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Induction of sperm-head abnormality in Swiss albino mice *Mus musculus* by administration of fresh and processed betel nut extracts

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Chewing betel nuts is a worldwide masticatory habit. However, these nuts have many harmful effects on human body. Sperm-head abnormality test is an easy and rapid *in vivo* technique to determine the ability of an agent to introduce abnormal change in the process of spermatogenesis. In the present study this test has been adopted to compare the dose-dependent toxic potentialities of fresh betel nuts of Tripura and processed nuts (tambul) of Assam. Both are capable of inducing significant dose-dependent toxic changes as well. Aqua leaching of small pieces of fresh betel nuts or tambul for 24 h only, makes them much less harmful in terms of this potentiality.

Keywords: Betel nut, *Mus musculus*, spermo-head abnormality, toxic potentiality.

PLANTS and various plant products are no doubt the main dietary ingredients of animals, including human being. However, in recent years, different reports are accumulating on the hazardous roles of different consumed plant products. One of these is the betel nut[1], which many people almost throughout the world consume as a masticatory habit[2]. Many oriental people use this nut[3,4] with or without betel leaves (leaves of *Piper betle*) along with other ingredients like slaked lime and tobacco[3,4]. The nuts are also used in different ways: in raw as well as processed form. In Tripura, it is generally used in raw form, while in Assam it is mainly processed and known as 'tambul'[5].

The main objective of the present study is to expand the existing pool of information regarding betel nut extract and to give a fresh insight into the toxic potential in terms of inducing significant and dose-dependent sperm-head abnormalities of two different types (unprocessed and processed) of betel nut collected from two different states of Northeast India.

In this study we adopt the assay of sperm-head abnormalities in mice. This *in vivo* technique assists in the rapid identification of the ability of an agent to cause an increase in the incidence of abnormality of the sperm-head in animals, regardless of the mechanism involved. When a compound induces a positive response in the sperm-head abnormality assay, it indicates that the compound may likely induce heritable genetic changes in

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whole mammals[6]. Thus the induction of faulty differentiation of spermatozoa in male mice treated with an agent may be an useful *in vivo* model for the induction of faulty differentiation in somatic cells that leads to carcinogenesis. This particular system of testing is important in assessing whether a chemical is safe or not. There is some evidence in humans that chemically induced sperm-head abnormalities are associated with infertility[7].

Healthy, sexually matured male mice, *Mus musculus*, 10–12 weeks old having an average body weight of 25 g were used for studying the sperm-head abnormality.

Two different types of extracts of *Areca catechu* nuts were taken for the experiment: fresh betel nuts of Tripura (BT) and tambul (processed betel nuts) of Assam (TA), NE India. Three different extracts were made with each type, extracting them using ethanol and water. (i) Ethanolic extract (EE): Small pieces of dehusked ripe fresh nuts were macerated in 50% ethanol (in the ratio of 1 g : 5 ml) and kept for 24 h. Then the solution was filtered and the filtrate was first air-dried and then under reduced pressure to a constant weight. (ii) Aqueous extract (AE): The process was similar to EE; however distilled water, instead of 50% ethanol, was used and the pieces of nuts were not macerated. (iii) Ethanolic extract after aqua-leaching (EEA): The process was similar to EE, but with the residual pieces of nuts after extracting with distilled water instead of the fresh nuts.

With each of these extracts three groups of solutions for treatment were prepared, viz. 2.5%, 5% and 10% diluting with sterilized distilled water.

These solutions were injected only once interperitoneally at a rate of 1 ml/100 g of body weight of the animals. This was chosen over oral administration with the assumption that it may act quickly. A suitable control was simultaneously prepared by injecting specimens with sterilized distilled water at the same rate. For each time interval (1 week, 3 weeks and 5 weeks) of each dose (2.5%, 5% and 10% extract) of each type of extract (EE, AE and EEA), five animals were taken for treatment. Controls were prepared for three time intervals with five animals each.

For this study, the methodology of Wyrobek and Bruce[8] was followed. The epididymides of treated animals were dissected out after one, three and five week(s) of betel nut exposure, representing treatments of spermatozoa, spermatid and spermatogonial cells respectively[9,10]; altogether 5000 sperms were studied from five mice.

Photomicrographs were taken an Olympus PM-6 photomicrograph using Orow 85 mm and AGFA black and white film.

The data obtained due to exposure of different extracts were compared with those of control by Student's *t*-test. Analysis of variance (ANOVA) was also done in order to determine any significant differences between the effects of different nut extracts for different doses and duration.

The criteria described by Wyrobek *et al.*[11] have been followed for scoring sperm-head abnormalities.

Figure 1 shows the frequency of spermatozoa with abnormal head morphology induced by both varieties (BT and TA) of betel nut extracts at different doses and time intervals as well as the results of control experiments. The major sperm-head abnormalities induced by extracts of both BT and TA included banana-shaped, hookless, amorphous, balloon-shaped, small headed, big headed and double-headed sperms (Figure 2). The abnormalities, however, observed in control mice consisted of sperms mainly with amorphous and hookless head, but in no case banana-shaped and double-headed forms were encountered.

In no case did any of the extracts of BT and TA varieties display lower percentage of abnormalities than the control mice.

Extracts of both the varieties showed maximum effect at 10% dose level regardless of the exposure period (Figure 1).

In all cases, the percentage of abnormalities increased with the longer duration at which the animals were sacrificed, except in two cases – the three-week exposure was less than the one-week exposure in case of 5% EE of BT, and the five-week exposure was less than the three-week exposure in case of 5% EEA of TA.

In all cases, the percentage of abnormalities was significant either at 1% or at 5% level, except in case of one-week exposure in case of 5% AE of BT. This was non-significant.

The data concerning the effect of AE of BT and TA on sperm-head morphology further provided evidence in support of the view that AE possesses a certain degree of toxic potentiality in every dose and duration of exposure.

The EEA of BT and TA were found to induce abnormal sperms significantly at a higher rate in comparison to the control, though the occurrences of faulty sperms were certainly less than those produced by EE.

Also the rate of abnormalities due to exposure of AE of either variety was obviously less than that of EE as well as EEA. But the abnormalities induced by this extract of both varieties were significantly higher than the control animals.

A comparative analysis of the data (Figure 1) shows a direct correlation between the dose exposure and rate of abnormalities. Moreover, out of three time intervals, exposure for a period of five weeks seemed to be more vulnerable with regard to the action of betel nut extract, irrespective of dose concentration and nut variety.

Statistical analyses of the data of sperm-head abnormality induced by different types of betel nut extracts of Tripura and Assam varieties, on the basis of Student's *t*-test, revealed significant sperm-head deformities in the experimental series, compared to control (Figure 1).

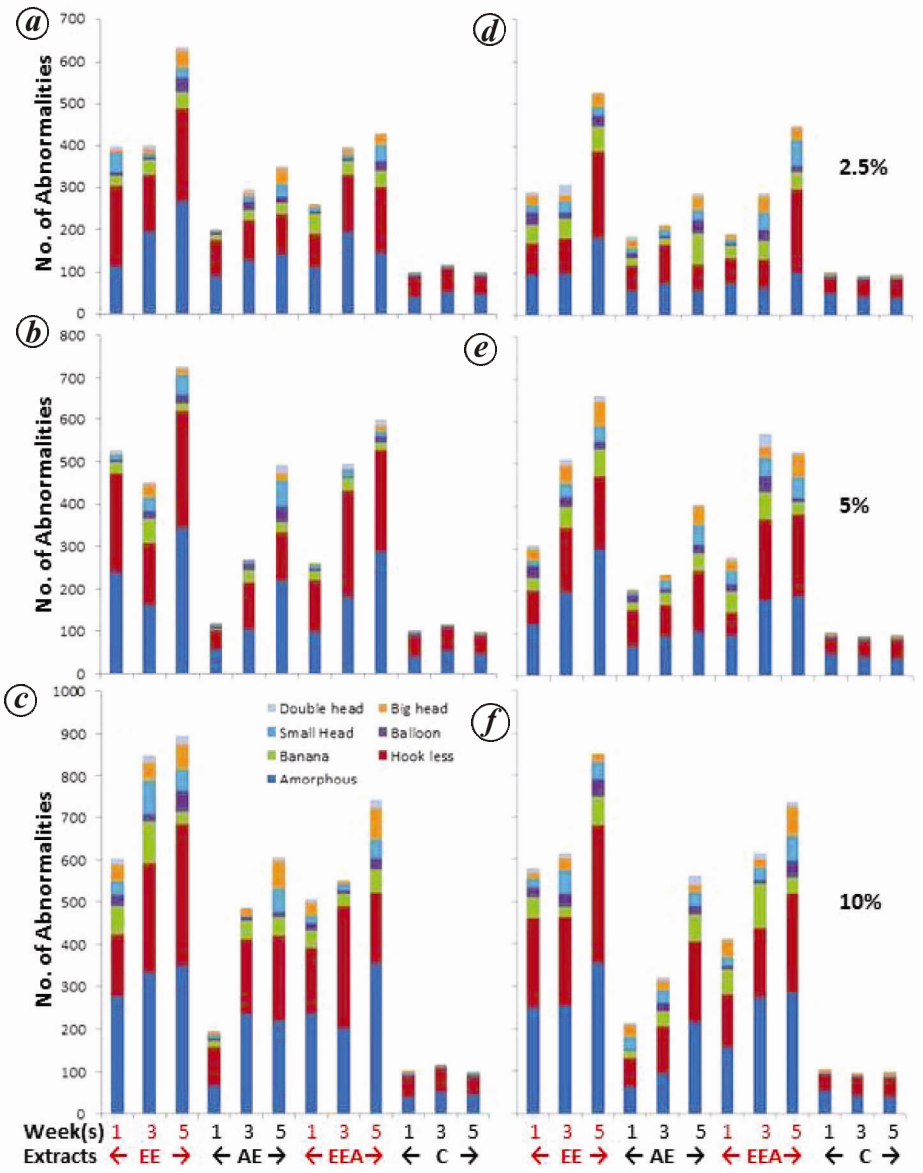

Figure 1. Frequency distribution of abnormal sperm-heads of mice induced by 2.5%, 5% and 10% of EE, AE and EEA of BT (***a***–***c***) and TA (***d***–***f***) with control (C) at different stages of spermatogenesis, viz. 1, 3 and 5 weeks. Five animals were taken for each treatment period; altogether 5000 sperms were studied.

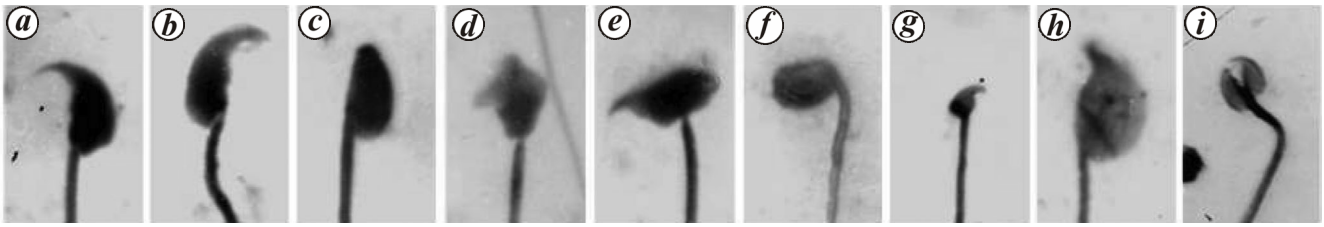

Figure 2. Sperm-head abnormalities: (***a***) Normal, (***b***) banana, (***c***) hookless, (***d***, ***e***) amorphous, (***f***) balloon, (***g***) small-head, (***h***) big-head and (***i***) double head.

All the treatments were significant at 1% level, except the following cases: 5% of AE for one week interval, which was non-significant, whereas 2.5% and 10% AE for one week interval and 5% AE for three weeks interval, which were significant at 5% level in case of BT. In case of TA also all the treatments were significant at 1% level, except the following cases: 2.5% AE for one and three weeks, 5% AE for three weeks interval, 2.5% of

Table 1. Two-way ANOVA for sperm-head abnormalities by the extracts of fresh betel nuts keeping the extract fixed

Sources of variance	Sums of squares	Degrees of freedom	Mean sums of squares	*F*	*P*
For ethanol extract					
Between doses	716,846.25	3	238,948.75	25.34*	0.0008
Between period of exposure (weeks)	70,568.00	2	35,284.00	3.74 NS	0.088
Error	56,578.00	6	9,429.67		
Total	843,992.25	11			
For aqueous extract					
Between doses	158,968.25	3	52,989.42	5.15 **	0.042
Between period of exposure (weeks)	108,475.17	2	54,237.58	5.27 **	0.048
Error	61,749.5	6	10,291.58		
Total	61,749.50	11			
For extract after aqua-leaching					
Between doses	387,470.91	3	129,156.97	20.02 *	0.0015
Between period of exposure (weeks)	68,835.50	2	34,417.75	5.33 **	0.047
Error	38,691.84	6	6,448.64		
Total	494,998.25	11			

*Significant at 1% level; **Significant at 5% level; NS, Not significant.

Table 2. Two-way ANOVA for sperm-head abnormalities by the extracts of BT keeping the dose fixed

Sources of variance	Sums of squares	Degrees of freedom	Mean sums of squares	*F*	*P*
For 2.5% dose					
Between extracts	220,248.00	3	73,416.00	16.79*	0.0025
Between period of exposure (weeks)	38,085.50	2	19,042.75	4.35 NS	0.068
Error	26,232.50	6	4,372.08		
Total	284,566.00	11			
For 5% dose					
Between extracts	363,045.66	3	121,015.22	11.11*	0.0073
Between period of exposure (weeks)	105,669.50	2	52,834.75	4.85 NS	0.056
Error	65,349.83	6	10,891.63		
Total	534,064.99	11			
For 10% dose					
Between extracts	744,178.25	3	248,059.41	25.21*	0.0008
Between period of exposure (weeks)	111,227.16	2	55,613.58	5.72**	0.041
Error	58,333.50	6	9,722.25		
Total	913,738.91	11			

*Significant at 1% level; **Significant at 5% level; NS, Not significant.

EEA for one week interval and 5% of EEA for one week interval. The analysis of data using two-way ANOVA (keeping the dose concentration fixed), showed that the occurrence of differential sperm-head abnormalities due to different types of extracts of BT and TA is statistically significant at 1%, 2.5% and 5% level of significance (Tables 1–4).

Thus, the occurrence of significant amount of abnormal sperms is revealed in all three stages of germinal cell treatment. The incidence of significant amount of sperm-head abnormalities in the mice group, exposed three weeks before being sacrificed can be explained by the fact that betel nut extracts of either variety might have imparted its action during the period of spermatid differentiation[9,10,12]. A similar significant occurrence of abnormal-shaped sperm in the mice group exposed to betel nut extract for a period of one week representing the treatment in the spermatozoa cell stage, may be explained by the action of betel nut extract in the development of young sperms that have not completed the process of differentiation. It is suggested that the betel nut extracts exerted maximum effects for the remarkably high

Table 3. Two-way ANOVA for sperm-head abnormalities by the extracts of tambul (TA) keeping the extract fixed

Sources of variance	Sums of squares	Degrees of freedom	Mean sums of squares	*F*	*P*
For EE					
Between doses	534,953.33	3	178,317.77	24.28*	0.0009
Between period of exposure (weeks)	96,563.17	2	48,281.58	6.5**	0.031
Error	44,055	6	7,342.5		
Total	675,571.67	11			
For AE					
Between doses	112,132.32	3	37,377.44	6.29**	0.0278
Between period of exposure (weeks)	56,538.50	2	28,269.25	4.75 NS	0.058
Error	35,682.18	6	5,947.03		
Total	204,353.00	11			
For EEA					
Between doses	386,226.66	3	128,742.22	10.08*	0.0093
Between period of exposure (weeks)	87,501.5	2	43,750.75	3.43 NS	0.102
Error	76,623.84	6	12,770.64		
Total	550,352.00	11			

*Significant at 1% level; **Significant at 5% level; NS, Not significant.

Table 4. Two-way ANOVA for sperm-head abnormalities by the extracts of TA keeping the dose fixed

Sources of variance	Sums of squares	Degrees of freedom	Mean sums of squares	*F*	*P*
For 2.5% dose					
Between extracts	128,870.91	3	42,956.97	9.91*	0.001
Between period of exposure (weeks)	47,820.50	2	23,910.25	5.52**	0.043
Error	25,996.83	6	4,332.80		
Total	202,688.25	11			
For 5% dose					
Between extracts	283,198.25	3	94,399.41	6.70**	0.024
Between period of exposure (weeks)	78,041.16	2	39,020.58	2.76 NS	0.141
Error	84,537.50	6	14,089.58		
Total	445,776.91	11			
For 10% dose					
Between extracts	605,176.66	3	201,725.55	25.31*	0.0008
Between period of exposure (weeks)	112,761.16	2	56,380.58	7.07**	0.026
Error	47,806.83	6	7,967.80		
Total	765,744.66	11			

*Significant at 1% level; **Significant at 5% level; NS, Not significant.

induction of abnormal sperm-head morphology at five weeks when the cells were in spermatogonial stage. It has been, however, argued that the induction of abnormal sperm-head morphology is the consequence of changes in the genes controlling the process of spermatogenesis[8,13–17].

The exact mechanism of abnormal-shaped sperm production is not clearly understood. As betel nut contains some alkaloids, it has been speculated that a probable non-disjunction event may occur in the germ cells, because many plant alkaloids act as spindle poisons which may cause non-disjunction of chromosomes in the dividing cells leading to abnormal-shaped sperm production[7,18]. Again it is assumed that arecoline, an alkaloid of betel nut, being a monofunctional alkylating agent[19] and having binding reactivity with nucleic acid and proteins[20] *in vivo*, may react with the germ cell DNA in its nucleophilic sites and thereby may elicit the lesions recognizable by the repair system of the early spermatid stages of the mouse.

It is interesting to note that the EEA produced less aberration in comparison to EE. It, therefore, implies that nuts cut into pieces and kept in water overnight may lose some of their active ingredients in water (AE), which

in turn are responsible for causing chromosomal damage to a certain extent. It is further noted that such an approach imparts no remarkable change in the morphology and quality of the nuts and presumably may not hamper any trade interest.

A comparative analysis of the data of betel nut exposures of both BT and TA varieties reveals no remarkable variation in their toxic potentialities. Since tambul is prepared by keeping the nuts under the soil for a period of 4–6 months, it may be assumed that during processing some of its chemical constituents may be degraded[21] to toxic chemicals as indicated by the appearance of a pungent odour[21]. So, there is a possibility that tambul is rather less toxic than raw yellow nuts of Tripura. But its almost equal toxic status suggests that the very processing method may allow the colonization of certain microbes which probably impart a deleterious role, directly or indirectly, on the chromosomal complements. The pungent odour of tambul represents a condition of tannin degradation, which is probably due to fungal spoilage[22]. It is argued that the inner soft endoplasm of the nut may be colonized by a range of fungi[23–25], and many of these fungi are mycotoxin producers. Further, some researchers have pointed out more specifically that betel nuts are often infested with aflatoxin producing fungus, *Aspergillus flavus*[26].

Betel nut/betel quid chewing is a socially and religiously accepted habit, though it has many adverse effects. Thus necessary steps should be adopted to educate the people about the consequences of discriminate use of betel nut and its components. It may be further suggested that both fresh and processed nuts, before use may be kept in water (preferably in small pieces), so as to minimize their harmful effects to some extent.

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