Ameliorative Effects of Nano-Selenium Against Fluoride Stress Induced Hepatocytes Autophagy and Apoptosis in Mice

Yajing Wang  
South China Agricultural University College of Economics and Management

Bingxian Liu  
South China Agricultural University College of Economics and Management

Qingyue Han  
South China Agricultural University College of Economics and Management

Khalid Mehmood  
The Islamia University of Bahawalpur Pakistan

Fazul Nabi  
Cornell University

Yung-Fu Chang  
Cornell University College of Veterinary Medicine

Zhaoxin Tang  
South China Agricultural University College of Economics and Management

Ying Li  
South China Agricultural University College of Economics and Management

Hui Zhang (✉ hz236@scau.edu.cn)  
South China Agricultural University College of Economics and Management  https://orcid.org/0000-0002-1700-5065

Research

**Keywords:** Fluorosis, Nano-Se, Autophagy, Apoptosis, Hepatotoxicity

**DOI:** https://doi.org/10.21203/rs.3.rs-44205/v1

**License:** Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background:** Fluorine is widespread in the environment, and the injurious impacts of fluoride underscore its significance for public health. The long-term presence of fluoride in environment could be a risk in hepatotoxicity for both human beings and animals. Important role of selenium in mitigation of heavy metal toxicity via regulating autophagy and apoptosis is well-known. Further, nano-Se is a common artificial nano material, with higher biological activity and lower toxicity. The aim of the current study was to examine whether nano-Se supplementation can reduce the effects of fluoride-induced hepatocytes autophagy and apoptosis.

**Results:** Here, we report that fluoride exposure induces apoptosis and autophagy with nucleus broken, dissolved and disappeared of hepatocyte, contributing to its hepatotoxicity. More importantly, Cyt-C and Beclin-1/Bcl-2 pathways are involved in the regulation of autophagy and apoptosis via targeting Caspase-9, Caspase-3, P53, Bax, LC3, ATG-5, P62 and mTOR expressions.

**Conclusion:** Nano-Se is capable to alleviate fluoride-induced hepatocyte damage, that selenium can be prefer to prevent chronic fluorosis-induced autophagy and apoptosis by regulating Cyt-C and Beclin-1/Bcl-2 signaling pathway. In precisely, NaF-induced the liver injury by activating autophagy and apoptosis, which indicate that fluoride exposure, pose an ecological risk to human beings and animals. Nano-Se has protective effects against fluoride-induced hepatocytes.

Introduction

Fluorine is an important trace element for humans and in animals, which is widely distributed in natural rock, soil, and in industrial waste. However, the excessive long-term fluorine exposure in the air (industrial exhaust), food, water, and even excessive use of toothpaste could be a risk for human beings [13]. Drinking water is main source of fluorine exposure [16], that why cut value for fluoride is 1.5 mg/L[16] according to World Health Organization (WHO). WHO reports indicated that about 200 million residents worldwide are using unsafe drinking water which has concentration well above the standard [16; 26]. Fluorosis is a worldwide endemic chronic systemic disease closely related to the long-term intake of excessive fluorine in the geographical environment [29], mainly manifested as dental fluorosis and skeletal fluorosis [36]. The endemic fluorosis is widely spread in many countries, like Thailand, India, Egypt, China, etc. [16; 32]. Human populations in many regions (e.g. Northwest, Southwest, Loess Plateau) of China suffer from the fluorosis due to the long-term intake of fluorine [21; 36; 40]. Previous study indicated that almost 19 million people suffered from fluorosis, of which the skeletal fluorosis has afflicted about 1.2 million people in China caused mainly by fluoride from coal-burning [35], which also disturb crops and livestock sector. Long-term consumption of food or water with high fluoride content will also cause chronic poisoning of fluorine (inflammation and degeneration of kidney, liver, intestine, brain, and lung) [9]. Among them, fluoride has toxic effect on the liver, so excessive intake of fluoride can lead to extensive degenerative changes in the liver ultimately reduce its detoxification function.
Oxidative Stress (OS) lead to the unbalanced oxidant–antioxidant status, and OS is initiating factor for the florosis [31]. Abnormal imbalance in the redox state of normal cells will produce peroxides and free radicals which result in damaging the proteins, lipids and nucleic acids of cells. Fluorine can directly interfere with oxygen metabolism, leading to the increase in ROS. ROS can activate the release of Cyt-C in mitochondria, damage DNA molecules, and regulate the expression of apoptosis related genes [2; 3]. Previous report indicated that sodium fluoride can promote Bax gene expression and inhibit the expression of BCI-2 gene to accelerate cell apoptosis, and the rate of cell apoptosis is positively correlated with fluorine concentration [5]. Zhao et al., found that NaF induced mitochondrial damage and ROS contribute to hepatotoxicity and cell damage [47].

Autophagy, a highly conserved biological process that degrades damaged organelles and double-membrane vesicles (autophagosomes), and then transported to lysosomes for future degradation, and considered important process in an array of cellular events (cell development, death, oxidative stress, toxic injury) [10; 19]. In the process of studying how fluoride induces autophagy in rat HAT-7 cell, Zhang et al. found that the expressions of autophagy genes LC3, Beclin-1 and Alg5 were up-regulated and the phosphorylation level of mTOR protein was reduced in mouse Leydig cells exposed to fluoride, which proved that fluoride could affect cytotoxicity via autophagy [45]. In addition, NaF-induced autophagy via regulating the autophagy related genes expression, like p62, Beclin1 and LC3 [48].

Selenium is involved in the synthesis of various selenoenzyme and selenoproteins, including glutathione peroxidase, which catalyzes the transformation of peroxides into water or various alcohols in the body, and protecting biofilms from oxidative damage [9; 37]. Selenium has an alleviation effect on many heavy metals toxic elements such as cadmium, arsenic, mercury, lead and silver, etc. At the same time, and selenium can antagonize cell damage caused by florosis, promote urinary fluoride excretion, correct free radicals and lipid metabolism disorder and significantly inhibit fluorine-induced lipid peroxidation. Previous study showed that the protective effect of selenium on fluoride-induced cell injury is closely related to its antioxidant capacity [34; 37; 39]. Feng et al., reported that selenium eliciting antagonistic effects in florosis by promote the removal of fluoride, repair lipid peroxidation, and enhances autoxidation [6]. In particular, selenium produced antioxidant effects on fluoride, relieves the toxic effect of fluoride in liver, and inhibits apoptosis [23]. Nanomaterials have been widely used in human and animal clinical practice due to diverse properties including increase absorption rate, decrease toxicity, high bioavailability and strong oxidation resistance, as compared with other therapeutic materials. As the point of interest in nanotechnology treatment, selenium nanoparticles (SeNPs) have been used as protective agents in different murine experimental models due to unique biological activities and low toxicity. However, the research regarding mechanism of action of nano-Se in relieving the toxic effect of fluoride has less reported, and its relevant mechanisms and biological functions connected with autophagy and apoptosis need to be further investigated.

Previous study reveals that fluoride has hepatotoxic effects, and selenium has vital role in mitigation of heavy metal toxicity via regulating autophagy and apoptosis is well-known. Still, the underlying molecular mechanisms of nano-Se in reversing fluoride-induced toxicity has rarely reported. The present study
aimed to explore the autophagy and apoptosis in hepatocytes as well as the effects of nano-Se against fluoride-induced hepatocytes damage to demonstrate the mechanism by which selenium prevents chronic fluorosis-induced autophagy and apoptosis.

Materials And Methods

Chemicals and Reagents

A total of 40 twenty-eight-day-old healthy mice (Kunming) were obtained from the Laboratory Animal Center of Southern Medical University (Guangzhou). Nano-selenium (Nano-Se) was purchased from the Macklin Biochemical Co. Ltd. in Shanghai (purity ≥ 99%, an average particle size of 40 nm). Sodium fluoride (NaF) was provided by South China Agricultural University (AR, 99%). Mouse monoclonal antibodies against Caspase-3 (35KD), ATG-5 (32KD), Beclin-1 (60KD), Cyt-C (15KD), LC3II/I (16KD/18KD), P62 (42KD) and rabbit polyclonal antibodies against GAPDH were purchased from Bioss (Shanghai, China), and other chemicals and agents (analytical grade) were obtained from Servicebio Co. Ltd (Wuhan).

Experimental Groups and Mice Treatment

Total of 40 healthy Kunming mice were divided into four equal groups (n = 10) randomly. All animals were offered ad libitum regular diet and housed in a controlled environment (12-h light/dark cycle; temperature 23 ± 2 °C) as suggested by the Laboratory Animal Center of South China Agricultural University. First group was control group (n = 10) administered with a standard normal diet. Second group (n = 10) was treated with 24 mg/kg NaF dose via intragastric route, third group (n = 10) was treated with 1 mg/kg nano-Se dose via same route, and lastly group (n = 10) was treated with NaF + nano-Se with same doses. After 28 days, animals were sacrificed by cervical dislocation. Liver samples were quickly fixed in paraformaldehyde (4%) for histopathology and immunofluorescence, and remaining samples were frozen in liquid nitrogen, until subsequent use and further analysis.

H&E Staining and Immunohistochemistry

The liver samples (n = 10) from each treatment group were processed for histological analysis via hematoxylin and eosin (H&E) according to our previous study [30; 44]. Briefly, the liver tissues were fixed in paraformaldehyde (4%) for 48 h, and then dehydrated in gradients of ethanol, subsequently embedded in paraffin. The tissue sections with 4–5 µm-thick sections were placed on polylysine-coated slides for H&E staining and IHC. For H&E, the above slides were dried for 12 h at 37 °C, then stained with hematoxylin/eosin, and observed under a light microscopy. For immunohistochemical analysis, the slides were blocked by 3% hydrogen peroxide for 10–20 min after washed in PBS. After washing with PBS, horse serum (10%) was used on sections for 1 h at 37 °C, then the slides were incubated with primary antibody against Cyt-C and LC3 for 1.5–2.5 h at 25 °C and washed with PBS. Thereafter, the sections were incubated for 1.5 h with secondary antibodies at 37 °C to fit the primary antibodies. The photomicrographs were obtained by using microscope (Olympus, Japan). For expression analysis of
proteins, six fields of slides were selected and observed randomly, and the expression values were analyzed quantitatively using the Image-Pro ® Plus 6.0.

**TUNEL analysis for apoptosis of hepatocytes**

For the hepatocytes apoptosis assay, the TUNEL staining was used according reported protocol [38; 46]. Briefly, the paraffin sections were de-waxed with xylene and ethanol, and then slides were digested with proteinase K (20 µg/ml) at 37 °C for 15–30 min. The slides were filled with TdT and dUTP (1:9) for 2 h at 37 °C and washed with PBS three times. Finally, sections were stained with 4, 6-diamido-2-phenylindole dihydrochloride, and observed under a fluorescence microscope. The value of positive nuclei was measured using the Image-Pro ® Plus 6.0.

**RT-qPCR analysis**

Specific primers for evaluating the apoptosis (Caspase-3, Bax, Bcl2, P53, Cyt-c, Caspase-9, Bak1 and APAF1) and autophagy (Parkin, Pink1, Beclin-1, Atg-5, LC3, mTOR and P62) genes were chosen and designed according to published mice sequences (Table S1) using the Primer Premier 6.0 software (Premier, USA), and the housekeeping gene (GAPDH) was served as the control. Total RNA from the liver tissues were extracted using the TRIzol (Invitrogen). The 50 µl cDNA was obtained by using the commercial reverse transcription cDNA kit (AT301-02, TransGen Biotech, China). Then, the real-time qPCR (RT-qPCR) was performed in quadruplex with Step One-Plus™ Real-Time PCR System. Relative mRNA expression level was calculated using delta Ct (2^−ΔΔCt) method as described by our previous study [43].

**Western Blot analysis**

Liver samples were collected and washed with PBS three times on ice, and homogenized in RIPA lysis buffer with 1% PMSF, and then centrifuged at 12,000 rpm for 10–15 min for collecting the supernatants for further experiments. The concentration of the total protein in liver samples was determined by BCA assay kit. The samples were boiled for 10 min with SDS-PAGE loading buffer. The protein was separated by SDS-PAGE on 10% polyacrylamide gel and then transferred to PVDF membrane according to the laboratory protocol. The membrane was incubated in 5% skimmed milk for 1.5 h at 25 °C and then incubated with diluted primary antibodies against ATG-5, Beclin-1, Caspase-3, Cyt-C, LC3, P62 and GAPDH (1:1000) at 4 °C for 12 h. Then, the membranes were washed for 3–5 times with TBST for 5 min each, followed by incubating with corresponding secondary antibody for 30 min at 25 °C. The images of proteins band were photographed with an imaging system (TransGen Biotech Co., China). Proteins levels were analyzed by detecting the optical density value via the Image J software (Bethesda, MD, USA).

**Statistical Analysis**

The statistical analyses were performed using Student’s t-test by using SPSS 19.0 software. The results were shown the means ± standard deviation. *P < 0.05 was set as level of significance.

**Results**
Effects of nano-selenium against fluoride-induced hepatocytes histological changes in liver

Autopsy affected the liver development (volume and weight decreased) of mice in the sodium fluoride (NaF) group more prominently than the control group. However, non-significantly change in the nano-Se group was observed as compared with the control group, and the abnormal liver development in NaF induced mice by was alleviated by nano-Se treatment. As shown in Fig. 1A, the hepatocytes were arranged precisely without any visible structural damage in live condition in the normal mice. In the NaF-treated mice, hepatocytes were disturbed, and observed changes were disordered arrangement, swelling, granulosa degeneration, vacuolar degeneration and necrosis of hepatocytes, and even nucleus broken, dissolved and disappeared. However, nano-Se treatment enhances the degree of hepatocyte resist oxidative damage or apoptosis, that protect hepatocyte damage induced by NaF. These results imply that fluoride induced hepatocytes apoptosis, and nano-Se is capable to alleviate fluoride-induced hepatocyte damage that selenium can be chosen to prevent chronic fluorosis-induced apoptosis.

TUNEL staining of liver tissues were analyzed to evaluate the hepatocyte apoptosis (Fig. 1B&D). No evident signs of hepatocytes damage were found, and TUNEL-positive cells were rarely detected in the control and nano-Se groups. In contrast, NaF treatment enhances the degree of hepatocyte apoptosis, and the percentages of TUNEL-positive increased by more than 7 fold as compared with normal mice in control. Fortunately, nano-Se could alleviate fluorosis-induced hepatocytes injury in mice (Fig. 1C).

Cytochrome C (Cyt-C) important factor in mitochondria pathway in fluoride-induced apoptosis

Immunostaining showed that the Cyt-C protein level in NaF group was highly expressed (P < 0.05) vs control and nano-Se groups, while the expression level of Cyt-C was decreased after nano-Se plus NaF treatment group (Fig. 2A&B). Cyt-C expression by RT-qPCR and western blot were depicted in Fig. 2C&D. As well, the expression of Cyt-C in NaF group was significantly higher as compared to control and nano-Se groups, and nano-Se possibly could reduce Cyt-C expression in fluorosis-induced hepatocyte apoptosis.

Beclin-1/Bcl-2 regulates autophagy and apoptosis in fluoride-induced hepatocyte damage

To assess whether the ameliorative effects of nano-Se against NaF-induced hepatocyte damage was associated with the activation of the Beclin-1/Bcl-2 pathways, the expression of the key marker protein LC3 of autophagy was studied. In comparison with control, the LC3 expression level was elevated in NaF group, and down-regulated in nano-Se group (Fig. 3). Together, the ratio of LC3II/LC3I protein expression was increased in NaF groups, while mRNA level of LC3 was increased in both NaF and nano-Se groups. Forehead results indicate that the hepatocytes undergo autophagy after fluoride treatment; in context the role of Beclin-1/Bcl-2 pathway remains unclear. For this purpose, the Beclin-1 and Bcl-2 mRNA and protein...
expression levels was further investigated. In comparison with control, the protein expressions of Bcl-2 and beclin-1 was increased in NaF treated hepatocytes. NaF vs nano-Se groups, Bcl-2 level was elevated and Beclin-1 level was reduced (P < 0.05), respectively. In conclusion outcomes spotlight the role of Beclin-1/Bcl-2: in regulation of autophagy in fluoride-induced liver damage, and in nano-Se against defective autophagy in the anesis of liver damage. These results could contribute to understanding the mechanism of fluoride-induced hepatotoxicity and the effects of nano-Se in alleviating fluorosis.

**Ameliorative effects of nano-selenium against fluoride-induced hepatocytes apoptosis**

Fluorosis is a major public health problem, and excessive fluoride exposure can induce apoptosis, and selenium has been used to alleviate this problem. To evaluate the ameliorative effects of nano-Se on hepatocyte apoptosis in fluorosis-affected mice, the Caspase-3, Caspase-9, Bax, P53, Pink1, and Parkin genes expression were analyzed. In comparison with control, the expression levels of Caspase-3, Caspase-9, and Bax mRNA were significantly elevated in the NaF and nano-Se treated groups; while Bax mRNA expression was up-regulated in nano-Se group (Fig. 4). In addition, the apoptosis proteins (Caspase-3, P53, Pink1, and Parkin) highly expressions were observed in treatment groups. Conversely, compared to the NaF group, the above abnormally expressed genes were decreased with the supplementation of nano-Se. These results suggested that NaF exposure impaired the liver damage and apoptosis, as confirmed by Caspase-3, Caspase-9, Bax, P53, Pink1, and Parkin genes expression. Fortunately, nano-Se could prevent chronic fluorosis-induced liver injury, in which anesis in apoptosis may also be considered as a key process in alleviating fluorosis.

**Nano-selenium alleviate autophagy in liver induced by fluoride**

Reported studies suggested that excessive fluoride exposure induced liver damage and autophagy. In current study, the autophagy was observed in hepatocytes by confirming protein LC3 (autophagy key marker) after fluoride treatment. However, the underlying mechanism of fluoride-induced autophagy in hepatotoxicity remains unknown. Compared to control and nano-Se groups, non-significantly differences were noticed in ATG-5, mTOR, and P62 mRNA expressions in NaF treatment group; while genes expression was decreased in nano-Se treatment. Surprisingly, the protein expression of ATG-5 was decreased after NaF exposure, and was increased after nano-Se treatment. In conclusion, NaF triggered the autophagy in hepatocytes and produced hepatotoxicity, and nano-Se play protective role in reversing toxicity.

**Discussion**

Fluoride common pollutant in the environment, imitated from the fluorine industry and used in the daily life, accompanied by the widespread presence in various geographical locations [13; 17]. Human population living in fluoride contaminated environment (water, soil, food, inusterial waste, drugs, cosmetics) suffer from chronic skeletal fluorosis and dental fluorosis [49]. Rather than dense tissues,
recently more attention has been paid to fluoride toxicity connected with kidney, liver, and reproductive system. Liver is a major detoxifying organ for the metabolism in the body, so in vulnerable way damaged by poisons and pollutants. Reported that excessive exposure of fluoride has hepatic toxic effect, including reduction in detoxification function, and studies have found that excessive fluorine exposure can cause significant liver damage, like hepatocyte swelling, degeneration and even necrosis, central venous distention and congestion, cell organelle disturbance, [1; 33; 41; 50] along with accompanied by apoptosis [33; 41; 49].

High fluoride exposure can inhibit the activity of cathepsin, trypsin and streptomycin, accompanied by promotion in the activity of transaminases (GOT and GPT); in addition, fluoride can significantly increase the malondialdehyde (MDA) level, NO content and free OH radical production [28]. Meanwhile, the previous studies have confirmed that significantly higher ROS and MDA levels were found in the ovaries of the fluoride treated mice, suggesting that fluoride has led to oxidative damage in the ovaries of zebrafish [20]. According to the previous results, we know that of SOD, CAT, and GSH-Px were decreased, and their mRNA expressions down-regulated, which may lead to reduce in antioxidant activities in fluoride exposure [20], and these effects can ultimately lead to changes in cell structure and function. The antioxidant enzymes (SOD, CAT, GPx and GSH), along with malondialdehyde (MDA), were studied as potential biomarkers in oxidative damage [51]. Meanwhile, previous report found that the oxidative stress genes (Gstp1, Ncf1, and Cygb) were significantly elevated in liver tissue in sheep exposed to chronic fluoride, while GPx, SOD 1, and SOD 2 genes were significantly decreased, which suggested that fluoride can induce hepatocyte injury [5]. In present study, we found that hepatocytes showed a disordered arrangement, swelling, granulosa degeneration, vacuolar degeneration and necrosis of hepatocytes, and even nucleus broken, dissolved and disappeared in the excess fluoride exposed hepatocytes. Meanwhile, the NaF enhances the degree of hepatocyte apoptosis, and the percentages of TUNEL-positive increased by more than 7-fold. Serum antioxidant levels suggested that fluoride could significantly reduce the level of SOD and GSH-PX, while MDA content was increased in the livers of fluoride exposed mice.

Current reports indicate that the mechanism of fluorosis accumulation induced-pathological damage is related to the increase of free radical level, manifested as disorder of free radical metabolism, decreased antioxidant capacity and increased oxidative stress level [14; 42]. Excessive intake of fluorine directly attacks oxygen, which leads to the production of oxygen free radicals (OFR). Meanwhile, studies have shown that fluorine attack antioxidant enzymes to inhibit their activity, which increased the oxygen free radicals and further leads to lipid peroxidation damage and cell death [42; 51]. Presently, free radical damage is considered as the main cause of cell death induced by fluorosis. Selenium is an effective antioxidant and an important component of GSH-PX, which is considered as an essential element for both animals and humans. Selenium was reported to improve the antioxidant capacity in both animals and humans with fluorosis; but the cell damage caused by fluoride exposure is antagonized by selenium via scavenging OFR [7; 6]. Current study found that the liver injure caused by fluorosis was characterized by the abnormal changes in antioxidant capacity with hepatocyte apoptosis, suggesting that fluorosis induced liver damage may be caused by increasing the free radical levels to induce lipid peroxidation, reduce the activity of antioxidant enzymes, and promote liver cell apoptosis. Fortunately, nano-Se
treatment enhances the degree of hepatocyte resist oxidative damage or apoptosis that protect hepatocyte damage induced by sodium fluoride. These results imply that fluoride induced liver apoptosis, thereby causing liver damage in the mice. Nano-Se is capable to alleviate fluoride-induced hepatocyte damage, that selenium can be preferred to prevent chronic fluorosis-induced apoptosis.

Oxidative stress causes damage to cell components, especially mitochondria, which are the site of ROS production and also the target of oxidative stress-induced damage [8]; previous reports have shown that ROS accumulation induced the mitochondrial respiratory chain damage [4]. Excessive fluorine intake increases the accumulation of ROS in the liver, suggesting that fluorine destroys the mitochondrial respiratory chain, which is mainly characterized by the occurrence of oxidative stress and the decrease of ATP content [33]. It has been reported that sodium fluoride exposure stimulates the production of ROS, causing oxidative stress [11; 25]. With the discovery and further understanding of mitochondrial role in apoptosis, the role of ROS in apoptosis has been paid more and more attention. Previous study showed that fluoride exposure induced apoptosis has also been occurred in kidney and liver of carp, *Cyprinus carpio* [20]. Apoptosis and oxidation pathways are particularly important in the study of the cell-dependent molecular basis of liver and kidney damage induced by fluoride [14]. Selenium may regulate the expression of apoptosis related factors, like Caspase-3, Caspase-9, Bcl-2 and Bax by enhancing the activity of Bcl-2 in cells, inhibit the activity of death regulating gene Bax, Caspase-3 and Caspase-9, for future delay the cell death regulating, thus protecting cells and extending cell life. Consistent with this result, the current study has been done in vivo concerning the apoptosis induced by fluoride found that fluoride has cytotoxic effects via affecting cell proliferation and even causing severe cell death. At the same time, our study also found that nano-Se can antagonize the apoptosis of hepatocytes in mice induced by fluorine, and inhibit apoptosis signal transduction and antagonize apoptosis through the expression of apoptotic factors, such as Caspase-3, Caspase-9, Bax, P53, CYT-C and Bcl-2, as well as the activity of antioxidant enzymes.

Autophagy is best characterized as a survival response, it is a widespread mechanism of a mechanism of programmed cell death, and an important pathway of intracellular component degradation, which is crucial for cell activity and body health. Cells can remove damaged organelles through autophagy to maintain cell homeostasis and provide energy [22]. Most recent studies have reported that autophagy involves many biological events, such as cell proliferation and development, oxidative stress, cell survival, cell senescence, and cell death [49]. For a complete autophagy pathway, each step is regulated by autophagy-related genes, such as ATG5, P53, mTOR, LC3, Bcl-2 and Beclin1. As an important detoxifying organ, liver is vulnerable to damage by poisons, among which autophagy plays an important role in the process of hepatocyte damage[15]. LC3 is crucial for the formation of autophagosomes, and the level of LC3 can reflect the number of autophagosomes. LC3-I can be specifically bound to the membrane of autophagosomes after transformation into LC3-II, so it is also considered as a marker protein for autophagy [24].

In our study, the expression of Beclin-1 was increased in fluoride group, which was in agree with other report by Guo et al. who reported that 100 mg/L NaF increased the autophagy-related proteins in mice.
In particular, we observed that nano-Se could regulate the expression of Cyt-C and Beclin-1/Bcl-2 signaling pathway to target apoptosis and autophagy and mediate the liver damage affected by fluorosis. Therefore, nano-Se has protective effects against fluoride-induced hepatocytes and can play key role as a protective agent to target autophagy. Beclin-1 is the first identified gene mediating autophagy in mammals and considered as a key regulator of autophagy. The autophagy precursor can be prevented by Beclin1 interacting proteins via regulating the activity of autophagy in cells [27]. Various autophagy regulatory proteins combine with Beclin1 to form protein complexes to regulate autophagy. The Bcl-2 interacts with Beclin1, resulting in preventing Beclin1-dependent autophagy leading to apoptosis. Therefore, Beclin1 can bind with autophagy-related proteins to regulate autophagy or apoptosis [18].

The current studies provide a novel evidence that fluoride exposure induces apoptosis and autophagy, and results in hepatotoxicity. Particularly, we have demonstrated that the related regulatory mechanisms are mediated by Cyt-C and Beclin-1/Bcl-2 signaling pathways. In addition, nano-Se has protective effects against fluoride-induced hepatocytes apoptosis, thereby reduce liver damage. The future studies should be investigated for nano-Se antidotal effect on fluoride toxicity. Moreover, our findings provide novel insights into the control mechanism of chronic fluorosis hepatotoxicity, and further studies among the fluoride, DNA damage and ROS are required to explore the fluoride induced oxidative stress in hepatocytes.

Declarations

Ethics approval and consent to participate

All the experiments were approved by the Animal Welfare and Institutional Animal Care Committee of South China Agricultural University Animal Care Committee, Guangzhou, China.

Consent for publication

“Not applicable”

Availability of data and materials

Yes, it will be provided by corresponding authors.

Competing interests

None of the authors has any conflict of interest.
Funding

The study was supported by National Key Research and Development Program of China (Zhaoxin Tang, Project No. 2017YFD0502200) and the Outstanding Young Talents Project of South China Agricultural University (Hui Zhang, 30004751).

Authors' contributions

ZT, YL, HZ developed idea, arrange funding and guided the students. YW, BL, QH, HZ arrange reagents, experiments and data analysis. KM, FN, YFC, HZ analysis and initial draft writing.

Acknowledgment

We are thankful to South China Agricultural University for providing Outstanding Young Talents Project (Hui Zhang, 30004751).

References

1. Alhusaini A, Faddaa L, Ali HM, Hassan I, El ON, Bassiouni Y. Amelioration of the protein expression of cox2, NFkappaB, and STAT-3 by some antioxidants in the liver of sodium Fluoride-Intoxicated rats. Dose Response. 2018;16(3): 712718311. doi:10.1177/1559325818800153.

2. Anuradha CD, Kanno S, Hirano S. Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL-60 cells. Free Radic Biol Med. 2001;31(3): 367-73. doi:10.1016/s0891-5849(01)00591-3.

3. Buccellato LJ, Tso M, Akinci OI, Chandel NS, Budinger GR. Reactive oxygen species are required for hyperoxia-induced Bax activation and cell death in alveolar epithelial cells. J Biol Chem. 2004;279(8): 6753-60. doi:10.1074/jbc.M310145200.

4. Cheraghi G, Hajiabedi E, Niaghi B, Nazari F, Naserzadeh P, Hosseini MJ. High doses of sodium tungstate can promote mitochondrial dysfunction and oxidative stress in isolated mitochondria. J Biochem Mol Toxicol. 2019;33(4): e22266. doi:10.1002/jbt.22266.

5. Efe U, Dede S, Yüksek V, Çetin S. Apoptotic and oxidative mechanisms in liver and kidney tissues of sheep with fluorosis. Biological trace element research. 2020. doi:10.1007/s12011-020-02121-y.

6. Feng P, Wei J, Zhang Z. Intervention of selenium on chronic fluorosis-induced injury of blood antioxidant capacity in rats. Biol Trace Elem Res. 2011;144(1-3): 1024-31. doi:10.1007/s12011-011-9087-9.

7. Feng P, Wei JR, Zhang ZG. Influence of selenium and fluoride on blood antioxidant capacity of rats. Experimental & Toxicologic Pathology. 2012;64(6): 565-8.
8. Gaignard P, Liere P, Therond P, Schumacher M, Slama A, Guennoun R. Role of sex hormones on brain mitochondrial function, with special reference to aging and neurodegenerative diseases. Front Aging Neurosci. 2017;9(406). doi:10.3389/fnagi.2017.00406.

9. Gao J, Wang Y, Xu G, Wei J, Chang K, Tian X et al. Selenium attenuates apoptosis and p-AMPK expressions in fluoride-induced NRK-52E cells. Environmental Science & Pollution Research. 2019.

10. Gu X, Han D, Chen W, Zhang L, Lin Q, Gao J et al. SIRT1-mediated FoxOs pathways protect against apoptosis by promoting autophagy in osteoblast-like MC3T3-E1 cells exposed to sodium fluoride. Oncotarget. 2016;7(40): 65218-30. doi:10.18632/oncotarget.11573.

11. Gu X, Wang Z, Gao J, Han D, Zhang L, Chen P et al. SIRT1 suppresses p53-dependent apoptosis by modulation of p21 in osteoblast-like MC3T3-E1 cells exposed to fluoride. Toxicol In Vitro. 2019;57(28-38. doi:10.1016/j.tiv.2019.02.006.

12. Guo Q, Sun Z, Niu R, Manthari RK, Yuan M, Yang K et al. Effect of arsenic and/or fluoride gestational exposure on renal autophagy in offspring mice. Chemosphere. 2020;241(124861. doi:10.1016/j.chemosphere.2019.124861.

13. Guth S, Huser S, Roth A, Degen G, Diel P, Edlund K et al. Toxity of fluoride: Critical evaluation of evidence for human developmental neurotoxicity in epidemiological studies, animal experiments and in vitro analyses. Arch Toxicol. 2020;94(5): 1375-415. doi:10.1007/s00204-020-02725-2.

14. Güven A, Kaya N, Karş. Effect of fluoride intoxication on lipid peroxidation and reduced glutathione in Tuj sheep. Fluoride. 2005;3838(139).

15. Han J, Bae J, Choi CY, Choi SP, Kang HS, Jo EK et al. Autophagy induced by AXL receptor tyrosine kinase alleviates acute liver injury via inhibition of NLRP3 inflammasome activation in mice. Autophagy. 2016;12(12): 2326-43. doi:10.1080/15548627.2016.1235124.

16. Hewavithana PB, Jayawardhane WM, Gamage R, Goonaratna C. Skeletal fluorosis in Vavuniya District: An observational study. Ceylon Med J. 2018;63(3): 139-42. doi:10.4038/cmj.v63i3.8723.

17. Johnston NR, Strobel SA. Principles of fluoride toxicity and the cellular response: A review. Arch Toxicol. 2020;94(4): 1051-69. doi:10.1007/s00204-020-02687-5.

18. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ. 2011;18(4): 571-80. doi:10.1038/cdd.2010.191.

19. Lei S, Zhang Y, Zhang K, Li J, Liu L. Effects of fluoride on the expression of beclin1 and mTOR in ameloblasts. Cells Tissues Organs. 2015;200(6): 405-12. doi:10.1159/000441052.

20. Li M, Cao J, Zhao Y, Wu P, Li X, Khodaei F et al. Fluoride impairs ovary development by affecting oogenesis and inducing oxidative stress and apoptosis in female zebrafish (Danio rerio). Chemosphere. 2020;256(127105. doi:10.1016/j.chemosphere.2020.127105.

21. Li M, Qu X, Miao H, Wen S, Hua Z, Ma Z et al. Spatial distribution of endemic fluorosis caused by drinking water in a high-fluorine area in Ningxia, China. Environ Sci Pollut Res Int. 2020;27(16): 20281-91. doi:10.1007/s11356-020-08451-7.

22. Lv SX, Qiao X. Isovitexin (IV) induces apoptosis and autophagy in liver cancer cells through endoplasmic reticulum stress. Biochem Biophys Res Commun. 2018;496(4): 1047-54.
doi:10.1016/j.bbrc.2018.01.111.

23. Miao K, Zhang L, Yang S, Qian W, Zhang Z. Intervention of selenium on apoptosis and Fas/FasL expressions in the liver of fluoride-exposed rats. Environ Toxicol Pharmacol. 2013;36(3): 913-20. doi:10.1016/j.etap.2013.08.003.

24. Nguyen TN, Padman BS, Usher J, Oorschot V, Ramm G, Lazarou M. Atg8 family LC3/GABARAP proteins are crucial for autophagosome-lysosome fusion but not autophagosome formation during PINK1/Parkin mitophagy and starvation. J Cell Biol. 2016;215(6): 857-74. doi:10.1083/jcb.201607039.

25. Ni J, Zhong Z, Zhang W, Liu B, Shu R, Li Y. Fluoride resistance in fibroblasts is conferred via reduced susceptibility to oxidative stress and apoptosis. FEBS Open Bio. 2020;10(3): 362-70. doi:10.1002/2211-5463.12786.

26. Saeed M, Malik RN, Kamal A. Fluorosis and cognitive development among children (6-14 years of age) in the endemic areas of the world: A review and critical analysis. Environ Sci Pollut Res Int. 2020;27(3): 2566-79. doi:10.1007/s11356-019-06938-6.

27. Salminen A, Kaarniranta K, Kauppinen A. Beclin 1 interactome controls the crosstalk between apoptosis, autophagy and inflammasome activation: Impact on the aging process. Ageing Res Rev. 2013;12(2): 520-34. doi:10.1016/j.arr.2012.11.004.

28. Sarkar C, Pal S, Das N, Dinda B. Ameliorative effects of oleanolic acid on fluoride induced metabolic and oxidative dysfunctions in rat brain: Experimental and biochemical studies. Food Chem Toxicol. 2014;66(224-36. doi:10.1016/j.fct.2014.01.020.

29. Srivastava S, Flora S. Fluoride in drinking water and skeletal fluorosis: A review of the global impact. Curr Environ Health Rep. 2020;7(2): 140-6. doi:10.1007/s40572-020-00270-9.

30. Tian X, Zhang H, Zhao Y, Mehmood K, Wu X, Chang Z et al. Transcriptome analysis reveals the molecular mechanism of hepatic metabolism disorder caused by chromium poisoning in chickens. Environ Sci Pollut Res Int. 2018;25(16): 15411-21. doi:10.1007/s11356-016-7630-1.

31. Tian Y, Xiao Y, Wang B, Sun C, Tang K, Sun F. Vitamin E and lycopene reduce coal burning fluorosis-induced spermatogenic cell apoptosis via oxidative stress-mediated JNK and ERK signaling pathways. Biosci Rep. 2018;38(4). doi:10.1042/BSR20171003.

32. Vithanage M, Bhattacharya P. Fluoride in the environment: Sources, distribution and defluoridation. Environmental Chemistry Letters. 2015;13(2): 131-47. doi:10.1007/s10311-015-0496-4.

33. Wang HW, Liu J, Wei SS, Zhao WP, Zhu SQ, Zhou BH. Mitochondrial respiratory chain damage and mitochondrial fusion disorder are involved in liver dysfunction of fluoride-induced mice. Chemosphere. 2020;241(125099. doi:10.1016/j.chemosphere.2019.125099.

34. Wang YX, Xiao X, Zhan XA. Antagonistic effects of different selenium sources on growth inhibition, oxidative damage, and apoptosis induced by fluorine in broilers. Poult Sci. 2018;97(9): 3207-17. doi:10.3382/ps/pey192.

35. Xu Y, Huang H, Zeng Q, Yu C, Yao M, Hong F et al. The effect of elemental content on the risk of dental fluorosis and the exposure of the environment and population to fluoride produced by coal-
burning. Environ Toxicol Pharmacol. 2017;56(329-39. doi:10.1016/j.etap.2017.10.011.
36. Yang JY, Wang M, Lu J, Yang K, Wang KP, Liu M et al. Fluorine in the environment in an endemic
fluorosis area in Southwest, China. Environ Res. 2020;184(109300.
doi:10.1016/j.envres.2020.109300.
37. Yang SY, Zhang L, Miao KK, Qian W, Zhang ZG. Effects of selenium intervention on chronic fluorsis-
induced renal cell apoptosis in rats. Biol Trace Elem Res. 2013;153(1-3): 237-42.
doi:10.1007/s12011-013-9649-0.
38. Yeh YL, Lu MC, Tsai BC, Tzang BS, Cheng SM, Zhang X et al. Heat-Killed lactobacillus reuteri GMNL-
263 inhibits systemic lupus Erythematosus-Induced cardiomyopathy in NZB/W f1 mice. Probiotics Antimicrob Proteins. 2020. doi:10.1007/s12602-020-09668-1.
39. Yoon I, Werner TM, Butler JM. Effect of source and concentration of selenium on growth
performance and selenium retention in broiler chickens. Poult Sci. 2007;86(4): 727-30.
doi:10.1093/ps/86.4.727.
40. Yuan L, Fei W, Jia F, Jun-Ping L, Qi L, Fang-Ru N et al. Health risk in children to fluoride exposure in a
typical endemic fluorosis area on Loess Plateau, north China, in the last decade. Chemosphere.
2020;243(125451. doi:10.1016/j.chemosphere.2019.125451.
41. Zhan XA, Wang M, Xu ZR, Li WF, Li JX. Evaluation of caspase-dependent apoptosis during fluoride-
induced liver lesion in pigs. Arch Toxicol. 2006;80(2): 74-80. doi:10.1007/s00204-005-0019-3.
42. Zhan XA, Xu ZR, Li JX, Min W. Effects of fluorsis on lipid peroxidation and antioxidant systems in
young pigs. Fluoride. 2005;38(2): 552-6.
43. Zhang H, Chang Z, Mehmood K, Abbas RZ, Nabi F, Rehman MU et al. Nano copper induces apoptosis
in PK-15 cells via a Mitochondria-Mediated pathway. Biol Trace Elem Res. 2018;181(1): 62-70.
doi:10.1007/s12011-017-1024-0.
44. Zhang H, Mehmood K, Jiang X, Yao W, Iqbal M, Waqas M et al. Effect of tetramethyl thiuram disulfide
(thiram) in relation to tibial dyschondroplasia in chickens. Environmental Science and Pollution
Research. 2018;25(28): 28264-74. doi:10.1007/s11356-018-2824-2.
45. Zhang J, Zhu Y, Shi Y, Han Y, Liang C, Feng Z et al. Fluoride-Induced autophagy via the regulation of
phosphorylation of mammalian targets of rapamycin in mice leydig cells. J Agric Food Chem.
2017;65(40): 8966-76. doi:10.1021/acs.jafc.7b03822.
46. Zhang S, Niu Q, Gao H, Ma R, Lei R, Zhang C et al. Excessive apoptosis and defective autophagy
contribute to developmental testicular toxicity induced by fluoride. Environ Pollut. 2016;212(97-104.
doi:10.1016/j.envpol.2016.01.059.
47. Zhao WP, Wang HW, Liu J, Tan PP, Lin L, Zhou BH. JNK/STAT signalling pathway is involved in
fluoride-induced follicular developmental dysplasia in female mice. Chemosphere. 2018;209(88-95.
doi:10.1016/j.chemosphere.2018.06.086.
48. Zhao Y, Li Y, Gao Y, Yuan M, Manthari RK, Wang J et al. TGF-beta1 acts as mediator in fluoride-
induced autophagy in the mouse osteoblast cells. Food Chem Toxicol. 2018;115(26-33.
doi:10.1016/j.fct.2018.02.065.
49. Zhao Y, Li Y, Wang J, Manthari RK, Wang J. Fluoride induces apoptosis and autophagy through the IL-17 signaling pathway in mice hepatocytes. Arch Toxicol. 2018;92(11): 3277-89. doi:10.1007/s00204-018-2305-x.

50. Zhou BH, Wei SS, Jia LS, Zhang Y, Miao CY, Wang HW. Drp1/Mff signaling pathway is involved in fluoride-induced abnormal fission of hepatocyte mitochondria in mice. Sci Total Environ. 2020;725(138192. doi:10.1016/j.scitotenv.2020.138192.

51. Zhou BH, Zhao J, Liu J, Zhang JL, Li J, Wang HW. Fluoride-induced oxidative stress is involved in the morphological damage and dysfunction of liver in female mice. Chemosphere. 2015;139(504-11. doi:10.1016/j.chemosphere.2015.08.030.

Figures
Figure 1

Histological analyses and TUNEL staining in liver of mice. (A) in histological structure of liver, the apoptotic cells increased in the Nano fluoride (NaF) group; (B) liver apoptosis was done by TUNEL assay the green color indicates apoptotic cells under microscope; (C) histogram shows the percentage of TUNEL positive; the TUNEL positive value was calculated by Image Pro plus 6.0 and repeated as quintuplicate (means ± standard deviations). TUNEL-positive (green), and DAPI nuclear staining (blue). (D) apoptotic cells under high magnification. *P ≤ 0.05, **P ≤ 0.01.
Fluoride-induced apoptosis as revealed by evaluating the Cyt-C expression. (A&B) Immunostaining of Cyt-C expression was increased in NaF treatment mice, and Nano-Se could alleviate fluorosis-induced Cyt-C expression; (C) Western blotting showed that the Cyt-C protein expression increased in the NaF treatment, while decreased in Nano-Se treatment; (D) qRT-PCR quantification revealed that the level of Cyt-C mRNA expression in the NaF group was significantly higher than control and Nano-Se groups, and Nano-Se decreasing impact observed on Cyt-C expression in fluorosis-induced hepatocyte apoptosis.
Figure 3

NaF induces autophagy and apoptosis associated with Beclin-1/Bcl-2 pathway. (A) the autophagy marker LC3 protein detection in liver by immunohistochemical staining. The brown punctate staining intensities represents LC3 protein expression level in different treatment groups. (B&C) images are representative of the relative mRNA and protein expression level of LC3 in current study by using RT-qRCR and western blot assay. (D) the ratio of LC3II/LC3I protein expression. (E&F) the relative mRNA expression levels of Beclin-1 and Bcl-2. (G, H and I) the relative protein expression levels of Beclin-1 and Bcl-2.
Figure 4

Effects of nano-selenium on fluoride exposure-induced hepatocytes apoptosis. (A) the mRNA expression levels of Caspase-3, Caspase-9, and Bax. (B) quantitative analyses of the band density revealed that the level of the apoptosis proteins of Caspase-3, P53, Pink1, and Parkin expressions normalized to GAPDH in mice liver. (C) representative images of western blotting band associated apoptosis proteins in mice liver. All values are presented as mean ± SD.
Figure 5

Effects of nano-selenium on fluoride exposure-induced hepatocytes autophagy. (A) expression levels of the autophagy marker genes (ATG-5, mTOR, and P62). (B) a quantitative analysis of the band density revealed that the level of ATG-5, mTOR, and P62 proteins in the fluoride exposure-induced liver damage was highly notable, while nano-selenium partially alleviates the autophagy. All values are presented as mean ± standard deviation (M ± SD). *P < 0.05.
Figure 6

Model of Nano-Se against fluoride-induced hepatic injury. NaF exposure impaired hepatocytes by regulating the expression of Cyt-C and Beclin-1/Bcl-2 signaling pathway to target apoptosis and autophagy, and Nano-Se has protective effects against fluoride-induced hepatocytes and can serve a key role as a protective agent against apoptosis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx