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Enzyme modulation studies of fumaronitrile on fresh water fish *Oreochromis mossambicus*

Chinnadurai Kuppusamy[1], Arul Ganesh[1], Karthikeyeni Sambanthan[2], Eyini Muthukumarasamy[3], Balaji Paulraj[1]*
[1]PG and Research Centre in Biotechnology, MGR College, Hosur, Tamilnadu, India.
[2]Department of Biotechnology, Aringar Anna College, Krishnagiri, India.
[3]Centre for Research and PG Studies in Botany, Thiagarajar College, Madurai, India.

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Abstract: The present study was directed to analyse the potential enzymatic variation efficiency of fumaronitrile on fresh water fish tilapia (*Oreochromis mossambicus*). To study the acute toxicity of xenobiotic five different concentrations (20, 40, 60, 80 and 100ppb) of fumaronitrile along with control group was maintained for over 96 hrs. At every 1 hr. fishes was monitor both control and treatment groups. The LC_{50} value was found to be as 60ppb. To study the chronic toxicity of xenobiotic three different concentrations (2ppb, 4ppb and 6ppb) of fumaronitrile along with control group was maintained for over 100 days. At 60, 80,100 days' fishes were sacrificed both control and treatment groups and the organs such as liver were collected to analyse the changes in testicular enzyme activities and an increasing pattern for both antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) was observed. Enzyme assay is one of the important methods to evaluate the toxicity of xenobiotic. At present, no report is available on the effect of fumaronitrile on antioxidant enzymes and testicular enzyme activities. This study focus light on the effects of fumaronitrile in antioxidant enzymes CAT, SOD and testicular enzymes ALP, ACP, LDH and SDH in *Oreochromis mossambicus*.

Key words: Fumaronitrile; *Oreochromis mossambicus;* Acute-toxicity; Chronic-toxicity; Enzymes

Introduction

The natural aquatic systems were the ultimate recipient of the pollutants (Fleeger *et al.,* 2003). Aquatic ecosystems were contaminated with a wide range of pollutants has become a matter of concern over the last few decades (Vutukuru, 2005). The accumulation and persistence of pollutants by contaminants and toxicants, released from weathering of geological matrix, or from anthropogenic sources, such as industrial effluents and mining wastes (Ebrahimpour and Mushrifah, 2010) represents a major threat to the biological life. Aquatic animals were the key stone species in many ecosystems (Lonsdale *et al.,* 2009). Fishes are one of the most widely distributed organisms in the aquatic ecosystem and reflect the biological effects of environmental pollution. The contamination of aquatic system was attracted the attention of researchers all over the world (Dutta and Dalal 2008).

O.mossambicus is a medium sized laterally compressed fish that has long dorsal fins with 10-13 rays and spines. Its scales are large along the snout and fore head and become smaller along the body. The coloration is a dull greenish yellow with weak banding pattern along the body (Froese & Pauly, 2007). In their natural range, Mozambique tilapia is extensively used as food by traditional fishers. The species has also been spread widely around the world as an aquaculture fish, as part of the ornamental fish trade and, to a limited extent, as a biological control agent. Mozambique tilapia is attractive for aquaculture largely because they can be successfully farmed at high densities and in often poor quality water (Luna 2012).

Aquatic pollution is one of the current global environmental issues. Due to rapid industrialization and unplanned urbanization many rivers in India are experiencing complicated problems of pollution. It causes reduction in the quality of water. Thus, water bodies are frequently stores for a large variety of xenobiotics which cause the biochemical alternations in fish. These enzymes, which remove peroxides, and superoxide radicals including SOD (EC 1.15.1.1, converts superoxide anion radical to hydrogen peroxide), CAT (EC 1.11.1.6, reduces hydrogen peroxide to water) are of the essence in oxidative stress to deal with free radicals causing several disturbances (Pinto *et al.,* 2003; Tripathi *et al.,* 2006). The activity of ALP is related to the mitosis of spermatogenic cells and glucose transport. ACP located in lysosome of leydig cells is involved in the protein

***Corresponding Author**:
Dr. Balaji Paulraj,
PG and Research Centre in Biotechnology,
MGR College, Hosur,
Tamilnadu, India.
E-mail: balaji_paulraj@yahoo.com

synthesis by abduction of sex hormones. Changes in the activity of ALP and ACP may be used as an indicator of spermatogenesis function (Zhang and Lin 2009).

LDH and SDH are widely distributed and located in the seminiferous tubules and germ cells, which is associated with the maturation of spermatogonia cells, testis and spermatozoa and the energy metabolism of spermatozoa (Yang, *et al.,* 2010).

Measurement of serum biochemical parameters can be especially useful to help in identifying target organs of toxicity as well as the general health status of animals. This measurement has been advocated to provide early warning of potentially damaging changes in stressed organisms (Folmar 1993; Jacobson- Kram and Keller 2001). Plasma and serum reflect the physiologic state of an animal because they are the products of intermediate metabolism (Artacho *et al.,* 2007).

Besides, a number of chemicals used by pharmaceutical industries are not degradable. The pharmaceutical effluents may contain some components or moieties that could be toxic, carcinogenic or mutagenic to aquatic life. Fumaronitrile ($C_4H_2N_2$) is one of the most common chemical agent used in the pharma industries. Fumaronitrile is a basic nitrile compound, derived from fumaric acid. When heated to decomposition this compound emits toxic fumes of carbon monoxide, carbon dioxide and nitrogen oxides (NOx). Fumaronitrile is insoluble in water but highly reacts with strong acids and bases (NTP, 1992). Xia *et al.,* (2011) reported that quaternary ammonium compounds are the potent inhibitors of hERG potassium channels which may results in cardio toxicity in vivo. No information available on acute or chronic toxicity effects of fumaronitrile on aquatic organisms. Hence, this study is aimed to address the Eco toxicity of fumaronitrile on fresh water fish *Oreochromis mossambicus* through acute toxicity, antioxidant and testicular enzyme activities.

Therefore, enzyme assay is one of the important methods to evaluate the toxicity of xenobiotic. At present, no report is available on the effect of Fumaronitrile on testicular enzyme activities. This study focus light on the effects of Fumaronitrile in testicular enzymes ALP, ACP, LDH, SDH, in *O.mossambicus*

Materials and Methods

Fumaronitrile (98%) was purchased from Sigma-Aldrich, hydrogen peroxide (H_2O_2), riboflavin, EDTA, sodium phosphate and other chemicals used in this assay were purchased from HiMedia (AR grade). All the reagents were prepared using double distilled water.

Acute toxicity

Juveniles of *Oreochromis mossambicus* was collected and acclimatized under laboratory conditions. The fishes were maintained in plastic tubs filled with 15 liters of dechlorinated tap water. The acute toxicity test of fumaronitrile was carried out in five different concentrations (20, 40, 60, 80 and 100ppb) along with control for 96 hrs. Each tub contains 10 fishes. The LC50 value of fumaronitrile on (*Oreochromis mossambicus*) were determined.

Chronic toxicity

Juveniles of *Oreochromis mossambicus* was collected and adapted under laboratory conditions. The fishes were kept in plastic tubs filled with 20 litres of dechlorinated tap water. The chronic toxicity test of fumaronitrile was carried out in three different concentrations from LC50 value (2, 4 and 6ppb) along with control for 100 days. Each tub contains 15 fishes. The toxic effects of fumaronitrile on (*Oreochromis mossambicus*) were determined by Behrens-Karber's method.

Antioxidant enzyme activities

Catalase activity

The reaction mixture (2ml) contained 50µl of tissue (liver) homogenate in 50mM phosphate buffer and 100µl of 50mM of H_2O_2 in phosphate buffer at pH 7.0. The specific activity of catalase was expressed as micromoles of H_2O_2 reduced per minute per mg protein.

Superoxide Dismutase activity

The reaction mixture (3ml) contained 50mM sodium phosphate buffer pH 7.6, 20µg riboflavin, 12mM EDTA,100mg NBT and 100µl of tissue homogenate was used to initiate the reaction. Enzyme activity was calculated by units measuring the change in absorbance at 480nm.

Testicular enzyme assay

Testicular enzymes such as Sorbitol dehydrogenase (SDH) and lactate dehydrogenase (LDH) activities were assayed by Gerlach (1983) and Vassault (1983), Acid phosphatase (ACP) and alkaline phosphatase (ALP) activities were assayed by Estiarte *et al.,* (2008) and Michell *et al.* (1970.

Estimation of acid phosphatase and alkaline phosphatase

Acid and alkaline phosphatase activity was estimated according to Michell *et al.,* (1970) and Estiarte *et al.,* (2008). The reaction medium for acid phosphatase contained 0.7 ml sodium acetate buffer (pH 5.0), 0.25 ml p-nitrophenyl phosphate (pNPP, 5mM) as substrate and 0.05 ml of enzyme totaling to 1 ml was incubated for 30 minutes at 37°C. The reaction was stopped by adding 4 ml NaOH (0.1 N) and incubated for another 30 minutes at 37°C. The reaction medium for alkaline phosphatase contained 0.5 ml glycine buffer (pH

7.8), 0.2 ml magnesium chloride (10 mM), 0.25 ml p-nitrophenyl phosphate (pNPP, 5mM) as substrate and 0.05 ml of enzyme totaling to 1 ml was incubated for 30 minutes at 37ºC. The reaction was stopped by adding 4 ml NaOH (0.02 N) and incubated for another 30 minutes at 37ºC. The estimation involves measurement of yellow colour of p-nitrophenol at 420nm (Synergy HT Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA).

Estimation of sorbitol dehydrogenase and lactate dehydrogenase

Sorbitol dehydrogenase (SDH) and lactate dehydrogenase (LDH) activity was measured according to Gerlach (1983) and Vassault (1983). In a 3.00ml reaction mix, final concentrations include 78mM triethanolamine, 183 mM D-fructose, 0.2mM ß-nicotinamide adenine dinucleotide, reduced form, 0.033% (w/v) bovine serum albumin and 0.055 - 0.075 unit sorbitol dehydrogenase. SDH activity was measured at 320nm (Synergy HT Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). LDH activity was measured by the spectrophotometric method of Vassault (1983). Tissue sample (0.1 ml), NADH (0.24mM), and Tris (81mM)/NaCl (203mM) buffer (pH 7.2) were incubated for 15 min at 30ºC. The reaction was initiated by the addition of 0.5 ml pyruvate (9.8mM) to make a total volume of 3.0 ml. The activity of LDH was measured at 320nm (Synergy HT Multi-Mode, Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA).

Statistical Analysis

The obtained values are expressed as mean ± SE. Differences between groups were evaluated by one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) software package for windows (version 16.0). Post-hoc testing was performed for inter group comparisons using the least significant difference (LSD) test $P<0.05$ was considered statistically significant.

Results

Acute Toxicity: In this study, every 12 hours the tubs were observed for the activity of fish. During the observation, every fish showed restlessness, rapid body movement and difficulty in respiration. No mortality was noted till 48 hrs on all fumaronitrile treatment groups whereas on 72 and 96 hrs increased rate of mortality was observed above 60 ppb concentrations. In control, there was no mortality of fishes. The acute toxicity of fumaronitrile on juveniles of *O.mossambicus* was found to be as 60ppb (LC_{50}) on 96 hrs.

Antioxidant enzyme activities: The antioxidant enzyme CAT activity of fumaronitrile treated *O.mossambicus* was depicted in Figure.1. The gradual increase in CAT enzyme was observed in all the treatment groups when compared with control. The result shows that the increased level of CAT enzyme after 60 days with increasing concentration of fumarotrile. The antioxidant enzyme SOD activity of fumaronitrile treated *O.mossambicus* was depicted in Figure.2. The increased level of SOD on increasing concentration of fumaronitrile as like CAT was observed during the experimental period. There were no significant changes observed in the control fishes for both CAT and SOD activities.

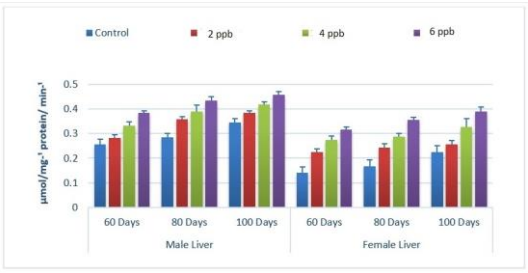

Figure 1. Catalase activity of fumaronitrile treated *O.mossambicus*

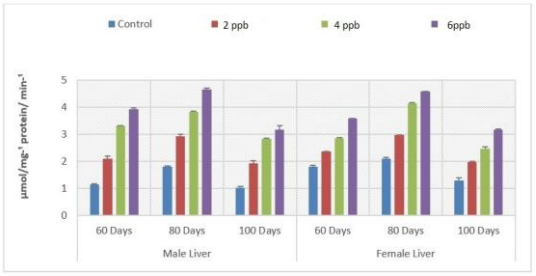

Figure 2. SOD activity of fumaronitrile treated *O.mossambicus*

Testicular enzyme activity

Acid phosphatise activity: Fig. 3 represents the specific activity of ACP in the testis of *Oreochromis mossambicus* exposed to fumaronitrile. The ACP activity in testis of *Oreochromis mossambicus* is low in all tested concentration of fumaronitrile when compared to that of control testis. The activity is decreased with increasing concentration. When compare with control a significant ($P< 0.05$) reduction in ACP activity is found in testis of *Oreochromis mossambicus* exposed to fumaronitrile.

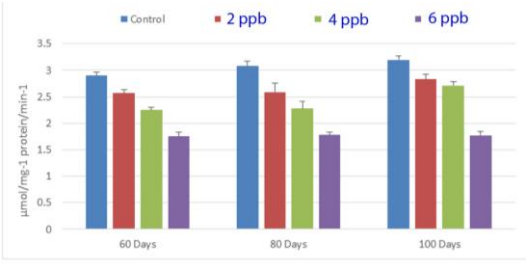

Figure 3. ACP of fumaronitrile treated *O.mossambicus*

Alkaline phosphatise activity: Content of ALP activity is reduced in higher concentration of fumaronitrile exposed *Oreochromis mossambicus* which is recorded (Fig 4). However, the activity shows a noticeable change at 4ppb and 6ppb than those of other exposure 2ppb. Decreased ALP activity in testis of *Oreochromis mossambicus* exposed to fumaronitrile is statistically significant ($P< 0.05$) when compared to control.

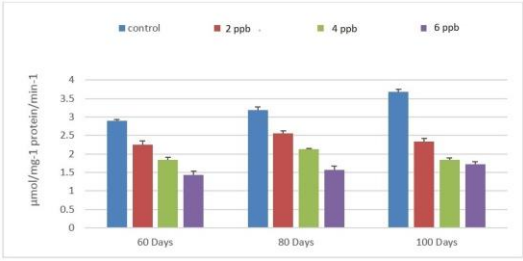

Figure 4. ALP of fumaronitrile treated *O.mossambicus*

Lactate Dehydrogenase activity: The activity of LDH in different organs of *Oreochromis mossambicus* is depicted in Fig (5). LDH activity observed in the fumaronitrile exposed fish testis is low when compared to control. In compared with that of control, a significant ($P< 0.05$) decrease in LDH activity was found in 100days of exposed *Oreochromis mossambicus*. However, the activity is significantly ($P< 0.05$) very low in testis of *Oreochromis mossambicus* exposed to higher concentration of fumaronitrile.

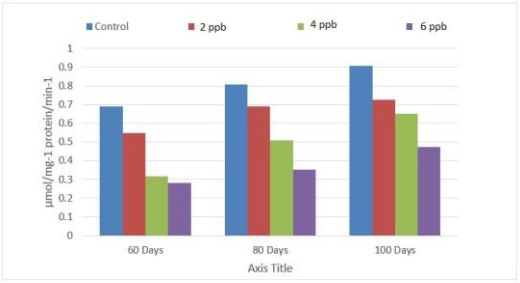

Figure 5.LDH of fumaronitrile treated *O.mossambicus*

Sorbitol Dehydrogenase activity: The activity of SDH is decreased at a lower concentration of fumaronitrile and its decrement was significant ($P< 0.05$) at a higher concentration. A prominent reduction in SDH enzyme activity is noticed in 80days and 100days when compared to 60days exposure of fumaronitrile. A decreased SDH enzyme activity is found in testis in all the concentrations when compared to control. Significant ($P< 0.05$) decrease in SDH activity is both dose and duration dependant in testis of *O.mossambicus* exposed to three different concentration of fumaronitrile (Fig.6).

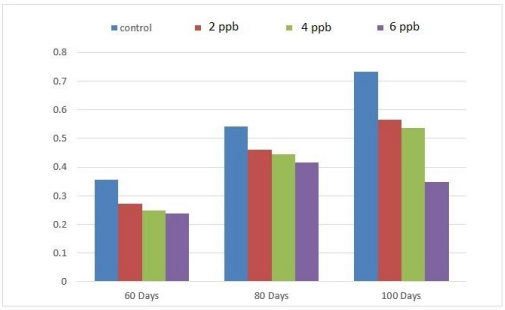

Figure 6. SDH of fumaronitrile treated *O.mossambicus*

Discussion

Related study on other xenobiotics was done and observed where every fish shows the movement on the surface to gulp air intense opercula movement, accumulation of mucus on the body (Anbu and Ramaswamy, 1991; Vasanth *et al.,* 2013). Vasanth *et al.,* (2012) reported that the similar dose dependent increasing activity of antioxidant enzymes on anthracene treated teleost fish *Labeo rohita*. Studies of metal-induced alterations in antioxidant enzyme activities reported that especially SOD and CAT, two major antioxidant enzymes, are affected both in vitro and in vivo (Almeida *et al.,* 2002; Atli *et al.,* 2006; Hansen *et al.,* 2006). In this study when fishes exposed to fumaronitrile it starts synthesizing free radicals. To overcome from the toxicity effect of free radicals, fishes starts to secrete the enzymes like SOD and CAT. Rueda-jasso *et al.,* (2004) reported that activity levels of the antioxidant enzymes CAT and SOD were higher in livers of fish *Solea senegalensis* with a high lipid level.

In this research ALP and ACP enzyme activity in the testis of *O.mossambicus* exposed to all concentration of fumaronitrile is decreased when compared to control. The activities of ACP and ALP enzyme are decreased gradually in low concentration to higher concentration.

A statistically significant decreased in ALP enzyme activity is found in the testis of *O.mossambicus* exposed to all fumaronitrile concentrations. In this study, decreased ACP enzyme activity in testis is observed in treated groups when compared with control. A decrease in the ACP and ALP activity in fumaronitrile treated *O.mossambicus* indicated that fumaronitrile administration produced a state of decreased steroidogenesis where inter and intracellular transport is reduced as a result in decreased steroidogenesis. Zhang and Lin (2009) observed all most equal level of ACP and ALP enzymes activity in rat. In earlier reports decreased ACP and ALP enzymes activity show decrease in the activity of phosphatase in nucleus of the spermatocytes during spermatogenesis. Decreased enzyme activity of testicular ALP and ACP of 3, 4-DCA-treated rats also reflect testicular

degeneration, which may be a consequence of suppressed testosterone and indicative of lytic activity (Kaur *et al.,* 1999).

However, in this study there is a decrease in LDH and SDH activity. The increased enzyme activity in testis and liver under normal condition, a statistically significant decrease in SDH and LDH activity ($P<0.05$), was found in the testis of *O.mossambicus* exposed to all fumaronitril concentrations. The decreased in SDH and LDH enzyme activity is statistically differ from control. The decreased activity of LDH and SDH will affect the process of spermatogenesis and may induce infertility.

These findings have shown that fumaronitrile disrupts the steroidogenesis functions. Thus, the present study concluded that exposure of fumaronitrile results in increased oxidative stress, altered antioxidant status and testicular enzyme activity. Therefore, these parameters can be used as indicators of fumaronitrile toxicity.

From the results, it is evident that one of the pharma industrial waste chemical called fumaronitrile causes damage to the fish tissues by generating reactive oxygen species (ROS). The elevation in the antioxidant enzyme results also confirms the free radicals formation and testicular enzymes modulation by fumaronitrile in fish *O.mossambicus.* Hence, further chronic toxicity study is needed to determine the bioavailability and fate of fumaronitrile on fishes as a bio indicator for Eco toxicity studies.

Ethical Approval

There is no ethical statement to use the fish as animal model. But all applicable international, national, and /or institutional guidelines for the care and use of animals were followed

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