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Chromium III Exposure Inhibits Brain Na^+K^+ATPase Activity of *Clarias batrachus* L. Involving Lipid Peroxidation and Deficient Mitochondrial Electron Transport Chain Activity

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Abstract The present study elucidated the role of lipid peroxidation and diminished mitochondrial electron transport chain activity in partial dysfunction of brain Na^+K^+ATPase of *Clarias batrachus* exposed to chromium III ions. The fish were exposed to 10% and 20% of the derived 96 h LC_{50} value, 5.69 mg/L and 11.38 mg/L, respectively, and sampled on 20, 40 and 60 days. Exposure to chromium III on fish brain demonstrated an increased lipid peroxidation, production of protein carbonyl and reactive oxygen species and loss of protein thiol groups in synaptosomal fraction with decreased activity of Na^+K^+ATPase, partial inactivation of mitochondrial electron transport chain activity and energy depletion.

Keywords Lipid peroxidation · Mitochondrial electron transport chain activity

Chromium (Cr) and its compounds are ubiquitous water pollutants which come to the natural water resources from the effluents of a variety of chemical industries. Cr occurs predominantly in two oxidation states—Cr 3 (III) and Cr 6 (VI). CrVI enters the cell through the sulphate-anion channel and undergoes a reductive metabolism to form stable trivalent species (CrIII) (Kadiiska et al. 1994). Compared to CrVI, CrIII is not well absorbed and its in vivo toxicity has not been well demonstrated (DeFlora et al. 1989). However, few reports have shown that CrIII accumulates in animals and humans and predominate within the cell (Bridgewater et al. 1994; Sterans et al. 1995). Few studies have shown that Cr III toxicity may be due at least in part to an oxidative stress induced by the production of reactive oxygen species (ROS) (Hassoun and Stohs 1995) leading to cell death (Balamurugan et al. 2004).

In brain among the membrane bound enzymes Na^+K^+ATPase, an energy dependent heterodimeric enzyme composed of α and β subunits, plays a crucial role in the maintenance of Na^+ and K^+ gradients across the cell membrane (Rakowski et al. 1989). The inactivation of Na^+K^+ATPase leads to diverse alterations in the neurons such as partial membrane depolarization, Ca^{2+} influx, altered neurotransmitter release and even apoptosis and all these processes can be related to functional deficits in the brain (Lijnen et al. 1986). Because of the central importance of Na^+K^+ATPase in brain function we have tried to explore in this in vivo study the impact of CrIII induced oxidative stress on Na^+K^+ATPase activity and to elucidate the mechanisms involved in it.

Among the various cell organelles, mitochondria may be identified as a prime target of cytotoxic action of CrIII possibly through increased generation of reactive oxygen species (ROS) and their subsequent oxidative injuries. But the effect of CrIII induced oxidative stress on mitochondrial electron transport chain activity (ETC) along with oxidative deterioration of proteins and lipids in fish brain have not

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been addressed previously. Accordingly, in the present study, we have investigated the propensity of CrIII to induce oxidative stress in brain of fish following sublethal exposure to CrIII ions. Our results suggest that oxidative stress induced by CrIII in subcellular fractions of brain is associated with partial inactivation of Na^+K^+ATPase activity through propagation of lipid peroxidation and deficient mitochondrial electron transport chain activity.

Materials and Methods

Animal use protocols have been approved by the University of Kalyani Animal Care Committee in accordance with national guidelines. Healthy adult specimens of *Clarias batrachus* L. (58 ± 2.24 g body weight, 14.3 ± 1.54 cm in length) were collected from a local hatchery and were acclimatized for 2 weeks in dechlorinated tap water in large glass aquaria in the laboratory. They were fed ad libitum on alternate days and the water with requisite Cr III salt renewed after every 48 h, leaving no faecal matter, unconsumed food or dead fish, if any. Prior to the commencement of the experiment, 96 h median lethal concentration (96 h LC_{50}) of chromium chloride hexahydrate (E. Merck, India) was estimated by probit analysis.

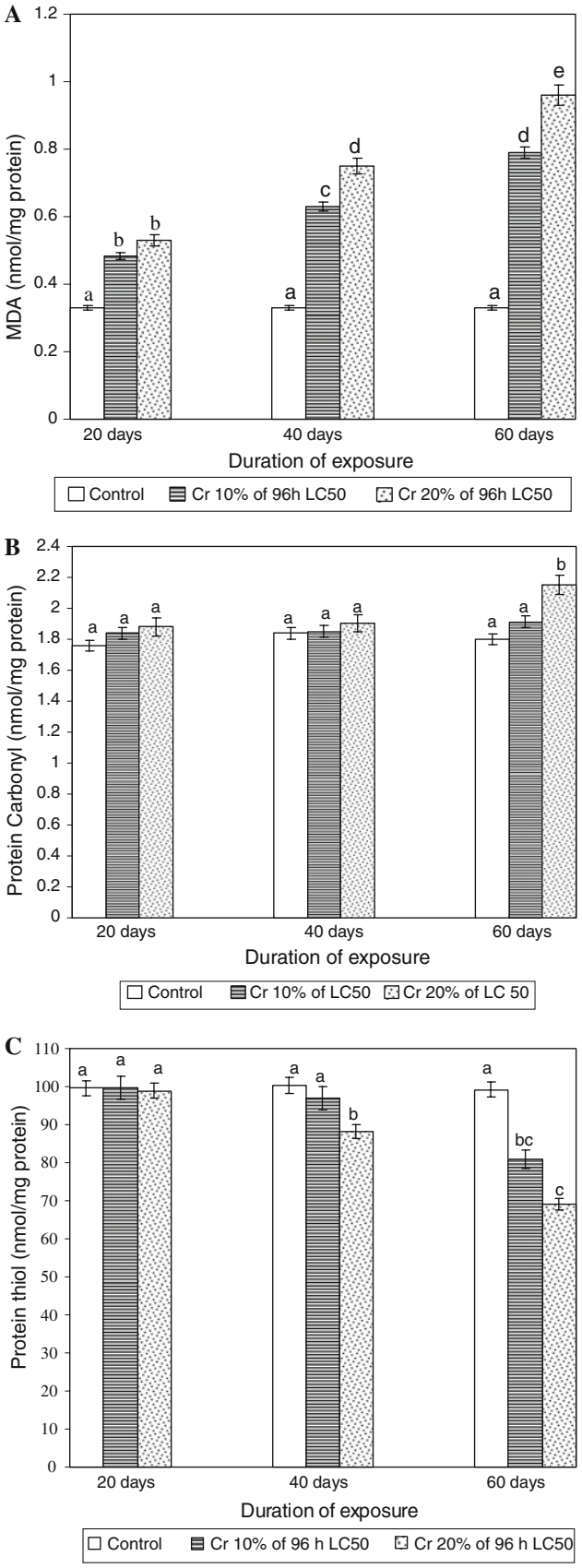
Adult *Clarias batrachus* were exposed to chromium chloride ($Crcl_3{\cdot}6H_2O$) treated water at 10% (5.69 mg/L Cr) and 20% (11.38 mg/L Cr) of the 96 h LC_{50} value (56.9 mg/L Cr). Eight fishes were randomly assigned for each aquaria containing 30 L of $CrCl_3$ treated water, prepared in tap water (having dissolved O_2 6.1 mg/L, pH 7.10, water hardness 23.2 mg/L and water temperature 25 ± 2°C). Identical groups of eight fishes each were kept in separate aquaria containing 30 L of plain dechlorinated tap water (without chromium salt) as controls. After each of the exposure periods of 20, 40 and 60 days, fishes from the respective experimental, as well as control aquaria were sacrificed and the brain tissues were utilised for various biochemical experiments. Atomic absorption spectrometry was used to measure the actual concentration of CrIII in experimental water during the exposure periods of 20, 40 and 60 days and was found very near to the desired concentration levels.

The brain synaptosomal and mitochondrial fractions were prepared and were utilized for biochemical assays. The protein carbonyl content of synaptosomal fraction was estimated by 2,4-dinitrophenylhydrazine assay following the method of Levine et al. (1990). The sulphydryl content of protein of the synaptosomal fraction was estimated using Ellman's reagent following the method of Habeeb (1972). Brain synaptosomal fraction was used for the assay of lipid peroxidation according to the method of Ohkawa et al. (1979) followed by measurement of Na^+K^+ATPase activity as adapted from the method of Mallik et al. (2000). The activity of complex I was assayed by using ferricyanide as the electron acceptor as adapted from Hatefi (1978). The activity of complex IV was assayed by noticing the oxidation rate of reduced cytochrome c (ferrocytochrome c) at 550 nm as mentioned in Wharton and Tzagoloff (1967). Mitochondrial ATP production was carried out using luciferin-luciferase bioluminescent assay following the method of Hays et al. (2003). DCFH-DA dye was used for measurement of ROS production as mentioned in the method of Dreiem et al. (2005). For all parameters mean ± SE were calculated. All data were subjected to analysis of variance and Duncan's multiple range test (DMRT) was used to determine significant differences among means at 5% level of significance.

Results and Discussion

The involvement of oxidative stress induced mechanisms in diminution of fish brain Na^+K^+ATPase activity exposed to CrIII ions have been evaluated in the present study. From the result it was observed that lipid peroxidation (as measured by MDA production) occurred in the fish brain in an enhanced manner with respect to increase in time and concentration of CrIII exposure showing the occurrence of oxidative stress in fish brain (Fig. 1a). Of the various modes of oxidative injury lipid peroxidation is of prime importance which is triggered and promoted by different radical and non-radical members of reactive oxygen species (ROS) family or by the catalytic decomposition of lipid hydroperoxides in tissues by several agents including most notably the microsomal cytochromes (Halliwell and Gutteridge 1989). The brain is prone to lipid peroxidation because of its high rate of oxygen utilization, availability of oxidizable substrates like polyunsaturated fatty acids and catecholamines and a deficient antioxidant defense system (Halliwell and Gutteridge 1989). An important feature of the present study was the incorporation of protein carbonyl, an important marker of protein damage induced by oxidative stress (Fig. 1b). The significant generation of protein

Fig. 1 Variation of malondialdehyde (**a**), protein carbonyl (**b**) and ▶ protein thiol (**c**) of fish synaptosomal fraction exposed to 0% (0.00 mg/L), 10% (5.69 mg/L) and 20% (11.38 mg/L) of 96 h LC_{50} of CrIII at different duration (20, 40, 60 days) of exposure. The malondialdehyde (nmol/mg protein), protein carbonyl (nmol/mg protein) and protein thiol (nmol/mg protein) were assayed as described in "Materials and Methods". Data are mean ± SEM of eight observations. Results of DMR test have been represented by small letters. Common letter between any two bars indicates their similarity while two different letters indicate significant difference at 5% level



carbonyl was only marked in the 60 days treatment period at a dose of 20% of 96 h LC_{50} of CrIII exposure ($p = 0.05$). The incorporation of protein carbonyl results from a primary damage inflicted by ROS or by secondary modifications due to formation of stable adduction compounds between reactive aldehyde end products of lipid peroxidation and amino acid side chains of protein molecules (Halliwell and Gutteridge 1989).

But other than protein carbonyl incorporation, different other forms of oxidative stress induced protein damage caused due to CrIII toxicity cannot be ruled out. The loss of free –SH groups is one such variable which has been included in our study. Under our experimental conditions, the oxidative stress induced by CrIII caused a gradual loss of –SH groups in fish synaptosomal fraction with increase in time and concentration of CrIII exposure (Fig. 1c). In this regard it may be noted that Na^+K^+ATPase enzyme, also being a –SH group containing enzyme gets affected due to CrIII exposure suggesting that –SH groups are essential for the enzyme activity. Maximum loss of Na^+K^+ATPase activity was observed at the 60 days duration at 20% of 96 h LC_{50} of CrIII exposure ($p = 0.05$, Fig. 2). Our finding, however, was not in conformity to a recent report that suggested lipid peroxidation had no effect on total thiol protein membrane content though it signifi-

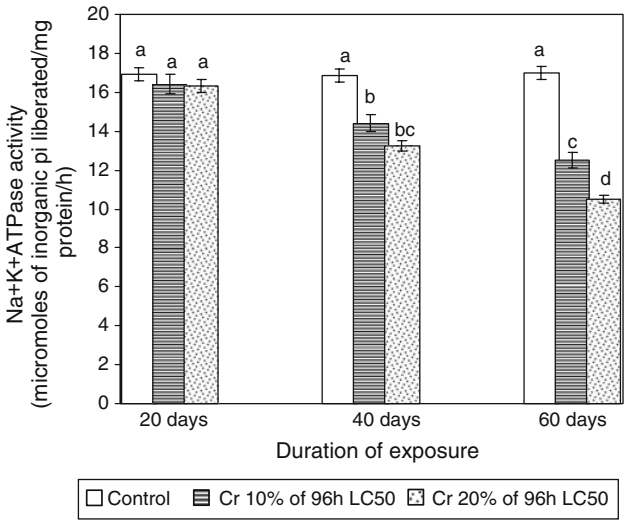


Fig. 2 Variation of Na^+K^+ATPase activity (μmoles of Pi liberated/mg protein/h)of fish synaptosomal fraction exposed to 0% (0.00 mg/L),10% (5.69 mg/L) and 20% (11.38 mg/L) of 96 h LC_{50} of CrIII at different duration (20, 40, 60 days) of exposure. The Na^+K^+ATPase activity was assayed as described in "Materials and Methods". Data are mean ± SEM of eight observations. Results of DMR test have been represented by small letters. Common letter between any two bars indicates their similarity while two different letters indicate significant difference at 5% level

cantly decreased Na^+K^+ATPase activity (Bavaresco et al. 2007).

However, other mechanisms affecting Na^+K^+ATPase activity due to CrIII toxicity cannot be ruled out. The peroxidative damage inflicted due to lipid peroxidation causes structural and functional derangement of phospholipid bilayer of membranes whereby important enzymes embedded in the plasma membrane gets dysfunctional. Na^+K^+ATPase, a key transmembrane enzyme responsible for maintaining the ionic homeostasis of the cell, may get partially inactivated due to such peroxidative damage inflicted by CrIII exposure. Besides, the treatment of Na^+K^+ATPase with lipid peroxidation derived aldehyde end products may induce alterations in the conformation of the enzyme molecule affecting its activity (Miyake et al. 2003).

The level of ATP production in brain mitochondria may be an important criteria in the CrIII induced inhibition of

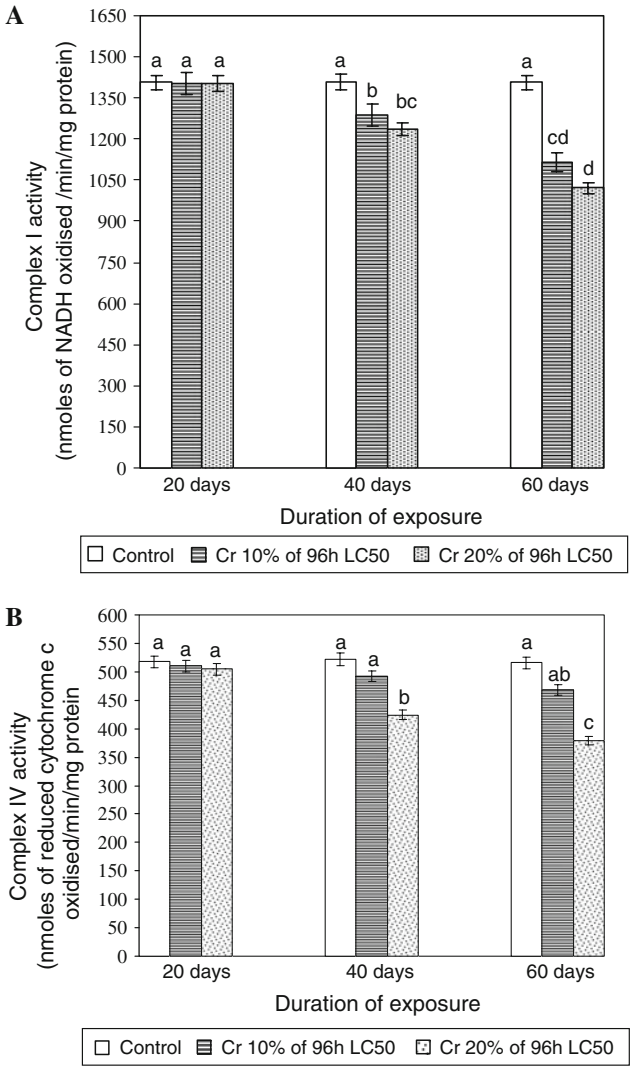


Fig. 3 Variation of complex I assay (**a**) and complex IV assay (**b**) of fish brain mitochondrial fraction exposed to 0% (0.00 mg/L), 10% (5.69 mg/L) and 20% (11.38 mg/L) of 96 h LC_{50} of CrIII at different duration (20, 40, 60 days) of exposure. The complex I assay (nmoles of NADH oxidized/min/mg protein) and complex IV assay (nmol reduced cytochrome c oxidized/ min/mg protein) were assayed as described in "Materials and Methods". Data are mean ± SEM of eight observations. Results of DMR test have been represented by small letters. Common letter between any two bars indicates their similarity while two different letters indicate significant difference at 5% level

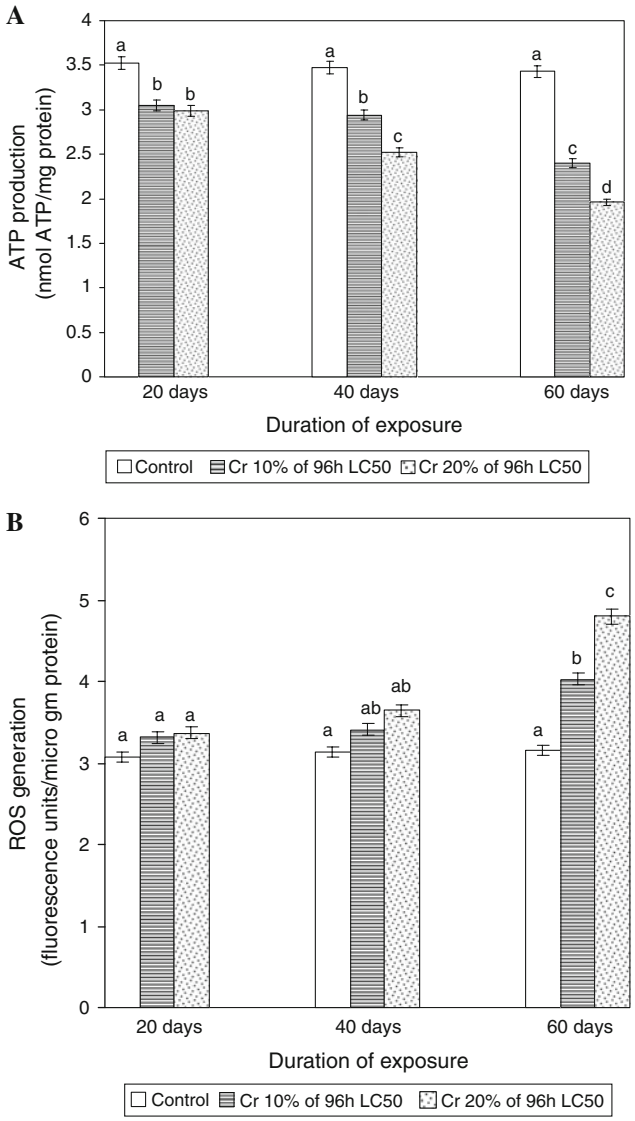


Fig. 4 Variation of adenosine triphosphate (ATP) production (**a**) and reactive oxygen species (ROS) production (**b**) of fish brain mitochondrial and synaptosomal fraction exposed to 0% (0.00 mg/L), 10% (5.69 mg/L) and 20% (11.38 mg/L) of 96 h LC_{50} of CrIII at different duration (20, 40, 60 days) of exposure. The ATP production (nmol ATP/mg protein) and ROS production (fluorescence units/µg protein) were assayed as described in "Materials and Methods". Data are mean ± SEM of eight observations. Results of DMR test have been represented by small letters. Common letter between any two bars indicates their similarity while two different letters indicate significant difference at 5% level

Na^+K^+ATPase activity of fish brain. Na^+K^+ATPase, an energy dependent antiport protein gets inactivated due to diminution in ATP production which may result from a deficient mitochondrial electron transport chain activity. The present study has shown a decline in ATP production in mitochondria, the drop in energy production was profound with increase in CrIII concentration and time of exposure period (Fig. 4a). We have demonstrated that due to CrIII exposure the activity of mitochondrial complex I (NADH-ferricyanide reductase) and complex IV (cytochrome c oxidase) were significantly affected with increase in time and concentration of CrIII exposure (Fig. 3a, b) and it is suggested that the inhibition of mitochondrial electron transport chain may stimulate enhanced production of ROS in fish brain mitochondria (Fig. 4b).

From the above discussion we can assume that the partial inactivation of Na^+K^+ATPase may be the end result of a number of biochemical mechanisms acting individually or cooperatively. Oxidative stress induced lipid peroxidation along with its aldehyde end products seem to have an inhibitory effect on Na^+K^+ATPase activity. The direct interaction of CrIII ion with the –SH group of Na^+K^+ATPase enzyme cannot be ruled out. This impounding effect on Na^+K^+ATPase is further aggravated by a vulnerable mitochondrial electron transport chain activity whereby neuronal ATP production is diminished which results in further impairment of Na^+K^+ATPase with associated toxic consequences. So it can be suggested that CrIII exposure induces oxidative stress in the fish brain and the reduction of Na^+K^+ATPase activity was partially mediated by lipid peroxidation and deficient mitochondrial electron transport chain activity.

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