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Exposure to Fluoride induces apoptosis in liver of ducks by regulating Cyt-C/Caspase 3/9 signaling pathway

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ABSTRACT

Fluorine being a well-known and essential element for normal physiological functions of tissues of different organisms is frequently used for growth and development of body. The mechanisms of adverse and injurious impacts of fluoride are not clear and still are under debate. Therefore, this study was executed to ascertain the potential mechanisms of sodium fluoride in liver tissues of ducks. For this purpose, a total of 14 ducks were randomly divided and kept in two groups including control group and sodium fluoride treated group. The ducks in control group were fed with normal diet while the ducks in other group were exposed to sodium fluoride (750 mg/kg) for 28 days. The results showed that exposure to sodium fluoride induced deleterious effects in different liver tissues of ducks. The results indicated that mRNA levels of Cas-3, Cas-9, p53, Apaf-1, Bax and Cyt-c were increased in treated ducks with significantly higher mRNA level of Cas-9 and lower levels of the mRNA level of Bcl-2 as compared to untreated control group ($P < 0.01$). The results showed that protein expression levels of Bax and p53 were increased while protein expression level of Bcl-2 was reduced in treated ducks. No difference was observed in protein expression level of Cas-3 between treated and untreated ducks. The results of this study suggest that sodium fluoride damages the normal structure of liver and induces abnormal process of apoptosis in hepatocyte, which provide a new idea for elucidating the mechanisms of sodium fluoride induced hepatotoxicity in ducks.

1. Introduction

Fluorine, is one of the important environmental toxicants and is routinely used in our daily life. Fluorine is also one of the essential elements required for normal physiological functions in the body. It is widely found in water, soil, atmosphere and other natural environments including animals and plants. In nature, fluorine is often stable in the form of compounds (Abbas et al., 2017). According to the report, the natural range of fluorine in Polish sand (20–63 mg/kg), levistic soil (168–196 mg/kg), clay soil (250–323 mg/kg) and in loam (750–1660 mg/kg) has been measured (Bombik et al., 2020). Fluorine is widely and routinely used as an additive in toothpastes, mouthwashes and drinking water to increase the resistance against the damage caused by plaque forming bacteria and high oral sugar contents and prevention of tooth decay (Shuang et al., 2016). But drinking water that contains more than a certain amount of fluoride can lead to freckling and brittle teeth. It can also lead to induction of skeletal disorders like skeletal fluorosis. Therefore, in spite of an essential trace element for human body, long-term exposure to environment containing fluoride or excessive intake of fluoride results to develop fluorosis. Chronic fluorosis is prevalent in many parts of the world due to accidental or long-term intake of fluoride (Bouaziz et al., 2006). The permissible concentration (1.5 mg/L) of fluoride has been recommended by The World Health Organization (WHO) in drinking water (Li et al., 2020). Studies have shown that fluorosis is a systemic toxic problem and the mean concentration (6.03 mg/L) of fluoride has been recorded in ground water

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(Demelash et al., 2019). Studies have highlighted that in addition to dental caries and skeletal fluorosis, fluorine usually causes damage to the heart, liver, kidneys, reproductive system and central nervous system (Liang et al., 2020). Liver is one of the important visceral tissue responsible for detoxification in the body because of its active metabolic function. Liver is the main and one of the target organs affected by fluoride and is extremely sensitive to fluoride contents. Previous studies in adult rats have shown that fluoride can induce liver abnormalities including degenerative and inflammatory BBB changes, hepatic sinus dilatation, and hepatocellular hyperplasia BBB (A et al., 1995). Reports are available about the induction of toxic effects of fluorosis on liver metabolic dysfunctions and pathological changes in exposed mice (Choubisa, 2014). It has also been investigated that long-term exposure to fluoride induces deleterious effects in liver (Cook et al., 2021).

Recently, it has been reported that fluorine acts as a cytotoxic substance that can induce apoptosis via oxidative stress-mediated mitochondrial dysfunctions and triggering the activation of caspase 3 (Shuang et al., 2016). Apoptosis involves activation, expression and regulation of a series of genes responsible to maintain stability, growth, development and tissue repair (Wyllie, 2010; Ola et al., 2011; Aziz et al., 2020; Ijaz et al., 2020; Latif et al., 2020). The mechanism of apoptosis is mainly including the following stages like receiving of the apoptotic signal, interaction between apoptotic regulatory molecules, and activation of proteolytic enzyme (Caspase) leading to continuous reaction process. The sequential reaction process causes the transcription and translation of a series of genes that damage certain components closely related to cell survival resulting in cell death. The apoptosis-related genes include Bcl-2 family, caspase family and oncogenes such as c-YTC, tumor suppressor gene P53 (Antonio et al., 2017). Although the mechanisms of apoptosis are still not fully understood however, studies have confirmed that Caspase plays an important role in induction of apoptosis. The caspases involved in process of apoptosis can be divided into two categories including initiators and effectors. Caspases are involved in cell processing, apoptosis and play important role in upstream and downstream of transduction of signals of death. It is reported that hepatocyte apoptosis is most common and is an important mechanism of hepatocyte death during different process of pathological damage like liver immune cell-mediated cytotoxicity, chemical drugs/poisonings, viral diseases and autoimmune diseases. Zhao et al. (2018) found that fluoride can influence the trace elements that constitute antioxidant enzymes, increase the generation of free radicals, reduce antioxidant capacity of cells and enhance lipid peroxidation leading to cell damage. In previous published literature, reports indicated that fluorine can cause cell apoptosis through a variety of ways but still the detailed mechanism of fluorine regarding induction of apoptosis is poorly understood.

Different earlier studies have determined toxic impacts of fluorine on liver functions in humans, rabbits, calves and adult rats. Scanty information is available about the toxic of fluoride on ducks and other waterfowl (Zuo et al., 2018). Due to continuous and persistent release of different environmental pollutants into terrestrial and aquatic ecosystem attention towards monitoring of toxic effects of industrial wastes including fluoride and fluorine is of great importance to minimize the toxic effects in different exposed organisms including public health (Bombik et al., 2020; Yuan et al., 2019). Therefore, purpose of this study was to investigate the effects of NaF on liver metabolism and histological changes in ducks to better understand the mechanism of hepatotoxicity of fluorosis.

2. Materials and methods

2.1. Experimental animals and treatment

A total of 14 one-day-old Sanshui white ducks having similar body weight were obtained from the Experimental Animal Center of South China Agricultural University in Guangzhou. After 7 days of domestication, the ducks were randomly divided into two equal groups each containing 7 ducks. Standard diet was provided to all the experimental ducks kept in metal cages in accordance with the recommended environmental conditions by SCAU Laboratory Animal Centre (temperature 34 ± 1°C). The ducks in experimental group (NaF group) were fed standard diet containing (750 mg NaF/kg) fluoride (NaF, Sinophenol Group, 99%) and fluorosis was induced on day 7 after acclimatization. All the experimental research work was performed after the approval of the Institutional Animal Welfare and Research Ethics Committee of South China Agricultural University, Guangzhou, China.

2.2. Clinical observation

During the experiment, the ducks were carefully monitored daily for any obvious clinical and behavioral ailments.

2.3. Histopathological observation

After 28 days, the ducks were sacrificed by injection of pentobarbital (25 mg/kg) and liver tissue was collected. For histopathological investigation small portion of liver was cut from each duck and fixed in neutral paraformaldehyde solution (10%) and processed using ascending grades of ethanol (80%, 90%, 95%, 100%). According to previous method (Li et al., 2021), after dehydration and clearing thin microscopic slice were cut with help of microtome and stained with Hematoxylin and Eosin stain. All the tissues were observed under optical microscope and images were captured.

2.4. The mRNA expressions of Apaf-1, Cas-3, Bax, Bcl-2, Cyt-c and P53 by RT-qPCR

Total RNA was extracted from liver tissues of meat ducks for real-time quantitative PCR. Housekeeping gene GAPDH, which was stativity expressed in tissues was selected as the internal reference gene. The relevant primer sequences and primers were selected (Table 1) according to earlier protocol (Wang et al., 2021). Total RNA in liver tissue was extracted using Trizol method and kept in liquid nitrogen. Briefly, a small portion of liver was homogenized and centrifuged under 4 °C for 15 min. After centrifugation the supernatant was removed and chloroform was added and sample was placed at 4 °C for 15 min. Then isopropyl alcohol was added and placed on the ice and centrifuged after 15 min. The supernatant was removed and ethanol was added and centrifuged for 5 min. After that suitable amount of DEPC water was added for precipitation and finally the samples were preserved at - 20 °C. RNA reverse transcription was performed according to the instructions of Novizam kit. The cDNA obtained after inversion was used as a template for RT-qPCR.

According to our previous protocol (Liu et al., 2021), we used LightCycler 480 Real-Time System (Roche, Germany) for amplifying cDNA with a SYBR Premix Ex Taq II kit (Vazyme Biotech Co., Ltd, Nanjing).

Table 1
Primers for RT-qPCR.

Genes name	Forward sequence (5′→3′)	Reverse sequence (5′→3′)
Caspase-3	CGGGTACGGATGTAGATGCT	GGGGCCATCTGTACCATAGA
P53	ACAGCAGACTCCTGGGAAGA	GGGGTATTCGCTCAGTTTCA
Caspase-9	GAACTGGATCCGATGTGGAC	TTCCGTCCGTTCCATAAATC
APAF-1	TGGAATTGGCAGTTGAATGA	AGGAAAGAACAGCACCTCCA
Bax	CTTCTGCTTCCAGACCAAGG	TCAGCGTGTTCTTCCTGTTG
Bcl-2	GAGTTCTCCCGTCGCTACC	CGGTTCAGGTACTCGGTCAT
Cyt-c	AAGTGCTCCCAGTGCCATAC	CCGCGAAAATCATCTTTGTT
GAPDH	GAGGGTAGTGAAGGCTGCTG	CACCACACGGTTGCTGTATC

2.5. The protein expression levels of Cas-3, p53, Bax, Bcl-2 and Cyt-c were detected by Western blot

Between groups the extraction of total protein in the cell, samples, protein content, SDS-page electrophoresis, the 80 V program 1 (30 min) run enrichment glue, 120 V program 2 (60 min) separation of glue, turn 60 V constant pressure membrane 90 min, closed 5% skimmed milk powder solution 1 h, incubation under 4 °C a fight for the night, two resistance to 1 h incubation, join the developer ECL color rendering, the film scanning, with purpose of protein bands of gray and gray ratio of GAPDH protein expression level according to previous study (Chen et al., 2020).

2.6. The expression level of Cas-3 and Cyt-c in tissues by immunohistochemical staining

According to our previous research procedures (Yang et al., 2021), the liver tissue was fixed in 4% paraformaldehyde solution for 24 h. After HE staining, the liver tissue was dehydrated, embedded, sectioned and placed at 37 °C overnight. The tissues were then kept at 55 °C for 40–60 min and then placed at room temperature for 20 min. After that the samples were placed in xylene I, II and dimethylbenzene xylene III for 10 min and then in 100%, 90%, 80%, 70%, 50% alcohol for 5 min for dehydration. After that samples were washed with 1X PBS for 10 min and this process was repeated 3 times. Antigen repair was carried out, washed once, treated with 27 ml 85% methanol + 3 ml 30% hydrogen peroxide for 15 min. Wash with distilled water once, draw wax rings, wash with 1X PBS, 5 min, 3 times. 1X PBS 10% horse serum was sealed at 37 °C for 1 h. After that, the antibodies were diluted with 1% BSA (1:200) and incubated overnight at 4 °C. 1X PBS was used as negative control. The samples were then washed 3 times with 1X PBS for 5 min. The secondary antibody was diluted with 1%BSA (1:200) and incubated at room temperature for 40 min. Again the samples were washed 3 times with 1X PBS for 5 min. Chromogen solution was added for development of color at room temperature and observed under microscope. The reaction was terminated by 1X PBS. Staining of nucleus was made with hematoxylin for 1 min (adjust the time according to the actual situation), then 1X PBS was used to wash the hematoxylin. 1% hydrochloric acid and alcohol were separated for 10 s and washed with tap water for 15 min. Again respectively 50%, 70%, 80%, 90% alcohol dehydration 2 min, then II I with 100% alcohol and 100% alcohol dehydration 3 min, with xylene I finally, xylene II transparent 5 min. The neutral gum was sealed and observed under a microscope and photographed.

2.7. Statistical methods

SPSS 23.0 statistical software was used for statistical analysis. Experimental data were expressed as mean ±standard deviation ($x \pm s$). The *t*-test was used to compare the mean between the two groups and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Physical parameters

The results on physical parameters showed that exposure to sodium fluoride induced lower body mass, liver weight and liver coefficient in meat ducks (Fig. 1A, B and C).

3.2. Effects of sodium fluoride on liver

The results on gross changes (Fig. 2A) in liver of meat ducks and histopathological changes (Fig. 2B) of ducks are presented. The results showed liver lobules were normal and clear in ducks of control group. Microscopic observation of liver of untreated control ducks exhibited that liver cells were arranged like cords, with obvious nucleoli and clear nuclear membranes. In the sodium fluoride treated group, microscopic examination showed that the structure of liver lobules was not clear, increased sinusoid space between the liver cells, liver cells were swollen, cytoplasm was cloudy, liver cells were abnormally arranged, liver cells showed granule and vacuolar degeneration.

3.3. Sodium fluoride induces hepatocyte apoptosis through the mitochondrial pathway

The results on internal mechanisms of hepatocyte apoptosis due to sodium fluoride including expression levels of Bax, Bcl-2 and p53, classical signals of mitochondrial apoptosis pathways (Fig. 3) showed mRNA expression level of Cas-9 was significantly increased in the sodium fluoride group ($P < 0.01$). The mRNA levels of p53, Bax, Cyt-c and Cas-3 were increased and the mRNA expression level of Bcl-2 was decreased in treated ducks. The mRNA levels of P53H and APAF-1 were not significantly different in treated and control group. Results revealed that the expression levels of apoptosis-related proteins in the sodium fluoride treated group were (Fig. 4A, B) different in treated and untreated control group. The protein expressions of p53, Bax, and Cyt-c were increased and protein expression of Bcl-2 was decreased. The protein expression level of Cas-3 was increased with no significant difference compared with the control group. Results showed positive expression of CYT-C by immunohistochemical method on liver of meat ducks due to exposure to sodium fluoride. The positive expression levels of Cyt-C and Cas-3 in sodium fluoride treated group were higher compared to control group (Fig. 5). The results showed that sodium fluoride could induce hepatocyte apoptosis by changing the expression levels of mitochondrial apoptosis-related genes and proteins.

4. Discussion

Liver is the important tissue in the body of different organisms and is responsible for different metabolic reactions, process of oxidation, synthesis and storage of proteins and variety of other physiological

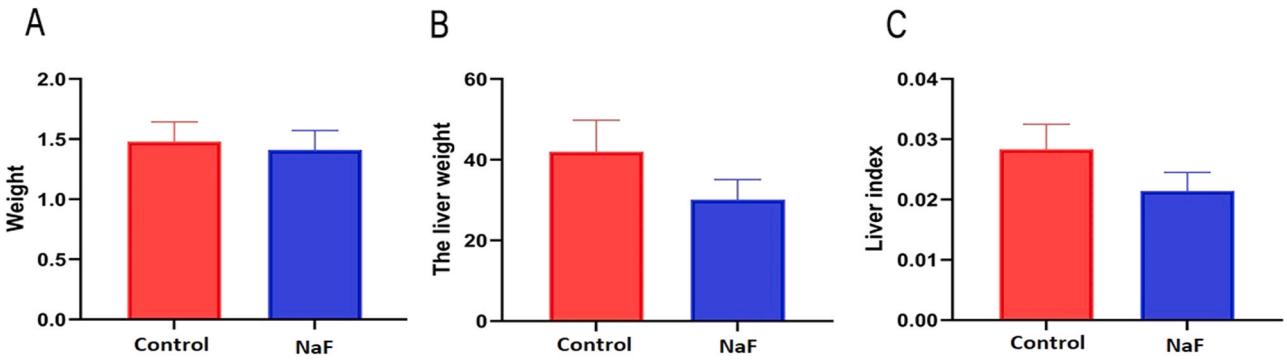

Fig. 1. Effect of sodium fluoride on liver injury. A&B&C) Effects of sodium fluoride on body weight, liver weight and liver index of meat ducks.

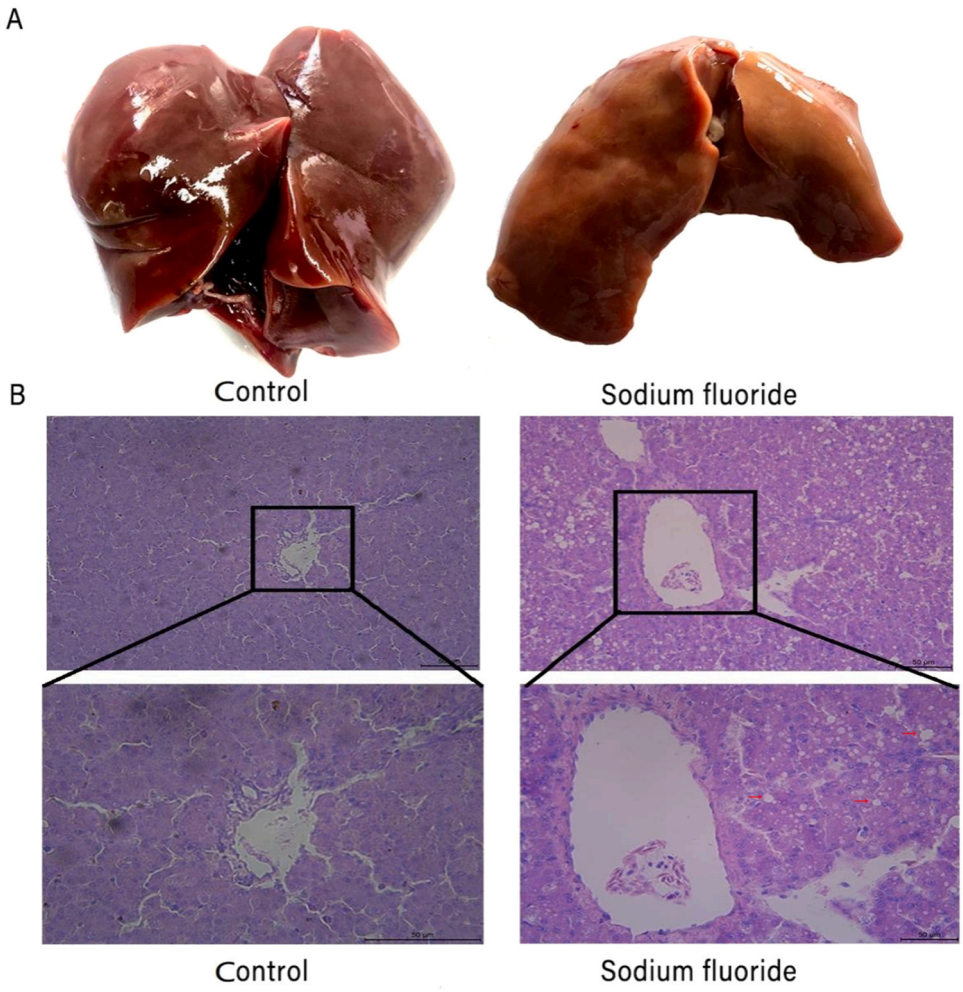

Fig. 2. Histopathological changes in the liver after exposure to sodium fluoride. A) ocular changes of liver. B) Histopathological changes of sodium fluoride after 28 days of exposure, the red arrow indicates cavitation degeneration.

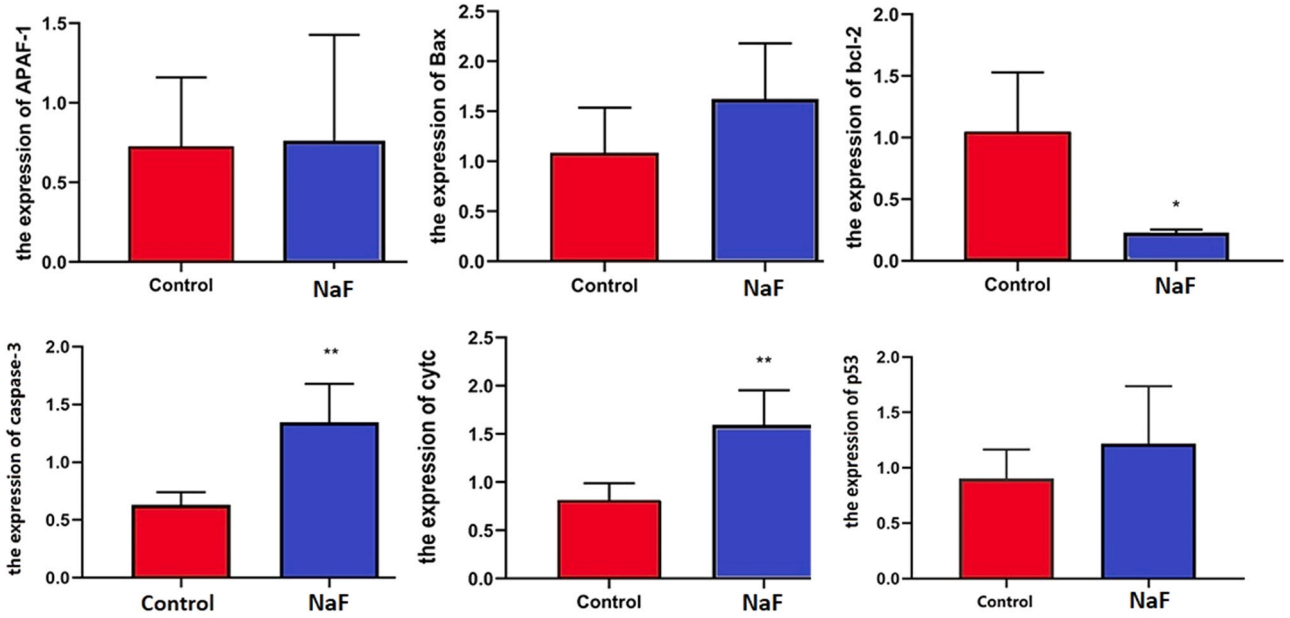

Fig. 3. Effect of sodium fluoride on mitochondrial apoptosis pathway. Effect of sodium fluoride on mRNA expression level of apoptosis-related genes in hepatocytes. (*$p < 0.05$, **$p < 0.01$).

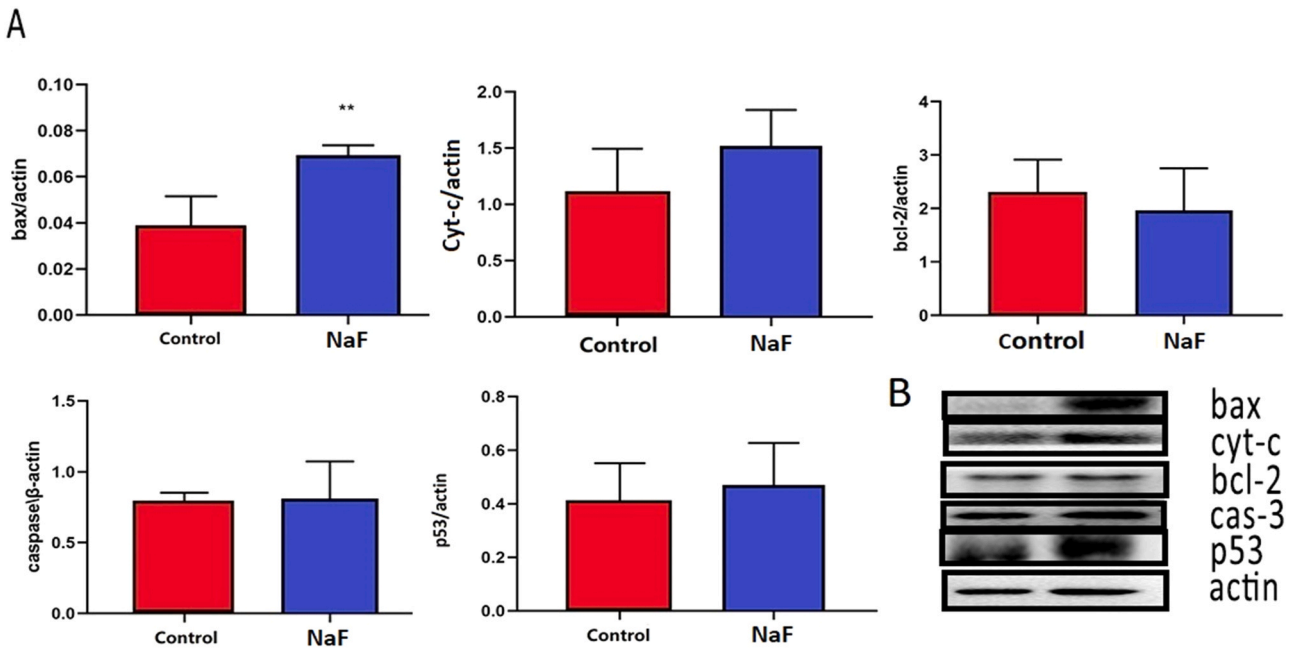

Fig. 4. Effect of sodium fluoride on mitochondrial apoptosis pathway. A) Detection of Bcl-2, Cyt-c, Cas-3 and P53 proteins by Western blot. B) Effects of sodium fluoride on protein expression levels of Bax, Bcl-2, Cyt-c, Cas-3 and P53 in liver. (*$p < 0.05$, **$p < 0.01$).

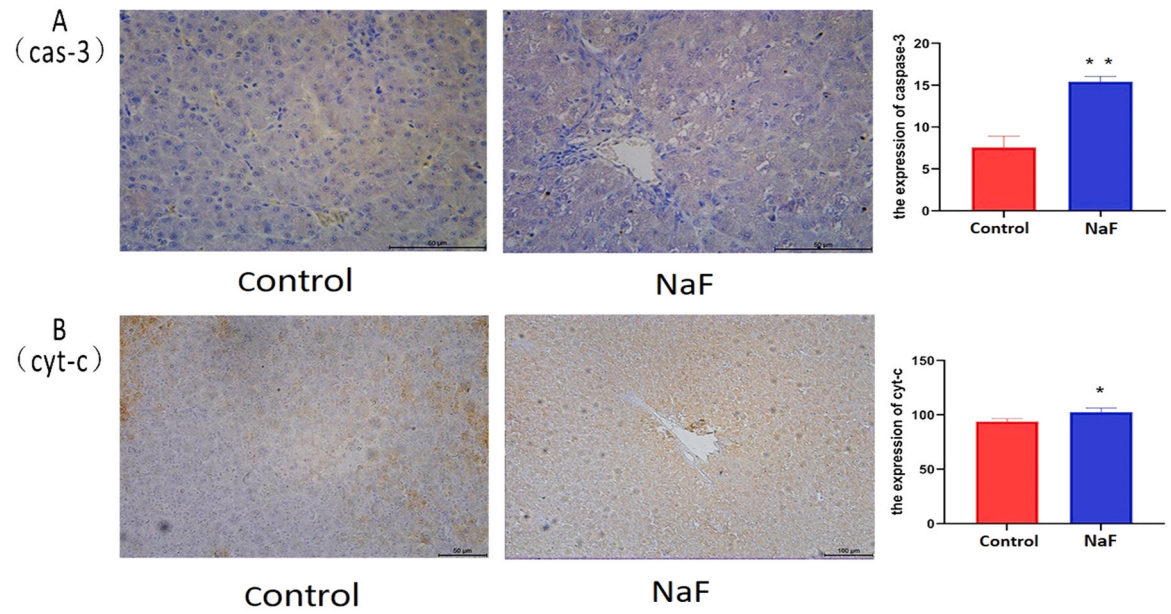

Fig. 5. Effect of sodium fluoride on mitochondrial apoptosis pathway. A) Caspase-3 protein expression was detected by immunohistochemical staining, which was significantly increased compared with the control group. B) The expression of Cyt-C protein was detected by immunohistochemical staining, and compared with the control group. (*$p < 0.05$, **$p < 0.01$).

functions to maintain the life. Different environmental toxicants, metabolic wastes produced in the body and drugs can impair the normal mechanism of detoxification in liver. Fluorosis can be divided into acute fluorosis and chronic fluorosis. Acute fluorosis may cause severe nausea, vomiting, abdominal pain, diarrhea and different other clinical ailments. Studies have indicated that human and livestock animals are more prone to chronic fluorosis which can cause skeletal fluorosis, tooth plaque and pigmentation of black brown enamel (Wang et al., 2018). Moreover, different studies have shown that sodium fluoride is toxic to the liver and can induce disorders in liver leading to metabolic disruption in exposed organisms including human and domestic animals. The results showed impair growth and development of liver, reduced volume, altered organ index, and motor disorders like weak legs and ataxia.

Previous studies have reported that fluoride can enhance liver free radicals, lower values of antioxidant capacity, increase lipid peroxidation, poor activity of hepatocyte in the liver leading to cell damage (Zuo et al., 2018; Rashid et al., 2013). Lu et al. (2017) found that fluoride can damage liver functions through oxidative stress and apoptosis in rats due to exposure to fluoride at 12, 24 and 48 mg/kg for 42 days. Miao et al. (2013) treated mice with 50 mg/L sodium fluoride and found increased apoptosis in liver cells. These results might be related to activation of apoptotic genes due to exposure to fluoride. It is reported that foods rich

in antioxidants can reduce the toxicity caused by fluoride (Jaiswal et al., 2020). Gao et al. (2021) found that selenium (an important component of glutathione peroxidase) plays a significant protective role against sodium fluoride-induced oxidative stress and apoptosis. Selenium treatment enhanced antioxidant enzyme activity, mitochondrial membrane potential and Bcl-2 protein expression and altered the expression of Bcl-2 / Caspase family. Londero et al., 2021 determined that adding rutin (a naturally occurring flavonoid glycoside) to catfish diet enhanced glutathione reductase, decreased lipid peroxidation, and reduced cleaved Caspase-3 expression levels to prevent apoptosis (Londero et al., 2021).

Studies have investigated that apoptosis plays an important role in the development and morphogenesis of embryos, the stability of normal cell populations in tissues, body's defense and immune responses, the cell damage caused by diseases or poisoning, aging and occurrence and growth of tumors. Removal of dead cells is mainly through the molecular mechanisms of nuclear fragmentation, pigment concentration and Caspase activation (Cui et al., 2018). Apoptosis can be divided into four stages: induction initiation, intracellular regulation, implementation, phagocytosis and transportation of apoptotic cells. Bcl-2 family, caspase family, oncogenes such as C-Myc and tumor suppressor gene p53 are key genes in cell apoptosis (Elmore, 2007). Among these caspase gene plays an essential role in the process of apoptosis, Bal-2 is an anti-apoptotic gene which can prolong cell life and Bax gene can promote cell death (apoptosis) (Martinou and Youle, 2011; Xiu et al., 2006). Zhan et al. exposed pigs to 400 mg/kg fluoride for 50 days and observed that fluoride activated CAS-9 and CAS-3 genes and induced apoptosis through oxidative stress (Xiu et al., 2006). Zhao et al. reported that exposure to 25, 50 and 100 mg/L NaF in mice induces significantly increased mRNA expression levels of CAS-3, CAS-8, CAS-12 and p53 while decreased the mRNA expression level of Bcl-2 (Mittal and Flora, 2006; Hong et al., 2013). In this study, the meat ducks were exposed to 750 mg/kg sodium fluoride exhibited that the mRNA expression levels of CAS-3, Bax and CAS-9 were significantly increased, the protein expression of CYT-C was significantly increased, and the protein expression of Bcl-2 was significantly decreased. In addition, previous study has demonstrated that NaF can induce the liver inflammatory responses by activation of NF-κB signaling pathways and the production of pro-inflammatory mediators related to the activation of NF-κB (Chen et al., 2019). NaF increased the expression of genes downstream of NF-κB in the kidneys such as nitric oxide synthase (iNOS), tumor necrosis factor -α (TNF-α), and interleukin-1 β (IL-1β), to induce renal inflammation (Luo et al., 2017). Furthermore, studies are needed to explore the mechanisms of induction of different changes due to sodium fluoride including NF-κB signaling pathways and release of pro-inflammatory factors in duck liver.

Sodium fluoride may also cause other biochemical damage in the liver, suggesting liver damage and functional disorders. Several studies in rats (Zhao et al., 2020), mice (Miranda et al., 2018) and goats (Qu et al., 2008) also reported similar results. Previously different histological changes in livers of mouse exposed to sodium fluoride like extensive degenerative changes ranging from ballooning degeneration, to complete cell necrosis and infiltration of mononuclear cells in the hepatic lobules have also been observed (He et al., 2015). Earlier literatures reported excessive intake of fluoride induces degenerative changes in liver such necrosis of nuclei, disorganized hepatocytes and other pathological changes in liver of rats. In current experimental study, body weight and liver weight of meat ducks exposed to 750 mg/kg were significantly reduced. This result suggests that sodium fluoride induces liver injury, which is consistent with previous reports.

Therefore, it can be suggested from the findings of our experimental trial that sodium fluoride can cause liver damage by inducing cell apoptosis, and long-term intake of sodium fluoride in feed can adversely affect the liver functions in leading to poor growth and development in exposed organisms including ducks. In this study, the mechanisms of fluoride exposure on apoptosis and injury to liver cells provided a new theoretical basis for estimation of toxic effects of fluoride exposure in ducks. The results of this experimental research can be useful for the treatment of diseases caused by fluorosis in poultry, to increase the economy of poultry industry in China, and improve the vigilance of fluorine-containing drugs in poultry industry in China.

In this study, we found that sodium fluoride induces liver injury and affects cell apoptosis and autophagy by regulating proteins and genes related to mitochondrial apoptosis pathway.

Ethical approval

All the experiments were performed after the approval of the Institutional Animal Welfare and Research Ethics Committee of South China Agricultural University, Guangzhou, China.

Consent to participate and consent to publish

All authors have participated in this study and given the consent to publish this data.

CRediT authorship contribution statement

Zhuanxu Ouyang, Bijing Yang, Shanshan Zhu, Suge Lu, Yingwei Liu, Yangwei Li, Yuanliang Li, Jiangnan Yi, Khalid Mehmood, Riaz Hussain and Jianying Guo were responsible for study conception and design; Hui Zhang, Muhammad Ijaz, Zhaoxin Tang and Ying Li were involved in the drafting of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analyzed during this study are included in this manuscript.

Acknowledgment

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