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Evaluation of Estrogenic Activity and Effect of Endosulfan on Biochemical Constituents in Ovariectomized (OVX) Swiss Albino Mice

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Endosulfan is an organochlorine insecticide extensively used to control the pests of vegetables and fruits. Endosulfan has been identified in a variety of environmental media, fruits and vegetables (Haz Dat, 1998). Their residue level in foodstuffs is going to be increased as it is extensively used all over the world. Many of the treated areas are also irrigated with water from the rivers, tanks, reservoirs, etc. Return ducts filled with excess run of water empty into reverine and other aquatic bodies, habitats where a number of aquatic animals reside and water is used for different purpose. The pesticides have become problematic due to care less transport and application. Therefore, there is a need to gather more toxicologic data on the mammals that could be utilized for meaningful extrapolation of poisoning in humans. The xenoestrogens are known to mimic the female sex hormone estrogen or by inhibiting androgen effectiveness (Kelce et al.1995). It has been reported that organochlorine pesticides exhibit estrogenic activity in different species of animal which leads to reproductive toxicity and cause ovarian toxicity, decreased pool of healthy, large and medium sized follicles and increase in atretic follicles accelerates persistent vaginal estrus, advances puberty in female rats and mice (Martinez and Swartz, 1991; Jadaramkunti and Kaliwal, 1999). Earlier studies in animals exposed to endosulfan shows estrogenic activity in vitro studies as compared with that of the other chlorinated pesticides (Soto et al. 1994). In contrast, it was reported that endosulfan showed lack of estrogenic effect (Shelby, 1996). Since liver is the site for most of the vital reaction in the body, including the metabolism of the xenobiotics, many tissue biochemical changes are expressed to be associated with liver during the pharmacotoxicological state of intoxication (Srinivasan, 1981). Therefore, lack/ conflicting information on endocrine effects of endosulfan has prompted us to undertake the present investigation.

MATERIALS AND METHODS

Endosulfan technical grade (purity 98 %) obtained from Rallies India Ltd., Mumbai, used for the experiments. The doses were given orally in olive oilvehicle, below their acute LD_{50} level of intoxication according to their daily body weight.

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Female Swiss albino mice (12 – 17 weeks old) weighing between 22–25 g, showing at least 3 consecutive regular estrous cycles were selected randomly from the breeding stock. The animals were housed in separate cages bedded with paddy husk and had free access to pellet diet "Gold Mohar" (Hindustan Lever Limited, Mumbai) and water *ad libitum* throughout the study. They were kept under a lighting schedule of 12 : 12 h light and dark cycle at room temperature $26 \pm 1\ ^{0}$ C.

Estrogenic activity was determined using a standard bioassay method using uterine weight and vaginal cornification as end points in ovariectomized mice (Lerner, 1981; Kaliwal and Rao, 1983). Mice displaying cornified vaginal smear at estrus smear in the morning were bilaterally ovariectomized for the experiment. The ovaries of the mice were exposed to a venterolateral angled incision below the last rib under mild ether anesthesia. The mesovarian blood vessels were ligated and the exposed ovaries were carefully removed after opening the bursa with minimal trauma and bleeding. Sham operation was performed by exposing the ovaries and replacing back into the body cavity. In both procedures the incisions were closed with sutures using sterilized cotton thread. The ovariectomized mice were randomly divided into five groups containing at least 10 mice each. An effective dose of 4 mg / kg body weight / d endosulfan was administered orally to the ovariectomized mice for 30 days. Antiestrogenic activity of endosulfan in ovariectomized mice was assessed by administering 4 mg / kg body weight / d endosulfan and 5 μg /g / kg body weight / d estradiol- 17 β for comparison for 30 days from the day of operation. Sham operated and ovariectomized mice treated with an equivalent volume of olive oil served as control groups. Daily the vaginal smear and the body weight were recorded throughout the experiment.

All animals were killed by cervical dislocation and necropsied on 31st day, 24 hours after the last oral dose, and soon after the vaginal smear observation. The liver and uterus were dissected out, freed from adherent tissues and weighed to the nearest milligram. The uterus was fixed in Bouin's fluid and embedded in paraffin, sectioned at 5 μm thickness and stained with hematoxylin and eosin for microscopic examination.

Freshly removed liver and uterus tissues were weighed to required milligram for the biochemical analysis of protein, glycogen and lipids. The net weight of the tissues were estimated gravimetrically. Protein estimation was performed as per the method described by Lowry et al. (1951), glycogen by Scifter et al. (1950) and lipid by Folch et al. (1957). Statistical significance between the control and experimental data were subjected to analysis of variance (ANOVA) together with Dunnett's test ($P<0.05$).

RESULTS AND DISCUSSION

Sham operated control mice showed regular estrous cycle and a normal 30 day duration estrous cycle. Ovariectomized control mice treated with olive oil showed

a prolonged 30 days of diestrus with the result the diestrus index was 100. Administration of 4mg /kg/d endosulfan to ovariectomized mice did not show vaginal cornification and continuous diestrus without any appearance of estrus indicating its non-estrogenic activity; the diestrus index was 100. There is also no significant change in the uterine weight. In contrast, the ovariectomized mice treated with 5μg/kg/d estradiol 17-β as a positive control shows vaginal cornification and prolonged estrus with concomitant significant decrease and the diestrus index was 17.2. The uterine weight is significantly increased. the administration of 4 mg/kg/d endosulfan conjointly with 5μg estradiol 17-β to ovariectomized mice shows a significant increase in the duration of estrus with concomitant significant increase in the duration of diestrus and the diestrus index was 16.6. The uterine weight is also significantly increased. This indicates that the endosulfan neither show the synergetic estrogenic effect nor antiestrogenic effect in mice. (Table 1).

Table 1. Evaluation of estrogenic / antiestrogenic activity of endosulfan (ES) in ovariectomized (OVX) mice.

Groups	Treat-ment (mg/kg/d)	No. of mice	Duration in days (mean ± S.E)		Diestrus index	Change in body weight (g)	Relative Uterine weight (mg/ 100g body weight)
			Estrus	Diestrus			
A	Sham + oil	8	6.62± 0.18	14.0 ± 0.53	46.66	1.75 ± 0.16	468.25 ± 35.5
B	OVX + oil	8	0	30.0 ± 0.00	100.0	1.62 ± 0.26	279.62 ± 0.62
C	OVX + ES (4 mg)	8	0	30.0 ± 0.00	100.0	1.12 ± 0.29	274.50 ± 2.66
D	OVX + Estradiol – 17β (5 μg)	8	23.1± 0.5*	5.2 ± 0.3*	17.2	1.62 ± 0.26	551.00 ± 7.26*
E	OVX ES (4 mg)+ Estradiol – 17β (5 μg)	8	24.0± 0.50*	5.0 ± 0.3*	16.6	1.87 ± 0.12	556.00 ± 5.86*

- = significant $P < 0.05$ Compared to OVX control ,

$$\text{Diestrus index} = \frac{\text{Number of days with clear diestrus smear}}{\text{Total duration of treatment (days)}} \times 100$$

The data obtained in the present study reveals that the administration of endosulfan to mice in all groups did not change the weights of body, liver, kidneys, adrenals, thyroid and thymus (data not shown). In the present study treatment with endosulfan and estradiol 17-β to ovariectomized mice revealed that the levels of protein were not significantly changed in liver and significantly increased in uterus (Table 2, 3). Earlier reports on the possible effects of endosulfan showed dose and age related marginal increase in the protein content in the liver produced body tremors, muscular contraction, as well as hyperglycemia and reduction in the liver glycogen content (Kiran and Verma, 1988) Recently it has been reported that administration of mancozeb produced significant changes in the level of protein, glycogen and lipids in the liver, uterus and ovary in rats (Baligar and Kaliwal, 2001)

Table 2. Biochemical changes in liver following the administration of endosulfan (ES) and 17β -estradiol –in ovariectomized mice.

Groups	Treatment (mg /kg/d)	No of mice	(µg / mg wet weight of tissue ; mean ± S.E)		
			Protein	Glycogen	Total lipids
A	Sham + oil	5	143.8 ± 0.58	3.74 ± 0.12	34.0 ± 0.59
B	OVX + oil	5	143.6 ± 0.52	3.70 ± 0.13	34.4 ± 0.52
C	OVX + ES (4 mg)	5	146.4 ± 0.33	3.36 ± 0.16*	32.8 ± 0.47
D	OVX + Estradiol – 17β (5 µg)	5	146.0 ± 0.42	3.32 ± 0.11*	32.4 ± 0.33
E	OVX + ES (4 mg) + Estradiol – 17β (5 µg)	5	143.0 ± 0.46	3.40 ± 0.13*	31.8 ± 0.23

* = significant $P < 0.05$ Compared to OVX control

The results of the present study reveals that the levels of glycogen were significantly decreased in liver with endosulfan treatment or estradiol 17-β or both in ovariectomized mice (Table 2, 3). In contrast, the levels of glycogen were significantly increased in the estradiol 17-β treated ovariectomized mice whereas there was no change in the levels of glycogen in endosulfan treated ovariectomized mice showing its no-estrogenic activity as compared to with the positive control estradiol 17-β treated mice. Recently it has been reported that the administration of mancozeb resulted in a significant decrease in the levels of glycogen in ovary and uterus (Baligar and Kaliwal, 2001).

Table 3 . Biochemical changes in uterus following the administration of endosulfan (ES) and estradiol –17β in ovariectomized mice.

Groups	Treatment (mg / kg / d)	No. of mice	(µg / mg wet weight of tissue ; mean ± S.E)		
			Protein	Glycogen	Total lipids
A	Sham + oil	5	113.2 ± 0.32	3.48 ± 0.11	41.8 ± 0.23
B	OVX + oil	5	103.8 ± 0.50	3.16 ± 0.10	40.2 ± 0.32
C	OVX + ES (4 mg)	5	103.2 ± 0.45	3.12 ± 0.11	39.6 ± 0.12
D	OVX + Estradiol – 17β (5 µg)	5	110.0±0.67*	5.12 ± 0.18*	36.6 ± 0.12
E	OVX + ES (4 mg) + Estradiol – 17β (5 µg)	5	108.8±0.57*	5.64 ± 0.10*	36.0 ± 0.63*

* = significant $P < 0.05$ Compared to OVX control

The data obtained in the present study revealed that the levels of lipids were not significantly changed in liver and significantly decreased in uterus in endosulfan and estradiol 17-β treated ovariectomized mice (Table 2, 3). It has been reported that the levels of total lipids, phospholipids and neutral lipids were significantly increased in ovary and liver and decreased in uterus with mancozeb treated rats (Baligar and Kaliwal, 2001). The changes in the levels of protein, glycogen and lipids with endosulfan and estradiol 17-β treatment suggests either the increased catabolism of the biomolecules to meet the enhanced energy demand of the animals under the stress or their reduced synthesis due to impaired tissue functions (Ivanova, 1982).

In the present study the observations revealed that the treatment with endosulfan to ovariectomized mice failed to show estrogenic response in vaginal cornification or uterine wet weight or glycogen content in the uterus. The endosulfan treatment conjointly with estradiol 17-β do not show a synergistic estrogenic response nor anti-estrogenic response of the dose and duration of the treatment in ovariectomized mice. However, further investigation in this regard is essential to know the estrogenic activity of the endosulfan in mice.

With growing concern those estrogenic chemicals in the environment either naturally occurring or man made may adversely affect the health of humans, domestic animals and wild life (Colborn et al. 1996). Need for the meaningful standardized and widely accepted methods for reliably detecting and

characterizing estrogenic chemicals has gained importance. Many assays for estrogenicity have been proposed and several are in broad use. Treatment with several organochlorine pesticides to female rats shows estrogenic activity (Bulger and Kupfer, 1985). The enhanced uterine wet weight in immature, intact and ovariectomized rats and mink has been observed after giving DDT analogs showing estrogenic activity (Welch et al.1969). However, the earlier studies with endosulfan with showed neither increased wet weight nor the glycogen content in uterus of the ovariectomized rats and mice. Both the above studies report that the lack of estrogenic effects of endosulfan in ovariectomized rats and mice might be due to its rapid metabolism in the body. It is also felt that the doses and animal size of studies were very limited to state that endosulfan lacks estrogenic activity (Shelby et al.1996).

In the present study the endosulfan treatment to ovariectomized mice failed to show estrogenic response in uterine wet weight or glycogen content in the uterus. The endosulfan treatment conjointly with estradiol 17-β showed all the above estrogenic responses positive. Hence, from our studies the endosulfan is neither estrogenic nor antiestrogenic of the given doses in mice. In contrast to our findings it has been reported that endosulfan, toxaphene and dieldrin have shown estrogenic response / effects on human estrogen sensitive cells in an *in vitro* study (Soto et al.1994). The rodent assay measures the increase of uterine wet weight / vaginal cornification / glycogen content; this is only a crude estimate of estrogen action because it represents the combination of effects viz. water imbibition hypertrophy, which is also produced by estrogen antagonists and hyperplasia (Clark et al.1974). Hence, further study is essential.

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