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Effect of Ethanol on Membrane Stability in *Oreochromis Mossambicus* (Peters)

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Abstract

Excess production and usage of ethanol as an alternative fuel has resulted in frequent ethanol spillage, which brings about massive fish kills arising due to depletion in oxygen content by ethanol, and data available so far on the impact of ethanol on aquatic ecosystems are scarce. The present investigates the alterations in the membrane stability after *in vitro* and *in vivo* exposure of fish erythrocytes and lysosomes to ethanol at different sub lethal concentrations. *Oreochromis mossambicus* (Peters) was selected as an animal model and the LC50 value of ethanol was found to be 13.01 g/l. Ethanol brings about considerable damage to the RBCs when subjected to *in vitro* and *in vivo* conditions of different ethanol concentrations and it was found to be highly significant at 0.1% level. Similarly a significant decrease ($P<0.001$) in lysosomal fraction of â-glucuronidase and acid phosphatase activities were observed both in *in vitro* and *in vivo* studies. Ethanol induced mutilations to the membrane of RBCs and lysosomes when subjected to different concentrations of ethanol both in *in-vivo* and *in vitro* conditions are found to be very momentous.

Keywords: RBCs, *Oreochromis mossambicus*, Ethanol, Membrane, Lysosomes.

In the last ten years, the production of ethanol has increased dramatically due to the demand for ethanol-blend fuels (Shaw, 2011). Sampling and analysis of environmental media that has occurred in connection with ethanol spill response activities have shown harmful impacts of ethanol on aquatic environments. The breakdown of ethanol in surface waters through biological and chemical processes could potentially result in the consumption of significant quantities of dissolved oxygen, which in turn would adversely affect aquatic life, potentially leading to fish kills. An ethanol concentration of 56.4 mg/l in the water column causes acute toxicity to aquatic life, whereas 61mg/l in water column causes chronic toxicity to aquatic life (USEPA, 1995).

There had been several news reports stating the spillage of ethanol resulting in fish kills (Environment News Service, 2009). Report by Kris Bevill (2009) cited that derailment of Canadian national train resulted in the leakage of an estimated amount of 55,000 gallons to 75,000 gallons of ethanol into the surrounding soils and waterways. The breakdown of ethanol in surface water consumed dissolved oxygen from the water column, which caused stress or killed fish and mussels (Kris Bevill, 2009). This brings into notice that ethanol is toxic at high concentration and can oxidize to acetaldehyde which is toxic even at lower concentrations.

The indiscriminate usage of ethanol contributes to pollution of air, soil, water and global warming (Pimentel *et al.,* 2008). The hydrophilic nature of ethanol makes it difficult to extract it from water. Due to the high water solubility, it readily crosses important biological membranes, such as the blood brain barrier, which in turn affect a large number of organs and biological processes in the body. Fish erythrocytes were used to study adaptive responses to xenobiotics induced changes at the membrane level within short time spans as these cells are nucleated and express many functions as that of somatic cells. Erythrocyte membrane was often used as a model membrane in investigating the structure and functions of the biological membranes. Lysosomes, the highly conserved multi-functional cellular organelles, contain a battery of over sixty hydrolytic enzymes (acid phosphatase (ACP), β glucuronidase, cathepsin, aryl sulphatase etc.) which play an important role in breaking down the substances within a cell (autophagy) or substances that have been taken in from outside the cell (heterophagy) (Bozzola and Russell, 1992; Holtzman,1976). Lysosomal reactions appear to provide useful biomarkers that are diagnostic for cell injury and the measure of integrity serves as a simple and cost effective approach to study pollutant exposure (Moore, 1990).

There is no report is available on the effect of ethanol on lysosomal and erythrocyte membrane of the fresh water teleost, *O. mossambicus* so far. *Oreochromis mossambicus* is a hardiest fishes in aquaculture farms and it can tolerate a wide range of salinity from fresh water to waters of 30 to 48 ppt salinity (Panikkar and Thampi, 1954). Therefore, the present work is a baseline attempt to investigate and assess the toxicity of different sub lethal concentrations of ethanol.

Materials And Methods

Experimental Design

Fresh water fish, *Oreochromis mossambicus* (Peters) of almost similar size (10 ± 2g) was collected from local hatcheries. They were acclimatized to the laboratory conditions for 15 days in large tanks filled with dechlorinated water (500L). The physico chemical parameters of water (dissolved oxygen of 8.16 ppm, total hardness 13±2 mg/l, total alkalinity 4±2 mg/l, temperature 26 ± 2^0 C, P^H 7.0 ± 0.33 and salinity at 0 ppt) was estimated daily using standard APHA (1998) methods and were maintained constant throughout the experiment. 96 hrs LC50 value has been determined in different concentrations of ethanol ranging from 1.27g/L to 127g/L. A control without the toxicant was also maintained similar to that of test with out addition of ethanol (Bijoy *et al*., 2003). The LC_{50} value for 96 hour was found to be 13.01g/L and it was confirmed following Probit analysis method (Finney, 1971). Three concentrations of ethanol that corresponds to $1/20^{th}$,$1/10^{th}$ and $1/5^{th}$ of LC_{50} value (0.65g/L, 1.3g/L and 2.6g/L respectively) were taken as sub lethal dose.

In vitro RBC and lysosomal membrane studies

RBC membrane stability studies in *in-vitro* condition was done with blood drawn from the common cardinal vein of fish, using 1ml sterile plastic insulin syringe (26mm gauge size) containing sodium citrate as an anticoagulant (5mg/ml) (1:5 dilution) (Oser, 1976 and Smith *et al*., 1952). Lysosomal fraction of hepatic tissues was isolated by homogenization in isotonic sucrose (0.33M) and centrifugation. 0.5 ml of ethanol was directly added into the tubes having specific volumes of RBCs and lysosomal fraction of hepatocytes, in which the final ethanol concentrations in the tubes were 0.65 g/l, 1.3 g/l and 2.6 g/l respectively. The RBC membrane stability was determined by measuring the absorbance of hemoglobin released by hemolysis in the supernatant at 540 nm. Activity of lysosomal enzyme, acid phosphatase and β-glucuronidase in the various sub cellular fractions of liver tissue of *O. mossambicus* (*in vitro* and *in vivo* conditions) were determined according to Plummer, (1987). Acid phosphatase activity was carried out by the method of King and Armstrong (1934); King *et al*., (1937, 1942). β—glucuronidase activity was estimated by the method of Kawai and Anno (1971). Total protein content in tissues was estimated by the method of Lowry *et al*. (1951). The enzyme activity thus obtained were expressed as units/mg protein.

In vivo RBC and lysosomal membrane studies

Ten numbers of *O. mossambicus* (10±2g each) were kept in three separate tubs having desired concentration of ethanol (0.65 g/L, 1.3 g/L and 2.6 g/L respectively) along with tap water. Six replicates were kept for each experiment. A control was also maintained in the water without the addition of ethanol. While in the sub lethal toxicity study, water was changed daily and the test solutions were renewed every 24 hours to maintain the dissolved oxygen concentration at optimum level (USEPA, 1975). The fishes were fed on the same commercial diet *ad libitum*. The exposure period such as 7 and 21 days were selected as per Organization for Economic Cooperation and Development (OECD, 2000) guideline programme meant for aquatic organisms. During the experimental period of 21 days, the animals were fed on the same diet to avoid the effects of starvation on normal physiological processes and antioxidant stress. Any other factor likely to influence toxicity was nullified by maintaining the suitable control. RBCs and lysosomal fraction of hepatic tissues were isolated from those fishes, which were exposed to respective ethanol concentrations for 21 days followed by a periodical sampling at 7 days. Membrane stability of RBC and lysosomes are determined as described above.

Statistical analysis

Data analysis was done with Microsoft Excel XP and SPSS version 15.0, for statistical evaluation. Results are presented as mean ± standard deviation (S.D). Data distributions were examined to fit a normal distribution and homogeneity of variance was tested using ANOVA supplemented by multiple comparison test using Dunnett's and Tukey's post hoc method (significance at $P<0.001$).

Results And Discussion

The 96 hr LC_{50} value of ethanol was found to be 13.107g/L (range between 12.786 and 13.382 g/L) with 95% confidence limit. Fishes exposed to different sub lethal concentrations of ethanol exhibited erratic movements, loss of equilibrium, grouping, increase in respiratory rhythm, excess secretion of mucus followed by a gradual onset of inactivity. Observations made by Yadav *et al*. (2007) corroborate the above statement. Frequent surfacing and gulping shows the effort of the animal to cope with the deficiency of oxygen, and fill the two lateral vascular air sacs with fresh air for accessory respiration. On initial exposure to ethanol, the fish *O. mossambicus* exhibited characteristic avoidance behavior by rapid swimming, stretching half of their body out of water surface and trying to jump out. Fish secreted copious amount of mucus, a defense mechanism to neutralize the effect of ethanol in the terminal phase of intoxication, the fish lost their balance and equilibrium and died.

The functions of membrane are determined by membrane composition and organization (Levin *et al.*, 1990). Membrane lipid composition directly reflects the membrane properties (Yeagle, 1985). The erythrocyte membrane has long been served as a convenient model system employed for studying the chemical and physical properties of cell membrane due to its relative simplicity (Salil and shyamali, 1999). Many xenobiotics evoke alteration directly in the bounding membrane of the lysosomes (Moore and Lowe, 1985). Weeks and Svendsen (1996) stated lysosomal fragility as a promising biomarker in environmental toxicity studies. Lysosomal alterations in fish hepatocyte have been recommended as potential cytological biomarkers for environmental pollutants. Lysosomal stability is a good indicator of physiological fitness in fish liver (Allen and Moore, 2004).

Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l of ethanol on erythrocyte and lysosome membrane stability in *O. mossambicus* is given in Figure 1 to figure 5. The results obtained were analyzed statistically using ANOVA of the raw data, followed by Dunnett's and Tukey's post hoc method. *In-vitro* and In vivo studies conducted on the RBC membrane stability revealed that ethanol had a labilising effect on the erythrocyte membrane and was found to be depended upon dosage. The present study confirms that, ethanol brings about an increased disintegration of erythrocytes. Findings of Cunha et al. (2007) corroborates the above statement. RBC hemolysis was observed when *O. mossambicus* was subjected to different concentrations of ethanol in *in-vitro* conditions (Figure 1) and indicates that erythrocyte membrane was damaged depending upon the concentration of ethanol. The levels of released hemoglobin serves as an indicator of hemolysis, caused by increased membrane fragility (Niranjan and Krishnakantha 2000). Chi and Wu (1991) observed similar state of increased rate of hemolysis of red blood cells when mediated by ethanol. The hemolysis rate increased depending upon the increased concentration of ethanol. The present investigation points out that ethanol bring about considerable damage to the RBCs when subjected to different concentrations of ethanol in *in-vitro* conditions. The ability of organic solvent such as ethanol to destabilize the membrane as well as the membrane proteins correlates

to cytotoxicity. This study proves that erythrocyte membrane exhibits increased rate of hemolysis when treated with ethanol. Membrane peroxidation leads to changes in membrane fluidity, permeability and results in the enhanced rates of protein degradation, which will eventually lead to cell lyses. The deformity of destructed RBC and increased rate of RBC hemolysis results in the increased production of free radicals. In addition, ethanol exhibits a denaturizing effect on erythrocyte membrane that is associated with abnormal RBC morphology resulting in an increased susceptibility to hemolysis (Prokopieva *et al*., 2000; Chi *et al*., 1990). From these results it can be concluded that RBC hemolysis and lipid peroxidation by ethanol can be one of the molecular mechanisms involved in ethanol induced toxicity (Armutcu *et.al.*, 2005).

RBC membranes are prone to peroxidative damage because they are rich in unsaturated fatty acids and are exposed to high oxygen concentration in the blood (Niki *et al*. 1988 and Hayam *et al*. 1993). The increase in RBC hemolysis (Figure 2) observed when *O. mossambicus* was exposed to ethanol for 7 days is due to membrane lipid peroxidation and may be due to increased activities of serum specific enzymes. Ivanov (2001) observed similar destruction of RBCs and hemolysis on the membrane when treated with organic solvents. The decrease in hemolysis observed at 1.3g/l (Figure 2) when *O. mossambicus* was exposed for 21 days indicates that RBC membrane has become rigid. This can be due to the slight increase in the cholesterol/ phospholipids ratio (Yeagle *et al.* 1990 and Kuypers *et al.* 1996). It also leads to the decrease in unsaturated fatty acids in the erythrocyte membrane followed by an increase in saturated fatty acids. A fall in polyunsaturated fatty acids (PUFA) followed by an elevation of cholesterol increases the rigidity of the phospholipid bilayer (Dobrestov *et al.* 1977). Oxidative cross-linking of membrane protein can also induce increased membrane rigidity and decreased RBC deformability. Levin *et al.* (1990) have proposed that the oxidation of membrane lipids results in the formation of peroxidative degradation products like malondialdehyde leads to the cross linking reactions of the lipid-lipid and lipid-protein type thereby making the membrane more rigid and hence less fluid. The increase in hemolysis observed at 0.65 g/l and 2.6 g/l on prolonged exposure can be due to the decrease in Hb that arises due to the increased fragility of the erythrocytes. Observations made by Patra *et al.* (2001) support this finding.

Highly significant (P<0.001) alterations are observed in all the three sub cellular fractions (nuclear, soluble and lysosomal) of the treatment groups when compared to control group of *O. mossambicus* in *in-vitro* (Figure 3). In the present study a significant decrease (P<0.001) in lysosomal fraction of acid phosphatase and â-glucuronidase activities are observed followed by an increase in soluble and nuclear fraction both in *in vitro* and *in vivo* (Figure 3 and Figure 4). Studies conducted by Kohler *et al*. (2002) and Wahli (2002) demonstrated a good dose-response relationship between the severity of lysosomal alterations in the liver of European flounder and the levels of xenobiotic pollution. The lysosomal enzyme release assay carried out *in vivo* (Figure 5) revealed significant increase in β-glucuronidase release with exposure period (P<0.001). Pair wise comparison employing Tukey's post hoc test explaines all possible interactions of time intervals and are found to be highly significant (P<0.001) (Figure 6).

Lysosomal membrane destabilization is a prognostic biomarker for toxicant-induced fish liver dysfunction in biomonitoring programs (Broeg *et al*., 1999; Kohler *et al*., 2001, 2002). Lysosomal membranes on immediate and prolonged exposure of ethanol are subjected to structural and physiological changes such as lysosomal fragility and release of acid hydrolases. These alterations are components of the inflammatory process that are followed by cell death (Cancio *et al*., 1995b). Studies conducted on lysosomes in mammals shows increased membrane fragility, which in turn may lead to cellular destruction and cell death upon heavy metal injury as reported by Sternlieb and Goldfischer (1976). Studies conducted by Tsvetkov

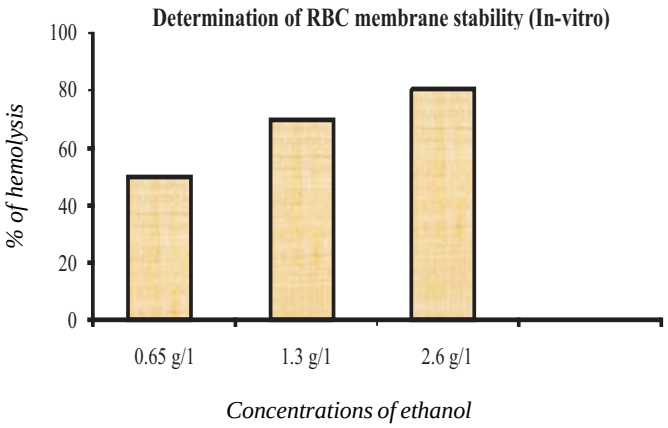

Fig 1: Percentage *hemolysis* in Q. mossambicus on exposure to different concentrations fo ethanol (*in vitro* conditions)

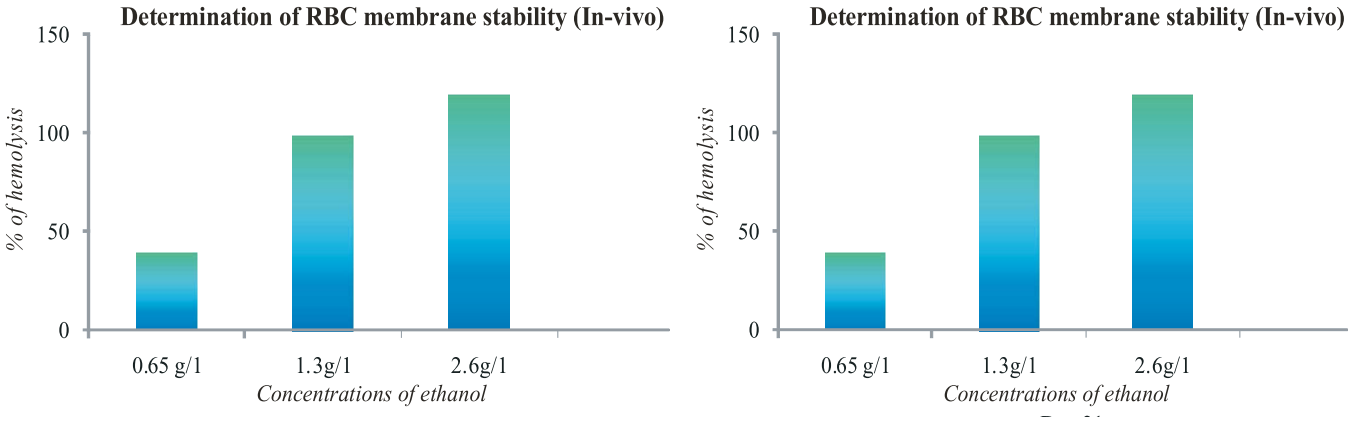

Fig 2: Levels of RBC Membrane stability (*in vivo*) in the blood of *O. mossamblcus* exposed for 7 day and 21 days to different concentrations of ethanol

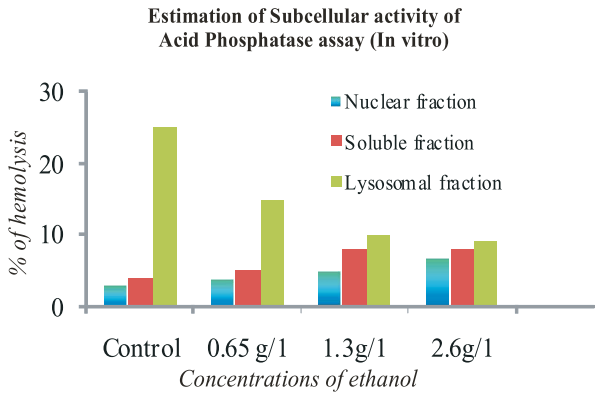

Fig 3: Levels of sub cellular acid phosphatase activity in the hepatic tissue of *O. mossambicus* (*in vitro*)

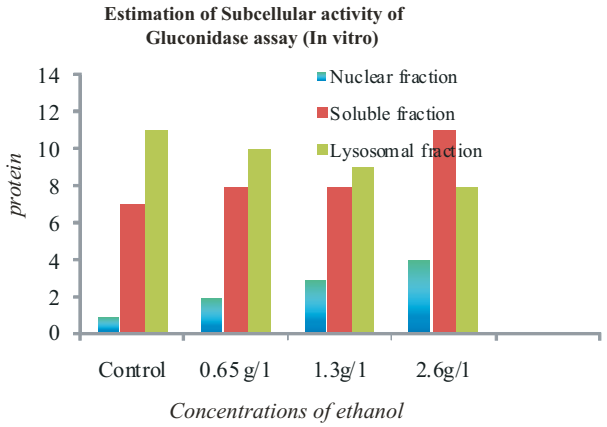

Fig 4: Levels of sub cellular β-glucuronidase activity in the hepatic tissue of *O. mossambicus* (*in vitro*)

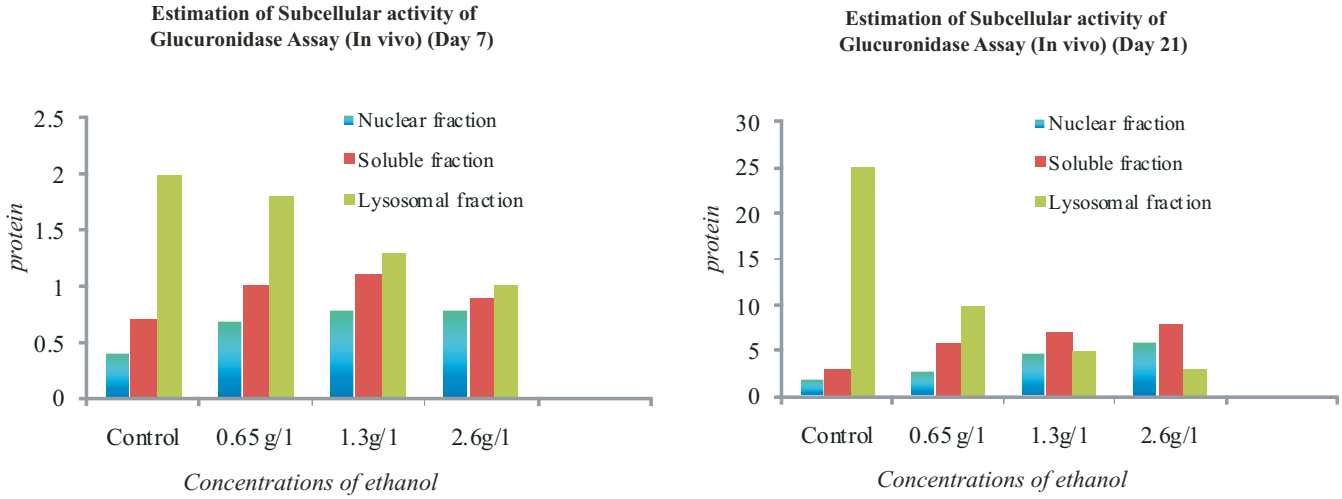

Fig 5: Lysosomal enzyme release as (β-glucuronidase) in the hepatic tissue of *O. mossambicus exposed* for 7 days and 21 days to different concentrations of ethanol (*in vivo*)

	Groups	β-Glucuronidase enzyme release assay
Tukey	0 min Vs 15 min	0.056[d]
	0 min Vs 30 min	0.000[a]
	0 min Vs 45 min	0.000[a]
	0 min Vs 60 min	0.000[a]
	15 min Vs 30 min	0.000[a]
	15 min Vs 45 min	0.000[a]
	15 min Vs 60 min	0.000[a]
	30 min Vs 45 min	0.003[b]
	30 min Vs 60 min	0.000[a]
	45 min Vs 60 min	0.000[a]

The values are significant at a = P<0.01, C=0.05 and not significant at d.

Fig. 6: Multiple Comparison Test (Time) (*In-vivo*) Subsequent pair wise comparisons by multiple comparison tests using Tukey is shown above

et al. (1997) shows similar changes in cathepsin D and acid phosphatase, which explains lysosome targeted stress.

Conclusions

The present study reveals that *O. mossambicus* is sensitive to sub lethal concentrations of ethanol. Ethanol brings about considerable damage to the RBCs when subjected to different concentrations of ethanol in in-vivo and in vitro conditions. To understand complete toxic responses in the liver and to apply this knowledge, we need to integrate molecular, biochemical, physiological and morphological characteristics from the cellular to organ level.

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