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Temporal Effect of Carbofuran, a Carbamate Insecticide in the Interruption of Estrous Cycle and Follicular Toxicity in Female Swiss Albino Mice

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Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol N-methyl carbamate), an anticholinesterase carbamate, is commonly used as an insecticide, nematicide and acaricide in agricultural practice around the world (Gupta 1994). The widespread carbofuran use, contaminations has been found in the soil, air, food water, and wild life. Carbofuran is highly toxic to humans and wildlife through the oral and inhalations routes of exposure (Baron 1991). It has been shown that members of this class, such as disulfiram and its metabolite dithiocarbamate, can interfere with catecholamine neurotransmitters metabolism by inhibiting the activity of dopamine-β-hydroxylase (DβH). This is an enzyme that converts dopamine to norepineprine and norepineprine then stimulates the release of GnRH (Maj et al. 1969; Przewlocka et al. 1975; Goldman et al. 1994). Therefore, the present investigation was undertaken to evaluate the temporal effect of carbofuran on estrous cyclicity and follicles in mice.

MATERIALS AND METHODS

Carbofuran (pure 98 %) was kindly provided by Rallis India Ltd., Mumbai and dissolved in olive oil as a vehicle for oral administration. Laboratory bred virgin female Swiss albino mice aged 12-17 weeks, weighing between 24-28 g, showing regular 4-5 days estrous cycle were selected randomly from the breeding stock. The animals were housed in separate cages bedded with paddy husk and had free access to synthetic pellet diet "Gold Mohar" (Hindustan Lever Ltd., Mumbai) and water *ad libitum* throughout the study. The lighting schedule was 12:12 h light and dark cycle at a room temperature 26° ± 1° C. Animals were divided into 5 groups having 10 animals in each group. In the previous study, 1/5th of the LD_{50} dose was fixed to 0.7, 1 and 1.3 mg. Treatment with 1.3 mg / kg/d carbofuran seems to be effective dose (Baligar and Kaliwal, 2002). Hence, the dose of 1.3 mg / kg/d was chosen to study the temporal effect of carbofuran on sexual periodicity and follicular dynamics. The Carbofuran was administered by gavage for 5, 10, 20 and 30 days respectively. The control group received an equal volume of olive oil. Daily vaginal smear and body weight were recorded throughout the experiment.

The phases of estrous cycle was determined by observing vaginal smear in the morning (08:00 h to 10:00 h) as described by Cooper et al. (1993). Animals were killed by cervical dislocation on day 31st, 24 h after the final exposure. Ovaries of 5 animals in each group were taken for follicular growth studies. The weight of ovaries of the animal nearest to the mean weight of the ovaries of respective group was selected. The

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ovaries were fixed in Bouin's fluid, embedded in paraffin, sectioned at 5μm thickness and stained with hematoxylin and eosin. All serial sections of the ovary were counted for various stages of development of follicles as described by Hiremath and kaliwal (2002). Follicles were classified according to Chen et al. (1981) into small, medium and large follicles. Healthy or atretic follicles were classified as described by Swartz and Mall (1989). By using these criteria, mean diameters of follicles have been measured at approximately < 20μm for small, 20-70μm for medium and >70μm for large follicles in mice. Follicles displaying the nucleus of the oocyte were measured by using a calibrated occular micrometer to avoid repeated counting. The maximum diameter and diameter at the right angle to it were used to obtain a mean diameter for each follicle. A follicle was considered to be undergoing atresia or to regressing whenever two or more pyknotic granulosa cells would be found in a single section or whether the oocyte showed signs of degeneration, such as fragmentation, loss of nuclear membrane, or thining of cumulus oophorus as proposed by Osman (1985).

The change in the body weight was calculated on the basis of the weight taken 1st day at the time of oral administration was considered as the initial body weight and the weight taken on 31st d before cervical dislocation was considered as the final weight. Ovary, uterus, kidney, adrenal, liver, spleen, thymus and thyroid were dissected out, freed from adherent tissue and weighed to the nearest milligram. To ensure normalization of data for statistical analysis, organs weight were expressed per 100 g body weight. In the preset study treatment with carbofuran in different durational groups, did not alter the weights of uterus, kidney, adrenal, liver, spleen, thymus and thyroid (data not shown). 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

The results obtained in the present study indicate that the control mice exhibited regular estrous cycle and normal duration of each phases of estrous cycle. Treatment with carbofuran for 30 d caused a significant decrease in the number of estrous cycle and duration of proestrus, estrus and metestrus with a concomitant significant increase in the diestrus phase. However, treatment with carbofuran for 5, 10 and 20 d caused no significant change in the number of estrous cycle and duration of each phases of estrous cycle (Table 1). The members of this class, such as disulfiram and its metabolite dithiocarbamate, can interfere with catecholamine neurotransmitters metabolism by inhibiting the activity of dopamine-β-hydroxylase (DβH). This is an enzyme that converts dopamine to norepineprine and norepineprine than stimulates the release of GnRH. The GnRH release is ultimately affected through the inhibition of DβH (Maj et al. 1969; Przewlocka et al. 1975; Goldman et al. 1994), this could be expected to effect estrous cyclicity.

Plowchalk et al. (1993) have reported that the quantitative assessment of follicle number is an indicator of normal function as well as toxic responses in the ovary. Follicles are the principle functional units of the mammalian ovary. The most important controllers of follicular development are follicle stimulating hormone (FSH) and luteinizing hormone (LH) produced from the pituitary and the ovarian steroid estrodiol produced by granulosa cells. Although all follicles are apparently exposed to the same fluctuations in these hormones, not all are equally responsive. Some ovulate and other become atretic, indicating the presence of intragonadal regulatory factors. The

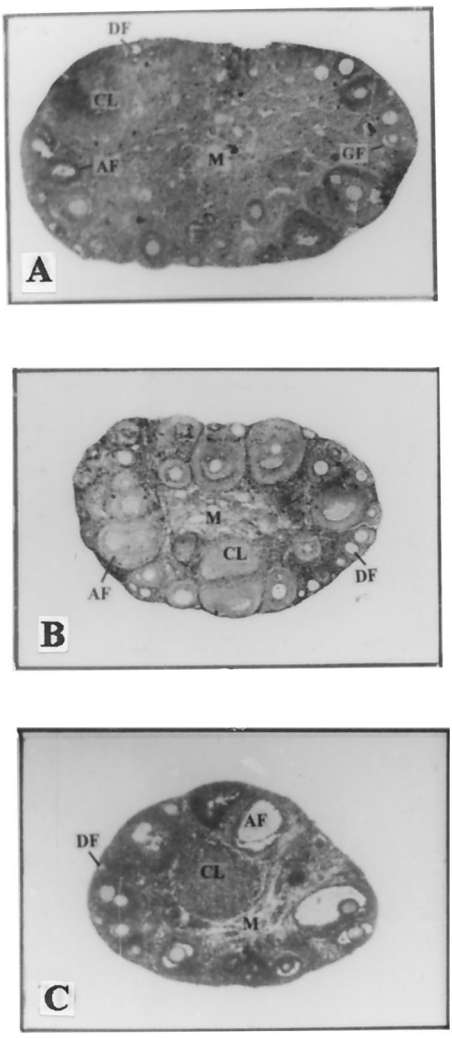

Figure 1. A through C: Temporal effect of carbofuran on ovary.

A - Section of the ovary of control mice showing the presence of GF, plenty of DF, few AF and many CL.

B - Section of the mouse ovary treated with carbofuran for days 20, showing the presence of few DF, CL and many AF.

C - Section of the mouse ovary treated with carbofuran for days 30 showing the presence of few DF, few small CL and many AF.

Abbreviations: GF = Graafian Follicle; DF = Developing Follicle; AF = Atretic Follicle; CL = Corpora Lutea; M = Medulla; (H & E; X 30).

Table 1. Temporal effect of carbofuran on estrous cycle, body and organs weight in albino mice

Groups[a]	Duration of treatment (days)	Number of Cycles	Duration in days (M ± S.E.)				Relative change in body and organ weight (M ± S.E.)	
			Proestrus	Estrus	Metestrus	Diestrus	Body weight (g)	Ovary weight (mg/100g B.Wt)
I	Control	5.9 ± 0.2	4.8 ± 0.3	7.6 ± 0.2	5.5 ± 0.2	11.9 ± 0.4	2.3±0.21	28.81±1.46
II	5	6.1 ± 0.2	4.9 ± 0.4	7.5 ± 0.2	5.3 ± 0.2	11.9 ± 0.5	2.4±0.27	29.98±1.40
III	10	5.7 ± 0.2	4.9± 0.3	7.1 ± 0.2	5.1 ± 0.2	12.3 ± 0.6	2.1±0.28	28.19±1.86
IV	20	5.6 ± 0.3	3.8 ± 0.4	6.9 ± 0.3	4.9 ± 0.3	14.2 ± 0.9	1.7±0.26	27.57±1.11
V	30	4.3 ± 0.3*	1.9 ± 0.3*	5.4 ± 0.3*	3.1 ± 0.2*	19.1 ± 0.5*	1.0±0.26*	18.84±1.88*

A = 10 animals in each group

* = Significant $P < 0.05$ compared to control

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

Table 2. Temporal effect of carbofuran on healthy and atretic follicles in albino mice

Groups[a]	Duration of treatment (days)	Number of follicles in the ovary by size classification (μm diameter); mean ± S.E.						
		Healthy Follicles				Atretic Follicles		
		Small < 20 μm	Medium 20-70 μm	Large > 70 μm	Total	Medium 20-70 μm	Large >70 μm	Total
I	Control	206.6 ± 2.7	65.2 ± 2.4	7.2 ± 0.4	279.0 ± 3.0	11.0 ± 0.9	2.2 ± 0.4	13.2 ± 1.1
II	5	205.0 ± 3.2	65.4 ± 1.2	7.4 ± 0.4	277.8 ± 3.9	11.2 ± 0.7	2.0 ± 0.3	13.2 ± 1.1
III	10	207.6 ± 4.9	62.4 ± 1.2	7.0 ± 0.3	277.0 ± 5.6	12.0 ± 0.6	2.4 ± 0.2	14.4 ± 0.5
IV	20	201.8 ± 1.9	60.2 ± 2.0	6.4 ± 0.2	269.0 ± 2.4	12.4 ± 0.81	2.6 ± 0.4	15.0 ± 0.8
V	30	196.2 ± 2.0*	52.2 ± 1.9*	4.2 ± 0.5*	252.6 ± 2.4*	21.4 ± 1.1*	3.8 ± 0.4*	25.2 ± 0.8*

a = 5 animals in each group
* = $P < 0.05$ compared to control

histologic observations of the control mice showed normal number of developing follicles, Graafian follicles, corpora lutea and atretic follicles (Fig. A). Histologic observations of the mouse ovary treated with carbofuran for days 5,10 and 20 showed the presence of developing follicles corpora lutea and many atretic follicles (Fig. B). Treatment with carbofuran for 30 d caused a significant decrease in the number of healthy follicles with concomitant significant increase in the atretic follicles when compared with that of control mice (Table2). Histologic observation of the mouse ovary treated with carbofuran for 30 d showed the presence of few developing follicles, few small corpora lutea and many atretic follicles (Fig. C). Treatment with carbofuran for days 5, 10 and 20 caused no significant change in the number of different stages of healthy and atretic follicles. This indicates that the effect of carbofuran on estrous cycle and follicular growth is duration dependent. Similar findings have been reported that the administration of number of organophosphate and chlorinated pesticides to adult rats increased the number of atretic follicles with concomitant decrease in the number of some of the follicular stages and total number of healthy follicles (Asmathbanu and Kaliwal 1997; Dhondup and Kaliwal 1997; Math et al. 1998). It has been shown that the ovarian androgen and inhibin secretion by follicles may be an important part in the regulation of FSH secretion and follicular growth (Evans et al. 1997).

In the present study, there is also a possibility that the decreased healthy follicles with concomitant increase in atretic follicles in mice may be due to affecting gonadotropin secretion via central nervous system mechanism, as it was observed in the dithiocarbamates (Goldman et al. 1994). In the present study there is also possibility that the disruption in the estrous cycle, decrease in the healthy follicles with concomitant increase in the atetic follicles may result from damage by toxicant at the level of hypothalamo-pituitary-gonadal axis. However, further investigation in this regard is essential to know the mechanism of action of carbofuran on follicular development and estrous cycle.

Treatment with carbofuran showed a durational related toxicity in terms of body weight (Table 1). There is a significant decrease in the body weight in long-term carbofuran treatment as there may be suppression towards food and water intake. Although food and water intake has not been measured in this study, this may be one of the reasons for low weight gain, which may alter the estrous cycle and folliculogenesis. This finding is important because nutritional deficiency have been shown to alter reproductive function (Simonich and Hites 1995; Hiremath and Kaliwal 2002).The ovary weight was decreased significantly with prolonged carbofuran treatment (Table 1). Similar observations were made in rats treated with monocrotophos and have reported that decrease in weight and size of the ovaries is due to the extensive fibrosis and atretic follicles (Adilaxamamma 1994). Therefore, the reasons may be due to the hormonal imbalance in any of the stages in hypothalamo-hypothysial ovarian axis or by insensitising the follicular receptors to the available gonadotropins thereby led to the retardation of further development of surviving follicles into next successive follicular stages and also arrest of estrogen, production which may effect the estrous cycle or directly on the ovary.

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