Fish Physiol Biochem (2015) 41:625–634
DOI 10.1007/s10695-015-0033-1

Neurotoxic effects of nickel chloride in the rainbow trout brain: Assessment of c-Fos activity, antioxidant responses, acetylcholinesterase activity, and histopathological changes

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Received: 19 November 2014 / Accepted: 3 February 2015 / Published online: 10 February 2015

Abstract The aim of this study was to determine the biochemical, immunohistochemical, and histopathological effects of nickel chloride (Ni) in the rainbow trout brain. Fish were exposed to Ni concentrations (1 mg/L and 2 mg/L) for 21 days. At the end of the experimental period, brain tissues were taken from all fish for c-Fos activity and histopathological examination and determination of acetylcholinesterase (AChE), superoxide dismutase (SOD), catalase (CAT) enzyme activities, lipid peroxidation (LPO), and glutathione (GSH) levels. Our results showed that Ni treatment caused a significant increase in the brain SOD activity and in LPO and GSH levels ($p < 0.05$), but it significantly decreased AChE and CAT enzyme activities ($p < 0.05$). Strong induction in c-Fos was observed in some cerebral and cerebellar regions of fish exposed to Ni concentrations when compared with the control group. However, c-Fos activity was decreased in necrotic Purkinje cells. Brain tissues were characterized by demyelination and necrotic changes. These results suggested that Ni treatment causes oxidative stress, changes in c-Fos activity, and histopathological damage in the fish brain.

Keywords Nickel · Fish · Brain · c-Fos · Toxicity · Oxidative stress

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Introduction

Pollution by heavy metals is a worldwide problem because of the persistent and continuous accumulation of metals in the environment. There are generally trace amounts of heavy metals in aquatic environments. In recent years, heavy metals have been found in increasing concentrations because of factors such as rapid human population growth, industrialization, mining, agricultural, and industrial activities. As a result, aquatic organisms, including fish, are affected by such increasing concentrations (De Mora et al. 2004; Hwang et al. 2006). Nickel and it compounds have many commercial and industrial uses, and the progress of industrialization has led to the increased

emission of pollutants into ecosystems (Cempel and Nikel 2006). Ni is a ubiquitous, naturally occurring trace metal (0.0086 % of the earth's crust), with increased concentrations in water bodies (Kienle et al. 2008). Water generally contains Ni at concentrations <10 μg/L (Cempel and Nikel 2006). Canadian rivers have been shown to carry concentrations from 0.001 to 0.01 mg Ni/L up to 0.5 and 2 mg Ni/L (Chau and Kulikovsky-Cordeiro 1995). Ni concentrations in highly contaminated fresh waters may reach as high as several hundred to 1000 μg/L (Eisler 1998; Pane et al. 2003). It has been reported that nickel chloride has teratogenic, carcinogenic, immunotoxic, and genotoxic effects in living organisms (Costa et al. 2005; Vijayavel et al. 2009; Adjroud 2013). Ni is a potential neurotoxic pollutant, and the nervous system is one of the systems affected by Ni toxicity (Xu et al. 2010). Ni affects fish behavior, including nervous impairment; however, it does not accumulate in the rainbow trout brain (Pane et al. 2003; Ololade and Oginni 2010). Kubrak et al. (2014) detected no accumulation in the goldfish brain when the fish were exposed to Ni. They discovered that Ni accumulated in goldfish kidneys, which provided efficient urine excretion of nickel (Kubrak et al. 2014). In another study, Tjälve et al. (1988) found Ni in cerebrospinal fluid and spinal cord of brown trout exposed for 1 and 3 weeks to 0.1 and 10 mg/L of Ni (Tjälve et al. 1988). Nickel chloride can cause adverse effects in fish. For example, it can decrease their locomotory activity and inhibit of antioxidant enzymes and apoptosis (Kienle et al. 2008; Zheng et al. 2014). Rainbow trout comprise one of the most sensitive fish species exposed to nickel chloride (Brix et al. 2004). Therefore, it is important to investigate the adverse effects of nickel chloride on fish in the aquatic environment.

Environmental contaminants can alter biochemical and physiological parameters in fish. In particular, these toxic contaminants cause cytotoxic effects by producing a reactive oxygen species (ROS) (Li et al. 2010a). For example, Ni can cause oxidative damage by inducing ROS (Zheng et al. 2014). Contaminant-stimulated ROS and oxidative stress may be a mechanism of toxicity for aquatic organisms living in polluted areas (Monteiro et al. 2006). The main antioxidant enzymes for the detoxification of ROS in all organisms are superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH)-dependent enzymes (e.g., GSH-Px, GST, and GR), which use GSH as a substrate. The activities of these enzymes have been used to quantify oxidative stress in cells (Van der Oost et al. 2003; Olsvik et al. 2005; Xing et al. 2012a, b). Many aquatic pollutants can cause the formation of ROS, such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals which damage biological processes (Livingstone 2001; Modesto and Martinez 2010; Ciji et al. 2012). Therefore, antioxidant enzymes can be used as biomarkers in environmental risk assessment (Li et al. 2010b). In addition, acetylcholinesterase (AChE) is one of the enzymes used as a biomarker, and it is responsible for cholinesterase activity in the nervous systems of vertebrates (Carr and Chambers 2001; Xu et al. 2011; Da Cuna et al. 2011). In cases of exposure to pollutants, it could be an appealing biomarker for the assessment of neurotoxic changes (Gholami-Seyedkolaei et al. 2013).

Ni toxicity has been investigated in mammalian model organisms, but the effects of Ni on rainbow trout have scarcely been studied. There is no study associated with c-Fos in fish exposed to environmental pollutants, especially heavy metals. Thus, the purpose of this study was to assess antioxidant responses, AChE activity, c-Fos activity, and histopathological changes in brain tissues of rainbow trout after 21 days of Ni exposure.

Materials and methods

Fish maintenance

Rainbow trout (150 ± 5.82 g, 14.62 ± 0.61 cm) were obtained from Ataturk University (Erzurum, Turkey), Faculty of Fisheries, and Inland Water Fish Breeding and Research Center. Experiments were carried out in three fiberglass tanks (each 400 L) with 15 fish in each tank under laboratory conditions in dechlorinated tap water (temperature 10–12 °C, pH 7.4, dissolved oxygen 7.1 mg/L, water hardness 173.5 mg/L, $CaCO_3$, SO_4^{-2} = 0.29 mg/L, PO_4^{-3} = 0.01 mg/L, NO_3^- = 1.40 mg/L, and NO_2^- = trace). Fish were acclimated for 15 days under laboratory conditions. Fish were fed 2.5 % body weight with commercial trout pellets (Sibal Group, Sinop, Turkey) every day during the acclimation period.

Experimental design

Nickel chloride ($NiCl_2$) was purchased from Sigma-Aldrich Co. A stock solution of $NiCl_2$ was prepared by dissolving it in distilled water. The nominal concentrations used for this study were 1 mg/L and 2 mg/L. These concentrations were chosen because they may occur in a polluted environment (Eisler 1998). Fish were separated into three groups: one control group and two $NiCl_2$-treated groups. Fish in the latter two groups were exposed to $NiCl_2$ concentrations (1 and 2 mg/L) for 21 days. $NiCl_2$ was administered by adding to water. All tests were conducted according to OECD guidelines. At the end of the experiment, 12 fish from each group were euthanized by cervical section, and brain tissues of both control and treated fish were immediately removed. A portion of the brain tissues was stored at −20 °C until analysis of AChE and antioxidant enzyme activities. The other portion of the brain tissues was fixed in 10 % neutral buffered formalin solution for c-Fos activity and histopathological examination.

AChE enzyme activity and protein determination

Brain tissues were homogenized (1 % v/v) in 0.1 M phosphate buffer (pH 7.4) containing Triton-X 100 using a homogenizer. The homogenate was centrifuged at 13,000×*g* for 30 min (4 °C), and the precipitate was removed. The supernatants were used as the enzyme source for estimating AChE activity (Chhajlani et al. 1989; Rosenfeld et al. 2001). AChE activity was determined according to the modified method of Ellman et al. (1961). The enzymatic reaction was measured in a total volume of 1.0 ml, containing 50 µL 0.5 mM DTNB in 1 % sodium citrate, 200 µL 0.5 M phosphate buffer (KH_2PO_4/K_2HPO_4; pH 8.0), 650 µL H_2O, 50 µL crude extract, and 50 µL 10 mM acetylthiocholine iodide. The control cuvette did not contain acetylthiocholine iodide. The changes in absorbance at 412 nm for 5 min were measured using a Beckman Coulter DU 730 UV/Vis spectrophotometer. One enzyme unit was defined as the amount of enzymes that catalyzes the hydrolysis of 1 µmol of acetylthiocholine iodide per minute at 25 °C. Protein concentration was determined spectrophotometrically at 595 nm according to the Bradford method with bovine serum albumin used as a standard (Bradford 1976).

Lipid peroxidation (LPO) determination

The level of LPO in brain tissues was determined by estimating malondialdehyde (MDA) using the thiobarbituric acid test (Ohkawa et al. 1979). The brain tissues were dissected, weighed, and homogenized in 10 ml of 100 g/l KCl. The homogenate (0.5 ml) was added to a solution containing 0.2 ml of 80 g/l sodium laurylsulphate, 1.5 ml of 200 g/l acetic acid, 1.5 ml of 8 g/l 2-thiobarbiturate, and 0.3 ml of distilled water. The mixture was incubated at 98 °C for 1 h. Upon cooling, 5 ml of n-butanol:pyridine (15:l) was added. The mixture was vortexed for 1 min and centrifuged at 4000*g* for 30 min. Subsequently, the absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1, 1, 3, 3-tetramethoxypropane. The recovery rate was over 99 %. The results were expressed as nmol MDA/g of tissue.

Superoxide dismutase (SOD) activity

SOD activity was measured according to Sun et al. (1988). SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with nitroblue tetrazolium (NBT) to form formazan. SOD activity was then measured at 560 nm by the degree of inhibition of this reaction and was expressed as mmol/min/mg of tissue.

Catalase (CAT) activity

Decomposition of H_2O_2 in the presence of CAT was measured at 240 nm according to Aebi (1984). The CAT activity was defined as the amount of enzymes required to decompose 1 mmol of H_2O_2 per minute, at 25 °C at pH 7.8. Results were expressed as mmol/min/mg of tissue.

Total glutathione (GSH)

The amount of GSH in brain tissues was measured according to the method described by Sedlak and Lindsay (1968), with some modifications. The brain tissues were homogenized in 2 ml of 50 mM Tris–HCl buffer containing 20 mM EDTA, at pH 7.5. After adding 2 ml ethanol (to precipitate the proteins), the homogenate was centrifuged at 3000*g* for 40 min at 4 °C. The supernatant was used to determine the GSH level using 5, 5′-dithiobis (2-nitrobenzoic acid)

(DTNB). The absorbance was measured at 412 nm. Subsequently, the GSH level of the brain was expressed as nmol/g of tissue.

Histopathology and immunohistochemistry

Brain tissue samples from cerebral hemispheres and the cerebellum were fixed in 10 % buffered formaldehyde solution and dehydrated in graded alcohol and xylol series, embedded in paraffin wax. Microtome sections in 4–5 μm (Leica Microsystems, Wetzlar, Germany) on normal slides (for hematoxylin–eosine, HE) and Poly-L-Lysine coated slides (immunohistochemistry for c-Fos) were obtained. The HE staining procedure was performed according to Presnell and Schreibma (1997).

Immunohistochemical reactivity of c-Fos in brain tissues was measured by an automated method on the VENTANA BenchMark GX System (Ventana Medical Systems, Inc.). After deparaffinization, the antigenic determinant sites for c-Fos were unmasked in citrate buffer with steam for 60 min. The primary antibody used for anti-c-Fos, an IgG class Rabbit polyclonal to c-Fos (Santa Cruz and sc-52, Lot D1411), was used at a dilution of 1:80. The slides were incubated in the antibody solution for 32 min at 37 °C followed by application of the ultraView Universal DAB detection kit (Ventana Medical Systems, Inc.). DAB was used as a chromogen and counterstained with hematoxylin. Similarly, processed sections from human tonsil tissues were used as positive controls for c-Fos immunostaining. The specificity of staining was confirmed by the inclusion of negative control slides processed in the absence of the primary antibody on tissue from the same animal. Histopathology and c-Fos immunoreactivity were semiquantitatively assessed. For this, tissue sections from 18 fish (six samples from each group) were randomly chosen. A total of 18 HE-stained slides (six from each group) and 18 immunhistochemistry slides (six fish from each group) were examined under the light microscopy. All microscopic areas on a slide were divided into three different regions. The distribution of c-Fos activity and histopathological lesions was assessed by two experts for all three regions in all slides. The positivity in each area was recorded. Finally, 0–6 positivity, 7–12 positivity, and 13–18 positivity in 18 fish were evaluated as +, ++, and +++. The comparison of histopathology and c-Fos immunoreactivity in the control and experimental groups is displayed in Table 1.

Statistical analyses

All data were expressed as mean ± SEM. Statistical analysis of data was performed using a one-way analysis of variance and Duncan test and analyzed using SPSS version 10.0 software. A value of $p < 0.05$ was considered statistically significant.

Results

In vivo AChE enzyme and antioxidant enzyme activities

Ni exposure of 1 and 2 mg/L caused a significant decrease in the brain AChE enzyme activities of rainbow trout. CAT activity of the brain was decreased in both experimental groups compared with the control group. ($p < 0.05$) (Table 2). In contrast, LPO levels in the brain were significantly increased by both Ni concentrations, and 2 mg/L Ni significantly increased the SOD activity in brain tissues ($p < 0.05$) (Table 2). Both Ni concentrations also increased GSH levels in brain tissues.

Histopathology

There were no histopathological lesions in the cerebral (Fig. 1a) and cerebellar (Fig. 2a) sections of the control group. Demyelination in limited areas was seen in the experimental groups in varying degrees (Fig. 1c, e). More prominent lesions were observed in the Purkinje layer of the cerebellum. Necrotic changes in Purkinje cells were detected in both experimental groups (Fig. 2c, e).

c-Fos immunoreactivity

c-Fos activity was observed in the nuclei and cytoplasm of many neuronal cells. In the cerebral region, strong positivity in c-Fos was observed in the molecular and external granular layers of gray matter of fish exposed to Ni concentrations when compared with the control group (Fig. 1b, d, f). This immunoreactivity was observed in decreasing degree in the Purkinje cell layer depending on the neuron necrosis

Table 1 Comparison of histopathology and c-Fos immunoreactivity in control and experimental groups

Histopathology and c-Fos activity	Control	1 mg/L	2 mg/L
Demyelinated areas in cerebral sections	–	–	+
Necrosis or loss of Purkinje cell	–	++	++
c-Fos activity in molecular and external granular layers	++	+++	+++
c-Fos activity in Purkinje layer	+++	+	+

Table 2 Changes in the brain AChE and antioxidant enzyme activities in fish exposed to nickel chloride

	Control	1 mg/L	2 mg/L
AChE (U/mg protein)	1.53 ± 0.09^{a}	0.62 ± 0.24^{b}	0.28 ± 0.17^{c}
LPO (nmol MDA/g tissue)	18.11 ± 0.353^{a}	21.19 ± 0.94^{b}	24.33 ± 0.37^{c}
SOD (mmol/min/mg)	1.04 ± 0.08^{a}	1.47 ± 0.09^{b}	3.45 ± 0.5^{b}
GSH (nmol/g tissue)	5.59 ± 0.19^{a}	6.59 ± 0.21^{b}	6.30 ± 0.21^{b}
CAT (mmol/min/mg)	0.50 ± 0.033^{a}	0.31 ± 0.008^{b}	0.21 ± 0.02^{c}

Nickel groups were statistically compared with control group in each parameter. Different letters indicate statistically significant differences ($p < 0.05$)

(Fig. 2b, d, f). Strong induction was detected in the granular layer of the cerebellum in control and experimental groups.

Discussion

We investigated the biochemical, immunohistochemical, and histopathological effects on brain tissues of $NiCl_2$ in rainbow trout. The effects of Ni on rainbow trout have scarcely been studied in the literature, but effects of Ni on different fish species have been reported in many studies (Kubrak et al. 2013, 2014; Pane et al. 2003; Kienle et al. 2009; Zheng et al. 2014). For example, Zheng et al. (2014) indicated that Ni induced oxidative stress and apoptosis in the liver of *Carassius auratus*. In another study, Ni caused hyperglycemia and glycogenolysis and affected the antioxidant system in the liver and white muscle of *Carassius auratus* (Kubrak et al. 2012). Pane et al. (2003) tested Ni on rainbow trout tissues and observed respiratory toxicant effects. Kienle et al. (2008) reported that Ni lead to reduced vitality and locomotory activity in *Danio rerio* embryos and larvae. Ni toxicity can cause oxidative damage by inducing ROS generation (Zheng et al. 2014). Enhanced ROS amounts damage biomolecules such as lipids, proteins, and nucleic acids. Therefore, LPO is one of the major indicators of oxidative stress (Kaplan et al. 2012). It has been shown that LPO is a major contributor to the loss of cell function during oxidative stress, and environmental contaminants may cause LPO production (Monteiro et al. 2006; Kumtepe et al. 2010). In the present study, LPO levels in the brain were significantly increased after exposure to Ni concentrations in a dose-dependent manner. Apparently, Ni caused ROS formation, and this event can be one of the main reasons for the heavy metal's toxic effects. Histopathological results confirmed that Ni can cause toxic damage in brain tissues, indicating that Ni induced demyelination in limited areas in varying degrees and necrotic changes in the Purkinje layer of the cerebellum.

SOD is the first step of the antioxidant defense system. An increase in SOD activity indicates enhanced production and/or the presence of superoxide radicals (Oruç and Uner 2000; Halici et al. 2012). SOD activity could be attributed directly to antioxidant defense and indirectly to free radical levels and oxidative stress (Unal et al. 2011 and Halici et al. 2012). In the present study, elevated SOD activity in brain tissues after Ni exposure was observed. This indicates that an organism activates SOD as a reflex to prevent the damaging effects of (Ni-induced) oxidative damage. It has been reported that aquatic contaminants can enhance the intracellular formation of ROS (Ciji et al. 2012) and Ni can lead to the depletion of intracellular free radical scavengers such

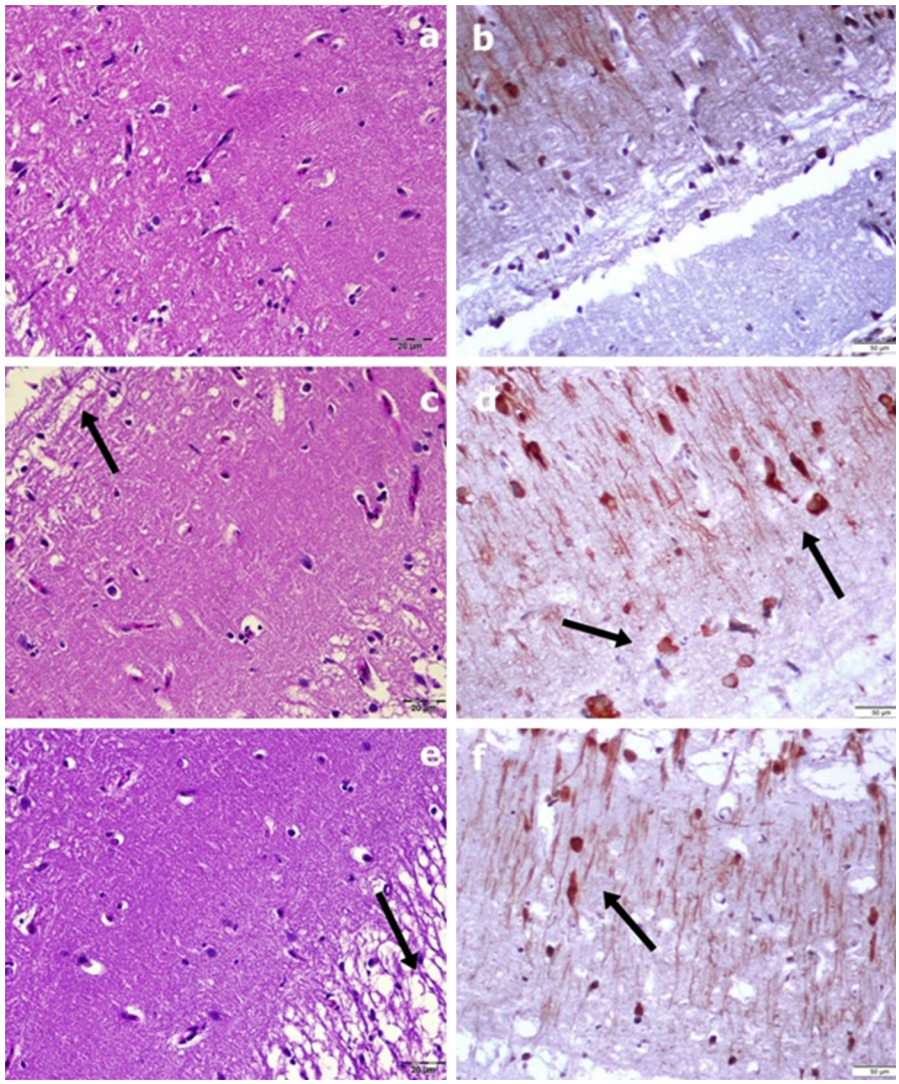

Fig. 1 Normal cerebral cortex (**a**) and demyelination in limited areas in fish exposed to 1 mg/L (*arrow* in **c**) and 2 mg/L Ni (*arrow* in **e**) HE. Strong c-Fos expression in external layers of cerebellum of fish exposed to 1 mg/L (*arrows* in **d**) and 2 mg/L Ni (*arrow* in **f**) when compared to group control (**b**)

as glutathione (GSH) (Kubrak et al. 2013). In the present study, Ni concentrations caused a significant increase in GSH levels of brain tissues. This increase can be explained by the protective role of glutathione against oxidative damage (Monteiro et al. 2006). GSH levels can increase as an adaptive mechanism because of increased synthesis under oxidative stress. However, oxidative stress may also suppress GSH levels because of impairment of the adaptive mechanisms (Monteiro et al. 2006; Unal et al. 2011). However, this was not observed in the present study.

CAT activity can be considered as an indicator and sensitive biomarker for metal toxicity in fish (Atli et al. 2006). In the present study, CAT activity in the brain tissues of the experimental groups was significantly decreased compared with the activity in the control group. This decrease in CAT activity could be due to the flux of superoxide radicals, which have been reported to inhibit CAT activity (Sayeed et al. 2003). Inhibition of CAT activity may also occur due to the direct binding of metal ions to functional SH groups on the enzyme (Ates et al. 2008). A suppression of CAT caused by Ni has also been observed in earlier studies on goldfish (Kubrak et al. 2013).

Several experimental studies have indicated that detoxifying enzymes have very important roles in toxicology (Hao et al. 2008; Xing et al. 2013). As one of such enzymes, AChE is the target of many

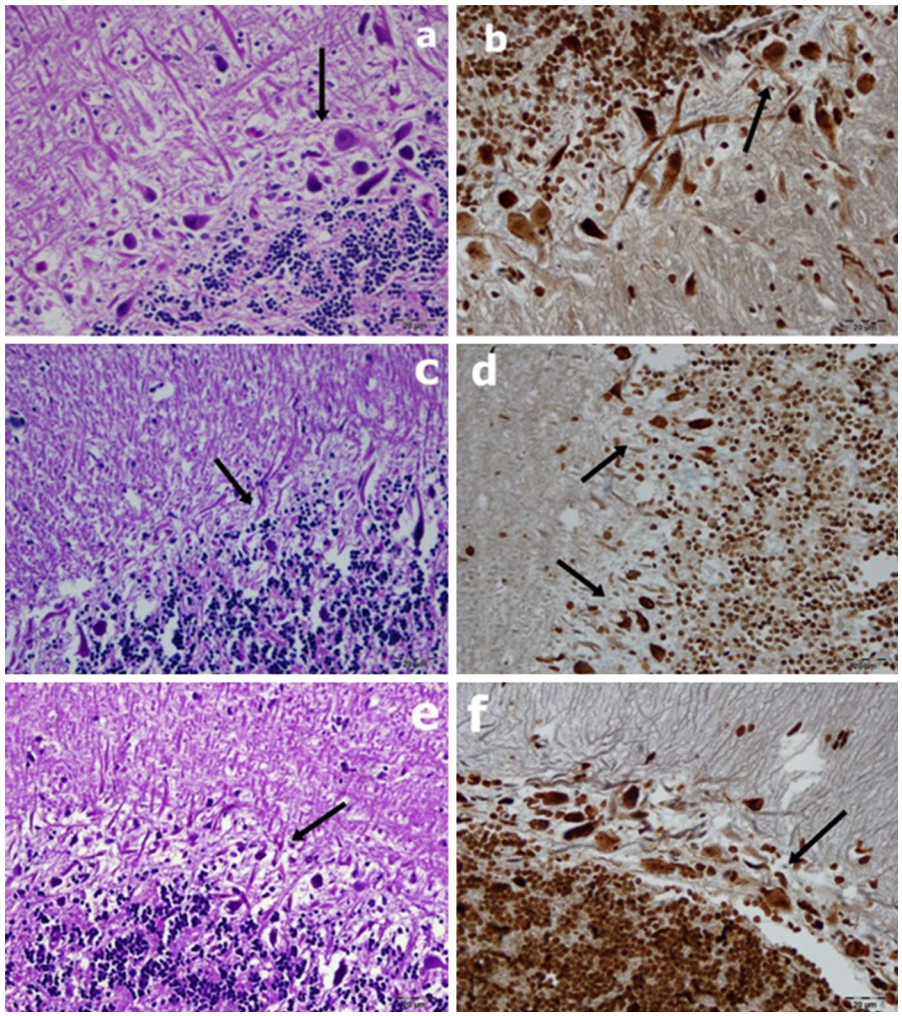

Fig. 2 Normal Purkinje layer in control group (**a**) and severe necrosis in Purkinje cells of fish exposed to 1 mg/L (*arrow* in **c**) and 2 mg/L Ni (*arrow* in **e**) HE. c-Fos expression in Purkinje layers of cerebellum of control group (*arrow* in **b**) and decreasing in c-Fos activity together with neuronal necrosis in fish exposed to 1 mg/L (*arrows* in **b**) and 2 mg/L Ni (*arrow* in **f**)

pollutants and is considered as a biomarker for neurotoxicity (Perez et al. 2013; Świergosz-Kowalewska et al. 2014). It is also known that pollutants such as metals inhibit AChE activity (De La Torre et al. 2002). In the present study, we found that Ni exposure caused a significant decrease in brain AChE enzyme activities of rainbow trout. These results are similar to those of studies performed with other heavy metals in fish (Guilhermino et al. 2000; Pretto et al. 2010). For example, AChE enzyme activity has been reported to decrease after cadmium exposure in silver catfish brain and muscle (Pretto et al. 2010). AChE enzyme inhibition leads to a degradation of acetylcholine. This will result in excessive stimulation of cholinergic nerves (Uner et al. 2006). When AChE activity is decreased, acetylcholine is accumulated within synapses, and this event can damage important behavioral functions in fish such as swimming and feeding (Glusczak et al. 2006). Also, AChE inhibition may be associated with cell damage and lower AChE expression in the nervous system (Zhang de et al. 2013; Xing et al. 2013). c-Fos is known to be induced in neurons of fish as a result of neuronal stimulation (Salierno et al. 2006). c-Fos is one of the members of the Fos family (Kovacs 1998) and is a transcription factor used as a biomarker of neuronal brain activity.

Various types of stressful stimuli such as neurotoxins and pollutants can induce c-Fos activation (Salierno et al. 2006; Varani et al. 2014). In the present study, c-Fos activity was observed in many neuronal cells. Strong induction was detected in the granular layer of the cerebellum and the cerebral region in the control and experimental groups. c-Fos activity decreased in necrotic Purkinje cells. The present study showed that c-Fos can be detected immunohistochemically in neurons that are related to the presented stimuli, suggesting that it is present (Bosch et al. 1995). Therefore, c-Fos activity can be induced by Ni toxicity.

In summary, we indicated that antioxidant responses, changes in c-Fos activity, and histopathological damage occurring in the relation to Ni exposure could lead to altered physiological functioning in the brain, indicating that Ni is neurotoxic to fish. Our results may provide useful data for future investigations. Fish in both cultured and natural environments are sensitive to this heavy metal, and contamination would disrupt the ecological balance.

Acknowledgments We would like to thank Ataturk University for financial support of the research (Project No: 2014/200) and Yunus Aktürk for technical support in the immunohistochemical study.

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