Bulgarian Journal of Veterinary Medicine (2008), **12**, No 1, 33–41

HISTOLOGICAL EXAMINATION OF ENDOSULFAN EFFECTS ON FOLLICULAR DEVELOPMENT OF BALB/C MICE

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Summary

Azarnia, M., H. M. Koochesfahani, M. Rajabi, Y. Tahamtani & A. Tamadon, 2009. Histological examination of endosulfan effects on follicular development of BALB/c mice. *Bulg. J. Vet. Med.*, **12**, No 1, 33–41.

The effects of endosulfan on ovary structures of 50-day-old BALB/C mice were assessed. Peanut oil was used as solvent. The vaginal smear and body weight of the mice were recorded on a daily basis. Mice were divided into three groups: untreated controls, peanut oil-treated and endosulfan-treated. Both treated groups received 10 mg/kg endosulfan 35% emulsifiable concentrate in the same amount of peanut oil, and peanut oil only, respectively, injected intraperitoneally once a day for 7 days. On the 8th day after the first injection the animals were sacrificed, the ovaries removed and studied macroscopically and microscopically. In the endosulfan-treated group no significant alteration in the relative weight of ovary was observed when compared with both peanut oil and control groups. Significant decreases in the number of primordial, primary, growing, and Graafian follicles, corpus luteum and increased number of atretic follicles were observed vs peanut oil-treated and control groups ($P<0.05$). The diameters of the Graafian follicles and their oocytes, and theca and granulosa cell layers were decreased in the endosulfan group when compared with peanut oil treated and untreated groups ($P<0.05$). Endosulfan-induced reduction in ovarian size in mice was associated with a decrease in healthy ovarian follicles and corpora lutea and an increase in atretic follicles.

Key words: BALB/c mice, corpora luteum, endosulfan, follicular development

INTRODUCTION

Endosulfan, a cyclodiene chlorinated hydrocarbon (6,7,8,9,10,10-hexachloro-1,5, 5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) is an insecticide with a relatively broad spectrum of activity. Technical endosulfan is a brown crystalline substance consisting of α- and β-isomers in the ratio of approximately 70:30 (Goebel *et al.*, 1982). Endosulfan has been identified in a variety of environmental media (air, surface water, ground water, soil, and sediment) collected at 89 of 1467 national priorities list (NPL) hazardous waste sites (HazDat, 1998). Endosulfan metabolites have been reported in foods including milk (Bennett *et al.*, 1997), chilli fruits (Pokharkar & Dethe, 1981), fruits, and vegetables (Mitchell, 1976; Pokharkar & Dethe, 1981). Earlier studies showed no persistence of endosulfan in biota and the pesticide does not readily bioaccumulate. The half-life

of endosulfan in water is estimated to be 4 days (Simonich & Hites, 1995). Endosulfan is well absorbed by ingestion, inhalation, and skin contact. The toxicity of endosulfan varies depending upon the route of administration, species, and sex of animal (Dikshith *et al.*, 1988). *In vitro* assays have demonstrated estrogenic responses to endosulfan (Raun Andersen *et al.*, 2002) similarly to other organochlorine pesticides as DDT, chlordecone, methoxychlor, and dicofol (Uphouse *et al.*, 1984; Osman, 1985; Gellert, 1978; Martinez & Swartz, 1992; Jadarmkunti & Kaliwal, 1999). Oral administration of the pesticides endosulfan, methyl parathion and mancozeb inhibits compensatory ovarian hypertrophy, decreases the number of healthy follicles, increases the number of atretic follicles, and affects the oestrous cycle in rats (Asmathbanu & Kaliwal, 1997; Dhondup & Kaliwal, 1997; Mahadevaswami *et al.*, 2000). There are no reports on the effects of endosulfan on follicular dynamics, oocytes and cell layers, and corpus luteum in BALB/c mice.

The purpose of the present study was to evaluate the effects of endosulfan on follicular development and structures in these laboratory mammals.

MATERIALS AND METHODS

Chemical

Endosulfan was applied as a commercial emulsifiable concentrate formulation containing 35% active ingredient and was obtained from Agricultural Struggle Center, Tehran, Iran. It was diluted in peanut oil for the final concentration. The LD_{50} of intraperitoneally injected endosulfan in mice is 14 mg/kg b.w. per day (Akhila *et al.*, 2007).

Animals and treatments

Eighteen BALB/c mature female mice (50-day-old) from Pasteur Institute, Tehran, Iran were used. They were housed in cages with *ad libitum* access to autoclaved standard mouse chow and water throughout the study. In order to maintain stable biological rhythms, 12 h of artificial light and 12 h of darkness were provided. All mice received humane care.

The mice were set in three groups (n=6 per group) as followed: endosulfan-treated group: they were injected intraperitoneally, once a day for 7 days with 10 mg/kg endosulfan dissolved in peanut oil; peanut oil-treated group: injected intraperitoneally, once a day for 7 days with the same volume of peanut oil only and control group: untreated.

Daily phases of the oestrous cycle and body weights were recorded throughout the experiment. The phases of estrous cycle were determined by observing the vaginal smear in the morning (08:00–10:00 h) as described by Zarrow *et al.* (1964). All animals were sacrificed under ether anesthesia on day 8, 24 h after the last injection, and soon after vaginal smear observation. All animals were found to be in dioestrus at the time of sacrifice. Ovaries were harvested freed from adherent tissue and were studied macroscopically and microscopically afterwards. Experimental procedures were approved by the Teacher Training University Animal Ethics Committee and followed National Institutes of Health and European Union guidelines for animal care.

The body weight gain percentage was calculated through comparing the weight of the animals 24 h before the treatment started and the respective weights by the 8^{th} day of treatment, before administering the anaesthetic.

Measurement of ovary weights and counting of corpora lutea and Graafian follicles

After weighing the ovaries and measuring their diameters, they were put into a human tubal fluid (HTF)-medium. To ensure normalization of data for statistical analysis, ovary weight was expressed per 100 g body weight as well. The number of corpora lutea and Graafian follicles in the ovaries was counted using a stereo microscope (Nikon SMZ800, Japan).

Ovary section preparation and morphological classification of growing follicles

The ovaries were fixed in 10% buffered formalin, embedded in paraffin wax, serially sectioned at 5 μm, stained with haematoxylin & eosin and analyzed under light microscope.

Follicle types in ovarian cross-sections were defined as followed: *Primordial follicles* comprised an oocyte surrounded by a single layer of cuboidal granulosa cells. *Primary follicles* comprised an oocyte surrounded by two or more layers of granulosa cells with no antrum. *Growing follicles* were distinguished by an antrum within the granulosa cell layers enclosing the oocyte F. *Atretic follicles* were those displaying two or more of the following criteria within a single cross-section: more than two pyknotic nuclei, granulosa cells within the antral cavity, granulosa cells pulling away from the basement membrane, and/or uneven layers of granulosa cells.

Quantification of early follicles

A fractionator/physical dissector design (Braendgaard *et al.*, 1990; Miller *et al.*, 1997) was used to estimate follicle number in the ovaries of BALB/c mice, as has been described previously for wild-type mice (Myers *et al.*, 2004). Briefly, follicle counts were made using the CASTGRID system (v2.1.4, Olympus Denmark A/S, Albertslund, Denmark) and a stereological design incorporating the fractionator and physical dissector. Estimates of follicle number per ovary were made by incorporating stereological sampling protocols on a predetermined fraction of ovarian serial sections. In this case, every 10^{th} and 11^{th} sections were used, and follicles (with visible nuclei) were counted if they appeared in one section but not the consecutive "look up" section, which ensured that follicles were counted only once.

Graafian follicles and their oocytes

Oocyte diameters of all counted Graafian follicles were measured. Profiles of Graafian follicles and oocytes were measured by calculating the geometric mean of the long and short axes which was measured using the CASTGRID system.

Statistical analysis

Data was analyzed using SPSS computer based software (SPSS for Windows, version 11.5, SPSS Inc, Chicago, Illinois). The significance was calculated using one-way analysis of variance (ANOVA) and followed by Tukey multiple comparison procedure to calculate the significance. A value of $P<0.05$ was taken as statistically significant.

RESULTS

There were no deaths in any of the groups. A significant decrease in the body weight of treatment group when compared both to peanut oil-treated and control groups ($P<0.05$; Table 1) has occurred. The daily administration of 10 mg/kg endosulfan to mice caused a significant reduction in the ovarian size. In treated groups, no significant alteration in

Table 1. Body weight, ovary weight, follicular development and structures in BALB/c mice treated i.p. once daily for 7 days with either 10 mg/kg endosulfan in peanut oil (n=6) or peanut oil only (n=6) and in untreated controls (n=6). Data are presented as mean ± SEM

	Groups		
	Endosulfan	Peanut oil	Control
Body weight gain (%)	12.55 ± 3.81[a]	25.73 ± 2.89[b]	27.98 ± 2.31[b]
Ovary weight (mg)	2.52 ± 0.05[a]	3.11 ± 0.06[b]	3.40 ± 0.81[b]
Relative ovarian weight (mg/100g b.w.)	16.10 ± 2.30	15.40 ± 1.40	14.90 ± 2.10
Ovarian diameter (mm)	1.49 ± 0.03[a]	1.54 ± 0.03[b]	1.69 ± 0.04[b]
Number of corpora lutea	1.71 ± 0.21[a]	3.68 ± 0.39[b]	3.92 ± 0.24[b]
Number of follicles			
Primordial	15.11 ± 0.91[a]	16.82 ± 0.58[b]	28.70 ± 0.71[b]
Primary	6.18 ± 0.22[a]	9.01 ± 0.52[b]	10.31 ± 0.31[b]
Growing	5.49 ± 0.59[a]	8.91 ± 0.72[b]	9.42 ± 0.60[b]
Graafian	4.05 ± 0.59[a]	4.14 ± 0.40[b]	5.22 ± 0.61[b]
Atretic	3.31 ± 0.30[a]	2.28 ± 0.08[b]	2.17 ± 0.12[b]
Graafian follicles			
Diameter (mm)	0.34 ± 0.02[a]	0.42 ± 0.02[b]	0.43 ± 0.03[b]
Oocyte diameter (μm)	51.93 ± 2.71[a]	48.19 ± 3.09[b]	41.07 ± 3.47[b]
Granulosa cell layers diameter (μm)	36.51 ± 2.11[a]	58.39 ± 1.94[b]	61.75 ± 2.03[b]
Theca cell layers diameter (μm)	25.15 ± 0.21[a]	28.71 ± 0.91[b]	31.41 ± 2.31[b]

[a, b] statistically significant difference at $p<0.05$ between different superscript letters for the same row.

the relative weight of ovary was observed when compared to the control group.

The treatment with 10 mg/kg endosulfan per day significantly decreased the number of primordial, primary, growing, and Graafian follicles, the number of corpora lutea, and significantly increased the number of atretic follicles when compared to the peanut oil-treated and control mice ($P<0.05$). There was a significant reduction in the diameter of the Graafian follicles and their oocytes and theca and granulosa cells layers compared to the peanut oil-treated and control mice ($P<0.05$).

Histological sections of representative ovaries are shown in Figs. 1 and 2. Some changes in follicular size and form in treated ovary section (Fig. 2) were clearly observed compared to those of the normal ovary section (Fig. 1).

DISCUSSION

Toxicants that interfere with ovarian function can act directly on the ovary or indirectly through demonstrating its influence at the hypothalamic or/and pituitary gland level (Smith, 1983). In the present study, endosulfan produced a significant ovarian hypotrophy in mice.

The endosulfan-associated decrease in ovarian growth was identified when compared to the ovary of either peanut oil-treated or control mice. A reduction in the number of healthy follicles was seen after endosulfan treatment, perhaps due to the suppression of secretion or release of gonadotropins or due to desensitization of

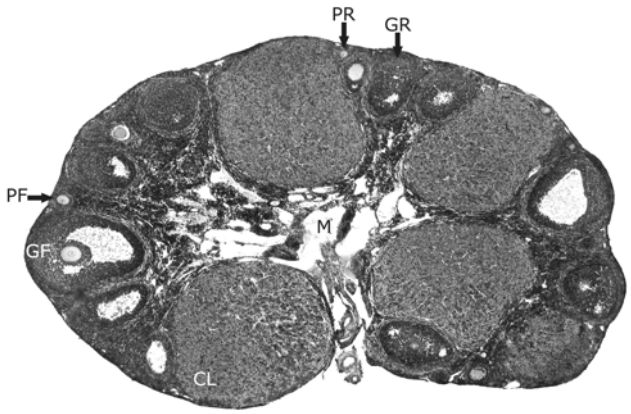

Fig. 1. Transverse section through the hilus of the ovary taken at autopsy on day 8 from a mouse of control (untreated) group showing the presence of many primordial follicles (*PR*), primary follicles (*PF*), growing follicles (*GR*), Graafian follicle (*GF*), and corpora lutea (*CL*). *M*= medulla. The animal was in dioestrus at the time of death. Hematoxylin and eosin, 30×.

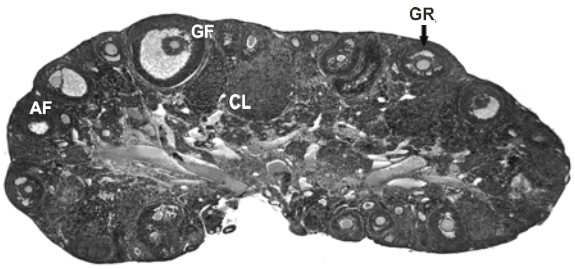

Fig 2. Transverse section through the hilus of the ovary taken at autopsy on day 8 from a mouse treated daily with 10 mg/kg endosulfan for 7 days showing the presence of growing follicles (*GR*), Graafian follicle (*GF*), corpora lutea (*CL*), and many atretic follicles (*AF*). The animal was in dioestrus at the time of death. Hematoxylin and eosin, 30×.

the ovary to gonadotropins as suggested by Peterson *et al.* (1964). Ovarian toxicity of 2-bromopropane in the rats also showed dose-dependent ovarian follicular atresia accompanied by a decreased number of normal antral and growing follicles in high dose treated rats (Kamijima *et al.*, 1997).

Analysis of differential follicular composition in this study indicated that endosulfan treatment for 8 days led to a significant decrease in the number of healthy follicles with a concomitant significant increase in the number of atretic follicles in the ovary. A reduction of different types of healthy follicles with increased follicular atresia has been reported in rats treated with different pesti-

cides (Asmathbanu & Kaliwal, 1997; Dhondup & Kaliwal, 1997; Mahadevaswami *et al.*, 2000; Hiremath & Kaliwal, 2002). The present investigation is comparable to the case of follicular toxicity caused by organophosphate pesticides in that endosulfan increased follicular atresia in hemicastrated mice (Hiremath & Kaliwal, 2002). In another experiment, Ataya *et al.* (1988) reported that cyclophosphamide inhibited the development of antral follicles in rats thereby increasing atretic follicles by interfering with normal ovarian follicular development and reducing estradiol. Oral doses of zineb retarded the development of splenic follicles in young rats (Dinoyeva, 1974). It has been suggested that polycyclic aromatic hydrocarbon ovarian toxicity was directed towards small oocytes (Krarup, 1979). Gellert (1978) reported an increased incidence of cystic follicles in ovaries exposed to DDT neonatally. It has been suggested that chlordecone induced follicular toxicity by reducing the pool of healthy follicles with an increase in atretic follicles (Swartz & Mall, 1989). According to Martinez & Swartz (1992), methoxychlor-treated adult rats showed an increase in the number of regressing follicles and reduced the total number of healthy follicles. It has been suggested that the ovarian toxicity of 2-bromopropane in the rat is associated with ovarian follicular atresia accompanied by decreased number of normal antral and growing follicles (Kamijima *et al.*, 1997).

In the present study, treatment of the mice with endosulfan produced a significant decrease in ovarian size, a decrease in the number of healthy follicles and corpora lutea, and a concomitant increase in atretic follicles, which may have been due to alteration of ovarian hormones or to altered gonadotropin secretion through a central nervous system mechanism, as observed in rats following administration of dithiocarbamates (Lu, 1995; Goldman *et al.*, 1994; Goldman *et al.*, 1997). Goldman *et al.* (1990) have reported that the insecticide chordimeform may suppress GnRH release. It has also been reported that toxicants may act directly on the hypothalamus to alter gonadotropin synthesis and secretion, or indirectly by altering pituitary responsiveness to GnRH or gonadal steroids. Both actions will result in alteration in serum FSH and LH levels (Dickerson *et al.*, 1992). It has been reported that some xenobiotics such as delta 9-tetrahydrocannabinol, cyclophosphamide and 7,12-dimethylbenz(a)anthracene may impair reproductive function by directly influencing the gonads resulting in impaired feedback to the hypothalamus and pituitary (Asch *et al.*, 1981; Jarrell *et al.*, 1987; Pasqualini *et al.,* 1990). Furthermore, endosulfan, toxaphene, and dieldrin have shown estrogenic response/ effects on human estrogen sensitive cells in an *in vitro* study (Soto *et al.*, 1994).

The administration of endosulfan for 8 days decreased the body weight. This finding is important because nutritional deficiencies have been shown to alter reproductive function (Dikshith *et al.*, 1988; Simonich & Hites, 1995; Baligar & Kaliwal, 2001). Reduction in glucose, glycogen, lipid, and protein synthesis have been shown in endosulfan toxicity in tissues of the freshwater snail *Bellamya dissimilis* (Padmaja Rambabu & Balaparameswara Rao, 1994). There were also observable changes in the weights of the ovary following a 8-day exposure to endosulfan.

The administration of endosulfan for 8 days decreased the size of the oocytes of Graafian follicles in diameter. The structural damages caused by the pesticides are dose- or time-dependent and are caused

either by autolysis induced by the cell's own enzymes or by rapid lysis induced by direct action of the toxicant. Both factors may have played a part in bringing on the damages to the oocytes, granulosa cells and theca cells. Bajpayee *et al.* (2006) have reported that endosulfan and its isomers and metabolites are able to induce DNA damage in mammalian cells. Sahai (1989) observed fusion of oocytes in the immature ovary of Ticto barbs (*Puntius ticto)* after several days of endosulfan exposure.

In conclusion, the endosulfan-induced decrease of ovarian size in mice was associated with a decrease in healthy ovarian follicles and corpora lutea, and increased number of atretic follicles. Whether this endosulfan effect is due to a direct effect on the ovary or an indirect effect on the hypothalamus or pituitary, or to a desensitization of the ovary to gonadotropins, cannot be ascertained from this study. Further investigation on mechanisms of endosulfan ovarian toxicity are therefore necessary.

ACKNOWLEDGEMENTS

Part of the costs of this research, as MS thesis, has been provided by the Department of Biology, Faculty of Science, Teacher Training University, Tehran, Iran.

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Paper received 14.07.2008; accepted for publication 10.11.2008

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