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Full Length Research Paper

Fetal neurohistopathology of chloropyrifos in mice

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Chlorpyrifos (CPF) was tested for feto-neuro-histopathological manifestations on fetal central nervous system (CNS) in mice at 3 maternally sub-toxic oral doses - 0, 9 and 18 mg/kg. Each dose group was further categorized as: single (gestation day (GD) 6) and triple exposures (9, and 12 respectively). Fetuses were exteriorized on GD18. No obvious signs of toxicity were seen in the dams at these exposures. Mean fetal weight showed a dose (9 and 18 mg/kg) and exposure (single and triple) dependent decrease compared to that of the 0 mg/kg group while the litter size remained unaffected. The neurohistopathological abnormalities include vacuolations of the medullary region along with cortical lesions in CNS in 9 and 18 mg/kg groups on triple exposure only. These neurohistopathological manifestations were considered as the indicatives of neuroglial cells necrosis apoptosis. Our findings suggest that gestational exposure of CPF at motherly safe dose levels in mice induce neuroglial cells apoptosis in fetal CNS.

Key words: Chlorpyrifos, neuroteratology, neurotoxicity, neurohistopathology.

INTRODUCTION

Chlorpyrifos (CPF) [*O*, *O*-diethyl-*O*-(3, 5, 6-trichloro-2-pyridyl) - phosphorothioate], an extensively used organo-phosphate (OP) insecticide, was tested for its effects on litter size, fetal weight and neurohistopahological abnormalities in developing mice.

Although, due to its exposure risks in children, use of CPF in domestic sector was restricted in 2000 in US (EPA, 2000), it is still being used extensively worldwide (Tian et al., 2005; Dowagro 2006) especially in developing countries like Pakistan. Metabolism of CPF, *in vivo,* releases CPF-oxon- a very potent cholinesterase inhibitor (Cometa et al., 2007). Timchalk et al. (2006) demonstrated age-dependent variations in capability to detoxify environmental chemicals both in humans and animals. Thus, it is not surprising to see that young animals are more sensitive to OP insecticides exposure than adults (Pope and Liu, 1997; Moser and Padilla, 1998). This greater sensitivity to the toxic effects of OP insecticides in young animals has been related to their partial incompetence in detoxification (Mortensen et al., 1998; Atterberry et al., 1997; Vidair, 2004).

An *in vitro* study showed that OP insecticides and their oxons particularly target astroglial cell proliferation (Guizzetti et al., 2005); thus, it seems logical to speculate that OP insecticides like CPF may accumulate and inflict severe toxicity during the prenatal development and early postnatal life than adulthood. Thus, repeated sub-toxic low dose exposures of CPF particularly target developing brain during the critical period of cell division causing abnormalities at cellular and synaptic levels (Whitney et al., 1995).

Most of the animal studies conducted so far have been restricted to the analysis of the feto-morphic teratology of CPF exposure, typically from the stand point of dose response model (Farag et al., 2003; Tian et al., 2005; Akhtar et al., 2006). There is a dearth of available literature reporting the *in vivo* neurohistopathology caused by low dose CPF exposure in fetal life. In present study, we investigated the fetal neurohistopathological

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Abbreviations: CPF, Chlorpyrifos; **CNS,** central nervous system; **GD,** gestation day.

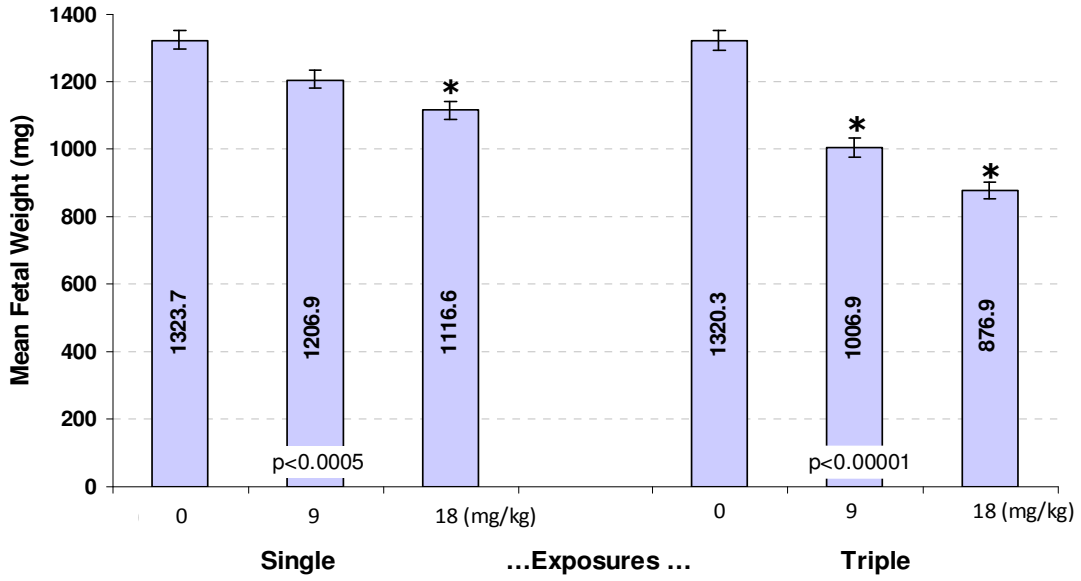

Figure 1. Effect of chlorpyrifos on mean fetal weight {p values: level of significance at two exposure levels; ± bars indicate standard error of the means and * indicate post hoc significant difference from control (p< 0.05).

manifestations of CPF at the maternally sub-toxic doses of exposure.

MATERIALS AND METHODS

Animals

A total of 90 pregnant females, of the Swiss Webster strain of albino laboratory mice *Mus musculus* were used in this research work. They were kept in 12" × 18" × 12" wire-mesh covered steel cages under 12 h light-dark cycles. Room temperature was maintained at 23±2°C. Food and water were provided *ad-libitum*. Males were caged with estrus females for 2 to 3 h. Thereafter, the dams were observed for the presence of vaginal plug as a confirmatory sign for successful coitus. Each mated female was parted from the male and the day of coitus was marked as day 0 of gestation.

Experimental groups and dose regimen

Bred females were randomly divided into 3 groups of 30 animals each, named as 0, 9 and 18 mg/kg groups. Each group was further divided into 2 subgroups of 15 animals each; respectively named as single [exposure on GD6] and multiple [exposure on GD6, 9, 12] exposure subgroups. The dose dilutions of CPF were made in corn oil. The animals in 0 mg/kg received pure corn oil in every treatment. The doses were delivered orally to the experimental animals by gavage.

Recovery and processing of the fetuses

Gravid uteri were exteriorized through a median incision on the abdominal wall from the euthanized dams on GD18. Fetuses were carefully removed, weighed and fixed in Bouin's solution for 48 h and finally transferred in 70% alcohol for storage and further study. Mean litter size and fetal weights / litter were obtained for each group and analyzed statistically.

Histology

Thirty randomly selected fetuses (one from each litter) of the three experimental groups were processed for wax embedding and microtomy. The serial sections (5 µ thick) were stained with Haematoxylin and Eosin for the neurohistopathological studies. Digital photomicrographs (40× and 100×) of the selected sections were obtained to report histopathological signs.

RESULTS

Mean litter size and fetal weight

There were no significant variations in mean litter size among the experimental groups. Mean fetal weight showed a dose (9 and 18 mg/kg) ($p< 0.0005$) and exposure (single and triple) related significant ($p< 0.0001$) decrease. Post-hoc analysis of the data indicate a significant ($p< 0.05$) difference between 0 and 18 mg/kg groups at single exposure and 0 mg/kg group to that of the 9 and 18 mg/kg groups at triple exposure (Figure 1).

Neurohistopathology

Vacuolations in the medullary region (an indication of neuroglial cells apoptosis) and cortical lesions(indicative of the damaged tracts),in CNS were seen in 8/15 fetuses

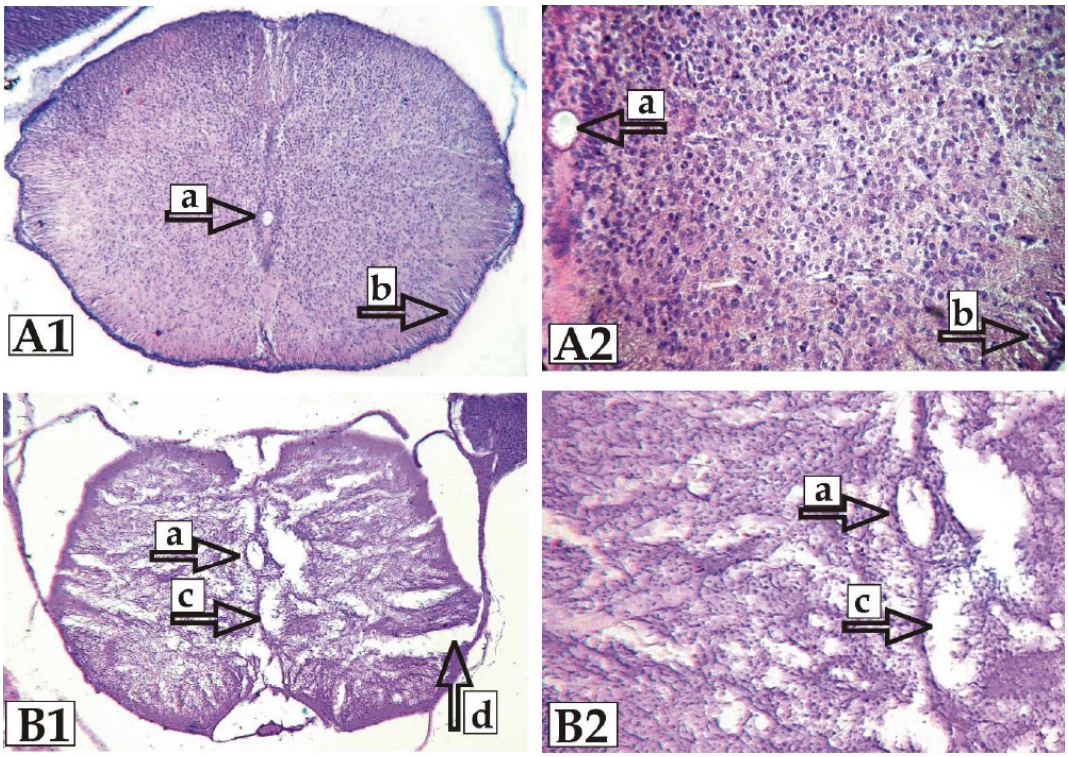

Figure 2. A1: selected section through the spinal chord of the fetus in 0 mg/kg triple exposure group (40×); A2: a closer look of the selected portion of A1 (100×); B1: selected section through the spinal chord of the fetus in 18mg/kg triple exposure group (40×); B2: a closer look of the selected portion of B1 (100×); a: neurocoele; b: cortical tracts; c:medullary vacuolations; d: cortical lesions.

processed from 18 mg/kg group at triple exposure; whereas 3/15 fetuses from 9 mg/kg group at triple exposure showed these signs. In 9 and 18 mg/kg single exposure sub-groups and 0 mg/kg group, no signs of medullary vacuolations or cortical lesions were seen (Figure 2).

DISCUSSION

Chlorpyrifos is a very potent neurotoxic agent (Ehrich et al., 2004); in rats, the gestational exposure to CPF causes persistent inhibition of brain acetylcholinesterase and suppression of choline-acetyltransferase (Richardson and Chambers, 2003). Results obtained from rat embryo cultures indicated that it particularly targets brain development (Roy et al., 1998). It has also been claimed that CPF promotes neurobehavioral abnormalities throughout the embryonic and neonatal development (Roy et al., 2005). The histopathological outcomes of prenatal CPF exposure obtained in this study are well in line with these developmental neurotoxicological studies.

Comparative analysis of the mean fetal weight in 0 mg/kg group to that of the 9 and 18 mg/kg groups at single and triple exposures clearly indicates that *in utero* CPF exposure caused dose and exposure-dependent growth retardation. In this context, it was reported earlier that levels of CPF in umbilical cord plasma are inversely proportional to the neonatal length and birth weight in humans (Whyatt et al., 2004).

The ability of CPF to interfere in the critical cellular processes such as cell division (mediated through inhibition of DNA replication), differentiation, gene expression and regulation are now well documented (Qiao et al., 2001). Whitney et al. (1995) pointed out that CPF inhibit DNA synthesis within a few hours of systemic administration to neonatal rats. Similarly, Samarawickrema et al. (2008) concluded that low dose chronic exposure to CPF leads to an increased oxidative stress and high DNA fragmentation in the fetuses. Moreover, CPF has been claimed to be a proven neurotoxic agent in young animals as it alters the replication and differentiation of neurons and bring about alterations in synaptic transmission independent to that of cholinergic stimulation. Induced oxidative stress of the insecticide exposure seems to play a key role in these neurotoxic outcomes (Saulsbury et al., 2009). Recently, it has been reported that CPF can induce apoptosis in well differentiated cells like human T cells (Li et al., 2009). Continued administration of CPF to neonatal rats is found to elicit a shortfall in cell numbers throughout the brain (Slotkin, 1999). Chlorpyrifos has also been found to interfere in the development of axonal projections thus logically affecting cell differentiation (Das and Barone,

1999; Li and Casida, 1998). The most critical effects involve the ability of CPF to interfere with the function of nuclear transcription factors that control cellular fate, including their expression, phosphorylation and capacity to bind on their DNA promoter recognition sites (Crumpton et al., 2000; Garcia et al., 2001; Schuh et al., 2002). Slotkin and Seidler (2007) have pointed out that CPF induces major transcriptional alternations in the genes controlling neural cell growth, development of glia and myelin, neural cell differentiation, cAMP-related cell signaling, oxidative stress, excitotoxicity and apoptosis. In the same context, Buratti et al. (2006) suggested that organophosphorothionate pesticides such as CPF induced neuro-developmental effects due to their *in situ* bioactivation by fetal enzymes. Caughlan et al. (2004) reports that independently from its proven neurotoxic effects mediated through inhibition of cholinesterase, CPF exposure may lead to apoptosis in the nervous system. Vacuolations, the medullary wide hollow spaces and the cortical lesions in the present study are respectively considered as the signs of the neuro-glial cells apoptosis and axono-dendronal/synaptotoxicity (Figure 2). These observations provide an *in vivo* indication of the similar previous findings (Buratti et al., 2006; Caughlan et al., 2004; Das and Barone, 1999; Li and Casida, 1998; Slotkin and Seidler, 2007; Samarawickrema et al., 2008; Saulsbury et al., 2009; Slotkin, 1999; Whitney et al., 1995).

The neurohistopathological defects observed in this study seem to be the logical outcomes of the afore cited broad range of developmental toxic effects of CPF. Chlorpyrifos and particularly CPF-oxon (produced by partial metabolism in maternal liver) readily cross the placental and blood-brain barriers (Parran et al., 2005). The developing fetal brains are more vulnerable and deficient in detoxification of these chemicals (Vidair, 2004; Timchalk et al., 2006). Thus, during the course of co-gestational triple exposures, CPF and CPF-oxon might have stayed longer in the fetal tissues, particularly brain (Guizzetti et al., 2005; Whitney et al., 1995), thereby causing huge oxidative stress. This oxidative stress must have been the most logical cause of apoptosis of the neuroglial cells (Franco et al., 2009; Verma et al., 2007; Yu et al., 2008). In this connection, an in-depth study of the estimation of CPF and CPF-oxon concentrations in the developing fetal brains is recommended.

Conclusion

On the basis of the results obtained in this study, it is concluded that the developmental exposure of CPF brings about several neurohistopathological changes; while the dose and exposure (single and triple) dependent decrease in the fetal weight is seemingly the out comes of the general growth retardation induced by the gestational CPF exposure.

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