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Respiratory Surveillance and Ca^{2+}-ATPase Enzyme Activity Studies of *Clarias gariepinus* Exposed to Acute Toxicity of Cyanide

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ABSTRACT. Potassium cyanide, a highly contaminating and toxic aquatic ecosystems pollutant was investigated for acute toxicity on the freshwater fish *Clarias gariepinus.* Its effect on the Ca^{2+} -ATPase activities in the liver, gills, muscle and intestinal tissues and oxygen consumption index was studied. Short-term toxicity test was carried out by static renewal bioassay test over a 96 h period using a lethal concentration (LC_{50}) value of 0.361mg/mL. Potassium cyanide was highly toxic to the animal tested. Results reveal that normal respiratory activity (O_2 consumption) of the fish was significantly affected and there was significant decreased in the Ca^{2+} - ATPase activities at the end of exposure periods (24, 48, 72 and 96 h). Correlation analysis reveals a strong relationship between oxygen consumption index and ATPase enzyme activity of *Clarias gariepinus* exposed to the toxicant. This study reflects the toxic effect of potassium cyanide to the freshwater fish, *Clarias gariepinus* and suggestion on the possible application of Ca^{2+} -ATPase activities and oxygen consumption index as possible biomarkers for early detection of cyanide poisoning in aquatic bodies.

1. INTRODUCTION

With the present industrialization of the world economy, there is an upsurge in the amount of industrial waste discharge into the ecosystem especially water bodies. Major sources of cyanide release to streams, lakes and rivers are discharge from gold mining, public owned water treatment plant, iron and steel production plants, organic chemical industry and notably effluent and waste from the processing of cassava and its products. Cyanide is a potent respiratory poison in aerobic organism which combines irreversibly with ferrocytochrome a/a_3 thereby inhibiting electron transport, mitochondrial oxygen uptake and cellular respiration (Jones *et al.,* 1984; Greer and Jo 1995; Okolie and Audu, 2004; Kadiri,2015). Ca^{2+}ion is an ions required by ATPase for their activity which involves cleavage of ATP to ADP/AMP and inorganic phosphate with liberation of energy. Disruption of this process can have dare consequence in the enzyme function and metabolic processes in living cells. Fish are an important model system for the evaluation of the extent of aquatic pollution (Shwetha and Hosetti, 2009; Kadiri, 2015). *Clarias gariepinus*, the African Catfish is an edible freshwater fish of great economic to the continent and other part of the world and are ideal animals for studying the abnormalities caused by the effects of toxic chemicals in aquatic environment.

Information on the acute toxic effects of potassium cyanide to fishes is rare and likewise its effect on *Clarias gariepinus,* which is of great economic importance in the aquatic food chain. In the present study, the author demonstrated the acute effect of potassium cyanide (KCN) on the oxygen consumption index and activities of the adenosine triphosphate enzyme 'Ca^{2+} -ATPase' within the liver, gills, muscle and intestinal tissues of the freshwater fish, *Clarias gariepinus.*

2. MATERIALS AND METHODS

Fish specimen collection and maintenance

Cat fish, *Clarias gariepinus* were obtained from the fish pond of the Fishery department of the University of Benin, Nigeria and acclimatized to laboratory conditions before the start of the experiments. Only active specimens (6.5±0.4 cm, 10.9±0.5g) with no signs of stress and injury were used in the study. Dechlorinated tap water (temperature 25.9±1.0 ^{0}C, dissolved oxygen 6.5 ± 0.5 mg/L, carbon dioxide 6.3±0.4 mg/L, hardness 23.2±3.4 mg/L as $CaCO_3$, phosphate 0.38±0.005 µg/L, salinity 0.01 ppt, specific gravity 0.001, conductivity less than 10 µS/cm and a light period of 11 h/day) was used throughout the experimental period and water was renewed every 48 h. The experimental fish were reared in aquaria of dimension (45cm×30×25cm). Fish were fed regularly with commercial fish food pellets during acclimatization and test periods. After 15 days, if fishes were in normal behavioural activity and good health conditions, those species were selected for experiment purpose and feeding was stopped two days prior to exposure to the test medium for acute toxicity test. All water quality parameters (Dissolved oxygen, temperature, pH, salinity, alkalinity, conductivity) were monitored throughout the duration of the experiment except for minimal variation tolerated by the fish in its natural habitat. In order to understand the influence of time over toxicity effect of lethal concentration of potassium cyanide on *Clarias gariepinus,* study at different periods of exposure of 24h, 48h, 76h and 96h was carried out. After acclimation, the following steps were carried out.

Preparation of potassium cyanide stock solution

Stock solution was prepared by dissolving KCN (99% purity) in double distilled water in standard volumetric flask. The required quantity of sodium cyanide was drawn from this stock solution using a micropipette. The concentration of test compounds used in short term definitive tests were between the lowest concentrations at which mortality was 100% and the highest the concentration at which mortality was 0% in the range finding tests.

Determination of LC_{50} of cyanide for *Clarias gariepinus*

Ten concentrations of cyanide (0.65, 0.55, 0.47, 0.44, 0.41, 0.368, 0.322, 0.2, 0.18, and 0.1mg/L) were prepared in 10 aquaria of different tanks of dimension (45cm×30×25cm) containing 90L of aerated dechlorinated tap water. A simultaneous control group was prepared with only aerated dechlorinated tap water after which ten healthy fish were stocked in each aquarium. Water with its fixed dose was changed daily within the period of the experiment (96 h). After 96h from exposure time, the percentage mortality rate was calculated in each aquarium according to probit analysis method (Finney, 1971). The percentage of fish mortality that were exposed to selected concentration of cyanide in aquatic environment followed the linear equation (193.35x-17.345) with R^2= 0.9578. The experiment was repeated twice and the average lethal concentration (LC_{50}) was found to be 0.361 mg/ml.

Oxygen Consumption Determination

In the present study, the respiration rate of the fish was measured from 24 h to 96 h with a 24 h interval. In other to reduce the effect of low oxygen level and metabolite accumulation, the experiment duration was regulated so that the oxygen concentration by the end of experiments was above 80% of its initial concentration. The dissolved oxygen was determined by the Winkler's iodometric method of Moran *et al.* (1980). Respiratory measurements were made in diffused daylight and the time of the experiment was kept constant in other to avoid the effect of time of day on the respiration of the fish. The temperature and pH during the course of the experiments were as stated previously.

Assay of Ca^{2+} - ATPase

To study the effect of cyanide on the enzyme activity of ATPase within the liver, muscle, gills and intestinal tissues, 40 healthy fish were stocked in each aquarium (10 fish/ aquarium) and subjected to LC_{50} of cyanide (0.361 mg/ml) for a time duration of 24h, 48h, 72h and 96 h. Likewise, 10 fish were also stocked in an aquarium containing only 90L of aerated dechlorinated tap water for

the same time period to act as control. The fish were sacrificed to collect muscle, liver, gill and intestinal tissues after each exposure period. Each tissue was finely ground in a hand mortar with 1.0ml of ice-cold physiological saline (0.9% NaCl w/v) for three minutes. The homogenate was then transferred into a vial and preserved in a refrigerator at $4^{O}C$. All tissue extracts were analysed within 24 hours. Ca^{2+} - ATPase activity was measured by estimating the amount of inorganic phosphate liberated following incubation of aliquots of tissue homogenate with disodium ATP (Heskett *et al.,* 1978). The assay mixture contained 0.1ml of 1mm EDTA; 0.4ml of 30mM $MgCl^2$; 0.4ml of 5mm $CaCl^2$; 0.3ml of 160mM tris buffer, pH 7.4 and 0.4ml of 0.3mM disodium ATP. Reaction was started by the addition of 0.4ml tissue homogenate. The mixture was vortexes and incubated at room temperature ($\approx 30^{O}C$) for 30 minutes, after which 4.0ml of 5% (v/v) trichloroacetic acid was added to stop the reaction. The mixture was subsequently clarified by centrifugation, and inorganic phosphate estimated in the supernatant using ammonium molybdate. Corrections were made for endogenous inorganic phosphate arising from inherent ATP hydrolysis by incorporating a homogenate ATP blank in the assay. CA^{2+}-ATPase was expressed in terms of µg inorganic phosphate liberated/ml of tissue extract.

Statistical Analysis

The detailed oxygen consumption and ATPase activities were recorded and subjected to one-way analysis of variance (ANOVA) and Duncan's significant difference test was used for mean separation. Significance level was set at $p<0.05$.

3. RESULTS AND DISCUSSION

Change in respiration rate is a common physiological response index used in evaluating metabolic changes in response to environment toxicities. Table 1 shows the Oxygen consumption index expressed as $mgO_2/g/h$ and in terms of % change. Mean oxygen consumption at different time intervals of potassium cyanide exposure were significantly different from the control group. Oxygen consumption was observes to increase in the first 24 h of exposure. However, oxygen consumption decreases subsequently after a 24 h exposure.

Table 2 shows the pattern of changes in Ca^{+} - ATPase enzyme activity in the gills, liver, muscle and intestine of *Clarias gariepinus* exposed to potassium cyanide solution. While the Ca^{+} - ATPase enzyme activity of fishes in the control group showed normal activities, enzyme activities of cyanide exposed specimen showed decreased enzyme activities which decrease progressively with duration of exposure.

Although many biological early warning systems monitor abnormal opercula movement as an indicator of respiratory stress, a more direct measurement of stress in this sense necessitates the quantification of oxygen consumed by the fish (Dube and Hosetti, 2010). Oxygen consumption measurements provide a robust indicator of whole animal stress and concomitant water quality (Dube and Hosetti, 2010). Cyanide acts on cytochrome oxidase by competing with oxygen for the heme site in the mitochondria of cells. This cause disruption in the mitochondrial membrane potentials and stalls the production of ATP due to the inhibition of the final transfer of electrons to oxygen.

Table 1: Oxygen consumption (mg of oxygen consumed/ g wet wt. of fish/h) of the freshwater fish, *C. gariepinus* following exposure to 96 h LC_{50} (0.361mg/ml) of Potassium cyanide

	Exposure period in hours				
O_2 Consumption	**Control**	**24h**	**48h**	**72h**	**96h**
$mgO_2/g/h$	0.738^{a}	0.805^{b}	0.267^{c}	0.173^{d}	0.087^{e}
% change	-	+ 9.08	-63.82	-76.56	-88.21
SD±	0.001	0.005	0.001	0.020	0.025

* Values are mean ± S.D (n=5). Those having different superscripts in same rows differ significantly ($p<0.05$)

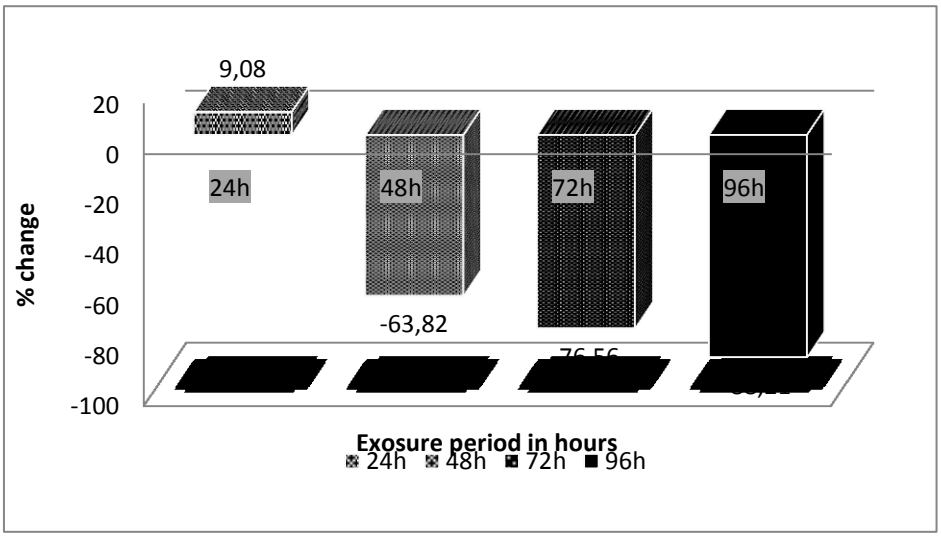

Figure 1: The % change in dissolved oxygen of *Clarias gariepinus*

Table 2: Ca^{2+}-ATPase (µmoL Pi liberated/mg protein/h) in different tissues of *Clarias gariepinus*

	Exposure (LC_{50}) period in hours (h)				
Tissues	**Control**	**24h**	**48h**	**72h**	**96h**
Liver	8.90 ± 0.03^{a}	7.55 ± 0.05^{b}	6.43 ± 0.02^{c}	6.04 ± 0.08^{d}	4.25 ± 0.20^{e}
Muscles	6.87 ± 0.03^{a}	5.54 ± 0.33^{b}	4.10 ± 0.22^{c}	3.89 ± 0.12^{d}	2.05 ± 0.54^{e}
Gills	10.23 ± 0.25^{a}	8.98 ± 0.01^{b}	8.00 ± 0.01^{c}	6.33 ± 0.23^{d}	5.19 ± 0.01^{e}
Intestine	4.56 ± 0.01^{a}	2.80 ± 0.02^{b}	1.35 ± 0.02^{c}	1.11 ± 0.30^{d}	0.58 ± 1.45^{e}

* Values are mean ± S.D (n=5). Those having different superscripts in same rows differ significantly ($p<0.05$)

Increased surface activity of the fish exposed to potassium cyanide was observed. The surfacing phenomenon might either be due to hypoxic condition of the fish as reported by Radhaiah and Jayantha (1988), Shwetha and Hosetti (2009) and Prashanth *et al.* (2011). This fact was clearly evidenced in the present study. A shift in the metabolic activities from aerobic to an anaerobic condition involving glycolytic oxidation with enormous amount of lactic acid cannot be ruled out. Decrease in oxygen consumption of *C. gariepinus* exposed to potassium cyanide is an indication of the beginning of acute hypoxia brought by oxidative stress created by the toxicant. Shwetha and Hosetti (2009) suggested that drop in the metabolic rate of the fish to be a form of protective and adaptive measure aim to ensuring a reduce intake of toxic substance. Increased in oxygen consumption rate in initial 24 h exposure to lethal concentration of potassium cyanide with corresponding decrease in oxygen consumption in subsequent time duration is in an agreement with the earlier report of Shwetha and Hosetti (2009). The high value recorded in the initial 24 h of lethal exposure to exposures, may be attributed to the initiation of specific protein synthesis or increased in its activity in response to detoxification of the toxicant (Connell *et al.,* 1999). This correlate in increase in respiration rate within that time duration. Thus, the increase in protein synthesis was reflected in an increase in respiration (oxygen consumption) rate as observes with 24 h of exposure. Furthermore, gills are the major respiratory organs in fish. Metabolic pathways depend upon the efficiency of the gills for their energy supply and damage to these organs results in chain of destructive events which affects respiratory function. Dube and Hosetti (2010) stated that

pronounced secretion of mucus layer over the gill lamellae observed during cyanide stress curtails the diffusion of oxygen, which ultimately reduce the oxygen uptake by the animal. Also, it was said that if gills were destroyed due to xenobiotic chemicals (Grinwis *et al.,* 1998) or the membrane functions are disturbed by a changed permeability (Hartl *et al.,* 2001), oxygen uptake rate would rapidly decrease. Thus, it can be inferred that decrease oxygen consumption observed might be as a result alteration in membrane or damage to gills membrane by potassium cyanide molecules of exposed fish specimens. Likewise, decreased in the oxygen consumption of *C. gariepinus* exposed to cyanide is an indication of the onset of acute hypoxia as stated previously. Cyanide molecules, been a toxicant and as well an inhibitor might also have inhibited respiratory enzymes which would have result in this drop in oxygen consumption. Isom *et al.* (1974) and Solomonson (1981) observes that in a situation of acute cyanide poisoning, there is rapid inhibition of the cytochrome c oxidase which results in energy deficit within target tissues. Cyanide is able to impair both oxidative metabolism and the associated process of oxidative phosphorylation by inhibiting cytochrome c oxidase in the respiratory oxidative phosphorylation (Holland, 1983; Dreisenbach and Robertson, 1987). Furthermore, inhibition of enzymatic process and stimulation of neurotransmitters in the nervous and peripheral nervous are other factors that results in the displayed acute toxicity syndrome (Ardelt, 1989; Isom and Borowitz, 1995) and possible cause for drop in oxygen consumption index with duration of exposure to the toxicant under study.

It was shown that when fish was exposed to lethal concentration, the mean concentration of Ca^{2+}-ATPase (µmoL Pi liberated/mg protein/h) in all tissues assay (Liver, gills, muscles and intestine) for the enzyme activity were significantly different and lower compared to the control after 24h, 48h, 72h and 96h, respectively. Drop in Ca^{2+}-ATPase enzyme activity might be due to the inhibition of oxidative phosphorylation reactions degradation of products of lipid peroxidation of the enzyme molecule (Tiwari *et al.,* 2002; Unnisa and Devaraj *et al.,* 2007). Observation on the decrease in Ca^{2+}-ATPase enzyme activity as a result of cyanide is in agreement with earlier reports of Kadiri (2015) which reported similar effect of potassium cyanide on the Na^{+}/K^{+} ATPase enzyme activity of Catfish. Likewise, Shwetha *et al.,* (2012) and Ramzy (2014) reported similar findings on the effect of sub lethal concentration of zinc cyanide and toxic concentration of sodium cyanide on the ATPase activity of *Cirrhinus mrigala* and the freshwater fish- Nile Tilapia. Okolie and Audu (2004) had earlier observed that cyanide, specifically inhibits the enzyme activity of Ca^{2+}-ATPase in lens and vitreous humour in rats resulting in visual impairment via the disruption of ocular calcium homeostasis, a confirmation of the toxic effect of cyanide to the Ca^{2+} - ATPase of animals, rat and fish inclusive. Thus, Ca^{2+}-ATPase of the test specimens is sensitive to cyanide and will be a useful biomarker for detection and mechanistic studies of the toxicant.

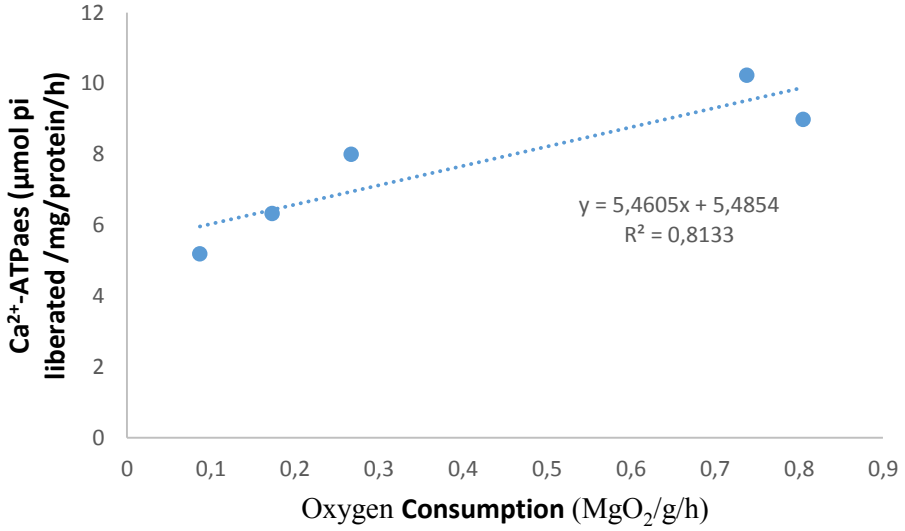

Figure 2: Correlation of ATPase enzyme activity with Oxygen consumption over a 96h time duration

Table 3: Pearson Correlation Coefficient Index between Oxygen Consumption and ATPase Enzyme Activity

		O2 Consumption	ATPase Enzyme Activity
O2	Pearson Correlation	1	.902*
	Sig. (2-tailed)		.036
	N	5	5
ATPase	Pearson Correlation	.902*	1
	Sig. (2-tailed)	.036	
	N	5	5

*. Correlation is significant at the 0.05 level (2-tailed).

A correlation analysis was performed to determine the likely interactions between the effects of potassium cyanide on oxygen consumption and ATPase enzyme activity (Fig. 2). Correlation between both factors is significant ($p<0.05$) using a 2-tailed test analysis. Oxygen consumption of the test specimen over a 96h duration was found to exhibit a linear correlation ($R^2 = 0.8133$) to the ATPase enzyme activity within the same duration. The correlation between these two values which is oxygen consumption and ATPase enzyme activity over the time duration is very high (Table 4.0). This implies that an inhibition in the biological activities of one of the factor which could be either ATPase enzyme activity or oxygen consumption by the toxicant affects the overall function of the other.

Table 4: Correlation coefficients analysis**.**

Correlation analysis	**Values**
The correlation is very high	$(0.9 <\|r\|\leq 1)$
The correlation is high	$(0.7<\|r\|\leq 0.9)$
The correlation is substantial	$(0.5 <\|r\|\leq 0.7)$
The correlation is insignificant	$(0.2 <\|r\|\leq 0.5)$
The correlation is low	$(\|r\|\leq 0.2)$

Source: Cartell (1996)

4. CONCLUSION

The study shows that the reduction of the ATPase results in disruption cellular metabolism, leading to histologic hypoxia in the expose fish. Significant correlation between the oxygen consumption rate and ATPase enzyme activity of specimen exposed to potassium cyanide was also established. The dysfunction in the respiratory and ATPase can thus serve as index of cyanide toxicity in the detection of potassium cyanide poisoning in aquatic bodies. It is therefore imperative to treat water and waste waters with cyanide content before been discharge into water bodies. Further studies on the chronic toxicity of cyanide to other living creatures are still required. Likewise, the relationship between oxygen consumption index and ATPase enzyme activity of fish exposed to cyanide needs to be investigated further in other to established scientific basis for this judgement.

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