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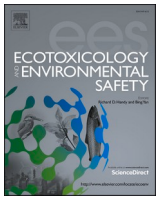

Long-term exposure to the fluoride blocks the development of chondrocytes in the ducks: The molecular mechanism of fluoride regulating autophagy and apoptosis

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ABSTRACT

Long-term exposure to excessive fluoride causes chronic damage in the body tissues and could lead to skeletal and dental fluorosis. Cartilage damage caused by excessive fluoride intake has gained wide attention, but how fluoride accumulation blocks the development of chondrocytes is still unclear. Here, we report a negative correlation between the length and growth plate width after NaF treatments via apoptosis and autophagy, with shrinkage of cells, nuclear retraction, dissolution of chondrocytes. Whereas, fluoride exposure had no significant effect on the number and distribution of the osteoclasts which were well aligned. More importantly, fluoride exposure induced apoptosis of tibial bone through CytC/Bcl-2/P53 pathways via targeting Caspase3, Caspase9, Bak1, and Bax expressions. Meanwhile, the Beclin1, mTOR, Pakin, Pink, and p62 were elevated in NaF treatment group, which indicated that long-term excessive fluoride triggered the autophagy in the tibial bone and produced the chondrocyte injury. Altogether, fluoride exposure induced the chondrocyte injury by regulating the autophagy and apoptosis in the tibial bone of ducks, which demonstrates that fluoride exposure is a risk factor for cartilage development. These findings revealed the essential role of CytC/Bcl-2/P53 pathways in long-term exposure to fluoride pollution and block the development of chondrocytes in ducks, and CytC/Bcl-2/P53 can be targeted to prevent fluoride induced chondrocyte injury.

1. Introduction

Fluoride is widely distributed in nature in various forms and is extensively used. Fluoride can be absorbed rapidly through the intestinal mucosa and interfere with the main metabolic pathways in humans (Singh et al., 2018; Zhou et al., 2020). As a semi-essential trace element for animals, low levels of fluoride are beneficial to dental health, prevent tooth decay and improve bone strength (Singh et al., 2018; Zhou et al., 2020). However, in areas with excessive fluoride contents in the water, fluoride is a severe threat to human and animal health, causing severe health problems worldwide (Liu et al., 2021a). The World Health Organization (2004) recommends an upper limit of 1.5 mg/L fluoride in drinking water; however, long-term improper use of fluoride through water or food causes discoloration of the teeth and decreases the chewing ability (Solanki et al., 2020). It is well known that excessive fluoride intakes can adversely affect bones, liver, kidneys, and reproductive organs; among them, skeletal dysplasia caused by excessive fluoride exposure is the most significant (Li et al., 2020a, 2020b; Liang et al., 2020; Solanki et al., 2020). Reports indicated that about 260 million people in 20 countries suffer from fluorosis threat caused by

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endemic fluoride pollution or improper use. China is one of the countries with the most serious epidemic of fluorosis in the world (Herath et al., 2018). Fluoride pollution is divided into natural pollution and industrial pollution. Among them, natural pollution results from mineralization of fluorinated minerals, rainfall, emission of volcanic gases, and spread of soil dust. Industrial fluoride pollution mainly comes from industrial aluminum electrolysis, fossil combustion, phosphate fertilizer, ceramics, electronics, glass and other industrial wastewater discharge (Addison et al., 2020; Bibi et al., 2017; Solanki et al., 2020). Fluoride pollution in the water has become a common phenomenon, and drinking such water is associated with an increase in health problems. Therefore, fluoride has aroused broad public concern in recent years.

Endochondral ossification is the main stage of bone development, which is a unique biological event for bone (Sheehy et al., 2013). The development of cartilage has four developmental processes, which are divided into resting, proliferative, hypertrophy and calcification zones. The cells at different periods have different shapes and secrete different proteins. These chondrocytes differ in expression of intracellular enzymes, extracellular matrix components, hormone receptors, morphology, growth factors, transcription factors, and secretory capacity (Mehmood et al., 2018; Qamar, 2019, 2020; Zhang et al., 2018a, 2018b, 2020). The proliferation rate of chondrocytes in the resting zone is low, and the Sox9, parathyroid hormone-related polypeptides (PTHrP), collagen II (Col II), and Aggrecan are the landmark molecules of cell secretion in this zone (Tian et al., 2013; Yao et al., 2020). In the proliferating zone, chondrocytes are arranged in a columnar and flat shape, with enhanced ability to secrete the cartilage matrix proteins (CMP), and the expression of Aggrecan, collagen (Col XI) and Col II are further increased. Aggrecan and Col X are the matrix components synthesized by the chondrocytes in the hypertrophic zone (Liu et al., 2021b; Tian et al., 2013; Yao et al., 2020). The surrounding capillaries invade the mineralized tissue area by the periosteum in the calcification zone, while gergenbauer cells and osteoclasts secrete bone matrix to replace the mineralized cartilage matrix gradually and finally complete the calcification. Simultaneously, blood vessels in the metaphysis penetrate into the growth plate, and the rich distribution of blood vessels in the growth plate ensures the transport of nutrients to the bone and the excretion of waste products of the cell metabolism (Zhang et al., 2018a, 2018b). However, some reports have shown that cartilage damage is more common among the bone injuries caused by fluorosis, including chondrocyte necrosis, metabolic changes of cartilage proteoglycan, decreased ability of collagen synthesis and imbalance in the enzyme activity of the cartilage tissue (Death et al., 2018; Gao et al., 2020a, 2020b; Wu et al., 2018). Wu et al. (2018) found that a high dose of fluoride causes IHH downregulation, depresses the early chondrocyte development, which leads to inhibition of the endochondral ossification. The contents of HS in the cartilage matrix of the growth plate in rats increased abnormally with exposure to fluoride. The expression of PTHrP increased and the expression of IHH decreased in the chondrocytes of the growth plate in the rats with fluorosis, suggesting that fluoride affected the proliferation and differentiation of the chondrocytes by inhibiting the IHH/PTHrP negative feedback (Donkelaar and Huiskes, 2007; Gao et al., 2020a, 2020b; Wu et al., 2018). Fluoride also upregulated the FGFR3 and STAT1 expression, thereby inhibiting the chondrocyte proliferation (Gao et al., 2020a, 2020b). Although much research has been done to date on the role of fluoride in bone formation, the exact molecular mechanism by which fluoride may affect bone development is not clear.

In the present investigation, we aimed to explore the autophagy and apoptosis in the tibial cartilage induced by long-term fluoride exposure and the effects of fluoride toxicity on proliferation and differentiation of tibial osteoclasts and osteoblasts to demonstrate the mechanism of fluoride exposure on the bone development.

2. Materials and methods

2.1. Ethics approval

The current study has been approved by the Ethics Committee of the South China Agricultural University (SCAU), Guangzhou, China.

2.2. Groups and treatment

A total of 14 ducklings with similar initial weight were selected from the experimental animal center of SCAU, Guangzhou. Then, the ducks were randomly divided into two equal groups after acclimatization for 1 week. All ducklings were offered a standard diet freely and housed in the metal cages with the recommended environment according to the suggestion of the experimental animal center of SCAU. The control group was given a standard diet ad libitum. The F group was offered NaF at 750 mg/kg in feed. After 28 days, ducks were sacrificed by injecting pentobarbital (25 mg/kg). Samples of the tibia were immediately collected and fixed in 4% paraformaldehyde (Aladdin, 95%), and remaining samples were frozen in liquid nitrogen for further use. The weight of the liver, lung, heart, bursa, kidney and tibia was determined by electronic balance. The length and weight of the tibia, and width of growth plate (GP) were measured by the electronic balance, and Vernier calipers, respectively.

2.3. H&E staining

According to our previous study, the tibia samples of ducks were processed histological analysis (Zhang et al., 2018a, 2018b). Briefly, the tibial tissues were fixed in 4% paraformaldehyde for at least 24 h and decalcified in 10% ethylenediamine tetra-acetic acid (Servicebio, Wuhan). Subsequently were embedded in paraffin. Tissue Sections (4–5 μm) were cut and placed on 3-Aminopropyl-Triethoxysilane--coated slides, and then the slides were dried overnight (37 °C) and stained with hematoxylin/eosin stain.

2.4. Transmission electron microscope analysis of chondrocytes

For the apoptosis assay and the micromorphological observation, the transmission electron microscope analysis was conducted according to the previously reported protocol. Briefly, the resting area of the tibia was cut approximately two-centimeters and then fixed in glutaraldehyde-paraformaldehyde (2.5%) overnight at 25 °C, and the samples were washed with phosphate-buffered saline. Afterward, the tissue samples were fixed in 1% osmium tetroxide, dehydrated with graded ethanol (30%, 50%, 70%, 80%, 90%, and 95%), and embedded in the epoxy resin. The mitochondrial structure was observed by using the transmission electron microscope.

2.5. RT-qPCR analysis

The specific primer sequences for evaluating the apoptosis, autophagy, and cartilage development genes expressions were designed by using the Primer Premier 6.0 and are listed in Table 1. Total RNA from the growth plate of tibia was obtained using the TRIzol (Invitrogen, 15596-026), and then reverse transcribed into cDNA (50 μl) by using the reverse transcription cDNA kit (Vazyme, R323-01). Then, the Step One-Plus™ Real-Time PCR System was performed to determine the mRNAs expression level. Firstly, RT-qPCR was carried out with 1 μl cDNA, 10 μl SYBR reaction mix (Transgen Biotech), 1 μl each of forward and reverse primers, and 7 μl distilled water. The reactions were performed with 95 °C for 30 s, 35 amplification cycles at 95 °C for 8 s, 55–58 °C for a time of 30 s and 70 °C for 30 s. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as per a previous study (Zhang et al., 2018a, 2018b).

Table 1
Primers used for the RT-qPCR.

Genes name	Forward sequence (5 →3)	Reverse sequence (5 →3)
Caspase-3	CGGGTACGGATGTAGATGCT	GGGGCCATCTGTACCATAGA
Bax	CTTCTGCTTCCAGACCAAGG	TCAGCGTGTTCTTCCTGTTG
Bcl2	GAGTTCTCCCGTCGCTACC	CGGTTCAGGTACTCGGTCAT
Cyt C	AAGTGCTCCCAGTGCCATAC	CCGCGAAAATCATCTTTGTT
P53	ACAGCAGACTCCTGGGAAGA	GGGGTATTCGCTCAGTTTCA
Caspase-9	GAACTGGATCCGATGTGGAC	TTCCGTCCGTTCCATAAATC
Bak1	CCGCTACCAACAGGAGAGAG	GCGTCGTACCGCTTGTTAAT
APAF1	TGGAATTGGCAGTTGAATGA	AGGAAAGAACAGCACCTCCA
Parkin	TGATGGGCTTTGTGAAATGA	TTCAGCGTGACACAGAGGAC
Pink1	CACGCTCTTCTTGGTGATGA	GAGGTCTCTGTGCGCTATCC
Beclin1	GCTCCCCTTGTACTGCTCTG	TTAGGGCTTTTGTCCATTGC
mTOR	AGTGGTCCAGTGGAAACAGG	GATCTCGAGCCATGGGATTA
Col II	GAGCGGAGACTACTGGATCG	TTCTTGTCTTTGGCCTTGCT
ALP	TTCACCTCCATCCTCTACGG	TGACTGTGCCTGGTAGTTGG
GAPDH	GAGGGTAGTGAAGGCTGCTG	CACCACACGGTTGCTGTATC

2.6. Western blot analysis

Tibia samples were washed with Phosphate Buffer Saline for 3–5 times to remove the blood, and the protein samples of tibial tissues were obtained by using the Total Protein Extraction Kit, and denaturation treatment for 10 min, then the BCA protein assay kit (Dingguo, Beijing, China) was used to check the total protein concentration. The SDS-PAGE was done to separate the proteins, then transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membrane was incubated in 5% skimmed milk, and then incubated with the diluted primary antibodies against CytC (1:300; Bioss, China), p53 (1:300; Bioss, China), Bcl-2 (1:300; Bioss, China), Beclin1 (1:300; ABclonal, China), mTOR (1:300; Bioss, China), Parkin (1:500; Wanleibio, China), Pink (1:300; Bioss, China) and GAPDH (1:1500; Bioss, China) at 4 °C overnight, and incubated with secondary antibody at room temperature for 30 min. The images of proteins band were captured with an imaging system (TransGen Biotech Co., China).

2.7. Statistical analysis

In the current study, the experiments were performed at least three times, and the student *t*-test were analyzed by using SPSS 19.0 software and Graph Pad Prism 6.0 (Graph PadInc., La Jolla). The results were presented as the means ± standard deviation. The difference between means at $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Toxic effects of NaF on organs index, tibial bone indicators

The results showed a decrease in ducks' body weight in NaF treatment group compared to the control group. Fluorosis caused major gross changes in the liver, including enlarged liver with yellow color (Fig. 1A). Simultaneously, the morphological examination of the proximal tibial growth plate showed that the proximal tibial growth plate's morphological examination showed less-mineralization and less-vascularization in cartilage mass (white cartilage wedge), which was remarkably increased in some ducks compared to control group. To further study, the severity of the "white cartilage wedge", we conducted quantitative statistics (SCORE) based on our previous study. The NaF fed ducks showed a score from 1 to 4, while 6 samples had a score ≥ 1. However,

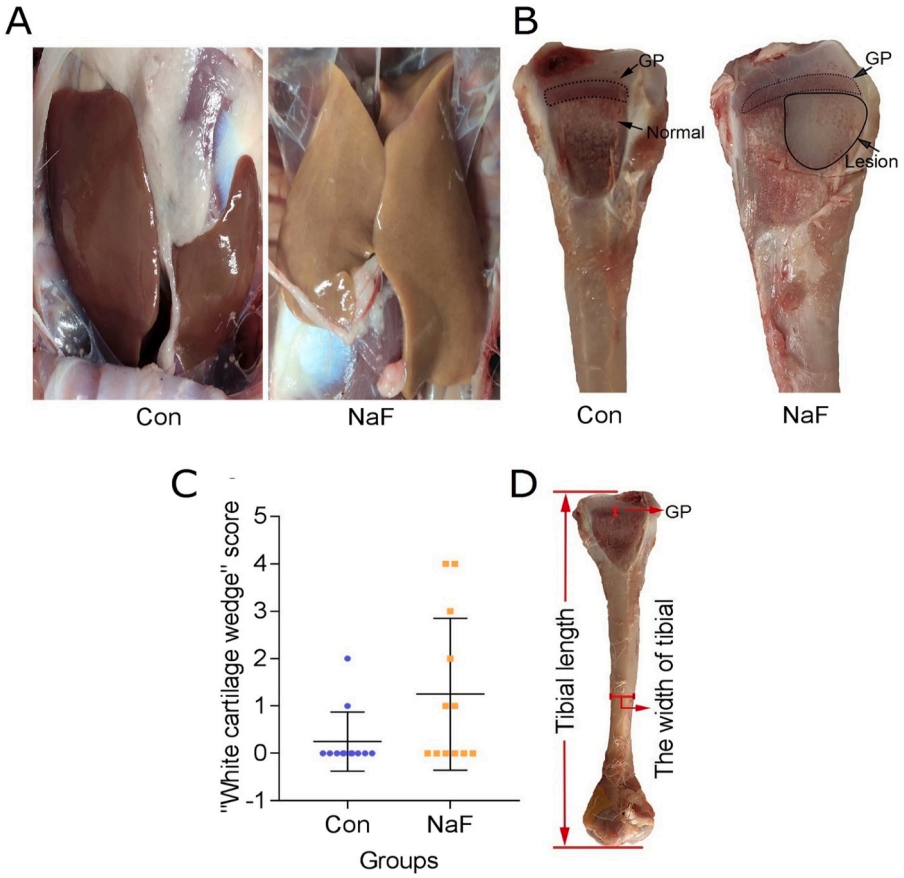

Fig. 1. Gross photographs of liver and tibia with the toxic effects of NaF. (A) Physical examination of the liver showed a poor condition in the NaF treatment group; (B) the changes of tibial metaphysis; (C) the white cartilage wedge; (D) changes of tibial bone-related evaluation indexes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

only 2 samples in the control group showed the score ≥ 1 (Fig. 1B,C). The tibial bone performance indicators (including length, width, weight, and width of the tibial growth plate) are presented in Fig. 1D and Fig. S1. The results indicated that the length of tibia and GP width were decreased in the NaF group, but the difference was not significant between normal ducks and NaF group ($P > 0.05$), while the weight and width of the tibial bone showed no significant change between two groups. The results indicated a perfect negative correlation between tibial length and GP width after the NaF treatment.

3.2. Fluoride-induced chondrocyte histological changes in the tibia

The clinical symptoms of the ducks showed no obvious clinical symptoms in the NaF group ducks and the normal ducks. The osteoclast assay is shown in Fig. S2. The trap staining results indicated that the fluoride exposure had no significant impact on the number and distribution of osteoclasts that were well aligned. Our previous studies have shown that fluoride reduces the tibial length and GP width (Fig. S2). Does fluoride play a toxic role by regulating chondrocyte development? Therefore, we made morphological observations on the chondrocytes in the metaphysis of the tibia. The results showed that the chondrocytes in the GP of tibia metaphysis were light and widened, the nuclei were condensed and some of the cells were lysed as observed by electron microscopy (Fig. 2). The number of hypertrophic chondrocytes decreased with the nuclear retraction, dissolution and apoptosis in the NaF-treated ducks as compared with normal ducks (Fig. 2).

3.3. Fluoride exposure alters chondrocyte development

The ALP is an essential enzyme in new bone formation, it is involved in the bone and cartilage calcification, tissue metabolism, mineralization of the bone, and collagen II (Col 2), which is the main component of the articular cartilage matrix. It plays a key role in maintaining the integrity and normal function of the cartilage structure. Here, our study showed that ALP and Col 2 expression were significantly lower in long-term fluoride exposed ducks than the normal ducks (Fig. 3).

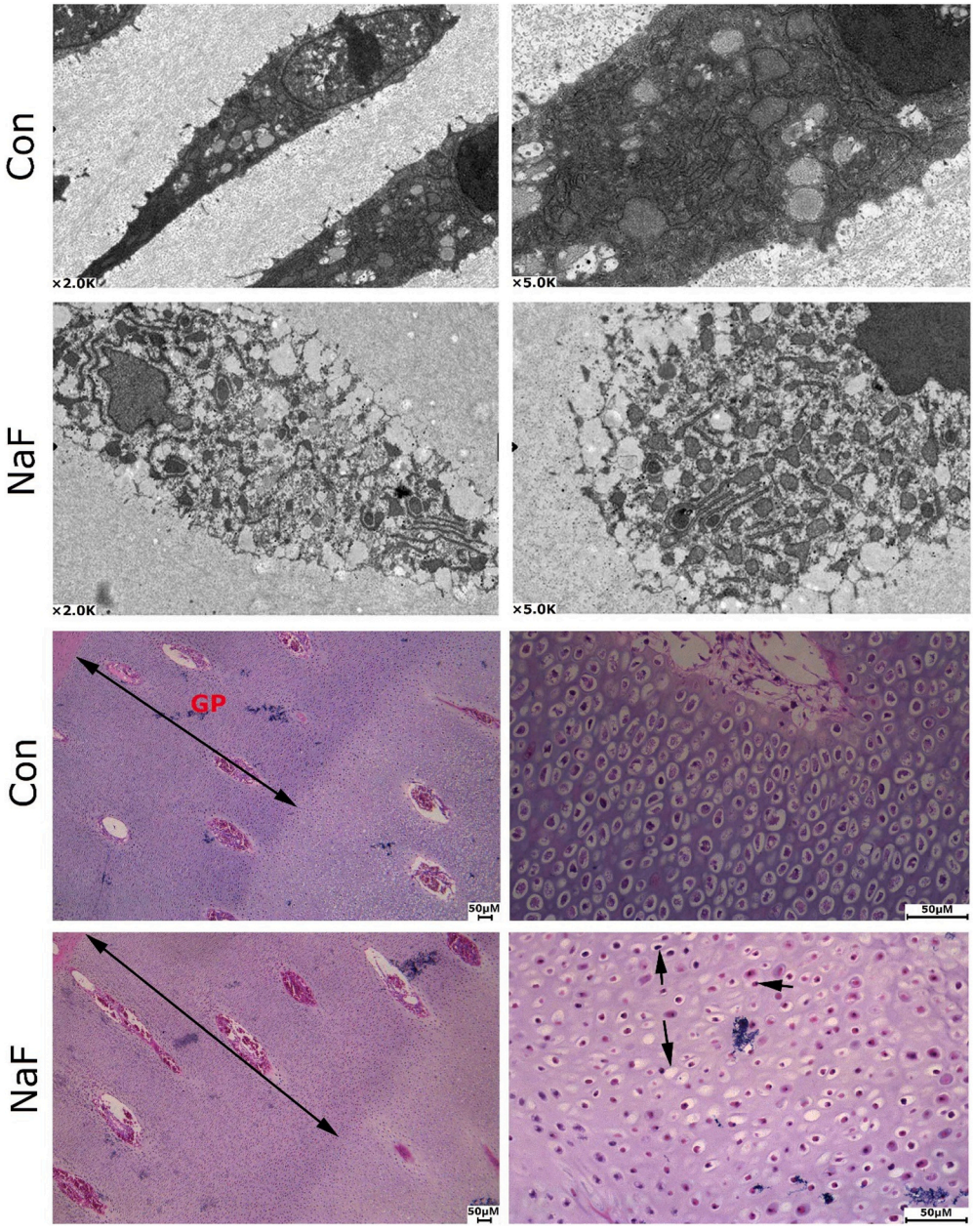

Fig. 2. Micromorphological observation of tibial bone from different experimental groups. (A) Trap staining of osteoclast showed no significant change; (B) gross changes and histopathological observation of resting area in tibial bones, and the projection electron microscope observation of chondrocyte in different experimental groups; (C) the histopathology examination of chondrocyte and cell morphological in the proliferative and hypertrophic zone of tibial bone between normal and NaF treated ducks.

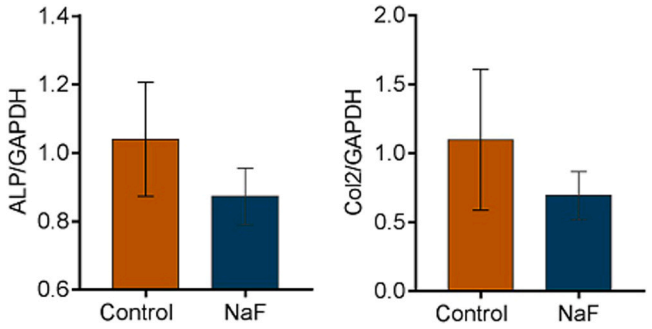

Fig. 3. Effects of fluoride exposure on chondrocyte development of tibial bone in ducks.

3.4. Fluoride exposure induces apoptosis of tibial bone, associated with CytC/Bcl-2/P53 pathway

Effects of NaF exposure on the mRNA levels of apoptosis-related genes in the chondrocyte of tibial bone are shown in Fig. 4. As compared with normal ducks, the mRNA levels of P53, Caspase-3, Bak1, and Caspase-9 were significantly increased in the fluoride-exposed group ($P < 0.05$); while the mRNA expression levels of Bcl2 and Bax was decreased in the fluoride group. The effects of NaF exposure on the protein's expressions of apoptosis-related genes in chondrocytes are shown in Fig. 5. Results showed that the apoptosis proteins CytC and p53 were highly expressed in NaF group, especially the p53 ($P < 0.01$). Conversely, compared to the NaF group, the Bcl2 expression levels were high in normal ducks. The current study indicates that long-term fluoride exposure impaired the chondrocyte functioning, which results in tibial bone damage and apoptosis.

3.5. Fluoride exposure-induced chondrocyte autophagy of tibial bone in ducks

The underlying mechanism involved in fluoride induced autophagy in chondrocyte was studied. In the current study, autophagy was observed in chondrocytes by confirming mRNA levels of Beclin1, mTOR, Pakin, and Pink after fluoride treatment, as shown in Fig. 6A. Results showed that the mRNA levels of Beclin1, mTOR, Pakin, and Pink were elevated in NaF treated group compared with the normal group. Whereas, the protein expressions of Beclin1, Pink1, and p62 were up-regulated in NaF treatment group compared to normal groups (Fig. 6B).

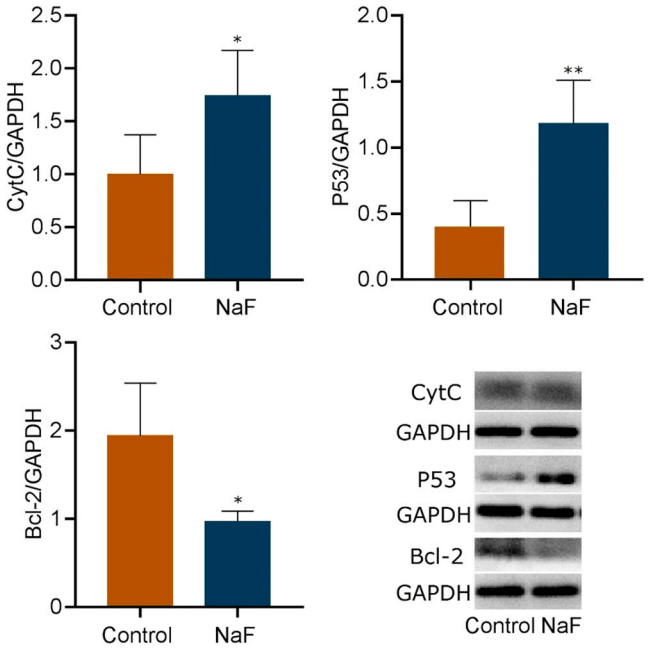

Fig. 5. The effects of NaF exposure on the protein expressions of apoptosis-related genes in chondrocytes. (A) Quantitative analyses of the band density revealed that the level of the apoptosis proteins of CytC, P53, and Bcl-2 expressions normalized to GAPDH in duck chondrocyte of tibial bone; (B) representative images of the western blotting band associated apoptosis proteins in chondrocyte. All values are presented as mean ± SD. *P < 0.05; **P < 0.01.

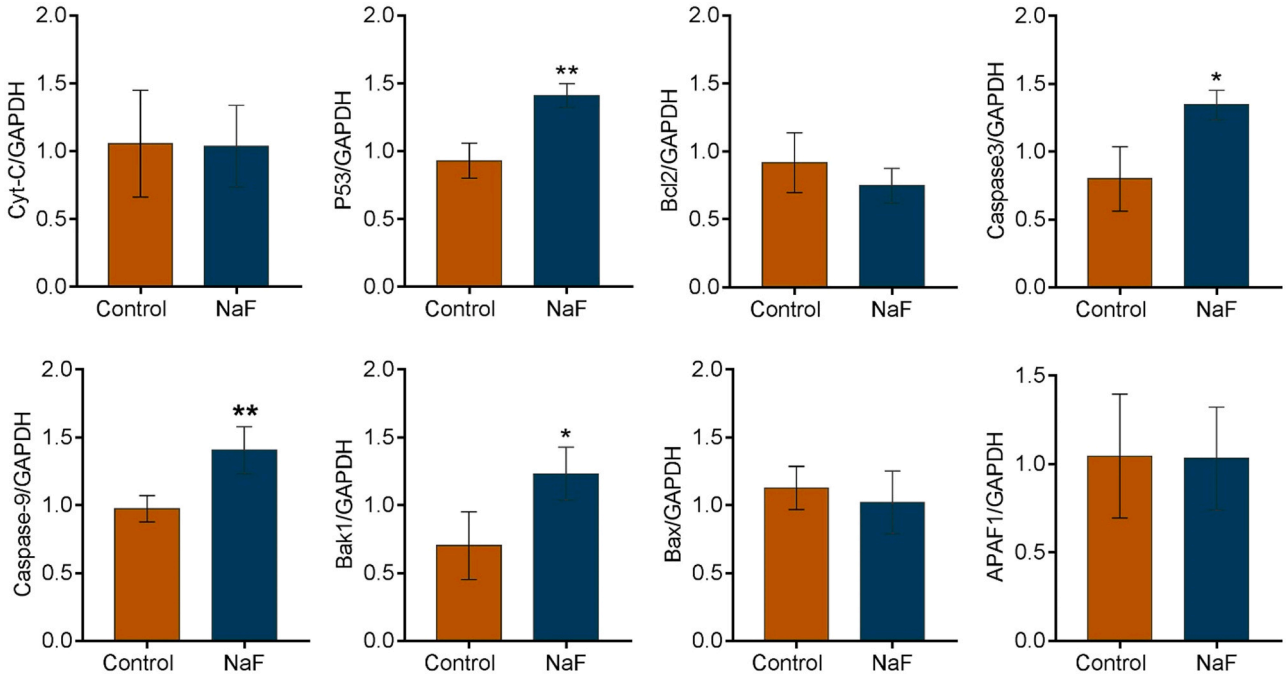

Fig. 4. Fluoride exposure induces apoptosis of tibial bone associated with CytC/Bcl-2/P53 pathway. The mRNA expression levels of CytC, P53, Bcl2, Caspase-3, Caspase-9, Bak1, APAF1 and Bax. All values are presented as mean ± SD.*P < 0.05; **P < 0.01.

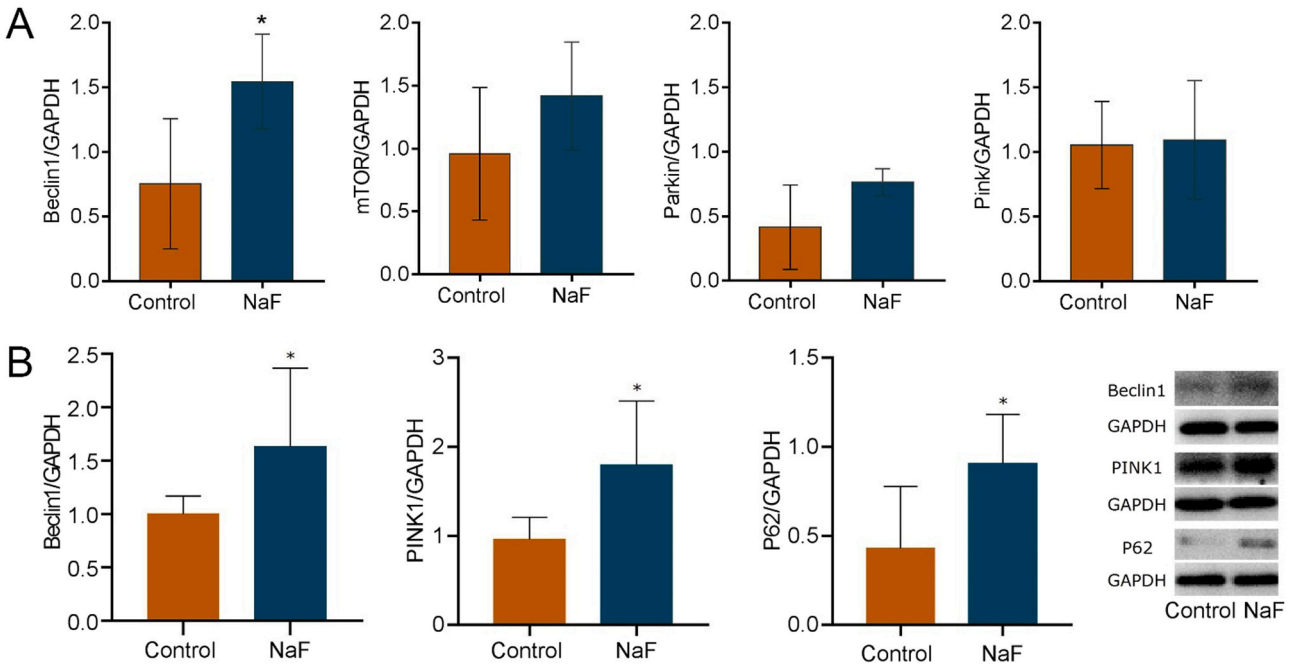

Fig. 6. Fluoride exposure-induced chondrocyte autophagy of tibial bone in ducks. (A) Expression levels of the autophagy marker genes (Beclin1, mTOR, Parkin, and Pink). (B) A quantitative analysis of the band density revealed that the level of Beclin1, Pink1, and p62 proteins in the fluoride exposure-induced chondrocyte damage was highly notable. All values are presented as mean ± standard deviation (M ± SD). *P < 0.05.

4. Discussion

A long-term excessive intake of fluoride causes systemic physiological and pathological changes called endemic fluorosis (Johnston and Strobel, 2020; Zhao et al., 2018). The long-term living of human and animals in fluoride contaminated environments (such as industrial waste, water/soil pollution, food/drugs inappropriate use, etc.) cause nerve damage, skeletal toxicity, immune dysfunction and reproductive injury (Suzuki et al., 2015). The previous study indicated that long-term contact of fluoride has typical hepatotoxicity and nephrotoxicity, which lead to renal tubular degeneration, inflammatory changes in rats, as well as apoptotic changes such as chromatin agglutination, endoplasmic reticulum dilatation, mitochondrial swelling or dissolution, cell membrane rupture (Etin et al., 2020; Ito et al., 2009; Li et al., 2020a, 2020b) accompanied by apoptosis. Fluoride is one of the semi-essential trace elements in animals for metabolism. Low dose fluoride is beneficial to the normal development of bones and plays an integral role in the activities of the nervous system (Sharma et al., 2017). However, an excessive intake of fluoride can produce intense local stimulation, adversely affect the synthesis of cartilage tissue protein, which leads to osteogenesis and chondrocyte metabolism disorder and promote the necrosis of cartilage tissue (Chao et al., 2018). Fluoride is a practical element for preventing caries, but long-term excessive fluoride exposure can cause environmental health hazards (Etin et al., 2020). A previous study reported that excessive fluoride intake in the body would seriously affect the function of dental ameloblasts, inhibit the proliferation and differentiation of ameloblasts, and then affect the formation of tooth enamel and cause dental fluorosis (Li et al., 2016).

Fluoride exposure of longer time can cause DNA damage in cells at high doses, which activate DNA-dependent protein kinase, phosphorylate p53 protein, promote the activation and expression of downstream pro-apoptotic genes, and then induce apoptosis (Etin et al., 2020; Ito et al., 2009; Li et al., 2020b, 2020a). In the present study, we found that the chondrocytes in the growth plate of tibial metaphysis were sparse and widened, the nuclei were condensed, and some of the cells were lysed as observed by electron microscopy. The number of hypertrophic chondrocytes decreased with the nuclear retraction, dissolution and apoptosis in NaF-treated ducks as compared with normal ducks. Current research showed that fluorosis induces tibial bone injures which was characterized by abnormal changes in the chondrocyte leading to apoptosis. Previous study indicated that fluoride induces endochondral ossification suppression mainly due to the inhibition of chondrocyte proliferation and hypertrophy (Wu et al., 2018). Rats treated with fluoride showed abnormal morphology of chondrocytes with decreased cartilage matrix and cartilage septae thickness and reduced the matrix volume between chondrocyte columns in growth plates and irregular and thinned trabeculae lamella (Xiu et al., 2006). Fluoride inhibits the endochondral ossification in long bones. Studies have shown that excess fluoride exposure increases the Bax, Caspase3, Caspase8 and Caspase9 expression levels and reduces the bcl-2 expression in the liver (Cao et al., 2013; Simon et al., 2014). In the current study fluoride-induced apoptosis showed that CytC, p53, Caspase3, Bak1, and Caspase9 expression levels were increased significantly in fluoride exposed ducks. At the same time, the Bcl-2 and Bax were decreased, which indicated fluoride exposure impaired the chondrocytes of tibial bone, caused damage and apoptosis.

Apoptosis is programmed cell death, and autophagy can inhibit oxidative stress, so it plays a protective effect on cells, but excessive autophagy can activate apoptosis (Salminen et al., 2013). According to the expression of the autophagy pathway, autophagy is regulated by mTOR, Beclin1, P53, p62, and Bcl-2 that can reflect the condition of apoptosis. Moreover, there is a specific connection between fluoride-mediated autophagy and apoptosis (Salminen et al., 2013; Suzuki and Bartlett, 2014). Beclin1 is considered an inducer of autophagy and an important bridge between autophagy and apoptosis via regulating autophagy activity in cells (Cui et al., 2019; Li et al., 2013). Meanwhile, Beclin1 and LC3 proteins were increased in naive chondrocytes. In our study, the mRNA level of Beclin1, mTOR, Pakin, and Pink was elevated in NaF treated group, which indicated that NaF triggered the autophagy in tibial bone and produced chondrocyte injury. Unexpectedly, the expression of p62 was also significantly elevated in the current study, suggesting that the accumulated autophagosomes resulted from impaired autophagy degradation rather than increased formation. Zhang et al. (2016) found that the expression level of p62 was

significantly elevated in rat after NaF exposure, consistent with the strong accumulation of p62 in testicular cells. These results indicated that the decrease of tibial cartilage development ability of ducks exposed to fluoride is closely related to fluoride-induced apoptosis and autophagy of chondrocytes, which will provide new insights for the study of bone injury caused by fluoride exposure.

5. Conclusions

The present study first provided novel evidence that long-term excessive fluoride exposure induces chondrocytes apoptosis and autophagy in the tibial bone of ducks and results in chondrocyte injury. Notably, we have revealed that the important regulatory mechanisms are mediated by CytC/Bcl-2/P53 pathways via targeting Caspase3, Caspase9, Bak1, and Bax expressions. Importantly, our study provides the original insights of fluoride-induced chondrocyte apoptosis by activating autophagy-related genes, i.e., Beclin1, mTOR, Pakin, Pink, and p62 expression, which demonstrate that fluoride exposure is a risk factor of cartilage development.

CRediT authorship contribution statement

Yajing Wang: Conceptualization, Visualization, Methodology, Writing - original draft. **Aoyun Li, Khalid Mehmood, Rao Zahid Abbas, Riaz Hussain, M. Tariq Javed and Ying Li**: Investigation. **Yung-Fu Chang, Lijun Shi and Zhaoxin Tang**: Writing - review & editing. **Hui Zhang**: Writing - review & editing, Resources, Project administration, Supervision, Funding acquisition.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112225.

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