

*Full Length Research Paper*

# Evaluation of the effects of gestational exposure to chlorpyrifos on implantation and neonatal mice

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Accepted 23 March, 2022

Exposure to some organophosphate (OP) compounds during pregnancy has been associated with adverse health consequence on both the mother and the offspring. The aim of the study was to evaluate the effect of exposure to chlorpyrifos (CPF) during gestation on implantation and on some neonatal parameters in mice. Twenty one virgin Swiss albino mice divided into 3 groups of 7 mice each served as subjects for this study. Mice in group 1 were dosed with corn oil (control), while those in groups 2 and 3 were exposed to CPF at a dose of 15.9 mg/kg (~15% LD<sub>50</sub>) and 21.2 mg/kg (~20% LD<sub>50</sub>), respectively. All the dams were dosed between gestation days (GD) 6 - 15 and monitored for signs of toxicity and gestation length. At birth, the litter size and weight, and the anogenital distance of the pups were measured. The pups were evaluated for physical characteristics and death. The dams were sacrificed at postnatal day 22, and the uterine horns evaluated for number of implantation sites. The results showed a significant decrease in the litter size and weight, and anogenital distance in pups exposed to CPF *in utero* compared to the control. In addition, all the pups prenatally exposed to CPF were born weak and died few days postpartum. A dose-dependent increase in percentage post-implantation loss was observed in mice dosed with CPF. In conclusion, exposure of pregnant mice to CPF caused increased gestation length, and postimplantation loss, decreased litter size and weight, survivability and anogenital distance.

**Key words:** Chlorpyrifos, implantation, gestation length, anogenital distance, litter size, weight, mice.

## INTRODUCTION

Experimental studies have suggested that animals exposed to pesticides have a greater risk of adverse reproductive outcomes, including embryonic and foetal death (Hayes and Laws, 1991). Epidemiologic studies have suggested associations between organophosphate (OP) exposure and reproductive disorders such as infertility, birth defects, adverse pregnancy outcomes (spontaneous abortions and foetal death) and perinatal mortality (Baldi et al., 1998; Sanborn et al., 2002; Recio et al., 2005). A previous study has established a positive association between occupational exposure to pesticide during early pregnancy and the risk of still births (Pastore et al., 1997). *Significant increase in cases of miscarriages has been observed* in a Polish study following agricultural pesticide exposure (Pastore et al., 2001), while maternal

residential proximity to pesticide applications was found to increase the risk of late foetal death due to congenital anomalies in California (Bell et al., 2001). Several pesticides have been considered endocrine disruptors because of their capacity to block or activate hormone receptors and/or to affect sex hormone levels (Vinggaard et al., 2000). OP still remains one of the most widely used insecticides, accounting for 50% of global insecticidal use (Cassida and Quistad, 2004). OPs are suspected to alter reproductive function by reducing brain acetylcholinesterase (AChE) activity and secondarily influencing the gonads (Recio et al., 2005).

Chlorpyrifos is an OP pesticide commonly used in various part of the world including Nigeria to control many types of insect pests in a wide range of crops and ornamentals. It is also used to control flies, mosquitos and household pests (Sultatos et al., 1982; Richardson and Wing, 1998). Despite the restriction of some of its domestic and agricultural uses by the

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United State Environmental Protection Agency (US EPA) in 2000, CPF still remains one of the most widely used OP insecticides. Because of its wide-spread agricultural and domestic uses, the present study is aimed at evaluating the effect of sublethal exposure of CPF to pregnant mice on the pregnancy status (post-implantation loss and gestation length) and the effect of prenatal exposure on some developmental parameters (litter size and weight, survival and anogenital distance) in neonates.

## MATERIALS AND METHODS

### Chlorpyrifos acquisition and preparation

Chlorpyrifos (Termicot<sup>®</sup>, Sabero Organics Gujarat Ltd. India), a 20% emulsifiable concentrate was obtained from a reputable commercial agrochemical store in Kaduna. They were reconstituted in corn oil as a 1% solution.

### Experimental animals

Twenty one 14 weeks old virgin female Swiss albino mice weighing 26 - 28 g were obtained from the Animal house of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria. They were kept in metal cages in the Teaching Laboratory of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria for a week prior to the commencement of the experiment for acclimatization. They were fed on pellets made from commercially purchased growers mash (Rebson Feeds Ltd, Zaria), maize bran (dussa) and groundnut cake in the ratio of 4:2:1 with wheat flour as binder. Fresh clean water was provided *ad libitum*.

### Experimental protocol

Twelve hours prior to the commencement of the study, the mice were selected at random into three groups of seven mice per group. The mice in each group were then allowed to cohabit with 3 proven fertile male mice. The mice were then checked for presence of copulatory plugs or spermatozoa in the vaginal orifice as evidence of mating, hence pregnancy. After mating the males were removed from the cages. The day when the vaginal plugs or spermatozoa were observed becomes gestation day (GD) 0. The animals were dosed with CPF starting from GD 6 to GD 15 (period of organogenesis), using the following protocol: Mice in group 1 were dosed with corn oil at a dose rate of 2 ml/kg. Mice in group 2 and 3 were dosed with reconstituted CPF at 15.9 (~15% LD<sub>50</sub>) and 21.2 mg/kg (~20% of LD<sub>50</sub>), respectively. The median lethal dose (LD<sub>50</sub>) of CPF (106.3 mg/kg) was obtained from a previous study (Ambali et al., 2007). The dams were monitored for clinical signs of toxicity and weighed daily until parturition to ascertain pregnancy status. At birth, the litter size and weight of pups, physical status and survival were evaluated. The anogenital distance of each pup was measured on post natal day (PND) 1. The dams were allowed to raise the pups up to weaning at PND 21. At PND 22, the dams were euthanized in a chloroform chamber, and the uterine horns were dissected, rinsed and kept in a phosphate-buffered saline in a Petri dish to prevent drying. The number of implantation sites was determined by staining the uterine horns with few drops of 10% ammonium sulfide solution for 10 min as described by Salewski (1964). Thereafter, the implantation sites appearing as dark rings were counted. The number of implantation sites in each dam was

then correlated with the litter size to determine if there had been post implantation loss, using the following formula (US EPA, 1996).

$$\% \text{ Post-implantation loss} = \frac{\text{No. of implantation sites} - \text{No. of litters}}{\text{No. of implantation sites}} \times 100$$

### Statistical analyses

Values obtained as mean  $\pm$  SEM were subjected to one-way analysis of variance (ANOVA) followed by Dunnett test using the Graph pad prism (www.graphpadprism.com). Values of  $P < 0.05$  were considered to be statistically significant.

## RESULTS

### Clinical signs

No clinical sign was observed in mice in the control group. The clinical signs observed in groups 2 and 3 exposed to CPF included hyperexcitability, tremor, arching of the back, piloerection, urination and respiratory distress.

### Effect of chlorpyrifos on gestation length

The gestation length for mice in group 2 was significantly longer ( $P < 0.05$ ) when compared to the control. On the other hand, the gestation length of mice in group 3 was not significantly different from the control, although it was slightly longer (Figure 1).

### Effect of chlorpyrifos exposure on litter size

There was a significant increase ( $P < 0.05$ ) in the litter size of mice in the control group compared to those groups exposed to CPF (Figure 2).

### Effect of chlorpyrifos exposure on litter weight

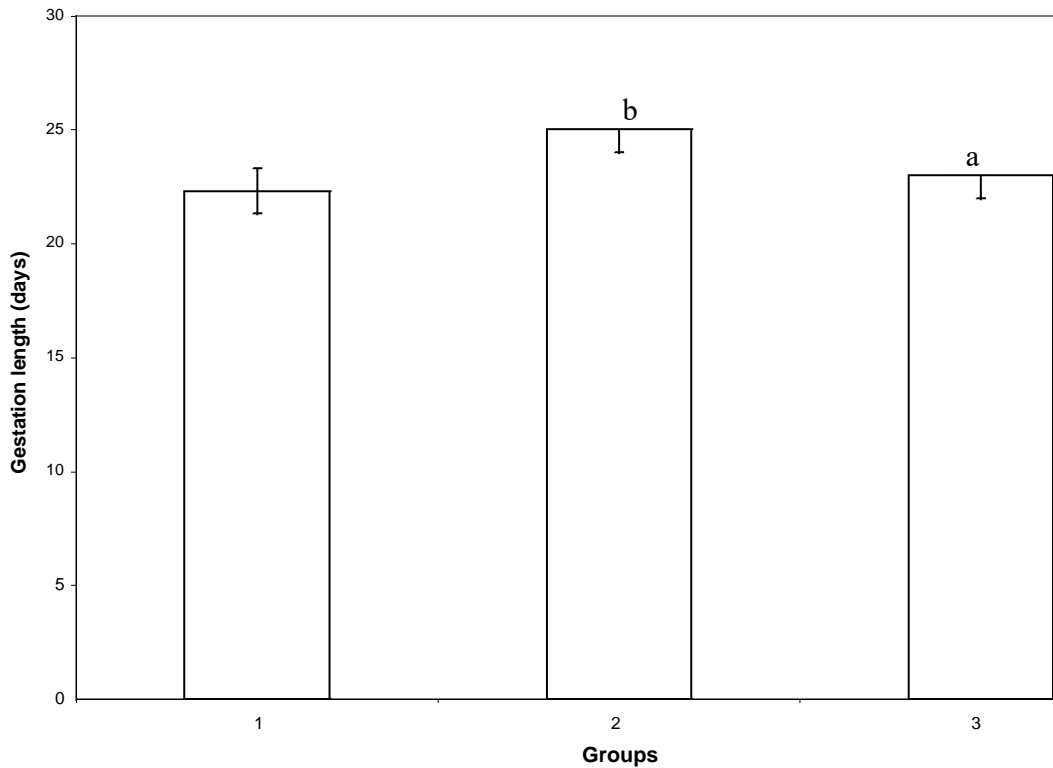
There was no significant change in the litter weight of pups between the control and those in group 3. However, the litter weight of pups in group 2 was significantly lower than those in the control (Figure 3).

### Effects on chlorpyrifos on pups' survival

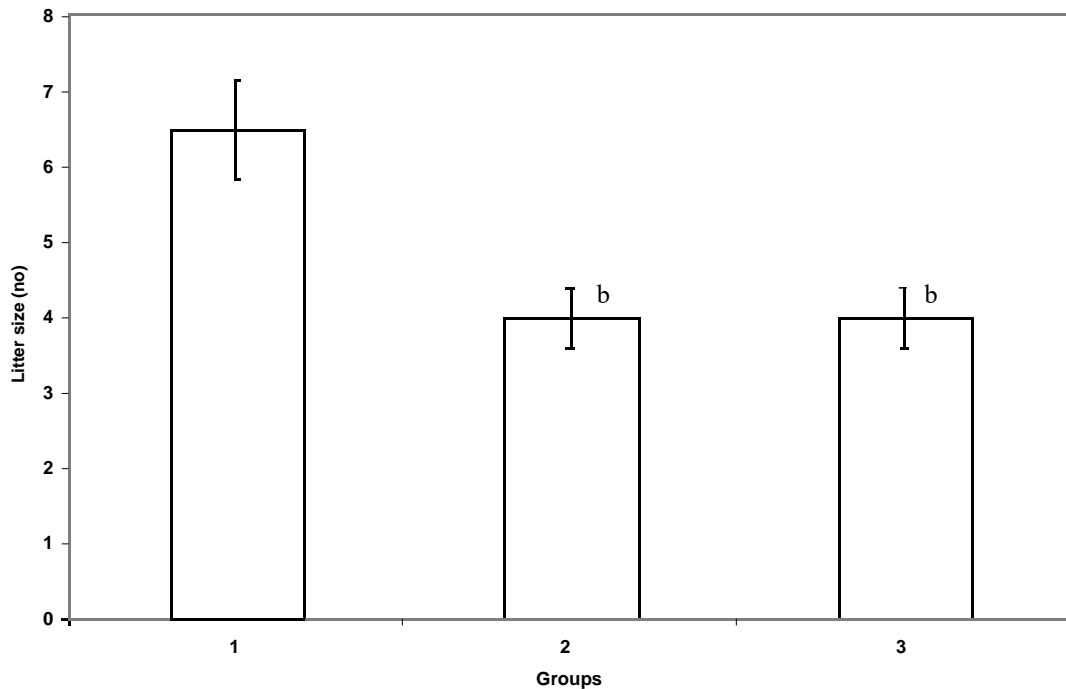
All the pups delivered by mice in groups 2 and 3 were born weak and died 2 - 3 days post delivery. On the other hand, pups from mice in the control group were stronger and they all survived.

### Effects of gestational exposure to chlorpyrifos on anogenital distance

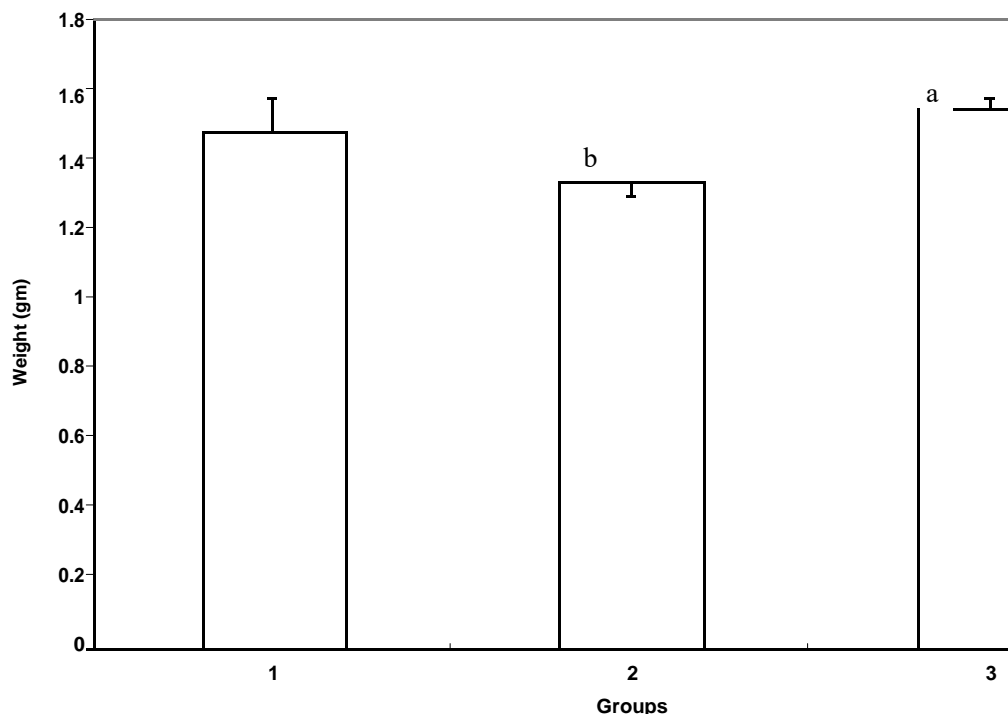
The anogenital distance of pups in the control group was not significantly different from those obtained in groups 2



**Figure 1.** Effects of *in utero* exposure to chlorpyrifos on gestation length: Group 1- mice in the control group exposed to corn oil only; group 2- mice exposed to CPF at a dose of 15.9 mg/kg (~15% LD<sub>50</sub>); group 3- mice exposed to CPF at a dose of 21.2 mg/kg (~20% of LD<sub>50</sub>); a= No significant difference compared to group 1 (control); b= significant difference (P < 0.05) compared to group 1 (control).



**Figure 2.** Effects of *in utero* exposure to chlorpyrifos on litter size: Group 1- mice in the control group exposed to corn oil only; group 2- mice exposed to CPF at a dose of 15.9 mg/kg (~15% LD<sub>50</sub>); group 3- mice exposed to CPF at a dose of 21.2 mg/kg (~20% of LD<sub>50</sub>); b= significant difference (P < 0.05) compared to group 1 (control).



**Figure 3.** Effects of *in utero* exposure to chlorpyrifos on litter weight: Group 1- mice in the control group exposed to corn oil only; group 2- mice exposed to CPF at a dose of 15.9 mg/kg (~15% LD<sub>50</sub>); group 3- mice exposed to CPF at a dose of 21.2 mg/kg (~20% of LD<sub>50</sub>); a= No significant difference compared to those in group 1; b= significant difference (P < 0.05) compared to those in group 1.

and 3. However, there was a comparative decrease in the anogenital distance of pups in the control group compared to those in groups 2 and 3 (Figure 4).

#### Effects of gestational exposure to chlorpyrifos on post implantation loss

The number of implantation sites observed in the uterus of mice was significantly higher (P < 0.01) in the control group compared to those in group 2. There was no significant difference in the number of implantation sites in the control group compared to those in group 3. There was no significant change in the number of implantation sites and litter size in both the control and group 2, though in the latter group, there was a marginal but non-significant reduction in the litter size compared to number of implantation sites. However, a significant reduction (P < 0.05) in litter size compared to the number of implantation sites was observed in mice in group 2. Correlation of the implantation site with the litter size as shown in Figure 5 indicated that no post implantation loss was recorded from mice in the control group, while 14.9 and 33.3% post implantation losses were recorded in mice in groups 2 and 3, respectively.

#### DISCUSSION

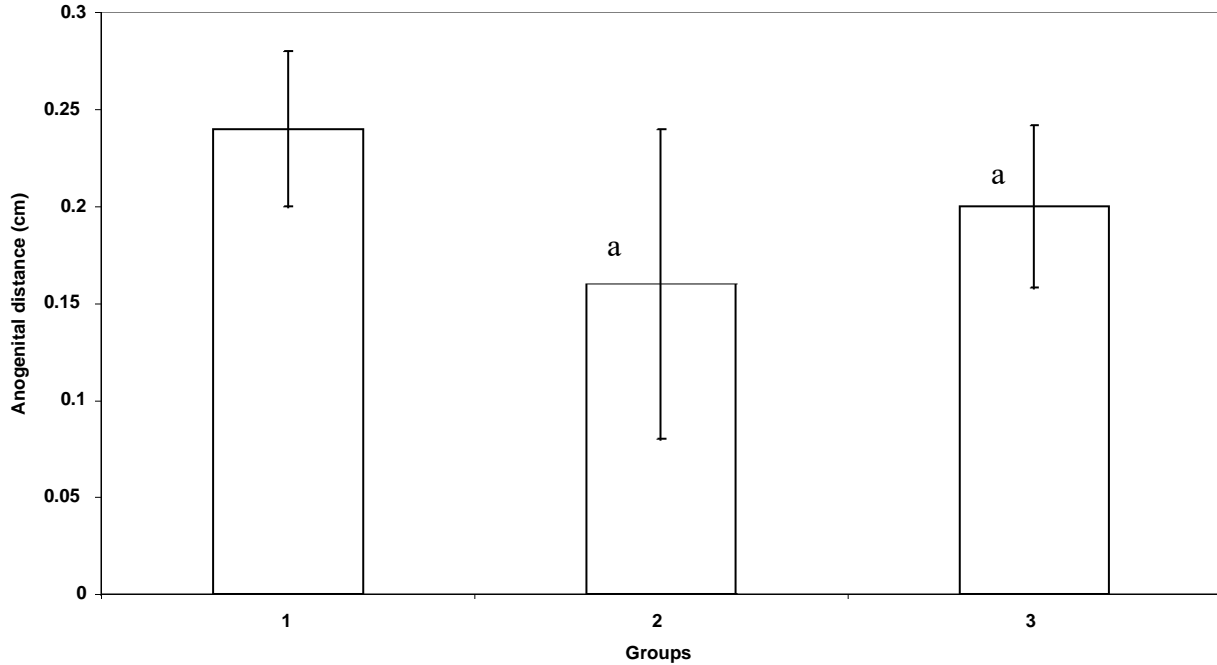
The clinical signs observed following maternal adminis-

tration of CPF were consistent with the cholinergic effects observed in OP poisoning. This results from the inhibition of AChE, an enzyme essential for the termination of the activity of acetylcholine.

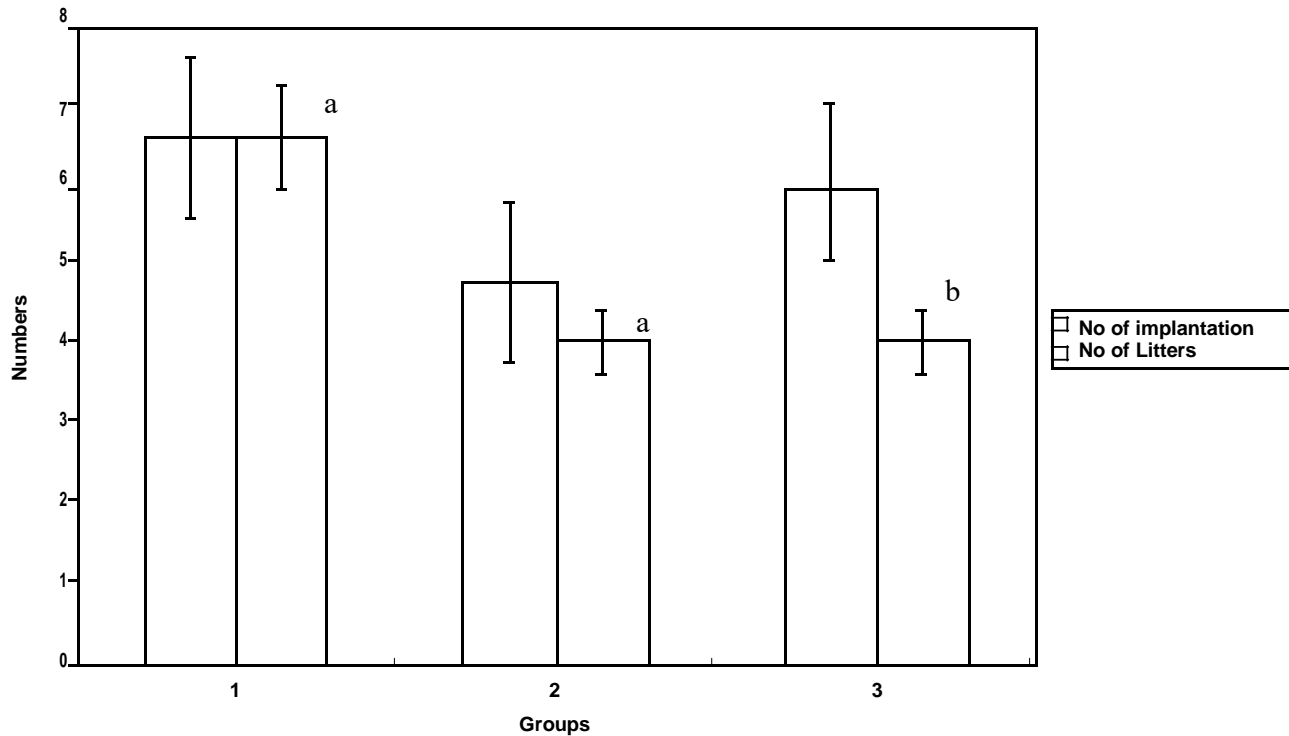
The study has shown that exposure of pregnant animals to CPF increases the gestation period. This was contrary to the findings of some other workers that gestational exposure to pesticides including CPF shortens the gestational length resulting in preterm delivery (Restrepo et al., 1990; Kristensen et al., 1997; Xiang et al., 2000; Dabrowski et al., 2003; Eskenazi et al., 2004). Some others studies have however found no association between gestational exposure to pesticides and duration of pregnancy (Grether et al., 1987; Fenster and Coye, 1990; Thomas et al., 1992). The inconsistency in CPF exposure and duration of pregnancy may be due to differences in their timing of exposure. The increased gestational length observed in this study may be due to CPF -induced changes in hormonal content of both the dam and the foetus, and the reduced foetal weight.

The present study has shown that gestational exposure to CPF causes reduction in the litter size. This is in agreement with the results obtained from a study by Gregory et al. (1993) where dermal exposure of pregnant dam to CPF resulted in the birth of fewer litters and litters with smaller pups. These results, however, contravened those by Deacon et al. (1980) and Oulette et al. (1983), which observed no effect on litter size in similar studies.

The present study has also shown that *in utero* expo-



**Figure 4.** The effect of *in utero* exposure to chlorpyrifos on anogenital distance: Group 1- mice in the control group exposed to corn oil only; group 2- mice exposed to CPF at a dose of 15.9 mg/kg (~15% LD<sub>50</sub>); group 3- mice exposed to CPF at a dose of 21.2 mg/kg (~20% of LD<sub>50</sub>); a= No significant difference compared to those in group 1.



**Figure 5.** Comparison of number of implantation sites to litter size following gestational exposure to chlorpyrifos: Group 1- mice in the control group exposed to corn oil only; group 2- mice exposed to CPF at a dose of 15.9 mg/kg (~15% LD<sub>50</sub>); group 3- mice exposed to CPF at a dose of 21.2 mg/kg (~20% of LD<sub>50</sub>); a= No significant difference compared to those in group 1; b= significant difference ( $P < 0.05$ ) compared to those in group 1.

sure of mice to CPF resulted in lower birth weight. This agreed with previous studies that showed that *in utero*

exposure to CPF causes reduction in the birth weight of pups (Deacon et al., 1980; California EPA, 1993). Simi-

larly, a study found an association between CPF exposure and low birth weight among African-American population (Perera et al., 2003). Whyatt et al. (2004, 2005) found an association between foetal length and weight and concentration of CPF and daimio in the cord of new borns in USA. On the other hand, Berkowitz et al. (2004) found no association between the urinary metabolite of CPF, 3,5,6-trichloro-2-pyridinol and birth weight. The low-birth weight observed in the present study may have been due to the induction of oxidative stress by CPF and alteration of the biochemical environment of the foetus arising from changes in the hormonal pattern of the dam.

The pups exposed *in utero* to CPF have been shown by this study to have lower chances of survival as all the pups were born weak and died as newborns. This is in agreement with other earlier studies (Deacon et al., 1980; California EPA, 1993; Gregory et al., 1993). This effect may be due to the effect of CPF on the liver, where it impedes the activity of ATPase, an important enzyme in cellular respiration (Sakai, 1990). In addition, chlorpyrifos-oxon has been shown to inhibit cholesterol ester hydrolyase, an enzyme important in promoting normal response to stress (Civen, 1977). Inhibition of this enzyme eliminates one of the mechanisms of response of the pups to CPF-induced stress.

Results obtained from this study also showed that *in utero* exposure to CPF caused a decrease in the anogenital distance of pups. This may indicate the anti-androgenic effect of CPF. Anti-androgens are chemicals that interfere with the normal function of testosterone and similar male hormones. Exposure to such chemicals, particularly during periods of vulnerability prenatally, can result in feminization of males (Tamura et al., 2001). In rodents, perineal growth is dihydrotestosterone dependent (Bowman et al., 2003), and males have a greater anogenital distance than the females (Marty et al., 2003). Although the mechanisms of anti-androgenic effect of CPF have not been elucidated, fenitrothion, an OP insecticide has been shown to bind avidly to the androgen receptor in cell culture, and blocks androgen receptor activity (Tamura et al., 2001). The developing fetus is extremely sensitive to the hormonal environment in the uterus, and natural differences in hormone levels surrounding rat or mouse fetuses of only 10 - 12 M has been shown to influence the timing of sexual development and the behaviour of the animal in adult life (Tyler et al., 1998).

The postimplantation losses observed in groups exposed to CPF were apparently due to foetal death and resorption. Sebe et al. (2005) reported that prenatal exposure to CPF in humans causes foetal death. The CPF-induced post-implantation losses recorded in mice exposed to CPF in the present study may have been due to *in utero* exposure of the pups to the chemical. It may have also resulted in alteration of the maternal hormonal levels either from the effect of CPF on the CNS, suppressing the brain release of gonadotropins, apparently due to AChE activity (Lyons, 1999). This alteration may result

in changes of the uterine biochemical content, hence alteration in intrauterine environment, leading to foetal death and resorption. Furthermore, CPF at low doses has also been shown to induce oxidative stress by increasing lipid peroxidation (Gultekin et al., 2001; Goel et al., 2006; Ambali et al., 2007; Verma, 2007), which may have altered the uterine environment through direct effect on the uterus and indirectly through its activity on the brain (Ambali, 2009), particularly the hypothalamus and the pituitary gland. This is complicated by the fact that pregnancy, due to the mitochondrial-rich placenta is a condition that favours oxidative stress (Myatt and Cui, 2004). The post-implantation losses observed in the groups administered with CPF in the present study were dose dependent. Pesticides have been shown to pass through the blood-brain barrier and placenta and have also been found in amniotic fluid (Bradman et al., 2003) indicating direct contact with the foetus. Recent studies have shown that fetuses and neonates have lower than-adult levels of detoxifying enzymes (chlorpyrifosoxonase) that deactivate OPs (Furlong et al., 2006; Holland et al., 2006) thus suggesting that they may be more vulnerable to these exposures. The uncondusive intrauterine environment resulting from alteration in hormonal levels, increased oxidative stress and the direct effect of CPF on the foetus may have contributed to the increased gestation length and reduced litter size and weight observed in CPF-exposed animals in the present study.

In conclusion, the present study has shown the ability of CPF to increase the gestation length and post-implantation losses, reduce the litter size, weight and survivability and anogenital distance. Therefore, measures targeted towards decreasing contact of pregnant animals or humans with CPF should be put in place in order to minimize the risk of adverse effects associated with *in utero* exposure to CPF on neonates.

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