

Environmental Acidification Impact on Fisheries by Changing Oxidative Markers of Liver and Intestine of Freshwater Fish *Cyprinus Carpio*.L

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Received date: January 27, 2016, Accepted date: March 26, 2016, Published date: March 30, 2016

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Abstract

The oxygen consumption rate provides a critical index of altering the oxidative metabolic enzymes in freshwater fish *Cyprinus carpio*. The present study emphasized to understand the increased anaerobic metabolic rates due to lowering the oxygen levels by the acidification of aquatic environment. where the oxidative enzymes like glucose-6-phosphate dehydrogenase (G-6-PDH), lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) in liver and intestine of *Cyprinus carpio*.L exposed at 1 to 7 and 1-15th days of sub lethal concentration of acidic media pH5.0 besides controls, the activities of G-6-PDH, SDH and MDH decreased at day 7th and 15th in the liver and intestine of experimental fishes with a corresponding increased in the activities of LDH and GDH of experimental fish over control. But in between the two organs the degree of decreased activity was observed greater in liver than the intestine of fish. Probably, more destruction of oxidative metabolism occurs in liver than the intestine of fish. Remarkably, in the case of GDH activity was high in liver than the intestine of fish.

Keywords: Acidification; *Cyprinus carpio*; Oxidative enzymes; Fish

Introduction

Aquatic environment is subject to number of variations in temperature, humidity, light, wind, pH, salt concentration and oxygen concentration [1] regards pH as one of the major factors of aquatic environment for the survival and distribution of organisms. Acidification is an indirect result of sulphur and nitrogen pollutants which are discharged from modern industries in large quantities [2,3]. The occurrences of alterations in the environmental pH are very frequent, which result in the rapid disappearance of fish population [4]. The studies on the alterations in the pH and resultant effects on fish population have attracted the attention of several investigators throughout the world. As a result of acidification, fish communities have suffered significant changes in community composition attributed to high mortality, reproductive failure, reduced growth rate, skeleton deformities, and increased uptake of heavy metals. Atlantic salmon fry have been observed to die when water with pH<5 was introduced interbreeding pools. In fish embryos, death appears to be due to corrosion of epidermal cells by the acid. Acidity also interferes with respiration and osmo regulation, in all fish at a pH of 4-5 the normal ion and acid base balance is disturbed. Sodium ion uptake is inhibited in low pH waters with the low salinity. Small fish are especially affected in this way because due to their greater ratio of body and liver and intestine surface area to overall body weight, the determined ion flux proceeds faster. In all fish low pH water causes extensive liver damage. Reactive oxygen species (ROS) initiated and causes the tissue damage my continuous exposure of liver tissue due to oxidative stress induced by the acidified media and hypoxia [5].

Energy a vital force in a biological system, occupies a key position in the metabolic machinery. The free energy liberated by the breakdown of organic constituents, mainly carbohydrates, as a rule, transforms

itself into the phosphate bond energy (ATP) before its utilization in any work [6]. The ATP synthesis in any biological system mainly depends on the phosphorylation of ADP associated with glycolysis and biological oxidation involving the citric acid cycle and electron transport system [7]. Any change in these cycles of cell leads to alterations in the energy budget [8]. Pollutants affect not only the rate of carbon flow in a given metabolic pathway but also the contribution of different metabolic pathways to the total metabolism of an animal.

Changes in mortality, protein metabolism was observed in both liver and intestine tissues of *Cyprinus carpio* under altered pH medium [2,3,9]. However, the literature is scanty on oxidative enzymes alterations in liver and intestine tissues in *Cyprinus carpio* on exposure to acidic media. In view of this, an attempt has been made in the present investigation to study the effects of acidic media on different oxidative enzyme levels in both tissues of fish.

Materials and Methods

Selection of an animal

The common fresh water carp, *Cyprinus carpio* (Linnaeus) is an economically important edible fish having great commercial value. It is abundantly available in fresh water tanks, ponds in and around Tirupati. Besides its wide availability and commercial importance, this fish is known for its adaptability to laboratory conditions and suitability to acidic media studies [2]. Hence this fish is selected as the experimental model for the present investigation. A brief account on the biology of its may constitute a suitable preamble.

Procurement and maintenance of experimental

The experimental material consists of the fish, *Cyprinus carpio* (L) with a weight of 25g ± 2g. They were collected as for the

recommendation of the local department of fisheries. Government of Andhra Pradesh, India. The adult stage was kept in large aquaria with continuously flowing dechlorinated water to acclimatize them to laboratory conditions ($27^{\circ}\text{C} \pm 2$, $\text{pH } 7.0 \pm 0.2$ and light period of 12 h per day) for fifteen days before they were used for experimentation.

Procurement of chemicals

All the chemicals used in this study were of analar (AR) grade and obtained from the following scientific companies:

- Sigma (St. Louis, MO, USA).
- Fisher (Pittsburg, PA, USA)
- Merck (Mumbai, India).
- LOBA
- Qualigens (Mumbai, India).

Biochemical analysis

Glucose-6- Phosphate Dehydrogenase (G-6-PDH) (E.C: 1.1.1.49): Glucose-6-phosphate dehydrogenase activity was assayed by the method of [10]. 10% (W/V) liver and intestine homogenates were prepared in ice cold sucrose 0.25 M solution and centrifuged at 1000 g for 15 min at 4°C . The reaction mixture in a total volume of 2.5 ml contained 1.0 ml of sodium phosphate buffer (pH 7.4), 200 μ moles of glucose-6-phosphate (disodium salt), 300 μ moles of INT and 3.5 ml of NADP. The reaction was initiated by the adding 0.5 ml containing 50 mg of tissue as an enzyme source. The reaction mixture was incubated at 37°C for 30 minutes and the reaction was stopped by the addition of 5 ml glacial acetic acid. The formazan formed was extracted with 5 ml of toluene. The activity was expressed in μ moles of formazan formed / mg protein / hour.

Lactate Dehydrogenase (LDH) (E.C:1.1.1.27): (L-lactate: NAD+ oxidoreductase)

Lactate dehydrogenase activity was determined by the method described by Lee and Lardy [11]. 10% (w/v) homogenates of the liver and intestine tissues were prepared in ice cold 0.25 M sucrose solution and centrifuged at 1000 g for 15 minutes at 4°C . The supernatant fraction was used for enzyme assay. The reaction mixture in a final volume of 2 ml contained 400 (μ moles of sodium lactate, 1000 μ moles of phosphate buffer (pH 7.4), 100 μ moles of NAD and 300 μ moles of INT. The reaction was initiated by the addition of 0.2 ml of homogenates containing 20 mg of tissue as an enzyme source and incubated for 30 minutes at 37°C and the reaction was stopped by the addition of 5 ml of glacial acetic acid. Zero time controls (ZTC) were maintained by addition of 5 ml of glacial acetic acid prior to the addition of the enzyme source to the incubation mixture. The formazan formed was extracted overnight into 5 ml of toluene at 5°C . The colour developed was measured at 495 nm in a spectrophotometer against the toluene blank. The enzyme activity was expressed in μ moles of formazan formed / mg protein / hour.

Succinate Dehydrogenase (SDH) (E.C: 1.3.99.1): (Succinate acceptor oxidoreductase)

The specific activity of SDH was assayed by the method of [12] as modified by Bhaskar and Govindappa [3] with slight modifications. 10% (W/V) homogenates of the liver and intestine tissues were prepared in 0.25 M sucrose solution and centrifuged at 1000g for 15 minutes at 4°C . The supernatant fraction was used for Enzyme assay. The reaction mixture a final volume of 2 ml contains of 1.0 ml of

phosphate buffer (pH 7.0), 400 μ moles of sodium succinate, 100 μ moles of NAD and 300 μ moles of INT. The reaction was initiated by adding 0.2 ml of homogenates containing 20mg of tissue as an enzyme source. The incubation was carried out for 15 minutes at 37°C and the reaction was stopped by the addition of 5 ml of glacial acetic acid. The subsequent steps were followed same as described for LDH. The activity was expressed in μ moles of formazan formed / mg protein / hour.

Malate Dehydrogenase (MDH) (E.C: 1.1.1.37) (L- Malate NAD+ Oxidoreductase)

Malate dehydrogenase activity was assayed by the method of Lee and Lardy [11]. Tissue homogenates like liver and intestine were prepared in ice-cold 0.25 M sucrose solution and centrifuged at 2000rpm for 15 minutes. The supernatant fraction was used for the assay of the enzyme. The final 2.0 ml volume reaction mixture containing 400 μ moles of sodium malate, 1.0 ml of phosphate buffer (pH 7.4), 100 μ moles of NAD, and 300 μ moles of INT. The reaction was initiated by the adding of 0.2 ml of enzyme source. The incubation was for 30 min at 37°C and the reaction was blocked by the addition of 5 ml of glacial acetic acid. The formazan formed was extracted overnight in 5 ml of toluene at 5°C . The colour developed was measured at 495 nm in spectrophotometer against a toluene blank and the activity of MDH was expressed as μ moles of formazan formed / mg protein / hour.

Glutamate dehydrogenase (GDH) (EC:1.4.1.3) (L-Glutamate; NAD + Oxidoreductase)

Glutamate dehydrogenase (GDH) activity was assayed by the method of Lee and Lardy [11]. 5% (W/V) of liver and intestine tissue homogenates were prepared in ice cold sucrose (0.25M) solution and the contents were centrifuged at 1000 g for 15 minutes at 4°C . The supernatant part was used as an enzyme source. The reaction mixture in a total volume of 2 ml contained 1.0 ml of phosphate buffer (pH 7.4), 400 μ moles of sodium glutamate, 100 μ mole of NAD, 300 μ moles of INT and 0.2 ml containing 10 mg of tissue as an enzyme source. The reaction mixture was incubated at 37°C for 30 minutes. The reaction was arrested by the addition of 5 ml glacial acetic acid and the formazan formed was extracted into 5 ml of toluene. The intensity of the colour was read at 495nm against the toluene blank. The enzyme activity was expressed as μ moles of formazan formed / mg protein / hour.

Statistical analysis

All the data are expressed as mean \pm SD. One-way ANOVA using SPSS. 16.0v software was used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

Glucose-6-phosphate Dehydrogenase

The glucose-6-phosphate dehydrogenase levels of experimental fishes were decreased in liver and intestine over control. The percent change of glucose-6-phosphate dehydrogenase level of experimental fish was decreased in liver at day 7 (-33.00) and at day 15 (-49.00) and also same trend was day 7 (-38.5) and at day 15 (-51.00) over control fish. But in between the two organs the degree of decrease was greater in liver than the intestine of fish and also the impact of pH on G-6-

PDH were more with increase in the duration of exposure days i.e. 7th and 15th day

Lactate dehydrogenase

The lactate dehydrogenase (LDH) levels of experimental fish were increased in the liver and intestine over control animals. The percent change in the LDH level of experimental fish was increased in liver at day 7th (+48.275) and at day 15th (+197.93) and also same trend was observed in intestine of experimental fish at day 7 (+20.262) and at day 15 (+49.125) over control fish. But the degree of increase was more with increase the duration of experimentation. In between the two organs the LDH activity was more in liver than the intestine of fish.

Succinate dehydrogenase

The Succinate dehydrogenase (SDH) activity of experimental fish was decreased in liver and intestine over control fish. The percent change in the SDH level of experimental fish was decreased in liver at day 7 (-58.653) and at day 15 (-68.269) and also same trend was observed in intestine of experimental fish at day 7th (-34.459) and at day 15th (-58.108) over control fish. But the degree of decrease was greater with increase the duration of experimentation. In between the two organs the SDH activity was more in the liver than intestine of fish.

Malate dehydrogenase

The malate dehydrogenase (MDH) activities of experimental fishes were decreased in liver and intestine over control animals. The percent change in the MDH level of experimental fish was decreased in liver at day 7 (-55.681) and at day 15 (-82.954) and also same trend was observed in intestine of experimental fish at day 7 (-29.069) and at day 15 (-70.930) over control fish. But the degree of decrease was more with increase in the duration of experimentation. In between the two organs the MDH activity was more in the liver than the intestine of fish.

Glutamate dehydragenase

The glutamate dehydragenase (GDH) activity of experimental fish was increased in both the liver and intestine over control fish. The percent change in the GDH level of experimental fish was increased in liver at day 7 (+44.00) and at day 15 (+128.00) and also same trend was observed in intestine of experimental fish at day 7 (+52.380) and at day 15 (+133.809) over control fish. But the degree of increase was more with increase the duration of experimentation. In between the two organs the GDH activity was high in liver than the intestine of fish (Table 1).

Enzyme	Control	Sub lethal exposure	
		7 days	15 days
G-6-PDH	0.300 ± 0.024	0.201 ± 0.031 -33.00 P<0.001	0.153 ± 0.004 -49.00 P<0.001
LDH	0.145 ± 0.015	0.215 ± 0.014 + 48.275 P<0.001	0.432 ± 0.048 +197.93 P<0.001

SDH	0.312 ± 0.013	0.129 ± 0.004 -58.653 P<0.001	0.099 ± 0.008 -68.269 P<0.001
GDH	0.025 ± 0.002	0.036 ± 0.003 + 44.00 P<0.001	0.057 ± 0.008 +128.00 P<0.001
MDH	0.088 ± 0.006	0.039 ± 0.003 -55.681 P<0.001	0.015 ± 0.001 -82.954 P<0.001
Values are mean ± S.D. of 20 individual fishes Values in the parenthesis are % change from that of control			

Table 1: Showing Oxidative enzymes activities (mg protein/minutes) in liver of fish.

Enzyme	Control	Sub lethal exposure	
		7 days	15 days
G-6-PDH	0.200 ± 0.030	0.123 ± 0.002 -38.5 P<0.001	0.098 ± 0.001 -51.00 P<0.001
LDH	0.686 ± 0.051	0.825 ± 0.043 +20.262 P<0.001	1.023 ± 0.025 +49.125 P<0.001
SDH	0.444 ± 0.011	0.291 ± 0.016 -34.459 P<0.001	0.186 ± 0.016 -58.108 P<0.001
GDH	0.021 ± 0.002	0.032 ± 0.004 + 52.380 P<0.001	0.049 ± 0.005 +133.809 P<0.001
MDH	0.086 ± 0.006	0.061 ± 0.005 -29.069 P<0.001	0.025 ± 0.001 -70.930 P<0.001
Values are mean ± S.D. of 20 individual fishes Values in the parenthesis are % change from that of control			

Table 2: Showing Oxidative enzymes activities (mg protein/minutes) in intestine of fish.

Discussion

The inland fisheries resources of India (rivers, wetlands, lakes, ponds, reservoirs) have a rich production potential: however,

suboptimal water quality or detrimental ecological conditions have limited fish production by creating stress to the resident fish [12]. Presence of chronic stress in fishes due to fluctuations in pH, dissolved oxygen and unionized ammonia in water has been reported by Das et al., [13]. Several physiological changes occur in the fishes due to the aquatic stressors. These changes are a direct or indirect result of the physiological response to the aquatic stress and can be quantified and used as predictive indices Dutta et al., [14]. Liver assists in digestion by secreting enzymes that break down the fats, storage and carbohydrates. It also is important in the devastation of old blood cells and in sustaining proper blood chemistry, as well as playing a role in nitrogen (waste) excretion.

Glucose - 6 - phosphate dehydrogenase (G-6-PDH) is the key enzyme of hexose monophosphate pathway and is used to generate NADPH and ribose - 5 - phosphate. If energy needs a high, this pathway serves, to generate glycolytic intermediate compound for the production of energy [3, 15]. In the present study, the greater decrease in G-6-PDH activity in the liver and intestine of the fish, *Cyprinus carpio* exposed to the acidic pH media in different days (1 to 7 and 1 to 15 days) control, may indicate the decreased oxidation of glucose through HMP shunt. In addition, as the liver are central metabolic network of blood circulation and the HMP pathway is predominantly found in RBC, whereas in the case of liver the degree of decrease was greater than the intestine at different days of acidic pH. It may be due to more suppression of oxidative metabolism occurring in liver than the intestine. Elevation of glucose - 6 - phosphate dehydrogenase and inhibition of SDH activity reflect the effect of aquatic stress, which leads to metabolic alteration in the fish [2,3,16]. Further, the increased glucose - 6 - phosphate dehydrogenase activity in the liver of fish suggests the increased synthesis of nucleic acid (RNA and DNA) and greater utilization of NADP for its maintenance and viability under acidic pH.

The glycolytic enzymes in the cell break down glucose resulting in the formation of pyruvate. It is the key enzyme located at the vital point between glycolysis and TCA cycle. Because of its strategic location and its relation to coricycle, it is likely that any fluctuation in the cellular environment alters the activity of this enzyme. Hence, it is selected in the present study to evaluate the effect of pH (5.0) on oxidative metabolism by analyzing the LDH which is an end product of glycolytic pathway.

Increased in LDH activity was observed in the liver and intestine of experimental fish in both the days of experimentation over control. This indicate that the fish under acidic pH stress might have relied more on anaerobic glycolysis in meeting the energy requirements, as the energetically more efficient oxidative metabolism is suppressed. Decrease in the rate of oxygen consumption warranted the fish to switch over from aerobic oxidative metabolism to anaerobic glycolysis. It is also support for the elevation of LDH activity in the organs of fish. Bhaskar (1982) [2] also correlated the magnitude of increase in LDH activity in Tilapia tissues to the tissue damage and organ necrosis. Elevation in LDH was also reported in tissues of metal - intoxicated Tilapia mossambica[17,3,18]. The reported increase in plasma lactic acid for carp due to oxygen shortage as a result of hypoxia was shown by Thomas et al., [19]. It is also support for the elevation of LDH activity in the liver and intestine of fish *Cyprinus carpio* under acidic pH. In between the two organs the LDH activity was greater in the liver than the intestine of fish. It could be due to more anaerobic glycolysis occurring in the liver and the intestine of fish.

The activity of SDH in mitochondria is greater than the other enzymes of TCA cycle, an insight into the alterations of this enzyme activity may be taken as an index to assess the function of TCA cycle in different fresh water animals [20]. As liver of the fish are central metabolic organ plays an important activities of oxidative metabolic enzymes. In the presented study, decrease in SDH activity in both the organs of experimental fishes at day 7 and 15 than the control animals. It could be due to the inhibition of oxidative metabolism in them by the effect of acidic pH media. Decrease in oxygen consumption and with an increase in LDH activity in the organs of fishes support for the inhibition of oxidative metabolism in them by the acidic pH media.

Bhaskar and Govindappa [2] in Tilapia an exposure to acidic media and [21] the reduction of SDH activity in *Cyprinus carpio* induced by the copper at different pH were stated that a synergistic impact of the acidic pH on the fish there by the oxidative metabolism is greatly suppressed. In addition to this the acidic pH might have even damaged the epithelium of the liver there by a decrease in oxidative metabolism is possible. Under acidic condition more energy is required for maintenance of basic function than under non-acidic conditions [22]. It is also support to the decrease in SDH activity in the organs of fish under acidic pH. Insufficient energy production in this organ could lead to the failure of osmo-and-iono regulatory functions [23-25] reported that decrease in SDH activity under deltamethrin exposed to freshwater fish *Labeo rohita*. The effects of acidic pH media also exert stress condition in fishes which in turn might have been responsible for the inhibition of SDH activity in the organs of fish. [18, 17, 26]. The decreased activity of SDH may be due to the dis-organization of mitochondria affecting enzymes of TCA cycle. But the SDH activity has greater decrease in liver than the intestine of fish. Probably more suppression of oxidative metabolism occurs in liver than the intestine of fish.

Glutamate Dehydrogenase (GDH) is one of the important mitochondrial matrix enzymes with a key role in nitrogen glutamate metabolism and the energy homeostasis. It cleaves glutamate to oxoglutarate (α -ketoglutaric acid) which is an important by product required for the operation of citric acid cycle. GDH also know to pay a crucial role in oxidative metabolism and its level alters with the influence of a variety of effectors [27]. It has several metabolic functions with great physiological significance. In the present study increase in GDH activity in the organs of the fish expose to acidic pH at 7th day and 15th day of experimentation. It could suggest increased production of glutamate in order to eliminate ammonia, as a decrease was observed in the level of ammonia in the organs of the fish exposed to acidic pH. The presence of ATP also plays an important role in the function of GDH activity. The low ATP causes inhibition through the GTP binding site where as the high ATP causes activation mediated through the ADP effectors sites. Hence, the high ATP presence might be responsible for elevated GDH activity in the organs of fish. Increase in amino acid levels as well could partly responsible for the rise in GDH activity [26,28,29]. The GDH activity was less in the liver when compared to intestine. The degree of decrease was more with increase in duration of experimentation.

Malatedehydrogenase (MDH) enzyme oxidizes malate into oxaloacetate in the presence of NAD⁺, which was the last step of citric acid cycle and plays an important role in citric acid cycle. Decrease in MDH activity in both the organs of fish at day 7 and 15 under pH 5.0 than the control fish. It suggested a decrease in the oxidative metabolism of the fish under acidic pH. As such reliance on anaerobic glycolysis as an energy source might have been increased to meet the

required energy demands. This is well evident by the increased activity of LDH in the organs of fish exposed to acidic pH.

Decrease in MDH activity in the organs of fish, since liver are the major metabolic organ and all other metabolic pathways depend upon the efficiency of liver for their energy supply and also liver interference of the ions on the activity of oxidative metabolic enzymes may be more and hence the animals might have switched over to glycolytic pathway [30] addition, the liver tissue plays an important role on digestion, metabolism, immunity, and the storage of nutrients within the body. These functions make the liver a vital organ; this signifies the key role it plays at the interface with the environment. The decrease in MDH activity may also suggest the reduced SDH activity which in turn lowers fumarate – malate conversions. It is also evident from the results that the liver organ is most affected than the intestine of fish.

Conclusion

From the above results it is evidenced that the effect of acidic pH may disturbs all the enzymes related to oxidative metabolism and also dependent on the organ specificity of fish. G-6-PDH is an important enzyme in hexose monophosphate (HMP) shunt, it is not only an alternate pathway for glucose oxidation but also produces pentose sugars and reduced NADP which are much needed for the synthesis of nucleic acids, fatty acids and amino acid. The G-6-PDH activity of was decreased which slows down the pentose phosphate pathway in acidic conditions. LDH activity levels were increased in both experimental liver and intestine. Increased LDH activity is a characteristic feature of a shift from aerobic to anaerobic metabolism leading to an elevated rate of pyruvate conversion into lactate. Diminished cellular oxidations leading to anaerobiosis could be one of the reasons for the elevation of LDH activity in both liver and intestine tissues. Decreased SDH activity might be observed due to depletion in the oxidative metabolism at the mitochondria level. The decrement in the SDH activity denotes fluctuations of oxidative metabolism and also reflects the turnover of carbohydrates and energy output. GDH plays a key role in oxidative metabolism to form glutamate to ammonia. The glutamate dehydrogenase activity (GDH) was found to be elevated in liver and intestine tissues of fish. The elevated GDH activity levels indicate its contribution to ammonia production and glutamate oxidation under acidic media. Decreased MDH activity over control level envisages active conversion of malate into oxaloacetate might be favorable for the operation of gluconeogenesis in the tissue. The elevated MDH activity level of liver suggests the active operation of citric acid cycle to release the energy. In view of the increased MDH levels for the mobilization of amino acids into TCA cycle. This form of mobilization of amino acids towards the operation of gluconeogenesis in the tissue exposure to acidic media. The data indicates that reactive oxygen species may be associated with the metabolism of acidic media leading to peroxidation of membrane lipids of the respective organs. The observed lipid peroxidation resulting possibly from ROS generated by the compound may lead to cell apoptosis; ROS and acidic stress have been shown to be triggers of apoptosis.

Acknowledgement:

The author is thankful to DBT for sanctioning the major research project No: DBT.No:BT/PR-14482/FNS/20/460/2010, dated: 11-02-2011 under which the present study was carried out.

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