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PROTEIN PATTERN IN CHLORPYRIFOS TREATED ZEBRAFISH EMBRYOS

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ABSTRACT

Chlorpyrifos a broad spectrum product has been in use for many applications in home and agriculture against the damages caused by pests around the globe. This extreme usage has lead to detrimental effects on biological life. The goal 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, 400µg/L, 600µg/L, 800µg/L and 1000µg/L of CP and observations were made at 24hpf, 48hpf, 72hpf and 96hpf. Changes in the protein content was estimated and proteins were resolved by SDS-PAGE. The results indicate that chlorpyrifos evoked alterations in the protein content during development. SDS-PAGE gels have revealed seven bands at molecular weight marker at 205kDa, 97kDa and three bands between 43-29kDa, and 20.1kDa showed increase/decrease in their intensity at different developmental stages. These bands comprise of detoxifying enzymes, vitellogenins, nuclear, membrane, centrosomal, mitochondrial, cytosolic and heat shock proteins. From these results it can be said the CP causes modulations in protein profile.

INTRODUCTION

The organophosphorus (OP) compounds which are effective pesticides were developed at beginning of this century by chemical manipulation of normal gases. 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 (Lemus and Abdelghani, 2000). There is a growing concern about CP because of its proven toxicity. Early in this decade Abdel-Halim *et al.,* (2006) have reported fish kill incidents in association with CP in water reaching several hundred ppb. Added to this, CP residues were detected in various food products and vegetables (Baig *et al.,* 2009; Kobayashi *et al.,* 2011), water and sediments (Rahmanikhah *et al.,* 2011; Otieno *et al.,* 2012) in different parts of the world. In India also CP residues were detected in water samples (Mukherjee and Arora, 2011), breast milk of nursing mothers (Sanghi *et al.,* 2003) and tissues of fish (Amaraneni and Pillala, 2001). 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 (Sledge *et al.,* 2011).

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Alteration of neuronal development, synaptic stability and growth in rat and human neuronal cells was shown earlier due to CP (Howard *et al.,* 2005; Roegge *et al.,* 2008). It was also demonstrated 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. Zebrafish (*Danio rerio*), a small tropical fresh water fish has been selected in the present study is it is a good experimental model for vertebrate embryogenesis and general development (Hill *et al.,* 2005). Their embryos in particular have because important model as they are optically transparent during early life stages. The transparency of these embryos has allowed visualization of all developmental stages with great clarity. Also this fish has high fecundity and its organogenesis occurs rapidly; all vertebrate specific body features can be seen within two days. The eggs can be easily collected as it breeds almost all the year round and has a short generation time. Using the zebrafish embryos we have shown that CP elicit several malformations like yolk sac and pericardial edema, dorsal curvature of the spine and decreased pigmentation (Sreedevi *et al.,* 2014). In view of these alterations, deeper investigation into CP effects other than the well known AChE inhibition was needed. The present study attempts to determine the possible alterations brought about by CP on protein profile during early development of *Danio rerio*.

MATERIALS AND METHODS

Zebrafish maintenance and egg collection: Maintenance of wild type adult Zebrafish (*Danio rerio*) used in this study and collection of eggs from the breeding stock was given in detail in our earlier publication (Sreedevi *et al.,* 2014).

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 50mg 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 (4hpf) were collected and exposure experiments were carried out by placing 100 embryos/larvae in 500ml of filtered tap water in glass chambers. To this 200µg/L, 400µg/L, 600µg/L, 800µg/L and 1000µg/L concentration of chlorpyrifos was added and stirred for uniform distribution of the toxicant. Controls were also maintained. All exposure experiments were carried out in triplicate. The toxicant was added everyday to maintain exact concentration. After 24hr, 48hr, 72hr and 96hr of exposure, the embryos/larvae were frozen in liquid nitrogen and kept -80°C till used for analysis.

Sample preparation and estimation of proteins

The pellet was discarded and the concentration of protein was estimated in the supernatant according to Lowry *et al.,* (1951).

Sodium dodecyl sulphate Polyacrilamide gel electrophoresis (SDS-PAGE)

Separation of proteins on SDS-PAGE was carried out using 12.0% running acrylamide gel according to Laemmli (1970). Protein samples were mixed with equal volumes of sample loading buffer (50mM Tris-Cl (pH 6.8), 100mM dithiothreitol, 2% (w/v) SDS (electrophoresis grade), 0.1% bromophenol blue, 10% glycerol) and kept in a boiling water bath for 3 minutes. The amount of proteins loaded was 20µg for each sample. Wide range (3-205kDa) protein molecular weight marker was loaded in lane 1.

Statistical analysis: Mean, Standard deviation and level of significance was calculated for data obtained from three independent experiments. One-way ANOVA was carried out by Duncan's test using SPSS software version 16.0 and P value < 0.05 was considered statistically significant.

RESULTS

Estimation of total protein

The biochemical response of zebrafish embryos (*Danio rerio*) to CP was analyzed and presented in Table 1 and Fig 1. From these it is understood that the amount of protein was more at 24hpf and decreased progressively with development. At 24hpf stage protein concentration showed a steady percent decrease with increase in concentration of CP. At 48hpf stage increase in protein concentration was noticed when exposed to 200µg/L, 400µg/L and 1000µg/L, where as it was found to decrease compared to control after exposure to 600µg/L and 800µg/L at this stage. Same trend was also noticed at 72hpf and 96hpf stages. As noticed at 48hpf the protein content increased when exposed to 1000µg/L at 72 and 96hpf stages. At these two stages decreased protein content was observed in 400µg/L exposed group, but in 200µg/L exposed group a slight increase and decrease over control was noticed at 72hpf and 96hpf stages respectively. The data were analyzed statistically for their significant increase/decrease at $P < 0.05$ level. ANOVA test showed significant difference over control in all the concentrations selected at four different developmental stages. The exceptions to this were 400µg/L and 600µg/L groups of 48hpf stage and 800µg/L group of 96hpf stage.

Protein analysis by SDS-PAGE

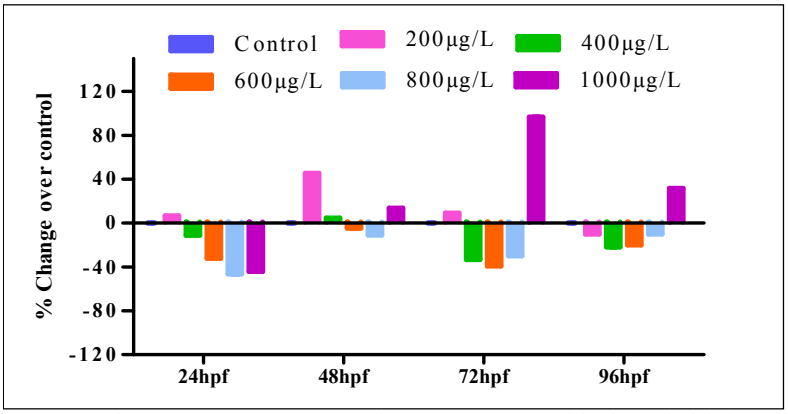
Homogenates of 24hpf, 48hpf, 72hpf and 96hpf embryos of control and five different concentrations of CP exposure groups were resolved through SDS-PAGE. Proteins were resolved on 12% gels with marker in the molecular weight range of 3-205kDa and are presented in Fig. 2 (A, B, C and D). In control 24hpf embryos, six prominent bands were observed. These are, one band each at 205kDa, 97kDa and 20.1kDa and three bands between 43-29kDa. Whereas at 48hpf and 72hpf stages only four prominent bands are noticed; one band each at 205kDa and 97kDa and two bands between 43-29kDa. Surprisingly at 96hpf stage six bands were noticed but these are different compared to six bands of 24hpf embryos. The bands noticed at 96hpf are, one each at 205kDa, 97kDa, 66kDa, 20.1kDa and two between 43-29kDa. The 205kDa and 97kDa bands noticed at all four developmental stages have shown decrease in the thickness with developmental progression. With regard to the 20.1kDa band, which was only noticed at 24hpf and 96hpf stages, was more intense at 24hpf compared to 96hpf. The three bands noticed between 43-29kDa markers are labeled as 'a', 'b' and 'c'. Band labeled 'a' was noticed only at 24hpf. Bands labeled 'b' and 'c' noticed at all developmental stages showed an initial decrease at 48hpf but showed same intensity as 24hpf at 72hpf and 96hpf stages.

Protein profiles of CP exposed samples showed varied response at each developmental stage. Comparison of these four/six bands between control and experimental groups at each developmental stages exhibited different trends. The band noticed near 205kDa marker was either normal or little up-regulated at the first concentration of 200µg/L CP at all developmental stages. But after this concentration the protein showed a trend in down regulation from 400µg/L group at all stages. Surprisingly at the highest concentration of CP tested (1000µg/L) this protein concentration increased at all developmental stages (Fig. 2-A, B, C and D). With regard to the protein band noticed at 97kDa marker, it did not either change or little increase in concentration was noticed in 200µg/L group at all developmental stages.

Table 1. Measured total protein concentration [mg/100embryos/larvae] in control and CP exposed embryos/larvae of Danio rerio at four different stages of development

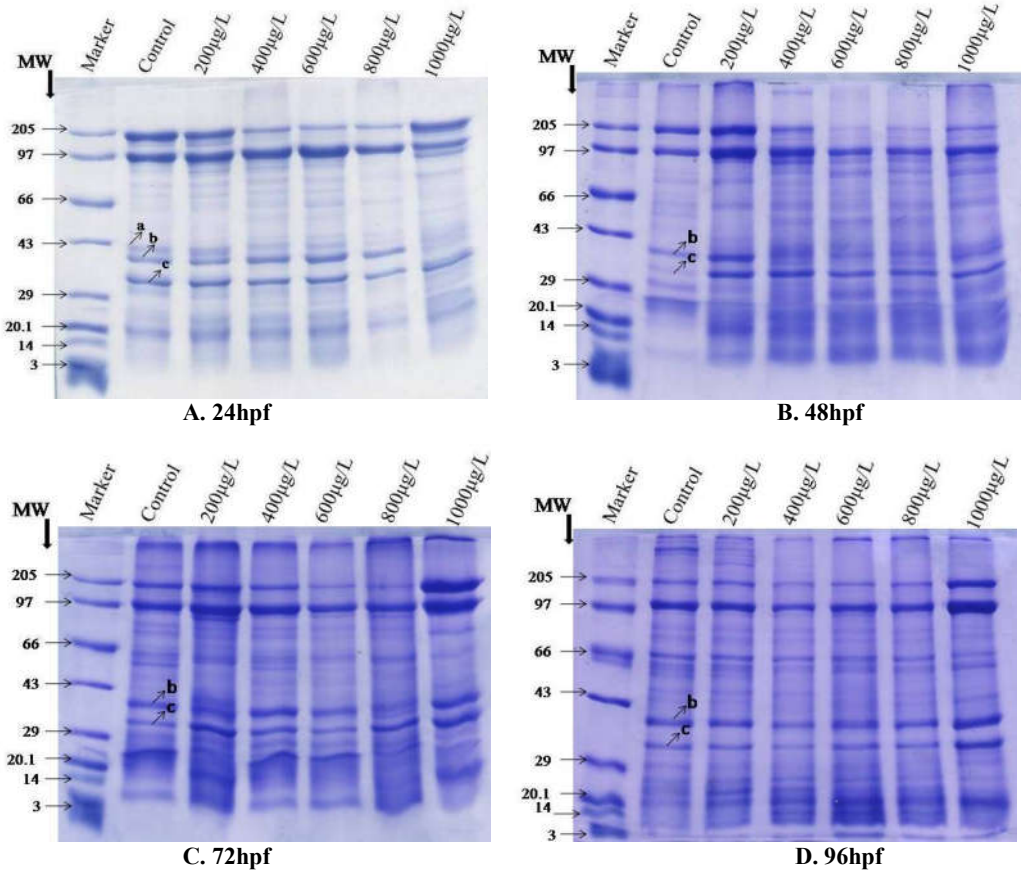
S. No	Chlorpyrifos concentrations	Observations made at			
		24hpf	48hpf	72hpf	96hpf
1	Control	2.24±0.01[a]	1.10±0.1[a]	0.87±0.01[a]	0.60±0.13[a]
2	200µg/L	2.09±0.03[b] (-6.69)	1.60±0.1[b] (+45.45%)	0.95±0.01[b] (+9.19%)	0.54±0.02[a] (-10%)
3	400µg/L	1.99±0.01[c] (-11.16%)	1.15±0.01[a] (+4.54%)	0.58±0.01[c] (-33.33%)	0.47±0.01[b] (-21.66%)
4	600µg/L	1.52±0.03[d] (-32.14)	1.05±0.15[a] (-4.54%)	0.53±0.01[d] (-39.08%)	0.48±0.01[b] (-20%)
5	800µg/L	1.2±0.1[e] (-46.42%)	0.98±0.02[d] (-10.90%)	0.61±0.02[e] (-29.88%)	0.54±0.01[a] (-10%)
6	1000µg/L	1.25±0.01[e] (-44.19%)	1.25±0.01[e] (+13.63%)	1.71±0.02[f] (+96.55%)	0.79±0.02[c] (+31.66%)

Data are expressed as mean ± SD (N=100). Values of each group with identical alphabets shown were not significantly different (P<0.05)



Data are expressed as mean ± SD (N=100). Values of each group with identical alphabets shown were not significantly different (P<0.05)

Fig. 1. Percent change of protein concentration in embryo/larvae of Danio rerio exposed to different concentrations of CP at different developmental stages



A. 24hpf **B. 48hpf**

C. 72hpf **D. 96hpf**

Fig. 2. Changes in the protein profile of early embryonic zebrafish exposed to different concentrations of chlorpyrifos at different developmental stages

At 96hpf this protein showed a steady increase in concentration with increase in concentration of CP. whereas at 48hpf and 72hpf stages down regulation was noticed with increase in concentration from 400µg to 800µg/L but was up regulated at highest concentration (1000µg/L). A different trend was noticed at 24hpf. Increased concentration of this protein band was observed at 400µg/L and 600µg/L but at 800µg/L and 1000µg/L the concentration of protein band decreased (Fig. 2-A). At 96hpf stage a prominent band was noticed near 66kDa marker. This band was not noticed at other developmental stages (Fig. 2-D). With regard to the intensity of the band, not much difference is noticed between control and experimental groups except at 1000µg/L group where it appears to be down regulated. Three intense bands noticed in the molecular weight range 29-43kDa in 24hpf control were also noticed in the embryos exposed to all five concentrations of CP at this stage. But they decreased in the intensity of the three bands with increasing concentration of CP (Fig. 2-A). Similar to controls of 48hpf, 72hpf and 96hpf developmental stages, band labeled 'a' between the molecular weight range of 29-43kDa was also not noticed at all exposure groups (Fig. 2-B, C and D). In 48hpf, up-regulation of two distinct bands, band 'b' and 'c' were noticed in 200µg/L. These bands decreased with increase in concentration. At 72hpf and 96hpf stages intensity of the two bands decreased with increasing concentration from 200µg/L to 800µg/L but bands intensity increased in1000µg/L group.

DISCUSSION

It is a well established that the proteins are among the most abundant biological macromolecules; extremely versatile in their function and interact with other molecules in various biochemical physiological processes (Wilson 1975). Since these molecules are involved in major events and plays a key role during development, assessment of protein content is considered a good diagnostic tool to determine the role of proteins during different stages of development. In the present study reduction in total protein content was noticed in majority of the exposure groups at different developmental stages. In support of our studies, reduction in protein content in the presence of the organophosphate pesticide was shown earlier (Tilak *et al.,* 2005; Tripathi and Shasmal, 2011). Among the different developmental stages the amount of total protein content was more at 24hpf. By this time cleavage, blastulation, gastrulation and segmentation periods were completed and most of the vital organs are seen (Kimmel *et al.,* 1995). Still there could be unutilized yolk and hence more protein content at this stage. By 96hpf the larvae slowly starts feeding on external diet and most of the embryonic yolk could be utilized. Hence the less amount of protein. The decreased trend of protein content during development of *Danio rerio* in the present study may be due to metabolic utilization of keto acids in the synthesis of glucose or for the osmatic and ionic regulation. In majority of the exposure groups at different developmental stages, the protein content showed decreasing trend. Under conditions of stress many organisms will mobilize proteins as an energy source via the oxidation of amino acids. Decreased protein level may be attributed to stress mediated immobilization of these compounds to fulfill an increased element for energy by the fish to cope with environmental condition exposed to the toxicant (Jenkins *et al.,* 2003). The depletion in total protein content may be due to augmented proteolysis and possible utilization of their products for metabolic purposes as reported by Ravinder et al. (1988). At the highest concentration tested (1000µg/L) the increase in protein content over control at all four developmental stages indicate that during stress, the embryo/larvae needed more energy to counter toxicity as well as detoxify the toxicant. Since there is very little amount carbohydrates in the eggs/embryos the alternative source of energy is protein to meet the increased energy demand. CP was known to induce changes in protein metabolism in various regions of the brain of *Tilapia mossambica* exposed for 4days (Subburaju and Selvarajan, 1989).

Protein expression profile is an excellent approach to visualize the pattern and the level of proteins expressed under defined environmental conditions. SDS-PAGE examination of zebrafish embryonic/larval proteins was carried out in control and after exposure to CP to have insight into the changed protein profile under CP toxicity. Early embryonic stages are the vulnerable stages to environmental stress like temperature, oxygen content and increase in pollution (Cook *et al.,* 2005). Therefore proteins of 24hpf, 48hpf, 72hpf and 96hpf stages were resolved by SDS-PAGE to visualize directly the effects of CP. At different concentrations of CP the energy demands of different developmental stages are different. For this reason we noticed up regulation/down regulation of these proteins at different concentrations. The treated samples were compared with their controls at each developmental stage to know the increase/decrease in the relative band intensity of four/six bands. Only these were selected as they were prominent.

Only one distinct high molecular protein band was noticed at 205kDa marker. Not much work has been done with regarding to the high molecular weight proteins hence there is no information in literature. For this reason it cannot be said clearly which is the protein/s that is showing up here. A prominent band was noticed near 20.1kDa marker at 24hpf and 96hpf stages only. In all experimental groups this band appeared with very low intensity. This band was not noticed in control at 48hpf, 72hpf and 96hpf and experimental groups at 48 and 72hpf. From literature it can be said that the thick intense band noticed at 97kDa region could be different centrosomal and mitochondrial proteins. There is intense cell division and rapid metabolic activity which requires energy during development. Hence this band is noticed at all developmental stages and at 24hpf stage of development this band is more intense. The intensity of this band decreased with increase in concentration till 800µg/L exposure group; but at the highest concentration (1000µg/L) there was increased in the intensity of the band. This trend indicates the breakdown of proteins at lower concentrations but surprisingly it appeared that the embryos/larvae stepped up the synthesis of centrosomal and mitochondrial proteins. Only at 96hpf stage a distinct protein band was noticed at 66kDa marker. In an earlier study with CP, Liu *et al.,* (2015) have shown that this band comprises of cytoskeletal and vitellogenin proteins. At this stage most of skeletal parts like notochord, skull in the head region are well developed along with the bronchial arches. For this reason it became prominent at this developmental stage. The appearance of these proteins only at this 96hpf stage corroborates with development of skeletal

structure. The three distinct bands noticed between 27-43kDa marker regions could be various nuclear proteins, membrane proteins, cytosolic proteins and proteins of translation missionary (Tay *et al.,* 2006). It has been shown by Gundel *et al.,* (2007) that different isoforms of vitellogenin is also seen in this region. At 24hpf there could be vitellogenin still unutilized and hence we noticed three bands-'a', 'b', 'c' at this stage. As the development progressed the vitellogenin is completely utilized as such only two bands were noticed band 'b' and 'c'. It appears that under CP stress there was more exposure periods. From these studies it can be said that CP as effect on early embryonic development of zebrafish by altering the protein profile.

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