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Role of Ascorbic acid in the brain and gills of fish, *Labeo rohita* and *Cirrhinus mrigala* following the sub- acute exposure of methyl parathion

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ABSTRACT Asccorbic acid is a key antioxidant in the brain and gill of fish. In the brain, ascorbic acid scavenges reactive oxygen species (ROS) generated during synaptic activity and brain metabolism where it is oxidized to dehydroascorbic acid and released into extracellular space. Intrinsic properties of ascorbic acid beyond acting as an antioxidant are important in its role as a key molecule in the metabolism of fish. Ascorbic acid can switch brain in glucose metabolism from glucose consumption, uptake and use of lactate as metabolic substrate to sustain synaptic activity. A sub- acute exposure to methyl parathion causes hypoxic condition due to the detachment of secondary lamellar epithelium from underlying pillar cell system. Results in the present study show that ascorbic acid content in the brain and gill was higher in *Cirrhinus mrigala* than *Labeo rohita*. Following the sub-exposure of methyl parathion, the brain ascorbic acid significantly increased ($p<0.04$; 0.02 and 0.08) in 24, 48 and 96h respectively in the *Labeo rohita* while the significant increase ($p<0.04$, 0.06, 0.002) in 24, 48 and 96h in *C. mrigala* was observed. Similar pattern was observed in the gill ascorbic acid in both of the fishes under study. The physiological significance of ascorbic acid a wonder and miracle molecule is reported herein for the first time.

KEYWORDS : Ascorbic acid, Brain, Gill, Labeo rohita, Cirrhinus mrigala

INTRODUCTION

Excessive use of pesticides has resulted in serious problems as well as health hazards (Olea and Fernandez, 2007). Many pesticides are known inducers of oxidative stress by directly producing reactive oxygen species (ROS) and impede the natural antioxidant or oxygen free radical scavenging system (Geter*et al*, 2008). Pesticides disturb the prooxidant- antioxidant system of the cells, thereby leading to the generation of free oxygen radical and reactive oxygen species (El- Gendy*et al*, 2010) causing oxidations in chain. All the biomolecules in the cell (lipids, proteins, polysaccharides and nucleic acids) are potential substrates for ROS (Manduzio *et al*, 2005). Such an effect may be at cellular or molecular level but ultimately it would lead to physiological, pathological and behavioural disorders that may prove fatal to the organisms (Ray and Sinha, 2016a).

Biological complex antioxidant system includes antioxidant enzymes and non-enzymatic antioxidants such as ascorbic acid and vit E acting against intracellular oxidative stress (Agarwal *et al*, 2003). Ascorbic acid has potential role to reduce the activity of free radical induced reactions (Holloway and Peterson, 1984). Ascorbic acid is a very valuable scavenger in biological system. It can reduce the toxic effects of environmental toxicants (Achary *et al*, 2008). Therefore, increasing the bioavailability of ascorbic acid may reduce the effects of environmental toxicants in fish. Ascorbic acid has a simple chemical structure and small molecular weight despite its high density and negative charge due to the presence of acid and carbonyl groups. Ascorbic acid is a water soluble free radical scavenger. Moreover, it regenerates Vitamin E in cell membrane in combination with GSH or compounds capable of donating reducing equivalents. Ascorbic acid changes to ascorbate radical by donating an electron to lipid radical in order to terminate the lipid peroxidation chain reaction. The pairs of ascorbate radicals react rapidly to produce one molecule of ascorbate and one molecule of dehydroascorbate. The dehydroascorbate does not have any antioxidant capacity. Hence, dehydroascorbate is converted back to ascorbate by addition of two electrons. The last stage of the addition of two electrons to the dehydroascorbate has been proposed to be carried out by oxidoreductase. Its precursor is glucose (Sinha, 1966).Biochemical studies on the organisms inhabiting polluted aquatic habitats indicate the extent of physiological stress experienced by them and also their efforts to resist the toxic effect of the pesticides. Under the pesticidal stress, the energy demand in the organisms becomes high (Ray and Sinha, 2014).

Methyl parathion has become the most widely used class of pesticides in the world replacing the persistent problematic organoclorine compounds. Methyl parathion is widely used because of its low toxicity to mammals and high sensitivity for insects compared with other organophosphate pesticides. The literatures regarding the changes in the ascorbic acid content during pesticidal stress on animals are very scanty. Therefore, in the present investigation effect of methyl parathion on the ascorbic acid content in the brain and gill of the fish, *Labeo rohita* and *Cirrhinus mrigala* because of their wide availability and suitability for toxicity testing.

MATERIALS AND METHODS

Ethical Statement:

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

Maintenance of Animals and the treatment:

Labeo rohita, and *Cirrhinus mrigala*, both common carps were 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 96h were performed on the fishes following the renewal of bioassay. The fishes were exposed intracoelomatically with 1/3rd of LC_{50} of the pesticide methyl parathion. After 24, 48, 72 and 96 h of exposure fishes were sacrificed for biochemical study of tissue ascorbic acid content.

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 (Finney, 1971). The 1/3rd of LC_{50} of the pesticide methyl parathion was 16.8 ppm for *Labeo* rohita and 14.0 ppm for *Cirrhinus* mrigala.

Determination of Ascorbic acid in tissue:

The method for extraction of ascorbic acid from the tissue was that of Kanungo and Patnaik (1969) and determined according to the 2,4- Diphenyl hydrazine method of Roe (1954). This method is a spectrophotometric method of measurement of extinction of colour developed in the reaction mixture. To read the extinction of the colour developed was read in Shimadzu UV-1800 spectrophotometer at 540 nm.

Statistical Analysis:

The statistical analysis of the results wasmade using a statistical software package Systat v. 7.0 (Spss. Inc., Michigan, Chicago, USA). The results were expressed as mean ± s.d. The sample size in each group was 5 (n=5).

RESULTS

In the present study Figs. 1,2,3& 4 show that ascorbic acid content in the brain of *Labeo rohita* increases till 48h and then decrease during 72h and 96h as compared to 48h. On the contrary, in the fish *Cirrhinus mrigala* ascorbic acid content increases till 96h following the exposure of methyl parathion. Figs. 3 & 4 show that ascorbic acid content of the gills of *C. mrigala* is relatively higher than *L. rohita*. Following the exposure of methyl parathion there is a marked increase of ascorbic acid content in the gills of both the fishes during 24h and thereafter no increase till 96h in *Labeo rohita* but on the contrary, in *Cirrhinus mrigala* ascorbic acid increases till 72h (Figs. 3 & 4).

DISCUSSION

Fishes protect themselves from xenobiotics generating oxidative stress by evolving a complex antioxidant defence systems. The systems are beginning to receive attention as a biological means to reduce damage to fishes (Wedderburn *et al*, 2000; Lionetta *et al*, 2003; Pandey*et al*, 2003). As mentioned earlier ascorbic acid is one of the non-enzymatic antioxidants that can protect the cells from reactive oxygen species (ROS), thus preventing tissue damage. Ascorbic acid is involved in the first line of antioxidant defence protecting lipid rich membranes and different cellular proteins from oxidative damage. Ascorbic acid is considered an important neuroprotective agent since it is a potent reducing agent, scavenging ROS, production and sustaining SOD and catalase activities. Ascorbic acid is an essential micronutrient in the brain. Neurons are highly sensitive to ascorbate deficiency; perhaps they have 10 fold higher rates of oxidative metabolism. A neuroprotective role of ascorbate is suggested by the existence of homeostatic mechanisms that maintain the high concentrations of ascorbate in cerebrospinal fluid (CSF) and in neurons (May, 2012).

It is known that brain is involved in processing and regulating several physiological functions of the fish explaining its elevated energetic demand (Ray and Sinha, 2016a). In fact, even at rest the brain accounts for upto 25% of O_2 consumption in adult human. Most of the energy utilized can be attributed to neuron and synapse related process. Restoration of membrane potential after depolarization could be the main energy demand of neurons (Attwell and Laughlin, 2001).

The central nervous system is especially sensitive to the damage caused by free radicals. In the brain, there is a high content of polyunsaturated fatty acids (PUFA) which are the main substrates for lipid peroxidation (Ray and Sinha, 2016a). The brain has a low content of antioxidant enzymes and is represented mainly by ascorbic acid and SOD. The brain is predominantly perfused by blood because it needs high amount of O_2 for its functional and metabolic processes. There are reasons why oxidative brain injury can be developed negatively influencing cell integrity and functions by lipid peroxidation as well as by damage of DNA and proteins under pathological conditions (Ray and Sinha, 2016a).

The brain relies on glucose metabolism to sustain the energetic cost of synaptic activity. Glucose is good source of energy making it an essential metabolic fuel and its metabolism correlates with the brain function and as such that there is a continuous flow of glucose to the brain for normal functioning. It is evident from the Figs. 1 & 2 that the ascorbic acid content is higher in brain of *Cirrhinus mrigala* than *Labeo rohita*. With the exception of muscle, the tissues of high metabolic activity have higher concentration of ascorbic acid (Harper, 1969). Therefore, the higher content of ascorbic acid in the brain of *Cirrhinus mrigala* is indicative of the fact that it is more active than *Labeo rohita*. It has also been observed in our laboratory that it was difficult to catch *Cirrhinus mrigala* in aquarium than *Labeo rohita*. It has been observed in the present study that following the exposure of methyl parathion there was an almost uniform increase in ascorbic acid content till 96h in *C. mrigala* while the increase in *L. rohita* was significant only till 48h and then decreased till 96h. The differential increase/ decrease following the exposure of the pesticide suggest that there is a differential mode of action of methyl parathion in the two fishes under study.

There is a very little oxygen dissolved even in best aerated water as compared to the oxygen content of air. Additionally water is an extremely dense medium as compared to air and therefore, a very large quantity of water must be inspired and expired to extract oxygen to maintain life functions. In comparison with land animals, fishes must extract the oxygen they need from the medium about 840 times dense and 55 times less oxygen and in which diffusion through gill membranes takes about 300,000 times longer than in air (Schumann and Piiper,1966). Therefore, it is not surprising that a large proportion of metabolic energy of fish is needed for respiration. This problem is solved by large gills present in the fish, but at the same time very large gills offer increased resistance to the flow of water and amount of energy must be diverted to overcome this resistance. This resistance, to some extent is overcome only by constant swimming with open mouth. In order to fulfil various respiratory and excretory functions, the gill must be relatively open to the outer medium. This, on the other hand, makes them the first organ to be affected by the toxic substances in the aquatic medium. For this reason, the gill also represents the weakest link in the fish's line of defence against osmotic stress and other toxicants.

To study the effect of methyl parathion on the respiratory processes of the fishes understudy, histopathological studies were also conducted. Disturbances in the gaseous exchange in both fishes were due to the gill damage, induced by methyl parathion. The main effect of the pesticide was detachment of the secondary lamellar epithelium from the underlying pillar cell system. Hyperplasia was subsequent to the epithelial cells which suggested that the former was a consequence of the later. The hyperplasia was characterized by the cellular proliferation in the inter-lamellar region of the respiratory lamellae, decreasing the surface area and making gaseous exchange more difficult (Ray *et al*, unpublished data). The fishes try to compensate the lower level of oxygen in their tissues by an increase in respiratory frequency which was evident from the opercular movement. Since, the gulping of air was more, the possibility of gaseous exchange due to the microturbulence was more.Gulping of air may help to ease the respiratory stress and avoid contact of toxic medium. It was also observed during the experiment that the surfacing phenomena were more following the exposure of methyl parathion which might increase the oxygen uptake. Following the exposure of sub-acute dose of methyl parathion, the fishes undergo hypoxic condition (Ray and Sinha, 2014). In the hypoxic condition there is a great need of ascorbic acid for preventing the propagation of lipid peroxidation or scavenging free radicals, increasing the tensile strength of the cell membrane, thus preventing the rupture and destruction of gill cell membrane and also various tissues (Ray and Sinha, 2016a; Kumari *et al*, 2011).

The response to a wide variety of stressors is controlled by adrenocorticotropic hormones (ACTH) from the pituitary which controls the release of catecholamines (Cortisol). Earlier, the authors have reported the increase of cortisol (stress hormone) following

the exposure of methyl parathion in both the fishes under study (Ray and Sinha, 2016b). Among the physiological changes caused by these hormones are the dilation of gill filament's arteries, increase of stroke volume of heart, increase glycogen metabolism and depression of immune system (Mazeaud and Mazeaud, 1981; Ray and Sinha, 2016c). Sub lethal exposure of methyl parathion led to changes in ascorbic acid concentration in the gills of two fishes understudy but the pattern of fluctuation varied.

Gills represent a thin and extensive surface in intimate contact with water. Due to the constant contact with the external environment, gills are the first target of water borne toxicants (Fernandez and Mazon, 2003). Fish gills have large surface area covered by their epithelial cells and are well supplied with blood to facilitate gas exchange.

Ascorbic acid content increases during stress indicating its role in neutralizing the effect of peroxyfree radicals. Similar findings have been reported by Rao and Chinmoy (1986). It is suggested that ascorbic acid has a protective and therapeutic effect against the pesticidal intoxication. After 72h of exposure there was a recovery towards normalcy in the fish (Ray and Sinha, 2014) which could be due to ascorbic acid.

It is suggested that reactive oxygen species (ROS) is a part of normal life but during stress it significantly increases and their interaction with the host antioxidant defence appears to exert marked influence on cellular chemistry in the fishes under study. Brain is a logical target of free radical attack because of its large concentration of unsaturated fatty acids and high rate of oxidative metabolism (Ray and Sinha, 2016a). Similarly, gills have also large content of ascorbic acid for proper development and functioning. Gills are very prominent organs because they are major interface with the environment where most of the exchange between the fish and its environment takes place, the surface area of the gills being approximately twice that of skin. As such, high gill ascorbic acid content is essential in neutralizing the toxic effects of xenobiotics.

CONCLUSION

Generation of reactive oxygen species is a part of normal life and their interaction with host antioxidant defence systems appear to exert a significant influence on cellular chemistry in health and disease. Brain is a logical target of free radical attack because of its large concentration of unsaturated lipids and high rate of oxidative metabolism. Oxidative radicals have been implicated in a broad range of neuropathological conditions.

The bioregulatory role of ascorbic acid to protect extracellular protein function through gene expression has been highlighted (Griffiths and Lunec, 2001). The stimulatory action of ascorbic acid is indicated by increase in cell population, protein content and level of lysosomal enzymes, antioxidants and enhanced capacity for phagocytosis (Agarwal *et al*, 2003). Ascorbic acid is one of the most important defence systems against free radicals and peroxides that are generated during cellular metabolism (May, 2000). The preventive and curative properties of ascorbic acid against the pesticidal toxicity have been reported herein which have been corroborated by other authors.

In short, ascorbic acid has multifunctional properties as such it could be regarded as a wonder and miracle molecule that nature has provided to all the aerobic organisms for survival against free radicals induced oxidative stress by methyl parathion in particular and other xenobiotics in general.

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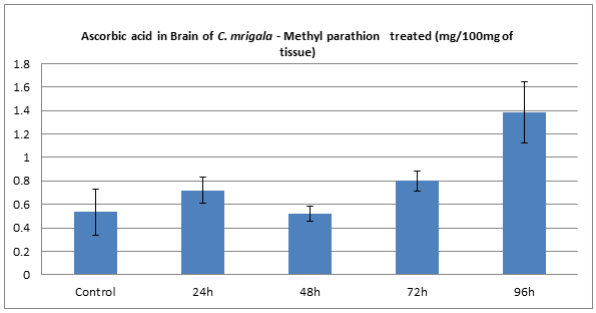

Fig. 1 Ascorbic acid in Brain of *C. mrigala* - Methyl parathion treated (mg/100mg of tissue)

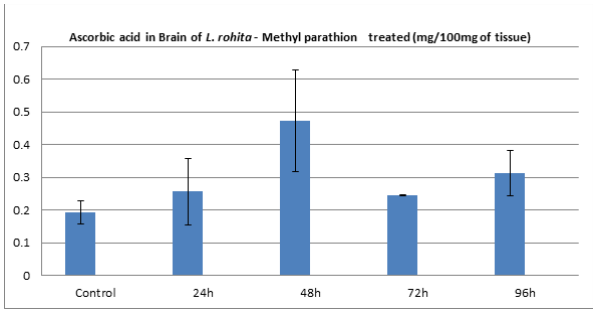

Fig. 2 Ascorbic acid in Brain of *L. rohita* - Methyl parathion treated (mg/100mg of tissue)

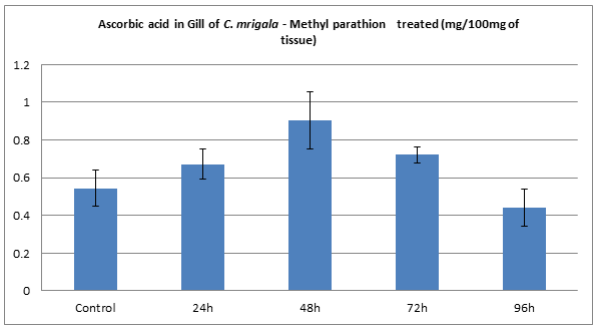

Fig. 3 Ascorbic acid in Gill of *C. mrigala* - Methyl parathion treated (mg/100mg of tissue)

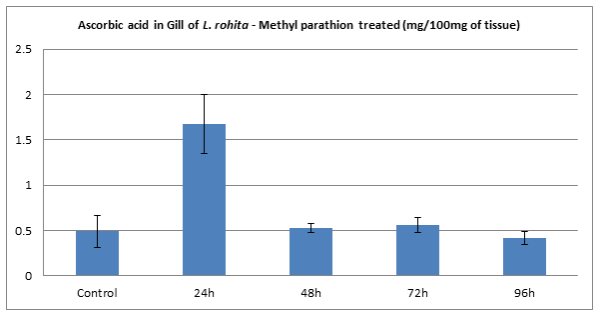

Fig. 4 Ascorbic acid in Gill of *L. rohita* - Methyl parathion treated (mg/100mg of tissue)

Table1. Physico-chemical characteristics of aquaria water

Sl no.	Parameter	Value
1.	Temperature	(24±2) ^{0}C
2.	pH	7.1± 0.2 at 24^{0}C
3.	Dissolved Oxygen	8.5 ±0.5 mg/L
4.	Total Hardness	23.4± 3.4mg $CaCO_3$/L
5.	Conductivity	<10 µs/cm

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