

Sodium fluoride causes oxidative stress and apoptosis in the mouse liver

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ABSTRACT

The current study was conducted to investigate the effect of sodium fluoride (NaF) on the oxidative stress and apoptosis as well as their relationship in the mouse liver by using methods of flow cytometry, quantitative real-time polymerase chain reaction (qRT-PCR), western blot, biochemistry and experimental pathology. 240 four-week-old ICR mice were randomly divided into 4 groups and exposed to different concentration of NaF (0 mg/kg, 12 mg/kg, 24 mg/kg and 48 mg/kg) for a period of 42 days. The results showed that NaF caused oxidative stress and apoptosis. NaF-caused oxidative stress was accompanied by increasing reactive oxygen species (ROS) and malondialdehyde (MDA) levels, and decreasing mRNA expression levels and activities of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GSH-PX) and glutathione-s-transferase (GST). NaF induced apoptosis via tumor necrosis factor receptor-1 (TNF-R1) signaling pathway, which was characterized by significantly increasing mRNA and protein expression levels of TNF-R1, Fas associated death domain (FADD), TNFR-associated death domain (TRADD), cysteine aspartate specific protease-8 (caspase-8) and cysteine aspartate specific protease-3 (caspase-3) in dose- and time-dependent manner. Oxidative stress is involved in the process of apoptotic occurrence, and can be triggered by promoting ROS production and reducing antioxidant function. NaF-caused oxidative stress and apoptosis finally impaired hepatic function, which was strongly supported by the histopathological lesions and increased serum alanine amino transferase (ALT), aspartic acid transferase (AST), alkaline phosphatase (AKP) activities and TBIL contents.

INTRODUCTION

Fluoride distributes very extensively in the natural environment, and is widely used among industry, agriculture as well as medicine [1]. Fluorine is one of the essential trace elements for human body [2]. Moderate levels of fluorine or fluoride ingestion can decrease the incidence of dental caries and promote the development of bones, but there are a number of adverse

effects on human health with fluorine or fluoride chronic ingestion at high doses [3]. The main pathway of fluoride poisoning is through drinking water, and high concentration fluoride is often associated with soft, alkaline, and calcium-deficient waters [4]. Other pathways of fluorine- or fluoride-entered body include food, industrial exposure, drugs, cosmetics, etc [5]. Fluoride toxicity targets to not only bone and teeth, but also soft tissues [6]. Previous studies have proved that

fluorine can induce genotoxicity, cytotoxicity, immunotoxicity, oxidative damage, apoptosis and lesions in the broiler peripheral blood [7-10], liver [11,12], kidney [13-15], thymus [16], spleen [17-19], bursa of Fabricius [20], cecal tonsil [21-25], and intestine [26-30], and in the mouse spleen and kidney [31-35].

Reactive oxygen species (ROS), as a byproduct of the metabolic process, can be scavenged by many anti-oxidative defense components under normal condition [36]. Imbalance between ROS and antioxidants is referred to as oxidative stress [37]. Fluoride is known to be an inhibitor of the antioxidant enzymes, which in turn promote the accumulation of ROS [38, 39]. NaF-altered ROS production levels and antioxidative parameters have been systematically observed in the mouse kidney [35]. Reports have indicated that fluoride exposure can induce oxidative stress in liver [12, 40, 41], kidney [35, 41], testicle [42], spleen [17], brain [43], heart [44] and cecal tonsil [25], and reduce the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX) and glutathione-s-transferase (GST) in the liver of broiler, fish, rabbit and rat [12, 40, 41, 45]. Zhou et al. has also found that fluoride can induce oxidative stress in the liver of female mice after 70 days of fluoride treatment [46]. Although there are reports on the relationship between fluoride and oxidative stress, very limited systematic studies are focused on the molecular mechanism of NaF-induced hepatic oxidative stress in mice.

It is well known that the ROS and oxidative stress can work as the inducer of apoptosis [47-49]. Apoptosis is an essential physiological process that plays a critical role in regulating growth and immune response via gene and/or protein expression [32]. Fluorine- or fluoride-induced apoptosis has been reported in vivo [11, 16, 20, 24, 33, 47-49]. Researches in liver have showed that fluoride increases caspase-3, caspase-8, caspase-9 and bax protein expression levels, and reduces the bcl-2 protein expression [50-52]. Death receptor pathway is one of the main apoptosis signal pathways [53], which belongs to extrinsic apoptosis pathway. Extrinsic apoptosis can be initiated by the binding of lethal ligands to various death receptors. For example, FasL binds to Fas, TNF α -related apoptosis-inducing ligand (TRAIL) can bind to TRAIL receptor-1/2 (TRAIL-R1/2), and TNF- α can interact with TNF-R1 and TNF-R2 [54]. Recent studies have indicated that fluoride can induce apoptosis in the liver, but there are no reports on the relationship between fluoride-induced hepatocyte apoptosis and death receptor pathway at present.

Based on the above-mentioned references, very limited

systematic reports are focused on the relationship between fluoride-induced oxidative stress and apoptosis in the mouse liver. Therefore, this study was conducted to observe the possible mechanism of NaF-induced oxidative stress and apoptosis as well as the relationship between NaF-induced oxidative damage and apoptosis by detecting ROS levels and MDA contents, and mRNA expression levels and activities of SOD, CAT, GSH, GSH-PX, GST as well as the mRNA and protein expression levels of tumor necrosis factor receptor-1 (TNF-R1), tumor necrosis factor receptor-2 (TNF-R2), Fas associated death domain (FADD), TNFR-associated death domain (TRADD), caspase-8 and caspase-3 using methods of flow cytometry, quantitative real-time polymerase chain reaction (qRT-PCR), western blot, biochemistry and experimental pathology.

RESULTS

Histopathological lesions in the liver

NaF resulted in histopathological lesions in a dose- and time-dependent manner. Lesions included hepatocellular granular degeneration, vacuolar degeneration and necrosis. In the granular and vacuolar degenerated hepatocytes, tiny particles and small or large vacuoles were appeared in the cytoplasm (Figure 1). Karyorrhexis, karyo-lysis and hypochromatosis were appeared in the necrotic hepatocytes (Figure 2). The above lesions were not observed in the control group.

Changes of hepatic functional parameters

Figure 3 showed that ALT, AST and AKP activities, and TBIL contents were increased ($p < 0.05$ or $p < 0.01$) in the 12, 24, 48 mg/kg groups when compared with those in the control group at 21 and 42 days of the experiment.

Changes of oxidative damage parameters in the liver

The MDA contents were significantly elevated ($p < 0.05$ or $p < 0.01$) in the 12, 24, 48 mg/kg groups in comparison with those in the control group at 21 and 42 days of the experiment. The SOD and GSH-PX activities were significantly lower ($p < 0.05$ or $p < 0.01$) in the 24, 48 mg/kg groups at 21 days of the experiment and in the 12, 24, 48 mg/kg groups at 42 days of the experiment than those in the control group. The GSH contents and the GST activities were decreased ($p < 0.05$ or $p < 0.01$) in the three NaF-treated groups at 21 and 42 days of the experiment. The CAT activities were obviously declined ($p < 0.05$) only in the 48mg/kg group at 42 days of the experiment. The results were shown in Figure 4.

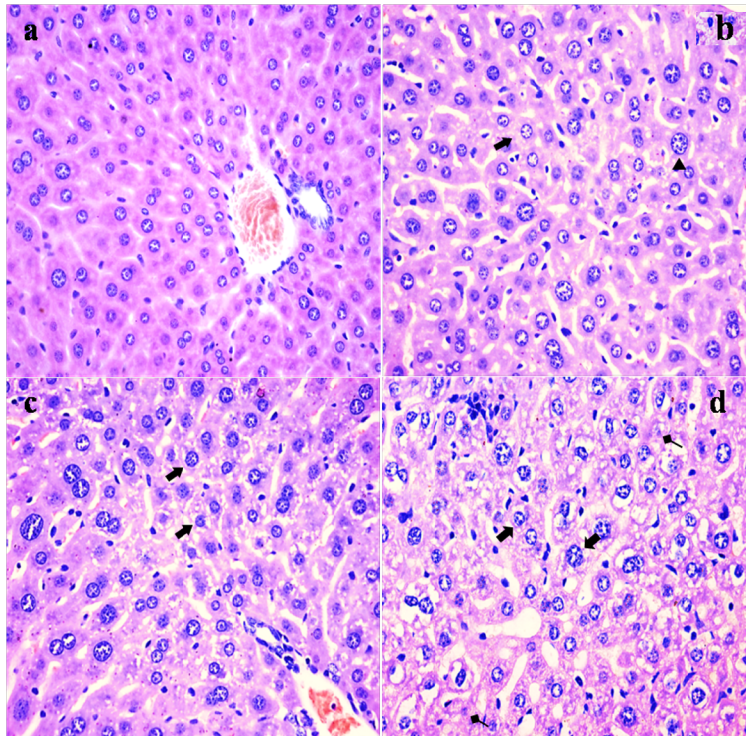


Figure 1. Histopathological changes in the liver at 21 days of the experiment. (a) The control group (H&E \times 400). (b) The 12 mg/kg group. Hepatocytes are swelled (\blacktriangle) and show slight granular and vacuolar degeneration (\blacktriangledown , H&E \times 400). (c) The 24 mg/kg group. Hepatocytes show granular and vacuolar degeneration (\blacktriangledown , H&E \times 400). (d) The 48 mg/kg group. Hepatocytes show obvious granular and vacuolar degeneration (\blacktriangledown). Necrotic hepatic cells (\blacktriangledown) are observed (H&E \times 400).

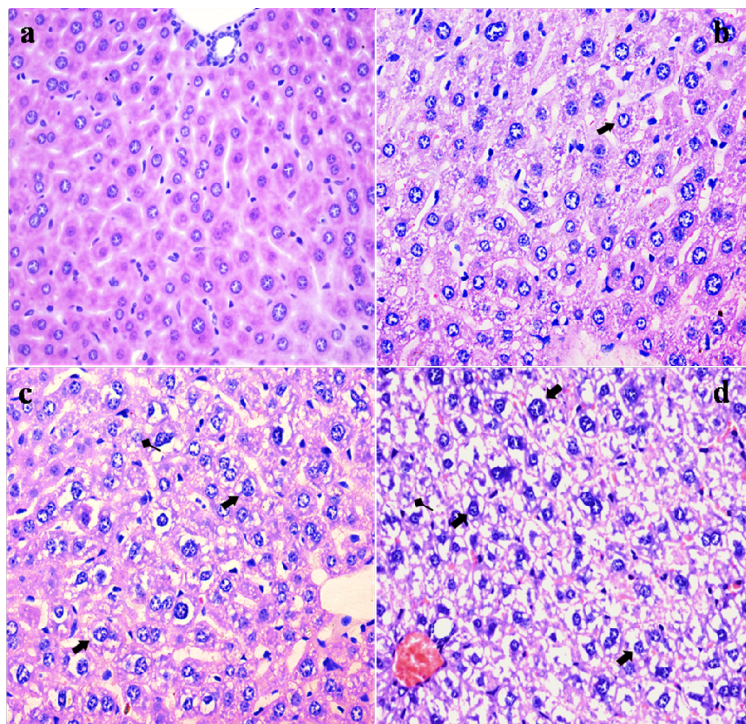


Figure 2. Histopathological changes in the liver at 42 days of the experiment. (a) The control group (H&E \times 400). (b) The 12 mg/kg group. Hepatocytes show granular and vacuolar degeneration (\blacktriangledown , H&E \times 400). (c) The 24 mg/kg group. Hepatocytes show obvious granular and vacuolar degeneration (\blacktriangledown). Also, necrotic hepatocytes are observed (\blacktriangledown , H&E \times 400). (d) The 48 mg/kg group. Hepatocytes show marked vacuolar degeneration (\blacktriangledown). Necrotic hepatocytes are observed (\blacktriangledown). And hepatic cords are disorganized or disappeared (H&E \times 400).

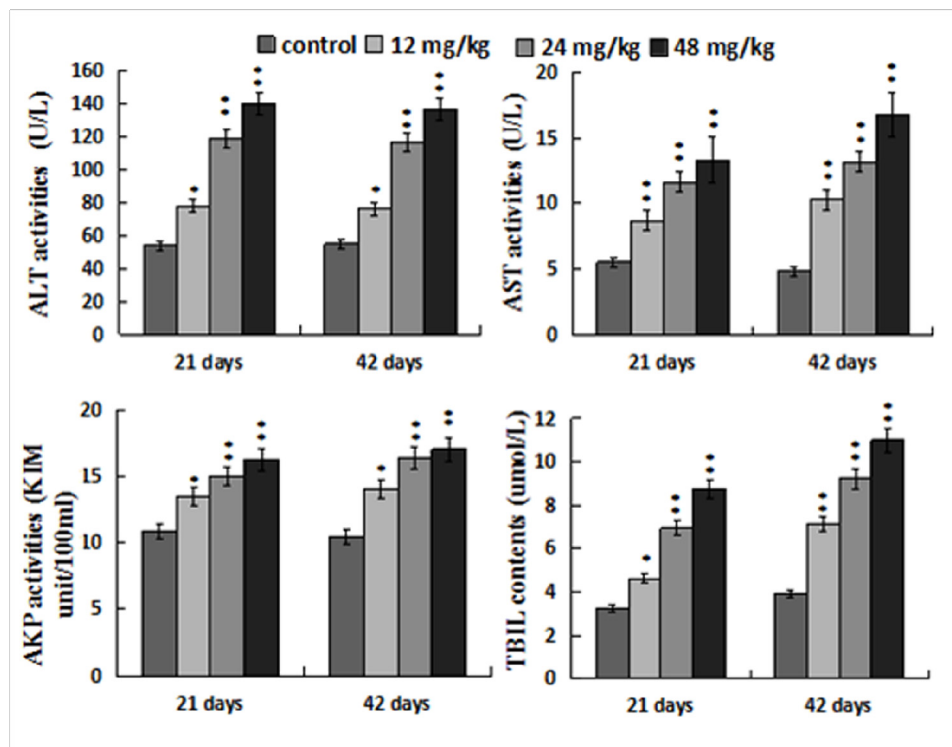


Figure 3. Changes of the serum ALT, AST, AKP activities and TBIL contents. Data are presented with the mean \pm standard deviation (n=8). * $p < 0.05$, compared with the control group; ** $p < 0.01$, compared with the control group.

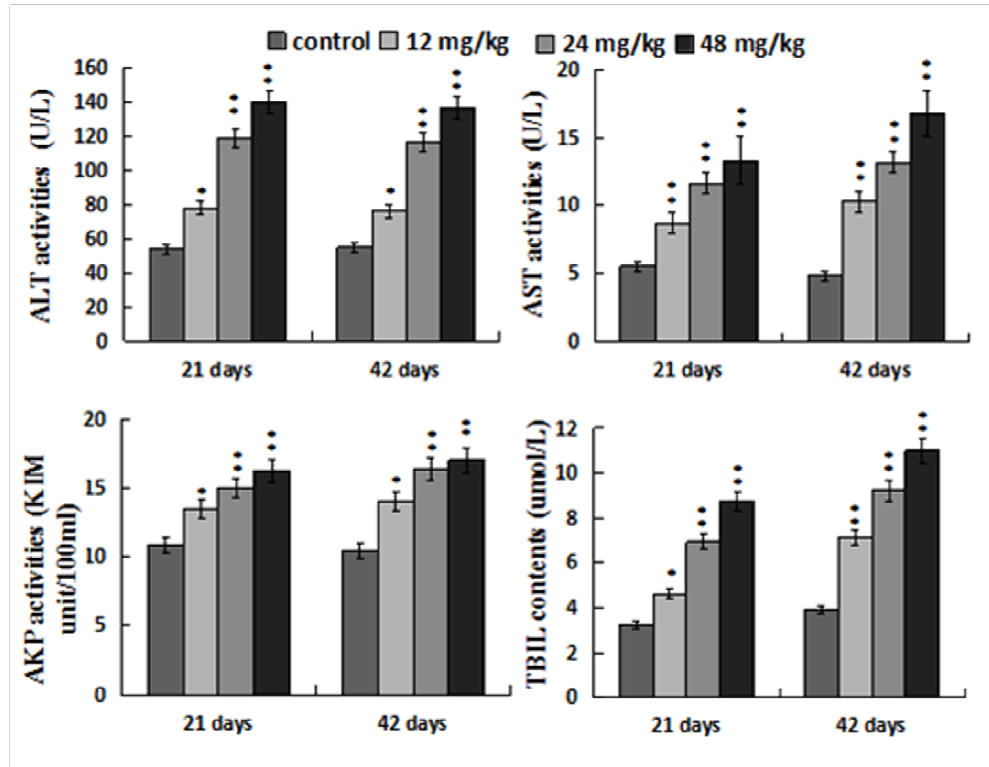


Figure 4. Changes of oxidative damage experiment parameters in the liver. Data are presented with the mean \pm standard deviation (n=8). * $p < 0.05$, compared with the control group; ** $p < 0.01$, compared with the control group.

Changes of antioxidant enzyme mRNA expression levels in the liver

The GST mRNA expression levels were decreased ($p < 0.05$ or $p < 0.01$) in the three NaF-treated groups at 21 and 42 days of the experiment. The GSH-Px mRNA expression levels were lower ($p < 0.05$) in the 48mg/kg group at 21 days of the experiment and in the 24, 48mg/kg groups at 42 days of the experiment than those in the control group. The Mn-SOD mRNA expression

levels were decreased in the 24, 48mg/kg groups at 42 days of the experiment, and the CuZn-SOD mRNA expression was decreased ($p < 0.05$ or $p < 0.01$) in the 12, 24, 48 mg/kg groups at 21 and 42 days of the experiment except in the 12mg/kg group at 21 days of the experiment when compared with those in the control group. The CAT mRNA expression levels were lower ($p < 0.05$) only in the 48 mg/kg group than those in the control group at 42 days of the experiment. The results were shown in Figure 5.

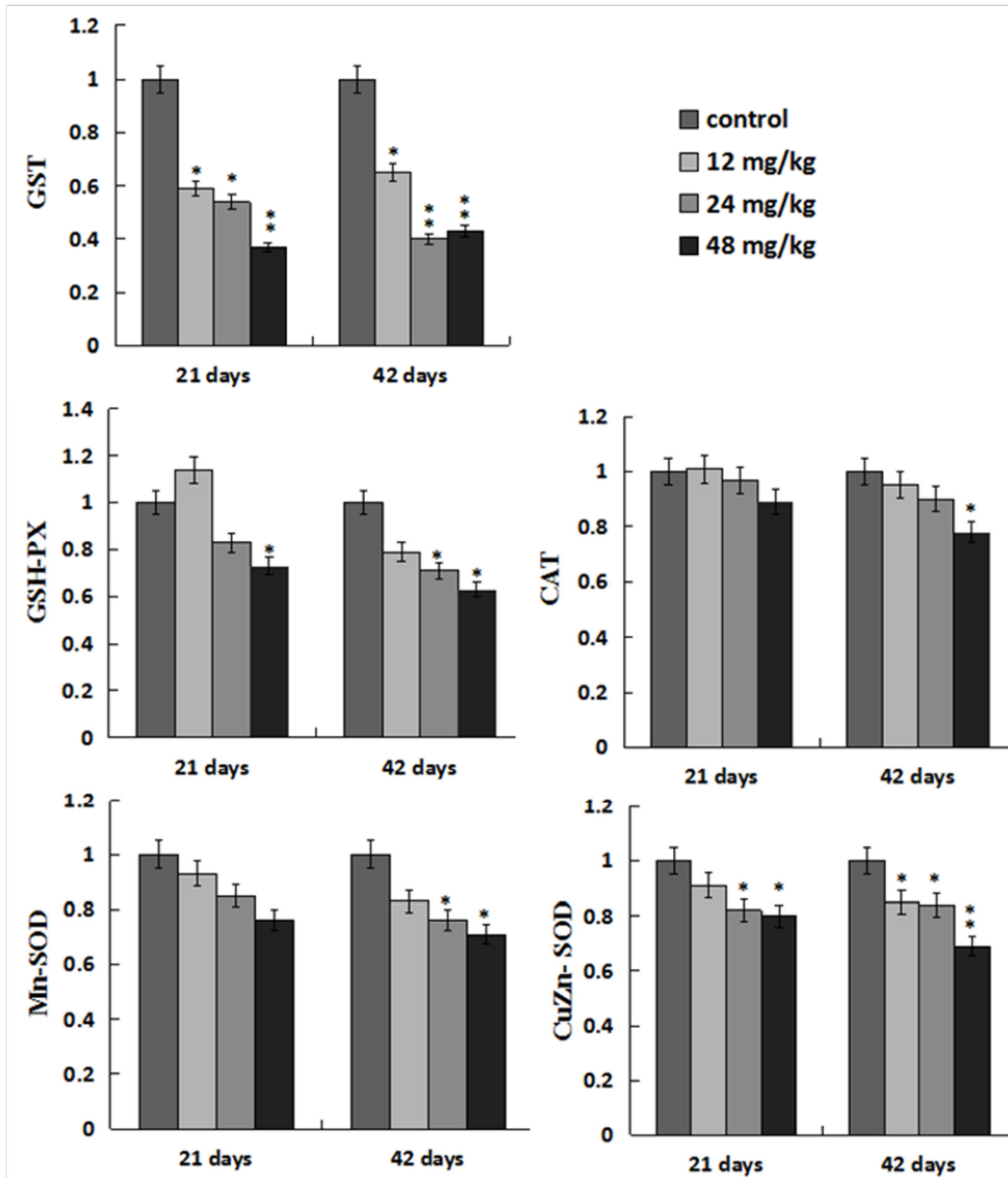


Figure 5. Changes of antioxidant enzymes mRNA expression levels in the liver. Data are presented with the mean \pm standard deviation ($n=8$). * $p < 0.05$, compared with the control group; ** $p < 0.01$, compared with the control group.

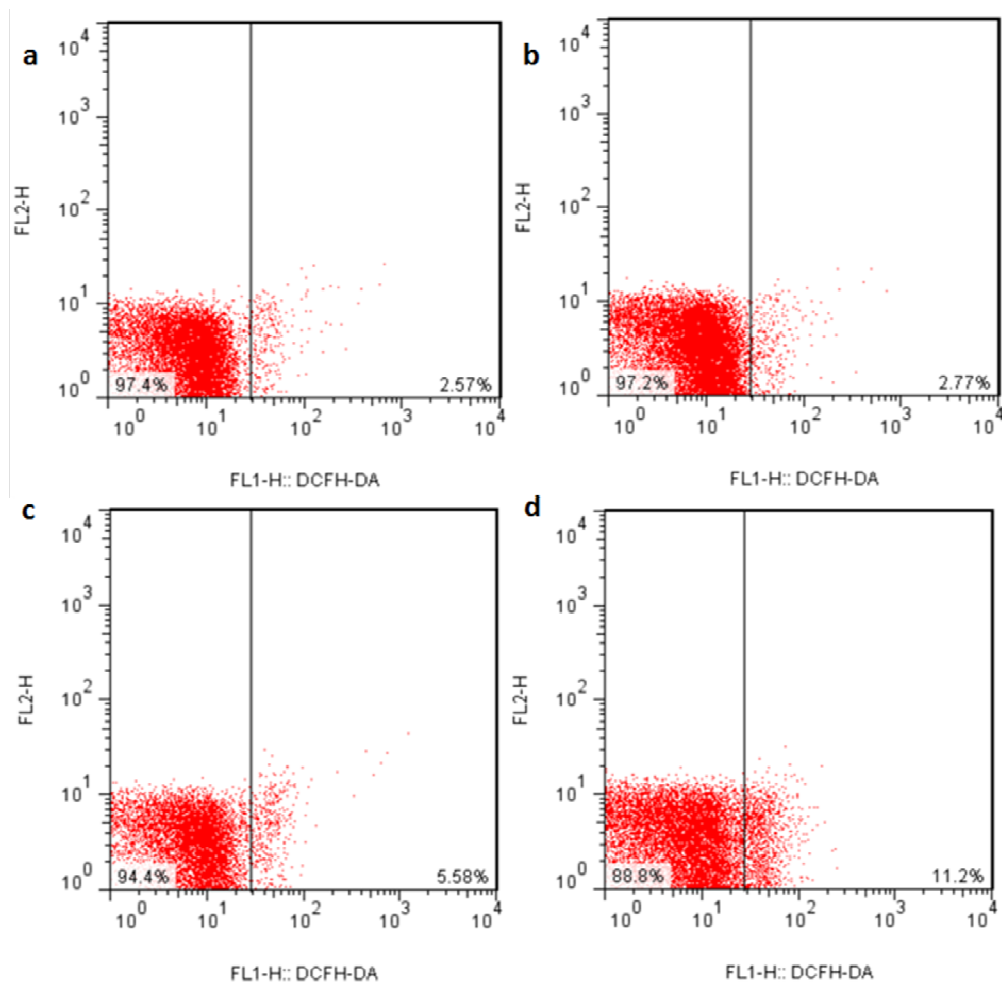


Figure 6. ROS production levels in the liver at 21 days of the experiment. Control group (a), 12mg/kg (b), 24mg/kg (c), 48mg/kg (d).

Changes of ROS production levels in the liver

The ROS production levels were significantly increased ($p < 0.05$ or $p < 0.01$) in the three NaF-treated groups at 21 and 42 days of the experiment except in the 12 mg/kg group at 21days of the experiment when compared with those in the control group. The results were shown in Figures 6, 7 and 8.

Changes of apoptosis percentages in the liver

The apoptotic percentages were significantly increased ($p < 0.05$ or $p < 0.01$) in the three NaF-treated groups at 21 and 42 days of the experiment in comparison with those in the control group. The results were shown in Figures 9, 10 and 11.

Changes of mRNA expression levels of parameters associated to death receptor pathway in the liver

The caspase-3 mRNA expression levels were higher ($p < 0.05$) in the 48mg/kg group at 21 days of the experiment and were significantly higher ($p < 0.05$ or $p < 0.01$) in the 12, 24, 48 mg/kg groups at 42 days of the experiment than those in the control group. The caspase-8 and TNF-R1 mRNA expression levels were increased ($p < 0.05$ or $p < 0.01$) in the 12, 24, 48 mg/kg groups at 21and 42 days of the experiment except in the 12 mg/kg group at 21 days of the experiment. However, the TNF-R2 mRNA expression levels were not obviously changed when compared with those in the control group. The FADD mRNA expression levels were elevated ($p < 0.05$) in the 24, 48mg/kg groups at 21 and 42 days of the experiment.

The TRADD mRNA expression levels were higher ($p < 0.05$ or $p < 0.01$) in the 12, 24, 48 mg/kg groups at 21 and

42 days of the experiment than those in the control group. The results were shown in Figure 12.

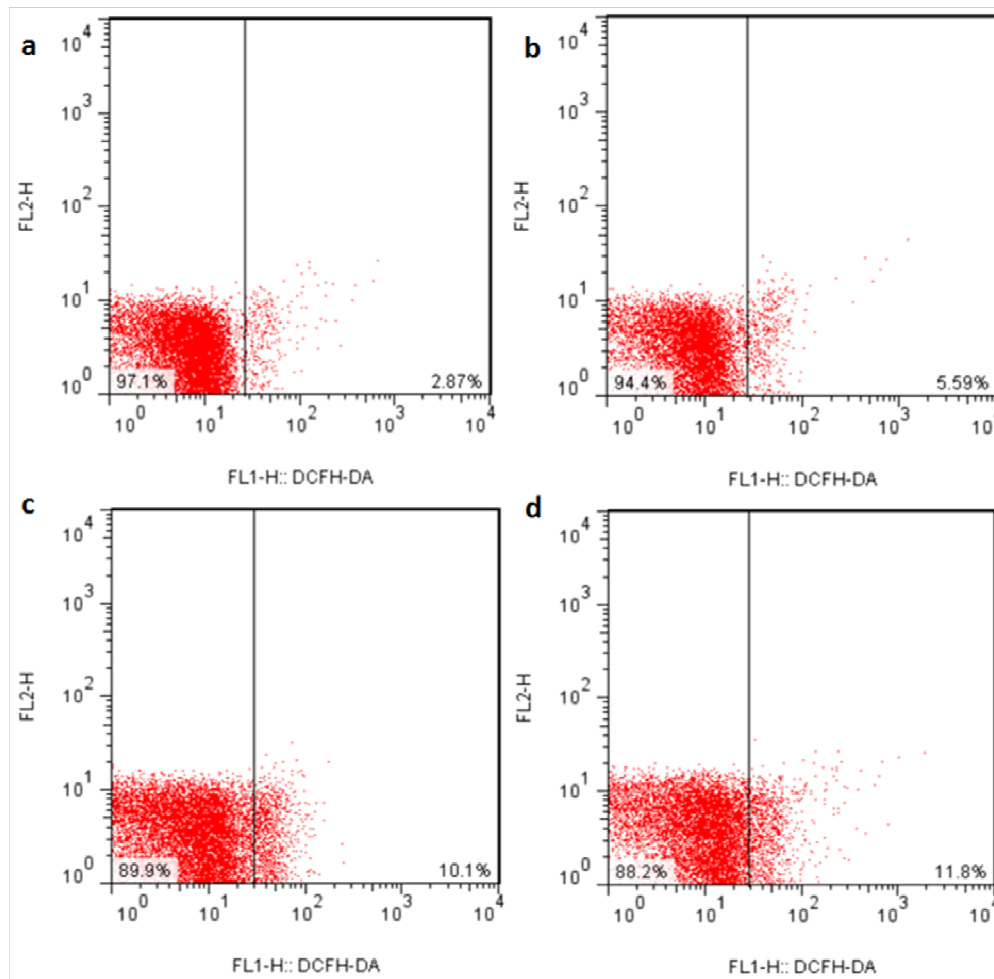


Figure 7. ROS production levels in the liver at 42 days of the experiment. Control group (a), 12mg/kg (b), 24mg/kg (c), 48mg/kg (d).

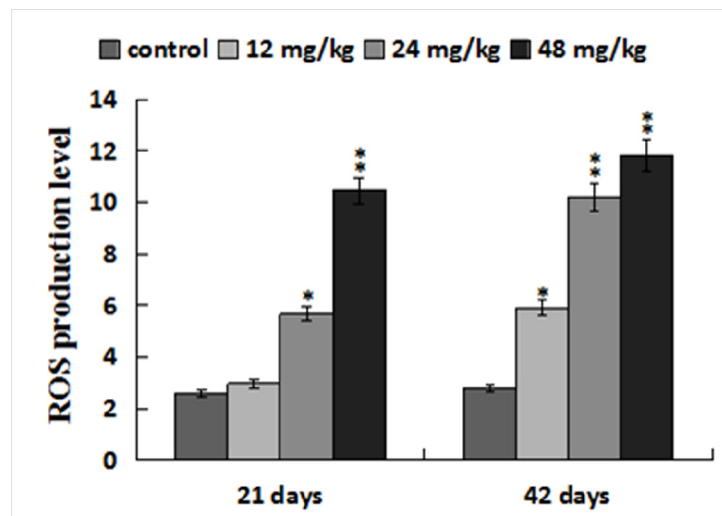


Figure 8. ROS production levels in the liver. Data are presented with the mean \pm standard deviation (n=8). * $p < 0.05$, compared with the control group; ** $p < 0.01$, compared with the control group.

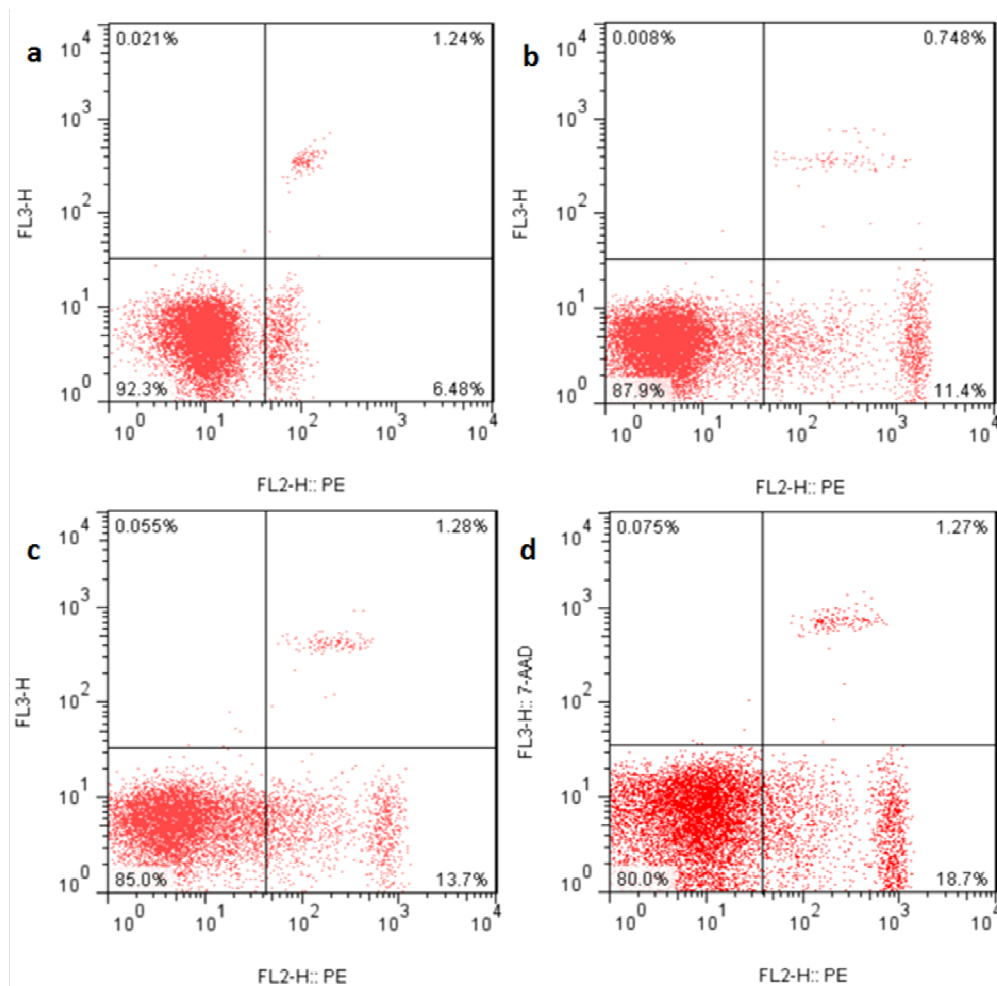


Figure 9. Apoptosis in the liver at 21 days of the experiment. Control group (a), 12mg/kg (b), 24mg/kg (c), 48mg/kg (d).

Changes of protein expression levels of parameters associated to death receptor pathway in the liver

The protein levels of cleaved caspase-8 and TRADD were significantly increased ($p < 0.05$ or $p < 0.01$) in the 12, 24, 48 mg/kg groups at 21 and 42 days of the experiment when compared with those in the control group. At the same time, the protein levels of TNF-R1 and FADD were higher ($p < 0.05$ or $p < 0.01$) in the 48 mg/kg group at 21 days of the experiment and in the 12, 24, 48 mg/kg groups at 42 days of the experiment than these in the control group. The cleaved caspase-3 protein levels were increased ($p < 0.05$ or $p < 0.01$) in the three NaF-treated groups at 21 and 42 days of the experiment except in the 12 mg/kg group at 21 days of the experiment. However, the TNF-R2 protein expression levels were not obviously changed. The results were shown in Figures 13 and 14.

DISCUSSION

This study defines the NaF-induced hepatic oxidative stress and apoptosis as well as their relationship. Also, NaF-induced apoptosis through TNF-R1 signal pathway has been firstly reported in the liver at present.

It is well known that fluoride toxicity is associated with ROS induction [55]. In the present study, the results have proved that NaF can enhance hepatocellular ROS production levels in a time- and dose-dependent manner. Excessive ROS production can lead to lipid peroxidation [56], and MDA is the important indicator of lipid peroxidation [57, 58]. Our results in this study showed that MDA contents were increased in the liver of NaF-treated groups, which were consistent with the increased ROS production levels. The imbalance between ROS and the antioxidants can cause oxidative stress. The increased ROS levels may indicate the

reduction of cellular antioxidant defenses. The current study showed that NaF decreased activities of antioxi-

dant enzymes (SOD, CAT, GST and GSH-PX), and GSH contents in the liver, which were in line with our

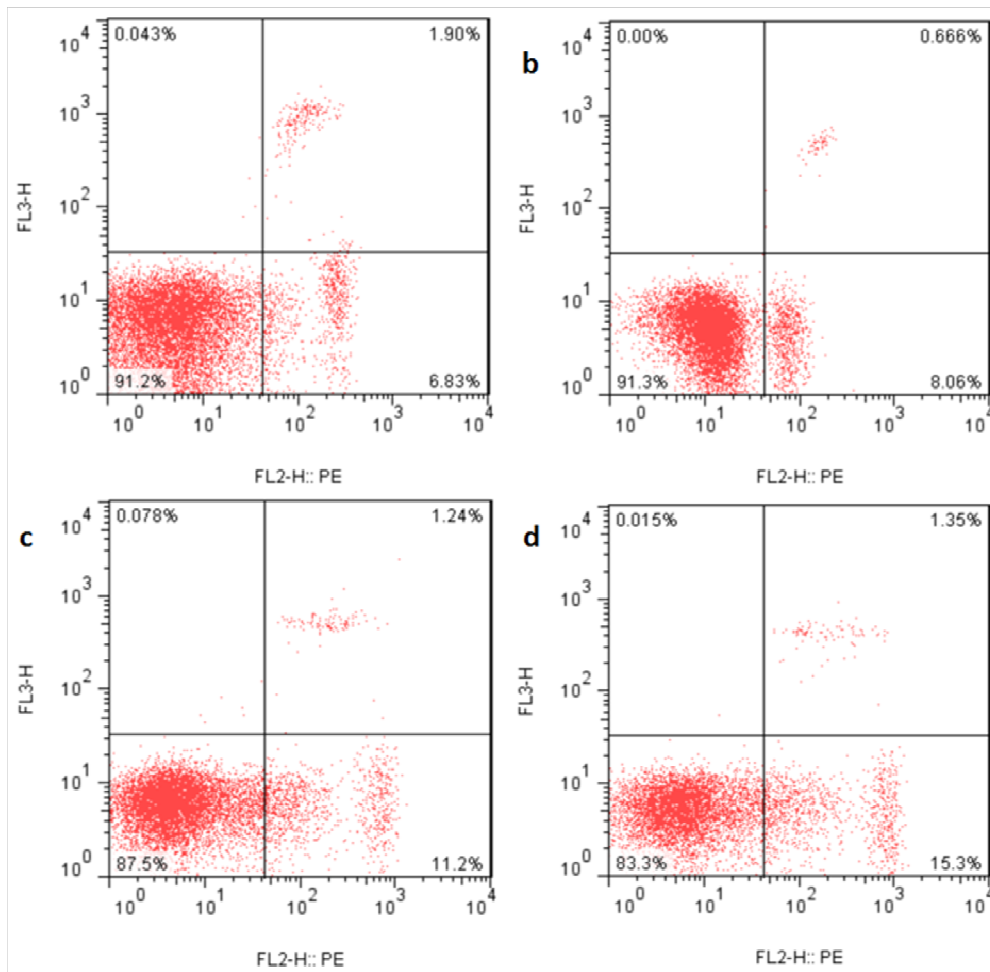


Figure 10. Apoptosis in the liver at 42 days of the experiment. Control group (a), 12mg/kg (b), 24mg/kg (c), 48mg/kg (d).

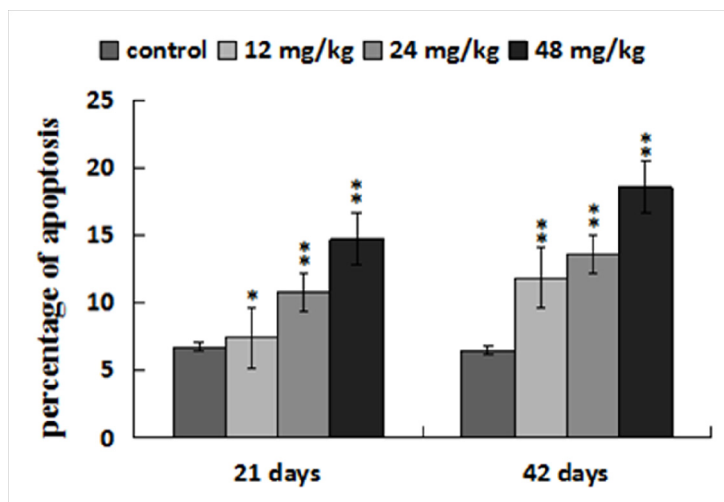


Figure 11. Percentage of apoptosis in the liver. Data are presented with the mean \pm standard deviation (n=8). * $p < 0.05$, compared with the control group; ** $p < 0.01$, compared with the control group.

earlier studies on the effect of NaF on the antioxidant enzymes [12, 16, 27, 34]. SOD and CAT are important antioxidant enzymes, playing a major role in ROS elimination [59]. Non-enzymatic scavengers such as GSH are also involved in scavenging ROS, and the GSH dysfunction could aggravate the organ injury [60]. GSH-PX can promote the reaction between GSH and H₂O₂ in order to achieve the purpose of eliminating peroxide [61]. Therefore, the decreased GSH-PX activi-

ties caused by NaF in this study are closely correlated to the reduction of GSH contents. To show the molecular basis of the changes of antioxidant enzyme activities, the mRNA expression levels of CuZn-SOD, Mn-SOD, GST, CAT, GSH-Px were detected in the present study. The results showed that these antioxidant enzyme mRNA expression levels were increased in the NaF-treated groups, which were consistent with the reduction of their activities. The above results clearly indicated

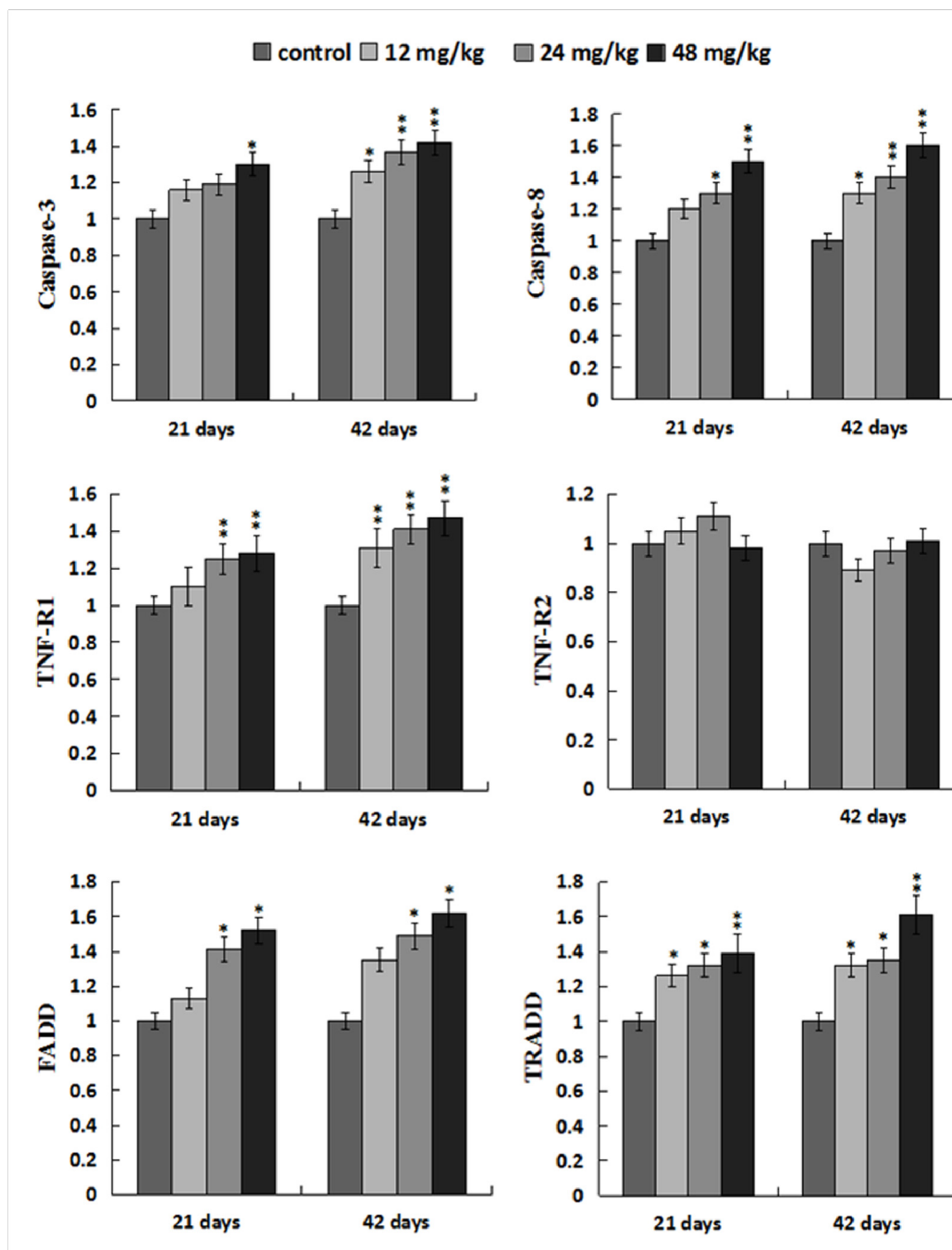


Figure 12. Changes of mRNA expression levels of apoptotic parameters associated to TNF- α signaling pathway in the liver. Data are presented with the mean \pm standard deviation (n=8). * p < 0.05, compared with the control group; ** p < 0.01, compared with the control group.

that NaF not only can promote the ROS production, but also inhibit the antioxidant enzyme mRNA expression in the liver. Then the imbalance between ROS and anti-

oxidative function leads to the oxidative stress, which contributes to the occurrence of hepatocellular apoptosis.

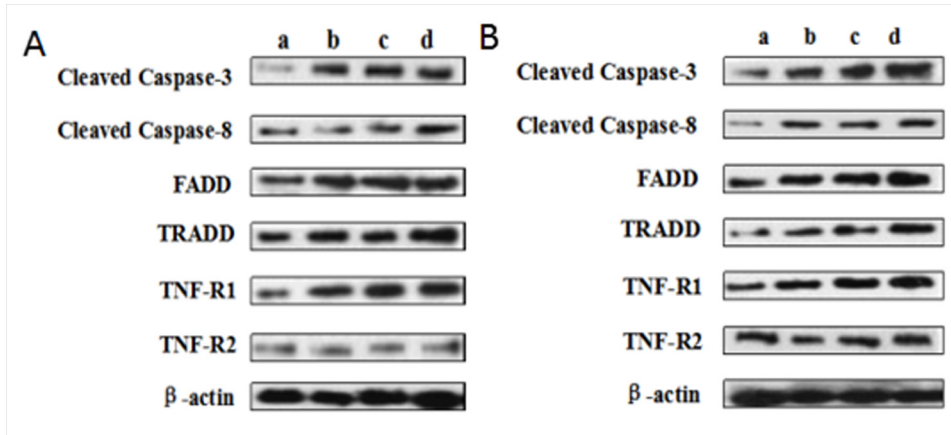


Figure 13. (A) The western blot assay at 21 days of the experiment. (B) The western blot assay at 42 days of the experiment. Control group (a), 12mg/kg (b), 24mg/kg (c), 48mg/kg (d).

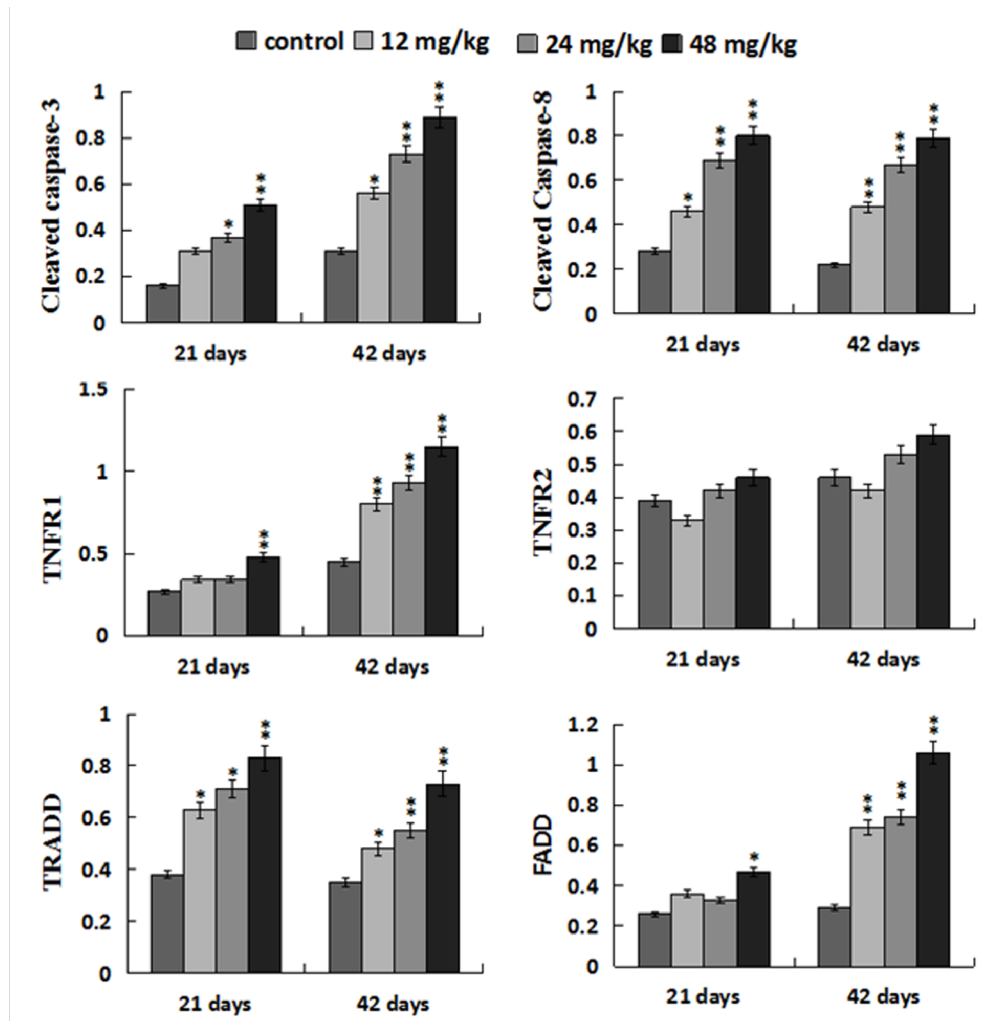


Figure 14. Changes of protein expression levels of apoptotic parameters associated to TNF- α signaling pathway in the liver. Data are presented with the mean \pm standard deviation (n=8). * $p < 0.05$, compared with the control group; ** $p < 0.01$, compared with the control group.

It has been accepted that oxidative stress is an apoptotic inducer and some agents that induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. Conversely, many apoptosis inhibitors have antioxidative activities or can enhance the antioxidative defense ability [62, 63]. Recently, there are studies on revealing the pivotal role of ROS and oxidative stress in inducing apoptosis [64, 65]. In the present study, the detection of flow cytometry demonstrated that percentages of apoptotic hepatocytes were higher in the NaF-treated groups than those in the control group. In order to reveal the apoptosis mechanism induced by NaF, we further observed the expression of death receptor signal pathway involved in hepatocellular apoptosis. Guicciardi et al. [66] has found that death receptors are widely expressed in all liver cells. In TNF-receptor family, TNF- α has two receptors, TNF-R1 and TNF-R2. TNF-R1 play an important role in inducing apoptosis [67, 68]. However, TNF-R2 mainly activates the anti-apoptotic pathway [69]. Combination of the ligands and receptors recruits the adaptor proteins FADD and TNFR-associated death domain (TRADD), and then initiates the formation of death-inducing signaling complex (DICS), which further activates caspase-8, as a central mediator of death receptor signal pathway [70]. Activated caspase-8 catalyzes the caspase-3 proteolysis and drives the cascade reactions of downstream. In this study, we found that NaF treatment increased mRNA and protein expression levels of TNF-R1, FADD, TRADD, caspase-3 and caspase-8, which confirmed that TNF-R1 signaling pathway played a pivotal role in NaF-induced hepatocellular apoptosis.

Based on the above-mentioned discussion and the results in the present study, NaF-caused oxidative stress and apoptosis finally impaired hepatocytes and hepatic function, which was strongly supported by the histopathological lesions and increased serum AST, ALT, AKP activities and TBIL contents.

CONCLUSIONS

The results show that NaF exposure induces hepatic oxidative stress and apoptosis. Oxidative stress is involved in the process of apoptotic occurrence, and can be triggered by promoting ROS production and reducing antioxidant function. NaF-caused oxidative stress and apoptosis finally impaired hepatocytes and hepatic function, which was strongly supported by the hepatocellular lesions histopathologically and increased serum AST, ALT, AKP activities and TBIL contents. Also, it has been demonstrated that NaF induces hepatocellular apoptosis through TNF-R1 signal pathway in mice.

MATERIALS AND METHODS

Chemicals

Sodium fluoride was purchased from Chengdu Kelong Chemical Co., Ltd. (Chengdu, China). ROS Assay kit (S0033) was obtained from Beyotime Biotechnology, China. Reagent kits for determination of biochemical parameters were purchased from Nanjing Jiancheng Bioengineering Institute of China (Nanjing, China). RNAiso Plus, Prim-ScriptTM RT reagent Kit and SYBR[®] Premix Ex TaqTM II were purchased from Takara Biotechnology (Dalian) Co., Ltd. (Dalian, Liaoning, China). All other chemicals used in the experiment were analytical grade.

Animals and treatment

240 ICR mice were provided by Chengdu Dossy Experimental Animals Co., Ltd. [License No. SCXK (Sichuan) 2008-24] and were randomly divided into 4 equal groups, and housed in separate cages. The control group received distilled water. The low-, medium-, and high-fluoride groups were oral administered with NaF at a dose of 12, 24 and 48 mg/kg body weight for consecutively 42 days, and the gavage volume was 1ml/100g body weight respectively. All of the mice had free access to food and water.

All experimental procedures involving the use of mice were approved by the Animal Care and Use Committee, Sichuan Agricultural University.

Histopathological observation

At 21 and 42 days of the experiment, eight mice in each group were humanely killed, and the livers were immediately taken out and fixed in 4 % buffered formaldehyde, dehydrated through graded alcohol, and routinely processed in paraffin. Thin slices (5 μ m) of each tissue were sliced through routine microtomy. Slices were stained with hematoxylin and eosin (H&E) and then were examined under optical microscope.

Detection of hepatic functional parameters

At 21 and 42 days of the experiment, blood was taken from retro-ocular artery of eight mice in each group without anticoagulant. Serum samples were collected after centrifuged at 3000 rpm for 15 min. Serum ALT, AST, AKP activities and TBIL contents were measured according to the directions of biological reagent kits purchased from Nanjing Jiancheng Bioengineering Institute of China (Nanjing, China).

Detection of oxidative stress parameters in the liver

At 21 and 42 days of the experiment, after eight mice in each group were sacrificed, the livers were immediately removed, and washed using chilled saline solution, weighed, homogenized in nine volumes of ice-cold 0.9% NaCl solution and centrifuged at 3500 rpm for 10 min at 4°C. The supernatants were collected for detecting the activities of CAT, SOD, GST and GSH-Px, and contents of GSH and MDA by biochemical methods following the instructions of the corresponding reagent experiment kits (CAT, A007-1; SOD, A001-1; GST, A004; GSH-Px, A005; GSH, A006-2; MDA, A003-1; total protein, A045-3, purchased from Nanjing Jiancheng Bioengineering Institute of Nanjing, China) after determining the total protein contents in the supernatant by the Bradford method [71].

Detection of antioxidant enzyme and apoptosis parameter mRNA expression by qRT-PCR

At 21 and 42 days of the experiment, livers from eight mice in each group were respectively stored in liquid nitrogen, and homogenized with liquid nitrogen for RNA extraction. The methods of RNA extraction were same as the described by Yin al. [72]. The total RNA of the liver were extracted using RNAiso Plus (9109; Takara, China). The cDNA was synthesized using a Prim-Script™ RT reagent Kit (RR047A, Takara, China) following the manufacture's instruction. The cDNA sequences of CuZn-SOD, Mn-SOD, GST, CAT, GSH-Px and cleaved caspase-3, cleaved caspase-8, TNF-R1, TNF-R2, FADD, TRADD were referred from NCBI,

and β -actin was used as reference gene. The primers were designed and synthesized by Sangon Biotech Biological Technology Company (Shanghai, China) (Table 1).

qRT-PCR reactions was performed on a Thermal Cycler (C1000, BIO RAD, USA) Using SYBR® Premix Ex Taq™II (RR820A, Takara, China). Gene expression values of the control group at 21 and 42 days of the experiment were used for gene expression calibration. The results were analyzed with $2^{-\Delta\Delta CT}$ method [73].

Detection of apoptosis parameter protein expression by western blot

Proteins were extracted with RIPA lysis buffer and the protein concentration was quantitated by BCA protein assay reagent. Protein samples were separated by (10%-15% gels) SDS-PAGE and transferred to nitrocellulose filter membranes. The membranes were blocked in 5% skim milk for 1h and incubated with the primary antibodies overnight at 4°C. The primary antibodies were cleaved caspase-3, cleaved caspase-8, TNF-R1, TNF-R2, FADD, TRADD. The membranes were then washed with PBST (PBS-Tween). Blots were visualized by ECL™ (BIO-RAD) and X-ray film. The statistical data of protein expression was done with imageJ2x software.

Detection of apoptosis by flow cytometry

At 21 and 42 days of the experiment, after eight mice in each group were sacrificed, livers were immediately

Table 1. The list of oligonucleotides used as primers in qRT-PCR analysis of gene expression.

Primer name	Primer sequence (5'-3')		Accession number	product size	Tm (°C)
	Forward primer	Reverse primer			
CAT	CCTATTGCCGTTTCGATTCTC	CCCACAAGATCCCAGTTACC	NM-009804	119bp	61
Mn-SOD	AACTCAGGTCGCTCTTCAGC	CTCCAGCAACTCTCCTTTGG	NM-013671	113bp	61
CuZn-SOD	GGGTTCCACGTCCATCAGTA	CAGGTCTCCAACATGCCTCT	NM-011434	113bp	61
GSH-Px	CCAGGAGAATGGCAAGAATG	AAGGTAAGAGCGGGTGAGC	NM-008160	102bp	57
GST	GGGATTGGCTGTGATGAGAT	AGGTAGGATGAATGGCAACTG	NM-019946	121bp	61
Caspase-3	TCTGACTGGAAAGCCGAAAC	GCAAGCCATCTCCTCATCA	NM-009810	103bp	57
Caspase-8	GCTGCCCTCAAGTTCCTGT	GATTGCCTTCTCCAACATC	NM-009812	118bp	61
TNF-R1	AATGCAGACCTTGCGATTCT	CATCTCCAGCCTCTCGATCT	NM-011609	114bp	59
TNF-R2	CATCACTGGGTCTGCTGATG	TCCTGGGATTTCTCATCAGG	NM-011610	124bp	57
FADD	CGTGAGAAACGAAAGCTGG	CTGCAGTAGATCGTGTCGGC	NM-010175	142bp	60
TRADD	TGGCTGACTGATGAAGAGCG	CACACGTCAGTTTGCAGAGC	NM-1033161	112bp	60
β -actin	GCTGTGCTATGTTGCTCTAG	CGCTCGTTGCCAATAGTG	NM-007393	117bp	59

moved and ground to form a cell suspension which could be filtered through the 300-mesh nylon. The cells were washed twice with cold PBS (phosphate buffer solution pH 7.2-7.4) and suspended in PBS at a concentration of 1×10^6 cells/mL, and 100 μ L cell suspension was transferred into a 5mL tubes, and stained with PE Annexin V and 7-aminoactinomycin (7-AAD). The mixture was gently vibrated and incubated for 15min in the dark place, and then 400 μ L of $1 \times$ binding buffer was added to each tube. Finally, the hepatic apoptosis was analyzed by FACSCalibur (BD FACS Calibur).

Detection of hepatocellular ROS production by flow cytometry

At 21 and 42 days of the experiment, after eight mice in each group were sacrificed, livers were taken to measure the levels of ROS production by flow cytometry. Livers were crushed, filtered with 350 mesh nylon membrane, centrifuged ($600 \times g$, 5min), and adjusted to a cell density of 1.0×10^6 cells/ml with phosphate-buffered saline (PBS). 300 μ L cell suspensions were taken and transferred to another centrifuge tube, and stained with 10 μ M DCFH-DA for 20 min at 37°C. Then the cells were washed with PBS and centrifuged ($600 \times g$, 5min) once more. The supernatant was discarded, and cells were resuspended in 0.5 ml PBS and counted by BD FACS Calibur flow cytometer within 45 min.

Statistical analysis

The significance of difference was analyzed by the SPSS version 17.0. The results were shown as means \pm standard deviation. The analysis was performed with the one-way analysis of variance (ANOVA). The differences between control and experimental group(s) at $p < 0.05$ or $p < 0.01$ were considered significant.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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