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ADVERSE EFFECTS OF CHLORPYRIFOS PESTICIDE IN AQUATIC ANIMALS ESPECIALLY IN DANIO RERIO

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ABSTRACT

Presence of pesticide residues in water or soil creates many biological, morphological and physiological abnormalities in the growth and development of some aquatic animals. Chlorpyrifos, an organophosphate pesticide in agriculture and urban house hold has been receiving growing environmental concern due to its abnormal biological activities. An attempt has been made to examine the potential developmental effects of non-lethal concentration of chlorpyrifos using zebra fish (*Danio rerio*) as an example. Different concentrations of chlorpyrifos i.e., 400 µg/L, 600 µg/L, 800 µg/L and 1000 µg/L were exposed to the fertilizesd eggs as well as adult Zebra fish and morphological, physiological abnormalities were observed and delay in hatching, 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. The suggestion made can bring awareness of usage of synthetic pesticides.

KEYWORDS: Chlorpyrifos, Zebra fish, Morphological Abnormalities, Vitellogenin Utilization.

INTRODUCTION

Usage of pesticides/ insecticide for agricultural yield in both developing as well as industrialized countries for a variety of agricultural and public health applications has increasingly occurred. Especially after the green revolution. Chlorpyrifos (O, O-diethyl O-

3,5,6-trichloro-2- pyr idyl phosphorothioate) (CP), one of the most widely used organophosphate pesticide, was detected in various food products like spinach, rice[1], Okra, egg plant[2] and green vegetables[3] in different countries.

Different concentrations of chlorpyrifos was also detected in water, sediments and suspended particles collected from Horqueta and Brown streams of Argentina[4], Laguna de Terminos, Mexico[5], Caspian Sea, Iran[6], Lake Naivasha, Kenya[7], Paddy field water samples, Bangladesh[8]. Fish kill an incident in association with CP in water reaching several hundred ppb was also reported earlier[9].

In India CP residues were also detected in water samples (0.003-0.006 µl/L) collected from Kaithal and Pant Nagar areas[10] and in tissues of fish (88.6 µg/g) collected from Kolleru Lake in Andhra Pradesh[11]. Exposure of different concentrations of CP was shown to cause some abnormalities in aquatic animals in general and fish in particular, like response latency, reduction in swimming activity and impaired learning[12-14], highly toxic to fish olfactory system[15], interfere with Hsp70 functioning, histopathology of organs[16] and metabolic enzymes[17] etc.,

Widespread exposure of children to different organophosphorus (OP) compounds was also noticed, low-level OP exposure has been linked to behavioral and cognitive problems in infants and school-aged children[18,19]. In rats also it was reported to be involved in multiple mechanisms causing genotoxicity, hepatic disfunction[20,21] etc.,

As per the recommendations of the International Organization for standardization and the organization for Economic Cooperation and Development (OECD) there is a significant genetic similarity between *Danio rerio* and humans, the abnormalities observed by chlorpyrifos exposure may be relevant for interpreting its impact on humans. So the present study was carried out by taking Zebrafish (Danio rerio) as a model to investigate the toxicity induced by CP exposure at the early stages of embryonic development and 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. Because this fish has high fecundity and its organogenesis occurs rapidly.[22]

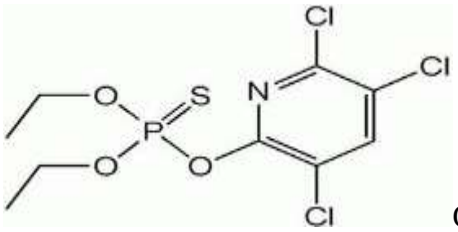
MATERIALS AND METHODS

Reagent and solutions

All chemicals used were of analytical-reagent grade of the highest purity available procured from Merck. Doubly distilled de-ionized water was used throughout the experiment. Glass vessels were cleaned soaking in acidified solutions of $K_2Cr_2O_7$ followed by washing with con. HNO_3 and were rinsed several times with high purity deionized water.

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.



Chemical structure of chlorpyrifos

Identification of Vitellogenin Cleavage Pattern in Zebrafish Embryos Exposed to Different Concentrations of Chlorpyrifos

Collection of fertilized eggs was done as mentioned in the first experiment i.e., fertilized eggs were collected from the spawning trays and embryos were rinsed several times with tap water and their quality was checked under the microscope being sure to select the healthy fertilized eggs for the experiment. Thirty fertilised eggs from control, 400, 600, 800 and 1000µg/L chlorpyrifos at 24 and 48hpf were collected for protein estimation and SDS PAGE analysis.

4.5.1. Protein Sample Preparation

Homogenisation of the frozen embryos or larvae was done in 250µl lysis buffer [8 M Urea, 2 % CHAPS, 0.5 % v/v IPG-Buffer 3-10 linear (GE-Healthcare, Uppsala, Sweden), 1 % (v/v) protease inhibitor cocktail (SIGMA, Taufkirchen, Germany. 30 embryos were homogenized using a disposable plastic pestle for 5 minutes.

The homogenates were centrifuged at 4 °C for 30 min at 9000 x g (SIGMA 2K15 Zentrifuge, Osterode, Germany). Total protein concentration in the supernatant was measured in

accordance to Lowry et al. (1995). Subsequently, protein samples were immediately used for SDS PAGE.

(a) Acrylamide solution (30 %)

100ml of acrylamide solution was prepared by dissolving 30gm acrylamide, 0.8gm N, N-methylene-bis-acrylamide in 70ml of distilled water. The contents were then filtered and the solution was finally adjusted to 100ml.

(b) Stacking gel buffer for SDS-PAGE

7.879gm of Tris was dissolved in the 75ml of double distilled water and the pH of the solution was adjusted to 6.8 using 1N HCl and finaly the volume of the buffer was made up to 100ml with distilled water.

(c) Running gel buffer for SDS-PAGE

23.638gm of Tris was dissolved in 75ml of double distilled water and the pH of the solution was adjusted to 8.8 using 1N HCl. Finally the volume of the buffer solution was made up to 100 ml using distilled water.

(d) Sample loading buffer for SDS-PAGE

1.2ml of 100 mM Tris (pH 6.8), 2ml of 4 % SDS, 1ml of 20 % glycerol, 0.5ml of β-mercaptoethanol, 0.001g of bromophenol blue were taken in to a 10ml reagent bottle, mixed well and volume was made up to 10ml and solution was stored at 4°C. When necessary 80µl of loading buffer was taken.

(e) Staining solution for SDS-PAGE

0.2gm coomassie brilliant blue was dissolved in 30 ml of methanol. To this 10 ml of acetic acid was added and finally the volume was made up to 100ml using distilled water.

(f) Destaining solution

30ml of methanol, 10ml of glacial acetic acid were mixed and volume was made up to 100ml using distilled water.

(g) Protein Markers

Medium Range marker supplied by MERCK Specialities Pvt Ltd, Mumbai was used. Size of the protein markers includes 205, 97.4, 66.0, 43.0, 29.0, 20.1, 14.3 and 3.5 KDa.

Recommended Procedure[23]

Microscopy and SDS-polyacrylamide gel electrophoresis technique

Simple microscopy and SDS-polyacrylamide gel electrophoresis technique are used in the present study. Microscopic studies were carried out for observing various stages of tissues, embryo, larval stage, etc. The protein samples were separated by SDS-polyacrylamide gel electrophoresis following the procedures, 12.0% running acrylamide gel solution was prepared by mixing 4.0ml 30 % acrylamide solution, 2.5ml running buffer (1.5M Tris-Hcl, pH-8.8), 3.3ml distilled water, 100 µl of 10% SDS, 10 µl of TEMED and 100µl of freshly prepared 10 % ammonium persulphate. The solution was mixed well and degassed before adding ammonium persulphate. The contents were then poured in between two sealed glass plates containing 1.5 mm spacers to form a slab. These contents were over-layered with 0.1 ml water saturated n-butanol and allowed to polymerize for 40 minutes at room temperature. After polymerization of running gel, butanol was removed by repeated washing and the traces of water were removed by wiping with filter paper strips. The 5 % stacking acrylamide gel solution was prepared by taking 0.83ml of 30 % acrylamide solution, 0.63ml of stacking gel buffer (0.5 M Tris-Hcl, pH-6.8), 3.40ml of water, 50µl of 10 % SDS, 5µl of TEMED and 50µl ammonium persulphate. The stacking gel solution was carefully over layered on the running gel. Immediately a comb of required size was placed to form the wells. The stacking gel was allowed to polymerize for 30 minutes. After 30 minutes the comb was removed and the wells were washed with tank buffer (50mM Tris-HCl, 14.4gm glycine, 0.1%SDS, pH 8.5).

Protein samples were mixed with equal volumes of sample loading buffer (100 mM Tris-Hcl, pH-6.8, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 10% β mercaptoethanol) and kept in a boiling water bath for 3 minutes. The contents were then briefly centrifuged before loading the sample in to the wells. The electrophoresis was carried out at 4°c and run at 50 volts initially. The sample entered in to resolving gel the voltage was increased to 100 volts. Once tracking dye reached the anode, run was stopped and the gel was carefully removed and proceded for staining. Staining solution containing 0.2 % W/V coomassie brilliant blue in methanol: water: acetic acid (30:60:10) for 6-8 hrs. The gel was then de-stained in the destaining solution containing methanol: water: acetic acid (30: 60: 10) for another 6-8hrs.

RESULTS AND DISCUSSION

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^{-1}, 600 µg L^{-1}, 800 µg L^{-1} and 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.

Effects of Chlorpyrifos on Zebrafish

Mortality and Hatching

Mortality/Survival was observed after exposing the fertilized eggs to different concentrations of CP up to 96 hpf. 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. 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. With regard to hatching (a critical period of embryogenesis, embryonic exit from the eggshell- (chorions)) 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. 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. 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 of10%and18% respectively. Whereas percentage of hatching in 800 µg/L and 1000 µg/L exposure groups was 90% and 86% with mortality38%and58%respectively.

Interference of Heavy metals

Heavy metals are known to interfere with the activities of various enzymes. Significant increase in activity of superoxide dismutase (SOD) in zebrafish muscle was observed after 2-hour of naphthalene exposure and 4 hour of cadmium exposure. Another metal, copper at a concentration of 8 and 15µg/L resulted in significant increases in gene expression of cytochrome C oxidase sub unit 17 (COX-17) and catalase, in the gill and liver after 48h indicating possible alterations in cell oxidative capacity and normal mitochondrial biogenic processes, possibly through metallochaperones like COX-17 in both gill and liver. Short-time exposures (24 and 72h) to sublethal dose (500µg/L) of nickel and lead, reduced brain monoamine oxidase (MAO), in the brain of *Danio rerio*

DISCUSSION

Results of the present investigation reveals that CP in vivo interfered with normal development and functioning of zebrafish. Observation of mortality has shown 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.. 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, has been critically used as endpoint in fish early life stage test. During 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.

Exposure of fertilized eggs/embryos to CP resulted in dose dependent malformations during development. Fish embryogenesis is sensitive to environmental factors including temperature, pH, nutrient levels, or chemicals such as pesticides. The effect on CP In the study zebrafish embryogenesis was also found to be sensitive to CP.

CONCLUSION

The result of the present study gives information regarding the toxic effects of Chlorpyrifos on Zebrafish at various stages of development and at different environmental as well as chemical and developmental conditions. The effect of CP on zebrafish embryogenesis was also found to be sensitive to CP and in this the most affected part by CP was yolk sac, pericardium. Spawn of adult female zebrafish exposed to 0.02µg/l decis for four days have shown delay in oocyte maturation as observed by decreased number of oocyte with Germinal

vesicle Breakdown (GV IV). This suggests that exposure to pesticides like CP causes morphological and physiological abnormalities in the development of Zebra fish and this brings awareness among the public regarding the usage of pesticides and insecticides especially made of prepared by chemical synthesis can disturb biochemical cycles and badly affect the ecological balance due to which instinct of many species takes place.

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