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Full Length Research Paper

Effect of sodium cyanide on the activities of some oxidative enzymes and metabolites in *Clarias gariepinus*

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The present study was conducted to determine the effect of sodium cyanide on oxidative metabolism, alterations in the activities of lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH), behavior, respiratory rate and metabolites (lactic and pyruvic acid) of the fingerling of *Clarias gariepinus*. The fingerlings were exposed to a sublethal concentration (0.75 mgL^{-1}) of sodium cyanide. Fish showed a gradual decrease in respiratory rate, increase in LDH and decrease in SDH levels, and lactic acid followed a similar trend with pyruvate in an eight-day trial. The changes in the levels of these enzyme activities may be as a result of impaired oxidative metabolism and cellular damage, which had effect on the release of these enzymes. Elevation in the level of lactic acid and decrease in pyruvic acid was due to shift from aerobic to anaerobic metabolism which resulted to a severe drop in the respiratory rate of the fish. It may be as a result of blockage of electron transfer from cytochrome-c oxidase to molecular oxygen which might lead to cellular hypoxia even in the presence of normal oxygenation of hemoglobin. Thus, inhibition of oxidative metabolism by sodium cyanide in *C. garipenus* was reconfirmed. Behavioral changes caused by sodium cyanide exposure in the fish were probably due to the combination of lactate acidosis with cytotoxic hypoxia, which might depress the central nervous system.

Key words: Succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), lactic acid, pyruvate, behavior, enzymes.

INTRODUCTION

Cyanide is a highly toxic compound that is readily absorbed and causes death by limiting the use of oxygen (Egekeza and Oehme, 1980). It is a naturally occurring substance as well as industrial products and is widely spread in the environment (Egekeza and Oehme, 1980). Its toxicity is well known but it is still used in the surgical dressing, metal-plating, mining, chemical and agricultural industries. Further, cyanide is a pivotal component of pharmaceuticals and is used in the production of the blood-pressure-reducing drug, nitroprusside and as an anticancer (Mark et al., 1999). The release of cyanide from various industries has been estimated to be more than 14 million kg/year (Rajesh et al., 2009). Being extensively used, the compounds of cyanide as industrial effluents are the major sources of toxic pollutants in an environment. Moran (2004) studied the chemistry of cyanide and its chemical effect on lakes, streams and sediments. It has been observed that it is complex and is influenced by various factors, including alkalinity and/or acidity of water bodies. Several physical-chemical methods are available for the treatment of total cyanides

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Abbreviations: LDH, Lactate dehydrogenase; **SDH,** succinate dehydrogenase.

in discharging liquid wastes by bringing them down to 0.2 mg/L (200 µg/L). However, even 0.2 mg/L of cyanide concentration in freshwater bodies is toxic to most of the life forms. There are reports in literature that cyanide concentration of 0.01 to 0.1 mg/L is able to kill some sensitive animal species present in waters (Blaha, 1976). Cyanide is also toxic to aquatic organisms (fish) as well as humans, at low concentrations.

Oxidation of pyruvate in the Krebs cycle under aerobic conditions produces more energy, which requires an abundant supply of oxygen. Succinate dehydrogenase (SDH) is one of the key enzymes that play an important role in energetics. Its activity is considered to reflect the rate of Kreb cycle operation in different organs of the fish under stress conditions. According to Singer et al. (1973), SDH is localized at the inner surface of the mitochondrial membrane, and contains a flavine adenine dinucleotide as the prosthetic group. Lactate dehydrogenase (LDH) is located at a strategic point between citric acid cycle and glycolysis, which catalyzes, lactate oxidation to pyruvate, in the terminal step.

Toxicity of cyanide and its derivatives is well-known as a metabolic inhibitor (Solomonson and Spehar, 1981). The present study was therefore, conducted to determine the effect of sodium cyanide on oxidative metabolism, alterations in the activities of LDH and SDH, behavior, respiratory rate and metabolites (lactic and pyruvic acid) of the fingerling of fish (*Clarias gariepinus*).

MATERIALS AND METHODS

Procurement of fish

Healthy *C. gariepinus* fingerlings were obtained from the Fisheries Department in the Kingdom of Saudi Arabia. Live fish was brought to the laboratory in large aerated plastic drums and were acclimatized in fish tanks (20 x 5 feet) for 30 days. Fish were fed with commercially available dry feed pellets (crude protein (CP) 30%).

The physico-chemical characteristics of the water used in the experiment were analyzed by following APHA (2005). During acclimatization and throughout the experimental period, a 12/12 h photoperiod was maintained. Water was renewed daily. Commercial fish food pellets were fed to fish regularly twice daily. Feeding of fish was stopped two days before the release of fish for sodium cyanide acute toxicity test.

Acute toxicity test

Sodium cyanide was dissolved in distilled deionized water for the preparation of stock solution. Fish were exposed to different concentrations of sodium cyanide in batches of 10 in 25 L water. Each of the treatments was applied in replicates of three. After every 24 h, mortality was recorded and dead fish was removed. Percentage killed was determined by using a probit analysis method (Finney, 1971) and Dragstedt-Beheren's equation (Carpenter, 1975) after 96 h of exposure. Half of LC50-96 h value was chosen as a sublethal concentration for sub acute studies (2, 4, 6, and 8 days). Sodium cyanide concentration was also quantified in the test medium by a spectroscopic method as described in APHA (2005).

Study of carp behavior

In order to study the stress in *C. garipenus*, both control and treated fish were used to evaluate the comparative behavioral changes under various concentrations by following the method described by David et al. (2009).

Estimation of respiratory rate

Welsh and Smith's method were used to measure the respiratory rate for sub lethal concentration as well as under control conditions (Shivakumar and David, 2008; Welsh and Smith (1961)). The oxygen inspired by sodium cyanide treated, and by the control fish was also determined. The wet fish weight of individual fish and their unit metabolism was calculated and expressed as milliliter of oxygen consumed/gram of fish/hour.

Assay of SDH activity

The SDH activity was recorded by following the method of Nacholas et al. (1960). Fish tissues were homogenized in ice cold 0.25 M sucrose solution to prepare 4% homogenates using glass-Teflon Homogenize. The extracts were centrifuged for 15 min in a controlled temperature centrifuge at 4000 rpm. The supernatants were used for assay purpose. The reaction mixture was composed of 40 µM of sodium succinate, 4 µM of 2-p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), 100 µM of potassium phosphate buffer (pH 7), and 0.5 ml of supernatant extract. The reaction mixture was incubated at 37°C for 30 min. 5 ml of glacial acetic acid was added to stop the reaction. The resultant iodoformazan was extracted overnight in 5 ml of toluene at 5°C. Spectrophotometer was used to determine its optical density at 495 nm using toluene blank. The enzyme activity was expressed as µM of formazan formed per milligram protein per hour.

Assay of LDH activity

The LDH activity was estimated by using the colorimetric method of Srikanthan and Krishnamoorthi (1955) modified by Govindappa and Swamy (1965). Tissues were homogenized in ice cold 0.25 M sucrose solution to prepare 4% homogenates using glass-Teflon Homogenizer. Homogenates (4%) were centrifuged for 15 min at 2500 rpm and the supernatant was used for the assay. The contents were incubated at 37°C for 30 min, and the reaction was stopped by the addition of 6 ml of glacial acetic acid. The formazan formed was extracted in 6 ml of toluene after overnight incubation at 5°C. The absorbance of developed color was determined at 495 nm using the spectrophotometer. The enzyme activity was expressed as micromoles of formazan formed per milligram protein per hour.

Estimation of pyruvate level

The pyruvate level was estimated by using the method explained by Friedemann and Haugen (1943). The resulting values were used to develop the standard curve and were expressed as micromoles of pyruvate per gram wet weight of fish tissue.

Estimation of lactic acid level

The lactic acid level was determined by the method of Barker and Summerson (1941) as modified by Huckabee (1961).

Table 1. Respiratory rate (milliliters of oxygen consumed per gram wet weight per hour) in the fingerlings of *C. garipenus* on exposure to sublethal concentration of sodium cyanide.

Parameter	Sublethal exposure periods in day (0.75 mg L^{-1})					
	Control	1	2	4	6	8
Respiratory rate	0.66a ±0.04	0.54b ± 0.03	0.43c ± 0.02	0.31d ± .03	0.23e ± 0.02	0.12f ±0.01

Data are means ± SD (n¼6), respiratory rate in a row followed by a letter are significant (P < 0.05) according to Duncan's multiple range test.

Estimation of protein contents

Protein contents were measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

The data were subjected to statistical analysis and was analyzed using analysis of variance and Duncan's multiple range tests (Duncan, 1955).

RESULTS AND DISCUSSION

Acute toxicity (LC_{50}-96h) of sodium cyanide for *C. garipenus* was determined as 1.6 mgL^{-1}. The upper and lower 95% confidence limits remained as 1.7 and 1.5 mgL^{-1}, respectively. Mortality was observed to be non significant during the experimental period, but *C. garipenus* showed various types of behavior under stress which are loss of equilibrium and appetite, dullness and erratic swimming. In the present study, the fish under sublethal concentrations of cyanide were observed under stress conditions. We believe that most of the absorbed sodium cyanide present probably reacted with thiosulfate, rhodanese resulting in the production of nontoxic thiocyanate. David et al. (2008) reported that rapid detoxification enables animals to ingest considerable sublethal quantity of sodium cyanide over a long period of time without causing any harm. These reports showed that fish may adapt sublethal concentrations of sodium cyanide. These findings were in line with that of the above-mentioned workers.

The rate of oxygen consumption of the fish decreased gradually from -40 to -83% at the termination of an experiment under various treatments in control and treated fish exposed to sublethal concentrations of sodium cyanide (Table 1). In the present experiment, this gradual decrease in the respiratory rate of fish was perhaps as a result of the inactivation of cytochrome c-oxidase, which caused hypoxia and cellular respiration even in the presence of normal hemoglobin oxygenation in *C. garipenus*. Tissue anoxia caused by the inactivation of cytochrome oxidase might have resulted in a shift from aerobic to anaerobic metabolism, hence requiring low oxygen. This was perhaps because of respiratory distress resulting from the impaired oxidative metabolism in fish. Similar findings were also reported by David et al. (2009).

SDH value varied in fish (*C. garipenus*) tissues under the various treatments. The lowest SDH activity was recorded in the muscles followed by gills, brain and liver. On the eighth day of the experiment, maximum decline in the SDH activity was observed in the gills (-66%), whereas on day one, this was -10% in liver tissues (Table 2). Further, continuous decline in SDH activity was observed in all the tissues throughout the experiment.

LDH activity appeared to be gradually increased with exposure of fish to sodium cyanide and maximum activity was observed in gills followed by muscle, liver and brain. LDH activity was maximum on day eight and minimum on day one in brain tissues (Table 2). The maximum LDH activity in gills was perhaps due to the direct effect of the sodium cyanide on disruption of the gill epithelium, resulting to the inhibition of cytochrome oxidase activity. This situation might favor anaerobic respiration due to the mild stress of hypoxia in *C. garipenus*; thus, aerobic processes might be operating at a very slow rate. Further, this increased LDH activity in fish might also result to lactate conversion into pyruvate at the expense of NAD. Resultantly, to fulfill the energy demands, there may be increase in the operation of glycolysis under sodium cyanide stress. We are of the opinion that the formation of a stable cytochrome c oxidase-CN complex in the mitochondria could also be the cause of variations in LDH and SDH activities. It may stop the cellular respiration by blocking the electron transport chain by transfer from cytochrome c oxidase to molecular oxygen, to meet the energy requirements of fish. Consequently, the decline in SDH activity shows a shifting of aerobic respiration to anaerobic respiration. Fenvalerate, 2-butenoic acid-3-[diethoxy phosphor-nothionyl] ethyl ester (RPR-V) and alphamethrin, were also observed to cause alterations in the activity of LDH and SDH in various tissues of *Cirrhinus mrigala* (Mushigeri and David, 2004), *Catla catla* (Singh and Singh, 2004) and *Tilapia mossambicus* (Rao, 2006), respectively. Findings of the present study in *C. garipenus* seem to be in line with that of the above-mentioned workers.

Pyruvate level also showed maximum decrease in muscle tissues (was recorded as -38%) followed by gill, brain and liver after eight days of exposure to sublethal

Table 2. SDH and LDH activities in the tissues of *C. garipenus* after exposure to sublethal concentration of sodium cyanide.

Parameter	Sublethal exposure periods in day (0.75 mg L^{-1})					
	Control	1	2	4	6	8
SDH activity						
Brain	0.71^{a}±0.05	0.62^{b}±0.06	0.55^{c}±0.04	0.46^{d}±0.05	0.38^{e}±0.02	0.29^{f}±0.03
Gill	1.12^{a}±0.05	0.97^{b}±0.04	0.85^{c}±0.03	0.65^{d}±0.07	0.41^{e}±0.05	0.34^{f}±0.04
Liver	1.88^{a}±0.10	1.691^{b}±0.08	1.46^{c}±0.10	1.28^{d}±0.07	1.17^{e}±0.07	1.08^{f}±0.06
Muscle	0.79^{a}±0.06	0.68^{b}±0.05	0.57^{c}±0.04	0.41^{d}±0.05	0.29^{e}±0.03	0.17^{f}±0.04
LDH activity						
Brain	0.41^{f}±0.05	0.49^{e}±0.04	0.55^{d}±0.04	0.59^{c}±0.05	0.66^{b}±0.06	0.71^{a}±0.05
Gill	0.66^{f}±0.07	0.76^{e}±0.05	0.83^{d}±0.04	0.89^{c}±0.08	0.98^{b}±0.10	1.08^{a}±0.08
Liver	0.44^{f}±0.04	0.55^{e}±0.06	0.64^{d}±0.07	0.69^{c}±0.05	0.72^{b}±0.06	0.81^{a}±0.04
Muscle	0.51^{f}±0.05	0.61^{e}±0.04	0.70^{d}±0.06	0.79^{c}±0.05	0.89^{b}±0.04	0.96^{a}±0.08

SDH and LDH recorded (micromole of formazon per milligram protein/hour). According to DMR test, means ± SD for a tissue in a row followed by the same letter are not significantly different ($p < 0.05$) from each other. LDH, Lactate dehydrogenase; SDH, succinate dehydrogenase.

Table 3. Effect of sublethal concentration of cyanide on lactic acid (mg / g wet weight) and pyruvate (micromoles / g wet weight) levels in the tissues of C*. garipenus*.

Parameter	Sublethal exposure periods in day (0.75 mg L^{-1})					
	Control	1	2	4	6	8
Lactic acid						
Brain	0.33^{f}±0.03	0.42^{e}±0.05	0.51^{d}±0.05	0.62^{c}±0.06	0.71^{b}±0.05	0.77^{a}±0.06
Gill	0.39^{f}±0.04	0.46^{e}±0.06	0.53^{d}±0.03	0.61^{c}±0.05	0.67^{b}±0.06	0.74^{a}±0.05
Liver	1.39^{f}±0.07	1.51^{e}±0.09	1.67^{d}±0.10	1.77^{c}±0.12	1.81^{b}±0.10	1.92^{a}±0.14
Muscle	0.83^{f}±0.08	0.91^{e}±0.07	0.98^{d}±0.09	1.11^{c}±0.12	1.28^{b}±0.14	1.39^{a}±0.18
Pyruvate						
Brain	1.08^{a}±0.09	0.97^{b}±0.11	0.83^{c}±0.14	0.71^{d}±0.10	0.60^{e}±0.11	0.49^{f}±0.08
Gill	1.41^{a}±0.06	1.29^{b}±0.07	1.17^{c}±0.05	1.04^{d}±0.04	0.89^{e}±0.03	0.73^{f}±0.05
Liver	3.28^{a}±0.15	3.10^{b}±0.18	2.82^{c}±0.16	2.56^{d}±0.13	2.33^{e}±0.11	2.01^{f}±0.17
Muscle	2.35^{a}±0.11	2.17^{b}±0.15	1.94^{c}±0.14	1.70^{d}±0.16	1.59^{e}±0.10	1.44^{f}±0.18

According to DMR test, means ± SD for a tissue in a row followed by the same letter are not significantly different ($p < 0.05$) from each other.

concentration of sodium cyanide. The minimum decreased in pyruvate level was recorded in muscle on day one (Table 3).

The maximum pyruvate level in *C. garipenus* was recorded in liver tissues followed by muscle, gills and brain under control treatment (Table 3). Pyruvate acts as indicator and a measure of aerobic conditions (based on the presence of oxygen) in the liver and muscle tissues as in both tissues, aerobic as well as anaerobic conditions may operate according to physiological requirements and presence of molecular oxygen, and within the physiological conditions both metabolic diversions are possible. However, the only pyruvate alteration cannot determine the aerobic and anaerobic conditions in different tissues as a keto group in pyruvate is unstable, which by decarboxylation may form acetyl-CoA or may be converted to lactic acid by taking two hydrogen ions, whereas, both phenomena are also controlled by the availability of oxygen. It also sums up that reduced oxidative decarboxylation of pyruvate caused the decrease in the level of pyruvate in different tissues (Tokarski and Reio, 1978).

The lactic acid contents were also estimated in the present experiment. It appeared to be higher in muscle tissues followed by liver, gills and brain after eight days of exposure of fish to sublethal concentrations of sodium cyanide (Table 3). Minimum lactic acid content was recorded in brain of fish on day one (Table 3). Under anaerobic conditions, the end product of glycolysis in tissues is lactic acid. The tissue lactic acid level is taken as an index of anaerobiosis, which might be beneficial to animal in tolerating hypoxic conditions (Thoye, 1971). It may also be presumed from these studies that muscle tissue is more vulnerable to anaerobiosis as compared to

other tissues in fish. From the above findings, it may also be inferred that predominance of anaerobic metabolism in the fish is probably due to the cytochrome b oxidase activity.

Behavioral changes under stress

The behavioral changes if any in *C. garipenus* under the exposure of sodium cyanide and control were also studied. It has been observed that the fish under control treatment remained active in feeding and alert to the slightest of the disturbance. No significant changes in the behavior of fish in control were observed. These findings may be considered as benchmarks for the entire experimentation. The loss of equilibrium followed by erratic and darting and swimming movements was observed together with imbalanced body activity and muscular incoordination. The behavioral changes in fish may be due to the inhibition of brain cytochrome c oxidase activity induced by cytotoxic hypoxia. It is well documented in the literature that the brain of the fish is a critical site for anoxia, which may be due to changes in electrical activity which cause damage to the region of the brain associated with the maintenance of equilibrium. Avoidance behavior of fish was also observed as they moved to the bottom of the tank when exposed to sodium cyanide. The erratic swimming, jerky movements and convulsions before death were evident and the serenity varied with pesticide concentration. It shows the signs of asphyxiation as shown by gasping to death when *Sarotherodan mossambicus* was exposed to dimethorate (Kalavathy et al., 2001). The avoidance behavior of the fish which is resultant to the exposure of sodium cyanide was also reported in trout (Murthy, 1987). The schooling behavior of the fish was also disrupted gradually during the experimental period and even at slight disturbance, the fish were found to be alert. The fish body leaned towards the abdomen position in the treated groups as compared to the control group. The control fish also showed reduced quantity of dietary protein consumption, perhaps because of sodium cyanide stress and/or depletion of muscle glycogen. Lactate acidosis and cytotoxic hypoxia in fish might have depressed central nervous system (CNS) and could be the major reason for altered behavior in *C. garipenus*. David et al. (2009) observed similar behavioral anomalies in common carp fish under sodium cyanide stress. The surfacing phenolmenon of fish observed under cyanide exposure might be due to hypoxic condition of the fish as reported by Radhaiah and Jayantha (1988). This fact was clearly observed in the present study. Metabolic shift from aerobic to an anaerobic condition involving glycolytic oxidation with an enormous quantity of lactic acid accumulation was also observed. Chronic exposure of finfish to aroclor was found to induce surfacing phenolmenon of fish as reported by Drummond et al. (1986).

Conclusion

It has been determined that sodium cyanide has been an asphyxiate in *C. garipenus*, as it severely affected the activities of LDH and SDH, respiration rate and metabolites. It is probably due to the predominance of anaerobic metabolism resulting from the irreversible inhibition of cytochrome c oxidase by sodium cyanide and respiratory distress because of impaired oxidative metabolism. Moreover, lactate acidosis in combination with cytotoxic hypoxia in *C. garipenus* might have depressed the CNS, and thus the principal reason for the behavioral anomalies observed under sodium cyanide stress.

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