evans et.al (1959) they used the MN frequency to measure the cytogenetic damage induced in root tips by fast neutron and gamma rays in the presence and absence of oxygen. It was found that all chromatid, chromosome and isochromatid break, as well as asymmetrical and incomplete symmetrical exchanges, will give rise to acentric fragments at mitosis and that these fragments are frequently excluded from the daughter nuclei and appear in the following interphase as MN.Begining about in 1970, Schmid and Co-workers and Heddle initiated studies to determine which parameters might serve as the most useful indicators of cytogenetic damage in bone marrow *in vivo* (Schmid and Staiger, 1969; Boller and Schmid 1970, Matter and Schmid 1971; Von Ledebur and Schmid,1973; Matter et.al,1973; Heddle 1973). This work led to the conclusion that the incidence of micro nucleated polychromatic erythrocytes (PCE) was a particularly useful index of *in vivo* bone marrow cytogenetic damage (Von Ledebur and Schmid, 1973).

About 43 years back Schmid (1976) and Maier and Schmid (1976) convincingly demonstrated in mouse that MN test is one of the dependable bioassay to detect the genotoxic potential of environmental agents. It is one of the efficient and rapid *in-vivo* method for the detection of cytogenetic damage. The first attempt to use micronuclei frequency to measure the cytogenetic damage induced in root tip cells of *Viva faba* was by fast neutrons and gamma rays in the presence and absence of oxygen. It was found that all chromatid, chromosome and iso chromatid breaks as well as exchange will give rise to acentric fragments at mitosis these fragments are frequently included in the daughter nuclei and appear in the following interphase as micronuclei. So the micronucleus test is comparable to or even more sensitive and reliable than metaphase scoring for the screening of chemical agents for mutagenicity (Matter and Schmid 1971, Maier and Schmid, 1976).

Concepts & Hypothesis

Micronuclei were previously known as Jolly bodies. There are some advantages of micronucleus test. These are-

1. Simple *in vivo* mammalian cytogenetic method.
2. Detect chromosome breaking agents of spindle poisons with a high degree of reproducibility.

3. Micronuclei can be observed throughout the cell cycle and the number of scorable cells is virtually unlimited.
4. A favourable karyotype is not required.
5. Micronuclei formed during cell division persist at least through the next interphase, so that the time of sampling is less critical.
6. It is not necessary to treat with any chemical other than that under test as spindle blocking agents are not required to obtain readable preparations.
7. The background frequency is low and is almost uniform among species.
8. The results correlate well with these obtained from more laborious mammalian techniques (e.g., metaphase method, dominant lethal test etc.)

Some limitations of MNTs are:

1. Does not necessarily detect the following types of compounds: those which
   i. Act tissue specific
   ii. Do not reach the target cells
2. Induce gene mutations only
3. Are strongly cytostatic
4. Does not distinguish between different types of chromosome aberrations.
5. Unsuitable for chronic mutagenicity testing.
6. The interpretation in quantitative terms (e.g. does response curves) is quite difficult.

Methodology

Test Chemical

The "Hooka" water used for different durations by the village folk was collected from Kanakpur village in the district of Purba Midnapore, West Bengal. During collection the "Hooka" smokers were questioned on different aspects, such as preparation of the tobacco, number of smoking per day, quantity of water used in "Hooka" containers and duration of the water level. The age of the water was determined on the basis of their declaration. In the present study 2,4 and 6 days old "Hooka" water was collected separately in vials and labelled. This was used as the test material. The water used for filling the container of "Hooka" served as control.

Test Animal

Adult individuals of both the sexes of common toad *Bufo melanostictus (Duttaphrynus melanostictus)* were collected from adjacent area of Zoology Department, Bishnupur, serve as the test animal in the present study. The toads were kept in the rearing chamber in the laboratory. The individuals were fed with small earthworms. Each individual was injected intraperitonially with 6 days old "Hooka" water at the rate of 4cc/100gms of the body weight. Animals were sacrificed after 1, 3, 7 and15 days following the treatment. For control series, the test animals were treated with water, used by the "Hooka" smoker and were sacrificed after the same intervals as in treated series.

Preparation of the Blood Smear Slides

After the specific duration of exposure four toads used per treatment group were anaesthetized with ether. The heart was opened by dissection and immediately blood was collected by puncturing the heart. A drop of blood was taken on each slide and blood smear was drawn with the help of either a cover slip or a slide. 4 to 5 slides were prepared per animal. So in total 16-20 slides were prepared per treatment group. Then the slides were dried in air for a short while.

Fixation and Staining

The tissue was fixed by putting the slides in absolute methanol for a period of 10-15 minutes. The slides were dried in air and kept overnight. Following day staining was done with 15-20 % Giemsa solution for one and half hours and washed in distilled water. The slides were dried in air and stored in dust free slide boxes.

Data Scoring

For each fixation time 4000 cells (1000 cells/ slides) were examined at random for the occurrence of micronucleus. Besides, micronuclei, other nuclear and cellular anomalies were also recorded. All anomalies were drawn with the help of camera Lucida and some of which were also photographed.

Findings

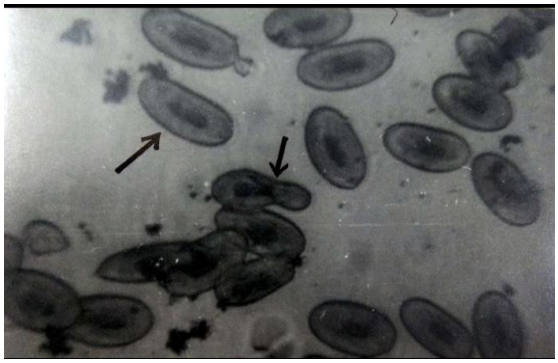
Control Series

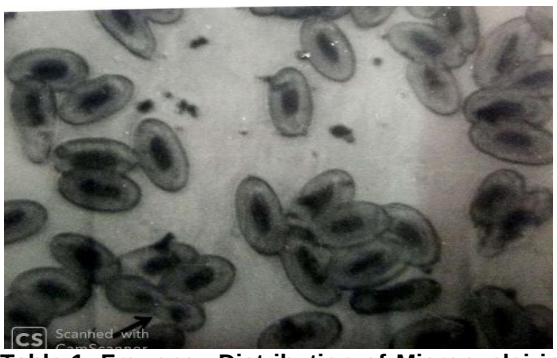
For all treated series, parallel controls were done. In this series not a single case of MN was recorded in all control hours. However, some other nuclear and cellular anomalies were recorded after 7 and 15 days exposure. (Table 1& 2)

Treated Series

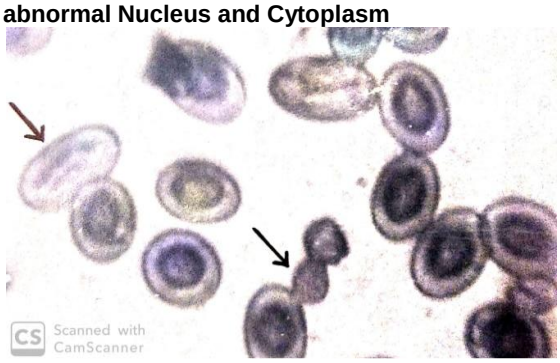
The treatment of "Hooka water" induced MN in all treated series except after one day exposure (Table-1). Qualitatively the size of the MN was dot shaped or small; while in some others these are relatively of larger size. In a number of erythrocytes the two halves of the nucleus, with respect to size, differs marginally. In one case the nucleus was divided into three parts (Figure 16, 30&32). The middle being larger than the other two. In some other cells the tendency of nucleus was to divide into three parts having two constrictions in between (Fig.11, 21).Besides all, this proportion of division of nucleus has been observed in many cases. The treated agent also induced different types of nuclear and cellular anomalies. Number of erythrocytes assumes the shape of dumbbell along with nuclear material. Number of cells were observed having protruded cytoplasmic material (Fig.18,22,23 & 29). A number of erythrocytes were also observed with pointed cytoplasmic protrusions assuming the shape of balloon (Fig. 25-28).Qualitatively "Hooka" water induced 0.187, 0.60 and 0.15 % of MN after 3, 7 and 15 days exposure respectively (Table-1). As regards other cellular and nuclear anomalies the said agent induced 0.03, 0.05, 0.008, and 0.343 after 1, 3, 7 and 15 days exposure respectively.

Photomicrograph showing Erythrocytes with Abnormal Nucleus and Cytoplasm





Photomicrograph showing Erythrocytes with abnormal Nucleus and Cytoplasm



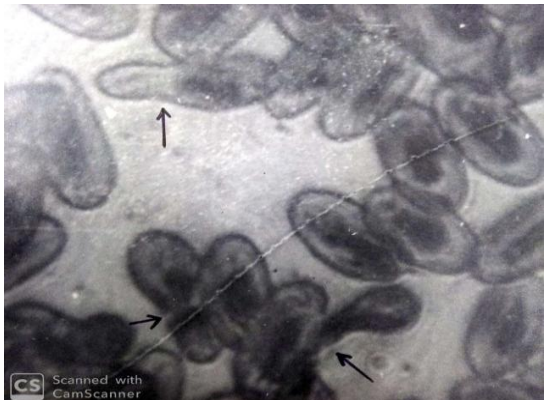


Table-1: **Frquency Distribution of Micronuclei in the Peripheral Blood Erythrocytes of common Toad, *Bufo melanostictus (Duttaphrynus melanostictus),*in control and treated series.**

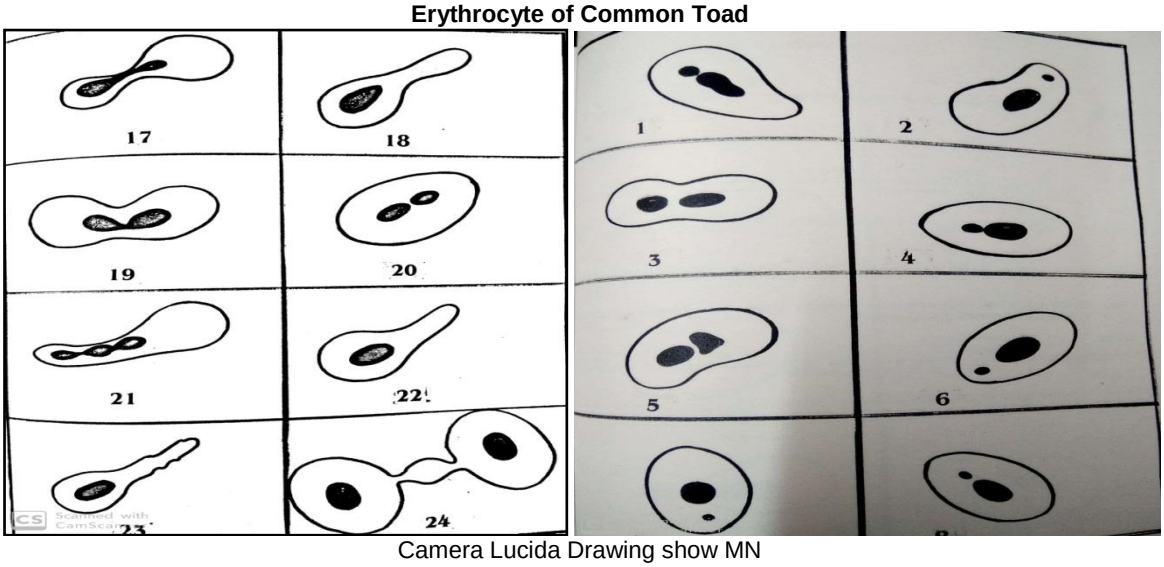
Sl.No	Dose	Time of Exposure	No. Of Individual	Total no.of cells studied	Cells with Micronuclei	% Mean	±SE
1	Treated	1 Day	4	16000	0.00	0.00	±0.00
	Control	1 Day	4	16000	0.00	0.00	±0.00
2	Treated	3 Days	4	16000	30	0.187	±0.645**
	Control	3 Days	4	16000	0.00	0.00	±0.00
3	Treated	7 Days	4	16000	96	0.60	±2.27**
	Control	7 Days	4	16000	0.00	0.00	±0.00
4	Treated	15 Days	4	16000	24	0.15	±0.912*
	Control	15 Days	4	16000	0.00	0.00	±0.00

** Results are significant at 1% level (P<0.01)
* Results are significant at 5 % level (P<0.05)

Table-**2: Frequency Distribution of Nuclear & Cellular anomaly in the Peripheral Blood**

Sl No	Dose	Time of Exposure	Total no of cells studied	Cells with nuclear abnormality	%Mean	±SE	Cells with cellular abnormality	% Mean	± SE	Cells with Nuclear & Cellular abnormality	% Mean	±SE	Cells without Nucleus	% Mean	±SE	Total
1	Treated	1 day	16000	9	0.056	±0.25**	12	0.075	±0.408	16	0.10	±0.408	5	0.03	±0.25**	42
	Control	1 day	16000	00	0.00	±0.00	00	0.00	±0.00	00	0.00	±0.00	00	0.00	±0.00	00
2	Treated	3 days	16000	19	0.118	±0.853*	20	0.125	±0.408*	18	0.112	±0.645**	8	0.05	±0.408**	65
	Control	3 days	16000	00	0.00	±0.00	00	0.00	±0.00	00	0.00	±0.00	00	0.00	±0.00	00
3	Treated	7 days	16000	45	0.281	±1.108*	30	0.187	±0.645**	40	0.25	±0.912**	14	0.008	±0.645**	129
	Control	7 days	16000	1	0.006	±0.25*	1	0.006	±0.25*	3	0.018	±0.25*	6	0.037	±0.288**	11
4	Treated	15 days	16000	14	0.087	±0.288**	41	0.256	±0.478**	17	0.106	±0.75**	55	0.343	±0.629**	127
	Control	15 days	16000	1	0.006	±0.25*	2	0.012	±0.288*	8	0.05	±0.408*	12	0.075	±0.408**	23

Erythrocyte of Common Toad



Camera Lucida Drawing show MN

Conclusion

The results from the present investigation indicate that the aqueous absorbent of tobacco smoke "Hooka water" could induce MN of erythrocytes of blood of toad. The indication of MN by any agent is indicative of its genotoxic, cytotoxic or aneugenic properties. Micronuclei may be formed due to chromosomal fragments or due to the elimination of a whole chromosome. It is known that time factor is critical for the induction of any mutagenic event in the

biological system. For such events especially in- vivo condition the physiological factors like absorption, elimination and retention of the chemical are largely responsible.

Genotoxic effects of some types of tobacco product have been studied earlier. Dash and Das (1992) studied the genotoxicity of "gudakhu" in mice in vivo and recorded significant increase of chromosome aberration, MN and sister chromatid exchanges. Earlier Stich and Anders(1989) reported higher incidence of MN in exfoliated cells of buccal mucosa of 7 habitual users of "gudakhu". Again Stich and his co- workers (1982), Brunnemann et.al(1987), Stich and Anders(1989) recorded a higher incidence of MN in buccal mucosa cells of habitual users of several tobacco preparation like "khaini", "snuffs", "nass", "betel quid" with tobacco. Similarly a higher incidence of sister chromatid exchange have been noted in peripheral lymphocytes of persons chewing tobacco (Ghosh and Ghosh 1984). So the "Hooka" water containing nicotine and other ingredients of tobacco preparation, proves to be genotoxic in the tested system.

Suggestion

The concentration of the chemical compounds in the target tissue of the cell is extremely important for the production of any mutagenic event, but the result of the present study is very interesting. The highest frequency of MN was produced after seven days exposure and lowest after 15 days exposure. While after three days exposure the result was intermediate. So it appears that after seven days the action of tobacco water decreased or cells with nuclear anomalities could not be accumulated in the peripheral blood probably due to cell death or elimination. Effect like other cytoplasmic anomalies indicate that "Hooka" water is not only genotoxic but also cytotoxic. So it is evident that direct smoking is far more injurious to health than indirect smoking especially when smoke passes through water. Hence it is suggested that "Hooka" smoking is less injurious to our health than other types of direct smoking such as bidi, cigarette etc. It also reveals that without knowing much about the adverse effect of tobacco on health long ago people could adapt "Hooka" smoking for which they didn't suffer in greater frequency from tobacco induced cancer disease.

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