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EFFECT OF THE FUNGICIDE (TRI-MILTOX FORT) ON SOME REPRODUCTIVE ASPECTS IN FEMALE RATS

By

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SUMMARY

Two main experiments were carried out to investigate the effect of chronic low dose oral administration of the fungicide Tri-Miltox Fort (TMF) on female reproduction in rats. In the 1st experiment, 24 non pregnant mature female rats were divided into 2 equal groups, the 1st one was kept as a control while , the 2nd one was given water containing 0.5 g/liter of the fungicide for a period of 2 months, thereafter, half of each group was sacrificed and the remaining half was allowed to drink tap water only for a period of another month then sacrificed. Blood and tissue samples were collected and subjected to biochemical, pathological and chromosomal studies. In the 2nd experiment, 14 pregnant rats were divided into 2 groups, the 1st one (N=6) was kept as a control while, the 2nd one (N=8) received the above mentioned dose of the fungicide during days 6-15 of gestation then killed on day 19 and foetuses were examined for morphological as well as visceral and skeletal malformations.

Results indicated that exposure of female rats to the fungicide TMF induced reduction of fertility, ovarian quiescence, adverse changes in serum biochemistry compared to control group. At the sametime, this fungicide induced pronounced pathological changes in ovaries, uteruses and thryroid glands together with different types of numerical and structural chromosomal aberrations in bone marow cells of exposed animals. All the aforementioned changes did not return back to normal status up to one month after cessation of TMF administration. Exposure of pregnant rats to this fungicide leads to reduction of foetal weights as well as skeletal and visceral malformations.

In conclusion, exposure to the fungicide TMF induced severe irreversible adverse, biochemical pathological chromosomal and teratogenic changes with consequent impairment of the reproductive function in female rats.

INTRODUCTION

Tri-Miltox Fort (TMF) is an agricultural fungicide related to carbamate. It is commonly used to prevent crop damage in the field and to protect the harvested crops from deteriorations during storage or transport (Lu and Kennedy, 1986). It is intensively applicated in Egyptian fields because of its low cost and good efficacy as broad spectrum antifungal agent (Ali, 1992 and Nebbia and Fink-Gremmels, 1996).

Exposure of male albino rats to TMF imparted the reproductive performance as manifisted by irreversible, blood biochemical changes, together with pathological alterations in the testes and accessory genital glands, and chromosomal aberrations (Ahmed et al., 1998). However, little information is available on the effect of TMF on female reproduction, therefore, the present investigation aimed to evaluate the effect of chronic low dose of TMF administration on some reproductive aspects of female albino rats taking in consideration the changes that might occur in some relevant blood biochemical values, pathological alterations of genital organs, chromosomal changes and teratogenic effect.

MATERIALS AND METHODS

Experimental animals:

The current study was carried out on a total number of 38 mature female albino rats, aged from 12 to 16 weeks and weighed from 150-200 g. These animals were obtained from The Animal Breeding House, National Research Centre.

Experimental design:

Fungicide:

The fungicide was obtained from Sandoz. Agro LTD. Basle, Switzerland, in a powder form under name Tri-Miltox Fort, which contains, mancozeb 20%, copper 21.5%, prussian blue 6.0%, and adjuvant and inerts 52.5%. The recommended dose is 250 g/100 liter for crops. The LD50 of the active ingredient of the fungicide (mancozeb) is 4000mg/kg body weight (Ali, 1992). In this experiment, rats were given 1/400 of the LD50 daily / kg body weight in drinking water (by dissolving of 0.5g TMF / liter of water / 5 days/ 12 rats, Ahmed et al., 1998).

Experimental procedures:

Two main experiments were carried out, the first experiment was done to study the effect of the fungicide on fertility in non pregnent rats with special reference to changes in some blood biochemistry, pathological alterations in the genital organs and chromosomal changes in bone marrow cells. The second experiment was done on pregnant rats to study the teratogenic effect of the fungicide.

Experiment No. 1:

Twenty four non pregnant mature female albino rats were divided itno 2 equal groups. 1 st group was given tap water and used as a control groups. The 2 nd group was drenched the fungicide daily for 2 months. Eight mature male rats (4 males for each group) were introducd to both groups after one month of administration of the fungicide to study the fertility rate in both groups. At the end of the second month, 6 female rats from each group were sacrificed, while the remaining rats

were provided with tap water only for one month as a period of recovery , thereafter rats were sacrificed.

Blood samples were collected just after sacrificing of rats, kept at room temperature for 2 hours then centrifugated at 1500 g/10 minutes. Sera were separated and kept at -20°C pending analysis. Progesterone (Abraham, 1981), thyroxine and triiodothyronine (Rodbard and Hult, 1974) levels were assayed by RIA. Assays have sensitivity, intra-and inter-assays CVs of 0.02 ng/ml, 4.65% and 5.15% for progesterone, 0.98 µg/dl, 4.63% and 7.66% for T4 and 0.09 ng/ml, 4.87% and 5.80% for T3, respectively. Total lipid, cholesterol, triglyceride, glucose, proteins, urea and creatinine were determined by chemical methods (Wilding and Kennedy, 1977). Zinc and copper concentrations were determined by atomic absorption spectrophotometry (Varley, 1976).

Postmortem examination was performed just after sacrificing the rats. Tissue specimens from ovaries, uteruses and thyroid glands were fixed in 10% neutral buffered formaline, washed, dehydrated, cleared and embedded in paraffine. Sections were prepared at 5-6 µ thickness, stained with haematoxyline and eosin stain and microscopically examined.

For chomosomal study, bone marrow samples were obtained from femurs of rats and chromosomes were prepared (Yosida and Amano, 1965), Fifty or more well spread metaphases were examined for each animal and the different types of aberrations were recorded.

Experiment No. II:

Forteen hand mated rats were used. pregnancy was confirmed by daily vaginal smears examinations and considering the day of the presence of vaginal plugs or sperms as the zero day of pregnancy (Hayes, 1986). Rats were divided into 2 groups; the 1st group (N=6) was given tap water and kept as control. The 2nd group (N=8) was drenched the fungicide (5.0 g/liter) during the period of organogenesis (days 6-15 of gestation). Animals were sacrificed on day 19 of gestation. The number of live and dead foetuses, resorption sites and implantation sites were counted and recorded (Kopf et al., 1964). The obtained living foetuses from each dam were counted, weighed and examined for morphological as well as visceral and skeletal abnormaltities (Hayes, 1986).

Statistical analysis:

Data were computed and statistically analysed using Student (t) test according to Snedecor and Cochran (1976).

RESULTS

Experiment No. I:

Administration of 0.5 g/liter of thh fungicide TMF in drinking water of rats for 60 days induced no clinical symptoms of toxicity or mortailities. Also, it affected the fertility, as indicated by absence of conception in comparison

to the control group (whereas 3 of 6 control rats get pregnant). Moreover, ovarian quesience as indicated by absence of cyclic changes (redness and swelling). in the vaginal mucous membrane and vaginal smears picture was evident.

Serum biochemical values:

The effect of administration of the fungicide TMF on some blood serum biochemical values is shown in table (1): Serum T4, T3, glucose and albumin values decreased (P<0.01), while, total lipid, cholesterol, urea, creatinine and zinc values increased at P<0.01, and triglyceride and copper values increased at P<0.05 in treated rats as compared with mean values of non pregnant rats (N=5) in the control group.

Cessation of the fungicide from drinking water of rats for 30 days post administration induced non encouragable results as no conceptions were recorded compared to the control group (4 out of 6 rats get pregnant) despite, the occurrence of some significant changes in serum constituents of the recovery group compared to the treated one especially in T3 and zinc values (P<0.01), but the values were still far from those of the control group (Table 1).

It was worthly noted that mean progesterone levels of the treated rats (N=6) were out of curve (<0.02 ng/ml) and were still low even after 30 days of cessation of TMF from the drinking water (8.19 ± 2.16 ng/ml, N=6). However, recorded progesterone levels in the control group were

Table (1): Effect of the fungicide Tri-Miltox Fort (5.0 g/l) on some serum biochemical values of mature female rats (Mean±SE)

Biochemical value	Control group• (N=5)	Treated group[a] (60 days, N=6)	Recovery group[b] (30days, N=6)
Thyroxine (µg/dl)	2.93±0.07	1.750.38**	1.92±0.12
Triodothyronine (ng/dl)	60.07±2.90	9.30±1.39**	17.87±0.30**
Total lipids (mg/dl)	325.27±18.79	398.20±5.20**	393.81±3.73
Cholesterol (mg/dl)	117.80±5.17	138.27±3.53**	129.97±2.85
Triglyceride (mg/dl)	71.21±1.41	91.27±8.98*	88.61±1.79
Glucose (mg/dl)	95.55±5.80	64.90±6.30**	73.20±5.27
Total protein (g/dl)	6.10±1.01	5.40±0.15	5.43±0.23
Albumin (g/dl)	4.35±0.05	3.63±0.09**	3.47±0.18
Globulin (g/dl)	1.75±0.60	1.77±0.12	1.97±0.41
Urea (mg/dl)	27.20±4.70	36.20±2.04**	36.03±2.13
Creatinine (mg/dl)	1.10±0.01	2.15±0.03**	2.10±0.01
Copper (µ/dl)	126.50±8.60	169.50±20.52*	136.33±10.78
Zinc (µ/dl)	141.20±8.17	194.17±13.20**	162.11±7.16**

* Significant at P<0.05
** Significant at P<0.01

a Compared with control group
b Compared with treated group
• Values of non pregnant rats

Table (2): Effect of the fungicide Tri-Milox Forton chromosomal aberrations in female rats (Mean±SE).

Group•	Total No. of examine metaphases	No. of abnormal metaphases	Mean %of abnormal metaphases		Metaphases with different types of chromosomal aberrations													
			Including gap	Excluding gap	Centromeric atenuation		Chromatide gap		Deletions		Ring		Fragment		Polyploidy			
					No.	%	No.	%	No.	%	No.	%	No.	%	No.	%		
Control	200	4	2.00±0.82	2.00±0.82	1	0.50	0	0.00	2	1.00	1	0.50	0	0.0	0	0.00		
Experimental (0.50g/l)	200	14	7.00±0.58*#a	7.00±0.58*#a	3	1.50	1	0.05	4	2.00	1	0.50	2	1.00	3	1.50		
			2.90±0.53	2.90±0.53	2	0.98	0	0.00	2	0.98	1	0.48	0	0.00	1	0.48		
Recovery	205	6																

* Significant at P<0.01

a compared with control group

• N=4 rats/group.

23.11±2,42 and 47.13±3.14 ng/ml for non pregnant (N=5) and pregnant (N=7) rats, respectively.

Histopathological findings:

Ovary:

Microscopical examination of ovaries of TMF treated rats revealed absence of mature Graafian follicles, decreased number of growing follicles associated with high incidence of follicular atresia (Fig. 1). Prominent interstitial gland cells around the degenerated ovarian follicles were observed. In the sametime, they showed necrobiotic changes whereas, cells appeared small in size and spindle in shape with small amount of light acidophilic cytoplasm with small, irregular outine pyknotic nuclei (Fig. 2). In most cases, old corpora lutea showed variable degrees of regression.

Table (3) Teratogenic effect of the fungicide Tri-Miltox Fort (0.5g/l) on pregnant rats during day 6-15 of gestation.

Item	Control (N=6)	Treated group[a] (60 days, N=6)
Dames		
Total number of foetuses	36	54
Viable foetuses (%)	100.00	98.10
Resorbed foetuses (%)	0.00	33.90
Dead foetuses (%)	0.00	1.90
Foetuses		
Mean weight (g)	5.99±0.63	1.43±0.03**
Mean crown rump length (cm)	37.2±0.40	22.1±0.70**
Foetal mal formations(%)		
A. Skeletal		
Skull	0.00	96.70
Ribs	0.00	16.07
Vertebral column	0.00	54.80
Sternebrae	0.00	99.90
Pelvic girdle	0.00	25.80
Fore & hind limbs	0.00	90.30
B. visceral	0.00	40.00
Palate	0.00	15.00
Eye	0.00	65.00
Brain	0.00	20.00
Thymus	0.00	20.00
Lung	0.00	20.00
Testis		

**Signific of P<0.01.

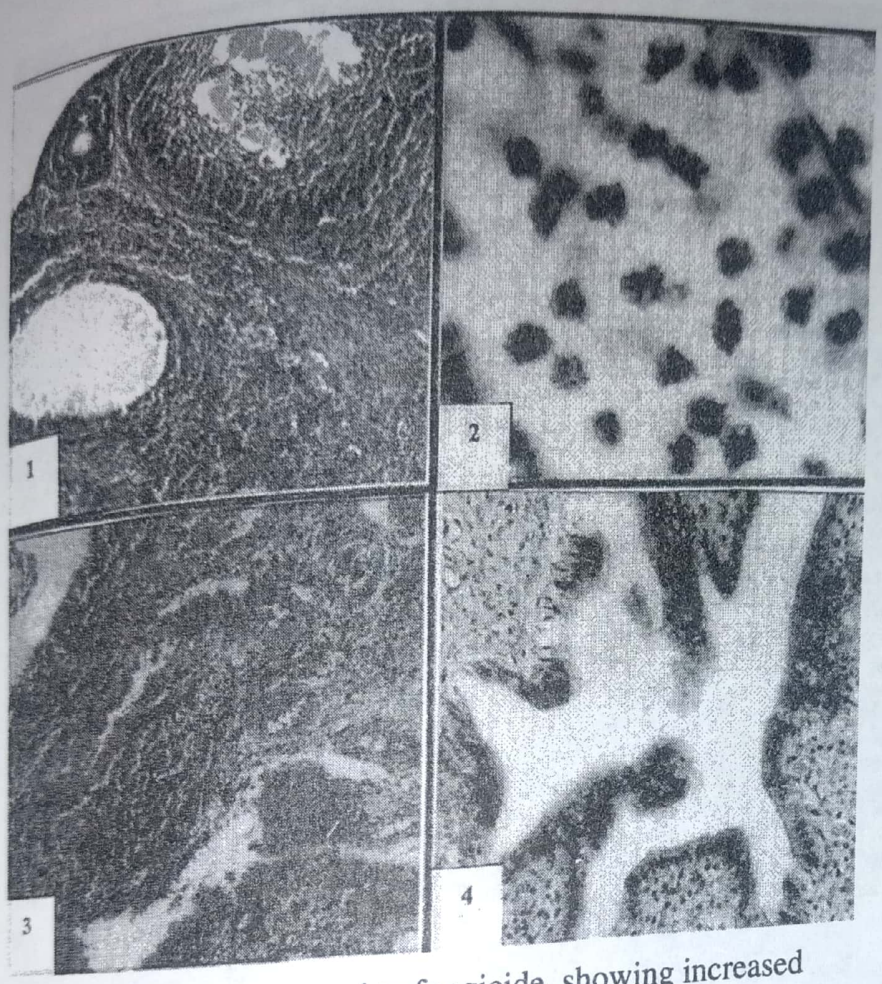


Fig.(1): Ovary of rat exposed to fungicide, showing increased number of atretic follicles (H&E x100)

Fig.(2): Ovary showing necrobiotic changes of interstetial cell gland (H&E x100)

Fig.(3): Ovary showing sever dilatation and conjetion of B.V. (H&E x100)

Fig.(4): Endometrium , showing focal hyperpllasia of the epitheli-um lining (H&E x 400)

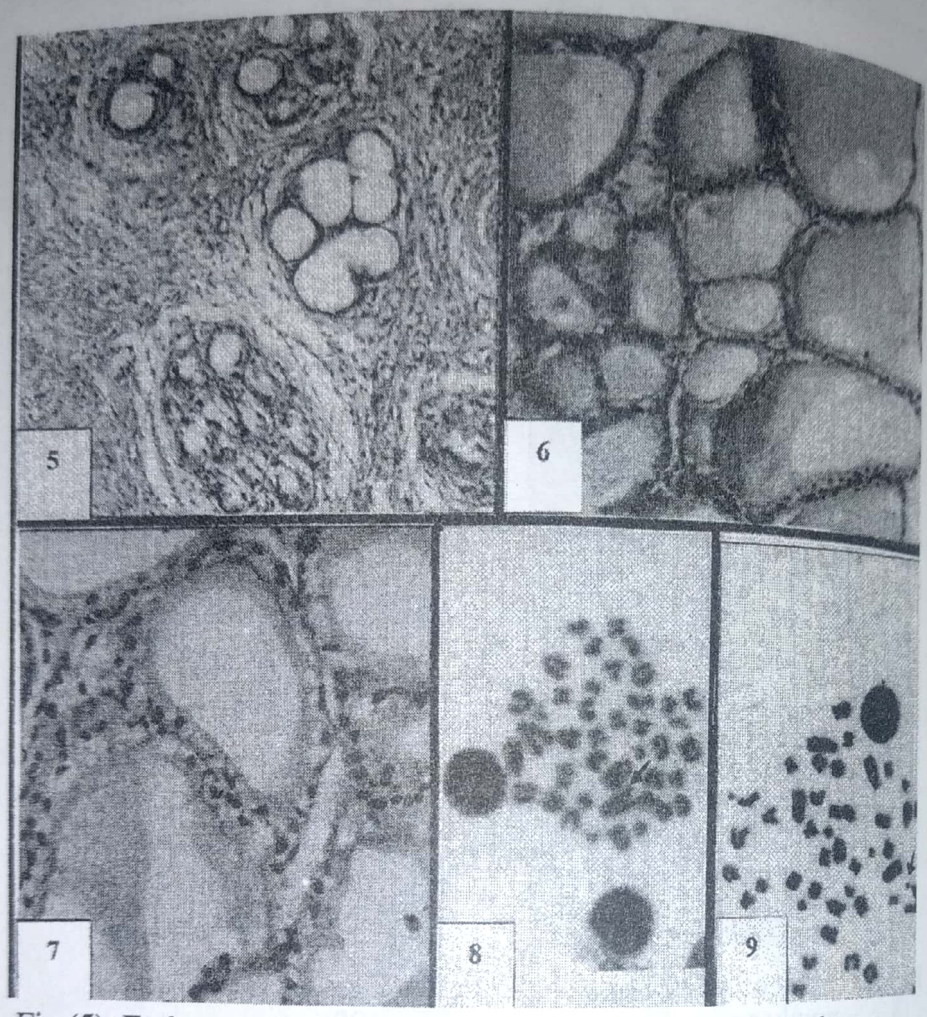


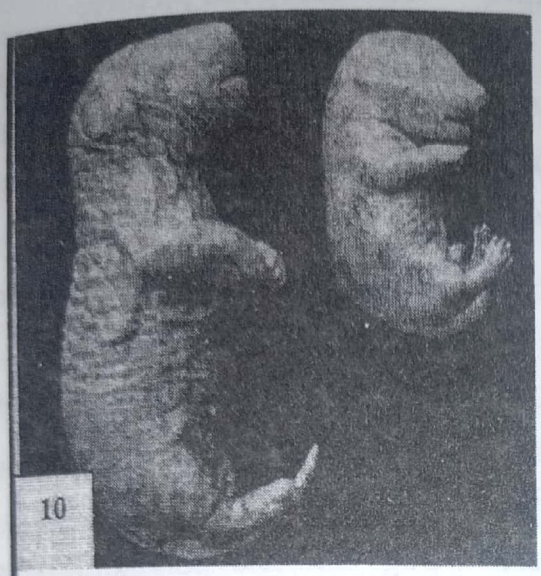
Fig.(5): Endometrium of rat exposed to fungicide, showing degeneration and cystic dilatation of uterine glands.(H&E x200)

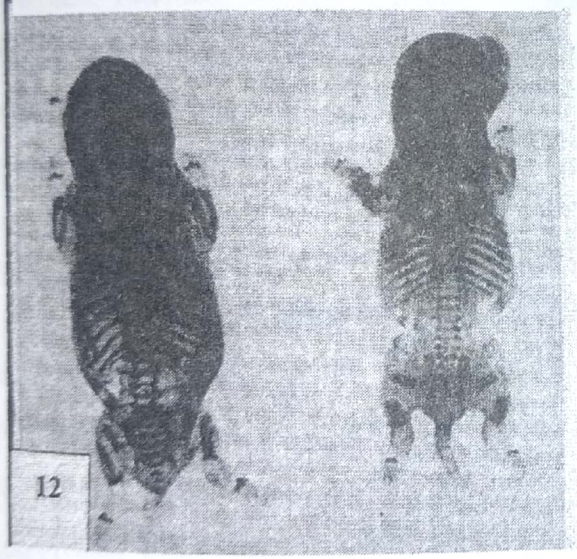
Fig.(6): Thyroid follicles, showing dilatation with faint esinophilic colloid (H&E x200)

Fig.(7): Thyroid follicles, showing cytoplasmic vaculation of the epithelial cells (H&E x200)

Fig.(8): Metaphase of female rat showing chromatid deletation (Arrow).

Fig.(9): Metaphase of female rat showing chromatidfragment (Arrow).





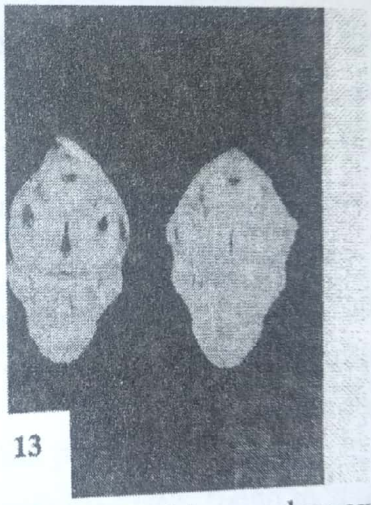


Fig.(10): Showing growth retardation in a foetus obtained from a dam exposed to fungicide (right) compared to control (left).
Fig.(11): Uterus of exposed mother, showing partial foetal resorption.
Fig.(12): Showing incomplete ossification of skull bones and abscence of caudal vertebrae (right) compared to control (left).
Fig.(13): Showing dilatation of 3rd. ventiricule in foetus obtained from exposed mother to fungicide (left) compared to control (right).

Congestion and severe interstitial haemorrhage among lutea cells were noticed. Moreover, clusters of erythrocytes and inflammatory cells mainly lymphocytes were seen in the central cavity of some old corpora lutea. In all cases, the ovarian blood vessels appeared dilated and congested (Fig. 3). Occasionally, subcortical haemorrhage was noticed. These changes were still observed even after recovery.

Uterus:

The surface epithelial cells showed focal hyperplasia associated with desquamation (Fig. 4), in some cases, cytoplasmic vacuolations were observed. Endometrial stroma revealed focal aggregations of mononuclear cells mainly lymphocytes, uterine glands showed degenerative and necrotizing changes of their epithelium linning. Meanwhile, some glands showed cystic dilatation associated with moderate fibroblastic proliferation (Fig. 5). Such alterations were evident after cessation of the fungicide administrations.

Thyroid glands:

Microscopical examination exhibited a relative increase in the numbers of macrofollicles which are mostly lined by low cuboidal to flattened epithelial cells. Moreover, the follicular colloid appeared faint eosinophilic or absent in other follicles (Fig. 6). The epithelial cell linning of some follicles showed cytoplasmic vacuolations (Fig. 7). Occasionally, hyperplasia of follicular epithelium was observed. In addition to these lesions, blood vessels were dilated and congested. These pathological pictures were present after 30 days from recovery of the fungicide.

Chromosomal picture:

The effect of the fungicide on induction of chromosomal aberrations in mature female albino rats bone marrow cells is shown in table (2). Treatment with the fungicide induced significant increase ($P<0.01$) in the percentage of chromosomal aberations even after excluding the number of metaphases with chromatid gaps. The most prevalent types of aberrations were centraomeric attenuation, deletions, ring, fragment and polyploidy (Figs, 8 & 9). Some chromosomal aberrations still observed one month later after stoppage of the fungicide adminsitration.

Experiment No. II:

Teratogenicity:

Administration of the fungicide TMF (0.5 g/liter) to pregnant rats from days 6-15 of pregnancy resulted in foetal malformations (Table 4). Signficant reduction ($P<0.01$) in the mean foetal weight and crown-rump length were evident in foetuses obtained from treated dams compared to those of control dams (Fig. 10). Moreover, dead and resorbed foetuses were observed (Fig. 11).

Incomplete ossification of skull bones, absence of phalanges, absence of sacral and caudal vertebrae, and wavy, short and fused ribs were the most predominant skeletal deformities recorded in foetuses obtained from treated dams (Fig. 12). While, cleft palates, hydrocephalus, absence of

thymus and testis were the most pronounced visceral malformation (Fig. 13).

DISCUSSION

Tri-Miltox Fort (TMF) is one of the intensively used antifungal agents for crop protection under Egyptian field condition. However, in the present study, chronic low dose administration (0.5 g/l) in rats were found to induce reduction of fertility, complete cessation of ovarian activity with significant changes in serum progesterone, thyroid hormones, lipids, glucose, albumin, urea, creatinine, copper and zinc vlaues. Pronounced pathological changes in ovaries, uteruses and thyroid glands were also recorded.

Exposure to carbamate was found to induce reduction of fertility and cessation of sexual behaviour and gonadal activity in rats (Kaloyanova and Ivanova-Chemishanska, 1989; Cummings et al., 1990; Ali, 1992), rabbits (Nebbia et al., 1995) and cattle (Nebbia et al., 1991). The condition was attributed to the direct cytotoxic action on gonads (Dunnick et al., 1984), antigonadotrphic action with consequently lack of sex steroid (Ali, 1992) as well as hypothyroidism (Nebbia et al., 1995). In the same time, the present disturbance of thyroid function was in agreemnet with the findings of Ahmed et al., (1980). Salem et al., (1989) and Nebbia et al., (1991 and 1995) who related the condition to hypothalamic pituitary influence as well as the target antithyroid effect of the fungicide. Moreover, Akhtar et al., (1996) added that fungicides in general decrease iodine binding protein and caused morphological abnormality of thyroid follicular cells , so induced hypothyroidism through, deficient iodine trapping or energy necessary for synthesis and conversion of T_4 into T_3. The increased serum lipids in the exposed animals was in agreement with the findings of Gupta et al. (1986), Szepvolgyi et al., (1989) and Nebbia et al., (1991). Changes in serum lipid spectrum was related to disturbed metabolism especially that of phospholipids (Ali, 1992). Moreover, Nebbia et al., (1991) found that toxic hepatitis, biliary cirrhosis and nephrotic syndromes resulted from administration of fungicides were mostly associated with thyroid hypofunction. Nebbia and Fink-Gremmels (1996) found a reverse relationship between the thyroid function and lipids following fungicide exposure. The changes of glucose might be due to liver involvement and consequently decreased glycogen content (Nebbia et al., (1991). A direct action on the pancreas might also be considered, whereas fungicides were found to accumulate in a relatively great extent to other body tissues (Nebbia and Fink-Gremmels, 1996). While, the changes in serum proteins in the current work were similar to the findings of Ahmed et al., (1980), Nebbia et al., (1991) and Ahmed et al., (1998) who explained the decreased albumin to liver involvement and the increase urea and creatinine to muscular destruction in the fungicide exposed rats. The high serum copper concentration may be related to the chemical structure of the fungicide (21.5% copper, Ahmed

et al., 1998), While, the high serum zinc concentration in the treated animals could be related to the high copper as Eltohamy et al., (1994) found a positive correlation between zinc and copper in the blood of bovines.

The main microscopical changes of the ovaries in the fungicide TMF treated rats herein were increased rate of follicular stresia associated with severe necrobiotic changes of interstitial cell glands and congestion and haemorrhages of ovarian tissue. Follicular, damage due to increase cellular lipid peroxidation would result in loss of structural and functional integrity of cellular membranes and consequently increase the rate of follicular atresia (Bourque et al., 1995). Also, the influence of the high copper content of the fungicide in inducing follicular atresia cannot be ruled out (Bires et al., 1995). The pronounced changes observed in the uterine tissue were focal hyperplasia of lining epithelial cells, cystic dilatation associated with degenerative changes of uterine glands. These changes may be either as a result of chronic cytotoxic irritation of the used fungicide on the female genital system or oestrogen like effect of the fungicide on genital tract. In this respect, Turner and Eliel (1978) and Eroschenko and Mousa (1979) reported that some pesticides show potent oestrogenicity. Moreover, the present pathological changes in the thyroid glands were increased number of macro follicles, cytoplasmic vacuolations and / or hyperplasia of the epithelium lining. This alterations in the thyroid glands of treated rats confirmed the aformentioned clinical findings and denoted the chronic cytotoxic effect of TMF (Ahmed et al., 1998).

It is obvious that the fungicide TMF iduced both numerical and structural chromosomal aberrations in treated femals rats. The most frequent aberrations were centromeric atenuation, deletions, ring and polyphoidy. Similar results were reported by Ali (1992) and Fahmy (1995). They attributed these changes to the genotoxic effect of the fungicide on chromosomes of mouse bone marrow cells. The mechanism of action of the fungicide on chromosomes may be resulted from breakage and rearrangement of the whole chromosome into abnormal form (Carrano and Natarajan, 1988) or affection on mitotic spindle with polyploidy fomation (Temtamy et al., 1982).

From clinical, blochemical and pathological points of view, it is evident that treating of female rats with the fungicide TMF induced irreversible changes due to direct cytotoxic effect or through pituitary and thyroid hormones imbalances (Ahmed et al., 1998). In this respect, it had been reported that manzozeb break down i mammalian tissue into ehtylene thiourea (ETU) metabolite which inhibits acelaldehyde dehydrogenase an enzyme vital to normal metabolism, resulting in build up of harmful compound in the cells of the organism (USEPA, 1992).

The results of this study demonstrate that TMF induced foetal growth retardation as well teratogenic effect when administrated to pregnant rats during the period of organogenesis. The

reduction in doetal body weight and crown rump length agreed with the previously reported data of Mathur and Bhatnagar (1991) who suggested that rarification of foetal bones may have lowered foetal weight. Also, they added that such reduction may be associated with maternal stress due to toxicity. Cummings et al., (1990) indicated that the reduction in the number of implantation sites may reflect the adverse effects of fungicides on both decidual growth and embryonic development. Janardhan et al., (1984) and Gray et al., (1988) found adverse effect on the number of offsprings per litter and foetal development following administration of maneb in rodents. On the contrary, Lu and Kennedy (1986) reported that inhalation of mancozeb in rat had no teratogenic effect. Variation in the obtained results may be related to route and dose of administration. However, the present teratogenic results may be related to direct embryotoxic effect of mancozeb metabolites.

In conclusion, exposure of animals to agricultural fungicides such as Tri-Miltox Fort induced adverse effects in the form of heath hazard, infertility, endocrine disturbance, mutagenisity and teratogenicity, therefore, great care should be taken in consideration in its application and searching for natural alternatives having minimum hazards for the environment.

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