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Evaluation of the Reproductive Toxicity of Emisan 6 in Female Rats

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Fungicides as a generic class include an array of compounds having diverse chemical structures for controlling fungus growth and related diseases on plants, seeds and grains. These can gain entry into the human body either by accidental ingestion or through cutaneous absorption. Additionally, they can gain entry into the human body inadvertently through the food chain. Furthermore, mercurial fungicides are highly toxic, immutable, non-biodegradable and undergo bio-transformation and bio-amplification during transfer through food chain (Takeuchi, 1972).

Toxicity of Emisan 6 has been evaluated by many workers in fishes and amphibian (Kirubagaran and Joy, 1988ab, 1989, 1991; Gill etal 1985, 1988; Chapparadhali and Kanamadi, 1995). However such investigations on mammals remain scarce. Considering the widespread use of this fungicide in India and the possibility of agro-environmental contamination, the present study was planned to evaluate possible *in vivo* reproductive effects of Emisan 6, an organomercurial fungicide, in female rats.

MATERIALS AND METHODS

Emisan 6 (methoxy ethyl mercury chloride) sold under the trade name Bagalol-6, was purchased from Excel Industries Ltd., Mumbai, India. Chemically it contains methoxyethyl mercury chloride: 50% w/w, inert material and colour : 50% w/w, containing 6% organic mercury. It was dissolved in distilled water such that 1ml contained either 1,2 or 3 mg of Emisan 6 and was given by gavage. Random-bred female albino rats that were 6-8 weeks old and had an average body weight of between 150-200 gm were used for this study. The animals were acclimatized for a week before the start of the experiment. They were maintained in our air-conditioned animal facility (University Central Animal House) with a 12hr light / dark cycle, and provided with standard food pellets (Hindustan Lever Ltd., Bombay, India) and tap water *ad libitium*.

In Experiment I, 20 animals were randomly assigned to 4 treatment groups for studying oestrous cycle, ovulation points or corpora lutea and histopathological alterations in the reproductive organs. The 4 groups (5 animals each) were dosed

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daily with the water vehicle or 1.0, 2.0 or 3.0 mg Emisan 6 / kg body weight for 30 days. Body weights of the rats were recorded weekly during the trial. Diets were withheld from the animals on the night prior to the day of termination of the experiment. Reproductive organs including ovaries and uteri were weighed. Oestrous cycle of rats was studied by examining vaginal smear from first day to the date of cessation of treatment. The number of ovulation points or corpora lutea in both the ovaries were counted and compared with the control group. The data were analysed by student's t-test and results were expressed as the mean ± SE. For pathological examination, the organs were fixed in bouin's solution for 24 hr and embedded in paraffin. Sections of 4μm were stained with haematoxylin and eosin and observed microscopically.

In experiment II, animals were divided into different groups and treated as described above for experiment I. However, after 30 days of treatment, the female rats were allowed to mate with males of proven fertility in individual cages. The presence of a copulatory plug was taken as evidence of a fertile mating and was counted as the first day of pregnancy. The mated females were lapratomized on the 10^{th} day of pregnancy and implantation sites in the uterus were recorded and the data obtained were statistically evaluated using the one-tailed Mann-Whitney U-test. Females that failed to show implantation sites were scored as infertile.

In experiment III, animals were divided into the 4 treatment groups as described above. After dosing daily with Emisan 6 for 30 days, dosing was discontinued for the next 30 days. Animals were then allowed to mate with fertile females and implantation sites were assessed as described above for experiment II.

RESULTS AND DISCUSSION

Body weight gain and genital organ weights measured at autopsy are shown in Table 1. Body weight gain was significantly decreased in the 3mg/ kg group in a dose- related manner. The absolute weights of ovaries and uteri in the 3mg/kg group were significantly lower than those of the control group. On the other hand, the relative weights of ovaries and uteri did not change due to the suppression of body weight.

Emisan 6 caused a reduction in the number of corpora lutea (Table 1). The number of corpora lutea reduced significantly in the 2 and 3 mg/kg group. It appears that the effect is due to the anti-oestrogenic properties in the Emisan 6 as oestrogen is a luteotrophic agent. (Bogdanov, 1966; Keyes and Nalbandov, 1967).

The average length of the dioestrous phase (Table 1) in the untreated group throughout the whole test was 3.45 days and after Emisan 6 administration it ranged from 4.60 to 8.60 days. Compared with the untreated this prolongation was statistically significant at 3.0mg/kg dose level. Cranston,1945; Lerner etal, 1958 and Holtkamp etal, 1960 considered this phenomenon to result from the inhibition of the secretion of oestrogen.

Table 1. The effect of Emisan 6 on body weight (gm), genital organ weight (mg), ovulation and oestrous cycle in female rats.

Parameter	Dose (mg/kg body weight)			
	Control	1.00	2.00	3.00
Final body weight	162.44±1.37	160.20±0.52	159.00±0.80	154.20±2.34*
Ovaries	63.0±1.21	60.7 ±1.70	60.0 ± 0.83	59.6 ±0.30*
Per body weight (%)	0.038±0.002	0.037±0.003	0.037±0.002	0.38±0.003
Uteri	103.1±1.79	100.3±1.11	98.6±1.14	95.0±1.99*
Per body weight (%)	0.063±0.004	0.062±0.006	0.062±0.003	0.062±0.006
Mean number of corpora lutea/animal	11.6±0.84	9.4±0.54	7.6±0.77*	4.8±0.69**
Average duration of dioestrous phase in days	3.45±1.58	4.60±1.80	5.50±1.00	8.60±1.22*

Data represented as mean ± S.E; Sample size equals 5.
Values significantly different from the control group, * $p<0.05$; ** $p<0.01$.

There were no specific alterations in the histoarchitecture of ovary and uterus evaluated in animals dosed with 1.0 mg/kg body weight of Emisan 6. In animals dosed with 2.0mg/kg, there were pathological alterations at some sites in the ovary such as degeneration of germinal epithelium and stroma and reduced number of developing follicles. With the highest dose of Emisan 6 (3.0mg/kg body weight), the pathological alterations noted above were more marked. No primary follicles were seen emerging from germinal epithelium. An increased follicular atresia was noticed. Follicular epithelium was irregular and not so active. Number of developing follicles greatly reduced and those which present were highly degenerated and showed nuclear pyknosis. A few corpora lutea and graffian follicles were visible. Stroma was highly vacuolated, large number of blood spaces within the stroma indicated the haemorrhage of blood vessels (Fig. 1). Treatment with 2.0 mg /kg of Emisan 6 revealed distended lumen and depletion in the population of uterine glands in the uterus. In contrast, with the high dose of Emisan 6 (3 mg/kg), the endometrium was fully distended, thin and lined by small inactive epithelial cells. The myometrium was atrophic with small elongated nuclei and scant cytoplasm. Endometrial glands were absent (Fig. 2).

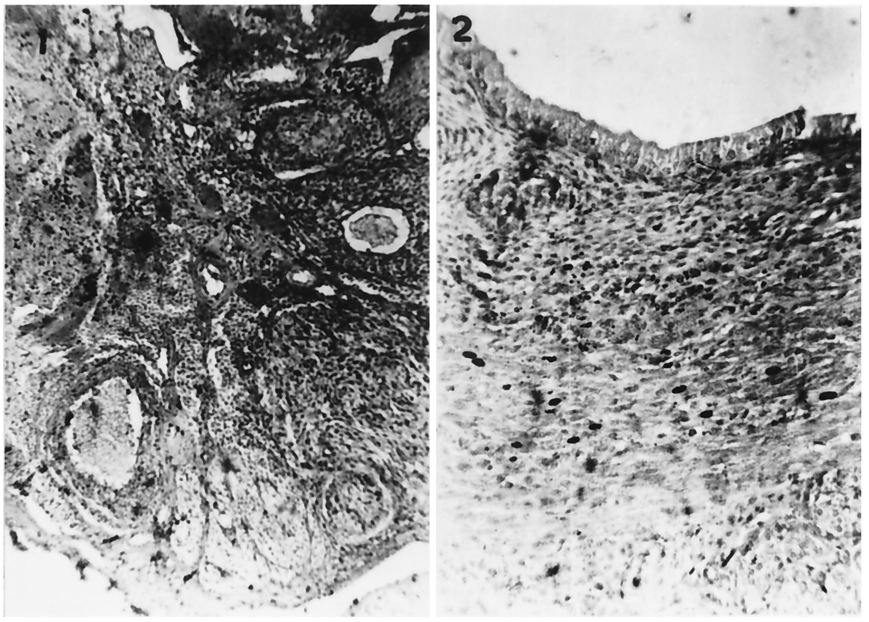

Figure 1. Ovary (3.0 mg). Degenerated germinal epithelium, stromal tissues and developing follicles (x160).

Figure 2. Uterus (3.0 mg) . Atrophic endometrium. The lumenal lining epithelial cells are small and the endometrial glands are absent (x 160).

Table 2. The effect of Emisan 6 on the fertility and implantation of female rats after 30 days of treatment.

Dose (mg/kg body wt)	No. of treated females	No. of normal male used	Fertile matings*	Total implants (mean ±SE)
0.0	5	5	5(100%)	47 9.4± 0.399
1.0	5	5	4 (80%)	31** 7.75±0.478
2.0	5	5	3(60%)	16*** 5.333±0.332
3.0	5	5	1(20%)	3*** 3 ± 0.00

* Those that resulted in pregnancy

Values significantly different from the control group, **p<0.05; ***p<0.01.

Thirty days of treatment of Emisan 6 did not affect mating behaviour. However, fertility was greatly affected. Only 20% of the rats that were treated with 1.0 mg/kg body weight of Emisan 6 had an infertile mating. The percentage of rats exhibiting infertile matings was as high as 40 and 80% following Emisan 6 treatments at dose levels of 2.0 and 3.0 mg/kg, respectively. The number of implants also decreased significantly at all dose levels (Table 2).

Table 3. The effect of Emisan 6 on the fertility and implantation of female rats mated 30 days after cessation of dosing.

Dose(mg/kg body wt)	No. of treated females	No. of normal Male used	Fertile matings*	Total implants (mean ± SE)
0.0	5	5	5 (100%)	47 9.4 ± 0.399
1.0	5	5	5 (100%)	45 9.0± 0.447
2.0	5	5	4 (80%)	28 ** 7.0 ± 0.408
3.0	5	5	1 (20%)	4*** 4 ± 00

*Those that resulted in pregnancy.
Values significantly different from the control group, ** $p<0.05$; ***$p<0.01$.

When fertility was evaluated thirty days after discontinuation of Emisan 6 treatment (Experiment III), there was a 20% increase in the 1.0 and 2.0 mg dose groups. However, the fertility did not increase in the 3.0 mg dose group compared to immediately after dosing (Table 3).

Emisan 6 caused a dose dependent decrease in the number of implants. Its anti-implantation effect appeared more potent at 3.0 mg/kg dose level. This may indicate that Emisan 6 possesses a strong anti-oestrogenic and anti-progesteronal activity which interferes the implantation. Since a perfect balance between oestrogen and progesterone is required for the implantation of an egg into uterus (Hensshaw, 1953; Hafez and Pincus, 1956; Mayer, 1963 and Psychoyos, 1966).

From the present study the hazards associated with use of this fungicide at high doses are evident and hence of considerable public health concern. The results obtained from the present investigation clearly depicted pathological lesions in the ovary and uterus by chronic ingestion of Emisan 6. It showed follicular atresia in the ovary which increased at higher doses. The process of atresia in mammalian ovary is due to lack of proper gonadotropin stimulation or due to imperfect balance of various hormones (Ingram, 1962 and Gauraya, 1973). These results are in agreement with the study of Kirubagaran and Joy (1988) who reported ovarian recrudescence in the catfish *Clarias batrachus* (L) by Emisan 6 and Ram and Sathyanesan (1986) who reported inhibition of gonadal development of the fish *Clarias batrachus* by the treatment of Emisan 6. Emisan 6 also showed inhibitory

effect on uterine histology as evidenced by absence of endometrial glands and atrophic myometrium. This may have occurred because of oestrogen (Allen etal 1937) which stimulates uterine histological structure in rats by increasing the number of uterine glands and stromal cells (Everett, 1962 and Euller and Heller, 1963).

The absolute weight of the investigated organs exhibited consistent dose dependent decrease with Emisan 6 treatment which was significant at the highest dose. The inhibitory effect of oestrogen on rat growth is known (Hart 1990). The effect has been attributed to depressed function of the anterior pituitary related to inhibition of the hypophysial growth promoting factor. Chapparadhali and Kanamdi (1995) have also reported significant decrease in weight of ovary and oviduct after the administration of mercurial fungicide, Emisan 6 to *Rana cyanophyctis*. Emisan 6 blocked follicular growth and resulted in enestrus in rats, possibly by altering both pituitary gonadotropin and ovarian steroid secretions. Since Kirubagaran and Joy (1991) documented disruption of adrenocortical pituitary activity in cat fish *Clarias batrachus* by Emisan 6, it seems plausible to speculate a similar mechanism operating in rats.

From the present investigation a dose of Emisan 6 as low as 3 mg/kg body weight can result in a deleterious effect in the reproductive system. It is necessary to further investigate toxicological profiles and other site specific effects associated with Emisan 6 intoxication.

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