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MORPHOLOGICAL AND PHYSIOLOGICAL ABNORMALITIES DURING DEVELOPMENT IN ZEBRAFISH DUE TO CHLORPYRIFOS

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ABSTRACT

Chlorpyrifos, a widely used organophosphate pesticide in agriculture and urban house hold has been receiving growing environmental concern due to its different biological activities. In this study we made an attempt to examine the potential developmental effects of non-lethal concentration of chlorpyrifos using zebrafish (*Danio rerio*) model. Fertilized eggs of the same developmental stage, 4 hours post fertilization were exposed to four different concentrations of chlorpyrifos namely 400 µg/L, 600 µg/L, 800 µg/L and 1000 µg/L. Hatching rate, morphological abnormalities were observed using Olympus microscope. Delay in hatching was noticed at higher concentrations of chlorpyrifos. Abnormalities like edema, difference in yolk sac size and decrease in pigmentation were observed in embryos before hatching, where as in larvae edema, shrinking of yolk sac and dorsal curvature of the body was noticed. From the studies carried out chlorpyrifos was shown to delay hatching and also caused different morphological abnormalities. These two could be due to slower utilization of yolk.

KEYWORDS : Chlorpyrifos, Zebrafish, Morphological Abnormalities, Vitellogenin Utilization

Chlorpyrifos (O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), a broad spectrum organophosphate pesticide has been one of the most widely used insecticide in both developing as well as industrialized countries for a variety of agricultural and public health applications. As such Chlorpyrifos (CP) was detected in various food products like spinach and rice (Ndengerio-Ndossi and Carm, 2005), Okra and egg plant (Baig et al., 2009) and green vegetables (Kobayashi et al., 2011) in different countries. Different concentrations of chlorpyrifos was also detected in water, sediments and suspended particles collected from Horqueta and Brown streams of Argentina (Jergentz et al., 2005), Laguna de Terminos, Mexico (Carvalho et al., 2009), Caspian Sea, Iran (Rahmanikhah et al., 2011), Lake Naivasha, Kenya (Otieno et al., 2012), Paddy field water samples, Bangladesh (Bhattacharjee et al., 2012), New Dqamietta drainage canal and EI-Embaby drain, Egypt (Malhat and Nasr, 2011). Fish kill an incident in association with CP in water reaching several hundred ppb was also reported earlier (Abdel Halim et al., 2006).

In India CP residues were also detected in water samples (0.003-0.006 µl/L) collected from Kaithal and Pant Nagar areas (Mukherjee and Arora, 2011), at measurable levels in breast milk of nursing mothers (Sanghi et al., 2003), in 16% and 20% of the made tea samples of Dooars and Hill regions, W.Bengal respectively (Bishnu et al., 2009) and in tissues of fish (88.6 µg/g) collected from Kolleru Lake in Andhra Pradesh, the state where the present study was carried out (Amaraneni and Pillala, 2001). The extent of CP residues found in different parts of India and the world in different systems has prompted us to undertake the assessment of ecotoxicological influence of CP.

Developmental exposure to different concentrations of CP was shown to cause significant spatial discrimination impairments, response latency, reduction in swimming activity and impaired learning (Levin et al., 2003, 2004, Eddins et al., 2010, Tilton et al., 2011), persisting behavioral effects in adult zebrafish like decreased habituation, decreased escape dividing response, increased swimming activity and lower learning rate (Sledge et al., 2011). It was also shown to be highly toxic to fish olfactory system (Sandahl et al., 2004, Tierney et al., 2007a), interfere with Hsp70 functioning, histopathology of organs (Scheil et al., 2010) and metabolic enzymes (Yang et al., 2011) during early developmental stages.

Widespread exposure of children to different organophosphorus (OP) compounds was also noticed earlier (Barr et al., 2004; Curl et al., 2003; Davis and Ahmed, 1988; Whyatt and Barr, 2001). Low-level OP exposure has been linked to behavioral and cognitive problems in infants and school-aged children (Kofman et al., 2006; Lizardi et al., 2008; Rosas and Eskenazi, 2008). In comparison to adults children have much lower levels of essential enzymes needed to break down OPs (Furlong et al., 2006). Thus exposure to OPs during critical periods of

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development can have severe long term neurobehavioral. In rats also it was reported to be involved in multiple mechanisms causing genotoxicity, hepatic dysfunction etc., (Poet et al., 2003; Mehta et al., 2008).

Zebrafish (*Danio rerio*) was used as a model in our study for investigation of toxicity induced by CP exposure at the early stages of embryonic development. The zebrafish embryo has emerged as an important model of vertebrate development as the embryos are optically transparent during early life stages, has clear chorion developed externally, which eliminates maternal toxicity as a confounding factor. Also this fish has high fecundity and its organogenesis occurs rapidly (Mc Grath and Li, 2008). As such it has found utility in screening of different toxic chemicals that inhibit or interfere with developmental processes (Hill et al., 2005; Scholz, 2008). Current study was conducted to determine whether there are any effects on hatching time, morphology and utilization of yolk for the process of development after early developmental exposure of 4 hours post fertilization (hpf) embryos to CP.

MATERIALS AND METHODS

Maintenance of Parental Fish

Wild type adult Zebrafish (*Danio rerio*) used in this study was bred in our aquarium facility for two generations. These were kept in aquaria filled with filtered tap water with the oxygen saturation of more than 80% and pH at 7.0 ± 0.3. The water temperature was maintained at 26 ± 1ºC at a 14h-10h light/dark cycle. Fish were fed with freshly hatched live brine shrimp (*Artemia nauplii*) once a day, supplemented with vitamin rich dried flake food once a day. The aquarium water was aerated continuously with stone diffusers connected to mechanical air compressor. Renewal of water was done daily by siphoning out 80% of water and refilling with fresh water and the aquaria screens were also cleaned daily.

Zebrafish Egg Collection

Eggs were collected from breeding stock of healthy, unexposed mature male and female zebrafish which were above the age of six months. The spawning glass trays covered with a fine nylon net with an appropriate mesh size for eggs to fall through were placed in the aquaria on the evening before the collection of eggs was required. Plant imitations made of plastic serving as spawning substrate were fastened to the nylon mesh. The fish were left undisturbed over night. Eggs were spawned synchronously at dawn of the next morning. After the lights were turned on the next morning embryos were generated by natural mating and then collected within 30 minutes after spawning. Fertilized eggs were collected from the spawning trays and they were rinsed several times with filtered tap water and their quality was checked under the microscope to select the healthy fertilized eggs for the experiment. Unfertilized eggs were identified by their milky white color and discarded. The dead appear white because of the coagulation and precipitation of proteins.

Chlorpyrifos: Procurement and Preparation of Stock Solution

Technical grade Chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate - 99% pure) was obtained from M/S Supelco, (Cat. No: PS - 418) USA. Stock solution was prepared by dissolving 50 mg chlorpyrifos in 5ml acetone. This is stored at 4°C and from this daily requirements are taken.

Experimental Design

Fertilized eggs at the same developmental stage 4 hpf were collected and exposure experiments were carried out by placing 100 eggs in 500 ml of filtered tap water in glass chambers. 400 µg/L, 600 µg/L, 800 µg/L and 1000 µg/L concentration of chlorpyrifos was added and stirred for uniform distribution of the toxicant. Controls were maintained with only acetone added to water. All exposure experiments were carried out in triplicate. The toxicant was added everyday to maintain exact concentration. Embryos and larvae were observed after 24 hpf, 48 hpf, 72 hpf and 96 hpf of exposure under a stereomicroscope (Magnus MLX) for mortality, hatching and morphological/ developmental abnormalities. The magnification used for observation was 10X and 4X for eggs and larvae respectively. Thirty eggs from the above experiment were frozen in liquid nitrogen after 24 hpf and 48 hpf to study the Vitellogenin cleavage pattern during development.

RESULTS

Mortality and Hatching

Mortality/Survival was observed after exposing

the fertilized eggs to different concentrations of CP upto 96 hpf (Figure 1). This was done by counting live/dead eggs/larvae in each of the exposure chambers. CP caused a dose related increase in mortality with significant death of embryos at a chemical threshold of 800 µg/L and 1000 µg/L and time threshold of 72 hpf and 96 hpf. No mortality was observed at 24 hpf stage in the control as well as in all the exposed groups. At 48 hpf stage there was no mortality in the control group and in 400 µg/L and 600 µg/L exposure groups (Figure 1). But there was 4% and 8% mortality in 800 µg/L and 1000 µg/L exposure groups respectively. 2%, 9%, 20% and 25% mortality was observed in the embryos exposed to 400 µg/L, 600 µg/L, 800 µg/L and 1000 µg/L after 72 hpf of exposure but there was no mortality in their controls.

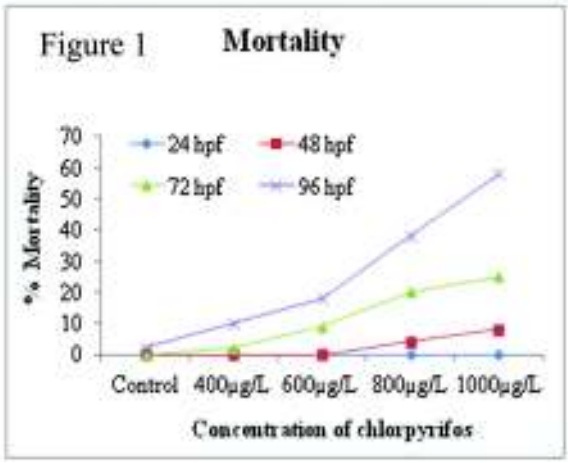

Figure 1. Graph Indicates Mortality at Different Time Periods After Exposing Fertilized Eggs to Different Concentrations of Chlorpyrifos.

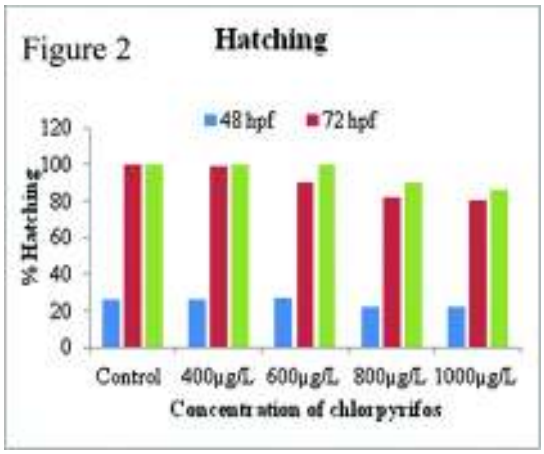

Figure 2. Percent Hatching Observed at Different Time Periods After Exposing Fertilized Eggs to Different Concentrations of Chlorpyrifos.

With regard to hatching at 48 hpf, 26% of embryos came out of their chorions in controls and 400 µg/L exposure groups, with 27% in 600 µg/L and 22% 800 µg/L and 1000 µg/L groups (Figure 2). After 72 hpf hatching percentage was 100% in controls and 400 µg/L groups with 90%, 82% and 80% in 600 µg/L, 800 µg/L and 1000 µg/L groups (Figure 2). All the fertilized eggs of control group hatched after 96 hpf exhibiting 2% mortality. All fertilized eggs completely hatched in 400 µg/L and 600 µg/L exposure groups after 96 hpf with mortality of 10% and 18% respectively. Whereas percentage of hatching in 800 µg/L and 1000 µg/L exposure groups was 90% and 86% with mortality 38% and 58% respectively.

Developmental abnormalities

Morphological abnormalities like yolk sac edema and less embryonic movements was noticed from 24 hpf onwards at higher concentration of CP i.e. 800 µg/L and 1000 µg/L (Figure 3-24 hpf D and E). At 48 hpf embryos exposed to all four concentrations of CP exhibited difference in body pigmentation and yolk sac edema was

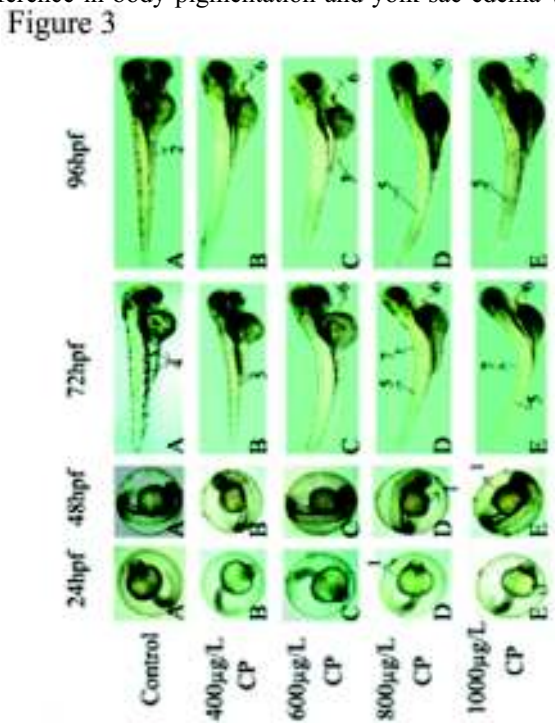

Figure 3. Developing Embryos of Zebrafish After 24 hpf, 48 hpf, 72 hpf and 96 hpf of Exposure to Different Concentrations of Chlorpyrifos (cp).

observed at 800 µg/L and 1000 µg/L CP (Figure 3-48 hpf D and E). Body pigmentation decreased with increased concentration of CP.

Abnormalities in hatched larvae at 72 hpf include elongated pectoral fin buds and edema in 400 µg/L treated group. Yolk sac started to shrink making the pericardial cavity more conspicuous and severe edema all around the body accompanied with growth retardation at other three concentrations namely 600 µg/L, 800 µg/L and 1000 µg/L. In these groups dorsal curvature of the body was also noticed. Pericardial edema started to increase with increase in concentration of CP in 72 hpf and 96 hpf groups (Figure 3-72 hpf D and E, 3-96 hpf D and E). In experimental groups at all stages decreased eye and body pigmentation and reduction in heart beats was noticed (Figure 3). With regarding to touch response, the larvae responded in 600 µg/L treated group normally but did not swim as long as the controls. Whereas the larvae elicited very little movement at concentrations of 800 µg/L and 1000 µg/L CP.

DISCUSSION

From the results of the present investigation, we provide information which demonstrate that CP in vivo interfered with normal development and functioning of zebrafish. Observation of mortality has shown a dose dependent increase. Different degrees of mortality of Zebrafish embryos was reported earlier when exposed to azacel (Ahmad and Ansari, 2011), colloidal silver and gold nanoparticles (Ilan et al., 2009), perfluorooctanesulfonate (Shi et al., 2008), Hexabromocyclododecane (Deng et al., 2009) and Arsenic (Li et al., 2009). The differences in susceptibility can be ascribed to the difference in the ontogenetic stage of the eggs and permeability of different chemicals through chorion.

Hatching, which is a critical period of embryogenesis, has been critically used as endpoint in fish early life stage test. Basically it classified into two types mechanical and enzymatic hatching. During normal hatching process, the chorion is digested by the hatching enzyme, chorionase secreted from the hatching gland cells of the embryo which in turn accumulated in the perivitelline space, reaches the chorion and induces its breakdown releasing the free-living larva. Delay in hatching especially at higher concentration of CP was clearly noticed indicating that CP could be inhibiting the release of chorionase/osmotic disturbances interfering with hatching enzyme activity. It was shown by Fan and Shi (2002) that the structure and function of the protease might be destroyed by toxicants and might block pore canals of the chorions, resulting in the shortage of oxygen supply to the development of embryos. Another reason for delay or failure to hatch may be due to developmental abnormalities observed in the present study (Figure 3), which may limit the developing embryos to mechanically break the outer chorion. Delay/Inhibition of hatching of zebrafish embryos was reported earlier due to SWCNTs (Cheng et al., 2007), perfluorooctanesulfonate (Shi et al., 2008), celastrol (Wang et al., 2010), chitosan nanoparticals (Hu et al., 2011), difenoconazole (Mu et al., 2013), nanomaterials (nC60), emodin (He et al., 2012), celastrol (Wang et al., 2010b), β-diketone antibiotics (Wang et al., 2013), genistein (Kim et al., 2009), metals like copper, lead, mercury and nickel (Dave and Xiu, 1991). And this delay in hatching could also be a strategy employed to prolong the residence of the embryo within the egg in response to adverse environmental conditions.

Exposure of fertilized eggs/embryos to CP resulted in dose dependent malformations during development. Development is a critically sensitive period where changes in environmental conditions can alter the normal programme of embryogenesis (Gilbert, 2001). Fish embryogenesis is sensitive to environmental factors including temperature, pH, nutrient levels, or chemicals such as pesticides. In our study also zebrafish embryogenesis was found to be sensitive to CP and in this the most affected part by CP was yolk sac, pericardium. Edema of yolk sac was noticed from 24 hpf (Figure 3-24 hpf D and E) and this yolk sac started to shrink after hatching making the pericardial cavity more conspicuous (Figure 3-72 hpf A). In fish eggs, the endogenous lipid reserves, mainly phospholipids and triglycerols are in the form of globules (Wiegand, 1996). Alteration of lipid synthesis and metabolism may cause yolk sac effect. Abnormalities relating to yolk in zebrafish like yolk sac edema, delayed yolk sac absorption, yolk syncytium, pericardial edema and embryonic malobsorption syndrome were observed in

zebrafish exposed to variety of compounds namely bifenthrin (Jin et. al., 2009), chitosan nanoparticals (Hu et al., 2011), difenoconazole (Mu et al., 2013), emodin (He et al., 2012), celastrol (Wang et al., 2010b), genistein (Kim et al., 2009), soxitocin (Lefebvre et al., 2004), cadmium and methyl mercury (Cheng et al., 2000; Yang, 2010).

The most notable malformation after hatching was dorsal curvature of the body with bending of the notochord, which is more pronounced at higher concentration of CP. The notochord is an axial structure common to the chordata phylum. In lower chordates and in larval stages of lower vertebrates it plays an important role as a structural element required for locomotion and coordinated movement. The notochord is also required for proper difference of adjoining tissues like nueroectoderm, muscle and vertebral elements in all vertebrates. Therefore, the primary axial structure upon which many other tissues depend for their proper formation and differentiation. It was shown by (Behra et al. 2002), that AChE place important role in the arrangement and maintenance of muscle cell integrity in older embryos and larvae. It is also known that AChE is required for neural and muscular developmental in zebrafish. Hence the abnormalities observed in the present study relating to dorsal curvature of the body along with bending of notochord may be due to inhibition of AChE, which was reported by same OP compound used in the present study i.e. CP. Literature shows example of notochord malformations in zebrafish, following early life stage exposure to varies toxic compounds such as dithiocarbamate (Haendel et al., 2004), perfluorooctanesulfonate (Shi et al., 2008), chitosan nanoparticles (Hu et al., 2011), trichlorfon (Coelho et al., 2011).

Kienle et al. (2009) used CP concentration of 0.25 & 0.5 mg and have shown unnatural bending of spine. It another concentration of 1µM CP Richendrfer et al. (2012) have shown curling of tails at much higher concentration of 1mg/L skeletal deformities were also reported (selderslaghs, 2010). Other OP compounds which were responsible for different abnormalities were dichlorvos (Sisman, 2010), malathion (Chemotti et al., 2006), trichlorfon (Coelho et al., 2011).

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