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RESEARCH ARTICLE

Sodium fluoride generates ROS and alters transcription of genes for xenobiotic metabolizing enzymes in adult zebrafish (*Danio rerio*) liver: expression pattern of Nrf2/Keap1 (INrf2)

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Abstract

Anthropogenic activities have resulted in an increase in the level of fluoride (F), a natural pollutant in water, causing great threat to the aquatic organisms including fishes. Earlier we reported that sodium fluoride (NaF) exposure alters histological ultrastructure in zebrafish (*Danio rerio*) liver evidenced by hyperplasia, cytoplasmic degeneration, heteropycnosis etc. In this study, zebrafish were exposed to 7.5, 15 and 30 mg NaF l^{-1} for 30 days as well as to 15 mg NaF l^{-1} for 90 days. In NaF treated fish, generation of reactive oxygen species (ROS), depletion of glutathione (GSH) and increase in malondialdehyde (MDA) content along with enhanced activities of oxyradical-scavenging enzymes like catalase (CAT) and superoxide dismutase (SOD) were recorded. Activity of GSH-metabolizing enzyme, glutathione-*S*-transferase (GST) was also enhanced. The mRNA levels of genes for xenobiotic metabolizing enzymes (XMEs) like cytochrome P450 1A (*Cyp1A*), NADPH Q Oxidase 1 (*Nqo1*) and Heme Oxygenase 1 (*Ho-1*) increased along with nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) whereas Kelch-like ECH-associated protein 1 (*Keap1*) decreased in the treated groups in comparison to their controls. The increase in Nrf2 protein levels in NaF treated fish confirmed its key regulatory role in F-induced oxidative stress. Chromatin condensation and nuclear fragmentations were evidenced in NaF-treated groups indicating possible induction of apoptosis. The modulation of these toxicological parameters at genetic and biochemical levels may be used as an early warning for the environmental risk assessment of F^- toxicity to aquatic organisms including fishes.

Keywords

Oxidative stress, reactive oxygen species, sodium fluoride, xenobiotic metabolizing enzymes, zebrafish liver

History

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Introduction

Fluorine (F^-), an important member of the halogen family, is strongly electronegative and highly reactive (Gillespie et al., 1989; Greenwood & Earnshaw, 1984). F^- is one of the most abundant elements in the earth's crust and is a natural pollutant in the aquatic ecosystem. The level of F^- in surface water depends on its geographical location and proximity to the source of emission. It may also be due to weathering of fluoride (F)-containing ores. Apart from this, various anthropogenic activities and industrial processes including manufacture of steel, production of primary aluminums, copper and nickel, processing of phosphate ore, enameling, production and use of phosphate fertilizers, glass, bricks, ceramics adhesives, F-containing pesticides, fluoridated dental preparations and controlled fluoridation of drinking water supplies lead to substantial increase in water F^- level in the last few decades (WHO 1999, 2006). Accumulating evidences of skeletal fluorosis in human in different parts of the world and subsequent reports on its harmful effects in various soft tissues in mammals have marked F^- pollution as a major threat to the modern world (Suma Latha et al., 1999). Although adverse effects of F in mammalian system(s) both *in vivo* and *in vitro* are reported (Chattopadhyay et al., 2011; Machalinska et al., 2002; Machalinski et al., 2003; Podder et al., 2010a,b), few studies have been made on the underlying mechanisms of its action in fish (Cao et al., 2014), an important component of aquatic ecosystem.

Fish can take up F^- directly from water (Nell & Livanos, 1988; Neuhold & Sigler, 1960). F^- level as low as 0.5 mg l^{-1} of water may be toxic to fish, particularly for those living in soft waters (Camargo, 2003). In unpolluted freshwater, the concentration of F^- ranges from 0.01 to 0.3 mg l^{-1} while in sea water this value increases up to 1.5 mg l^{-1} (Camargo, 2003; Datta et al., 2000; World Health Organization [WHO], 2002; Weinstein & Davison, 2004). US Geological Survey reported that F-level of Walker and Pyramids Lakes in Nevada were 13 mg l^{-1} whereas the same from Madison and

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Fire hole rivers in Yellowstone National park ranged between 12 and 14 mg l^{-1}. In Japan, F^- concentration in well water was reported to be 1.5–5.5 ppm (Kobayashi, 1951) and in New Zealand, natural thermal water contained 1–12 mg l^{-1} of F^- (Mahon, 1964; Sigler & Neuhold, 1972). Fluoride level may be as high as 25–50 mg l^{-1} in some hot springs (Neuhold & Sigler, 1960) whereas its concentration in the industrial waste water was estimated as high as 96.8 mg l^{-1} (Ding et al., 1998).

Zebrafish (*Danio rerio*), a fresh water teleost, is an excellent model for the study of reproductive and developmental biology (Bailey et al., 2013; Froehlicher et al., 2009) as well as for toxicological and physiological studies (Ali et al., 2011; Briggs et al., 2002; Craig et al., 2007; Neumann & Galvez, 2002). Effect of F pollution is more adverse in organisms dwelling in soft water compared to that of hard water, since bioavailability of F decreases with increase in hardness of water (Camargo, 2003). Therefore, study of F-toxicity in zebrafish has important relevance in assessing its impact on soft water ecosystem where fish is an important component. Majority of the F-toxicity studies in fishes incorporated parameters like histopathology and biochemical analysis. This study incorporated the mRNA expression patterns of XMEs genes related to phase I and phase II detoxification system which might act prior to the toxic effect at anatomy and biochemical level.

Cytochrome P450 (CYP) has been used as an important biomarker for assessing environmental exposures of fishes to halogenated compounds and members of CYP450 gene families 1–4 are considered as important in xenobiotic metabolism (Van der Oost et al., 2003; Whyte et al., 2000). Among all the families and types, CYP1A enzyme activity is regulated by different types of heavy metals as well as organic compounds (Anwar-Mohamed et al., 2009; Zhou et al., 2010). However, no report is available on its expression after NaF exposure. CYP1A converts both exogenous and endogenous compounds to polar and water soluble compounds. CYP1A is mainly expressed in the liver, although its localization in other tissues has also been reported (Stegeman et al., 1989).

Beside phase I XMEs, phase II detoxification system is associated with different enzymes known as Phase II xenobiotic metabolizing enzymes (Phase II XMEs). The Phase II XMEs include Heme Oxygenase-1 (HO-1), NADPH quinine oxidoreductase 1 (NQO1) and Glutathione-S-Transferase (GST). Reports on the induction of phase II XMEs' at mRNA and protein levels with various toxicants exist but their induction after F exposure particularly in fish model are scanty. An inverse correlation between *Cyp1A* and *Ho-1* was reported in rats, though the causality of this relationship is unclear (Abraham et al., 2000). NQO1 plays an important role in cellular protection through its anti-oxidative properties (Lim et al., 2008; Xiao et al., 2011). HO-1 enzyme, involved in heme catabolism, is expressed by different inducers including heavy metals and environmental pollutants. It is reported that HO-1 is present at low levels in liver and other soft tissues under normal conditions and is inducible by a wide range of stimuli that cause oxidative stress (Abraham et al., 2003). While the induction of *Ho-1* has been documented and is known to be primarily at the level of gene transcription, the molecular mechanism(s) underlying this response is poorly understood.

The transcription factor, known as nuclear factor (erythroid-derived 2)-like 2 (Nrf2), has been recognized as the master regulator of the cellular defense mechanism against toxic insults. Nrf2, a member of the cap ''n'' collar basic region-leucine zipper transcription factors, serves as the major transcription factor in defense against a range of toxicants. Under normal conditions, Nrf2 remains anchored to actin bound Kelch-like ECH-associated protein 1 (Keap1) or inhibitor of Nrf2 (INrf2), which negatively regulates Nrf2 through ubiquitination and proteasomal degradation in the cytoplasm (Itoh et al., 1999; Jaiswal, 2004). However, in response to ROS or oxidative/elecrophilic stimuli, Nrf2 is released from Keap1, translocates to the nuclei and transactivates the constitutive induction of detoxifying enzymes along with oxidative stress proteins (such as HO-1) through antioxidant responsive element (Itoh et al., 2004; Liu et al., 2013; Ma et al., 2004).

Liver is the major target organ for any toxic substance and plays an important role in the detoxification process. It is the chief site for xenobiotic metabolism. Pollutants and xenobiotics affect liver function and subsequent microsomal enzyme (Cytochrome P450) induction which in turn determine the degree of hepatotoxicity (Conney, 1967). Reports also suggest that these lead to the depletion of glutathione and ultimately induction of whole redox scavenging pathways (Gadgoli & Mishra, 1997). Similarly, fluoride is reported to be hepatotoxic and induced histopathology and stress protein synthesis in mouse (Chattopadhyay et al., 2011) and zebrafish (Mukhopadhyay & Chattopadhyay, 2014). Therefore, we attempted to look into the generation of ROS in liver induced by NaF, consequent hepatotoxicity using biochemical parameters and the effect on mRNA transcription of xenobiotic metabolizing enzymes (XMEs) as well as mRNA and protein expressions of Nrf2 and Keap1 genes in female zebrafish. The rationale for using only female zebrafish was to avoid any variation of expression pattern of XMEs, since the expression of antioxidant genes; housekeeping genes as well as antioxidant enzyme activities differ in male and female zebrafish (McCurley & Callard, 2008; Shao et al., 2012).

The goal of this study was to examine the dose and time dependent effects of F-exposure in zebrafish to elucidate the physiological and transcriptional endpoints of chronic hepatotoxicity. In this study, we investigated the regulatory role of *Nrf2/Keap1*on *Nqo1* and *Ho-1* and its subsequent effect on *Cyp1A* at mRNA level after NaF exposure. Quantitative real time PCR was employed to quantify the F-mediated induction of these five genes. The dose and time dependent oxidative stress was evaluated by determination of malondialdehyde (MDA) and glutathione (GSH) content, analysis of the activity of glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) along with the fluorescence microscopy study of hepatocytes. Western blots were used to study the expression of Nrf2 along with its inhibitor, Keap1. This study attempted to explore the effect of NaF on zebrafish that would help to assess the expression of XMEs-genes as an important biomarker in freshwater ecotoxicology of F and its risk assessment.

Materials and methods

Chemicals and reagents

Primary antibodies against Nrf2 and β-actin were purchased from Pierce (Thermo, Waltham, MA). Keap1 antibody was purchased from Abcam (Cambridge, MA). Mouse anti-rabbit (Abcam) and Goat anti mouse (Sigma, St. Louis, MO) ALP conjugated secondary antibody, BCIP/NBT, TRI reagent for RNA isolation, Hoechst 33342 and 2′,7′-dichlorofluorescin diacetate (DCF-DA) were procured from Sigma-Aldrich Corporation (St. Louis, MO). Reverse transcriptase and all chemicals for PCR mix were purchased from Fermentas (Hanover, MD). Sodium Fluoride (NaF, molecular weight 41.99) was purchased from Sisco Research Laboratories (SRL, Mumbai, India). All other reagents and chemicals used were of highest grade purchased from reputed manufacturers.

Zebrafish and sodium fluoride (NaF) exposure

Adult female zebrafish (average weight of 0.7 ± 0.01 g and length of 3.6 ± 0.05 cm) were procured from local supplier and acclimatized to soft water in the laboratory condition for 2 weeks prior to the experiments. Fish were kept in aquaria with uniform water temperature (26 ± 1 °C), pH (7.0–7.2) and a photoperiod (14:10 h light:dark cycle) and fed daily with commercial food. Fish (10 fish per group in triplicate sets) were randomly selected for experiments and exposed to NaF for 30 days with three concentrations (7.5, 15 and 30 mg L^{-1}) lying within the reported range of F^- levels in different water bodies across the world. Treatment with only 15 mg NaF L^{-1} was extended for 90 days, since it showed maximum hepatotoxicity as evidenced in liver histopathology. Two control groups were used for comparisons of 30 and 90 days treatment. Only female fish were used to avoid any gender based variation in the activity of antioxidant enzymes as well in gene expressions (McCurley & Callard, 2008; Shao et al., 2012). Water in aquaria was replaced daily for maintaining uniform quality. Rules of the ''Institutional Animal Ethics Committee'' of Visva-Bharati University were strictly followed during the entire period and steps were taken to protect the welfare of experimental animals. Liver samples of each set (10 fish) were pooled and data of three such sets were used for statistical analysis ($n = 3$).

ROS level in fish hepatocyte

Reactive oxygen species (ROS) levels in the fish hepatocyte were determined by the oxidation of 2′,7′-dichlorofluorescin diacetate (DCF-DA) according to the procedure described by Contreras et al. (2005). Samples of pooled tissues (0.1–1 g wet wt) were incubated in 100 ml of 10 μM DCF-DA in methanol for 30 min in a water bath at 37 °C. After incubation, fluorescence of DCF was determined in spectrofluorometer (SpectraMax, Molecular Devices, Sunnyvale, MA) at an excitation wavelength of 488 nm and emission wavelength of 525 nm. Fluorescence values were obtained using a standard curve prepared with 0–500 nM DCF.

Protein content

The liver tissues were homogenized and centrifuged at $10\,000 \times g$, 20 min at 4 °C. After centrifugation, the clear supernatants were pipetted out and kept in a fresh tube. These were used as protein samples and their concentrations were determined following the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard.

Measurement of GSH level

The level of GSH in liver was measured by the method of Akerboom & Sies (1981). Briefly, freshly collected tissues were homogenized and lysed by alternate freezing (10 min) and thawing (10 min) three times at 0 and 28 °C and centrifuged at 10 000 rpm for 5 min at 4 °C. The supernatant was deproteinized using 100 μl ice-cold 10% 5-sulphosalicylic acid with intermittent shaking and centrifuged at 10 000 rpm at 4 °C for 15 min. The supernatant was immediately used for GSH estimation. Fifty microliters of sample suspension was added to a reaction mixture containing 1 ml buffer (0.1 M EDTA phosphate buffer, pH 7.0); 50 μl NADPH (4 mg/ml); 20 μl DTNB (1.5 mg/ml); 20 μl GSH reductase (6 units/ml), mixed thoroughly and the optical density was measured continuously for 5 min at 412 nm in a UV–visible spectrophotometer (Beckman Coulter) against a sample blank.

Thiobarbituric acid reactive substances level in liver tissue

The level of lipid peroxidation as measured by the thiobarbituric acid reactive substances (TBARS) was determined according to the method of Buege & Aust (1978). Briefly, 1 ml of microsomal sample was mixed with 2 ml of TBA–TCA–HCl mixture thoroughly and heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at $1000 \times g$ for 10 min. The absorbance of the supernatant was determined at 535 nm. The MDA concentration of the sample was calculated using an extinction coefficient of 1.56×10^5/M/cm and expressed in terms of nM MDA/mg protein.

Enzyme extraction and biochemical assays

For enzymatic activities, liver was homogenized in phosphate buffer. Homogenates were centrifuged at $10\,000 \times g$ for 20 min at 4 °C. The supernatant of each sample was employed for biochemical measurements.

Catalase (CAT) activity was assayed following the procedure of Aebi (1984). The sample (20 μL) was added to 980 μL of an assay buffer containing 50 mM Tris-HCl (pH 8.0), 9 mM H_2O_2 and 0.25 mM EDTA to constitute the assay volume of 1 mL. The decrease in ΔOD/min of that assay mixture was recorded at 240 nm for 1 min. The results were expressed as U/mg protein.

Glutathione-*S*-transferase (GST) activity was determined in fish liver cytosolic fractions as described by Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate in the presence of excess GSH (5 mM). The rate of CDNB conjugation was estimated by direct spectrophotometry at 340 nm for 3 min. The result was expressed as nM GS-CDNB formed/min/mg protein.

Superoxide dismutase (SOD) activity was assayed following the procedure of Ewing & Janero (1995) using nitroblue tetrazolium (NBT). The specific activity was expressed in the

units of SOD per mg of protein. One activity unit was defined as the amount of enzyme required for the inhibition of initial rate of NBT reduction by 50% and expressed as U/mg protein.

Real time reverse transcription-polymerase chain reaction (Q-PCR)

Total RNA was isolated from frozen liver tissue using the TRIzol reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. The concentration of total RNA was quantified by absorbance at 260 nm using a BECKMAN COULTER Spectrophotometer (DU 730). The purity of total RNA was assessed by determining the A260/A280 ratio. Five micrograms of total RNA was reverse-transcribed into cDNA using the Revert Aid reverse transcriptase (Fermentas) following manufacturers protocol. Quantitative real-time PCR was performed on a BioRad CFX Manager qPCR system. The 20 μL reaction mixture contained 10 μL of 2× iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), forward and reverse primers (0.1 μM each), nuclease-free water, and 1 μL of cDNA template. As a house-keeping gene, β-actin transcript was used. The mRNA level was expressed as its ratio to β-actin mRNA. PCR was performed in triplicate and primer sequences used in this technique is shown in Table 1. The gene-specific PCR primer sequences were either designed or taken from published report (Liedtke et al., 2008). The amplification protocol was as follows: 95 °C for 10 s followed by 40 cycles at 94 °C for 5 s, 59 °C for 15 s and 72 °C for 10 s. The relative expression ratio (R) was calculated based on the following equation: $R = 2^{-\Delta\Delta Ct}$, where C_t represents the cycle in which the fluorescence signal is significantly different from background and $\Delta\Delta C_t$ is $(C_{t,target} - C_{t,actin})$ treatment $- (C_{t,target} - C_{t,actin})$ control. R is a ratio between the expressions in the treated sample versus the expression in the control sample in comparison to the β-actin gene (Pfaffl, 2001).

Western blot analyses

Protein (60 μg) from the lysates of control and treated cells was resolved on 10% SDS-PAGE at a constant voltage (60 V) for 2.5 h and then transferred onto a poly vinylidene fluoride (PVDF) membrane using blot apparatus (Bio-Rad, Hercules, CA). The membranes were first incubated with primary antibodies at a dilution of 1:1000 overnight at 4 °C, followed by 2 h incubation with corresponding ALP-linked goat anti-rabbit (for Nrf2 and Keap1) or anti-mouse IgG secondary antibodies (for β-actin) at 1:5000 (Sigma) dilutions with continuous rocking. The immunoreactive bands were detected using 5-bromo-4chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT). Densitometric quantification was done using ImageJ (NIH, Bethesda, MD) software.

Table 1. Primer sets employed for quantitative Real Time PCR (5′–3′).

Target gene	Primer sequences	GenBank Accession No.
CYP1A*	F: CCTGGGCGGTTGTCTATCTA R: AGGTTCGCCCTGTCAGATAA	AB078927
Nrf2	F: TGGCCCTGAAGAATTTAACG R: CCCGGTGAGAAGCTCTGTAG	NM_182889.1
Keap1	F: TGATGGACAAACCCAACTCA R: CACTGGACAGGAAACCACCT	NM_182864.2
HO-1	F: GGAAGAGCTGGACAGAAACG R: CGAAGAAGTGCTCCAAGTCC	NM_001127516.1
NQO1	F: CCATGCTTTCCTTCACCACT R: CGCAGCACTCCATTCTGTAA	NM_001204272.1
β-Actin*	F: AGGTCATCACCATTGGCAAT R: GATGTCCACGTCGCACTTCAT	AF057040

*Sequences for CYP1A and β-actin were used from Liedtke et al. (2008).

Hoechst 33342 staining

Freshly collected hepatocyte from fish was digested by two-step collagenase (Sigma Aldrich) method as described by Shimano et al. (2003) with some modifications. Collected hepatocytes were washed with PBS, fixed with 3.7% paraformaldehyde solution at room temperature and stained with bisBenzimide H 33342 trihydrochloride (Hoechst 33342; 2 mg/ml) and visualized under fluorescence microscope (Dewinter, Italy) within 30 min of adding the stain.

Statistical analysis

In each group, triplicate sets of pooled samples of ten fish livers were used for biochemical analyses, RT-qPCR ($\Delta\Delta C_t$ values) and Western blots. All results were analyzed for significant differences ($^*p < 0.05$; $^{**}p < 0.001$) using a one-way analysis of variance (ANOVA) followed by the Dunnett's test using SPSS Statistics 20.0 (SPSS Inc., Chicago, IL). Densitometric analysis was carried out using ImageJ software and expressed as arbitrary units. The data were presented as means with standard errors of the mean (mean ± SE).

Results

Reactive oxygen species generation

The intracellular ROS generation was estimated using non-fluorescent stain (DCF-DA) which converted into florescent DCF due to oxidation by cellular oxidants. In our study, all the treated groups showed elevated levels of ROS which was significant at 15 mg NaF l^{-1} treatment for 30 days (Figure 1a).

Effect of NaF on tissue GSH and MDA levels

Following NaF treatment, the GSH level of liver in all the treatment groups decreased though not in a dose-dependent manner. A significant decrease (2.1-fold, $p < 0.001$) was noticed in 15 mg NaF l^{-1} for 30 days treatment group (Figure 1b) and a moderate decrease was observed after treatment with 15 mg NaF l^{-1} for 90 days. MDA level increased in all the treatment groups, recording a significant value (3.4- and 3.9-fold, $p < 0.001$) after treatment with 15 mg NaF l^{-1} for 30 and 90 days, respectively (Figure 1c).

Effect of NaF on enzyme activities

Figure 2(a) shows the activity of CAT in the control and treatment groups. Significant increase was observed in all the treatment groups (7.5, 15, 30 mg NaF l^{-1} for 30 days and 15 mg NaF l^{-1} for 90 days; 1.7-, 1.8-, 1.4- and 1.3-fold, respectively). A significant increase in GST activity was also observed after 30 days (15 and 30 mg NaF l^{-1}) and 90 days

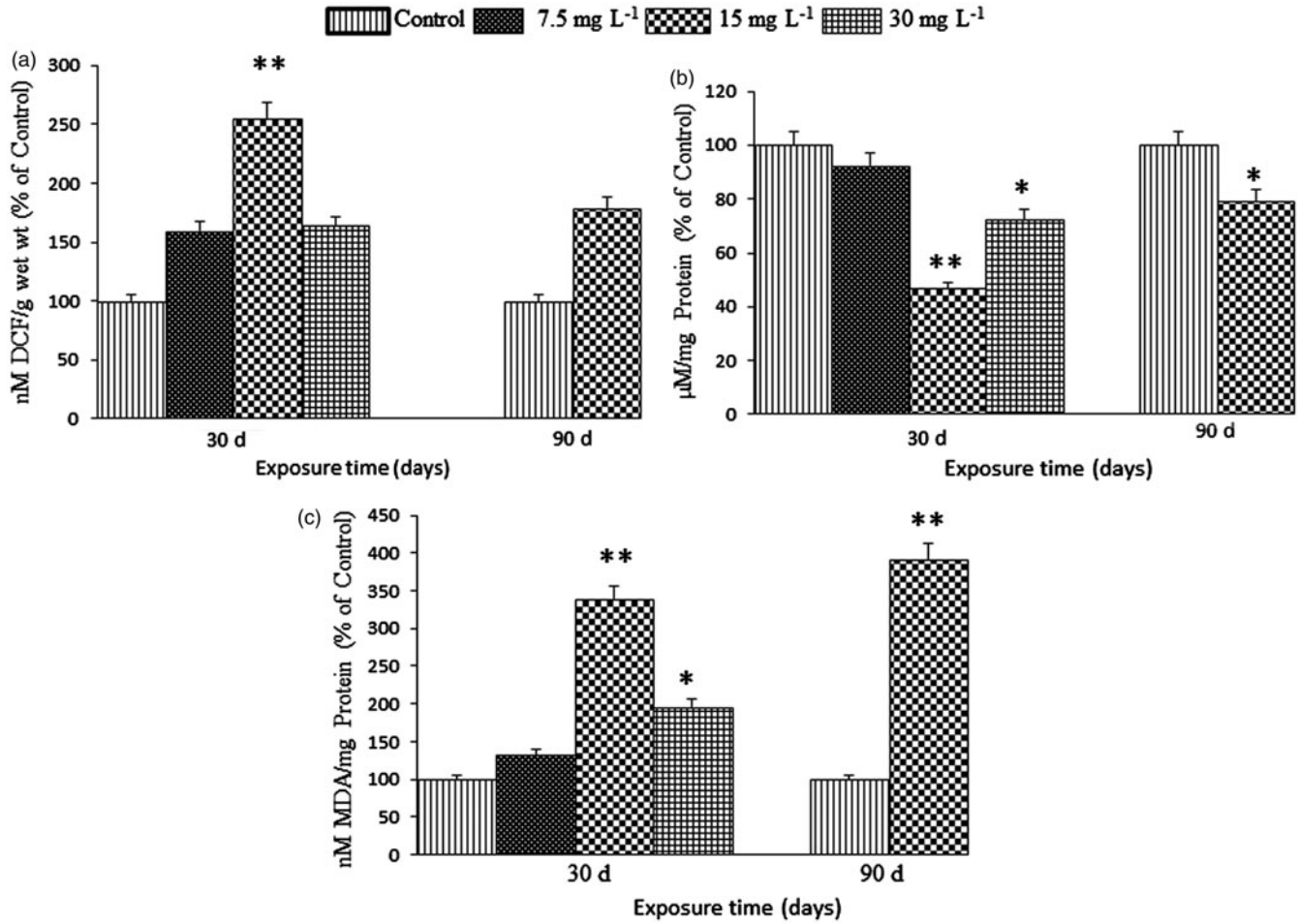

Figure 1. (a) ROS level (b) GSH content and (c) MDA production in the liver of different groups of fish ($n = 3$) exposed to different doses of NaF (mg l^{-1}) for 30 and 90 days. Values are expressed as mean ± SEM. Value is statistically significant at (*$p < 0.05$; **$p < 0.001$). d = days.

(15 mg NaF l^{-1}) treatment (1.7-, 1.5- and 1.6-fold, respectively) when compared to the control (Figure 2b). SOD activity increased in all the treatment groups. The increase was significant after treatment for 30 days but not after 90 days (Figure 2c).

Effect of NaF on gene expressions

Effect of NaF exposure on mRNA level of genes involved in *Nrf2/Keap1* pathway was determined by RT-qPCR (Figure 3). *Cyp1A* mRNA expression pattern increased in a dose dependent manner up to 30 mg NaF l^{-1} for 30 days treatment group (1.4-, 16.0- and 23.5-fold) but decreased in the 90 days treatment (15 mg NaF l^{-1}) group (1.2-fold; Figure 3a). The mRNA expressions after 15 and 30 mg NaF l^{-1} treatment for 30 days were significant ($p < 0.001$). This pattern was just opposite in case of *Ho-1* (Figure 3b) where the expression after only15 mg NaF l^{-1} for 90 days was significant ($p < 0.001$). *Nqo1* expression increased (2-, 3.8-, 2.4- and 2.6-fold) in all the treatment groups (Figure 3c) and was significant in the group treated with15 mg NaF l^{-1} for 90 days (3.8-fold, $p < 0.001$).

The mRNA level of *Nrf2* increased significantly only after 30 days treatment at the dose of 15 mg NaF l^{-1} (4.5-fold, $p < 0.001$) whereas *Keap1* significantly decreased (1.5-, 6.6-, 1.4- and 7.6-fold; $p < 0.05$) in all the treatment groups when compared to the control (C) (Figure 3d and e).

Effect of NaF on Nrf2 and Keap1 protein levels

Elevated expressions of Nrf2 protein was found in all the treatment groups compared to the control. Since the control group of 30 and 90 days had no differences in expression level, only one control group is represented in the Figure 4. Group treated with 15 mg NaF l^{-1} for 30 days had higher Nrf2 expression level than the rests in contrast to a consistent decrease in Keap1 level. This decrease was most prominent after 15 mg NaF l^{-1} treatment for 90 days. Alterations at protein level were not dose dependent.

Effect of NaF on chromatin condensation

Chromatin condensation was determined using Hoechst 33342 staining. Noticeable increase in the number of condensed and fragmented nuclei were observed In NaF (15 mg l^{-1} for 30 days) treatment group, where maximum cytotoxicity was observed (Figure 5).

Discussion

Besides having high similarity (~75%) with human genome, zebrafish is capable to tolerate soft water (Boisen et al., 2003; Craig et al., 2007). Consequently, it is a preferred model for *in vivo* screening of various toxic compounds and gene function analysis for medicinal applications (Alestrom et al., 2006; Hill et al., 2005). In India, zebrafish are found in the

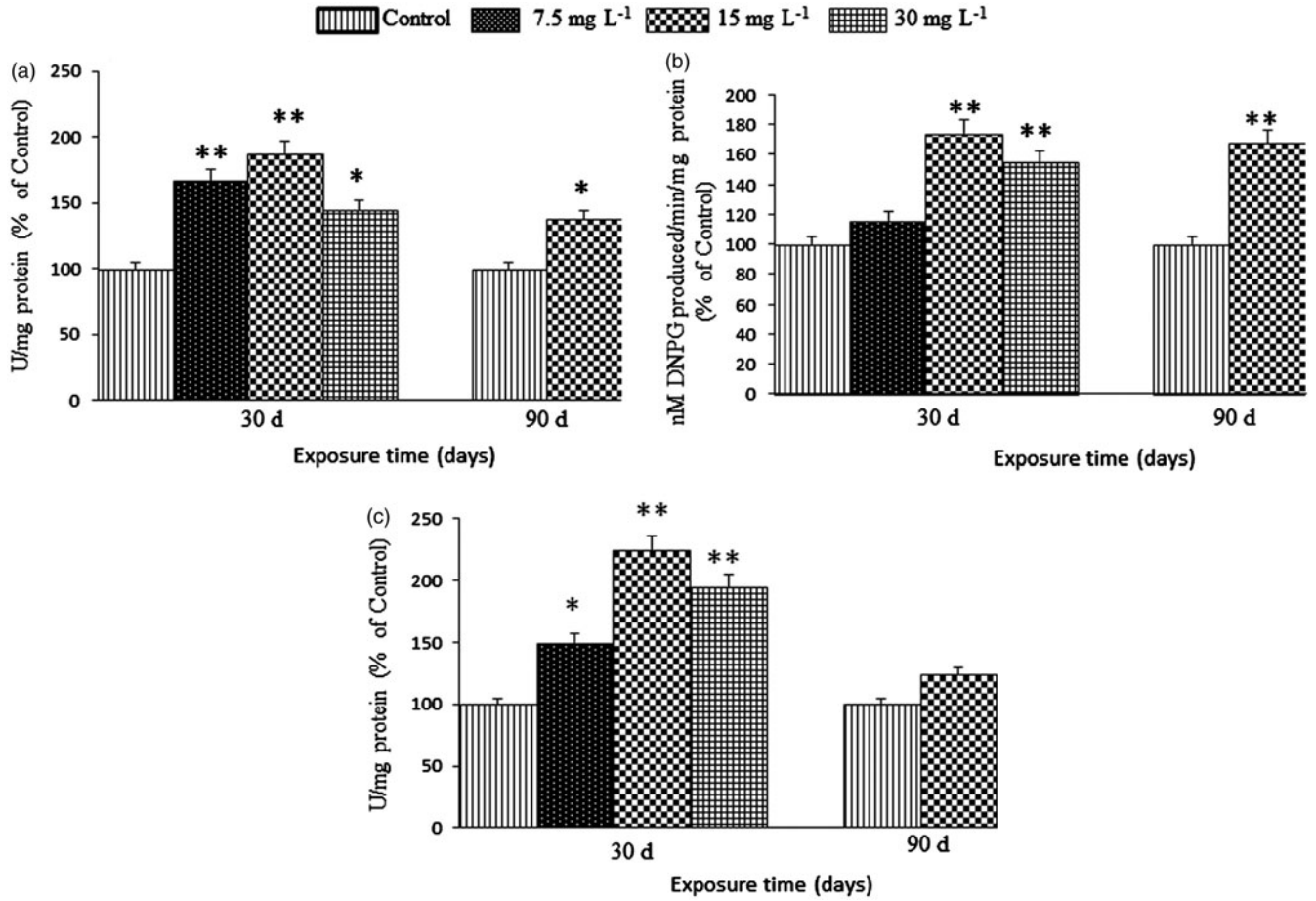

Figure 2. Activities of (a) CAT, (b) GST, (c) SOD in liver of female zebrafish ($n = 3$) exposed to NaF for 30 and 90 days. Asterisk (*) represents a statistically significant difference when compared to the controls; */** at $p < 0.05/0.001$ levels. d = days.

river Ganges flowing through the Indian subcontinent and exposed to a wide variety of xenobiotics including F.

Available literature suggests that F-concentration above $0.2\,mg\,l^{-1}$ is lethal to Salmon fish and also hinder its migration (Foulkes & Anderson, 1994). The 96 h LC_{50} value of NaF to fresh water fish *Puntius* was estimated as $126.12\,mg\,l^{-1}$ (Narwaria & Saksena, 2012). Therefore, the dose range used in this study fits well within the toxic level of F.

This study confirms that ROS generated by F, induce oxidative stress in zebrafish liver. ROS scavenging enzymes act to minimize the ROS induced damages. GSH is the major antioxidant and primary internal redox regulator during cellular oxidative stress. Any change in GSH level gives an indication of internal redox misbalance. Our results showed depletion of GSH level. On the other hand, elevation of MDA production due to lipid peroxidation was observed in all the treatment groups. Together, these data prove that generation of ROS is the major causal factor in fluoride toxicity. Besides this, ROS scavenging enzymes like CAT and SOD were also modulated in the treatment groups. In the redox regulating cascade, CAT is an important enzyme that catalyzes breakdown of hydrogen peroxide into oxygen and water. In this study, CAT activity increased in all the treatment groups to protect the organism from deleterious effect of H_2O_2. GST, a phase II enzyme is involved in detoxification of both endogenous substances and xenobiotics. It catalyzes GSH dependent conjugation to maintain redox homeostasis in an organism (Tang et al., 1998). It was observed that GST activity increased at 15 and $30\,mg\,l^{-1}$ NaF treatment. SOD, the endogenous scavenger, catalyzes dismutation of superoxide anion to hydrogen peroxide (Husain & Somani, 1998). Any alteration in the activity of the enzyme indicates internal redox misbalance. The results showed an elevated pattern of this enzyme. The depletion of GSH, elevation of MDA level, CAT, GST and SOD activities, misbalance of internal redox and generation of ROS is indicated during F-toxicity of fish. The generated ROS level corroborates our earlier study of DCFDA stained cells observed under fluorescence microscope (Mukhopadhyay & Chattopadhyay, 2014).

In order to correlate the ROS production and hepatotoxicity, the transcriptional effects of NaF on hepatic tissues were investigated. Though increasing numbers of researchers have focused on oxidative stress response in fish by targeting the changes in activities of antioxidant enzymes, the molecular basis of stress response is not yet fully understood. In general, oxidative stress activates the transcription of a number of antioxidant genes governed by key signaling pathways (Sen & Packer, 1996; Shi & Zhou, 2010).

Earlier we reported about the modulation of a number of antioxidant genes including *Gst, Cat* and *Sod*. It was observed that the mRNA levels of *Sod* and *Cat* did not correlate with their enzyme levels It was also observed that the mRNA expressions of *Cyp1a1, Gst, Hsp 70, Cat, Cu/ZnSod, MnSod*

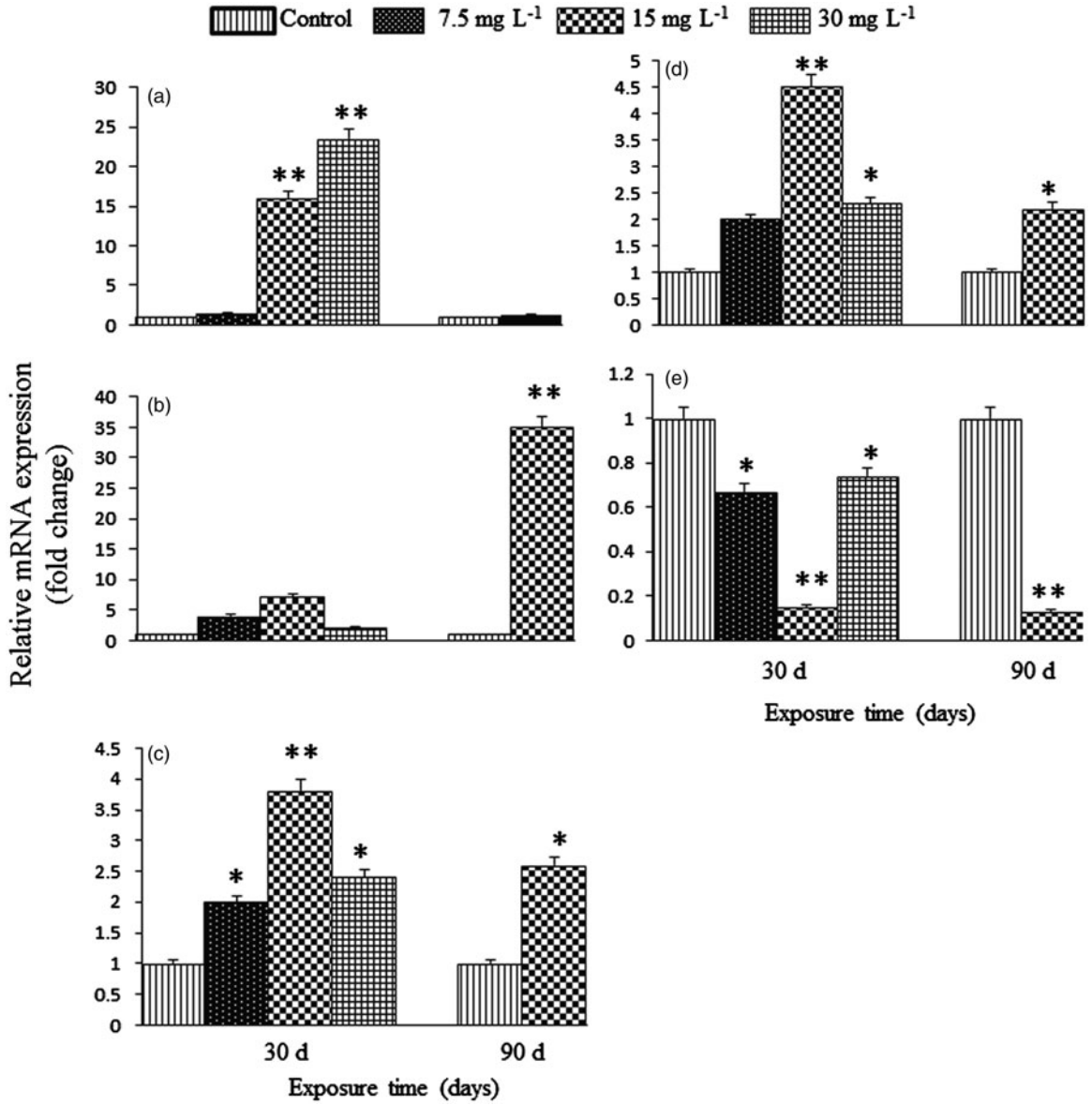

Figure 3. Real-time PCR analysis of hepatic mRNA expression levels of (a) *Cyp1A*, (b) *Ho-1*, (c) *Nqo1*, (d) *Nrf2* and (e) *Keap1* in female zebrafish of different NaF treatment groups. Gene expression levels represent the relative mRNA expression compared to the control. Values are presented as the mean ± SE of three different groups ($n = 3$) having 10 fish per group. Significance is indicated by */** for $p < 0.05/0.001$, respectively, compared to the control. d = days.

and Gpx increased after 15 mg NaF l^{-1} treatment for 30 days whereas that of *MnSod* and *Cat* showed moderate increase. The expression of Ucp2 decreased after 30 days which could be due to over-production of ROS (Mukhopadhyay & Chattopadhyay, 2014). Nrf2 is the key factor regulating the cellular oxidative stress response in majority of the cases (Kobayashi et al., 2004, 2009). Nrf2/Keap1 system is not only present in mammals but also in fish depicting that its role in cellular defense is conserved throughout the evolution of animal kingdom (Maher & Yamamoto, 2010). In zebrafish, a large number of antioxidant genes are identified which are solely influenced by Nrf2 (Craig et al., 2007; Liu et al., 2008). Therefore, in this study, we provide data for establishing the involvement of Nrf2/Keap1 pathway by studying their expression at mRNA and protein levels during fluoride induced stress condition. Under normal condition, Nrf2 is localized in the cytoplasm bound by Keap1 and Cullin 3 which can degrade Nrf2 by ubiqutination. In stressed condition, Nrf2 dissociates from Keap1 and translocates to the nuclei to activate several antioxidant genes (Jaiswal, 2004; Srivastava et al., 2013). In this study, mRNA level of Nrf2 showed a clear elevation in all the treatment groups followed by its protein level. Keap1 protein levels decreased in stressed condition but not at the transcriptional level. There may be several reasons for the absence of correlation. First, lack of unified post-transcriptional mechanism involved in turning mRNA to protein; second, proteins show different half lives at different time points and internal condition *in vivo* and third, sometimes protein as well as mRNAs give significant noise and error during experimental condition that prevents to get a clear cut conclusion between them (Baldi & Long, 2001; Szallasi, 1999). Reports are also available that the protein and mRNA levels of Nrf2 and Keap1 would increase in response to the oxidative stress induced by different chemicals (Buommino et al., 2012; Ho et al., 2005; Shi & Zhou, 2010; Srivastava et al., 2013). However, the activated Nrf2

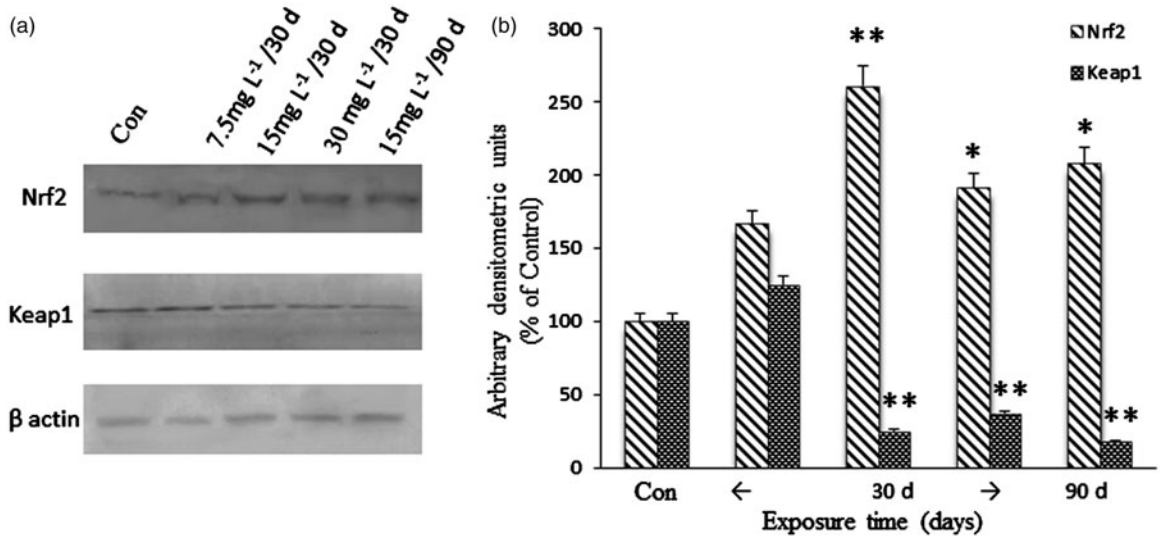

Figure 4. Changes in the expression levels of proteins in adult zebrafish ($n = 3$) liver tissue of control and treated groups. (a) A dose-dependent elevation of Nrf2 and Keap1 was evident in NaF-treated fish. Interestingly, the treatment caused a down regulation of Keap1 protein expression. β-actin served as protein loading control (b) A densitometry analysis of the bands was provided to mark the changes in the protein expressions in reference with that of control and asterisk (*) denotes the significant (*$p < 0.05$; **$p < 0.001$) level. Images are representative of three independent experiments. d = days.

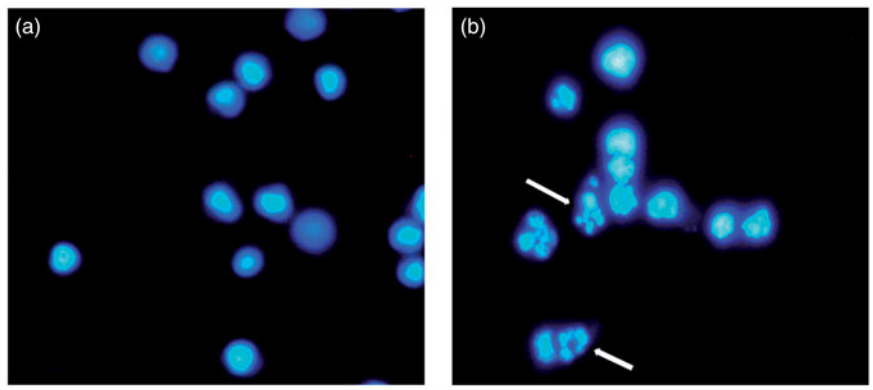

Figure 5. Fish hepatocytes showing condensed and fragmented nuclei (arrow) upon Hoechst 33342 staining after 15 mg NaF l^{-1} exposure for 30 days (b) with comparison to the control (a).

translocates to nuclei and in turn binds to ARE along with small molecule Maf to form a heterodimer (Kensler et al., 2007). ARE is present in the promoters of Nrf2 target genes which encodes both antioxidant and phase II detoxification enzymes. As the main target of Nrf2/Keap1/ARE pathway, the transcription patterns of *Nqo1* and *Ho-1* as well as its effect on Phase I detoxifying enzyme Cytochrome P450 1 A (*Cyp1A)* have been investigated to understand the interrelationship of both kinds of detoxification system. NQO1, a cytosolic flavoprotein, is constitutively expressed in different tissues and cell types (Aleksunes & Manautou, 2007; Zhu et al., 2007). In addition to drug metabolism, it catalyzes reduction and detoxification of highly reactive quinones that can cause redox cycling and oxidative stress (Siegel et al., 2004). Our data showed steady elevation of mRNA level of *Nqo1* in treated groups which corroborates previous reports with a variety of inducers (El Gendy et al., 2012; Ishii et al., 2002; Liu et al., 2011). Heme oxygenase-1 (HO-1) is an enzyme that catalyzes the breakdown of heme into antioxidant biliverdin, CO and iron. Our results showed that HO-1 initially remained at its basal level but elevated significantly after prolonged treatment (90 days) with 15 mg l^{-1} NaF. This pattern is just opposite in case of *Cyp1A* mRNA expression. Published data also showed the inverse relation between HO-1 and CYP1A expression but the causality is not clear yet (Abraham et al., 2000). This could be due to breakdown of cellular heme pool by Ho-1 that ultimately leads to down-regulation of Cyp1A at transcriptional level.

Fluoride has the tendency to bioaccumulate in the bone or exoskeleton of fish (Julshamn et al., 2004). The aquatic environment is the ultimate sink of different pollutants including NaF and other F compounds. Therefore, it is important to understand the likely impact and molecular mechanism of F^- toxicity in aquatic organisms including fish. In this study, hepatotoxicity induced by chronic exposure of F to female zebrafish was evaluated by estimation of ROS generation, induction of oxidative stress along with hepatocyte damage, transcriptional effects and western blotting. It was also elucidated that NaF can modulate the Nrf2/Keap1 signaling pathway. To the best of our knowledge, this is the first report on the expression of Nrf2 and its regulation on XME-genes related to phase I and phase II detoxification

processes of F in zebrafish *in vivo*. It should be also noted that all the genes and enzymes, employed for this study, could be used as sensitive and reliable biomarkers for environmental risk assessment in F-polluted aquatic systems.

Conclusion

NaF induced ROS could modulate the transcription of genes encoding both phase I and phase II XMEs as well as the expression of *Nrf2/Keap1*genes in the liver of female zebrafish. The understanding of Nrf2/Keap1 signaling events in response to NaF may provide a future perspective on cellular defenses against oxidative damages that may be important in combating F induced toxicity *in vivo*. Our findings provided, for the first time, the expression pattern of XMEs genes related to Nrf2–Keap1 signaling pathway in the zebrafish liver due to F toxicity at the dose range relevant to aquatic environment. Therefore, the information of this study will help to use these toxicology biomarkers for assessing F-toxicity in fish and environmental risk assessment.

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Declaration of interest

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