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Dose-Dependent Estrous Cycle, Ovarian Follicles and Biochemical Contents Reversal in Albino Mice after Exposure to Mancozeb.

M. D. Parimala and B. B. Kaliwal *
Post- graduate Department of Studies in Biotechnology and Microbiology, Karnatak University, Dharwad-580003, India.
** Corresponding Authors E-mail: b_kaliwal@yahoo.com*

ABSTRACT
Mancozeb, a fungicide of a manganese-zinc ethylenebisdithio-carbamate (EBDC), was administered by gavage at doses of 200, 400, 600 and 800 mg/kg/day to female virgin mice for 30 days. The mice were autopsied on 31st day. Mice were also treated with similar doses for a period of 30 days and the treatment was withdrawn for a further period of 30 days for reversible study. The mice were autopsied on 61st day. Estrous cycle and follicles were affected in all the mancozeb treated mice when compared to the controls. There was a recovery in number and phases of estrous cycle in the mice after withdrawal of 400 and 600 mg/kg/day mancozeb, however, recovery was not seen in 800 mg/kg/day mancozeb withdrawal mice. There was a recovery in the number of follicles in the mice after withdrawal of 400 mg/kg/day mancozeb. However, complete recovery of the follicles was not seen in 600 and 800 mg/kg/day mancozeb withdrawal mice. Mice treated with 600 mg/kg/day mancozeb showed significant decrease in the levels of protein and glycogen in the ovary, glycogen and total lipids in the uterus, glycogen in the liver but there was significant increase in the total lipids of the liver. Mice treated with 800 mg/kg/day mancozeb showed significant decrease in protein and glycogen but there was significant increase in total lipids in ovary and liver , however, there was complete decrease in the levels of protein, glycogen and total lipids of the uterus. There was recovery in the levels of protein and total lipids in the ovary and liver in 600 and 800 mg/kg/day mancozeb withdrawal mice, however, recovery was not seen in the level of glycogen. There was significant decrease in the relative weight of the ovary and liver whereas spleen and thyroid weights were increased significantly with 800 mg kg/day mancozeb treatment. The recoveries in the relative weight were observed in the ovary, uterus, thymus and thyroid however, recovery in the relative weight was not seen in liver and spleen in 800 mg/kg/day mancozeb withdrawal mice. The results of the present study indicated a marked effect in the recovery of the estrous cycle, follicles and biochemical contents of the ovary, uterus and liver in mice after exposure to mancozeb with lower doses and it was dose dependent

Keywords: Biochemical Contents, Estrous cycle, Female mice, Follicles, Mancozeb, Ovary, Reversal

INTRODUCTION

Pesticides are indispensable in modern agriculture by increasing food production by controlling agricultural pests and reducing vector born diseases. Carbamate pesticides are biodegradable with low mammalian toxicity and hence these pesticides have gained much more importance and are used to manage several pests. It has been reported that some of the organophosphorous and carbamate pesticides have been found to interrupt the estrous cycle and induce follicular toxicity in rats (Dhondup and Kaliwal, 1997; Asmathbanu and Kaliwal, 1997; Mahadevaswami *et al*. 2000; Baligar and Kaliwal, 2001). A compound related to mancozeb, sodium N-methyldithiocarbamate has been found to block the ovulation in rat (Goldman *et al*. 1994). Studies have been carried out on the toxicity of dithiocarbamates containing heavy metals. Mancozeb (Mn - and Zn - containing dithiocarbamate) and maneb (Mn – containing dithiocarbamate), two commonly used fungicides have been shown to induce tumors in rats (Subra-moniam *et al*. 1991) and cause teratogenic effects (Larsson *et al*. 1976). It has been reported that mancozeb

reduces its biological effects via its metabolites like ethylene thiourea (ETU) and carbon disulphide (CS2) (Thorn and Ludwing, 1962; Ivanova-Chemishanska L, 1962). Studies on mice also suggests that follicular count may provide a sensitive means of estimating the extent of ovarian toxicity in females exposed to xenobiotics (Mattison *et al*. 1989; Weitzman *et al*. 1992). Mancozeb is used either alone or in combination with copper or sulfur (Kurttio *et al*. 1990). Despite its low acute toxicity, mancozeb has been shown to produce adverse effects on reproduction, liver, kidney, central nervous system and chromosomes of bone marrow cells in mice (Sittig and Mane, 1991). Mancozeb is also known to cause maternal toxicity, embryonic toxicity and characteristic teratogenic effects in rat (Varnagy *et al*. 2000). It has been reported that the oral administration of mancozeb affects ovarian growth, follicular development, vaginal cyclicity, biochemical contents of the liver, uterus and ovary and on spermatogenesis in rats (Kackar *et al*. 1997; Mahadevaswamy *et al*. 2000; Baligar and Kaliwal, 2001; Baligar and Kaliwal, 2003; Ksheerasagar and Kaliwal, 2003), causes menstrual cycle disorder in women (Kaskevich *et al*. 1981) and also affects pregnancy in women (Neyko *et al*. 1974). It has been also reported that the exposure of pregnant mice to mancozeb inhibits implantation (Bindali and Kaliwal, 2002). Since, there are no reports on reversibility of induced gonadal toxicity in female mice after chronic exposure to a fungicide mancozeb, the present study was undertaken to elucidate the effect of carbamate fungicide mancozeb on the estrous cycle, the pattern of changes in the ovarian follicular kinetics, biochemical contents of ovary, uterus and liver and their recovery after withdrawal of mancozeb treatment in albino mice

MATERIALS AND METHODS

2.1. Chemical

Mancozeb (manganese-zinc ethelene bisdithiocarbamate) carbamate fungicide was made available from Indofil Chemicals Company, Mumbai [75% wettable powder (wp)]. The stock solution was prepared in olive oil with required concentration for oral administration. Doses were given according to the body weight of mice. The quantity of olive oil for each dose will never exceed 1ml/kg body weight/d. The doses were given below the acute LD50 level of intoxication. The LD 50 of mancozeb in rat is 8 g/kg.

2.2. Animals and treatments

Laboratory bred adult virgin female Swiss albino mice aged between 80-90 days and weighing 25-30 gms were selected for the experiment. However, the animals were matched with age and by body weight across treatment groups at the start of the experiment. The mice were maintained in the laboratory of the Post-graduate Department of Studies in Zoology, Karnatak University, Dharwad. The mice breed quite normally, almost throughout the year. They were housed in separate polypropylene cages containing sterile paddy husk as bedding material. The animals were provided with standard pellet diet "Gold Mohar" mice feed (Hindustan Lever Company, Mumbai) and water ad libitum. The mice were maintained under normal day/night schedule (12L: 12D) at room temperature 26° ± 1°C. Daily vaginal smear and body weight were recorded in the morning to determine the duration of estrous cycle throughout the experiment. Animals showing normal and regular estrous cycle (4 to 5 days) for at least three consecutive times were selected for the experiment. The mice were divided into 5 groups each consist of 20 mice. Graded doses of mancozeb from 200, 400, 600 and 800 mg/kg/day were administered orally for 30 consecutive days from the day of estrous. Olive oil treated mice served as controls. 10 animals in each group were used to study the effect of graded doses of mancozeb and remaining 10 animals were used to study the recovery after withdrawal of mancozeb treatment. The last part of the experiment was desisgned to study the recovery ability of exposed mice on estrous cycle, follicular dynamics and biochemical contents of the ovary, uterus and liver.

2.3. Estrous cycle

Vaginal smear was examined in the morning between 10.30 a.m. to 11.30 a.m. by the method described by Cooper *et al*., (1993). The mice were autopsied by cervical dislocation either on 31st day 24 hours after the last treatment or on day 61st for recovery study. All mancozeb treated animals were found to be diestrous at the time of sacrifice.

The control animals were found to be in diestrous but some of them showed variability in any one stages of the estrous cycle.

2.4. Morphometric analysis of follicular growth

Ovaries of five animals of each sub-group were taken for the follicular studies. Both the ovaries were sampled from each animal and there was no preference for right or left ovaries as both ovaries were used for the study. Ovaries were selected based on the stage and comparability of the weight with respective control ovaries. The ovaries were fixed in Bouin's fluid, embedded in paraffin and sectioned at 5 μm thickness. The sections were separated for every 10th section and stained with hematoxylin and eosin. Sections of the ovary were examined under a light microscope and the general histologic appearance of the ovary was assessed. All serial sections of the ovary were counted for various stages of development of follicles as described by Moawad *et al*. (1965) and Bolon *et al*. (1997). Follicles and atretic follicles were classified according to the method described by Swartz and Mall (1989) and Bucci *et al*. (1997). Three classes (Chen *et al*. 1981) of ovarian follicles (small, medium and large) were categorized using the relative cross sectional diameter of the follicle as measured from the outer margins of the granulosa cell layers, the number of margins of the granulosa cell layers and the nature of the antral space.These quantitative criteria represent a substantial simplification of Pedersen and Peters (1968) with eight stages and several substages to differentiate between primordial oocytes (type 1) to antral follicle (type 8).

1. Small follicles – (Pedersen and Peters types 1-3 b) consisted of an isolated oocyte or an oocyte surrounded by a partial or unbroken layer of granulosa cells.

2. Medium/growing follicles – (Pede-rsen and Peters Types 4-5 b) has the oocytes surrounded by multilayered, solid mantle of granulosa cells.

3. Large/antral follicles (Pedersen and Peters types 6-8) were characterized by central oocyte and fluid space bordered by number of granulosa cells.

By using these criteria, mean diameter of follicles have been measured at approximately < 20 μm for small, 20-70 μm for medium and >70 μm for large follicles in mice. Follicles displaying the nucleus of the oocytes were measured by using a calibrated ocular micrometer to avoid repeated counting. The maximum diameter at right angle to it was used to obtain a mean diameter for each follicle. A follicle was considered to be undergoing atresia or regressing whenever two or more pyknotic granulosa cells were found in a single section or whether the oocyte showed signs of degeneration, loss of nuclear membrane or thinning of cumulus oophorus as proposed by Osman (1985).

2.5. Biochemical estimation

Freshly removed ovary, uterus and liver tissue was weighed to the nearest milligram for biochemical analysis, such as protein glycogen and total lipids. The net weight of the tissue was estimated gravimetrically. Protein estimation was performed according to the method described by Lowry *et al*. (1951) glycogen by Sciefter *et al*. (1950) and total lipids by Folch *et al*. (1957).

2.6. Body and organs weight

The weight taken on the 1st day was initial body weight and the weight taken on day 31st or 61st, before autopsy was considered as the final body weight. The ovaries, uterus, liver, spleen, thyroid and thymus were dissected out and freed from adherent tissues and blood vessel were removed blotted free of mucous and weighed to the nearest milligram. The organs weights were expressed per 100 g body weight to ensure normalization of data for statistical analysis.

2.7. Statistical analysis

Data were subjected to ANOVA with post hoc Dunnett's test to compare the control group (olive oil) Vs. treated (mancozeb) groups ($p<0.05$). Data were tested for homogenecity prior to conducting ANOVA analysis.

RESULTS

3.1. Estrous cycle

The control mice showed regular estrous cycles and normal duration of each phases of estrous cycle. Treatment with 200

mg/kg/day mancozeb caused no significant changes in the number or phases of estrous cycle when compared with those of the corresponding parameters of the control mice. Estrous cycle was affected by showing significant ($P<0.05$) decrease in the number of estrous cycle, duration of proestrous, estrous and metestrous with concomitant increase in the diestrous phase in 400, 600 and 800 mg/kg/day mancozeb treated mice. There was recovery in the number and phases of estrous cycle in the mice in 400 and 600 mg/kg/day mancozeb withdrawal mice when compared with those of the corresponding parameters of mancozeb treated mice. However, recovery was not seen in the number and phases of estrous cycle in 800 mg/kg/day mancozeb withdrawal mice as compared with that of mancozeb treated mice. An increase in the diestrous index was observed with an increased concentration of mancozeb treatment and in mancozeb withdrawal mice when compared with that of controls (Table 1).

3.2. Follicular kinetics

The histologic observations of the ovaries of control mice showed number of developing follicles, corpora lutea and atretic follicles (Fig. 1). Treatment with 200 mg/kg/day mancozeb showed no significant change in the number of healthy and atretic follicles when compared with those of the corresponding parameters of the control mice (Table 2). The histologic observations of the ovaries revealed developing follicles, Graafian follicles, corpora lutea and atretic follicles (Fig. 2). Treatment with 400 mg/kg/day mancozeb showed significant decreases in the number of small and healthy follicles (Table 2). The histologic observations of the ovaries revealed developing follicles, corpora lutea and atretic follicles (Fig. 3). Treatment with 600 and 800 mg/kg/day mancozeb caused a significant decrease in the number of small, medium, large and total number of healthy follicles with concomitant significant increase in the number of atretic follicles. The histologic observations of the ovaries revealed few developing follicles, corpora lutea and many atretic follicles (Figs. 4, 5). Histologic structure of the ovaries revealed many developing follicles, Graafian follicles, corpora lutea and atretic follicles (Figs. 6, 7). There was recovery in the number of follicles in 400 mg/kg/day mancozeb withdrawal mice when compared with that of mancozeb treated mice (Tables 2, 3). The histologic structure of the ovary revealed developing follicles, corpora lutea and atretic follicles (Fig. 8). There was no recovery in the number of follicles in 600 and 800 mg/kg/day mancozeb withdrawal mice when compared with those of mancozeb treated mice (Tables 2, 3). The histologic structure of the ovaries revealed few developing follicles, corpora lutea and many atretic follicles (Figs. 9, 10).

3.3. Biochemical analysis

a. Ovary

There was no significant change in the levels of protein, glycogen and lipids with 200 or 400 mg/kg/day mancozeb treatment as compared with those of the corresponding parameters of controls. Treatment with 600 or 800 mg/kg/day mancozeb showed a significant decrease in the levels of protein and glycogen but there was a significant increase in the level of lipids only in 800 mg/kg/day mancozeb treated mice as compared with that of controls (Table 4). There was recovery in the level of protein and lipids in the mice after withdrawal of 600, 800 and only 800 mg/kg/day mancozeb withdrawal mice respectively when compared with those of mancozeb treated mice (Table 4). However, there was no recovery in the level of glycogen in 600 and 800 mg/kg/day mancozeb withdrawal mice when compared with those of mancozeb treated mice (Table 4).

b. Uterus

Treatment with 200 and 400 mg/kg/day mancozeb showed that there was no significant change in the levels of protein, glycogen and lipids when compared with those of the corresponding parameters of controls. Treatment with 600 and 800 mg/kg/day mancozeb revealed that there was a significant decrease in the levels of glycogen and total lipids, however, there was significant decrease in the levels of protein with 800 mg/kg/day mancozeb treated mice when compared with those of oil treated controls (Table 5). There was complete recovery in the levels of glycogen, lipids and proteins in 600, 800 and only in 800 mg/kg/day mancozeb withdrawal mice respectively when compared with those of

the corresponding parameters of mancozeb treated mice (Table 5).

c. Liver

There was no significant change in the levels of protein, glycogen and lipids with 200 and 400 mg/kg/day mancozeb treatment when compared with those of the corresponding parameters of controls. Treatment with 800 mg/kg/day mancozeb showed significant decrease in the level of protein but there was a significant decrease and increase in the levels of glycogen and lipids respectively with 600 and 800 mg/kg/day mancozeb treated mice when compared with those of controls (Table 6). There was a recovery in the level of glycogen and total lipids in the mice after withdrawal of 600 and 800 mg/kg/day mancozeb. However, the recovery was also seen in the level of protein after withdrawal of 800 mg/kg/day mancozeb when compared with those of the corresponding parameters of controls (Table 6).

3.4. Body and organs weight

The body weight gain in control mice was 2.30 g when compared with that of the initial body weight. A significant decrease in the body weight gain was observed in all the mancozeb treated mice (Table 7) when compared with those of controls. There was recovery in the body weight gain in the mice after withdrawal of 200 to 600 mg/kg/day mancozeb, however, recovery of body weight gain was not seen in 800 mg/kg/day mancozeb withdrawal mice (Table 7). The weight of the uterus was not changed significantly in all the mancozeb treated or withdrawal groups when compared with those of controls (Table 5). No significant change was seen in the weight of the ovary (Table 4), liver (Table 6), spleen, thymus and thyroid (Table 7) with 200, 400 or 600 mg/kg/day mancozeb treatment or withdrawal gropus as compared with those of the corres-ponding parameters of controls. However, treatment with 800 mg/kg/day mancozeb showed significant decrease in the weight of the ovary and liver whereas there was significant increase in the weight of the spleen, thymus and thyroid when compared with those of the corresponding parameters of controls (Tables 4-7). There was recovery in the weight of the ovary, thymus and thyroid, however, recovery was not seen in the weight of the liver and spleen in 800 mg/kg/day mancozeb withdrawal mice when compared with those of mancozeb treated mice (Tables 4-7).

DISCUSSION

An estrous cycle is a rhythmic reproductive cycle occurring in sexually mature female mammals which depend upon the periodic release of gonadotropic releasing hormones, gonadotropins and sex hormones (Lerner, 1969; Neguin *et al*., 1975) and gives a fair index of ovarian and uterine function. There was a significant decrease in the number of estrous cycle and duration of proestrous, estrous and metestrous phase with higher doses of mancozeb treatment with concomitant significant increase in the disestrous phase. Diestrous index was also increased in all groups following the administration of mancozeb. There was a recovery in the number and phases of estrous cycle in the mice after withdrawal of lower doses of mancozeb. However, recovery was not seen in higher dose of mancozeb withdrawal mice. This clearly indicated that the effect of mancozeb on estrous cycle and its phases was not transient and reversible with higher concentration of mancozeb. Recently, similar results have been reported that the rats treated with pesticides caused a significant decrease in the number of estrous cycle and duration of proestrous, estrous and metestrous with a concomitant increase in the diestrous phase (Budreau and Singh, 1973; Gowda and Sastry, 1979; Math and Kaliwal, 1996; Sortur and Kaliwal, 1999; Mahadevswami *et al*. 2000; Baligar and Kaliwal, 2001; Hiremath and Kaliwal, 2002). In the present study, the reason for interruption of estrous cycle may be due to hormonal imbalance.

The data obtained in the present study on follicular kinetics revealed that the treatment with higher doses of mancozeb caused a significant decrease in the number of healthy follicles with concomitant increase in the number of atretic follicles. The effect of mancozeb on follicular growth is dose dependent. Similar results were reported on the follicular growth after induction of mancozeb to female rats (Mahadevswami *et al*. 2000; Baligar and Kaliwal, 2001). There was a recovery in the number of follicles in

the mice after withdrawal of lower doses of mancozeb. However, complete recovery in the number of follicles was not seen in higher doses of mancozeb withdrawal mice. Similar findings have been reported on the reduction of different types of healthy follicular stages with concomitant increase in the atretic follicles in rats and mice treated with different pesticides (Ataya *et al*., 1988 ; Swartz and Mall, 1989; Martinez and Swartz, 1991; Jadaramkunti and Kaliwal, 1999).

In the present study, the interruption of estrous cycle and the decrease in healthy follicles with concomitant increase in atretic follicles in mice may due to inhibition of acetyl cholinesterase which alters the pituitary gonadotropins and could influence on gonadal function directly through the effect on the pituitary acetyl cholinesterase as it was observed in the rats treated with pesticide quinolphos (Sarkar *et al*. 2000). This might be due to imbalance in gonadal steroids which are essential for normal functioning of the gonads (Sharpe, 1983). Another possibility may be due to affecting catecholamine neurotransmitter metabolism by inhibiting the GnRH release through the inhibition of DβH (Maj and Vetulani, 1969; Przewlocka *et al*. 1975). In the present study, it has also been shown that there was a recovery in the estrous cycle and follicles after withdrawal of low doses of mancozeb may be due to detoxification of mancozeb by the liver. However, there was no recovery in the follicles after withdrawal of high doses of mancozeb due to its toxicity in mice. Therefore, the mancozeb that interfere with ovarian function could do so indirectly by acting at the level of hypothalamus or pituitary gland or both (Smith *et al*. 1991). The other reason may be that the direct effect of mancozeb on gonads may impair reproductive function by direct insult to the cell population within the gonads resulting in the impairment of the feed back mechanism to the hypothalamus and pituitary (Jarell *et al*. 1987; Asch *et al*. 1990; Pasqualine *et al*. 1990). It has also been reported that oocyte production in females of most mammalian species is believed to cease before birth, although males retain germline sperm cells for spermatogenesis throughout adult life. It was believed that females had fixed number of oocytes at birth and that this pool of germ cells was not renewable (Zuckerman, 1951; Anders-on and Hirshfield, 1992). In constrast to these results Johnson *et al*. (2004) have reported by histomorphometric findings that there was germ cells proliferation and follicle renewal in the postnatal mouse ovary. A previous investigation of adult female mice exposed to busulphan reported a similar decline in immature follicle numbers with complete oocytes loss occurring sometime between 2-17 weeks after injection due to cytotoxic actions of busulphan in follicle enclosed oocytes but hours to days in 9, 10-dimethylbenz[a] anthracene (DMBA) or cyclophosphamide treated mice (Genero-so *et al*. 1971; Shiromizu *et al*. 1984). However, complete recovery was not seen with high dose of mancozeb withdrawal mice. Hence, in the present study, the interruption of estrous cycle and follicles after exposure to mancozeb may be due to hormonal imbalance. Whether the observed toxicity occurred as a result of direct effect upon ovary or indirectly through the action on the hypothalamus and/ or pituitary or by desensitizing the ovary to gonadotropins cannot be ascertained from the present study. Hence, further investigation is needed to understand the mechanism of the action of mancozeb on ovary in mice.

The present study revealed that there was a recovery in the levels of protein and total lipids in ovary, uterus and liver in the mice after withdrawal of higher doses of mancozeb. However, recovery was not seen in the levels of ovarian glycogen in higher doses of mancozeb withdrawal mice. It has also reported that there was a significant change in biochemical contents with mancozeb treatment in rats (Mahadevswami *et al*. 2000; Baligar and Kaliwal, 2001; Ksheerasagar and Kaliwal, 2003). It has been suggested that increase in the levels of phosphoionositides and phosphotidic acid in liver suggest the likely involvement of phospholipase-c-pathway of signaling in the toxicity of mancozeb in different tissues varying levels (Subramoniam *et al*. 1991). It has also been recorded that mancozeb showed its biological effects through its metabolites like ethylene thiourea (ETU) and CS2 (Thorn and Ludwing, 1962; Ivanova-Chemishanska, 1962). Stott *et al*. (1997) have suggested that diethyle di-thiocarbamate inhibits hepatic cyt-P-450 dependent enzyme

activity in rats. Nebbia and ferrero (1991) have reported that there was a significant decrease in the levels of blood glucose and glubolin in mancozeb treated rats, due to low thyroxin level because of impaired thyroid function. It has also been demonstrated that the heavy metals like Cu, Zn, Cd, Mg and Fe showed decreasing in the spermatozoal glycolysis by a decrease in the concentration of glucose and fructose levels and also depressed oxidative metabolism. The changes in the levels of protein, glycogen and lipids with mancozeb treatment indicated either an increased catabolism of the biomolecules to compensate the energy demand under stress or their reduced synthesis due to impaired tissue function (Ivanova-Chemishanska, 1962). Manco-zeb, which is having acute mammalian toxicity, exhibits significant toxicological effects and this suggest that the use of mancozeb in agriculture and occupation practices should be judicious. The administration of mancozeb and its withdrawal mice affects the body weight gain. This observation is important because nutritional deficiencies have been revealed to alter reproductive function (Dikshith *et al*., 1988; Simonich and Hites, 1995). However, the animals exhibited the depressed activity after the immediate administration of mancozeb. Similar results have been suggested in mancozeb treated animals (Lu and Kennedy, 1986; Baligar and Kaliwal, 2001; Ksheerasagar and Kaliwal, 2003). The present data revealed that there was a significant decrease in the weight of the ovary and liver with higher dose of mancozeb treatment, whereas in the lower doses of mancozeb treatment, there was no significant change in the weight of the ovary, spleen and liver. There was a significant increase in the weight of the spleen with higher dose of mancozeb treatment. However, there was a recovery in the weight of the ovary after withdrawal of higher dose of mancozeb treatment. The recovery was not noticed in the weight of the liver and spleen after of higher dose of mancozeb withdrawal mice. Similar findings have also been recorded that there was a sign of poisoning, decrease in kidney weight and pathomorph-ological changes in liver, brain and kidney in rats after the administration of mancozeb with high dose (1500 mg/ kg/ body wt/ d) and chronic exposure (360 d) may be due to toxicity to the biological system of rats exposed to mancozeb (Kackar *et al*. 1999). Hore *et al*. (1997) have reported that the exposure of mancozeb caused pathological changes in liver, kidney and heart showed congestion and hemorrhage, spleen was slightly enlarged and showed congestion.

There was a significant increase in the thyroid and thymus weight with higher dose of mancozeb treatment, whereas in lower doses no significant change in the thymus and thyroid weight. However,the recovery was not observed in the weight of the thymus and thyroid in higher dose of mancozeb withdrawal mice. Similar effects have also been reported with mancozeb treatment in rats (Trevedi *et al*. 1993; Mahadevaswami *et al*. 2000; Baligar and Kaliwal, 2001; Ksheerasagar and Kaliwal, 2003). Increase in thyroid weight may be either due to increase in circulating thyroid stimulating hormone (Ivanova-Chemishanka *et al*. 1971) or due to hypertrophy and hyperplasia of follicular cells which may be due to ETU and CS2 which are major metabolites of mancozeb (Shrivastava and Shrivastava, 1998). In principle, iodide is transported from the blood across the follicular lumen by the follicles. Mancozeb is also involved in inhibiting the thyroid peroxidase, 125 I uptake PB125I and thyroid protein, along with T4 that has produced significant structural and functional changes in the thyroid as evidenced by inhibition of thyroid peroxidase, 125I, uptake, PB125I thyroid protein and T4 (Lu and Kennedy, 1986). In conclusion the dose-related altera-tion in the estrous cycle with prolonged diestrous decrease in healthy follicles with an increase in atretic follicles, changes in biochemical contents, changes in organs weight and their dose-related recovery seen in this study following treatment with mancozeb are perhaps due to a hormonal imbalance at any stage in hypothalamo-hypophysealovarian axis or by desensit-izing the ovary to gonadotropins or to a mutagenic and /or carcinogenic action, or direct effect. However, further investiga-tion is needed to understand the real mechanism of action of the mancozeb toxicity.

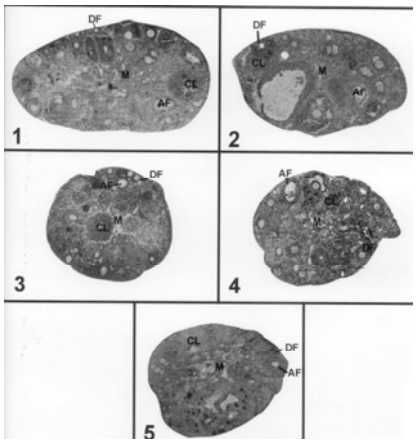

Fig. 1. Transverse section through the hilus of the ovary taken at autopsy on day 31st from a control mouse treated with olive oil showing the presence of developing follicles (DF), corpora lutea (CL), atretic follicles and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

Fig. 2. Transverse section through the hilus of the ovary taken at autopsy on day 31st from a mouse treated with mancozeb 200 mg/kg/d showing the presence of many developing follicles (DF), corpora lutea (CL), atretic follicles (AF) and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

Fig. 3. Transverse section through the hilus of the ovary taken at autopsy on day 31st from a mouse treated with mancozeb 400 mg/kg/d showing the presence of many developing follicles (DF), corpora lutea (CL), atretic follicles (AF) and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

Fig. 4. Transverse section through the hilus of the ovary taken at autopsy on day 31st from a mouse treated with mancozeb 600 mg/kg/d showing the presence of many developing follicles (DF), corpora lutea (CL), atretic follicles (AF) and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

Fig. 5. Transverse section through the hilus of the ovary taken at autopsy on day 31st from a mouse treated with mancozeb 800 mg/kg/d showing the presence of many developing follicles (DF), corpora lutea (CL), atretic follicles (AF) and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

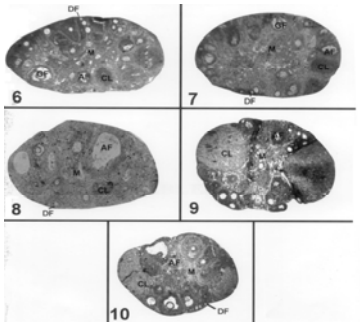

Fig. 6. Transverse section through the hilus of the ovary taken at autopsy on day 6-1st from a control mouse after withdrawal of olive oil showing the presence of developing follicles (DF), Graafian follicle (GF), Corpora lutea (CL), atretic follicles and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

Fig. 7. Transverse section through the hilus of the ovary taken at autopsy on day 61st from a mouse after withdrawal of mancozeb 200 mg/kg/d showing the presence of many developing follicles (DF), Graafian follicle (GF), Corpora lutea (CL), atretic follicles (AF) and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

Fig. 8. Transverse section through the hilus of the ovary taken at autopsy on day 61st from a mouse after withdrawal of mancozeb 400 mg/kg/d showing the presence of many developing follicles (DF), Graafian follicle (GF), Corpora lutea (CL), atretic follicles (AF) and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

Fig. 9. Transverse section through the hilus of the ovary taken at autopsy on day 61st from a mouse after withdrawal of mancozeb 600 mg/kg/d showing the presence of many developing follicles (DF), Graafian follicle (GF), Corpora lutea (CL), atretic follicles (AF) and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

Fig. 10. Transverse section through the hilus of the ovary taken at autopsy on day 61st from a mouse after withdrawal of ancozeb 800 mg/kg/d showing the presence of many developing follicles (DF), Graafian follicle (GF), Corpora lutea(CL), atretic follicles (AF) and medulla (M). The animal was in diestrous at the time of death. Harris hematoxylin and eosin, 30X.

Table 1. Effect of graded doses of mancozeb and its reversal on estrous cycle in albino mice.

Effects of mancozeb on						Withdrawal effects of mancozeb on					
Duration (days)						Duration (days)					
Mancozeb dose											
(mg/Kg/d)	Number of cycles	Proestrous	Estrous	Metestrous	Diestrous	Diestrous Index	Number of cycles	Proestrous	Estrous	Metestrous	Diestrous	Diestrous Index
Control (Olive oil)	5.50 ± 0.40	6.00 ± 0.16		7.60 ± 0.21	5.30 ± 0.21	10.90 ± 0.40	36.33	5.40 ± 0.24	5.90 ± 0.11	7.80 ± 0.12	5.20 ± 0.15	10.90 ± 0.00	36.33
200	5.30 ± 0.21	5.60 ± 0.25		7.10 ± 1.56	4.83 ± 0.60	12.40 ± 0.66	41.33	5.16 ± 0.30	5.56 ± 0.16	7.40 ± 0.42	5.10 ± 0.16	12.00 ± 0.51	40.00
400	3.23 ± 0.21*	4.60 ± 0.16*		5.60 ± 0.33*	4.70 ± 0.21*	15.00 ± 0.34*	50.00	4.12 ± 0.40	5.50 ± 0.22	7.60 ± 0.30	5.30 ± 0.42	11.30 ± 0.80	37.66
600	3.12 ± 0.21*	4.51 ± 0.40*		5.50 ± 0.61*	4.60 ± 0.50*	15.39 ± 0.90*	50.96	4.30 ± 0.33	5.60 ± 0.30	7.50 ± 0.22	5.00 ± 0.25	12.80 ± 0.33	42.66
800	3.16 ± 0.40*	4.60 ± 0.22*		5.00 ± 0.51*	4.70 ± 0.40*	15.60 ± 1.08*	52.00	3.83 ± 0.40*	5.50 ± 0.30	5.00 ± 0.30*	4.80 ± 0.30	14.60 ± 0.55*	48.66

Mean ± S.E.M., n=10 per group

P < 0.05 compared to control

$$\text{index} = \frac{\text{Number of days with clear diestrous smear}}{\text{Total days of treatment}} \times 100$$

Table 2. Effect of graded doses of mancozeb and its reversal on healthy follicles of the ovaries in albino mice

Mancozeb dose (mg/Kg/d)								
Effects of mancozeb on				Withdrawal effects of mancozeb on			
Number of healthy follicles by size classification (µm diameter)				Number of healthy follicles by size classification (µm diameter)			
	Small(< 20 µm)	Medium(20-70 µm)	Large (> 70 µm)	Total	Small(< 20 µm)	Medium(20-70 µm)	Large(> 70 µm)	Total
Control (Olive oil)	214.80 ± 1.44	69.00 ± 1.07	9.50 ± 0.76	293.40 ± 3.27	215.00 ± 1.04	68.10 ± 0.07	9.00 ± 0.76	292.00 ± 1.87
200	211.80 ± 1.74	65.60 ± 1.11	8.00 ± 0.51	285.40 ± 3.36	212.00 ± 1.00	65.50 ± 1.14	9.00 ± 0.36	286.50 ± 2.50
400	209.00 ± 0.57*	64.60 ± 0.61	7.50 ± 0.56	281.10 ± 1.74*	211.10 ± 0.87	66.00 ± 0.57	8.10 ± 0.60	285.20 ± 2.04
600	199.60 ± 1.11*	60.50 ± 1.14*	5.60 ± 0.49*	265.70 ± 2.74*	202.80 ± 0.74*	63.50 ± 0.76*	6.80 ± 0.60*	273.10 ± 2.10*
800	191.80 ± 0.94*	56.00 ± 1.59*	4.30 ± 0.42*	252.10 ± 2.95*	200.50 ± 0.61*	62.50 ± 0.92*	5.50 ± 0.22*	268.50 ± 1.75*

Mean ± S.E.M., n=5 per group (Ovaries examined were 10).

P < 0.05 compared to control.

Table 3. Effect of graded doses of mancozeb and its reversal on atretic follicles of the ovaries in albino mice.

Mancozeb dose (mg/kg/d)	Effects of mancozeb on: Number of atretic follicles by size classification (µm diameter).			Withdrawal effects of mancozeb on: Number of atretic follicles by size classification (µm diameter).		
	Medium (20-70 µm)	Large (>70 µm)	Total	Medium (20-70 µm)	Large (>70 µm)	Total
Control (Olive oil)	12.50 ± 0.61	2.60 ± 0.30	15.10 ± 0.91	12.04 ± 0.16	2.50 ± 0.30	14.54 ± 0.46
200	12.80 ± 0.74	2.50 ± 0.20	15.30 ± 0.94	12.50 ± 0.61	2.30 ± 0.20	14.80 ± 0.81
400	14.00 ± 0.57	3.30 ± 0.30	17.30 ± 0.87	13.60 ± 0.49	2.60 ± 0.40	16.20 ± 0.89
600	17.60 ± 0.49*	3.60 ± 0.20	21.20 ± 0.69*	16.80 ± 0.54*	2.80 ± 0.30	19.60 ± 0.84*
800	23.00 ± 0.85*	5.60 ± 0.40*	28.60 ± 1.25*	20.30 ± 0.42*	5.00 ± 0.30*	25.30 ± 0.72*

Mean ± S.E.M., n=5 per group (Ovaries examined were 10).
P < 0.05 compared to control

Table 4. Effect of mancozeb and its reversal on biochemical contents of the ovary in albino mice

Mancozeb dose (mg/kg/d)	Effects of mancozeb on: Weight (g) /100 g B. wt.	(µ g / mg wet weight of tissue)			Withdrawal effects of mancozeb on: Weight (g) / 100 g B. wt. M ± S.E.	(µ g / mg wet weight of tissue)		n ± S.E.)
	Ovary	Protein	Glycogen	Total lipids	Ovary	Protein	Glycogen	Total lipids
Control (Olive oil)	0.090±0.02	115.75 ± 7.14	6.65 ± 0.13	87.50 ± 1.32	0.089±0.03	118.45 ± 1.14	6.55 ± 0.01	86.50 ± 0.13
200	0.089±0.04	114.75 ± 1.41	6.58 ± 0.12	83.00 ± 1.29	0.089±0.01	120.00 ± 5.80	6.49 ± 0.14	85.25 ± 1.70
400	0.086±0.02	110.25 ± 1.93	6.60 ± 0.06	84.50 ± 1.55	0.087 ± 0.07	116.50 ± 4.19	6.56 ± 0.17	82.00 ± 1.20
600	0.084±0.02	107.75 ± 0.85*	4.79 ± 0.16*	84.50 ± 1.70	0.091 ± 0.05	114.00 ± 2.16	5.12 ± 0.11*	85.50 ± 1.20
800	0.075±0.02*	105.25 ± 1.10*	3.72 ± 0.29*	97.00 ±1.29*	0.086 ± 0.04	113.00 ± 5.25	5.65 ± 0.10*	86.50 ± 2.02

Mean ± S.E.M., n=10 per group.
P < 0.05 compared to control

Table 5 . Effect of mancozeb and its revirsal on biochemical contents of the uterus albino mice.

Mancozeb dose (mg/kg/d)	Effects of mancozeb on: Relative Weight (g) / 100 g B. wt.	(µ g / mg wet weight of tissue)			Withdrawal effects of mancozeb on: Relative Weight (g) / 100 g B, wt.	(µ g / mg wet weight of tissue)		n ± S.E.)
	Uterus	Protein	Glycogen	Total lipids	Uterus	Protein	Glycogen	Total lipids
Control (Olive oil)	0.367±0.01	92.00 ± 2.58	3.40 ± 0.14	60.75 ± 1.03	0.377 ± 0.04	89.00 ± 0.68	3.20 ± 0.41	62.75 ± 1.08
200	0.342±0.02	89.50 ± 0.64	3.12 ± 0.08	58.00 ± 0.70	0.367±0.04	87.50 ± 8.70	3.12 ± 0.11	59.50 ± 1.04
400	0.313±0.02	87.50 ± 0.95	3.07 ± 0.08	57.00 ± 0.91	0.368 ± 0.04	86.50 ± 8.42	3.10 ± 0.19	58.75 ± 1.03
600	0.333±0.05	88.50 ± 0.95	2.90 ± 0.10*	55.75 ± 0.85*	0.364 ± 0.03	91.50 ± 0.95	3.12 ± 0.08	59.00 ± 0.91
800	0.308±0.07	77.50 ± 0.40*	2.59 ± 0.10*	53.50 ± 0.64*	0.338 ± 0.02	84.50 ± 7.80	3.12 ± 0.11	58.75 ± 0.62

Mean ± S.E.M., n=10 per group.
P < 0.05 compared to control..

Table 6. Effect of mancozeb and its reversal on biochemical contents of the liver in albino mice.

Effects of mancozeb on					Withdrawal effects of mancozeb on			n ± S.E.)
Mancozeb dose (mg/kg/d)	Relative Weight (g) / 100 g B. wt.	(µ g / mg wet weight of tissue)			Relative Weight (g) / 100 g B wt	(µ g / mg wet weight of tissue)		
	Liver	Protein	Glycogen	Total lipids	Liver	Protein	Glycogen	Total lipids
Control (Olive oil)	5.910±0.00	93.00 ± 5.00	4.67 ± 0.08	41.25 ± 0.50	5.700±0.75 5.470±0.30	92.00 ± 2.00	4.87 ± 0.06	42.25 ± 0.81
200		92.50 ± 1.04	4.57 ± 0.08	43.25 ± 1.03	5.440±0.10 4.470±0.40*	88.00 ± 2.00	4.92 ± 0.08	43.25 ± 0.85
400	5.430±0.00	88.50 ± 6.50	4.60 ± 0.10	42.25 ± 0.64		91.00 ± 0.91	4.52 ± 0.17	43.00 ± 0.91
600	5.750±0.00	90.50 ± 2.87	3.77 ± 0.11*	50.75 ± 0.85*		90.00 ± 0.91	4.27 ± 0.08*	42.75 ± 0.85
800	4.870±0.00*	78.00 ± 1.41*	3.80 ± 0.14*	54.00 ± 1.29*		89.25 ± 0.85	4.00 ± 0.12*	45.25 ± 0.85

Mean ± S.E.M., n=10 per group.
P < 0.05 compared to control.

Table 7. Effect of mancozeb and its reversal on organs weight in albino mice.

Relative weight (g/ 100 g body weight)								
Mancozeb dose (mg/kg/d)	Body weight gain (g)	Effects of mancozeb on			Withdrawal effects of mancozeb on			
		Spleen	Thymus	Thyroid	Body weight gain (g)	Spleen	Thymus	Thyroid
Control (Olive Oil)	2.30 ± 0.90	0.505±0.02	0.124±0.05	0.016±0.00	2.36 ± 0.60	0.506 ± 0.04	0.123±0.06	0.016±0.09
200	1.60 ± 1.00*	0.503±0.01	0.127±0.01	0.017±0.00	2.16 ± 1.00	0.504 ± 0.04	0.123±0.01	0.015±0.02
400	1.50 ± 0.50*	0.500±0.03	0.121±0.05	0.019±0.00	2.33 ± 0.50	0.510 ± 0.01	0.118±0.07	0.015±0.00
600	1.50 ± 0.60*	0.504±0.01	0.124±0.05	0.021±0.00	2.60 ± 0.60	0.514 ± 0.03	0.120±0.02	0.015±0.00
800	1.50 ± 0.50	0.643±0.03*	0.129±0.02*	0.024±0.00*	1.60 ± 0.80	0.623±0.01*	0.117±0.06	0.015±0.00

Mean ± S.E.M., n=10 per group.
P < 0.05 compared to control.

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