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CHLORPYRIFOS SUPPRESSES FEMALE REPRODUCTIVE FUNCTION IN RAT.

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ABSTRACT

We report here the toxic effects of chlorpyrifos, a broad-spectrum organophosphorous insecticide, on female reproductive function in rat model. We observed significant alterations in the rhythmicity and phasic cellular characteristics of estrous cycle with a concomitant increase in diestrus index in chlorpyrifos treated female rats in a dose-dependent manner (5.4 and 8.1 mg/Kg.b.wt./day) compared to control rats. The levels of serum female reproductive hormones (LH, FSH and estradiol) were also decreased dose-dependently. Structural disorders and degenerations of ovarian and uterine tissues were observed in treated rats. The mean body weight and organ weight of ovary and uterus in treated female rats were also significantly decreased. From the results, we may conclude that chlorpyrifos suppresses the female reproductive functions in rat presumably by impairing the estrous cycle function and inducing oxidative stress induced structural disorders and depletions of ovarian and uterine tissues.

KEY WORDS: **Chlorpyrifos, Estrous cycle, Ovary, Uterus, Oxidative stress.**

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INTRODUCTION

Chlorpyrifos (CPF) [O, O-diethyl-(3, 5, 6-trichloro-O-pyridyl) phosphorothioate) is a class II broad-spectrum organophosphorous insecticide (moderately toxic). It is used extensively to control agricultural and other pestiferous insects[1,2]. It kills insects by blocking respiration and paralysing the muscles involved in the movement of the wings of insects. The blocking of respiration and movement of the wing muscles of the insects is due to chlorpyrifos induced inhibition of acetylcholinesterase (AChE), the enzyme that cleaves acetylcholine into choline and acetate, found on the plasma membrane of motor nerve terminals that innervates the muscles involved in respiration and movement of wing[3]. As a result of the inhibition of acetylcholinesterase enzyme, acetylcholine (ACh) acts on the muscle for a long period and causes paralysis of the muscles. It has been reported that the farmers involved in chlorpyrifos spray in the field, including the general people living in the vicinity of application field, are directly exposed to chlorpyrifos through aerosol inhalation and dermal absorption during the spraying of chlorpyrifos[4,5]. As a xenobiotic substance, chlorpyrifos may exert toxic effects on human physiological function of different organ systems of the body[6,7,8]. From some reports based on experiments in animal models, we understood that chlorpyrifos is an endocrine disrupter substance[9]. Moreover, it impairs fetal growth, induces maternal toxicity and oxidative stress induced structural alterations in some organ tissues[10,11,12,13,14]. There is every possibility of the suppression of male and female reproductive functions in humans as a result of the chlorpyrifos induced toxicity. We observed no reports about the toxic effects of chlorpyrifos on female reproductive functions in a coherent manner due to chlorpyrifos induced toxicity till to date. Therefore, the present study was designed to examine the possible effects of chlorpyrifos on the function of the female reproductive system in rat model for extrapolating the results in human female reproductive system.

MATERIALS AND METHODS

Chemicals and reagents

All the reagents used in the study were of analytical grade. Chlorpyrifos (CPF) was purchased from Devidayal (sales) Limited, Mumbai, India. Sodium chloride (NaCl), Paraffin wax (58˚-60˚C) and Xylene etc. were procured from E.Merck, India. Eosin and Hematoxylin were procured from Sigma, USA.

Experimental Animals

Laboratory bred adult female Charles Foster strain rats, aged 90-120 days, weighing between 110-120 gms were used to carry out the study. The animals were kept in cages and were fed standard laboratory chow and water. The animals were maintained as per the recommendation of Animal Ethical Committee of the University of Kalyani as per national guidelines.

Experimental design

After acclimatization to the laboratory environment, the rats were subdivided into 3 groups each comprising of 8 animals.

1. Group I- received distilled water for 30 days (Control group).
2. Group II- received 5.4 mg/kg.b.wt./day (i.e., 20% LD_{50}) for 30 days (Treated I).
3. Group III- received 8.1 mg/kg.b.wt./day (i.e., 30% LD_{50}) for 30 days (Treated II).

Vaginal smear of all animals was taken and observed regularly. The body weight was also recorded. The animals were sacrificed by cervical dislocation after completion of last dose and the absolute body and organ (ovary and uterus) weights were recorded.

Methodology for the study of estrous cycle

Vaginal smear histology is used as an index of ovarian functions. It is also used as a test of potency of various steroid hormones[15]. A detailed comparative estrous cycle study was carried out in female rats exposed to two designated doses of chlorpyrifos by the

modified method of Marcondes *et al.*, 2002 with slight modifications[16].

a. Collection of vaginal smear

The vaginal lavage was collected with a plastic pipette filled with 10μL of normal saline (0.9% NaCl) by inserting the tip into the rat's vagina in every morning (10.00-11.00a.m). Vaginal lavage was placed on glass slides. A different glass slide was used for each rat housed in a cage for the specific dose. One drop was collected with a clean tip from each rat.

b. Staining and microscopic observations

The unstained slides were studied under microscope to confirm the stages of estrous cycle. The slides containing the vaginal smear were stained with hematoxylin and eosin according to the method of Bancroft *et al.*, 2003[17] with slight modifications and mounted with DPX. All the stained slides were stored and subsequently examined in order to confirm the phases of estrus cycle. The diestrus index was calculated by the following formula:

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

Body weight and absolute organ weight

The body weight was calculated on the basis of the weight taken on the day of the application of first dose and it was considered as the initial body weight. The body weight taken on the 31st day was considered as the final weight. Ovary and uterus were dissected out, free from adherent tissue and weighed to get absolute organ weight. Organ weight was expressed per 100gm of body weight.

Hormonal assay

Levels of serum luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E_2) were estimated by enzyme-linked immunosorbent assay (ELISA) methods using kits of ERBA Diagnostic GmbH Mannheim Germany.

Histopathological study

Bouin's fixed and paraffin impregnated ovarian and uterine tissue sections were stained with normal hematoxylin-eosin stain according to the method of Bancroft *et al.*, 2003[17] with slight modifications. Each paraffin section about 5μm thickness was kept sequentially in xylene and graded ethanol, and stained with hematoxylin for 2 minutes. After removing the excess color, the slides were counterstained with eosin and then the stained slides were dehydrated with graded ethanol, cleared with xylene and mounted with DPX. Stained sections were observed under a compound microscope (Model No-Olympus OM 1) at 400X magnification. Images were obtained by SONY DSC W320 camera.

Statistical analysis

The data were expressed in terms of mean±SEM. The data were analyzed by Student's't' test, one way and two way analysis of variance (ANOVA) wherever applicable. $^{a}P \leq 0.05$, $^{b}p \leq 0.01$, $^{c}p \leq 0.001$ were considered significant. The number of each experiment is indicated by the alphabet 'n' in the results.

RESULTS

Effects of Chlorpyrifos on estrous cycle

1. Changes in duration

We found significant decrease in frequency of estrous cycle; and duration of proestrus, estrus and metestrus in treated groups of rats in a dose dependent manner for the exposure periods compared to control group of rats (Table-1).We also observed significant increase in the duration of diestrus phase and diestrus index of treated group of rats in a dose dependent manner (Table-1).

Table 1
Showing average duration of estrous cycle phases and diestrus index in control and chlorpyrifos treated rats.

Groups	Doses (mg/Kg/d)	No. of rats (n)	Duration in days(Mean±SEM)				Diestrus Index (DI)
			Proestrus	Estrus	Metestrus	Diestrus	
GroupI (control)	0	8	5.625±0.263	7.375±0.420	5.250±0.366	11.750±0.491	39.16
Group II	5.4	8	3.625±0.183[c]	4.125±0.295[c]	3.875±0.227[b]	18.375±0.498[c]	61.25
Group III	8.1	8	2.500±0.189[c]	3.125±0.295[c]	3.625±0.263[a]	20.750±0.366[c]	69.16

Values are represented as Mean±SEM. [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ vs. control groups of rat(Two way ANOVA).

2. Changes in cytology

We observed significant changes in the cellular characteristics of vaginal smear in component phases of estrous cycle in treated group of rats compared to control rats. In proestrus phase, the characteristic nucleated epithelial cells were replaced by non-nucleated cornified epithelial cells (Fig.1: Plate C1, T1-5 and T2-9). We found a significant increase in the number of cornified cells in estrous phase; and leucocytes, shorr cells, cornified epithelial cells and non-nucleated epithelial cells in metestrus phase of chlorpyrifos treated rats in a dose-dependent manner. Further, the slight thickness of smear in both estrus and metestrus phase, and changes in the morphology of cornified epithelial cells in estrus phase were observed in treated rats (Fig.1: Plate C-2,3,T1-6,7,T2-10,11). We also found significant thickening of smear, an increase in the number of leucocytes and aggregation of leucocytes in diestrus phase of the cycle in treated rats in a dose dependent manner (Fig.1: Plate C-4,T1-8,T2-12).

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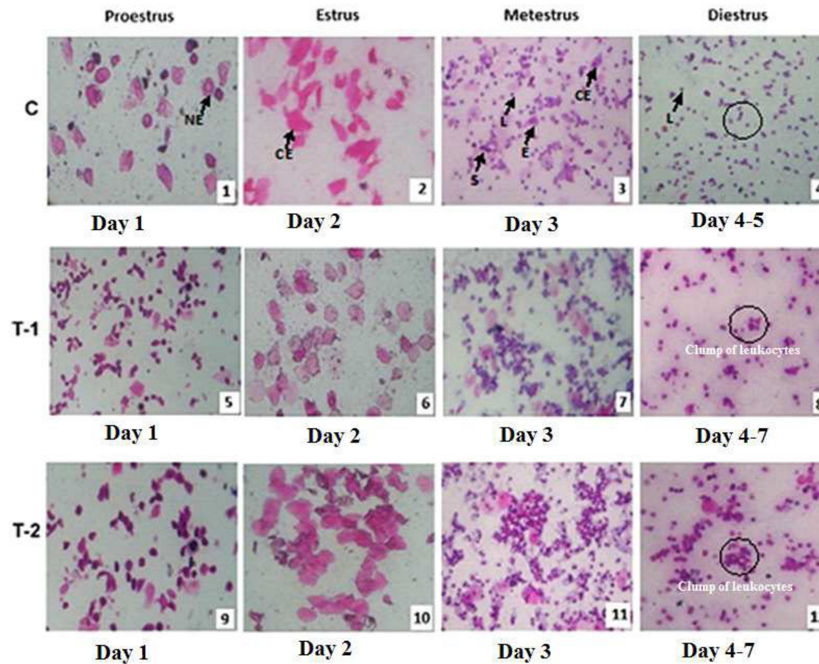

Figure 1

Photomicrographs (400X magnification) showing cytological characteristics of proestrus, estrus, metestrus and diestrus phase of a representative estrous cycle in chlorpyrifos treated (T1 and T2 series, Plates 5-8 and 9-12) and control (C series Plate 1-4) groups of rats. C: control, T1-Treated I (5.4mg/kg/d)and T2-Treated II (8.1mg/kg /d). (NE-Nucleated Epithelial cell, CE-Cornified Epithelial cell, L-Leucocytes, S -Shorr cells, E-Non Nucleated Epithelial cells.)

Effects of chlorpyrifos on body weight and organ weight of ovary and uterus.

The mean body weight of chlorpyrifos exposed groups of rats was decreased significantly in a dose-dependent manner compared to control group of rats (Table-2). The absolute and relative mean weights of ovary and uterus were also decreased significantly in exposed groups of rats (Table-3&4).

Table 2
Showing average body weight of control and treated rats.

Body Weight (gm)	Dose (mg/kg.b.wt./day for 30 days duration)		
	0	5.4	8.1
Initial	114.25±0.701	114.62±0.532	114.5±0.567
Final	116.62±0.680	111.87±0.549^{a}	108.7±0.620^{c}

Values are represented as Mean±SEM. ^{a} p<0.05, ^{b} p<0.01, ^{c} p<0.001 vs. control groups of rat (n=8).

Table 3
Showing absolute and relative weight of ovary of control and treated rats.

Experimental Groups (mg/kg.b.wt./day)	Absolute weight of ovary(gm)			Relative weight of ovary (gm/100g.b.wt.)		
	Left(L)	Right(R)	L+R	Left(L)	Right(R)	L+R
0	0.044±0.001	0.044±0.001	0.088±0.002	0.037±0.001	0.037±0.001	0.075±0.001
5.4	0.038±0.001	0.038±0.001^{c}	0.076±0.002^{c}	0.033±0.001^{c}	0.034±0.001^{c}	0.068±0.001^{c}
8.1	0.033±0.001^{c}	0.033±0.00^{c}	0.066±0.00^{c}	0.030±0.000^{c}	0.030±0.000^{c}	0.060±0.001^{c}

Values are represented as Mean±SEM. ^{a} p<0.05, ^{b} p<0.01, ^{c} p<0.001 vs. control groups of rat (n=8).

Table 4
Showing absolute and relative weight of uterus of control and treated rats.

Experimental groups (mg/Kg.b.wt./day)	Absolute weight of uterus (gm)			Relative weight of uterus (gm/100g.b.wt.)		
	Left(L)	Right(R)	L+R	Left(L)	Right(R)	L+R
0	0.44±0.006	0.44±0.008	0.88±0.013	0.37±0.003	0.37±0.005	0.75±0.008
5.4	0.40±0.005[c]	0.39±0.005[c]	0.79±0.010[c]	0.35±0.004[c]	0.34±0.007[a]	0.70±0.011[a]
8.1	0.30±0.005[c]	0.28±0.004[c]	0.58±0.009[c]	0.27±0.004[c]	0.25±0.002[c]	0.53±0.007[c]

Values are represented as Mean±SEM. [a] P<0.05, [b] p<0.01, [c] p<0.001 vs. control groups of rat (n=8).

Hormonal assay

The levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E2) were decreased significantly at diestrus phase of treated groups of rats than the respective control groups (Table-5).

Table 5
Showing female reproductive hormone levels in chlorpyrifos treated rats.

Dose(mg/kg.b.wt/day)	LH(mIU/ml)	FSH(mIU/ml)	Estradiol(pg/ml)
0	0.92±0.012	0.89±0.009	58.10±0.53
5.4	0.89±0.013[b]	0.75±0.004[b]	49.40±0.61[b]
8.1	0.62±0.01[b]	0.68±0.005[b]	32.30±0.46[b]

Values are represented as Mean±SEM. [a] p<0.05, [b] p<0.01, [c] p<0.001 vs. control groups of rat (one way ANOVA test) (n=8).

Histopathological studies

We observed remarkable structural disorders and degenerations of some tissues in ovary and uterus in diestrus phase of chlorpyrifos treated groups of rats in a dose-dependent manner (5.4 and 8.1 mg./Kg.b.wt./day) for the periods of the exposure (30 days) of chlorpyrifos compared to control groups of rats. We found depletion of thecal and granulosa cell layers in graafian follicles and inhibition of the progressive maturation of primordial follicles, as the primordial follicles outnumber the graafian follicles in the ovary. We also found significant increase in the number of atretic follicles in the ovarian sections of chlorpyrifos treated rats (Fig. 2a). In chlorpyrifos treated uterine sections, we found significant loss of uterine glands in uterine wall and reduction in the diameter of the uterine lumen. Further, we found thinness of the diameter of the endometrium, myometrium and perimetrium in the uterine wall due to damage of cellular architecture (Fig.2b).

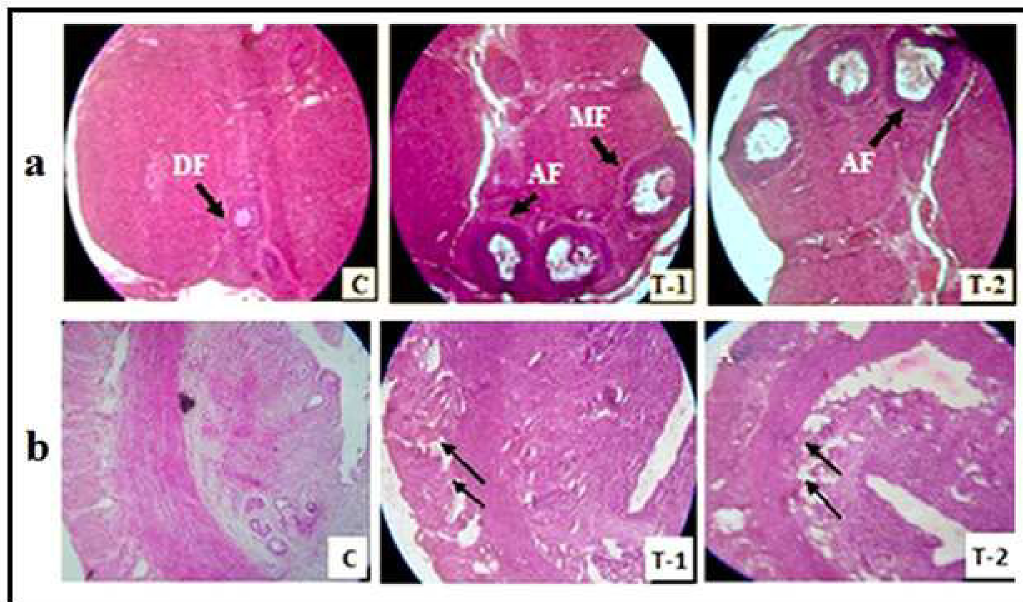

Figure 2

Photomicrographs (400X magnification) showing the histopathological changes in diestrus (a) ovary and (b) uterus in chlorpyrifos treated and control female Charles foster rats. C indicates control diestrus ovary and uterus in panel (a) and (b) respectively, showing normal ovarian and uterine architecture. T1 (Treated group 1) and T2 plate (Treated group 2) showing the disorder and structural depletion of ovary (in panel a) and uterus (in panel b). [DF-developing follicle, AF-atretic follicle, MF-mature follicle].In panel (b) the arrow heads of T1 and T2 plates indicates the absence of uterine glands and degeneration of uterine wall structure.

DISCUSSIONS

Mammals excepting the primates do not menstruate, and their sexual cycle is called an estrous cycle. Rat is a spontaneously ovulating species with estrous cycles and shows regular endocrine events that are essentially the same as those in the menstrual cycle. In this study we have examined the estrous cycle variables of chlorpyrifos exposed female rats as a holistic indicator about the toxic effects of chlorpyrifos on the female reproductive system function involving the component roles of hypothalamus-pituitary, ovary and uterus. We have observed significant decrease in the frequency of estrous cycle during the chlorpyrifos exposure period and the duration of proestrus, estrus and metestrus with a concomitant increase in the duration of diestrus in each estrous cycle in a dose dependent manner compared to control group of rats. Moreover, the diestrus index has been increased significantly in chlorpyrifos treated rats in a dose dependent manner. Our results are similar to the observations of Budreau and Sing et.al with some pesticides like fenthion and dimethoate[18]. From the results we may consider the effects of chlorpyrifos on the set-point secretion and function of some female reproductive hormones secreted from the anterior pituitary and ovary that control the estrous cycle function in female rat. We have observed significant reduction in the level of LH, FSH and estradiol in chlorpyrifos treated rats. This indicates that chlorpyrifos may inhibits the function of ovary and uterus as supported by the results from estrous cycle study presumably by inhibiting the secretion of LH and FSH from anterior pituitary and estradiol from ovary.

The depression of the secretion of LH and FSH might be due to direct action of chlorpyrifos on anterior pituitary gonadotrophs, responsible for the secretion of LH and FSH; or hypothalamic neurons, responsible for the secretion of gonadotropin-releasing hormone (GnRH) that exerts trophic action on anterior pituitary gonadotrophs. Thus, changes in

cellular characteristics in vaginal smear in different phases of an estrous cycle and increase in diestrus index might be due to chlorpyrifos induced depression of the secretion of LH and FSH from anterior pituitary; and estradiol from the ovaries in female rat. In order to ascertain the effects of chlorpyrifos on the follicular development in ovary and uterine preparedness for implanting the embryo, the histopathological examinations of the diestrus ovary and uterus in chlorpyrifos treated female rats have been considered. We observed significant decrease in matured ovarian follicle with a concomitant increase in the number of atretic follicles. Moreover, the most of the matured follicles showed depletion of thecal and granulosa cell layers; and aoocytic. The depletion of uterine wall has also been observed in chlorpyrifos treated female rats. We found absence of uterine glands and depletion of decidual cells in uterine wall structure. These results suggest that chlorpyrifos may promote the disorders of follicular maturation, structural degenerations of ovary and uterus presumably by lowering the level of gonadotropin and estradiol and by promoting the oxidative stress induced damage of the tissues of ovary and uterus, as corroborated by Samarawickrema et.al., 2008[14].

To find out the neural influence in chlorpyrifos induced inhibition of female reproductive function; we have taken the accounts of whole body weight and organ weight of ovary and uterus in control and chlorpyrifos exposed female rats. We observed significant decrease in whole body weight and organ weights of ovary and uterus compared to control rats. Moreover, we observed marked depression in food and water intake of chlorpyrifos treated rats. From the results it is suggested that chlorpyrifos may reduce the body as well as organ weights presumably by shifting the hypothalamic set-point controls for food and water intake toward satiation. This might be partially due to facilitation of synaptic transmission in neural circuits through chlorpyrifos induced irreversible inhibition of acetylcholinesterase (AChE), the key enzyme responsible for termination of synaptic transmission through acetylcholine hydrolysis at the synaptic transmission. This hypothesis may be corroborated by the report of Hancock et.al., 2007[19] who stated that chlorpyrifos inhibits acetylcholine esterase. The other reason for the chlorpyrifos induced loss of body and organ weights of ovary and uterus may be endocrine disruption. Inadequate female gonadotropins may reduce the body and organ weights by inducing the atrophy of somatic tissues and tissues of ovary and uterus. In addition to this, oxidative stress induced damage of tissues in chlorpyrifos exposed rats may be taken in accounts as one of the causes of the loss of whole body weight and organ weights of ovary and uterus. Thus, we may conclude from the results that chlorpyrifos suppresses the female reproductive function in rat presumably by impairing the estrous cycle; inhibiting the secretion of female reproductive hormones through the shift in set-point control of the function of hypothalamo-hypophyseal-ovarian axis; and promoting the oxidative stress induced structural disorders and degenerations of the tissues of ovary and uterus.

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