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Acute neurotoxicity effect of acrylamide in *Oreochromis niloticus* (Linnaeus, 1758)

CM Gopika, N Sumi, * KC Chitra
Toxicology and Endocrinology Laboratory, Department of Zoology, University of Calicut, Malappuram District, Kerala, India

Abstract
Acrylamide is a monomer widely used as an intermediate in the production of one of the organic chemicals, polyacrylamides. The present study was focused to determine the neurotoxic effect of acrylamide on the brain tissue of the fish *Oreochromis niloticus*. Sublethal concentration i.e., 8.96 µg/L of acrylamide was exposed to fish for 24, 48, 72 and 96 h maintaining control group. The weight of the animal and brain tissue remained unchanged after acrylamide exposure. The activities of superoxide dismutase, catalase and glutathione reductase showed significant ($P<0.05$) decrease than the control group. However, the levels of lipid peroxidation and hydrogen peroxide generation showed significant ($P<0.05$) increase in all treatment groups in time-dependent manner. The results suggest the generation of free radicals in brain tissue as a result of acrylamide exposure. The neurotransmitter enzyme, acetylcholinesterase, showed significant ($P<0.05$) reduction in the brain and serum of blood and this could prove the neurotoxicity of acrylamide. Histological damages like atrophy, infiltration, degeneration, and vacuolization in brain tissue were also observed thereby suggesting acute neurotoxic effect of acrylamide in the fish, *Oreochromis niloticus.*

Keywords: acrylamide, neurotoxicity, oxidative stress, antioxidant enzymes, *Oreochromis niloticus*

1. Introduction
Water is polluted by several sources that include agricultural practices, industrial and commercial effluents, daily human activities and accidental spills of transportation of certain substances. There is a great concern that millions of people around the world even lack access to safe drinking water. Another concern is the reduction in fish population due to pollutants, and fishes are cursed among the organisms as they are continuously exposed to toxic substances. Most fishes are consumed as food by human and thus harmful when the pollutants enter the body through the food chain. In recent years, many countries have introduced several restrictive laws to give particular attention to public to prevent and control aquatic pollution. New technologies and strategies are introduced in many developed countries to limit and eliminate aquatic contamination. International organizations such as Environmental Protection Agency (EPA), World Health Organization (WHO) have prescribed several standard legislative measures to improve quality of water, increase availability and to preserve the water resources. However, the pollution control measures seem to be useless as there are many countries, including India that no laws have been adopted yet to control water pollution. In this case, water pollution is supranational problem that seriously affect the natural water resources at world level. The most effective means of warning the people about the aquatic contamination is done by several eco toxicological studies that pinpoint on the toxic impacts of several toxicants that are exposed directly or indirectly to water bodies. It therefore results in serious damage to the health status of aquatic organisms, particularly fish and thereby affects the fish population as a whole.

Acrylamide is the low cost chemical with wide range of applications - in drinking water and wastewater treatment, crude oil production processes, paper and textile industries, ore, concrete and mineral processing, soil and sand treatment for stabilizing soil erosion, manufacture of dyes and cosmetic additives, and other miscellaneous uses photographic emulsion, adhesives and coatings. Therefore, there is a possibility of the release of certain amount of non-polymerised acrylamide in water bodies (Smith and Oeheme, 1991; EURAR, 2002) [1, 2]. Several observations led to the hypothesis that heating of food could be an important source for the release of acrylamide. Acrylamide that was formed while heating biological materials evaporates and fraction of formed acrylamide could be found in air both in laboratory and kitchen environments were up to about 4 µg/ cubic meter acrylamide could be measured above the fry pan during frying of potato. Acrylamide was found abundant in food production plants, like bakeries, potato chips factories, restaurant kitchens and other places where biological materials are heated, which contains components that could be precursors of acrylamide. This can be a source of acrylamide exposure, which has not been included in risk assessments reports so far (EURAR, 2002) [2]. Therefore, intake of certain fried foods or bakery products containing acrylamide may be of direct human health concern. Ecological studies are now greatly concerned with toxic effects of acrylamide on aquatic organisms and have been observed to serve as indicators of water pollution. The present study was thus designed to focus on the toxic effects of one of the contaminants, acrylamide in fish health and its population.

2. Materials and Methods
2.1 Chemicals
Acrylamide (2-propenamide) of 99.9% purity, malondialdehyde, NADPH, glutathione oxidized,

thiobarbituric acid, pyrogallol and dithiobisnitrobenzoic acid were obtained from HiMedia Research Laboratories Pvt. Ltd., Mumbai, India. Acetylthiocholine iodide was purchased from Alfa Aesar, England. All other chemicals were of analytical grade and obtained from local commercial sources.

2.2 Test animal

Freshwater fish, *Oreochromis niloticus* weighing 17±1.5 g and length 8±1.5 cm were collected from the fish farm, Aquafish Aquarium, B.H. Road, Kottakal, Malappuram District. Fishes were acclimatized to the laboratory conditions for four weeks with constant supply of water and good lighting system. They were maintained in well-aerated tubs (40 L capacity), which was dechlorinated and sustained with fresh water maintaining light and dark at 12: 12h.

2.3 Preliminary tests

The physico-chemical features of the tap water were estimated as per APHA (1998) [3]. Water temperature in the test ranged from 28 ± 2°C during the experiment, oxygen saturation of water ranged between 70 and 100 %, pH is 6.5 to 7.5 which were monitored using a standardized procedures.

2.4 Evaluation of median lethal concentration

The LC_{50} values in the respective time intervals were determined by probit analysis, with a confident limit of 5 % level (Finney, 1971) [4] for 96 h. In the experiment, the concentration of acrylamide at which 50 percentage of the exposed fish undergo mortality at a specific period, i.e., for 96 h is LC_{50}-96 h or median lethal concentration of acrylamide. Fishes were not fed a day prior to and during the test to reduce fecal and excess food contaminating the test solution. For determining LC_{50} concentration, separate tanks of tap water (40 L capacity) were taken, which was dechlorinated and aerated using tubed motorized pumps. Monofilament netting was used to cover the tanks so as to prevent the specimens from jumping out of test solutions. Seven different concentrations (70, 75, 80, 85, 90, 95 and 100 µg/ L) of acrylamide were added in each separate tank. In both control and experimental tanks, 10 fishes were introduced and mortality as well as the behaviour of fishes was recorded throughout the study. The lethal concentration for 50% killing (LC_{50}) values was computed on the basis of probit analysis for 96 h with a confident limit of 5 % level (Finney, 1971) [4].

The above acute toxicity tests were repeated three times in order to confirm the mortality and to reduce the statistical errors. Data were analyzed by Probit of regression analysis as statistical method using SPSS 19.0 statistical analysis software. The LC_{50} value (with 95% confidence limits) was calculated, and then the correlation between mortality against concentrations and the best-fit line were also obtained.

2.5 Experimental design

One-tenth of median lethal concentration of acrylamide was selected as sublethal concentration i.e., 8.96 µg/L. The test concentration was maintained for four durations i.e., 24, 48, 72 and 96 h, respectively along with control fish group. Single dose with different durations were used in present study and ten fish specimens were used for every test and also in control groups. The first group of fishes was maintained in toxicant-free water and was used as control and the treatment groups were exposed to acrylamide at 8.96 µg/L concentration for 24, 48, 72 and 96 h, respectively.

2.6 Preparation of tissue extract

The fish was caught very gently using a small dip net, one at a time with least disturbance. At the end of each exposure time, fishes were weighed and decapitated. Brain of both control and all treatment groups were dissected and stored at 4ºC until the biochemical analyses were performed. A 1% (w/ v) homogenate of brain was prepared in ice-cold normal saline with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 800*g* for 15 min at 4°C to obtain the supernatant, which was then used for the analyses. Total protein concentration in the tissue was estimated by the method of Lowry *et al.* (1951) [5]. Hydrogen peroxide generation was assayed by the method of Pick and Keisari, 1981 [6]. The levels of lipid peroxidation were measured according to the method of Ohkawa *et al.* (1979) [7]. Activities of antioxidant enzymes such as superoxide dismutase (Marklund and Marklund, 1974) [8], catalase (Claiborne, 1985) [9], glutathione reductase (Carlberg and Mannervik, 1985) [10] were assayed in supernatant of brain tissue. Acetylcholinesterase (Ellman *et al.*, 1961) [11] activity was assayed in both tissue and blood serum.

2.7 Histological analysis

At the end of every treatment period, brain tissue from control and treatment groups were dissected, rinsed in physiological saline to remove blood and debris and fixed in 10% buffered formalin for 24h. Tissues were dehydrated in ascending grades of alcohol and were cleared in xylene till the tissues become translucent. Tissues were then transferred to molten paraffin wax for an hour for complete impregnating with wax. The tissue blocks were made and were cut in sections of thickness 4 to 6 microns using rotary microtome. The sections were double stained with haematoxylin and eosin and mounted in DPX (Roberts and Smail, 2001) [12]. The slides were carefully examined and photographs were taken using Cannon shot camera fitted to the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope.

2.8 Statistical analysis

Statistical analysis were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against control group. Data are presented as mean ± SD for n=10. All biochemical estimations were carried out in duplicates.

3. Results and Discussion

Acute toxicity of acrylamide with respect to antioxidant defense system was evaluated in the brain of the fish, *Oreochromis niloticus*. The median lethal concentration for 96 h (LC_{50}-96 h) in the fish, *Oreochromis niloticus* observed by probit analysis was 89.68µg/L (Figure 1). According to OECD guidelines, the test method followed for acute toxicity is by static test, which means there is no flow of test solutions, where it remain static throughout the duration of the test. The mortality of the fish observed during the study showed a

steady step-wise increase according to the increase in the test concentrations. Thus a high degree of positive correlation (r = +0.98) was observed when a graph was plotted for the concentration against the mortality. One-tenth of the acute toxic concentration i.e., 8.96µg/L was used as sublethal concentration for the further evaluation of neurotoxicity of acrylamide.

Sublethal exposure of acrylamide (8.96µg/L) showed no significant changes in the weight of fishes in all treatment durations when compared to the control group (Figure 2). But the percentage of mucous deposition was found increased and this could be first line of defensive mechanism of the fish to escape from toxicant. Acrylamide exposure modified the behaviour of the test animal, which concerns the fate and effects of acrylamide on the aquatic ecosystem. Behaviour is the comprehensive variable to detect the early warning signals to pollution. Fish when exposed to all concentrations of acrylamide showed erratic swimming, rapid opercular movement, and surfacing and this could be to escape from the exposed toxicant. Acrylamide at 8.96µg/L showed no significant changes in the weight of brain in all treatment groups (Figure 3). Brain is the controlling organ and, therefore, vital to maintain normal physiological, metabolic and hormonal activities of the animal. The intelligence of fish is known to acquire by storing, retrieving, combining and comparing memory in new contexts (Humphreys, 1979) [13].

The alterations in antioxidant defense system in the brain of fish were evaluated by assessing the activities of various antioxidant enzymes. Fishes when exposed to acrylamide showed significant ($p<0.05$) decrease in the activities of superoxide dismutase, catalase and glutathione reductase in brain tissue in all treatment groups in time-dependent manner when compared with the corresponding group of control animal (Figures 4-6). The antioxidant enzymes plays an important role in the control, production and elimination of reactive oxygen species (ROS), which in excess can alter the normal functions of the cell and lead to oxidation of cell membranes as well as lesion in mitochondria, protein, DNA and other component of the cell (Stohs and Bagchi, 1995) [14]. ROS are produced by the cells as the result of normal metabolism and are of two groups, namely free radicals and non-radicals. The non-radical forms are created by the sharing of unpaired electrons by the two free radicals. The major ROS having physiological significance includes superoxide anion, hydroxyl radical and hydrogen peroxide (Birben *et al*., 2012) [15].

Superoxide dismutase is the first line of defensive enzyme that catalyses dismutation of superoxide radical to hydrogen peroxide and molecular oxygen (Miller *et al*., 1990) [16]. In the present study, the decrease in the activity of superoxide dismutase revealed the failure of antioxidant defense system in the brain of fish as a result of acrylamide exposure. This was further evident by the significant increase in the level of hydrogen peroxide generation in time-dependent manner. Catalase is another antioxidant enzyme that exists as a tetramer composed of four identical monomers, each of which contains a haeme group at the active site. Catalase also actively participates in the conversion of hydrogen peroxide into water molecule along with glutathione reductase/ peroxidase system (Kirkman *et al*., 1999) [17]. Exposure of acrylamide at sublethal concentration decreased the activities of catalase and glutathione reductase in the brain of the fish. The present results clearly state that acrylamide induced the production of reactive oxygen species and altered the normal functions of antioxidant defense system. The induction of reactive oxygen species has been shown to be associated with oxidative stress-related neurological damage in brain (Calabrese *et al*., 2000) [18]. Similar results have been observed when one of the environmental toxicants, chlordecone, was exposed to the freshwater fish, *Pseudetroplus maculatus* (Asifa and Chitra, 2017) [19].

The levels of hydrogen peroxide generation and lipid peroxidation increased significantly ($P<0.05$) in time-dependent manner after acrylamide treatment when compared to the corresponding control group (Figures 7 and 8) and this could reflect the destruction of membrane lipid bilayer arrangement in the brain tissue. Products of lipid peroxidation, such as malondialdehyde and unsaturated aldehydes, have been shown to inactivate many cellular proteins by forming protein cross-linkages (Siu and Draper, 1982) [20]. The measurement of the level of lipid peroxidation is widely used as biomarker for the induction of oxidative stress, and it was clear from the present observations that acrylamide induced oxidative stress in brain of the fish.

There was a significant ($p<0.05$) time-dependent reduction in the activity of acetylcholinesterase in the brain and serum of blood in all treatment groups when compared to control group (Figures 9 and 10). Acetylcholinesterase is widely used biomarker enzyme to detect the effect of toxicants in the nervous system. The enzyme terminates the nerve impulses by catalysing the hydrolysis of the neurotransmitter acetylcholine into choline and acetate (Tripathi and Srivastava, 2008) [21]. The enzyme is found abundant at central cholinergic synapses and neuromuscular junctions (Sancho *et al*., 1997) [22]. The inhibition of the acetylcholinesterase activity by the toxicant can affect locomotion and equilibrium of exposed organisms, which was evident by the behavioural modifications observed after acrylamide exposure. Acetylcholine acts as an excitatory transmitter for voluntary muscle in the somatic nervous system. It also serves as both a preganglionic and a postganglionic transmitter in the parasympathetic nervous system, and as a preganglionic transmitter in the sympathetic nervous system. In critical regions of the central nervous system, acetylcholine serves as an excitatory transmitter. Thus when cholinesterases are inactivated by the binding of toxicants such as acrylamide, then accumulation of acetylcholine may occur at the nerve synapse, interfering with the normal nervous system function. Therefore, acrylamide was proved as neurotoxic agent, which inhibited neurotransmission as evident by the decrease in the activity of acetylcholinesterase enzyme.

Control tissue showed no histopathological alterations (Figures 11A) whereas acrylamide treatment showed several pathological lesions as atrophy, infiltration, degeneration and vacuolization in brain tissues (Figures 11B-11E). Control brain tissue showed normal histoarchitecture whereas acrylamide-treated fish for 24, 48, 72 and 96 h showed structural modifications. Brain tissue when exposed to acrylamide showed atrophy, infiltration and vacuolization and this could be due to microsomal and mitochondrial

dysfunctions (Tilak *et al.*, 2005) [23]. The severity of histopathological changes increased with the duration of exposure. In the present study the possible reduction in the cholinergic activity of brain could be correlated to the neuronal damages induced as a result of acrylamide exposure. Thus acrylamide proved as potent neurotoxic agent that inhibited the activity of acetylcholinesterase enzyme in the brain of fish, which is also associated with the change in the behaviour of the fish. Similar observations were reported in the brain tissue when exposed to chlordecone in the fish, *Pseudetroplus maculatus* (Asifa and Chitra, 2017) [19]. Thus neurotoxicity of the toxicant, acrylamide was consequently proved by histological examination.

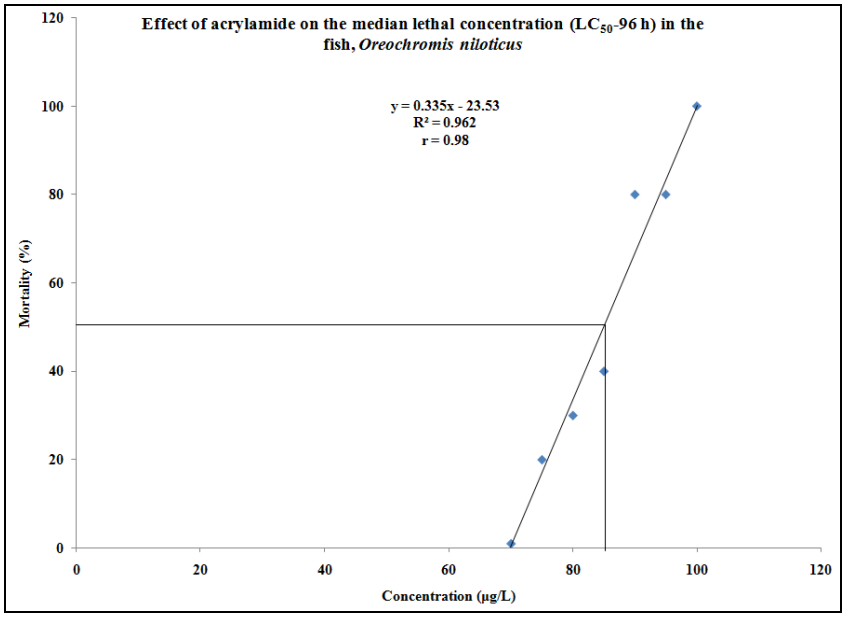

Fig 1

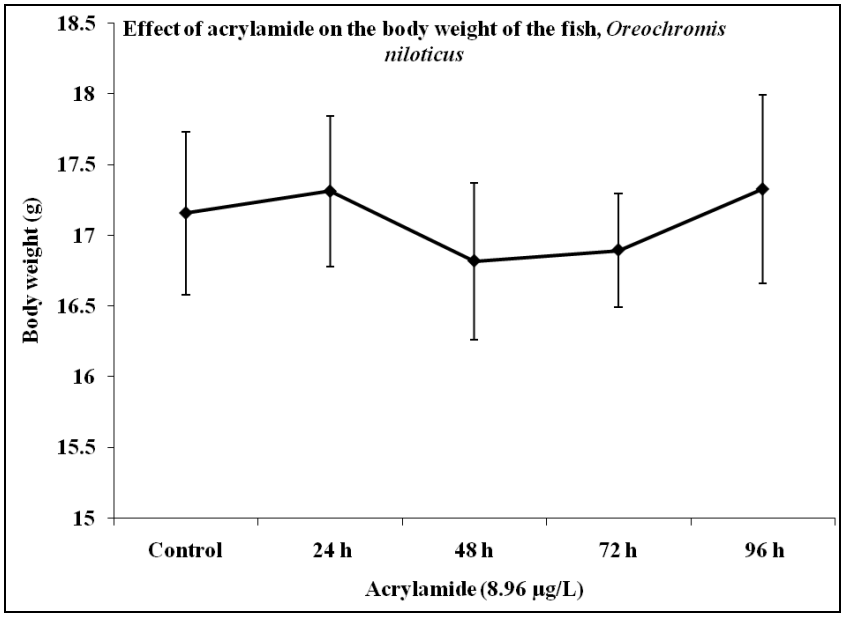

Fig 2

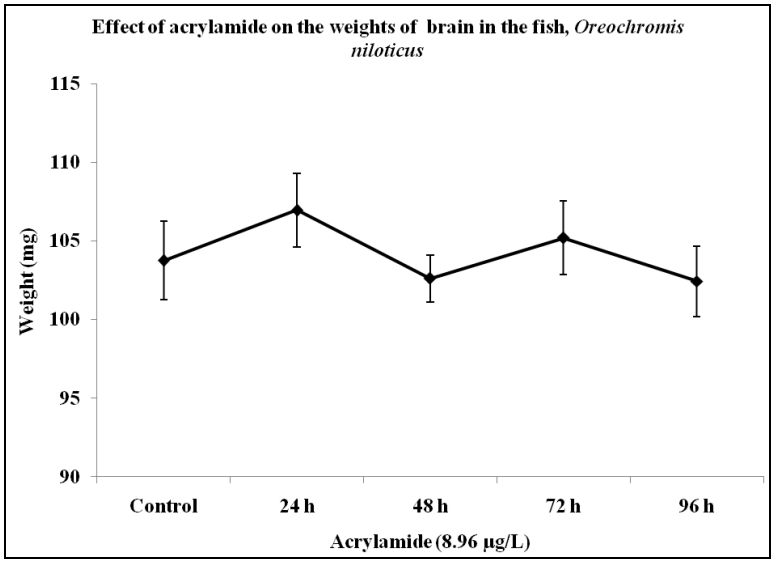

Fig 3

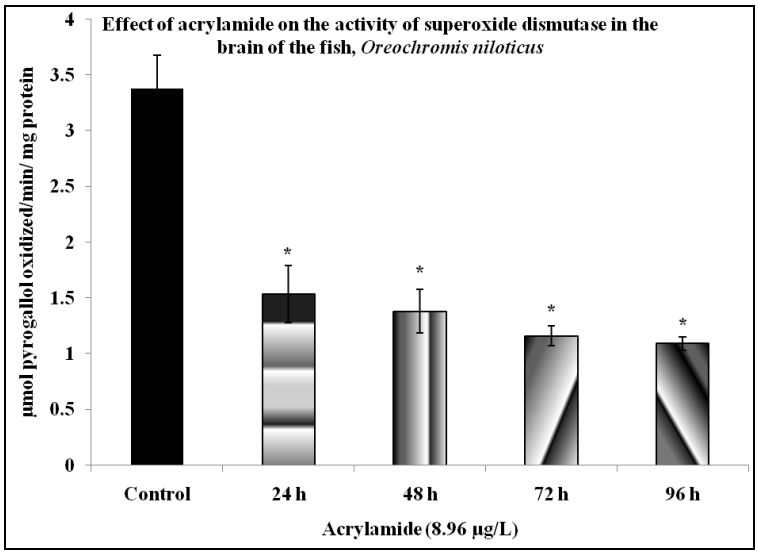

Fig 4

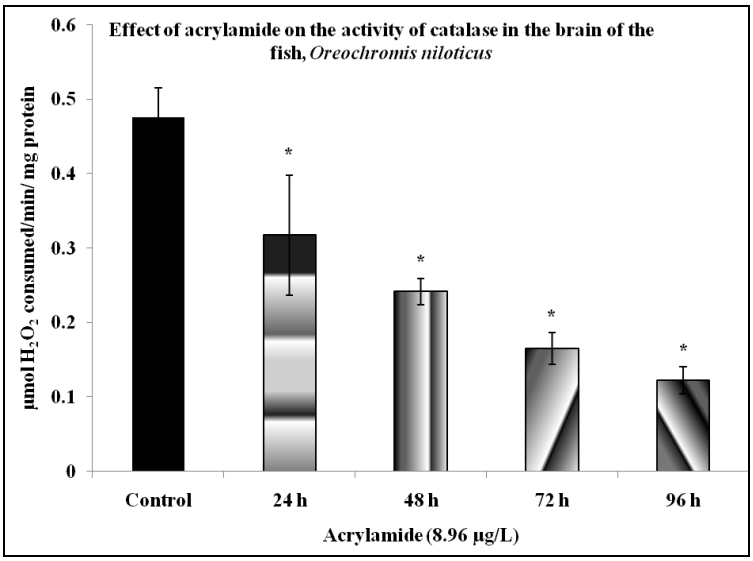

Fig 5

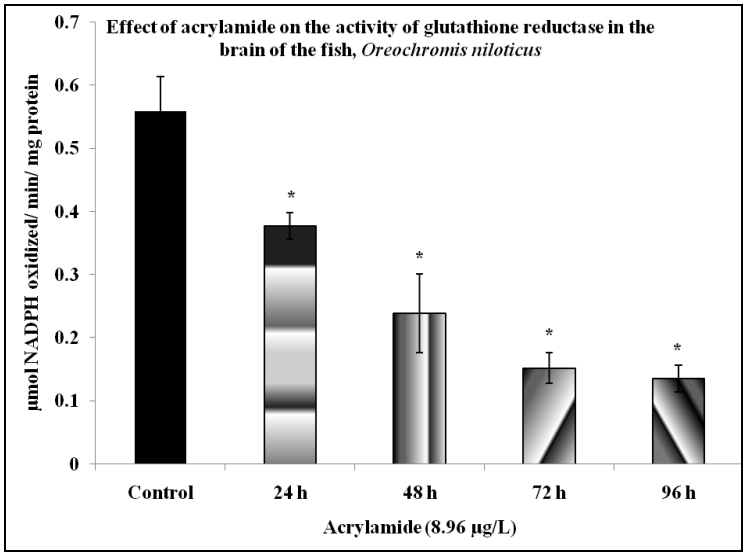

Fig 6

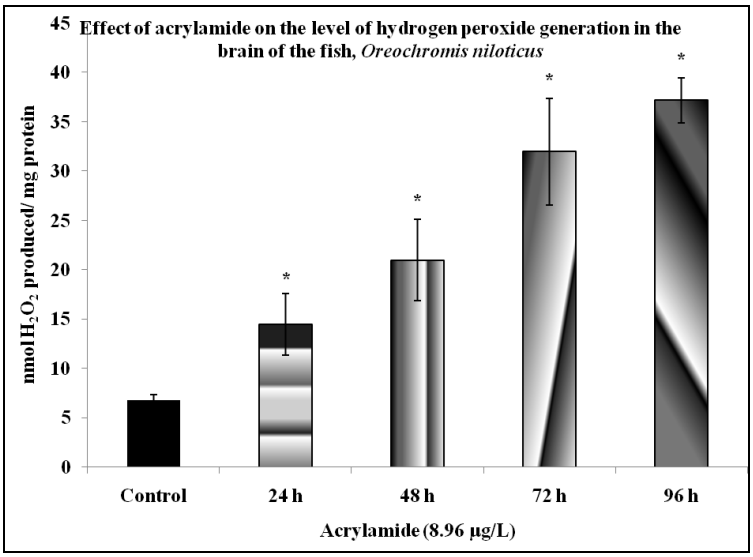

Fig 7

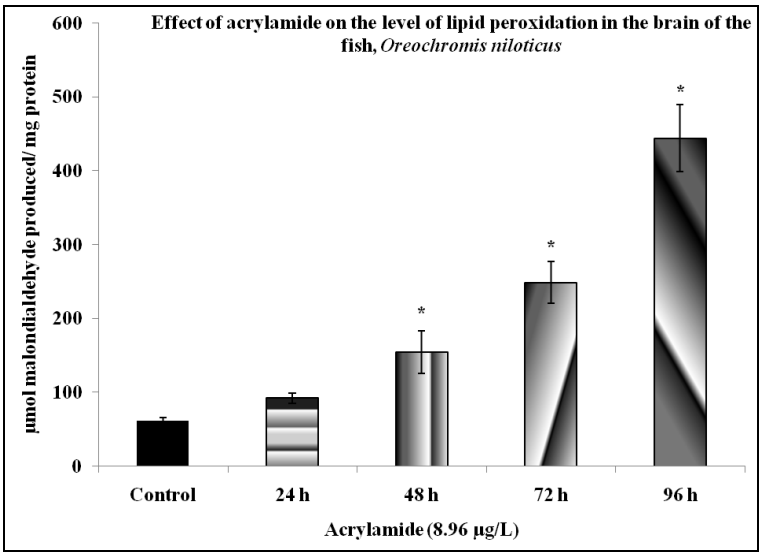

Fig 8

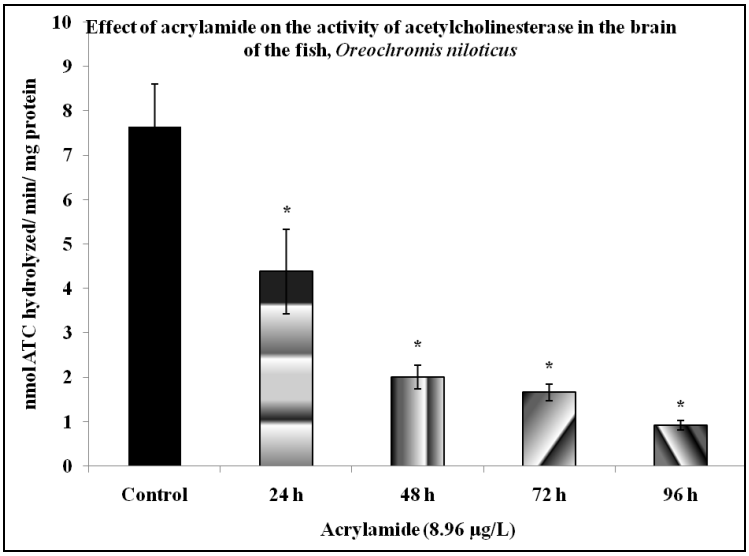
Effect of acrylamide on the activity of acetylcholinesterase in the brain of the fish, Oreochromis niloticus
nmol ATC hydrolyzed/ min/ mg protein
10
9
8
7
6
5
4
3
2
1
0
*
*
*
*
Control
24 h
48 h
72 h
96 h
Acrylamide (8.96 µg/L)

Fig 9

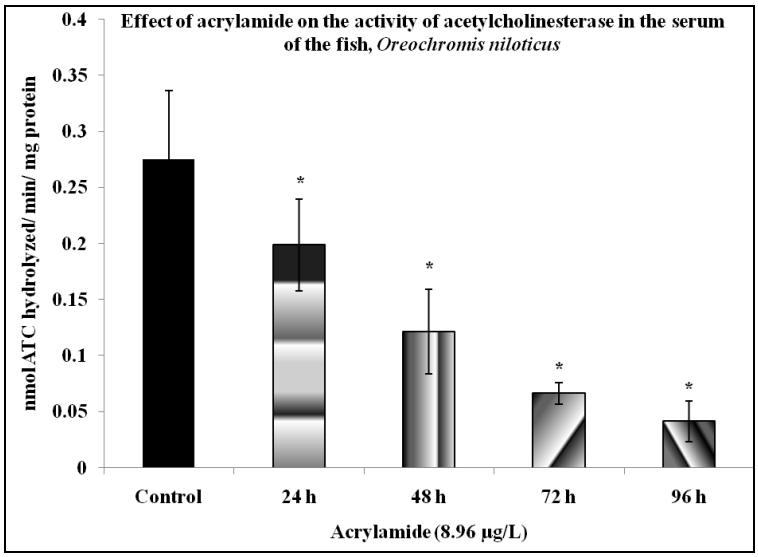
Effect of acrylamide on the activity of acetylcholinesterase in the serum of the fish, Oreochromis niloticus
nmol ATC hydrolyzed/ min/ mg protein
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
*
*
Control
24 h
48 h
72 h
96 h
Acrylamide (8.96 µg/L)

Fig 10

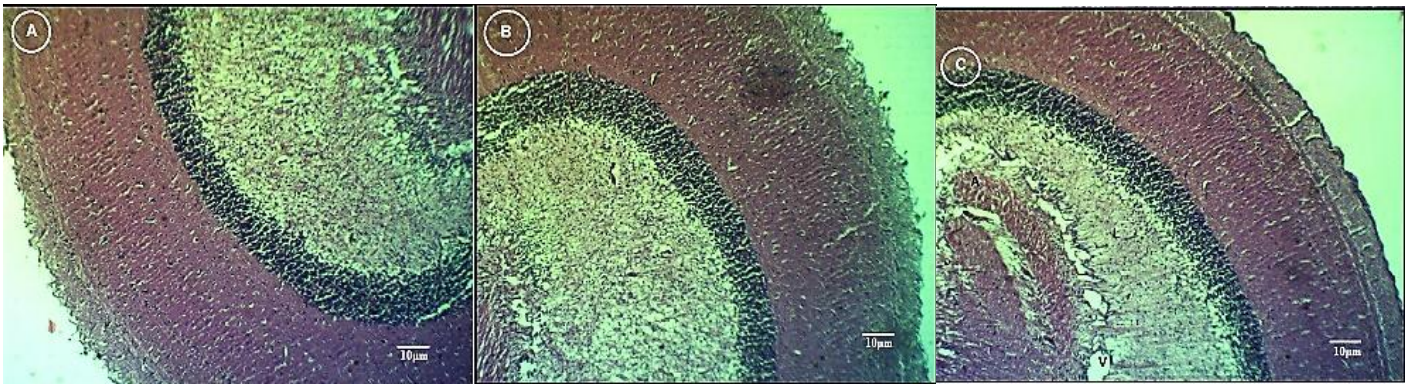
A
B
C
10µm
10µm
10µm

Fig 11: Photomicrographs of histological sections in A and B- control brain of *Oreochromis niloticus.* C-F: acrylamide-treated brain of *Oreochromis niloticus* for 24, 48, 72 and 96 h, respectively showing A-atrophy; L-infiltration; V-vacuolization (24 h); L-infiltration; V-vacuolization (48 h); A-atrophy; L-infiltration; D-degeneration V-vacuolization (72 h); A-atrophy; V-vacuolization (96 h).

4. Conclusion

The present study demonstrates that acute exposure of acrylamide induced oxidative stress by altering the activities of antioxidant enzymes and by induction of lipid peroxidation. It also caused neurotoxicity as shown by the decrease in the activity of nerve marker enzyme, acetylcholinesterase and the brain damage was also proved by histological examination in the fish, *Oreochromis niloticus*.

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