Genotoxicity of 'gudakhu', a tobacco preparation used in Orissa

'Gudakhu' is a paste-like tobacco preparation the ingredients of which, besides tobacco (10%), are molasses (35%), lime (7%), red soil (a particular type of reddish soil available locally, 28%) and water (20%). During use it is rubbed over teeth with a finger tip. Its use is highly prevalent in Orissa and neighbouring states; in Orissa every third person is addicted to it. Persons are known using gudakhu for 40 years or more, and as many as 20 times a day.

Experimental and epidemiological studies have conclusively proven carcinogenic, mutagenic and clastogenic potential of tobacco and tobacco products[1–5]. Recently, a significant increase in the incidence of micronuclei has been recorded in exfoliated cells of buccal mucosa of habitual users of gudakhu[6]. Acetone extract of gudakhu (AEG) has also been proven to be genotoxic by us in mouse bone marrow system using chromosome aberration, micronucleus and sister chromatid exchange assays[7]. In view of the widespread and chronic use of gudakhu, it is worthwhile to study its genotoxic effect following repeated treatment. Bone marrow is a proliferative tissue and hence fails to provide correct information regarding genotoxic efficacy of an agent following chronic exposure. Chromosomal damages recorded in bone marrow cells at a particular sampling time following chronic exposure constitute the damages caused during the cell cycle prior to harvest, not the cumulative damages for the entire period of exposure. Sometimes an agent may go undetected in bone marrow cytogenetic assay following single treatment, particularly if it has low effect. Analysis of micronuclei (MN) in peripheral RBCs of mice, however, provides a very simple and important assay for evaluation of genotoxic efficiency of an agent following chronic exposure[8–10]. The present study deals with the evaluation of genotoxic effect of AEG in mice following repeated treatment for different periods using the micronucleus test (MNT) in peripheral erythrocytes.

Seven albino Swiss mice (4 males and 3 females) aged 10–12 weeks were employed. Gudakhu of 'Samaleswari' brand was purchased from the local market. Details of the extraction procedure have been presented elsewhere[7]. In brief, gudakhu of known weight was homogenized in a mortar and pestle with acetone, extract was filtered and the filtrate evaporated to dryness through vacuum desiccation. A freshly prepared dry pellet of known weight of gudakhu extract was suspended in an appropriate amount of distilled water and the suspension fed to mice by gavage. Animals were fed with a dose of 300 mg/kg (the highest dose tested in our earlier work[7] and having no visual abnormal effect on behaviour of the animals) of the dry extract once daily for 4 weeks. Blood was collected from the tail vein of each animal, without killing it, at the end of each week during the course of treatment and also on day 0 just before starting the treatment, and smear was drawn on grease-free slides. Blood collected on day 0 served as control sample. Thus, the same animals provided blood samples for control as well as for different test weeks of the treated series. Air-dried smear was fixed in absolute methanol for 15 min and stained next day in Giemsa diluted (1.10) with buffer (pH 6.8) for 10–15 min. Erythrocytes (both polychromatic and normochromatic) were examined for the presence of MN and the frequency of micronucleated erythrocytes (MNRBCs) was determined. For each sampling point 35,000 erythrocytes were scored from 7 animals (5000 from each).

Micronuclei were invariably round in shape and were of varied size groups (Figure 1). Never was an erythrocyte found to contain more than one MN. The frequency of MN increased significantly, compared to the control value, in all the test weeks. For weeks 3 and 4 the values were about 3 times the control value. Further, the increase showed a good positive correlation with the time course of chronic treatment (Figure 2).

The results clearly reveal positive genotoxic effect of AEG and support our earlier findings[6,7]. MN are induced by clastogenic effect or spindle dysfunction. The former generally results in induction of single micronucleus while the latter in multiple micronuclei. As in the present study, no case of multiple MN was encountered; the MN are supposed to have resulted from the clastogenic effect of AEG, and this finding is in accordance with our earlier one[7]. Elevated incidence of micronucleated polychromatic erythrocytes (MNPCEs) was also noted in peripheral blood of

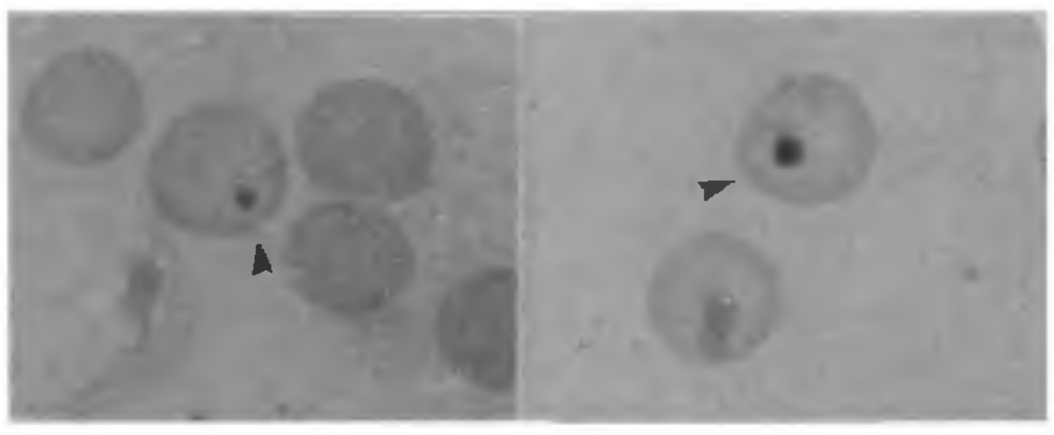

Figure 1. Micronucleated RBCs.

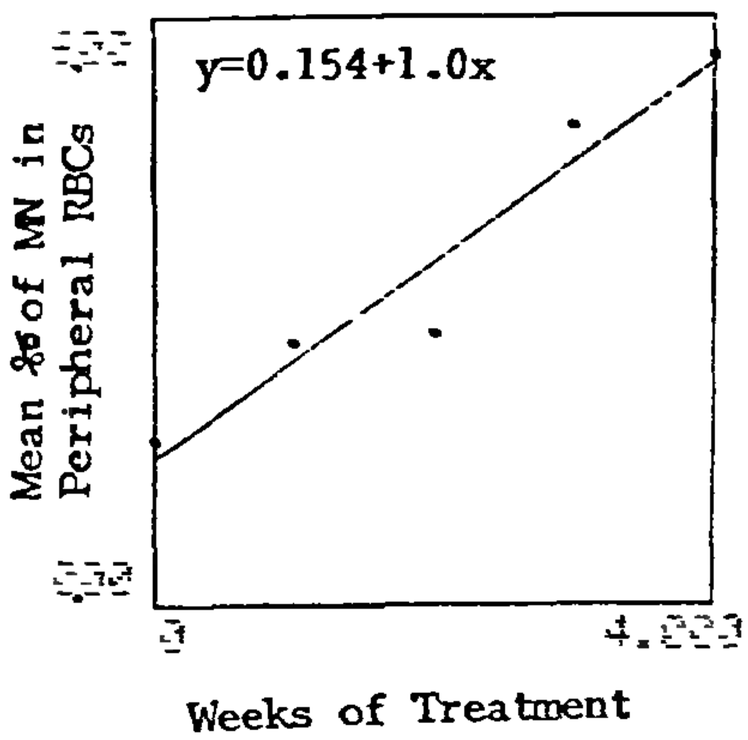

Figure 2. Linear regression curve showing the relation between the number of weeks of chronic treatment with gudakhu extract and the incidence of micronucleated peripheral erythrocytes ($r = +0.965$, $p < 0.001$, $a = 0.154$, $b = 1.00$)

new-born mice the mother of which had been exposed to tobacco smoke daily for 1 h during the last 6 days of pregnancy[11]. Recently, equal incidences of micronucleated peripheral reticulocytes (MNRETs) and bone marrow MNPCEs in mice treated with mitomycin C have been reported, though the induction of MNRETs was delayed by about 12 h compared to bone marrow MNPCEs[12].

In some strains of mice, unlike in most of the mammals, including humans, MNRBCs are not removed by the spleen; they remain in circulation[9,10,13,14]. As a result, MNRBCs get accumulated in their circulation. The RBCs of mice survive in the peripheral circulation for about 4 weeks[13]. Hence, repeated treatment is expected to exhibit gradual increase of the incidence of MNRBCs up to 4 weeks. Positive and significant correlation between the incidence of MNRBCs and the time course of chronic treatment obtained in the present study can be explained from such a behaviour of RBCs in this strain of mice.

Our results prove the uniqueness of this simple system[9,10], which is helpful in monitoring even weak clastogens following chronic exposure. Further, in this assay the same animals can provide blood samples, as in the present study, for control as well as treatment data for various periods. However, both polychromatic and normochromatic erythrocytes need to be considered.

Conclusion

Genotoxic effect of acetone extract of 'gudakhu', a paste-like tobacco preparation, has been evaluated using the micronucleus test in peripheral erythrocytes of mice following chronic exposure. A dose of 300 mg/kg of the dry extract in aqueous suspension was fed to mice once daily for 4 weeks. Micronucleated erythrocytes (MNRBCs) were scored from smears of tail vein blood collected just before beginning the treatment course and at the end of each week. The frequency of MNRBCs increased significantly in all the test weeks and showed a good positive correlation with the time course of treatment. The result has been discussed in the light of the fact that MNRBCs are not screened out by the spleen in mice, and this assay proved to be very important, particularly after chronic exposure.

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Azolla sp. from the Early Cretaceous of Cauvery basin, South India

A new record of Azolla sp. based on megaspores and massulae from the Late Albian (Early Creataceous) of the Pondicherry area in South India is presented in this study. The massulae are with characteristically circinate and septate glochidia. This is the oldest record of Azolla known to date.

During a palynological study of the Early Cretaceous carbonaceous shales from a depth of 32.4 m in bore hole BH/23, drilled by a private party for ground water, 1 3 km west of Kattarambakkam village in Pondicherry area in South India, the authors have come across a number of well-preserved massulae of *Azolla*, a water fern, along with varied spore and pollen taxa. One of the palynoslides also shows fortuitously a fairly large megaspore with a thick mat of hairy (tomentose) perine in which are seen entangled five massulae (Figure 1 *a*). All these massulae are characterized by circinate glochidia. Similar massulae have not been recorded to date from any horizon in India.

The following are the details of this new find of *Azolla* sp : (i) megaspores about 500 µm in diameter, extremely thick-walled, wall up to 30 µm thick; stratification not clear as the specimen is dark, dense and more or less opaque; (ii) outer layer of megaspore wall representing perine well developed and made up of thick felt of delicate hairs (tomentose) (Figure 1 *a–c*); (iii) number and nature of floats could not be deciphered, (iv) massulae attached to perisporal hairs of megaspore, oval to elliptical or irregular in shape, 85–214 µm in longest dimension, 54–125 µm in shortest dimension, (v)