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EXPRESSION OF GENES (*NTL, FLH, MOM* AND *DOC*) DURING THE DEVELOPMENT OF ZEBRAFISH UNDER CHLORPYRIFOS TOXICITY

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ABSTRACT

Chlorpyrifos is one of the most widely used organophosphate insecticides. It is effective against a broad spectrum of insect pests of economically important crops around the globe. This extreme usage has lead to detrimental effects on biological life. The aim of the present study was to determine the toxic effects of chlorpyrifos during early development in zebrafish. The embryo/larvae were exposed to 200µg/L and 1000µg/L of CP and observations were made at 24hpf, 48hpf and 72hpf. To understand the reasons for abnormalities four genes namely no tail (*ntl),* floating head *(flh),* momo *(mom)* and doc (*doc*) responsible for their role in development have been selected and analyzed by RT-PCR. We have shown induction in expression of all four genes. Of all the four genes induction of *flh* was more at 24hpf and doc was less at the same time. Comparing induction between the two treatment groups (200µg/L and 1000µg/L), higher expression was noticed at 1000µg/L for all genes except *flh* at 24hpf stage.

KEYWORDS: Chlorpyrifos, Zebrafish, no tail (*ntl),* floating head *(flh),* mom *(momo)* and doc (*doc*).

INTRODUCTION

Organophosphate (OP) compounds are used worldwide in agriculture and gardening to control insect pests. Among the OP compounds chlorpyrifos (CP) has been one of the most widely used throughout the world for control of a variety of agricultural pests[1] and is effective against a broad spectrum of insect pests of economically important crops. It is also used for the control of mosquitoes (larvae and adults), flies, termites and various soil and household pests. It has been used in agricultural and home formulations since the 1960s.[2]

Due to its extensive use, CP residues were detected in various food products like spinach and rice,[3] Okra and egg plant[4] and green vegetables[5] in different countries, water and sediments[6,7] in different parts of the world. In India also CP residues were detected in water samples (0.003-0.006µl/L) collected from Kaithal and Pant Nagar areas,[8] in 16% and 20% of the made tea samples of Doors and Hill regions, W. Bengal respectively,[9] breast milk of nursing mothers[10] and tissues of fish (88.6 µg/g) collected from Kolleru lake in Andhra Pradesh, the state where the present study was carried out.[11] In 2006 Abdel-Halim et al.,[12] have reported fish kill incidents in association with CP in water reaching several hundred ppb.

This insecticide, CP like other OPs inhibit acetylcholinesterase, which plays an important role in neurotransmission at cholinergic synapses. Number of other studies have shown that exposure to CP during development can cause persisting neurobehavioral dysfunction, even with low doses that do not elicit acute cholinergic toxicity.[13] Developmental exposures to different concentrations of CP were shown to cause significant spatial discrimination impairments, response latency and reduction in swimming activity and impaired learning.[14,15,16] It was also shown to interfere with Hsp70 functioning[17] and metabolic enzymes[18] during early developmental stages. In view of these deeper investigation into CP effects was needed. The present study attempts to determine the expression of four genes, *no tail* (*ntl), floating head (flh), momo* (*mom)* and *doc (doc)* involved in the formation of notochord after exposing 4hpf embryos to two concentrations of CP till 72hpf stage.

Zebrafish (*Danio rerio*), a small tropical fresh water fish has been chosen in the present study as it is a good investigational model for vertebrate embryogenesis and general development.[19] This animal model has great advantages like small size, easy maintenance, short breeding cycle, high fecundity, translucent embryos, and cheap cultivation. We have carried out few studies in our lab using CP. SDS-PAGE analysis of protein have profile shown alterations of seven bands of different molecular weights at different developmental stages (24hpf, 48hpf, 72hpf and 96hpf) in zebrafish.[20] We have

shown that several malformations like yolk sac and pericardial edema, dorsal curvature of the spine and decreased pigmentation in zebrafish.[21]

MATERILAS AND METHODS

Preparation of Stock Solution

Technical grade chlorpyrifos (99%) (O,O-diethylO-3,5,6-trichloro-2-pyridyl phosphorothioate) CAS-No: 5598-13-0 was obtained from Sigma Aldrich (USA). Stock Solution of chlorpyrifos (CP) was prepared by dissolving 50 mg of Chlorpyrifos in 5ml of Acetone. This was stored at 4ºC and from this daily requirements are taken.

Zebrafish egg collection

Fertilized eggs were collected from healthy unexposed male and female six months old zebrafish as described earlier.[22]

Embryo –larval toxicity treatment

To characterize toxic effects during development, zebrafish embryos of the same developmental stage (4hpf) were collected and rinsed with filtered water. Exposure experiments were carried out by placing 250 of these embryos in 500 ml of embryonic solution (NaCl 5mM, KCl 170µM, $CaCl_2$ 330µM, $MgSo_4$ 300µM). Embryos were exposed to two different concerntrations of chlorpyrifos namely 200 and 1000µg/L up to 72hpf. Solvent control embryos were maintained in embryonic medium with acetone and another set served as control (without solvent and toxicant). These were maintained at an ambient temperature of about 27±1°C in semi static conditions and fresh media were renewed daily to maintain even concentrations of chlorpyrifos. Occasional stirring was done to ensure even distribution of toxicant. Embryos/larvae were examined after 24, 48 and 72hpf for their health condition. Any dead embryo/larvae and detritus were removed daily at frequent intervals. Exposure experiments were carried out in triplicate.

RNA extraction and quantification

Total RNA was extracted from 100 embryos/larvae of 24hpf, 48hpf and 72hpf developmental stages of control and CP treated groups using TRI Reagent ® (Sigma). The protocol was followed as per manufacturer's instructions. RNA quality and quantities were assessed using the Nano Drop ND-2000 spectrophotometer (Thermo Scientifics) by measuring at A260/A280nm. This purified RNA was evaluated by Agarose Gel Electrophoresis.

cDNA Synthesis

Total RNA obtained was used for synthesizing DNA by first strand cDNA synthesis kit (Thermo Scientifics) according to the manufacturer's instruction. In brief 1µg of RNA sample was mixed with 1µl of oligo dT, 0.25µl of RNase inhibitor (10U/µl) and 10µl of DEPC water. The reaction mixture was kept at 65°C for 15 min and immediately chilled on ice. Then 4µl of 5x reaction buffer, 2µl of dNTP's (10mM), 1µl of Reverse Transcriptase enzyme (200U/µl) was added, mixed gently, centrifuged briefly and incubated at 37°C for 60 min. After incubation was completed, the reaction was terminated by heating at 70°C for min and immediately chilled on ice. Quantity and purity of cDNA were measured at A260/A280nm using Nano Drop ND- 2000 spectrophotometer (Thermo Scientifics). The obtained cDNA was confirmed by Agarose Gel electrophoresis and visualized using UV- transilluminator. cDNA efficiency was then normalized with RT-PCR.

Primer designing

In order to monitor PCR amplification with maximum specificity and efficiency, RT – PCR primers were designed using Primer 3 software. Criteria considered are: Melting temperature Tm > 25°C, GC content of 40 – 60%, Primer length 20 – 24 nucleotides and amplicon size of 100 – 200bp. Primer sequence for selected genes (Table 1).

RT-PCR (Reverse transcription Polymerase Chain Reaction) Analysis

In this study, 10 RNA samples were selected randomly with 10 samples each belonging to Control, 200 and 1000ug/L CP treatment group. RT-PCR analysis was carried out for *ntl*, *flh*, *momo* and *doc* as described by respectively. Reverse transcription of 1 µg of total RNA isolated was achieved by mixing the RNA with (10 µl of 2x PCR master mix), 1 µl of deoxynucleotides, 1 µl of oligodT, 0.25 µl RNAase inhibitor (10 U/µl), and 0.5 µl of Reverse Transcriptase (200 U/µl) in a 20 µl volume. This was followed by incubation of the mixture at 37 °C for 60 min, and then for 5 min at 95 °C. Four microliters of the RT product was taken and PCR was carried out in Applied Biosystem thermal cycler. The GAPDH primers served as control.

STATISTICAL ANALYSIS

Standard deviation and level of significance was calculated for data obtained from three replicates. Two-way ANOVA was carried out by using Graph Pad Prism software version 6.0.

RESULTS

We choose representative genes, which have two different functions. According to PCR studies these four genes were expressed as follows.

ntl showed significant increase of expression compared to control in 24hpf "Fig. 1" and 72hpf "Fig. 3" treated embryos in both exposure groups (200µg/L and 1000µg/L), but at 48hpf "Fig. 2" stage expression was not significant in 200µg/L treated embryos. In 200µg/L exposed embryos increased expression was more at 24hpf and almost same in 48hpf and 72hpf. Where as in 1000µg/L exposed group maximum expression was seen at 24hpf and 48hpf followed by 72hpf.

Expression of *flh* has increased significantly in CP treated embryos compared to control at two stages of

development 24hpf "Fig. 1" and 72hpf "Fig. 3" and no significant of increase in 200µg/L group at 48hpf "Fig. 2" stage. 200µg/L exposed embryos showed highest expression at 24hpf and then decreased slightly at 48hpf and increased in 72hpf over 48hpf. In 1000µg/L exposed embryos increased expression was almost same in all three exposed groups.

momo showed significant increase of expression compared to control in 24hpf and 72hpf in both treated groups (200µg/L and 1000µg/L) and no significant increase at 48hpf in 200µg/L treated embryos. In 200µg/L exposed embryos, increased expression was seen in 24hpf "Fig. 1" and then expression decreased at 48hpf "Fig. 2" and 72hpf "Fig. 3", but the level was more than control. Where as in 1000µg/L exposed group maximum expression was seen at 24hpf "Fig. 1" and decreased from then on with developmental stage.

Expression of *doc* was significantly higher in 24hpf and 72hpf (1000µg/L) but no significant of expression in 48hpf treated embryos in 200µg/L treated group. In 1000µg/L exposed group highest expression was noticed at 24hpf "Fig. 1" and 72hpf "Fig. 3" but expression was lower at 48hpf "Fig. 2". In 200µg/L exposed embryos also similar trend was noticed.

Among the four genes, increased expression of *flh* was maximum in 24hpf "Fig. 1" stage after exposure to 200µg/L and expression of *doc* was minimum at the same time. Induction of gene expression between the two treatment groups (200µg/L and 1000µg/L) showed higher expression at 1000µg/L of all genes except *flh* (24hpf).

Table I: Primers used for Reverse transcriptase – PCR analysis of the following genes.

Gene	Forward Primer	Reverse Primer	Anealing Temp	GC Content
ntl	AAGGAGGTTGCTGATCGTGG	CTCTGCACTCCAAGTCCCAT	54°C	20/20
flh	CGAAAGCAGCAGTTCATTCTC	CAGATGCCAACAGAAAGCGT	52°C	21/20
mom	CTGACCAAAACTGCCAGTGG	ATGTGCCTCTGAACACCGTA	54°C	20/20
doc	TCAGTGTGTGACGAGGACAA	TGCTGAAGTTCTTGGTCTGG	52°C	20/20

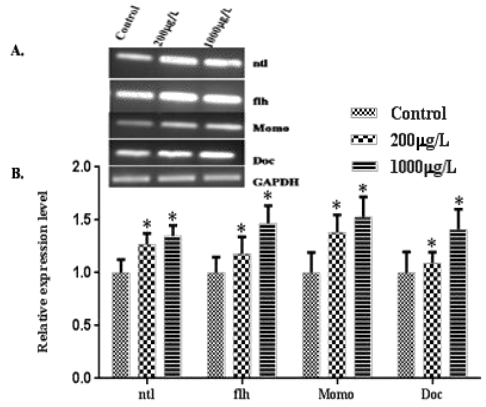

Figure 1: (A.) Embryo/larval samples of zebrafish after exposure to 200 and 1000µg/L of CP were analyzed by RT-PCR for expression of *ntl, flh, momo* and *doc* at 24hpf. (B.) Graph showing expression of four genes relative to GAPDH levels in 24hpf embryos of zebrafish (*Danio rerio*) exposed to 200 and 1000µg/L of Chlorpyrifos. Values are mean of embryos/larval samples (n = 100) ± SD. Decrement was statistically significant (P<0.05) over control when denoted with asterik (*).

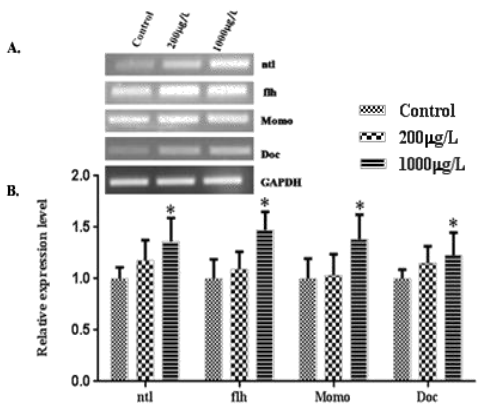

Figure 2: (A.) Embryo/larval samples of zebrafish after exposure to 200 and 1000µg/L of CP were analyzed by RT-PCR for expression of *ntl, flh, momo* and *doc* at 48hpf. (B.) Graph showing expression of four genes relative to GAPDH levels in 48hpf embryos of zebrafish (*Danio rerio*) exposed to 200 and 1000µg/L of Chlorpyrifos. Values are mean of embryos/larval samples (n = 100) ± SD. Decrement was statistically significant (P<0.05) over control when denoted with asterik (*).

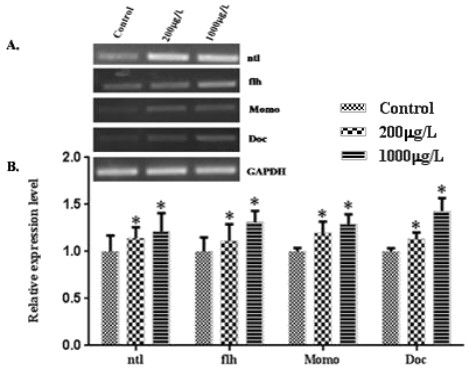

Figure 3: (A.) Embryo/larval samples of zebrafish after exposure to 200 and 1000µg/L of CP were analyzed by RT-PCR for expression of *ntl, flh, momo* and *doc* at 72hpf. (B.) Graph showing expression of four genes relative to GAPDH levels in 72hpf embryos of zebrafish (*Danio rerio*) exposed to 200 and 1000µg/L of Chlorpyrifos. Values are mean of embryos/larval samples (n = 100) ± SD. Decrement was statistically significant (P<0.05) over control when denoted with asterik (*).

DISCUSSION

Expression of four genes namely *ntl, flh momo* and *doc* were examined after treating 4hpf eggs with CP for three days. All four genes are known to be involved in the formation of notochord during development of zebrafish.[23,24,25,26] Notochord formation starts from 10.5hpf stage and is completed by the end of the day. For this reason this window of exposure was selected. Also studies from our lab have shown that CP has caused dorsal curvature of body.[21] In this study expression of all four genes at all the three stages of development i.e., 24hpf "Fig. 1", 48hpf "Fig. 2" and 72hpf "Fig. 3" after exposure to both (200 and 1000µg/L) concentrations of CP was enhanced. Very little research has been carried out showing the relationship between phenotypic abnormalities and pattern of expression of related genes.

Notochord is a defined structure of all chordates including zebrafish. This is an embryonic mid-line structure having two important functions. First, it produces secreted factors that signal to all surrounding tissues, providing position and fate information. Second, the notochord plays an important structural role, serving as axial skeleton of the embryo until other elements are formed. The notochord arises from the dorsal organizer. So the first major transition in the development of notochord is the formation of chorda mesoderm from dorsal organizer. It was shown that during early gastrula stages the chorda mesoderm becomes morphologically and molecularly distinct from other mesoderm. Genetic screens in zebrafish have identified two loci, floating head (*flh*) and bozozok (dharma-zebrafish; information network), as being essential for this transition to occur.[27,28,29,26]

Mutations in all four genes (*ntl, flh, momo* and *doc*) have resulted in embryos lacking muscle pioneer cells, which form following the induction of adaxial cells through signaling from the notochord and a horizontal myoseptum, which separates the somites into dorsal and a ventral part. Mutations in four genes were also known to affect the early specification of the notochord primordium.[30] They have further suggested that function for *doc* in the maintenance of *ntl* expression. Starting at 10 hours post fertilization (hpf), one pair of somites are formed every 20-30 minutes by formation of a new somatic furrow. Roughly 30 somite pairs form in a normal embryo; 7 above the yolk cell, 10 above the yolk extension and 13 posterior to the anus.

In zebrafish *ntl* and *flh* genes have been identified for notochord development. In our study, we observed that CP induced expression of *ntl*. Loss of function mutations in the *flh* gene was known to be the cause for lack of notochord along the entire length of the embryo, with somites in the trunk fused.[26] These somites defects could be due to an impairment of the specification of the adaxial cells during early stages of development as suggested by Odenthal et al.[31] They have also shown the expression of this gene in the notochord and floor plate of early zebrafish embryos. Expression studies suggested that cells lacking *flh* function can only differentiate into muscle but not notochord.[32] *ntl* along with its role in notochord development it also involved in tail formation.[33,34] The *ntl* gene is expressed in the germ ring of gastrulating embryos, in the developing notochord during somitogenesis (axial mesoderm) and in the prospective mesodermal cells of the tail bud.[35] Though *ntl* gene was induced under CP toxicity curvature tail is noticed and swimming behavior was also impaired.

In the zebrafish, the floor plate can be subdivided into single row of medial floor plate cells.[36,37] It has been shown that *ntl* and *doc* mutants have an undeveloped notochord in the trunk, and form somite lacking the myoseptum where as in embryos mutant for *ntl* or *doc* the floor plate is present. In case of *momo* mutants also notochord in the trunk was lacking.[30]

CONCLUSION

We have exposed 4hpf zebrafish embryos to two concentrations (200 and 1000 µg/L) of CP and analyzed genes which are responsible for notochord during development. In our study though we observed induction in expression of *flh* gene, still bending notochord was observed along with fusion of somites. This could be due to an impact on the translational machinery and also the mechanism operating between gene expression and formation of notochord/somites could be affected.

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