Integration of Cholinesterase, cortisol and blood glucose in the fish, Labeo rohita as biomarkers of pollution

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1. Abstract:
Signal transduction by xenobiotics in fish has gained much attention in the recent decade. Organophosphates and carbamates bind specifically to the membrane bound enzyme acetylcholinesterase affecting neural transmission. Since nervous system is one of the important physiological systems in animals, hypofunction of nervous system leads to secondary hypothalamic functions. Even low levels of xenobiotics are sufficient enough to bring about remarkable changes in the functional physiology of fish. In the present study, sub-lethal acute toxicity of methyl parathion in fish, Labeo rohita was assessed using an integration of biomarkers namely acetylcholinesterase, butyrylcholinesterase, cortisol and glucose. These biomarkers were selected as indicators of key physiological functions in the fish Labeo rohita as a function of methyl parathion. These biochemical end-points could therefore be recommended to the policy makers in India for using these biomarkers of pollution to be used as complementary parameters in the assessment of water quality.

Key words: Cholinesterases, Labeo rohita, Biomarker, Methyl parathion

2. Introduction:
India is an agricultural country, pesticide usage has increased to a great extent and as such environmental monitoring of the pesticides has become imperative. Contamination by pesticides is an important public health problem mainly in developing countries. The intensity and the amount of pesticide use for the pest control for the improvement of crops in intensive agriculture are posing serious hazards to terrestrial as well as fragile ecosystems and biota including fish [1, 2, 3, 4, 5].

The physiological stress response is commonly activated in experiencing potentially harmful toxicants in aquatic environment. The response to stress in fish is characterized by the stimulation of the hypothalamus which results in the activation of the neuroendocrine system and subsequently metabolic and physiological changes [6, 7].

Fish being strictly aquatic are directly exposed to these pesticides by absorption through the skin, breathing in and oral intake of pesticide contaminated prey [8]. Among various biomarkers of pesticide exposure the family of cholinesterases (acetylcholinesterase, AchE) and butyrylcholinesterase (BchE) have been widely used as biomarker to evaluate the noxious effects of pesticides especially organophosphates and carbamates. BchE is synthesized in the liver and present in the plasma, smooth muscles, skin, brain and heart. Although, its physiological function is not very clear, it is present as one of the most effective detoxifying enzymes and scavenges a broad range of xenobiotics compounds. Moreover, AchE and BchE are different concerning several aspects, while AchE has in-vivo half life of 120 days, BchE lasts for 7-12 days. AchE is inhibited by substrate excess but BchE is activated by substrate excess.
Fishes give the nippy signal of pesticide intoxication long before their death and therefore, they are taken as model organisms [9, 10, 11, 12]. AchE plays an important role in neurotransmission at cholinergic synapses and neuromuscular junction by rapid hydrolysis of neurotransmitter acetylcholine to choline and acetate. Butyrylcholinesterase is crucial for different parts of immune system. BchE is considered as one of the core detoxifying enzymes [13, 14, 15, 16, 17]. Some of the investigations hypothesize that BchE protect AchE against xenobiotics like pesticides [18].

Inhibition of AchE induces alteration in swimming behaviour, shaking palsy and other undesirable effects. Behavioural changes are the most sensitive indicators of the potential toxic effects. The behavioural and swimming patterns of the fish exposed to methyl parathion are because of the interference of the nervous system and sensory receptors [19]. The effect of certain insecticides on the activity acetylcholinesterase may lead to decreased mobility in fish [20]. In this way, the results may provide insight into the integrity of the ecosystem as a whole. Measurements of toxicity in sentinel species (Labeo rohita) can be used as an early warning of population decline and as an ecological relevant end point. Ecological risk assessment must aim at the preservation of the integrity of the ecosystem.

Plasma cortisol is widely used as a general indicator of stressful conditions in fish [21]. Under the condition of stress, the body of the fish emits immediate responses recognized as primary and secondary responses. The primary response is the perception of an altered state by the central nervous system (CNS) and the release of stress hormones, cortisol and catecholamines into the blood stream. Secondary responses occur as a consequence of the released stress hormone causing changes in the blood and tissue chemistry e.g. an increase in plasma glucose [22, 23]. This entire metabolic pathway produces a burst of energy to prepare the fish for an emergency situation [24, 25]. Since stress has been reported to increase plasma cortisol and glucose levels many researchers consider as a “rule of thumb” that fishes undergoing stressful situation exhibit plasmatic increase of cortisol and glucose [25, 26, 27]. Cortisol activates glycolysis and gluconeogenesis and modulates cardiovascular and respiratory functions [28]. The whole process increases the substrate level (glucose) to produce enough energy according to the demand of the animal [25].

The aim of the present study is to estimate the acute toxicity of the pesticidal stress of methyl parathion and integrate an array of biomarkers: acetylcholinesterase, cortisol and glucose in the most common edible and commercial fish, Labeo rohita.

3. Material and methods:

3.1. Ethical Statement: Presently, we do not have any Ethical Committee in our University. But however, we have followed the ethical norms, which are being followed elsewhere which is evident in the Materials & Methods Section.

3.2. Maintenance of animals: Labeo rohita, a common carp was obtained from the local hatchery. Fishes were acclimated to laboratory conditions for about 5-7 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 composed of ground shrimps available in the local market to avoid the possible effects of starvation. The feeding and maintenance of the fishes and physico-chemical characteristics of the aquaria water were measured (Tab. 1). Short-term test of acute toxicity over a period of 96 h were performed on the fishes following the renewal of bioassay. The fishes were exposed intra-coelomically
with 1/3rd of LC$_{50}$ value of the pesticide methyl parathion. After 24, 48, 72 and 96 h of exposure fishes were processed for further investigations.

3.3. Determination of LC$_{50}$: The experiments were 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 [29]. The LC$_{50}$ of methyl parathion for the fish *Labeo rohita* was 16.8 ppm.

3.4. Blood collection: The fishes were taken out of the aquarium water individually through fish net with a minimum possible disturbance. After preliminary investigations, the blood samples were collected from caudal fin as described by many authors. In the present study, the blood collection from caudal fin had to be abandoned because there was an unusual elevation in Lactate dehydrogenase (LDH) and Creatine phosphokinase (CPK) activities which were recorded due to leakage of these enzymes from the surrounding muscle tissues. Thus, cardiac sampling was the only suitable method available as an alternative to obtain blood under the present study. After the blood collection, the serum were separated and processed for enzymatic and hormonal assays.

3.5. Assay of cholinesterase: Estimation of acetylcholinesterase was done by the spectrophotometric method of Ellman *et al* (1961) [30] using DTNB (dithionitrobenzoic acid) as a chromogen and acetylcholine iodide as the substrate. The reaction was rapid and the assay was also sensitive (i.e. 10µl sample of blood was adequate). For brain cholinesterase activity, 100 mg of brain was homogenized in 5 ml of phosphate buffer (pH-7.4) and assayed for acetylcholinesterase activity.

3.6. Behavioural: The behaviour and condition of fishes in both control and treated were noted every 24h upto 96h. Behavioural manifestations of acute toxicity of methyl parathion were observed.

3.7. Assay of Cortisol: The concentration of serum cortisol was estimated by Chemiluminescence Immuno Assay Reader Neo-Lumax Model-4901 because it has measuring ranges over many other Immunoassay methods and excellent for detection and quantification.

3.8. Assay of blood glucose: Serum glucose was determined spectrophotometrically by GoD-PoD method [31].

4. Results:

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Parameter</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Temperature</td>
<td>(24±2)°C</td>
</tr>
<tr>
<td>2.</td>
<td>pH</td>
<td>7.1± 0.2 at 24°C</td>
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<tr>
<td>3.</td>
<td>Dissolved Oxygen</td>
<td>8.5 ±0.5 mg/L</td>
</tr>
<tr>
<td>4.</td>
<td>Total Hardness</td>
<td>23.4± 3.4mg CaCO$_3$/L</td>
</tr>
<tr>
<td>5.</td>
<td>Conductivity</td>
<td>&lt;10 µs/cm</td>
</tr>
</tbody>
</table>
Figure 1: Butyrylcholinesterase Activity (BchE) in Plasma and Acetylcholinesterase activity (AchE) in Brain

Figure 2: Cortisol and glucose levels in Serum

5. Discussion:

Cholinergic biomarkers are generally divided into two main classes: acetylcholinesterase and butyrylcholinesterase in most of the organisms which are extensively used as a diagnostic tool for ecotoxicological assessment of pesticides as well as xenobiotics in aquatic environment [10]. Inhibition of cholinesterase has been reported in several tissues and organs with a focus on brain tissues [32, 33]. In the present study, sensitivity of the fish, *Labeo rohita* to the exposure of methyl parathion has shown that acetylcholinesterase activity in the brain is inhibited significantly p<0.14, 0.002, 0.001 and 0.009 in 24, 48, 72 and 96h respectively. When, AchE activity decreases (Fig. 1) Ach is not hydrolysed and accumulates within the synapses which therefore cannot function in a normal way [34, 35]. Similarly, butyrylcholinesterase activity decreased significantly p<0.07, 0.01, 0.001 and 0.004 in 24, 48, 72 and 96h respectively. Following the exposure of methyl parathion, the inhibition of AchE in the brain has been observed in the present study which reflected in the behavioural pattern of the fish. Fish behaviour
provides a link between physiology and ecology and its environment [36]. It is an attempt to adjust to external and internal stimuli to meet the challenges of surviving in an altered surrounding. Behaviour represents an integrated response of the fish to the toxicant (methyl parathion) induced stress [37]. It was observed in the present study that there was a fast and abrupt movement of the fish in the experimental tank had a tendency to escape from toxic water. Thus, behaviour is a selective response that is constantly adapting through direct interaction with physical, chemical, social and physiological aspects of the environment. In the present study, the control fish behaved normally that is, they were active with well co-ordinated movements. They were alert to the slightest disturbances but in the toxic environment the fish exhibited irregular, erratic swimming movements and loss of equilibrium due to the inhibition of AchE activity leading to the accumulation of acetylcholine at the cholinergic synapses resulting in hyperstimulation and finally lethargic condition. Similar reports have been made by Munshigeri and David, 2005; Kumari et al, 2011 [38, 39]. Since inhibition of AchE is a typical characteristic of organophosphate [40, 41, 42, 43, 44] behavioural pattern changes. The fish secreted excess mucous all over their bodies which formed a barrier between the body and the toxic media thereby reduced contact with the toxicant to minimize their irritating effect and eliminating the toxicant through epidermal mucus. Similar observations were made by Kumari and Sinha, 2011; Rao et al 2003 [39, 45]. Gulping of air at the surface was observed. Surface phenomena and gulping of air may help to take in more air to meet the challenge of hypoxic condition. The behavioural responses can be used as a tool in biomonitoring programme to monitor ecotoxicity risk of methyl parathion to the test species.

It is clear from Fig. 1 that the percentage of inhibition of butyrylcholinesterase was 52.9%, whereas AchE in the brain was 76.7% after 96h of exposure as compared to the Control. Evidently, the inhibition in the plasma butyrylcholinesterase by methyl parathion was much less (Fig. 1) which could be due to the irreversible binding of BchE to the molecule of the pesticide and inactivated the methyl parathion molecule before it escaped from the circulation and reached the physiological target, the brain. Similar observation has been reported by Ravesh et al, 1997 [46]. It is known that loss of AchE function leads to muscle paralysis and may cause death by hypoxia. BchE can be considered as an endogenous scavenger of anti-cholinesterase compounds like methyl parathion because BchE detoxifies them before they reach AchE at physiologically important target sites. Thus, BchE acts as prophylactic agent against methyl parathion. As such, the methyl parathion is mediated by the inhibition of neurotransmission since nervous system has integrative and coordinative functions to maintain normal hormonal homeostasis dysfunction of the nervous system leading to cascade of inhibitory events in endocrine system.

Cortisol is the principal glucocorticoid secreted by the interrenal tissue located in the head of the teleost fish [47]. This hormone is released by the activation of hypothalamus-pituitary-interrenal axis (HPI axis) [48]. When the fish undergoes stressed condition, the hypothalamus releases corticotropin-releasing factor (CRF) towards blood circulation. This polypeptide further stimulates the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary [49] which finally activates the release of cortisol by the inter-renal tissue.

Serum cortisol has been widely used to assess the state of health of the fish exposed to stress [50, 51]. In the present study, since there is a significant increase in the cortisol concentration (p<0.003, p=0.66, p<0.26, p<0.002, p<0.006, p=0.16 in 1st, 2nd, 3rd, 4th, 5th and 6th h respectively) immediately after exposure (Fig. 2), thus it could be concluded that fishes exposed to the sub-lethal concentration of methyl parathion undergo an immediate stress and continue to be under stress till 6th h with a parallel increase of glucose concentration (Fig. 2). Many authors have reported that it is catecholamines which are known as “fight and flight” hormone which increase glucose initially and thereafter the cortisol further increases the blood glucose [25]. Glucose increase is a general response of fish to acute pollutant effects, including organophosphates [52]. Hyperglycemic effect of cortisol was due to glycogenolysis to
meet the energy demand during the pesticidal stress and secretion of cortisol was slower than catecholamines but its effects were more prolonged for the restoration of homeostasis [25]. An adverse role of cortisol was suggested to be linked to mobilization of energy reserves through catabolic functions. After stress, the cortisol level showed no increase (Fig. 2) to avoid tissue damage because it is well known that high level of cortisol could cause death in fishes by tissue degeneration and aberration in homeostatic mechanism. Thus cholinesterases, cortisol and glucose could be treated as biomarkers of pollution for acute stress.

6. Conclusion:

In conclusion, the importance of integrating biomarkers to assess the effect of methyl parathion in the fish, Labeo rohita has been confirmed by the results showing significant inhibition of cholinesterase activity and increase in the stress hormone, cortisol resulting in the increase in blood glucose level to meet the energy demand during stress. This showed the significance of integrating biochemical parameters in assessing methyl parathion toxicity in fishes. It is therefore recommended to the policy makers in India that these biochemical end points to be used as biomarkers as complementary parameters in the assessment of water quality.

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