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Assessment of Toxicity of Chlorpyrifos Insecticide on Fetuses and Suckling Pups of Rats

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Abstract: Background: The female reproductive toxicity and teratogenic risk of organophosphates had been a global concern because of the wide spread use of these compounds. Chlorpyrifos is a broad-spectrum organophosphate insecticide used extensively in agriculture and for residential pest control throughout the world. **Material and Methods:** The developmental toxicity of chlorpyrifos on fetuses and suckling pups of rats was assessed. Pregnant rats were divided into 5 groups (n = 10); one was used as a control (received vehicle) and two groups were given orally chlorpyrifos at 5 and 10 mg kg^{-1} b.wt. (corresponding to 1/20 and 1/10 of LD_{50}) from the day 6th through the 15th day of gestation. The other groups were given the same doses from the 15th day of gestation through the day 21st after delivery (weaning age). Rats were euthanized using ether anaesthetic, uteri were dissected out and fetuses were subjected to morphological, visceral and skeletal examinations. **Results:** It was found that chlorpyrifos caused embryotoxic and teratogenic effects with growth retardation of fetuses. The observed visceral malformations included microcephaly, dilated cerebral ventricles and hypoplasia of the heart and lungs. The skeletal abnormalities were incomplete ossification of skull bones and absence of the last rib. Chlorpyrifos significantly decreased the viability, gestation and weaning indexes of fetuses. Deaths of all sulking pups were reported before the end of weaning age. **Conclusion:** exposure to chlorpyrifos during pregnancy and lactation periods should be prohibited to avoid its teratogenic action and its lethal effect on suckling pups.

Key words: Insecticides, chlorpyrifos, teratogenicity, suckling pups

INTRODUCTION

Owing to the widespread use and the potential for human exposure, pesticides comprise a hazardous impact on human health and environmental pollution. Among pollutants organophosphate insecticides that are routinely used in agricultural purposes and for combating insects that infest man and animals (Akhtar *et al*., 2009). Organophosphates are potent inhibitors of serine esterases and hence their toxicities are attributed to the inhibition of acetylcholinesterase through binding of the phosphate moiety. Chlorpyrifos is a broad spectrum insecticide (organophosphates) which is extensively used in agriculture and to control pests (Hanley *et al*., 2000). Chlorpyrifos irreversibly inhibits acetylcholinestrase enzyme activity in the central nervous system and in turn lowers plasma, erythrocyte and brain cholinesterase (Ogawa *et al*., 1988; Sakai, 1991). Toxic symptoms include nausea, vomition, salivation, diarrhea, tremors and convulsions (Kamrin, 1997). Prolonged exposure to organophosphates causes chronic neurological syndrome, malignant tumors, immunosuppressive action, teratogenic effect, abortion and decreased male fertility in experimental animals (Cannon and Kimbrough, 1979; Nafstad *et al*., 1983). Chlorpyrifos induced developmental changes in the central nervous system of fetal and young rats (Lemus and Abdelghani, 2000). Insecticide contaminants can adversely affect the reproductive function either directly via their direct cytotoxic action and/or indirectly through their interfering with the hypothalamo-hypophysial control function (Mahadevaswami and Kaliwal, 2002). The present study was, therefore, carried out to investigate the adverse effect of chlorpyrifos on the fetal development and suckling pups till weaning age in rats.

MATERIALS AND METHODS

Insecticide: Chlorpyrifos (Kanzaril® 50% EC) was purchased from KANZA group, Egypt, as 50% Emusifiable Concentrate (EC) backed in brown bottles of 100 mL capacity each.

Animals: Sexually mature Sprague Dawley rats of both sexes weighing from 180-200 g b.wt. and 12-14 weeks old were used in the experimental work. Animals were housed

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plastic cages with steel mesh provided from the breeding unit of the National Research Center (Dokki, Egypt). Rats were maintained at a temperature of 25±2°C, relative humidity of 50±5% and photoperiod at 12 h dark/12 h light. Animals were fed on commercial standard locally prepared rat pellets (Cairo Agriculture Development Company, 6th October City, Giza, Egypt) and water was supplied *ad libitum*. Rats were allowed to acclimatize to the laboratory environment for 7 days before start of the experiments. The experiments were carried out according to the National regulations and rules for animal welfare and guidelines of Institutional Animal Ethical Committee (IAEC), Dokki, Cairo, Egypt. Virgin female rats were kept overnight with adult males (the same strain). In morning of the next day, vaginal smears were taken and females were considered mated if sperms and/or vaginal plugs were observed in the smears (Barcellona *et al*., 1977). The day at which evidence of mating was observed was defined as gestation day zero. Pregnant females were individually identified with numbers (metal ear tag) upon assignment to study.

Determination of LD_{50}: The LD_{50} was mathematically determined following oral administration of chlorpyrifos to adult rats according to Kerber method (Pershin, 1971) using the following formula:

$$LD_{50} = LD_{100} - \Sigma (z \times d)/m$$

where, z is a half of sum of animal quantity died from two successive doses, d is the interval between two successive doses and m is the number of animals per group.

Effect of chlorpyrifos on fetal development: Chlorpyrifos was given to pregnant rats during the day 6th through the day 15th of gestation (period of organogenesis). During this period, the different organs are developing and the fetuses became more sensitive to the administered agents (Tuchmann-Duplessis, 1975). Three groups of pregnant rats each of 10 animals were used to investigate effect of chlorpyrifos on the fetal development. The first group was kept as a control and received the vehicle, whereas, the other two groups were orally administered chlorpyrifos by a stomach tube at doses of 5 and 10 mg kg^{-1} b.wt., respectively, during the period of organogenesis. Rats in all groups were kept under observation till the 20th day of gestation at which they were euthanized by CO_2 asphyxiation and uteri were dissected out. Each fetus was individually identified, weighed, sexed and grossly examined for external malformations/variations including observation for palatal defects. All obtained fetuses were examined for any morphological, visceral and skeletal malformations.

Morphological examinations of fetuses: The horns of the gravid uterus were exteriorized, the uterus was opened using a scissor and then the fetuses were removed. The number of implantation and/or resorption sites, live and dead fetuses were counted. The resorption sites were numbered as described by Cook and Fairweather (1968). For clearing the sites of early fetal resorptions or implantation sites, the horns were impregnated in 10% ammonium sulphide solution for 20 min (Kopf *et al*., 1964). After impregnation, the implantation or resorption sites appear as black spots that could be easily detected and counted using a magnifying lens. The live fetuses were examined for external morphological abnormalities, weighed and measured (Crown-rump length).

Visceral examination of fetuses: The fetuses preserved in Bouin's fixative solution were rinsed with cold water and several sections were made throughout the fetal body. These sections were then grossly examined under a dissecting microscope for any visceral malformations (Staples, 1974; Hayes, 1986).

Skeletal examination of fetuses: The eviscerated fetuses were kept in 95% ethanol for 7 days for dehydration. The dehydrated fetuses were placed in 2% potassium hydroxide solution for 24-35 h according to the size of the fetus till complete digestion of the muscles. After clearing, the fetuses were immersed in Mallsch's solution with alizarin red stain for 24 h as described by Staples and Schnell (1964). The stained fetuses were kept in Mallsch's solution alone for 2 days, then rinsed with water and cleared by successive passage in graded concentrations of glycerin watery solution. (70, 80, 90, 100%). The fetuses were then examined under a dissecting microscope and the skeletal malformations were reported (Tuchmann-Duplessis, 1975). The heads of selected fetuses for visceral examination were removed, placed in Bouin's fixative and subsequently sectioned and examined for craniofacial defects (Wilson, 1965).

Effect of chlorpyrifos on suckling pups: Thirty pregnant rats were divided into 3 equal groups, each of 10 rats. The first group was kept as a control, whereas the other two groups were administered chlorpyrifos orally at doses of 5 and 10 mg kg^{-1} b.wt., daily from the 15th day of gestation to the 21st day after parturition (age of weaning). The method described by Hayes (1986) was

used for morphological examination of suckling newborns. Gestation, viability and weaning indexes were calculated as follow:

$$\text{Gestation index} = \frac{\text{No. of live fetuses}}{\text{Total No. of fetuses}} \times 100$$

$$\text{Viability index} = \frac{\text{No. of live newborns till the 4th day of age}}{\text{No. of live newborns}} \times 100$$

$$\text{Weaning index} = \frac{\text{No. of live newborns till the 21st day of age}}{\text{No. of live newborns till the 4th day of age}} \times 100$$

Statistical analysis: All statistical analyses were performed using SPSS statistical version 13 software package (SPSS® Inc., Chicago, Illinois, USA). Developmental differences between control and treated groups for any parameter within the same genotype were analyzed for significance using a two-tailed, unpaired Student's t-test with Mann-Whitney post hoc test according to Snedecor and Cochran (1986). Means were considered significantly different at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

RESULTS

Acute toxicity of chlorpyrifos: The calculated LD_{50} of chlorpyrifos was 100 mg kg^{-1} b.wt. after oral administration to female rats. Doses of 5 and 10 mg kg^{-1} body weight of chlorpyrifos which equal to 1/20 and 1/10 of the calculated LD_{50} were used in the present study.

Effect of chlorpyrifos on fetuses: Oral administration of chlorpyrifos at 5 and 10 mg kg^{-1} b.wt. from the 6th to the 15th day of gestation induced complete or incomplete fetal resorptions (Fig. 1a, b), deaths and growth retardation of the fetuses. The fetal resorptions and deaths were significantly increased whereas, the fetal body weight and the crown-rump length of live fetuses were significantly reduced (Table 1).

Visceral malformations of fetuses: Oral administration of chlorpyrifos at doses of 5 and 10 mg kg^{-1} b.wt. to pregnant rats caused brain malformations in 35.5 and 45.0% and heart malformations in 20 and 30%, of the examined fetuses, respectively (Table 2). The brain malformations of fetuses were microcephaly (Fig. 2a),

Table 1: Effect of oral administration of chlorpyrifos to pregnant rats from the 6 to the 15th day of gestation on the obtained fetuses

Groups	Doses (mg kg^{-1})	Viable fetuses (%)	Dead fetuses (%)	Resorbed fetuses (%)	Fetal body weight (g/100 g b.wt.)	Fetal length (mm)
Control (Vehicle)	0.0	88.0	0.0	0.0	3.2±0.08	32.4±1.5
Chlorpyrifos	5.0	76.0*	6.66	8.88	2.5±0.09**	28.2±1.6***
	10.0	75.0*	8.42	12.63	2.3±0.06**	25.8±1.4***

*,**,***Significant at $p \leq 0.05$, ≤ 0.01 ≤ 0.001, No. of pregnant rats per group = 10

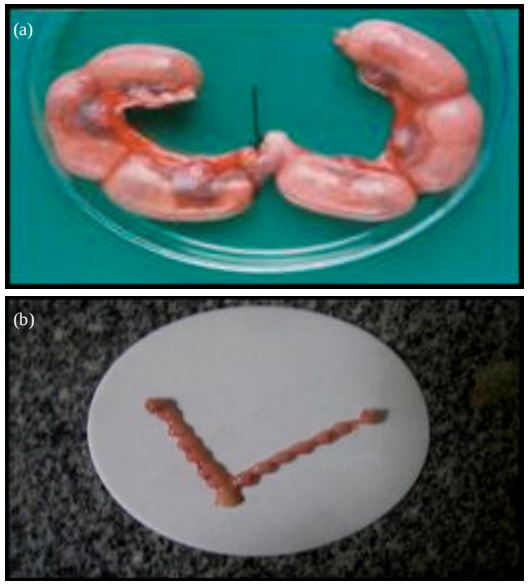

Fig. 1(a-b): (a) Incomplete and (b) Complete fetal resorptions in uterine horns (arrows) of pregnant rats given orally chlorpyrifos at 5 and 10 mg kg^{-1} b.wt., respectively from the 6 to the 15th day of gestation

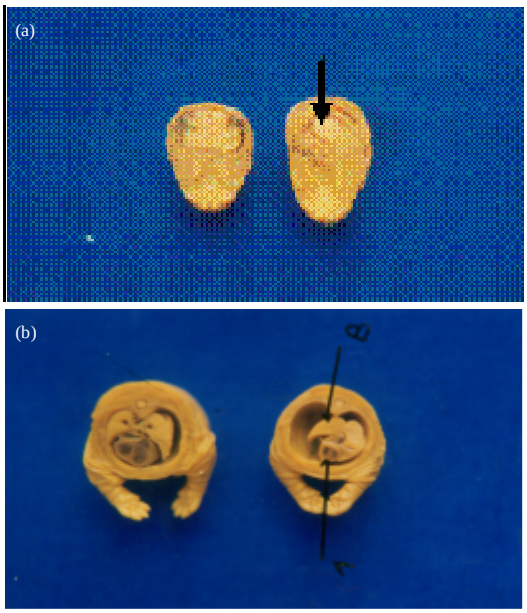

Fig. 2(a-b): (a) Microcephaly and (b) Hypoplasia of heart and lungs in a fetus obtained from pregnant rat given orally chlorpyrifos at 10 mg kg^{-1} b.wt. from the 6 to the 15th day of gestation (Left: controls)

Table 2: Effect of oral administration of chlorpyrifos to pregnant rats from the 6 to the 15th day of gestation on visceral malformations of fetuses

			Visceral malformations							
			Brain		Heart		Lungs		Renal pelvis	
Groups	Doses (mg kg^{-1})	No. of fetuses	No.	%	No.	%	No.	%	No.	%
Control (Vehicle)	0.0	55.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorpyrifos	5.0	45.0	16.0	35.5	9.0	20.0	3.0	6.6	7.0	15.5
	10.0	40.0	18.0	45.0	12.0	30.0	12.0	30.0	8.0	20.0

No.: Number of visceral abnormalities

Table 3: Effect of oral administration of chlorpyrifos to pregnant rats from the 6 to the 15th day of gestation on skeletal malformations of fetuses

			Skeletal malformations							
			Skull		Ribs		Forelimbs		Coccygeal vertebrae	
Groups	A	B	No.	%	No.	%	No.	%	No.	%
Control	0.0	33.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorpyrifos	5.0	17.0	4.0	23.5	6.0	35.2	3.0	17.6	2.0	11.7
	10.0	15.0	7.0	46.6	8.0	53.3	6.0	40.0	5.0	33.3

A: Doses (mg kg^{-1}), B: No. of examined fetuses, No.: Number of skeletal abnormalities

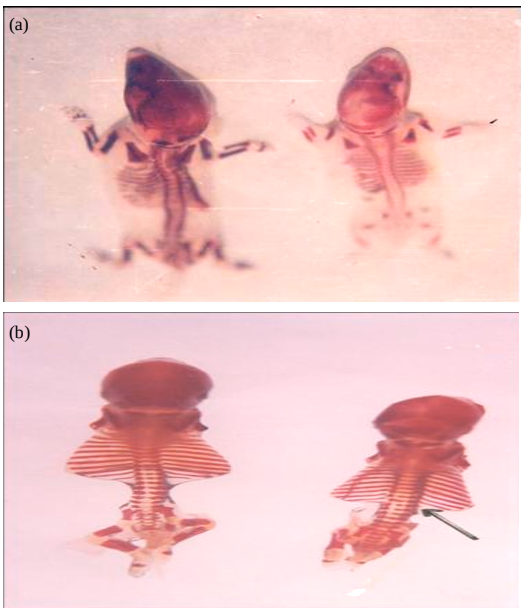

Fig. 3(a-b): (a) Incomplete ossification of skull bones and (b) Absence of the last rib in fetuses obtained from pregnant rats given orally chlorpyrifos at 10 mg kg^{-1} b.wt. from the 6 to the 15th day of gestation (Left: controls)

dilated cerebral ventricles and hypoplasia of the heart and lungs (Fig. 2b). The visceral malformations included hypoplasia of lungs in 6.6 and 30.0% and dilated renal pelvis in 15.5 and 20% of the examined fetuses for the small and large doses of chlorpyrifos, respectively (Table 2).

Table 4: Effect of chlorpyrifos on gestation index (GI), viability index (VI) and weaning index (WI) of suckling pups till the age of weaning

Groups	A	B	GI (%)	C	VI (%)	D	WI (%)
Control (Vehicle)	85.0	80.0	94.1	77.0	96.2	73.0	94.8
Chlorpyrifos (5.0 mg kg^{-1})	86.0	60.0	69.6	26.0	43.3	0.0	0.0
Chlorpyrifos (10.0 mg kg^{-1})	85.0	42.0	49.4	15.0	35.7	0.0	0.0

A: Total number of fetuses (viable+dead+resorbed) at birth, B: No. of viable fetuses at birth, C: No. of viable newborns till the 4th day of age, D: No. of viable newborns till the 21st day of age (weaning age)

Skeletal examination of fetuses: The percentages of skeletal malformations are complied in Table 3. The examined fetuses showed incomplete ossification of skull bones (Fig. 3a) and absence of the last rib (Fig. 3b). Additionally, the oral administration of chlorpyrifos caused absence of phalanges of forelimbs by 17.6 and 40.0% and some coccygeal vertebrae by 11.7 and 33.3%, of the examined fetuses, respectively.

Effect of chlorpyrifos on suckling pups: The effect on newborn pups whose their dams were given chlorpyrifos from the 15th day of gestation till the end of weaning age was presented in Table 4. The results denoted that chlorpyrifos decreased percents of the gestation and viability indexes of suckling pups. The weaning index of newborns was 0 vs. 94.8% in the control group. Neither visceral nor skeletal examinations were carried out for the pups born from dams because all newborn pups gradually died during lactation period and before the end of weaning age.

DISCUSSION

There has been a growing concern about the female reproductive toxicity and teratogenic risk of organophosphates because of the wide spread use of these compounds. The lipophilicity of these compounds allows the rapid crossing of placental barriers and

adversely reaches the mammalian fetuses (Harbison, 1975). It is well established that many organophosphates could produce some toxic and adverse effects on the fetuses and suckling newborns in a variety of experimental animals (WHO, 1995). Acute toxicity revealed that the LD_{50} of chlorpyrifos after its oral dosage was 155 mg kg^{-1} b.wt. The LD_{50} value varies according to species (mice, rats, rabbits), sex of experimental animals, the type of administered agent and the method (mathematical or logarithmic) used for the LD_{50} determination (Hayes, 1986). Our finding agrees with the preceding studies as they stated that the acute oral toxicity of chlorpyrifos is considered moderate, with acute oral LD_{50} values ranging from 85 to 155 mg kg^{-1} (McCollister *et al*., 1974; Uzun *et al*., 2010; Ambali *et al*., 2010). The observed toxic symptoms in female rats were depression, diarrhea, muscular tremors, convulsions, coma and death which occurred within 24-36 h post-administration. These symptoms were referred to inhibition of acetylcholinesterase enzyme and accumulation of acetylcholine in the synapse with subsequent signs of toxicity including autonomic dysfunction, involuntary movements and muscles fasciculation (Prope and Liu, 1997). In this respect, Breslin *et al*. (1996) found that chlorpyrifos at a small dose decreased plasma and erythrocyte cholinesterase and at a large dose decreased plasma, erythrocyte and brain cholinesterase levels. Furthermore, the most observed effect in animal and human studies after exposure to chlorpyrifos is the inhibition of the various types of acetylcholinesterase (Gibson *et al*., 1998; Clegg and Van Gemert, 1999).

Oral administration of chlorpyrifos in doses of 5 and 10 mg kg^{-1} b.wt to pregnant rats from the 6 to the 15th day of gestation caused early fetal resorptions and deaths. A marked decrease in the fetal body weights and mean crown rump lengths was also reported. These effects were found to be a dose dependant. These findings indicated that chlorpyrifos is embryotoxic and fetotoxic in rats. Concerning visceral and skeletal examinations of the fetuses, chlorpyrifos induced microcephaly; dilated cerebral ventricles and hypoplasia of heart and lungs. The fetal skeletal abnormalities were incomplete ossification of skull bones, absence of the last rib; and some coccygeal vertebrae. Similar findings have been reported on mouse (Deacan *et al*., 1980; Tian *et al*., 2005) and rat (Muto *et al*., 1992; Jackson *et al*., 1999; Farag *et al*., 2003). These reports support the interpretation that chlorpyrifos is embryotoxic and teratogenic. Additionally, the results of a toxicokinetic study by Hunter *et al*. (1999) suggested that the fetal nervous system may be exposed to a higher concentration of chlorpyrifos than the maternal nervous system when the dam was orally exposed to chlorpyrifos during late gestation. Fortunately, the toxicological database for chlorpyrifos indicates that humans are not more sensitive than the laboratory animals to its toxic effects (Cochran, 2002). The embryotoxic, fetotoxic and teratogenic effects of chlorpyrifos may be attributed to its direct cytotoxic action on the embryo and/or the fetus during gestation period.

Oral administration of chlorpyrifos at doses of 5 and 10 mg kg^{-1} b.wt. to bred rats from the 15th day of gestation to the end of weaning age decreased the numbers and weights of the newborns. Deaths of all newborns were observed at the 15 and 17th day post-parturition. The retarded growth of newborns whose their dams exposed to organophosphate compounds was attributed to an alteration of the activity milk lipase enzyme with a diminished secretary function in the mammary gland leading to interference with the nursing of the offsprings (Kalow and Warton, 1961). Similar results were reported by Carlton *et al*. (1987) who mentioned that oral administration of tricresyl phosphate (organophosphate insecticide) in doses of 100, 200 or 400 mg kg^{-1} b.wt. throughout gestation and lactation periods s ignificantly decreased the litter size and viability and survival rates of pups. Additionally, a decrease in the litter size, proportion and weights of live newborns was attained in Swiss CD mice fed on a diet containing tricresyl phosphate (Chapin *et al*., 1988). The toxic effect of chlorpyrifos on suckling pups could be explained by the greater susceptibility of newborns than adult rats to chlorpyrifos or its metabolites secreted in milk during lactation. In rat suckling pups, Mansour and Mossa (2010) suggested that the transfer of chlorpyrifos through the mother's milk has resulted in oxidative stress as well as some biochemical histopathological alterations.

CONCLUSION

It could be concluded that oral administration of chlorpyrifos to pregnant rats causes embryotoxic, fetotoxic and teratogenic effects as well as deaths of all suckling pups before the end of weaning period. Therefore, exposure to chlorpyrifos during both pregnancy and lactation periods should be prohibited to avoid its teratogenic action and lethal effect on fetuses and newly born pups.

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