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ORIGINAL ARTICLE

Mitochondrial respiratory chain inhibition and Na^+K^+ATPase dysfunction are determinant factors modulating the toxicity of nickel in the brain of indian catfish *Clarias batrachus* L.

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ABSTRACT

Nickel is a potential neurotoxic pollutant inflicting damage in living organisms, including fish, mainly through oxidative stress. Previous studies have demonstrated the impact of nickel toxicity on mitochondrial function, but there remain lacunae on the damage inflicted at mitochondrial respiratory level. Deficient mitochondrial function usually affects the activities of important adenosinetriphosphatases responsible for the maintenance of normal neuronal function, namely Na^+K^+ATPase, as explored in our study. Previous reports demonstrated the dysfunction of this enzyme upon nickel exposure but the contributing factors for the inhibition of this enzyme remained unexplored. The main purpose of this study was to elucidate the impact of nickel neurotoxicity on mitochondrial respiratory complexes and Na^+K^+ATPase in the piscine brain and to determine the contributing factors that had an impact on the same. Adult *Clarias batrachus* were exposed to nickel treated water at 10% and 20% of the 96 h LC50 value (41 $mg.l^{-1}$) respectively and sampled on 20, 40 and 60 days. Exposure of fish brain to nickel led to partial inhibition of complex IV of mitochondrial respiratory chain, however, the activities of complex I, II and III remained unaltered. This partial inhibition of mitochondrial respiratory chain might have been sufficient to lower mitochondrial energy production in mitochondria that contributed to the partial dysfunction of Na^+K^+ATPase. Besides energy depletion other contributing factors were involved in the dysfunction of this enzyme, like loss of thiol groups for enzyme activity and lipid peroxidation-derived end products that might have induced conformational and functional changes. However, providing direct evidence for such conformational and functional changes of Na^+K^+ATPase was beyond the scope of the present study. In addition, immunoblotting results also showed a decrease in Na^+K^+ATPase protein expression highlighting the impact of nickel neurotoxicity on the expression of the enzyme itself. The implication of the inhibition of mitochondrial respiration and Na^+K^+ATPase dysfunction was the neuronal death as evidenced by enhanced caspase-3 and caspase-9 activities. Thus, this study established the deleterious impact of nickel neurotoxicity on mitochondrial functions in the piscine brain and identified probable contributing factors that can act concurrently in the inhibition of Na^+K^+ATPase. This study also provided a vital clue about the specific areas that the therapeutic agents should target to counter nickel neurotoxicity.

KEY WORDS: mitochondrial respiratory chain, complex IV, sodium potassium ATPase, nickel, neurotoxicity

Introduction

Nickel (Ni) is an environmentally reactive toxic metal that exhibits a high degree of toxicity and carcinogenicity to living organisms including fishes (Brix *et al.*, 2004; Pane *et al.*, 2004). Previous studies have proposed oxidative stress as a possible mechanism involved in Ni cytotoxicity (Chen *et al.*, 2003; Ahmed *et al.*, 1999; Chen *et al.*, 1998). Among the various cell organelles, mitochondria

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have been identified as a prime target for cytotoxic action of Ni, possibly through increased generation of reactive oxygen species (ROS) and the subsequent oxidative injuries. Mitochondrial dysfunction involving loss of mitochondrial membrane potential, reduced ATP generation and decreased mitochondrial DNA content have been reported in previous studies exposed to Ni toxicity (Xu *et al*., 2015;He *et al*., 2011; Xu *et al*., 2010; Guan *et al*., 2007; M'Bemba-Meka *et al*., 2006; M'Bemba-Meka *et al*., 2005; Bragadin & Viola, 1997). However, the impact of Ni toxicity on the mitochondrial respiratory complexes especially in piscine brain has remained uninvestigated. There is a high possibility of alteration of mitochondrial respiratory complexes in Ni cytotoxicity as alterations in other parameters of mitochondrial function have been reported in previous studies. Alteration in mitochondrial membrane potential is most likely to disrupt mitochondrial structure and function increasing the possibility of disruption of mitochondrial respiratory chain activity (Desagher & Martinou, 2000).

In the brain, among the membrane bound enzymes, Na^+K^+ATPase, a known major energy dependent heterodimeric enzyme, 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 often leads to diverse alterations in the neuron leading to functional deficits in the brain (Xiao *et al*., 2002; DiPolo & Beauge, 1991; Lijnen *et al*., 1986; Archibald & White, 1974). As Na^+K^+ATPase occupies central importance in maintaining brain function, in this study we have also tried to explore the impact of Ni-induced neurotoxicity on Na^+K^+ATPase and to elucidate the likely mechanisms involved in the inhibition of this particular enzyme. Inhibition of mitochondrial respiratory complexes upon Ni exposure may lead to diminution of energy production that can disrupt the normal functioning of Na^+K^+ATPase. In addition, oxidative stress induced factors may also contribute for the inhibition of this enzyme. Thus there may be involvement of several contributing factors that could act simultaneously for the inhibition of Na^+K^+ATPase. In our study, *Clarias batrachus* has been chosen as a working model as fishes are now considered to be more sensitive to transition metals and act as good alternative to mammalian models for studying metal neurotoxicity. In the present study, there were two main objectives – first, to study the impact of Ni neurotoxicity on both mitochondrial respiratory chain and Na^+K^+ATPase functions in the brain of *Clarias batrachus* and second, to determine the contributing factors that might lead to the suppression of mitochondrial respiratory chain and Na^+K^+ATPase functions in piscine brain upon Ni exposure.

Materials and methods

Chemicals

All common chemicals were of analytical grade. 2,6-dichlorophenolindophenol (DCPIP), rotenone, ubiquinol, cytochrome c, EGTA, phenyl-methanesulphonyl fluoride (PMSF), JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolylcarbocyanine iodide) and diethylenetriaminepentacetic acid (DTPA) were purchased from Sigma Chemical Co.(USA). NADH, dimethyl sulphoxide (DMSO), sodium dodecyl sulphate (SDS), HEPES, 5,5V-dithiobis-2-nitrobenzoic acid (DTNB), dimethylformamide, trichloroacetic acid (TCA) and sucrose were from Sisco Research Laboratory (Mumbai, India).

Animals, Grouping and Experimental protocol

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* (60±1.19 g body weight, 15±0.79 cm in length) were collected from a single population 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 Ni salt was renewed after every 48 hr, leaving no fecal 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 $NiCl_2.6H_2O$ (E.Merck) was estimated by probit analysis (Finney, 1971) as in natural waters Ni^{2+} is the dominant chemical species.

Adult *Clarias batrachus* were exposed to $NiCl_2.6H_2O$ treated water at 10% (4.1 mg.l^{-1}) and 20% (8.2 mg.l^{-1}) of the 96 h LC_{50} value (41 mg.l^{-1}). Eight fishes were randomly assigned for each aquarium containing 30 l of $NiCl_2.6H_2O$ treated water, prepared in tap water (having dissolved oxygen 6.6 mg.l^{-1}, pH 7.23, water hardness 23.8 mg.l^{-1} and water temperature 26±2 °C).Identical groups of eight fish each were kept in separate aquaria containing 30l of plain dechlorinated tap water (without Ni 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. Atomic absorption spectrometry was used to measure the exact concentration of Ni in experimental water during the course of 20, 40 and 60 days and was found to be very near to the desired concentration levels.

Preparation of brain synaptosomal fraction

Each individual fish was killed, decapitated and the brain removed. The crude synaptosomal fraction was prepared by the method of Whittaker (1972). Briefly, the brain was cut into small pieces and minced with a sharp scalpel. Each minced brain was homogenized in a homogenizing buffer comprising of 9 volumes of 320 mM sucrose and 5 mM HEPES at pH 7.4 containing 0.1 mM phenylmethylsulphonyl fluoride. Post homogenization, the homogenate was centrifuged at 1000 x g for 10 min at 4°C. The supernatant obtained was collected and centrifuged at 12000 x g for 15 min. The supernatant obtained in this step was collected and stored for nitrite measurement. The remaining pellet was resuspended in the homogenizing buffer and was subjected to centrifugation at 12 000 × *g* for 15 min. The final pellet was resuspended in the desired buffer and later utilized for measurement of lipid peroxidation, protein thiol, protein carbonyl and Na^+K^+ATPase.

Assessment of lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid assay (Ohkawa *et al*., 1979). To each sample of 200 µl of synaptosomal suspension in 50 mM phosphate buffer, pH 7.4, was added in a sequence – 200 µl of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.85% TBA and 400 µl water followed by heating for 15 min in a boiling water bath. After cooling the tubes, the pink color developed was extracted with 5 ml of (1:15) n-butanol-pyridine. The organic layer was collected after centrifuging the tubes at 3000 rpm for 10 min. The absorbance was read at 532 nm and the amount of MDA calculated from the molar extinction co-efficient of MDA-TBA adduct (ε=1.56×10^5 M^{-1}.cm^{-1}).

Assessment of protein carbonyl content

The protein carbonyl content of brain samples was assessed by 2,4-dinitrophenylhydrazine assay (Levine *et al*., 1990; Shecter *et al*., 1996). Briefly, the sample proteins were derivatized with 10 mM 2,4-dinitrophenylhydrazine (DNPH) for 15 min. This was followed by addition of TCA (20%) and pelleting of the protein and three successive washings of the pellet with ethanol-ethylacetate (1:1) to remove the excess DNPH. The derivatized protein was finally dissolved in protein dissolving solution containing 2% sodium dodecylsulphate (SDS) and optical density measured at 370 nm against the appropriate blank.

Assessment of protein sulphydryl (-SH) content

The free sulphydryl content of protein sample was estimated using Ellman's reagent (Habeeb,1972).The sample proteins were dissolved in a solution containing 2% SDS and 0.5% EDTA in 0.08 M phosphate buffer, pH 8.0. To this solution, 5 mM dithiobisnitrobenzoic acid (DTNB) solution was added and the absorbance of the yellow color was measured at 412 nm.

Measurement of Na$^+$K$^+$ATPase activity

An aliquot (100 µl) of synaptosomal fraction containing 100–200 µg of protein was used for the assay of Na$^+$K$^+$ATPase activity in a reaction mixture containing 100 mM NaCl, 10 mM KCl , 6 mM $MgCl_2$ and 3 mM ATP in 25 mM Tris, pH 7.4 in the presence or absence of 2 mM ouabain as adapted from the method of Mallik *et al*. (2000).The activity of the enzyme was expressed as µmoles of inorganic phosphate liberated/mg protein/h.

Immunoblotting of Na$^+$K$^+$ATPase

Synaptosomal fractions obtained from piscine brain as discussed earlier were utilized for the immunoblotting of Na$^+$K$^+$ATPase**.** The proteins were subjected to SDS-PAGE (8% acrylamide for resolving gel, 4% acrylamide for stacking gel) under conditions as described by Laemmli (1970). The proteins were then electrophoretically transferred onto PVDF membranes using a semi-dry electroblotting system. After transfer membranes were blocked with 10% skim milk in TTBS (0.05% tween 20 in tris-buffered saline: 20 mM.l^{-1} Tris-HCl; 500 mM.l^{-1} NaCl, pH 7.6) for 1 h before being incubated overnight at 4 °C with anti-NKA antibody (α5, 1:400 dilution, DSHB, USA). The primary antibody was diluted in 1% bovine serum albumin in TTBS. The membranes were then incubated in goat anti-mouse horseradish peroxidase – conjugated secondary antibody (1:5 000 dilution; Santa Cruz Biotechnology, USA) for 1 h at room temperature followed by band analysis.

Isolation of piscine brain mitochondria

The brain mitochondrial fraction was isolated following the method of Berman & Hastings (1999), with minor modifications. Briefly, the brain was homogenized in 10 ml of buffer A (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/ml BSA, pH 7.4).The homogenate was brought to 30 ml with the same buffer followed by centrifugation at 2000 g for 3 min at 4 °C .The supernatant was preserved and the pellet resuspended in 10 ml of buffer A followed by recentrifugation as earlier. The supernatants were pooled and centrifuged in 4 tubes at 12,000 g for 8 min. The pellet in each tube containing synaptosomes and mitochondria was treated with 10 ml of buffer A containing 0.02% digitonin to lyse the synaptosomes. The mitochondria were pelleted down by centrifugation at 12,000 g for 10 min. The mitochondrial pellet was washed again in buffer A without EGTA and BSA and resuspended in an appropriate buffer for further experimentation.

Assessment of mitochondrial respiratory complexes

Frozen and thawed samples of mitochondrial suspension in 50 mM phosphate buffer, pH 7.4 (10–30 µg of protein), were used for mitochondrial complex I and complex IV enzyme assays. The activity of complex I was assayed by using ferricyanide as the electron acceptor as adapted from Hatefi (1978).The assay system at 30 °C contained 0.17 mM NADH, 0.6 mM ferricyanide, triton X-100 (0.1% v/v) in 50 mM phosphate buffer pH 7.4. The reaction was initiated by addition of mitochondrial suspension (10–30 µg protein) to the sample cuvette and the rate of oxidation of NADH was measured by the decrease in absorbance at 340 nm (Clark *et al*., 1997).

Complex II (succinate-ubiquinone oxidoreductase) activity was performed using 2,6-dichlorophenolindophenol (DCPIP) as acceptor and succinate as donor. The reaction mixture contained 30 µg mitochondria, 25 mM potassium phosphate, 5 mM $MgCl_2$, 2 mM KCN, 20 mM succinate, 3 µM antimycin A, 1 µM rotenone. 1 mM DCPIP was reincubated for 10 minutes at 37 °C the reaction was started with ubiquinone (0.1 mM) and the enzyme-catalyzed reduction of DCPIP to $DCPIPH_2$ was recorded for 5 min at 600 nm (ε=21,000 M^{-1}) (Bo *et al*., 2014).

Complex III (ubiquinone-ferricytochrome-c oxidoreductase) activity was measured by monitoring the reduction of cytochrome c at 550 nm in the assay medium (25 mM potassium phosphate, 5 mM $MgCl_2$, 2 mM KCN, 2 µg/ml rotenone, pH 7.2). Cytochrome c (15 µM) and ubiquinol (0.1 mM) were added to the assay medium, and the nonenzymatic rate was recorded for 1 min. Then

mitochondria were added, and the increase in absorbance was recorded for 3 min (Bo *et al*., 2014).

The activity of complex IV was assayed by following the oxidation of reduced cytochrome c (ferrocytochrome c) at 550 nm. Reduced cytochrome c (50 µM) in 10 mM phosphate buffer pH3097.4 was added to each of two 1 ml cuvettes. In the blank cuvette, ferricyanide (1 mM) was added to oxidize ferrocytochrome c and the reaction initiated in the sample cuvette by the addition of mitochondrial suspension (10–30 µg protein).The rate of decrease of absorbance at 550 nm was measured at room temperature. The activity of the enzyme was calculated from the first-order rate constant taking into account the concentration of reduced cytochrome c in the cuvette and the amount of mitochondrial protein added (Wharton & Tzagoloff, 1967).

Assessment of mitochondrial ROS generation

The assessment of mitochondrial ROS generation was carried out by following the method of Dreiem *et al*. (2005), using DCFH-DA dye. DCF fluorescence values were corrected for protein levels and autofluorescence of the samples, according to the formula Fco = (Fsa – Fbl)/ protein, where Fco is the corrected fluorescence value, Fsa is observed fluorescence in the sample and Fbl is observed fluorescence in the blank.

Assessment of mitochondrial ATP production

Mitochondrial ATP production was assessed by using luciferin-luciferase bioluminescent assay following the method of Hays *et al*. (2003). Mitochondrial samples were thawed and centrifuged at 10 000 × *g* for 5 min and the supernatant was used to determine ATP content. To prepare the luciferin-luciferase solution, 0.28 mg/ml luciferin and 1 mg/ml luciferase were combined with stabilizing buffer containing HEPES (0.025 M,pH 7.5). The sample supernatant of 10 µl was added to 200 µl of HEPES buffer and then 200 µl of this solution was placed into cuvettes. Next, 100 µl of luciferin-luciferase solution was added to each luminometer cuvette and placed in the luminometer. The results are expressed as nM ATP/mg protein.

Measurement of mitochondrial transmembrane potential

Aliquots of mitochondrial suspensions were incubated at 37 °C for 30 min in isotonic buffer A containing 10 mM pyruvate, 10 mM succinate and 1 mM ADP with 5 µM JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolylcarbocyanine iodide, CS0760, Sigma-Aldrich). After incubation, the dyed mitochondria were collected by centrifugation, washed with isotonic buffer A to remove excess dye and resuspended in the same buffer in appropriate dilution, followed by measurement of fluorescence intensity (λex 490 nm, λem 590 nm).

Assessment of caspase-3 and caspase-9 activities

Caspase-3 and caspase-9 assays were performed on the brain samples according to the manufacturer's protocol (Biovision, K106-100 and K119-100). The assays are based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA (for caspase-3) and LEHD-pNA (for caspase-9) respectively. Initially the harvested brain tissue was homogenized using a Dounce homogenizer in lysis buffer followed by centrifugation at 10 000 × *g* for 10 min to obtain the supernatant. For post-protein estimation approximately 50 µg protein was diluted to 50 µl lysis buffer for each assay followed by addition of 50 µl of 2× reaction buffer (containing 10 mM DTT). To each sample 5 µl of the 4 mM DEVD-pNA / LEHD-pNA substrate was added, followed by incubation at 37 °C for 1 hour. Post incubation samples were read at 405-nm using a 100-µl micro quartz cuvette and the results are expressed as absorbance at 405 nm.

Protein assessment

The protein was assessed after solubilizing the membranes in 1% SDS by the method of Lowry *et al*. (1951).

Statistical analysis

All data were subjected to Duncan's multiple range test (Duncan, 1955; Gomez & Gomez, 1984) to determine significant differences among means at 5% level of significance. In figures, data are mean ± SEM of eight observations. Results of DMR Test have been represented by small letters. Common letter between any two bars (in figures) indicate their similarity while two different letters indicate significant difference at 5% level.

Results

Nickel exposure increased lipid peroxidation and protein carbonyl content with reduced protein thiol level in fish synaptosomal fraction

The extent of lipid peroxidation in synaptosomal fraction was assessed by the amount of malondialdehyde (MDA), an important by-product of lipid peroxidation formed. It is apparent from the data presented in Figure 1A that among all the combinations of exposure of nickel with respect to duration and concentration of treatment, the combination of 60 days treatment with Ni 20% (of 96 h LC_{50}) exposure recorded the highest increase in MDA production (nearly 4fold vs. control). However, a general tendency of increase in MDA production was observed in the synaptosomal fraction with successive increase in duration and concentration of Ni exposure (Figure 1A).Significant incorporation of protein carbonyl, an important marker of oxidative stress induced protein damage, was also noticed in the synaptosomal fraction of fish brain (Figure 1B).There was a gradual increase in carbonyl content in the synaptosomal fraction from 20 to 60 days duration of nickel treatment with respect to increasing concentrations of Ni (10% and 20% of 96 h LC_{50} value) exposure. The highest incorporation of protein carbonyl in fish synaptosomal fraction was noticed at 60-day exposure period at Ni 20% (of 96 h LC_{50}) treatment (nearly two fold vs. control). With the exception of 20-day treatment, in both 40- and 60-day

treatment periods, statistically significant differences in protein carbonyl incorporation was obtained among control (0% of 96 h LC_{50}) and Ni treated (10% and 20% of 96 h LC_{50} value) groups ($p<0.05$) (Figure 1B). Under our experimental conditions, a gradual loss of sulphydryl (-SH) group content with increase in duration and concentration of Ni treatment was observed, as seen in Figure 1C. The maximum difference in -SH group content between control (0% of LC_{50}) and its respective Ni treated groups (10% and 20% of 96 h LC_{50} value) were observed in 60-day treatment period (by 38% and 45% respectively, $p<0.05$) (Figure 1C).

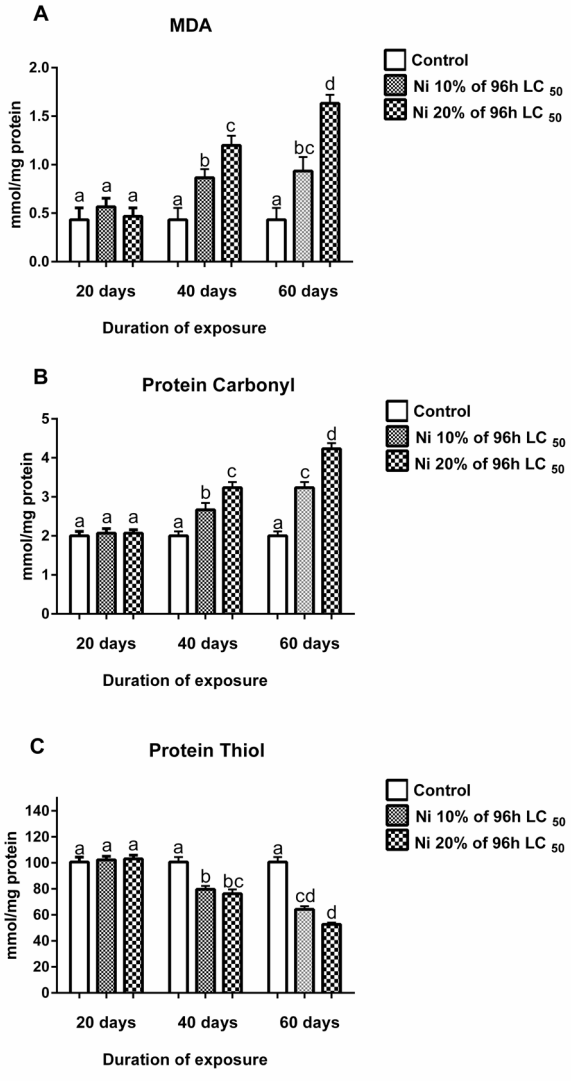

Figure 1. Nickel exposure induced alteration of malondialdehyde (A), protein carbonyl (B) and protein thiol (C) content of fish synaptosomes exposed to 0% (0.00 mg L^{-1}), 10% (4.1 mg L^{-1}) and 20% (8.2 mg L^{-1}) of 96 h LC_{50} of Ni at 20, 40 and 60 days of exposure. Data are mean ± SE of eight 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.

Loss of Na^+K^+ATPase activity and protein expression in nickel treated fish synaptosomes

In Na^+K^+ATPase activity measurement, in case of 20 days of Ni exposure, though no statistically significant difference in enzyme activity was observed between control (0% of 96 h LC_{50}) and Ni treated groups, yet in case of 40 day treatment, the Na^+K^+ATPase activity obtained at Ni 20% (of 96 h LC_{50}) exposure was found to be significantly lower compared to the control (0% of 96 h LC_{50}) and Ni 10% (of 96 h LC_{50}) exposure (by 18% and 22% respectively, $p<0.05$) (Figure 2A). In 60-day duration of Ni exposure, at Ni 20% (of 96 h LC_{50}) concentration, maximum decrease in Na^+K^+ATPase activity was observed, which was significantly lower compared to control (0% of 96 h LC_{50}) and Ni 10% (of 96 h LC_{50}) treated values (by 37% and 16% respectively, $p<0.05$) (Figure 2A).

However, considering the results obtained in the measurement of Na^+K^+ATPase activity, it was decided to focus solely on Ni 20% (of 96 h LC_{50}) dosage in immunodetection study as this particular dosage had a profound impact on the activity of the enzyme at 20, 40 and 60 days of exposure period compared to Ni 10% (of 96 h LC_{50}). The immunodetection of Na^+K^+ATPase indicated a gradual lowering of protein expression compared to control, as indicated by lowering of band intensity as the exposure period progressed from 20 days to 60 days; least band intensity was noticed in 60-day exposure period compared to other durations (Figure 2B).

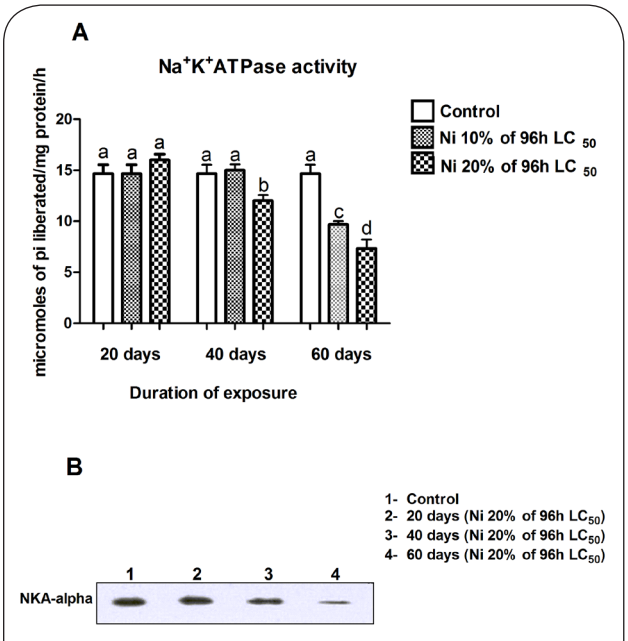

Figure 2. (A) Alteration of Na^+K^+ATPase activity (µmoles of Pi liberated/mg protein/h) of fish synaptosomes exposed to 0% (0.00 mg L^{-1}), 10% (4.1 mg L^{-1}) and 20% (8.2 mg L^{-1}) of 96 h LC_{50} of Ni at 20, 40 and 60 days of exposure. Data are mean ± SE of eight observations. Results of DMR Test 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. (B) Immunodetection of protein expression of Na^+K^+ATPase (alpha 5) exposed to 0% (0.00 mg L^{-1}) and 20% (8.2 mg L^{-1}) of 96 h LC_{50} of Ni at 20, 40 and 60 days of exposure.

Exposure to nickel caused mitochondrial dysfunction with suppression of mitochondrial respiratory complex IV in fish brain

In the mitochondrial respiratory enzyme complex studies, there was no diminution in mitochondrial complex I, II and III activities under Ni exposure (Figure 3A,B,C), however, significant inhibition of mitochondrial IV activity was observed in 40 and 60 days of exposure period (Figure 3D). In 60-day exposure period, significant difference in complex IV activities was observed between control (0% of 96 h LC_{50}) and Ni treated groups (10% and 20% of 96 h LC_{50} value) (by 26% and 41% respectively, $p<0.05$) (Figure 3D). In 40-day treatment period, the complex IV activity obtained at Ni 20% (of 96 h LC_{50}) exposure was found to be significantly lower than the control (0% of 96 h LC_{50}) value (by 19%, $p<0.05$) but insignificant to Ni 10% (of 96 h LC_{50}) exposure (Figure 3D).

Nickel treatment induced enhanced mitochondrial ROS generation accompanied by reduction of mitochondrial membrane potential and ATP generation

Regarding the status of energy generation in our experimental conditions, the present study has shown a decline in ATP generation in mitochondria. The drop in energy generation was profound in 60-day exposure period with significant difference observed between control (0% of 96 h LC_{50}) and Ni treated groups (10% and 20% of 96 h LC_{50} value) (by 20% and 37% respectively, $p<0.05$) (Figure 4A). In the 40-day exposure period, only significant difference was observed between control (0% of 96 h LC_{50}) and Ni 20% (of 96 h LC_{50} value) group (by 22%, $p<0.05$) (Figure 4A). Analysis of mitochondrial ROS generation clearly indicated the occurrence of oxidative stress upon Ni exposure with significant difference observed between control (0% of 96 h LC_{50}) and Ni treated groups (10% and

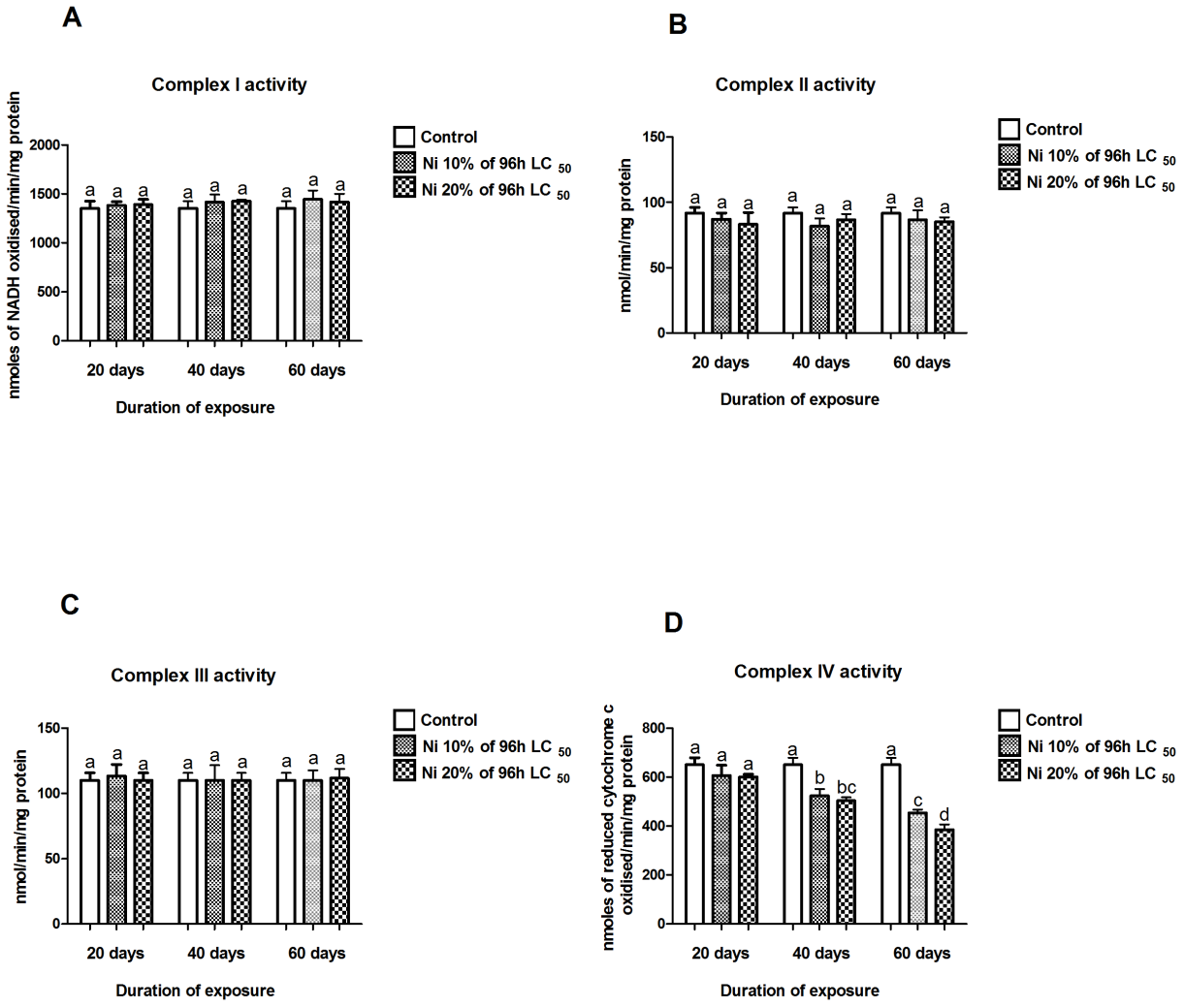

Figure 3. Impact of nickel exposure on mitochondrial complex I (A), complex II (B), complex III (C) and complex IV (D) of fish brain mitochondrial fraction exposed to 0% (0.00 mg L^{-1}), 10% (4.1 mg L^{-1}) and 20% (8.2 mg L^{-1}) of 96 h LC_{50} of Ni at 20, 40 and 60 days of exposure. Data are mean ± SE 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.

20% of 96 h LC_{50} value) at 40 and 60 days of nickel treatment (Figure 4B). For mitochondrial membrane potential measurement, severe loss of membrane potential was observed during 40 and 60 days of nickel treatment with significant difference observed between control (0% of 96 h LC_{50}) and Ni treated groups (10% and 20% of 96 h LC_{50} value) at 40 and 60 days of nickel treatment (Figure 4C). The maximum loss of membrane potential of 34% was observed at Ni 20% (of 96 h LC_{50}) exposure during 60 days treatment, indicating the maximum loss of mitochondrial function during nickel toxicity (p<0.05) (Figure 4C).

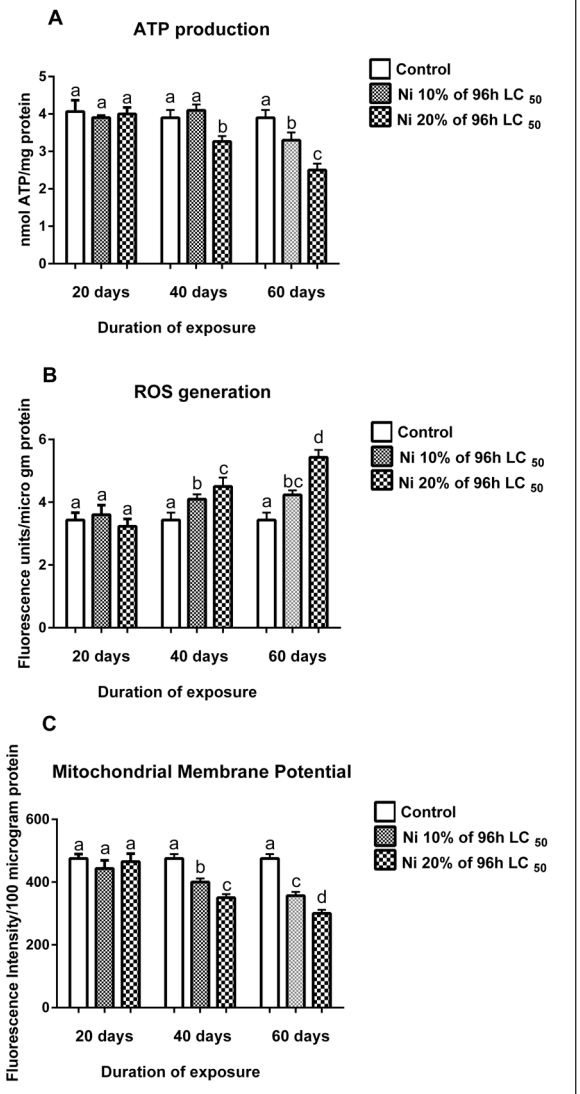

Figure 4. Variation of mitochondrial adenosine triphosphate (ATP) production (A), mitochondrial reactive oxygen species (ROS) generation (B) and mitochondrial membrane potential (C) of fish brain mitochondrial fraction exposed to 0% (0.00 mg L^{-1}), 10% (4.1 mg L^{-1}) and 20% (8.2 mg L^{-1}) of 96 h LC_{50} of Ni at 20, 40 and 60 days of exposure. Data are mean ± SE 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.

Nickel exposure induced enhanced neuronal death as evidenced by enhanced caspase-3 and caspase-9 activities

In caspase-3 activity measurement, in case of 20 days of Ni exposure, though no statistically significant difference in enzyme activity was observed between control (0% of 96 h LC_{50}) and Ni treated groups, however, in 40-day treatment, the caspase-3 activity obtained at Ni 20% (of 96 h LC_{50}) exposure was found to be significantly higher compared to control (0% of 96 h LC_{50}) (by 91% , p<0.05) (Figure 5A). In 60-day duration, significant increase in caspase-3 activity was observed at both Ni 10% and 20% (of 96 h LC_{50}) exposure compared to control (0% of 96 h LC_{50}) (by 101% and 117% respectively, p<0.05) (Figure 5A). A similar trend in activity was also observed for caspase-9 activity for 40 and 60 days of Ni exposure. Enhanced increase in caspase-9 activity was observed at 60-day exposure period at Ni 10% and 20% (of 96 h LC_{50}) exposure compared to control (0% of 96 h LC_{50}) (by 37% and 58% respectively, p<0.05) (Figure 5B).

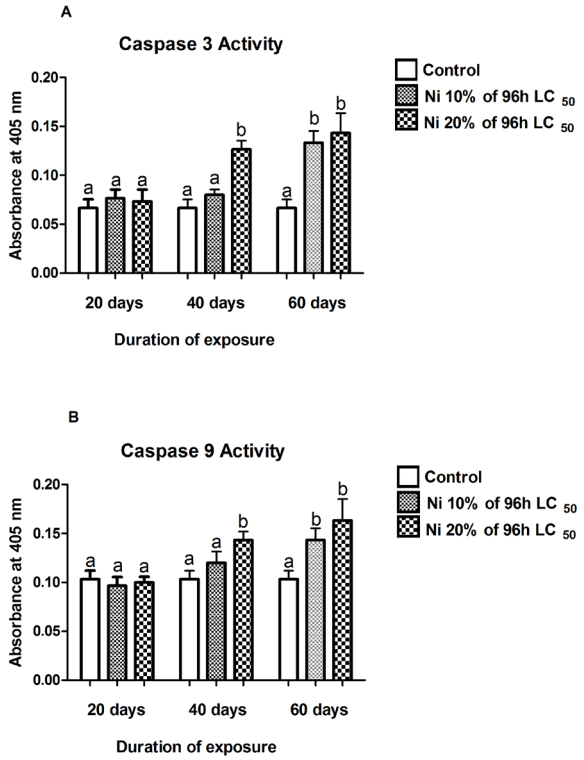

Figure 5 Nickel exposure induced variation of caspase-3 (A) and caspase-9 (B) activities in piscine brain exposed to 0% (0.00 mg L^{-1}), 10% (4.1 mg L^{-1}) and 20% (8.2 mg L^{-1}) of 96 h LC_{50} of Ni at 20, 40 and 60 days of exposure. Data are mean ± SE of eight observations. Results of DMR Test 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.

Discussion

In our current study, the involvement of oxidative stress induced mechanisms following Ni exposure leading to partial inhibition of mitochondrial respiratory chain and Na^+K^+ATPase dysfunction were observed in the brain of Indian catfish *Clarias batrachus*. The brain is prone to oxidative stress because of its high O_2 uptake as it accounts for the vast amounts of energy required to maintain neuronal intracellular ion homeostasis in face of all the openings and closings of ion channels associated with the propagation of action potentials and neurosecretion (Halliwell, 2006). Besides, the availability of oxidizable substrates (like polyunsaturated fatty acids and catecholamines) and a deficient antioxidant defence system (low levels of catalase, glutathione peroxidase, and vitamin E) makes the brain more vulnerable to oxidative stress (Halliwell & Gutteridge, 1989). Among cell organelles, mitochondria are most prone to oxidative stress as mitochondrial oxidative phosphorylation is a major producer of ROS directly attacking its own membrane lipids, proteins, and nucleic acids (Chance *et al*., 1979; Richter *et al*., 1988). Exposure to trace elements like Ni as used in our study, amplifies the generation of reactive oxygen species even further. Earlier studies suggest that Ni exposure can disrupt mitochondrial function by decreasing membrane potential, reducing ATP generation and lowering mitochondrial DNA copy numbers and mtRNA transcript levels (Xu *et al*., 2015; He *et al*., 2011; Xu *et al*., 2010). But the impact of Ni neurotoxicity on the individual mitochondrial respiratory complexes remains unexplored and uninvestigated. In the current study, we demonstrated for the first time that the activity of mitochondrial complex IV (cytochrome oxidase) in fish brain was significantly impaired with increase in time and concentration of Ni exposure, highlighting the sensitivity of piscine brain complex IV to Ni neurotoxicity. Mitochondrial complex I, II and III activities remained normal, however, the partial inhibition of complex IV was sufficient to lower the level of ATP generation in fish brain mitochondria, as seen in our study.

Regarding the cause of inhibition of complex IV activity, lipid peroxidation can be considered to be a major contributor. In our study increased generation of MDA in the fish synaptosomal fraction following Ni exposure has been observed, which is considered an important biomarker for lipid peroxidation. As respiratory enzyme complexes of the mitochondrial electron transport chain are embedded in the inner mitochondrial membrane, lipid peroxidation can cause a change in the lipid microenvironment of the membrane and thus the individual complexes of the mitochondrial respiratory chain may get affected in varying degrees. In our study, complex IV seems most affected and the direct involvement of lipid peroxidation in causing mitochondrial complex IV dysfunction cannot be ruled out. Besides, the numerous deleterious end-products of lipid peroxidation may also affect complex IV of the mitochondrial respiratory chain. In support of this concept, Picklo *et al*. (1999) have already demonstrated the deleterious effects of byproducts of lipid peroxidation (namely 4-hydroxy-2-nonenal), altering the mitochondrial complex I and complex III linked respiration. Apart from lipid peroxidation, alteration of mitochondrial membrane potential, as observed in our current study, may also affect the functioning of mitochondrial complex IV activity as this respiratory complex is known to translocate protons from the mitochondrial matrix to intermembrane space, contributing to the proton gradient later utilized by ATP synthase for the generation of ATP (Marchi *et al*., 2012; Desagher & Martinou, 2000). Moreover, the high sensitivity of complex IV to oxidizing agents may also result from the fact that complex IV contains heme groups and copper centers, all of which can be a site of direct ROS attack and their oxidative modifications can manifest with a decrease of enzymatic activity and dysfunction of the whole respiratory chain (Marchi *et al*., 2012; Brown *et al*., 1999; Bragadin & Radi, 1996; Cleeter *et al*., 1994).

A deficient mitochondrial electron transport chain can have a direct impact on the activities of important adenosinetriphosphatases, namely Na^+K^+ATPase as explored in our study. Na^+K^+ATPase is an enzyme implicated in neuronal excitability, metabolic energy production, as well as in the uptake and release of catecholamines, serotonin, and glutamate (Liapi *et al*., 2011; Lees *et al*., 1990; Swann AC, 1984; Mata *et al*., 1980). Our study clearly demonstrates the significant dysfunction of Na^+K^+ATPase activity and lowering of protein expression in fish brain. The degree of inhibition and lowering of protein expression increase with concentration and time of Ni exposure. This finding is in tune with that of Liapi *et al*. (2011), though in a different animal model, demonstrating a significant inhibition of Na^+K^+ATPase in rat brain following an *in vivo* short-term exposure of Ni. The Ni-induced inhibition of the piscine brain Na^+K^+ATPase as observed in this study is a novel finding as it highlights the neurotoxicological impact of long-term exposure of Ni mediated through oxidative stress. Like mitochondrial respiratory dysfunction, there may be involvement of several contributing factors that led to inhibition of Na^+K^+ATPase following Ni neurotoxicity. The conformation of Na^+K^+ATPase is likely to be severely altered by the structural and functional derangement of phospholipid bilayer of membranes in which this enzyme remains embedded. In addition, the lipid peroxidation-derived aldehyde products, namely 4-HNE, can also inflict severe damage to the normal conformation of this enzyme affecting its function (Miyake *et al*., 2003; Zamai *et al*., 2002). The loss of thiol (-SH) groups upon Ni exposure as observed in our study can be another important determining factor that leads to inhibition of this enzyme. Na^+K^+ATPase, being an –SH group containing enzyme, gets impaired due to Ni exposure, suggesting that –SH groups are essential for the enzyme activity. This finding is in conformity with earlier research reports that showed Ni toxicity causing

a decline in the thiol capacity of the cell (Petrushanko *et al*., 2012;Chakrabarti & Bai,1999;M'Bemba-Meka *et al*., 2006). The fact that Na^+K^+ATPase is highly susceptible to oxidative attack can also be proved by an increase in lipid peroxidation induced protein carbonylation in the synaptosomal fraction as observed in our study. Such oxidative damaged proteins usually enter the degradation pathway in a cell indicating the measure of irreversible and unrepairable modification of proteins induced by oxidative stress (Nyström, 2005). Though direct evidence in favor of conformational changes of Na^+K^+ATPase upon nickel exposure has not been provided in this study, there remains a high chance of involvement of different contributory factors, like deficiency in ATP generation due to inhibition of mitochondrial complex IV, loss of thiol groups and lipid peroxidation derived metabolites, all `contributing concurrently to the inhibition of Na^+K^+ATPase. These initial findings further encourage us to take up enzyme kinetics related work pertaining to Na^+K^+ATPase inhibition, which was not within the scope of the present study.

The direct implication of partial inhibition of mitochondrial respiratory chain and dysfunction of Na^+K^+ATPase is the neuronal death that was observed in the brain of *Clarias batrachus* in our study. To ascertain the occurrence of cell death upon Ni exposure, notably two cell death markers, i.e. caspase-3 and caspase-9, were studied as these markers are situated at pivotal junctions in cell death pathways. Interestingly, with the increase in duration and concentration of Ni exposure, enhanced activities of caspase-3 and caspase-9 were observed in the brain of *Clarias batrachus,* signifying the occurrence of neuronal death following Ni exposure.

Based on information obtained from this study and also from previous findings, it can be concluded that partial inhibition of mitochondrial respiratory chain and Na^+K^+ATPase dysfunction are the determinant factors modulating the toxicity of Ni in the brain of *Clarias batrachus* L .Though previous studies have dealt with the impact of Ni toxicity on mitochondrial function, there were certain lacunae concerning mitochondrial function especially at mitochondrial respiratory level which have been addressed in this study. Regarding the impact on Na^+K^+ATPase dysfunction, previous reports have demonstrated the dysfunction of this enzyme upon Ni exposure, however the factors contributing to the inhibition of this enzyme remained unexplored. Our current study clearly depicts the contributing factors that lead to the dysfunction of this enzyme, at the same time suggesting a possible link between mitochondrial dysfunction to that of Na^+K^+ATPase inhibition. So the rationale for targeting mitochondrial respiratory chain and Na^+K^+ATPase function in our study lies in the fact that the alterations in these basic functions can very well explain the loss of cell viability due to Ni neurotoxicity. In this connection, our study provides a vital clue about the specific areas that the therapeutic agents should target on in order to counteract the deleterious impact of Ni induced neurotoxicity.

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