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Sub-lethal Toxicity Impacts of Endosulfan on Some Biochemical Parameters of the Freshwater Crayfish (*Astacus leptodactylus*)

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ABSTRACT

Endosulfan, an organochlorine pesticide in surface waters of Iran near agriculture farms such as Anzli Lagoon has been reported by some authors. The present research aimed to evaluate the effect of sub-lethal concentrations of endosulfan on some biochemical parameters. After determining 96 h LC₅₀ of endosulfan, eighty one freshwater crayfish, *Astacus leptodactylus* were subdivided into three groups and were exposed to 0.0, 32.5 and 65 µg L⁻¹ of endosulfan. Biochemical parameters such as protein, succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), acid phosphatase (AcP) and alkaline phosphatase (ALP) activities in muscle tissue of crayfish after 15 and 30 days. The significant decrease in protein levels was observed in treated crayfish when compared with the control group (p<0.05). The SDH activity showed a significant decrease (p<0.05) in the crayfish exposed to 65 µg L⁻¹ and both concentrations of endosulfan on 15 and 30 days, respectively. A significant increase (p<0.05) was observed in LDH activity of crayfish exposed to 32.5 and 65 µg L⁻¹ of endosulfan after 15 and 30 days, respectively. After 30 days, a significant increase in the levels of ALP and AcP activities was observed in the treated crayfish by 65 µg L⁻¹ of endosulfan (p<0.05). In conclusion, exposure to endosulfan at sub-lethal concentrations induced biochemical alterations in crayfish. These biochemical parameters offer a rapid and sensitive means of monitoring towards the impact of pesticides on aquatic biota and ultimately whole of the ecosystem.

Key words: Endosulfan. crayfish, *astacus leptodactylus*, biochemical parameters

INTRODUCTION

Endosulfan is an organochlorine pesticide that is used to control pest on food and non-food crops and also as a wood preservative. It is extremely toxic to fish and aquatic invertebrates and is a main concern contaminant for international environmental agencies. It has also been detected in the air, soils, sediments, runoff waters, ground and surface waters (Funari *et al.*, 1995; Schultz *et al.*, 2001; Ayati, 2003; Chorom and Shrif, 2010). According to researcher's reports, levels of agrochemicals, in particular, diazinon, DDT and endosulfans, are a major cause for concern in the Caspian Sea and Anzali Lagoon (UNDP, 2004; FAO, 1990). Exposure to pesticides may have significant impacts on aquatic animals and human health.

Freshwater crayfish, *Astacus leptodactylus* with a limited geographical distribution in the Anzali Lagoon and the Aras River basin are the only crayfish existing in Iran (Fard *et al.*, 2011).

The amount of the local consumption of crayfish in Iran is very low; therefore, almost all catches are exported to European countries (Fard *et al.*, 2011). Nevertheless, a limited number of them are sold as aquarium species. Pollution of aquatic ecosystems in Iran is probably one important factor in the loss of these animals. In other words, the population of crayfish may be severely threatened in Iran. Whereas, crustaceans are sensitive to pesticides for example amphipod (Gibbs *et al.*, 1984), isopod crustacean (Brock *et al.*, 1992), copepod (Green *et al.*, 1996), cladocerans (Hanazato and Yasuno, 1990), mysid (Roast *et al.*, 1998), crabs (Rodriguez and Amin, 1991; Mian and Mulla, 1992), shrimp (Cripe, 1994; Suryavanshi *et al.*, 2009), prawn (Selvakumar *et al.*, 2005) and crayfish (Jarboe and Romaine, 1991).

Pesticides can cause serious impairment to physiological and health status of aquatic animals. The effect of pollutants on different aspects of biological and physiological aspects of crustacean was studied by several scholars (Selvakumar *et al.*, 1996; Valarmathi and Azariah, 2002; Vijayavel and Balasubramanian, 2006; Kumar *et al.*, 2007; Chourpagar and Kulkarni, 2009; Sekar *et al.*, 2009; Suryavanshi *et al.*, 2009; Thenmozhi *et al.*, 2009). Therefore, biochemical tests are routine laboratory tests useful in recognizing acute, sub-lethal or chronic toxicity of pesticides (Banaee *et al.*, 2008; Al-Kahtani, 2011) and can be a practical tool to diagnose toxicity effects in target organs and to determine the physiological status in aquatic animals. Biochemical test gives indicates what is happening in the body of animals exposed to pesticides. When different tissues are injured, the damaged cells release specific enzymes into plasma and we can recognize their abnormality levels in blood. Then it helps locate the lesions. Also, if certain organs are not eliminating certain waste products or not synthesizing certain important materials, this can tell us they are not functioning properly. In some cases due to the severity of the damage to tissues, particularly liver or hepatopancreas, synthesis of many biochemical parameters may reduce significantly in cells which can decrease some biochemical factors in blood or hemolymph of animals exposed to pesticides (Banaee, 2010). However, few studies have been done on the biochemical parameters of freshwater crustacean by pesticides exposure (Sreenivasan *et al.*, 2009, 2010, 2011a, b).

In this study, the biochemical parameters such as protein levels, succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), acid phosphatase (AcP) and alkaline phosphatase (ALP) activities in muscle tissue were measured after exposure to sub-lethal concentrations of endosulfan.

MATERIAL AND METHODS

Experimental animals: Crayfish (*Astacus leptodactylus*) (35.32±5.56 g, 16.70±1.95 cm) was purchased from a local fish dealer. They were acclimatized in the laboratory in dechlorinated tap water at 17±1°C with controlled photoperiods (10L:14D), pH: 7.2±0.1, DO: 6 mg L⁻¹ and total hardness 180±20 mg L⁻¹ and 20% water exchange rate/day for 15 days prior to use. The floor of the aquariums were covered with gravel, broken bricks and lengths of drainpipe, to act as shelters and limestone chippings in order to maintain calcium concentrations in the water. During acclimation, crayfishes were fed with prepared pellets according to commercial formulations at 10% of their body weight twice a day.

Acute toxicity experiments: One hundred and eighty freshwater crayfish, *Astacus leptodactylus*, (average weight and length 35.32±5.56 g; 16.96±1.92 cm, respectively) were used in acute toxicity tests. The acute toxicity test was conducted following the Organization for Economic Cooperation and Development (OECD) Guideline No. 203 under static-renewal test

conditions (OECD, 1992). Test solutions of endosulfan were prepared from a commercial endosulfan (35 EC), Thiodan brand, with the active molecule 6,7,8,9,10,10-hexachloro 1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide, purity 35% dissolved in technical solution were purchased from Partonar Co. Iran. Nominal concentrations of active ingredient tested were 0 (control), 0.25, 0.5, 1, 1.5, 2 mg L⁻¹ and triplicate aquariums (200 L) were designated for each concentration. Ten crayfishes were introduced in each aquarium. During the 96 h acute toxicity experiment, water in each aquarium was aerated and had the same conditions as the acclimation periods. The static-renewal tests exposed the crayfish for 96 h with replacement of the test solution every 24 h (all stock solutions were made fresh solution prior to use). The water was changed daily to reduce the build-up of metabolic wastes and to keep concentrations of endosulfan near the nominal level. Crayfish mortality was recorded 0, 24, 48, 72 and 96 h after exposure to endosulfan. LC₅₀ values were calculated by the Probit Analysis test (Aydin and Koprucu, 2005).

Sub-lethal toxicity experiments: Eighty one crayfish were randomly distributed in nine 200 L aquariums (3 treatments by triplicate) to perform the 30 day period sub-lethal toxicity tests. Every aquarium containing 9 crayfishes were exposed to test solutions with the following concentrations of endosulfan (35 EC): 0.0 (control), 32.5 µg L⁻¹ (1/20 96 h LC₅₀) and 65 µg L⁻¹ (1/10 96 h LC₅₀), respectively. During the experimental periods, crayfishes were fed with commercial pellets at 2% of their body weight twice a day. At the end of the experiment periods, 9 crayfish per treatment were captured and hemolymph was collected from the pericardium using a 1 cc syringe. After bleeding, they were sacrificed by removing head. The exoskeleton was carefully removed and the carcasses' crayfish (gutted body) were immediately washed in normal saline solution and then pooled, minced under sterile conditions. Samples were homogenized for 1 min in the ice cold phosphate buffer (pH 7.4; 1:10, w/v) using a glass homogenizer and then centrifuged for 15 min at 12000 g at 4°C to obtain the supernatant for biochemical analyses. Supernatants were immediately used to determine biochemical parameters by using a UV/VIS spectrophotometer.

Biochemical assay: Total protein levels are determined according to Biuret method by standard procedures used in clinical biochemistry laboratories based on manual biochemical kits (Parsazema Co, Iran). This method is based on the interaction of cupric (Cu²⁺) ion with protein in an alkaline solution and the resultant development of absorbance at 545 nm. Succinate dehydrogenase (SDH) activity is determined by measuring the formation of formazan due to the tetrazolium salt reduction (Munujos *et al.*, 1993). This reaction is monitored at 600 nm. Lactate dehydrogenase (LDH) is an NAD⁺-dependent enzyme that catalyses the inter conversion of pyruvate and lactate. The reaction velocity is determined by a decrease in absorbance at 340 nm resulting from the oxidation of NADH (based on manual biochemical kits (Parsazema Co, Iran)). Acid phosphatase (AcP) activity is assayed by utilizing para-nitrophenyl phosphate (pNPP) as a chromogenic substrate for the enzyme. In the first step, AcP dephosphorylates pNPP. In the second step, the phenolic OH-group is deprotonated under alkaline conditions resulting in p-nitrophenolate that yields an intense yellow color which can be measured at 405-414 nm (based on manual biochemical kits (Parsazema Co, Iran)). Alkaline phosphatase activity is measured by using p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which turns yellow color when dephosphorylated by ALP. The change absorbance change at 405 nm was recorded (based on manual biochemical kits (Parsazema Co, Iran)).

Statistical analysis: Statistical analyses were performed using SPSS (Release 15) software. Data are presented as Mean±SD. All the data were tested for normality (Kolmogorov-Smirnov test). Data were analyzed by one-way of variance analysis (ANOVA). The significant means were compared by Tukey's test and a p<0.05 was considered statistically significant.

RESULTS

Median lethal concentration (LC₅₀) was investigated in semi-static tests for 24, 48, 72 and 96 h. LC₅₀ values significantly decreased in accordance with the exposure time from 1.43±0.085 mg L⁻¹ at 24 h to 0.65±0.056 mg L⁻¹ at 96 h (Table 1). The reduced strength of the shell and destruction of gills are an important clinical signs observed in dead crayfishes. Although, soft shell of some crayfish exposed to sub-lethal concentrations of endosulfan was observed, no mortality was recorded for all treatment groups studied during experimental periods.

Alterations in muscle biochemical parameters are presented in Table 2. The significant decrease in protein levels was observed in 15 days treated crayfish when compared with the control group (p<0.05). The reduced levels of protein were also seen at 30 days of treatment in crayfish exposed to 65 µg L⁻¹ of endosulfan. In this study, the SDH activity significantly decreased in the crayfish exposed to 65 µg L⁻¹ of endosulfan on 15 days (p<0.05). The SDH in the muscle of treated crayfish

Table 1: Median Lethal Concentrations of endosulfan to freshwater crayfish, (*A. leptodactylus*). Acute endosulfan toxicity was determined in crayfish after 24, 48, 72 and h of toxicity. LC₅₀ was calculated by the probit analysis test. Results are expressed as Mean±SD with the maximum and minimum values

LC ₅₀ (mg L ⁻¹)	Duration of exposure (h)
1.43±0.085 (1.28-1.62)	24
1.10±0.070 (0.97-1.25)	48
0.81±0.059 (0.70-0.94)	72
0.65±0.056 (0.54-0.77)	96

Table 2: Biochemical parameters of freshwater crayfish (*A. leptodactylus*) exposed to sub-lethal concentrations of endosulfan after 15 and 30 days

Biochemical parameters	Concentrations of endosulfan (µg L ⁻¹)	15 day	30 day
Protein (mg/100 mg tissue)	Control	3.25±0.31 ^b	3.13±0.63 ^b
	32.5	2.20±0.62 ^a	2.67±0.64 ^{ab}
	65.0	2.40±0.54 ^a	2.05±0.61 ^a
SDH (mIU/min/mg protein)	Control	3.50±0.62 ^b	3.39±1.47 ^b
	32.5	2.73±0.88 ^{ab}	1.68±1.17 ^a
	65.0	2.16±0.62 ^a	1.59±0.55 ^a
LDH (U mg ⁻¹ protein)	Control	4.86±0.68 ^a	5.38±1.63 ^a
	32.5	9.95±3.51 ^b	6.68±1.76 ^a
	65.0	8.87±4.21 ^{ab}	11.04±1.87 ^b
AcP (U mg ⁻¹ protein)	Control	2.05±0.36 ^a	1.56±0.75 ^a
	32.5	2.53±0.52 ^a	1.99±0.75 ^a
	65.0	2.35±0.37 ^a	3.41±0.82 ^b
ALP (U mg ⁻¹ protein)	Control	6.75±0.58 ^a	5.81±1.23 ^a
	32.5	8.78±2.62 ^a	12.60±3.83 ^b
	65.0	9.95±3.11 ^a	14.42±6.44 ^b

Effects of sub-lethal levels of endosulfan (0.0 µg L⁻¹ (Control), 32.5 µg L⁻¹ and 65 µg L⁻¹) on muscle biochemical parameters were determined in crayfish (n = 9) after 15 and 30 days exposure. Different letters indicate significant differences between values (One-way ANOVA, p<0.05). Values are presented as Mean±SD

showed decrease when compared to control group on 30 days. The results showed a significant rise in LDH activity of crayfish exposed to $32.5 \mu\text{g L}^{-1}$ of endosulfan after 15 days ($p < 0.05$). Furthermore, a significant increase in the LDH activity was observed in the crayfish treated by $65 \mu\text{g L}^{-1}$ of endosulfan on 30 days ($p < 0.05$). After 30 days, a significant increase in the levels of ALP and AcP activities was observed in the treated crayfish by $65 \mu\text{g L}^{-1}$ of endosulfan when compared to control group ($p < 0.05$).

DISCUSSION

The present results show that endosulfan is highly toxic to freshwater crayfish, *Astacus leptodactylus*. The toxicity of endosulfan on *A. leptodactylus* increased with increasing concentration and exposure time.

When crayfishes were exposed to 0.25 mg L^{-1} endosulfan, only 26.7% died after 96 h, whereas all the fishes (100%) died after 72 h when were exposed to a concentration of 2 mg L^{-1} endosulfan. In addition, the 24, 48, 72 and 96 h LC_{50} values of endosulfan of crayfish were found 1.43, 1.10, 0.81 and 0.65 mg L^{-1} , respectively.

Sub-lethal toxicity testing was planned based on one tenth or more of LC_{50} dose in moderate periods. In sub-lethal toxicity, the organs or biological systems which may be affected at such exposure can be respiratory, hepatic, haematopoietic, nervous, cardiovascular, reproductive and immune systems. Different biomarkers of aquatic animals exposed to pesticides are usually evaluated in these experiments. Pesticides may lead to changes in the blood biochemical parameters and hematological profile of fish which can be investigated as biomarker in pollution monitoring (Mushigeri and David, 2005; Banaee *et al.*, 2008; Kavitha and Rao, 2009). In fact, these compounds may induce alterations in the activities or levels of a number of different enzyme systems, including those necessary for biochemical reactions in cells (Banaee *et al.*, 2011). So, biochemical studies are very important from a toxicological point of view. Biochemical parameters and physiological indicators can be used as diagnostic tools to recognize potential environmental problems before the health of aquatic systems is seriously changed.

Reduce strength of the shell or exoskeleton erosions were commonly observed in animals exposed to both concentrations of endosulfan. Signs of the exoskeleton erosions or soft shell include rickety, erosion and pitting of the exoskeleton that may be caused by a disorder in the structure of the chitin exoskeletons.

In the present study, protein levels in muscle of crayfish exposed to endosulfan was lower than those in the control group. The decrease of the muscle protein was due to supply of energy to meet the impending energy demand under toxic stress and alter protein biosynthesis process. These results have been reported by other scientists (Suryavanshi *et al.*, 2009; Thenmozhi *et al.*, 2009; Sreenivasan *et al.*, 2009).

Succinate dehydrogenase is an oxidative enzyme which plays an important role in Tri-Carboxylic Acid cycle (TCA). Therefore, any alteration in the SDH activity reflects change in TCA cycle operation and disorder in the pathway of carbohydrate metabolism. According to obtained results, the SDH activity in the muscle of crayfish exposed to $65 \mu\text{g L}^{-1}$ of endosulfan was significantly lower than the control group on 15 days ($p < 0.05$). A significant decrease in the muscle SDH activity of treated crayfish was also observed on 30 days. Decrease in SDH activity in the muscle of treated crayfish by endosulfan may indicate impairment in the Krebs's cycle resulting in the decreased synthesis of high energy phosphate molecules such as ATP and ADP as well as inability of cells to maintain the normal biological activities. Other researchers reported the same

biochemical alterations in different tissues of mud crab (*Sesarma quadratum*), freshwater crab (*Barytelphusa cunicularis*), freshwater field crab (*Spiralothelphusa hydrodroma*) treated with copper chloride (Valarmathi and Azariah, 2002), endosulfan (Venkateshwarlu and Shanmugam, 2005), Cypermethrin (Sreenivasan *et al.*, 2010, 2011a), respectively.

LDH is an enzyme participated in anaerobic pathway of carbohydrate metabolism. The increase of LDH activity is a diagnostic index widely used to recognize increases of anaerobic metabolism resulting from depletion of energy under anaerobic and environmental stress conditions. Results were showed a significant rising in LDH activity of crayfish exposed to 32.5 $\mu\text{g L}^{-1}$ of endosulfan after 15 days ($p < 0.05$). Furthermore, a significant increase in the LDH activity was observed in the treated crayfish by 65 $\mu\text{g L}^{-1}$ of endosulfan on 30 days ($p < 0.05$). The increase of LDH activity can be attributed to the conversion of accumulated pyruvate into lactate which is transported through muscle to hepatopancreas and regenerated glucose and glycogen to supply energy crayfish exposed to endosulfan. These changes were observed in mud crab (*S. quadratum*), freshwater crab (*B. cunicularis*), freshwater filed crab (*Spiralothelphusa hydrodroma*) in contact with copper chloride (Valarmathi and Azariah, 2002), endosulfan (Venkateshwarlu and Shanmugam, 2005), Cypermethrin (Sreenivasan *et al.*, 2010, 2011a, b), respectively.

Acid phosphatase plays an important role in carbohydrate metabolism. This enzyme can be found inside the membrane of lysosomes. So, any damage to the membrane of lysosomes can cause the release of this enzyme into muscle and increase its levels. In the present study, AcP activity in the muscle of crayfish exposed to 65 $\mu\text{g L}^{-1}$ of endosulfan was higher than control group on 30 days. These changes were observed in freshwater field crab (*Spiralothelphusa hydrodroma*) which were exposed to Cypermethrin (Sreenivasan *et al.*, 2011b).

The alkaline phosphatase plays a significant role in phosphate hydrolysis and in membrane transport as well as is a good bio-indicator of stress in biological systems. The importance of measuring alkaline phosphatase is to check the liver dysfunction (Banaee *et al.*, 2011) and the cellular membrane health. A significant increase in the levels of ALP activity may indicate disorder in tissue cellular membrane of crayfish exposed to 65 $\mu\text{g L}^{-1}$ of endosulfan after 30 days. Similar results were observed in freshwater field crab (*S. hydrodroma*) which was exposed to Cypermethrin (Sreenivasan *et al.*, 2010).

In conclusion, the biochemical parameters measured muscle in the present study was useful for monitoring the long-term effects of endosulfan, an organochlorine pesticide, on freshwater crayfish. One can infer that endosulfan was highly toxic to crayfish. Exposure to chronic sub-lethal concentrations of endosulfan resulted in significant biochemical alterations. These changes may be potentially disruptive for the survivability of this crustacean in contaminated aquatic ecosystems. This fact should be taken into consideration when this pesticide is used for pest control in agricultural fields surrounding surface waters.

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