Effect of endosulfan on the absorption ratio of α and β chains of hemoglobin and acetylcholinesterase activity in the fish, *Labeo rohita*

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Monitoring of environmental pollution can be achieved through the use of biomarkers. Fish biochemical and physiological patterns act as early warning signals for diagnosing the environmental pollution before ecosystem level damage occurs. Cholinesterase activity is a biomarker of exposure to endosulfan which has been legally permitted to be used in India. The aim of this research is to study the effects of endosulfan on the cholinesterase activity of blood and brain of the tropical fish, *Labeo rohita,* a bioindicator species. Fishes were exposed to sub-lethal concentration of endosulfan and after 24, 48, 72 and 96 hrs blood and brain were taken for enzymatic assays. Results show that absorption ratio of α and β bands of hemoglobin decrease significantly after 24, 48, 72 and 96 hrs ($p<0.005$, $p<0.004$, $p<0.002$ and $p<0.045$ respectively). The maximum inhibition of acetylcholinesterase activity (65.4%) in the brain is after 24 hrs following the exposure of endosulfan and thereafter inhibition became less in 48 & 96 hrs. In contrast to the brain the inhibition of cholinesterase activity in the blood is only 51.6% and thereafter the inhibition becomes less till 96 hrs. The physiological significance of these observations is discussed herein.

Key words: *endosulfan, Labeo rohita, acetylcholinesterase activity, biomarker.*

Влияние эндосульфана на соотношение абсорбции α и β цепей гемоглобина и активность ацетилхолинэстеразы у рыб *Labeo rohita*

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Мониторинг загрязнения окружающей среды можно проводить с использованием биомаркеров. Биохимические и физиологические показатели рыб могут предупредить о загрязнении окружающей среды до того, как оно проявится на уровне экосистем. Активность холинэстеразы является биомаркером действия эндосульфана, разрешенного к использованию в Индии. Цель данного исследования – изучить влияние эндосульфана на активность холинэстеразы в крови и мозге тропической рыбы *Labeo rohita* как вида-биоиндикатора. Рыбы подвергались действию сублетальной концентрации эндосульфана, и через 24, 48, 72 и 96 ч после воздействия проводился анализ ферментов крови и мозга. Результаты показали, что соотношение абсорбции α и β цепей гемоглобина значительно уменьшается через 24, 48, 72 и 96 ч после воздействия ($p<0,005$, $p<0,004$, $p<0,002$ и $p<0,045$ соответственно). Максимальное угнетение активности ацетилхолинэстеразы (65,4%) в мозге наблюдается через 24 ч после воздействия эндосульфана, затем угнетение ослабляется (через 48 и 96 ч.). В отличие от мозга, в крови угнетение активности холинэстеразы составляет только 51,6%, а через 96 ч. угнетение ослабляется. Обсуждается физиологическое значение указанных наблюдений.

Ключевые слова: *эндосульфан, Labeo rohita, активность ацетилхолинэстеразы, биомаркер.*

Introduction

In India, the misuse of pesticides in agricultural areas is detrimental to the quality of water and consequently could affect the health of the aquatic organisms. Pesticide mixtures, used for agricultural treatments often include some of the most poisonous persistent organic pollutants (POPs) such as aldrin, dieldrin, endrin and endosulfan. Endosulfan is semi volatile. It evaporates from the surface of the soil and plants after application. It has been reported from field studies that 70% of endosulfan is lost from the cotton fields through volatilization (Watts, 2008).

Endosulfan targets the prefrontal cortex of the brain which is involved in cognitive tasks, selective attention, response inhibition, behavioural flexibility. It is sensitive to muscle spasm and it alters brain levels of neurotransmitters, dopamine, noradrenalin and serotonin.

The primary acute effect is on the Central Nervous System (C.N.S.) as a result of endosulfan binding to GABA receptors in the brain causing hyper-excitation, and convulsions and nervous system mediated effects on respiration and the heart. The primary systemic targets are the liver and kidney but endosulfan also causes hematological and respiratory effects. It causes oxidative stress which is implicated in its neurotoxic effects. Endosulfan is an endocrine disruptor in mammals, birds, amphibians and fishes affecting both male and female reproductive hormones. It chronically depresses testosterone levels.

Organochlorine pesticides are widely used in Southeast Asia in rice farming. They are reported to be toxic to several non-target organisms including fish (Holden, 1973) and also contribute to ulcerative fish

disease (Tonguthai, 1985). The use of endosulfan, an organochlorine insecticide is increased in India due to ban on endrin and due to its comparatively lower toxicity for mammals. Further, as fishes are highly susceptible to endosulfan, it is used for the removal of weed fishes from ponds and lakes before their restocking with desired species (Gill et al., 1991). In fishes, at sub-lethal concentrations, endosulfan affects the reproductive physiology (Basak, Konar, 1976), reduces respiratory rate (Manoharan, Subbiah, 1982), causes deformity in developing embryos (Kulshrestha, Arora, 1984) and alters tissue proteins (John, Jayabalan, 1993). They are group of animals with highest number of multiple hemoglobins.

Hemoglobins are particularly important in studies of fish adaptations because the Hb molecule is poised between the metabolism of the organism and the environment and so it has to cope with both metabolic requirement and environmental constraints (Riggs, 1979). Krogh & Leitch (1911) showed that blood respiratory properties could be related to availability of environmental oxygen. After this, many authors showed that differences existed in functional properties of Hbs in fishes from different environments.

The ability of the aerobic metabolism of animals to satisfy the demands for oxygen is only possible due to hemoglobins contained in red blood cells which facilitate the dissolution of large quantities of O_2 and take it to the tissues where it functions as the final acceptor of electrons originating from oxidative catabolism reaction. Hbs seem to be adapted to different metabolic necessities of animals of greatest interest to research of adaptive processes due to their possible evolutionary chain and to their genotypic characteristics. These features guarantee enormous plasticity in the environments and capacity of some species to cope up with different conditions.

Expression of α and β chains and their post-translational assembly into $\alpha_2\beta_2$ tetramers are fundamental for the formation and function of most vertebrate hemoglobins. Rapidly swimming fishes have higher respiratory oxygen demands and can experience blood pH changes during periods of high activity. Most fishes contain tetrameric hemoglobins in which oxygen binding is highly pH sensitive (Root effect Hbs) and other Hbs with little or no pH sensitivity. Fishes have been reported to load and unload oxygen into and out of their swim bladders at the fastest rates (Bentley, Wiley, 1982). The expression of multiple types of α and β chains and their assembly into functionally distinct $\alpha_2\beta_2$ tetramers make these functional adaptations possible. The higher absorption rates of α and β chains is suggestive of higher oxygen affinity (Bugge, Weber, 1999).

Pesticides used in pest control program seem to produce many physiological and biochemical changes in freshwater fishes by influencing the activities of several enzymes (Sancho et al., 1998). Acetylcholinesterase (EC 3.1.1.7) activity is routinely used as a biomarker of exposure to certain group of contaminants, such as organochlorines and organophosphates. Low concentration of the compounds can inhibit AchE, which leads to the accumulation of acetylcholine at central cholinergic synapses and neuromuscular junctions (Sancho et al., 1997; Varo et al., 2003; Kumari, Sinha, 2006). The inhibition of AChE by pesticides can affect locomotion and equilibrium of exposed organisms (Sanglio et al., 1998; Kumari, Sinha, 2006). Thus the aim of the present study is to elucidate the adverse effects of sub-lethal concentration of endosulfan on the absorption ratio of α and β chains of Hb which is an index of oxygen affinity and AchE activity in the blood and brain of the most popular and tastiest fish *Labeo rohita.*

Material and methods

Labeo rohita, common carps were obtained from the local hatchery. Fishes were acclimated to laboratory conditions for about 5 days. They were kept in aquarium tank (250 L) and water was constantly aerated by a static system. During the acclimation period, they were given artificial (commercial) feed and ground shrimps available in the local market to avoid the possible effects of starvation on any parameters under study. The feeding and maintenance of the fishes and physico-chemical characteristics of the aquaria water were measured. Short term test of acute toxicity over period of 96 hrs was performed on the fishes following the renewal of bioassay. Fishes were exposed intracoelomatically with $1/3^{rd}$ of 0.003mg (LC_{50}) of technical grade endosulfan. After 24, 48, 72 and 96 hrs of exposure fishes were killed for different assays.

The behavior and condition of the fishes were noted every 24 hr up to 96 hrs. The fishes which failed to respond even to strong tactile stimuli were considered dead and removed immediately.

Determination of LC_{50}. The experiment was repeated several times and only arithmetic mean of the experiments at each concentration was taken to express the results. LC_{50} values were determined by EPA Probit Analysis Program (Finney, 1971).

Blood collection. The fish was caught individually with small hand net from the aquaria with minimum disturbance. After preliminary investigations of the length and weight, the blood samples were collected. Initially, blood samples were collected from the caudal fin as described by many authors but in the present investigation the collection of blood from the caudal fin had to be abandoned because there was marked increase of the enzyme activities mainly of creatinine phosphokinase (CPK) and lactic dehydrogenase (LDH)

leaked from the surrounding muscles. Thus, cardiac sampling was only suitable method of obtaining blood under study. After the blood sampling, tissues such as brain were taken for enzymatic assays.

Spectrophotometric analysis of hemoglobin derivatives. A known volume of blood was collected and centrifuged in cooling centrifuge (Model C-30) at 4°C for 15 minutes. Red blood cells were separated, purified and then hemolyzed. Then, hemolyzed sample was again centrifuged at 10000 x g for 15 minutes to have stroma free hemolysate. Then, the stroma free hemolysate was aerated for complete conversion of reduced hemoglobin to oxyhemoglobin, Thereafter, absorption spectra was recorded in UV-Vis Shimadzu Spectrophotometer (Model 160A) over the wavelength range 300–800 nm and the graph was plotted and absorption ratios of α at λ_{575} and β bands at λ_{540} were calculated therefrom.

Assay of acetylcholinesterase. Estimate of acetylcholinesterase was done by the spectrophotometric method of Ellman et al. (1961) using DTNB (dithiobisnitrobenzoic acid) as chromogen and acetylthiocholine iodide as the substrate. The reaction was rapid and the assay was also sensitive (i.e. 10 ul sample of blood is adequate). For brain cholinesterase activity, 100 mg of brain was homogenized in 5 ml phosphate buffer (pH 7.4) and assayed for acetylcholinesterase activity.

Observation. The behaviour and condition of fishes in both control and test solution were noted every 24 hrs up to 96 hrs. The fishes showed a marked change in their behaviour when exposed to sublethal concentrations of endosulfan. Behavioural manifestations of acute toxicity like excessive mucus secretion, surfacing and darting movements have been observed, and in the late stage fishes were seen flocking near the aerator suggestive of oxygen distress and at the end of 96 hrs the fishes also exhibited erratic swimming and loss of equilibrium. At the time of death transient hyperactivity was also observed.

Results and discussion

Fish hemoglobins (Hbs) have for many years attracted the attention of many researchers due to the wide spectrum of functional properties. As such, adaptive Hb variations are apparently necessary because fishes are influenced by very many varying conditions like temperature, pH, O_2 tension and others. To accommodate these environmental shifts, their Hbs must function over a great range of conditions than Hbs of higher vertebrates (Weber, 1992). Moreover, functions apart from normal respiratory cycle are served by Root effect Hbs of fishes. These extremely pH sensitive Hbs facilitate O_2 unloading to tissues such as swim bladder, even at great depths where O_2 pressure is high (Bentley, Wiley, 1982). Hbs along with high pH-sensitive Root effect Hbs are widely regarded as an elegant compensatory adaptation, one in which pH insensitive Hbs can provide a back up for O_2 uptake and delivery in cases where drops in blood pH render the Root effect Hbs ineffective.

In the present study, tab. 1 shows that the absorption ratio of α and β chains of hemoglobin decreases significantly following the treatment by sublethal concentration of endosulfan.

Table 1.

Effects of endosulfan on absorption ratio of α and β chains of hemoglobin of *Labeo rohita*

Parameters	Time	Control	Endosulfan	p< value
Absorption ratio	24 hrs	1.057±0.015	0.971±0.03	0.005
	48 hrs	1.057±0.015	0.935±0.045	0.004
	72 hrs	1.057±0.015	0.948±0.03	0.002
	96 hrs	1.057±0.015	0.999±0.037	0.045

Note: values are mean, ±standard deviation; number of samples is 5.

It has been observed in the present study that there was a marked change in fishes' behaviour by manifesting excessive secretion of mucus, surfacing and darting movements indicating hyperactivity due to initial stress condition (Kumari, Sinha, 2006).

Endosulfan, which is an organochlorine insecticide, is highly toxic to fishes and produces greater toxicity than other pesticides like carbamate and methyl parathion. The excitable locomotion (swimming) due to the treatment by endosulfan, leads to anaerobic mode of life. It has been observed in our laboratory that following the treatment by endosulfan LDH activity in the muscle greatly enhances (Ray, Sinha, 2009). If there is no O_2, glucose is converted to lactic acid whereby lowering the pH of blood. As such, during the stress condition, lowering of blood pH takes place resulting in quick release of O_2 from Hb to the tissues for metabolism which is known as the Root effect. Thus, the Root effect is a functional property of fish Hbs. This property consists in drastic reduction of hemoglobin O_2 affinity. This is substantiated further by the result of

absorption ratio of α and β bands of Hb where the absorption ratio decreases significantly following the exposure of endosulfan (tab. 1 & fig. 1) suggesting lower O_2 affinity of Hbs, thus releasing O_2 to meet the demand of needy tissues. This could be considered as an adaptation to environmental hypoxia which is often met in fishes. Fish Hbs display great flexibility in adapting to changing environmental conditions, metabolic demand and modes of life. These adaptations which involve modification of intra-erythrocytic environment display functional plasticity of Hb-O_2 in different conditions. Fishes respond to respiratory challenges through different behavioural and physiological mechanisms. Since Hb is a very sensitive molecule, the absorption ratio of α and β chains of Hb could be considered as a significant biomarker of pollution.

This could be considered as an adaptation of environmental hypoxia, very susceptible to Hb-O_2 association which is often met in fishes. It is concluded that adaptation and fine tuning, however, are not confined to macroscopic biology dealing with entire organisms and their parts but they are evident at the molecular level and govern the machinery of life as explained by the molecular behaviour of Hbs.

Acetylcholinesterase (AchE) is an enzyme responsible for hydrolyzing acetylcholine released at the neuromuscular function and is localized in the synaptic cleft interposed between the nerve terminals and the post-synaptic membrane where a substantial fraction of the enzyme is attached to the synaptic basal lamina (Massolie et al., 1993). The distribution of AchE molecules on the synaptic basal lamina closely matches the distribution of nicotinic acetylcholine receptors as well as other molecules on the pre- and post-synaptic membranes indicating high degree of organization of molecular components at the neuro-muscular junction (Hall, Sanes, 1993). Neuronal communications involve neurotransmitters released from the pre-synaptic nerves interacting with post-synaptic receptors resulting in responses in these target cells. Acetylcholine is the chemical that elicits the skeletal muscle contraction and relaxation.

Acetylcholine is metabolized to choline and acetate by the enzyme AchE found bound to post-synaptic membrane. AchE therefore regulates the level of Ach in cholinergic synapses. Choline produced from the extra-cellular metabolism of acetylcholine by AchE is brought back into pre-synaptic cholinergic terminal by a high affinity of receptive process.

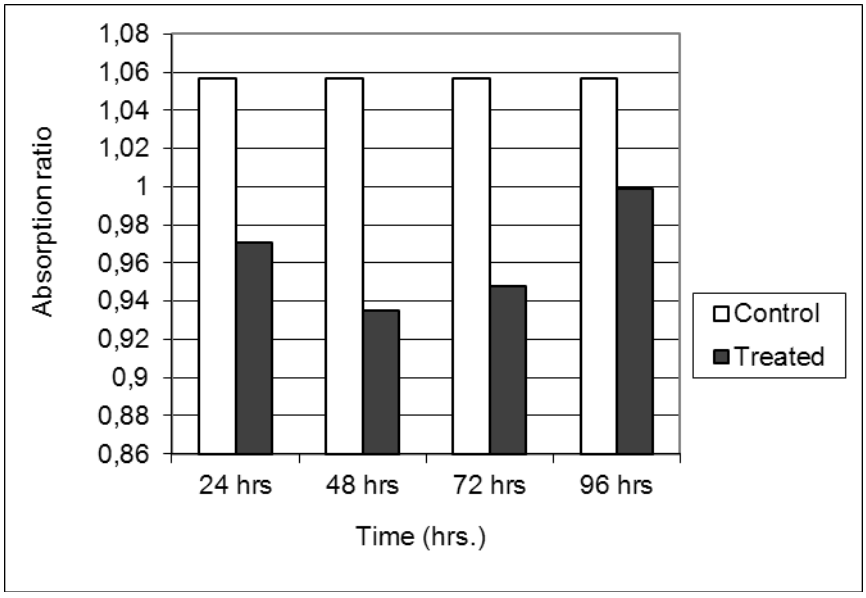

Fig. 1. Effects of endosulfan on absorption ratio of α and β chains of hemoglobin of *Labeo rohita*

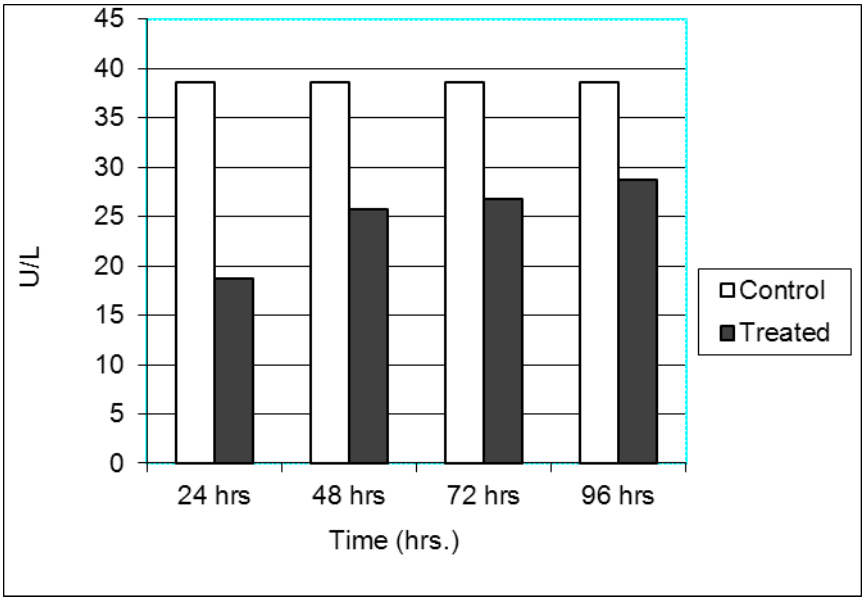

Fig. 2. Effects of endosulfan on cholinesterase activity in the blood of *Labeo rohita*

In the present study, the maximal inhibition of cholinesterase activity in the blood (52%) and brain (65.4%) is observed after 24 hrs following the exposure of endosulfan (fig. 2, 3) and thereafter inhibition becomes less in 48 hrs till 96 hours (tab. 2) showing signs of recovery. The primary acute effect is on the central nervous system as a result of binding endosulfan to GABA receptors in the brain causing hyper-excitation and copious secretion of mucus as well as nervous system mediated effects on respiration and heart. It was also observed that swimming activity was normal in control fishes, but at the sub-lethal concentration of endosulfan the fishes became hyperactive and later on became sluggish after 72/96 hrs, and they flocked mainly around the bubbling aerator suggesting oxygen stress.

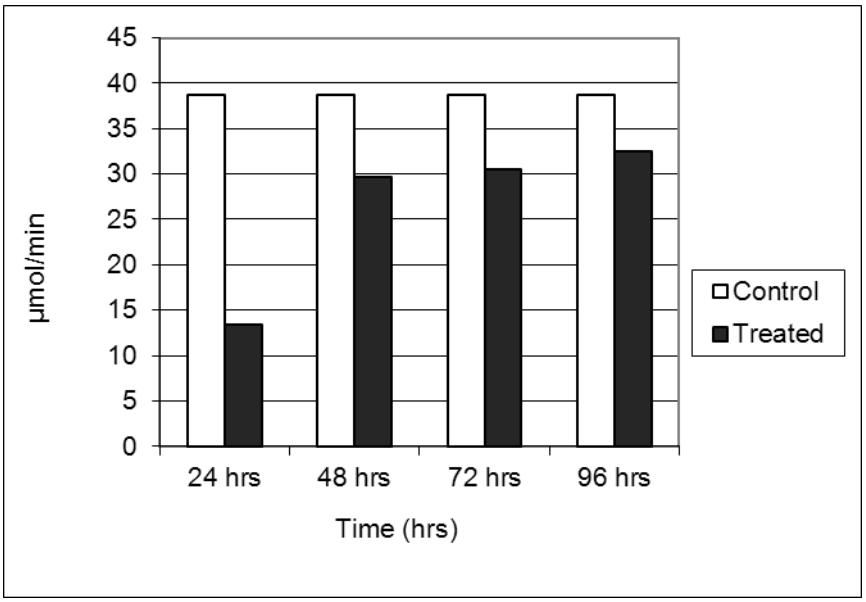

Fig. 3. Effects of endosulfan on cholinesterase activity in the brain of *Labeo rohita*

When AchE activity decreases (tab. 2) Ach is not broken and accumulates within the synapses which therefore cannot function in a normal way (Dutta, Arends, 2003; Kumari, Sinha, 2006). Following the treatment by endosulfan, changes in the brain and plasma cholinesterase activity observed in the present study (tab. 2) reflected in swimming activity like initially surfacing and darting movements and slowly

becoming lethargic and ultimately fishes become immobile. These adverse effects in swimming are due to AchE participating in neuronal or neuromuscular transmission. Endosulfan provoked higher AchE inhibition in the brain than the plasma (tab. 2). The inhibition of AchE consequently leads to excessive accumulation at the synapse and neuromuscular junction resulting in over stimulation of Ach receptors which could ultimately ends in death due to respiratory failure (Gupta, 1994).

Table 2.

Effects of endosulfan on cholinesterase activity in the blood and brain of *Labeo rohita*

Tissue	Time	Control	Endosulfan	p< value
Blood, U/L	24 hrs	38.63±8.87	18.7±3.54	0.009
	48 hrs	38.63±8.87	25.7±6.03	0.107
	72 hrs	38.63±8.87	26.8±1.8	0.03
	96 hrs	38.63±8.87	28.72±5.83	0.146
Brain, μmol/min	24 hrs	38.71±1.25	13.4±5.95	0.001
	48 hrs	38.71±1.25	29.6±12.7	0.168
	72 hrs	38.71±1.25	30.53±10.5	0.165
	96 hrs	38.71±1.25	32.44±8.76	0.219

Note: values are mean, ±standard deviation; number of samples is 5.

The interaction of the pesticides with AchE is widely accepted as key event of the mechanism of toxicity for anti-cholinesterse pesticides and inhibition of this cholinesterase in the blood/plasma creates the prescription that the chemical also causes inhibition of neural AchE. Chemicals are also absorbed in the blood and transported to the peripheral nervous system / outside of the C.N.S. (i.e. separated from the C.N.S. by the blood brain barrier). These blood measures of cholinesterase activity are viewed as a surrogate for the effects of AchE in the peripheral nervous system and C.N.S. Since the data on AchE inhibition in the peripheral nervous system have rarely been reported in humans, the blood cholinesterase inhibition measures are generally the only information available to assess the potential of chemicals to inhibit AchE in the peripheral nervous system. As such, blood cholinesterase inhibition serves as a surrogate for the effects in both the central and peripheral nervous systems because neither of the neural tissues is available for evaluating directly in humans. Therefore, blood cholinesterase data are considered in-the-weight of the evidence analysis of the entire database on the single pesticide where acetylcholinesterase inhibition is a common mechanism of toxicity.

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