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Sodium fluoride causes oxidative damage to silkworm (*Bombyx mori*) testis by affecting the oxidative phosphorylation pathway

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ABSTRACT

Bombyx mori was used to study the molecular mechanism of fluoride induced reproductive toxicity. In our previous study, we confirmed the physiological and biochemical effects of NaF on reproductive toxicity, and we found that the molecular mechanism of NaF induced reproductive damage may be associated with the oxidative phosphorylation pathway. To further study the function of NaF exposure on the oxidative phosphorylation pathway in the testis in *Bombyx mori*, and the relationship between oxidative phosphorylation and oxidative stress, we measured the changes in the main ROS (O_2^- and H_2O_2) in the testis, the activity of the main electron transport chain complex enzymes in the oxidative phosphorylation pathway and the transcription levels of the corresponding genes; we additionally performed pathological observations of the silkworm testis after exposure to 200 mg/L NaF solution for different times. The content of O_2^- and H_2O in the silkworm gonads increased significantly at 24 h, 72 h and 120 h after NaF stress. The activity of mitochondrial complexes I, III, IV and V in the silkworm testis was significantly greater than that in the control group. RT-PCR analysis suggested that the mRNA transcription levels of *NADH-CoQ1*, *Cyt c reductase*, *Cyt c oxidase* and *ATP synthase* genes were up-regulated significantly. Histopathological investigation showed that the damage to the silkworm testis was more severe with increasing NaF exposure times. These results indicated that NaF stress affects the NADH respiratory chain of the mitochondrial electron transport chain and increases the activity of related enzyme complexes, thus destroying the balance of the electron transport chain. Subsequently, the content of ROS in cells significantly increases, thus resulting in oxidative stress reactions in cells. These results enable better understanding of the testis-damaging molecular toxicological mechanism of NaF.

1. Introduction

Fluorine is one of the essential trace elements in animal bodies and is of great significance to the health of the body (Martinez-Mier, 2011). Intake of appropriate amounts of fluoride positively affects the prevention of dental caries (Carey, 2014), whereas excessive intake causes damage in animals, including invertebrates (Camargo, 2003; Pradesh et al., 2011; Ullah et al., 2017). According to the WHO, approximately 200 million people are at risk because they are continually exposed to fluoride-containing (over 1.5 mg/L) drinking water (Ayoob and Gupta, 2006). Excess fluoride has been shown to be toxic to many organs, including the brain, thyroid, kidney, liver and testis (Barbier et al., 2010).

Notably, the male reproductive toxicity of fluoride has received attention in recent years, particularly in vertebrates (Long et al., 2009; Dey and Giri, 2016; Zhang et al., 2016). Many epidemiological investigations and animal experiments suggest that increased levels of fluoride in the environment affect male reproductive function (Gupta et al., 2007; Long et al., 2009; Kim et al., 2015; An et al., 2019), by decreasing the total sperm count and sperm motility (Elbetieha et al., 2000; Sun et al., 2016; Chaithra and Sarjan, 2019); affecting the serum levels of reproductive hormones (An et al., 2019); decreasing testosterone concentrations (Tang et al., 2018a; Chaithra and Sarjan, 2019); causing structural changes in spermatozoa and functional incapacitation

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(Sun et al., 2014; Han et al., 2019), damage to the cytoskeleton and cytomembranes (Li et al., 2018) and alterations in the ultra-structure of sperm flagella (Liang et al., 2019); inducing mitochondrial impairment (Liang et al., 2020a, 2020b); and decreasing genitality (Kim et al., 2015). However, few reports have described the reproductive toxicity of fluoride in invertebrates, and the underlying molecular mechanisms are not clearly understood.

The silkworm is a potentially promising model organism used in assessment of environmental pollution and health safety, because of its sensitivity to chemical compounds, such as drugs, heavy metals and pesticides, in addition to other features including bio-safety, the ability to perform experiments quickly and easily, low cost and the availability of full genetic resources (Nouara et al., 2018). Therefore, we used the silkworm to study the reproductive toxicity of fluoride to males. Our previous studies have demonstrated that NaF can cause reproductive damage and oxidative stress to the reproductive glands of silkworms (Tang et al., 2016, 2018a), and digital gene expression profiling has shown that many differentially expressed genes are enriched in the oxidative phosphorylation pathway (Tang et al., 2018b).

Oxidative phosphorylation refers to the formation of ATP through the process of biological oxidation. The mitochondrial respiratory chain provides 95% of the energy for cellular life activities, and its machinery is mainly composed of five complexes located on mitochondrial membranes. Complex I, often referred to as NADH-Q reductase or NADH dehydrogenase, is the first protein in the electron transport chain. It is responsible for receiving electrons from NADPH and finally transferring them to the next complex through ubiquinone. Complex II consists of succinate dehydrogenase (a flavin protein with flavin adenine dinucleotide as the cofactor) and an iron sulfur protein, which transfers electrons from succinic acid to ubiquinone (coenzyme Q). Complex III, also known as cytochrome c reductase or cytochrome b-c 1, transfers electrons from reduced ubiquinone to cytochrome c, and cytochrome c transfers electrons to complex IV. Complex IV, also known as cytochrome c oxidase, is mainly responsible for transferring electrons from cytochrome c to O_2 molecules and generating H_2O. Complex V, also known as ATP synthase, is the final segment of the electron transport chain (ETC). Its main function is to complete the oxidative phosphorylation process together with the above four complexes to generate ATP (Elmore and Merrill, 2019). The ETC can be divided into two pathways: one is called the NADH respiratory chain involving complexes I through complexes III and IV, and the other is called the FADH2 respiratory chain involving complex II through complexes III and IV (Qu et al., 2013).

To further study the influence of NaF in inducing the oxidative phosphorylation pathway in the testis in *Bombyx mori*, and the relationship between oxidative phosphorylation and oxidative stress, we measured the content of the main ROS (H_2O_2 and O_2^-) in the testis, the activity of the main ETC complex enzymes in the oxidative phosphorylation pathway and the mRNA expression levels of the corresponding genes; we further performed pathological observation of the silkworm testis after NaF exposure for different times. Our results should aid in understanding of the testis-damaging toxicological mechanism of NaF and provide a theoretical basis for determining the reproductive toxicity of NaF in invertebrates.

2. Materials and methods

2.1. Chemicals

NaF with a purity of approximately 98% was obtained from the Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China), and 1 g/L mother liquor in distilled water was prepared. All other chemicals were of analytical reagent grade.

2.2. Silkworm strains and NaF exposure

The silkworm strain Xian 2 was selected for research, and the study design and treatment of the experiment followed the methods described in our previous publication (Tang et al., 2018b).

2.3. Sampling and total RNA extraction

Several male silkworms were randomly selected from the experimental and control groups after 24, 72 and 120 h of NaF exposure. The testes in each group were dissected and removed under an ice bath. A portion of each sample was added to 1 mL Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quickly placed in liquid nitrogen. Finally, the samples were stored at −80 ℃ for later RNA extraction. The other portion of each sample was weighed and placed directly at −80 ℃ for later determination of enzymatic activity. Three biological replicates per group were analyzed.

The total RNA from the testes was extracted with Trizol reagent (Invitrogen). The DNA was removed from the total RNA (5 μg) samples with DNase (CapitalBio, China), and the DNA-free total RNA (0.5 mg/sample) was reverse-transcribed to cDNA with M-MLV reverse transcriptase (Takara, Shiga, Japan) and a random hexamer primer according to the manufacturer's instructions. RNA purity and concentration were measured with a NanoPhotometer® spectrophotometer (Implen, Westlake Village, CA, USA). RNA integrity was analyzed on 1% agarose gels.

2.4. Quantitative real-time PCR analysis

Real time PCR was used to detect the mRNA expression levels of *NADH-CoQ1*, *Cyt c reductase*, *Cyt c oxidase* and *ATP synthase* genes in samples after 120 h of NaF exposure. The housekeeper gene *actin3* was used as the internal reference. The primers used in the experiment were designed in Primer Premier 5.0 (Biosoft, Palo Alto, CA, USA) and synthesized by GenScript Co., Ltd. (Nanjing, China) (Table S1). The specific steps and data processing of the qRT-PCR were performed according to the method described in our previous publication (Tang et al., 2018b).

2.5. Reverse transcription PCR (RT-PCR) analysis

RT-PCR was used to measure the mRNA expression levels of *NADH-CoQ1*, *Cyt c reductase*, *Cyt c oxidase* and *ATP synthase* genes in samples after 24, 72 and 120 h of NaF exposure. The *actin3* gene was used as the internal reference. The design and synthesis of primers were the same as in step 2.4 (Table S2).

RT-PCR was performed in a final volume of 25 μL containing 1 × PCR buffer (TaKaRa), 250 μM of dNTPs (TaKaRa), 0.6 U Taq DNA polymerase (TaKaRa r Taq, TaKaRa), 6 μM each of the primer pair and 1 μL of the cDNA template (1000 ng/μL). The program was 95 °C for 45 s, followed by 25 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s and finally 72 °C for 10 min. The PCR products were detected with 1% agarose gel electrophoresis, EB staining was performed after electrophoresis, and observation and imaging were performed with a gel imaging system.

2.6. Determination of the content of ROS

The production of ROS (H_2O_2 and O_2^-) in the testis tissues was assayed with a Hydrogen Peroxide and Superoxide Anion Content Assay Kit (Colorimetric Method, Sangon Biotech Co., Ltd, Shanghai, China) according to the manufacturer's instructions.

2.7. Determination of the enzymatic activity of ETC complexes

The activity of ETC complexes I, III, IV and V was measured with the relevant mitochondrial respiratory chain complex activity assay kits (Sangon Biotech, China). The enzyme activity of each sample was

determined at least three times with three biological repeats in each group. The procedures were performed according to the manufacturer's instructions.

2.8. Histopathological observation

2.8.1. Light microscopy

Testis tissue was fixed in 4% paraformaldehyde fixing solution (Sangon Biotech, China) and processed with routine paraffin techniques. Four-micrometer thick sections were cut, stained with H&E (hematoxylin and eosin), and then subjected to microscopic observation and photography.

2.8.2. Ultrastructure observation by transmission electron microscopy (TEM)

The ultrastructure of the testis of Bombyx mori exposed to NaF for different times was observed by transmission electron microscopy, and the specific operation steps followed the methods described in our previous publication (Tang et al., 2018a).

2.9. Statistical analysis

All data were analyzed in Excel 2016 (Microsoft Office Excel 2016 for Windows, Microsoft, Redmond, WA, USA) and SPSS19.0 software (SPSS Inc., Chicago, IL, USA). Differences between the control and treatment groups were determined with *t*-test analysis of paired samples. Data are expressed as means ± standard deviation (S.D.), and $*p < 0.05$ and $**p < 0.01$ were considered statistically significant.

3. Results

3.1. Differentially expressed genes of the oxidative phosphorylation pathway in silkworm testis after NaF-treatment

In our previous study (Tang et al., 2018b), we found that NaF treatment up-regulates the expression of the ETC-associated genes NADH dehydrogenase (Ndufa13, Ndufs3, Ndufs5 and Ndufb1), cytochrome c reductase (QCR8), cytochrome c oxidase (COX7A, COX6A2, COX17, COX7C, COX6A1 and COX6b) and ATP synthase (ATP-synt-g). DGE analysis indicated that the expression of 12 mitochondrial complex-associated genes was significantly up-regulated, and these genes were enriched in the "oxidative phosphorylation" term in KEGG pathway analysis (Fig. S1).

3.2. Verification of ETC-related genes with qRT-PCR

qRT-PCR was used to analyze the expression of ETC-related genes in this pathway after NaF exposure for 120 h (the sixth day of the fifth instar). The results are shown in Fig. 1. The qRT-PCR results of mitochondrial complexes NADH-CoQ1, Cyt c reductase, Cyt c oxidase and ATP synthase were consistent with the DGE results, and showed significant up-regulation relative to the control group results.

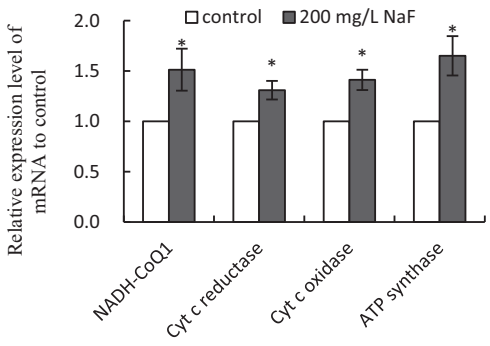

Fig. 1. Verification of oxidative phosphorylation genes with qRT-PCR. Values that are significantly different from the control are indicated by asterisks (*t*-test analysis, $*P < 0.05$).

3.3. RT-PCR analysis of mitochondrial complex-related genes

To further measure the changes in expression of ETC-associated genes after NaF induction, we used RT-PCR to measure the transcription of the NADH-CoQ1, Cyt c reductase, Cyt c oxidase and ATP synthase genes 24 h, 72 h and 120 h after NaF treatment. As shown in Fig. S2, the transcriptional levels of all genes in the control group and the NaF-treatment group differed at different times.

The results of RT-PCR were quantitatively analyzed with Quantity One 4.2.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). As shown in Fig. 2, after 24 h, 72 h and 120 h of NaF exposure, the mRNA transcription level of NADH-CoQ1 increased by 1.55-, 0.97- and 2.08-fold relative to the control group levels, respectively, and it increased significantly at 120 h with increasing NaF exposure time ($P < 0.05$) (Fig. 2A); the transcription level of Cyt c reductase was 1.67-, 1.12- and 1.27-fold that of the control group, respectively, it decreased significantly at 70 h ($P < 0.01$) and 120 h ($P < 0.05$) with increasing NaF exposure time (Fig. 2B); the transcription level of Cyt c oxidase was 6.21-, 4.59- and 2.36-fold that of the control group, respectively, it decreased significantly at 120 h ($P < 0.01$) with increasing NaF exposure time (Fig. 2C), whereas the transcription level of ATP synthase was 2.05-, 1.06- and 1.57-fold that of the control group levels, respectively, it decreased significantly at 70 h ($P < 0.05$) and 120 h ($P < 0.01$) with increasing NaF exposure time (Fig. 2D). Notably, the transcription level of Cyt c oxidase was most significantly increased ($P < 0.01$), as compared with the control group expression. The transcription levels of Cyt c reductase, Cyt c oxidase and ATP synthase changed the most after 24 h of NaF exposure ($P < 0.01$), and the transcription levels of NADH-CoQ1, Cyt c reductase and ATP synthase changed the least after 72 h of NaF exposure ($P > 0.05$), whereas the transcription level of NADH-CoQ1 changed the most after 120 h of NaF exposure ($P < 0.01$).

3.4. NaF-induced enzymatic activity changes in ETC complexes

To further measure the effect of NaF on the silkworm testis ETC, we determined the changes in the enzyme activity of respiratory chain complexes I, III, IV and V. As shown in Fig. 3, the enzymatic activity of complex I was significantly higher than that in the control group after 24 h of NaF exposure ($P < 0.01$), and that of complex I was significantly higher than that in the control group after 72 and 120 h of NaF exposure ($P < 0.05$), and it decreased significantly at 70 h ($P < 0.05$) and 120 h ($P < 0.01$) with increasing NaF exposure time (Fig. 3A). The enzymatic activity of complex III was significantly higher than that in the control group after 24, 72 s and 120 h of NaF exposure ($P < 0.05$), it decreased significantly at 70 h ($P < 0.05$) with increasing NaF exposure time (Fig. 3 B). The enzymatic activity of complex IV was significantly higher than that in the control group after 24 h of NaF exposure ($P < 0.05$), and that of complex I was significantly higher than that in the control group after 72 and 120 h of NaF exposure ($P < 0.01$), it increased significantly at ($P < 0.05$) with increasing NaF exposure time (Fig. 3 C). The enzymatic activity of complex V was significantly higher than that in the control group after 24 h of NaF exposure ($P < 0.01$), and that of complex I was significantly higher than that in the control group after 120 h of NaF exposure ($P < 0.05$), it decreased significantly at 70 h ($P < 0.05$) with increasing NaF exposure time (Fig. 3 D). The results suggested that the enzymatic activity of complex I, III and V in the silkworm testis showed the most significant increase, as compared with the control group levels, after 24 h of NaF exposure. Moreover, the enzymatic activity of complex IV in the silkworm testis showed the most significant increase, as compared with the control group levels, after 120 h of NaF exposure.

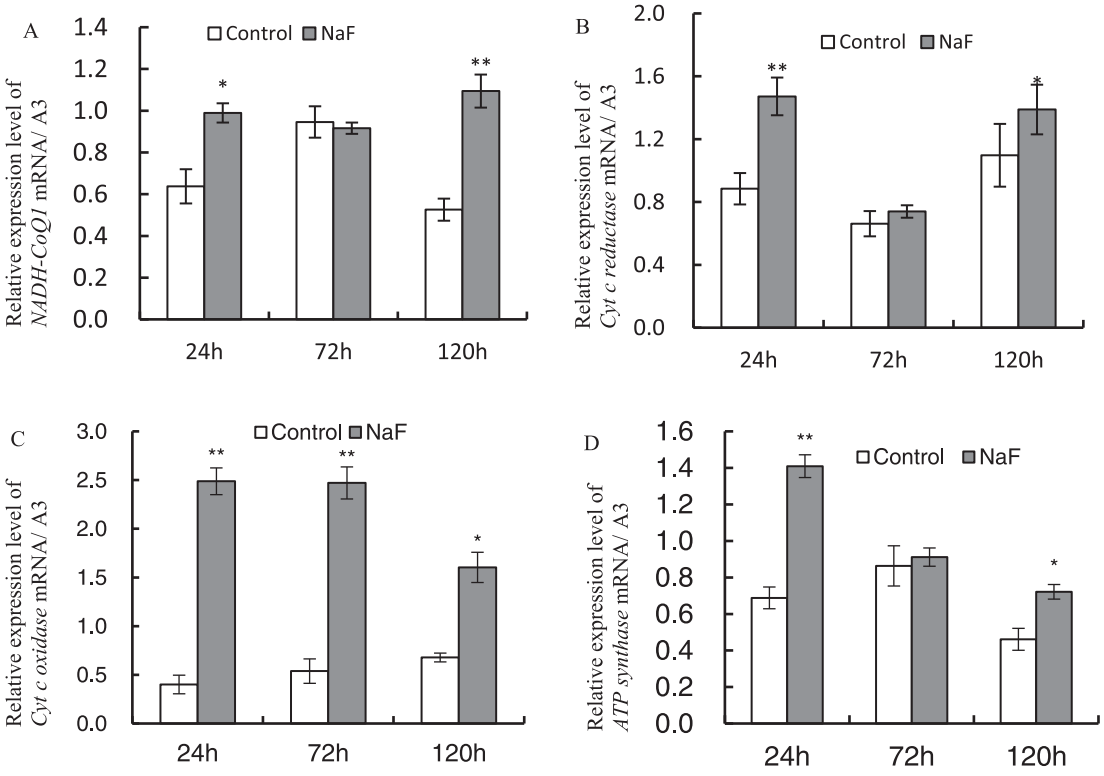

Fig. 2. The relative expression analysis of RT-PCR for oxidative phosphorylation genes in silkworm testis under NaF exposure. (A) *NADH-CoQ1*, (B) *Cyt c reductase*, (c) *Cyt c oxidase*, (D) *ATP synthase*. Values that are significantly different from the control are indicated by asterisks (*t*-test analysis, $*P < 0.05$, $**P < 0.01$). Values that are significantly different from the 24 h of NaF exposure are indicated by well number (One-way ANOVA, $\# P < 0.05$, $\#\# P < 0.01$).

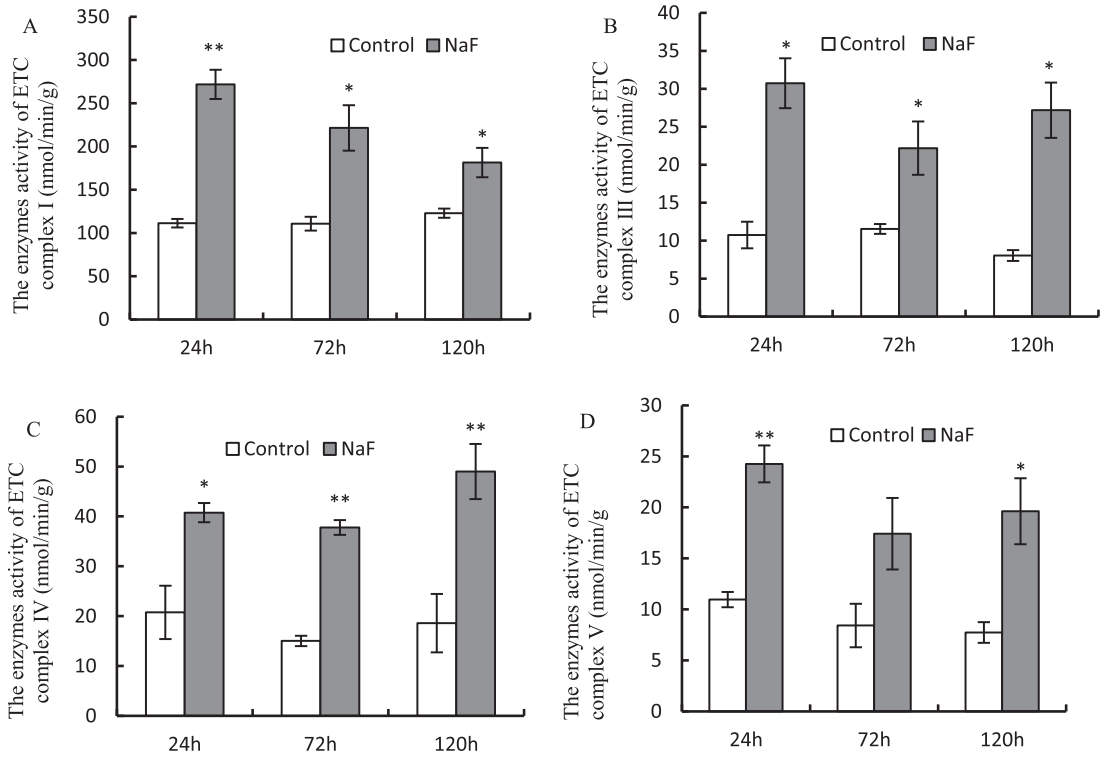

Fig. 3. The mitochondrial complex enzyme activity of silkworm testis under NaF exposure. (A) Mitochondrial complex I, (B) mitochondrial complex III, (C) mitochondrial complex IV, (D) mitochondrial complex V. Values that are significantly different from the control are indicated by asterisks (*t*-test analysis, $*P < 0.05$, $**P < 0.01$). Values that are significantly different from the 24 h of NaF exposure are indicated by well number (One-way ANOVA, $\# P < 0.05$, $\#\# P < 0.01$).

3.5. NaF-induced oxidative stress

To determine whether NaF induced testis tissue oxidative damage, we measured the content of major ROS (O_2^- and H_2O_2) in the silkworm testis. As shown in Fig. 4A, the O_2^- content in the silkworm testis was significantly ($P < 0.05$) higher than that in the control group after 24, 72 and 120 h of NaF exposure, and it decreased significantly at 120 h ($P < 0.05$) with increasing NaF exposure time; the O_2^- content showed a highly significant difference after 24 h of NaF exposure ($P < 0.01$). The H_2O_2 content in the silkworm testis increased after 24, 72 and 120 h of NaF exposure, as compared with the control group levels; the H_2O_2 content increased significantly ($P < 0.05$) after 120 h of NaF exposure and showed an extremely significant difference after 72 h of NaF exposure ($P < 0.01$) and it increased significantly at 70 h ($P < 0.01$) and 120 h ($P < 0.05$) with increasing NaF exposure time (Fig. 4B).

3.6. Histopathological investigations

3.6.1. Histopathological light microscopy

The testes in the control group displayed normal morphological characteristics, such as closely arranged, distinct cell borders and regular cell shapes (Fig. 5A). However, the spermatocytes had swelled slightly, and the cell membranes gradually disappeared after 24 h of NaF-treatment (Fig. 5B); after 72 h of NaF treatment, the spermatocytes showed clear swelling, the cell membranes gradually disappeared, and small vacuoles appeared (Fig. 5C). In addition, after 120 h of NaF exposure, the spermatocytes showed clear swelling, the cell membranes disappeared, and local necrosis and many vacuoles appeared (Fig. 5D).

3.6.2. Ultrastructural findings

TEM analysis revealed that the spermatocytes displayed slight endoplasmic reticulum distention and a small amount of mitochondrial turgescence in control silkworms (Fig. 6A). However, the spermatocytes showed mitochondrial swelling and endoplasmic reticulum expansion after 24 h of NaF exposure (Fig. 6B). In the group treated with 200 mg/L NaF for 72 h, the mitochondria appeared swollen, and ridges gradually disappeared, the endoplasmic reticulum was expanded and degranulated gradually, and vacuoles and lysosomes appeared in the cells (Fig. 6C). After 120 h of NaF exposure, the cells contained even more apoptosis, and necrosis and vacuoles were evident (Fig. 6D).

4. Discussion

Fluoride is a systemic poison that is toxic to many organs (Ullah et al., 2017). In recent years, the reproductive toxicity caused by fluoride has attracted much interest (Jiang et al., 2019; Chaithra et al., 2019). However, although much research has shown that fluoride can induce reproductive toxicity in vertebrates, few studies have reported the reproductive toxicity in invertebrates. The silkworm is an important lepidopteran model insect that has been widely used as a model animal in toxicology, medicine, physiology and other fields (Matsumoto et al., 2011; Tansil et al., 2011; Yuan et al., 2013; Nouara et al., 2018).

In our previous study, we confirmed the effects of 25, 50, 100 and 200 mg/L NaF on the biochemical and physiological reproductive toxicity of silkworms, and found that 200 mg/L NaF had an obvious effect on the testes of silkworms (Tang et al., 2018a). To further explore the testis-damaging toxicological mechanism of fluoride, we used RNA sequencing technology to measure whole-genome transcriptional responses in the testes of silkworms exposed to 200 mg/L NaF, and found large numbers differentially expressed genes enriched in the oxidative phosphorylation pathway (Tang et al., 2018b). However, the relationship between oxidative phosphorylation and oxidative damage caused by NaF is unclear.

However, in our experiment, with increasing exposure time, the content of O_2^- decreased significantly, whereas the content of H_2O_2 increased significantly. Relevant research has shown that superoxide dismutase (SOD) prevents cellular damage by catalyzing the conversion of O_2- to H_2O_2 and O_2 under emergency (Zhang et al., 2008). In addition, our previous studies have shown a significant increase in SOD activity after NaF exposure in the testes of silkworms (Tang et al., 2016). Therefore, we speculate that the decrease in O_2^- and the increase in H_2O_2 may be closely related to the production of SOD with increasing exposure time. Interestingly, the content decreased to ROS at 120 h, a finding that may be related to the elimination of antioxidant enzymes in *Bombyx mori* testes after 120 h of NaF exposure.

The redox system plays an important role in life activities in organisms. An imbalance in oxidation and antioxidation in vivo leads to oxidative stress (Kothari et al., 2010). Oxidative stress may be caused by excessive production of ROS or deficiencies in antioxidant systems (Wang et al., 2017). In our study, the content of major ROS (O_2^- and H_2O_2) was measured in the silkworm testis after 24, 72 and 120 h of 200 mg/L NaF exposure, and the content of both H_2O_2 and O_2^- increased significantly. The results also suggested that large amounts of ROS were produced and accumulated after exposure to NaF in the mitochondria in the silkworm testis. The production of ROS in mitochondria crucially plays a role in oxidative damage in many diseases and is important in retrograde redox signals from organelles to the cytoplasm and nucleus (Murphy, 2009). Therefore, we speculated that the NaF induced testis damage in silkworms might be closely associated with the accumulation of H_2O_2 and O_2^-.

The production of ROS is closely associated with the respiratory electron-transport chain. Because the electron transfer in the

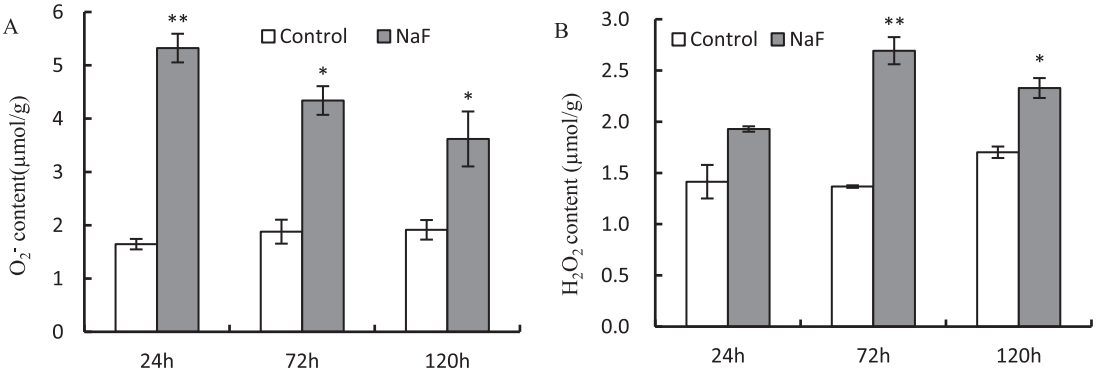

Fig. 4. The content of O^{2-} (A) and H_2O_2 (B) in testis of silkworm under NaF exposure. Values that are significantly different from the control are indicated by asterisks (*t*-test analysis, $*P < 0.05$, $**P < 0.01$). Values that are significantly different from the 24 h of NaF exposure are indicated by well number (One-way ANOVA, $\#\ P < 0.05$, $\#\#\ P < 0.01$).

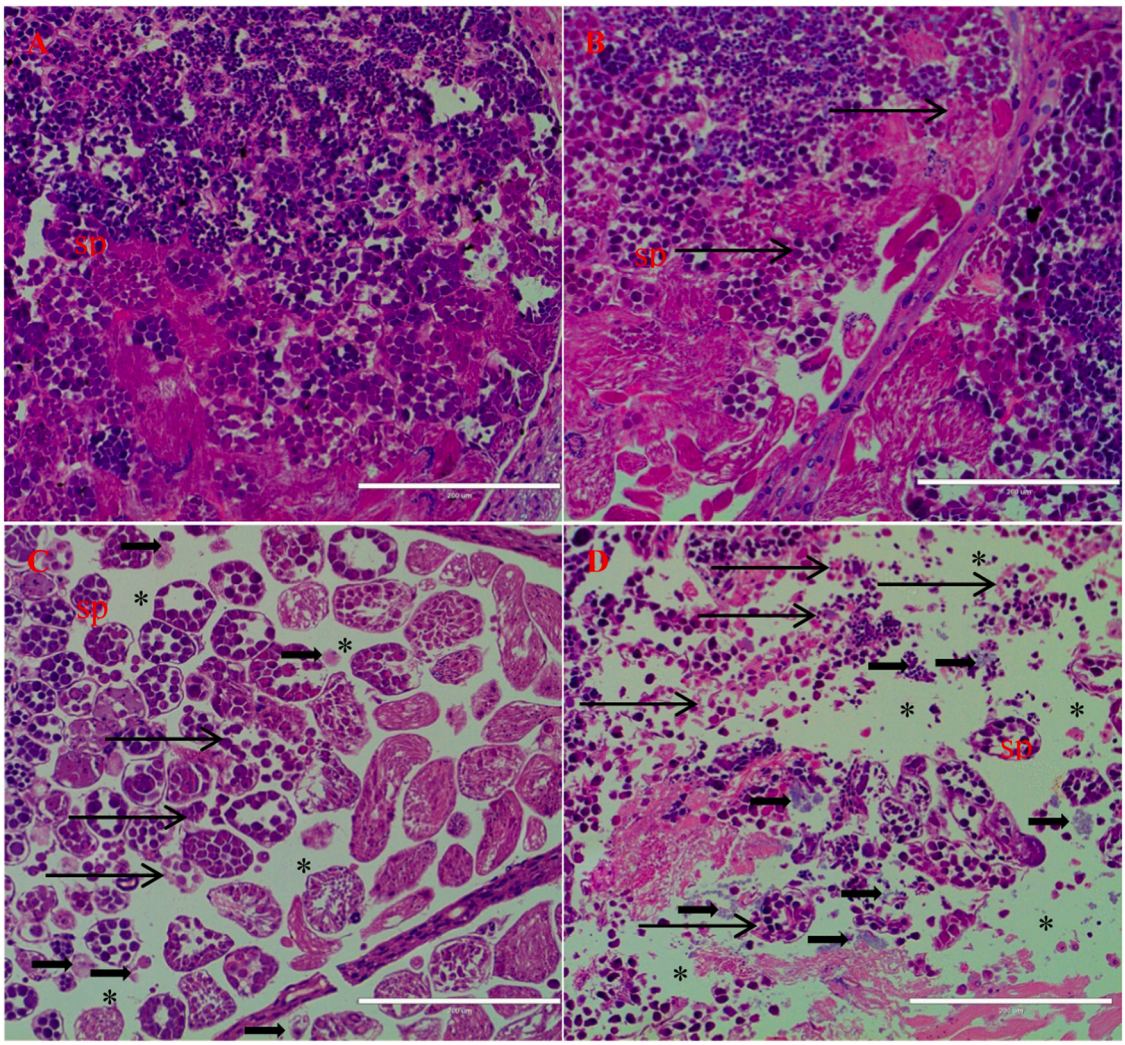

Fig. 5. Photomicrograph of H&E stained testis sections. (A) control groups. Spermatocytes are closely arranged; (B) 200 mg/L NaF-treatment group at 24 h. Spermatocytes swelled slightly and cell membranes gradually disappeared (thin arrows); (C) 200 mg/L NaF-treatment group at 72 h. Spermatocytes showed obvious swelling and cell membrane gradually disappeared (thin arrows), and small vacuoles appeared (asterisk); (D) 200 mg/L NaF-treatment group at 120 h. Spermatocytes showed obvious swelling and cell membrane disappeared (thin arrows), local necrosis (bold arrows) and many vacuoles appeare (asterisk). sp: spermatocytes.

mitochondria respiratory chain is not completely effective, electron leakage during transport is the main source of ROS (Castro et al., 2013). In particular, mitochondrial complex I and complex III are the main sites of ROS production (Chen et al., 2003; Gill and Tuteja, 2010). ETC complexes I and III are known to mainly release superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) into the matrix and intermembrane space in the chondriosomes, respectively (Bleier et al., 2015). Cytochrome c oxidase (complex IV), the terminal element of the ETC, converts one O_2 molecule to two H_2O molecules during four single electron reduction reactions (Kowaltowski et al., 2009). In the current study, we measured the activity of the main ETC complex enzymes in the oxidative phosphorylation pathway and the transcription levels of the corresponding genes after exposure to NaF. The results showed that the enzymatic activity and gene mRNA expression levels of mitochondrial complexes I, III, IV and V increased significantly. With increasing NaF exposure time, the changes in relative expression levels for all genes showed a time effect, except for *Cyt c reductase*, which decreased significantly at 72 h and increased to a certain extent at 120 h. In addition, the production of ROS in the testis reached a maximum 72 h after NaF exposure, and decreased at 120 h. Complex III is the main site of ROS production. We speculate that the increase in the relative expression level of *Cyt c reductase* at 120 h may be related to the decrease in ROS. Interestingly, the enzymatic activity of mitochondrial complexes II was not clearly changed after exposure to NaF. Thus, NaF exposure increases mitochondrial respiration and energy production through the influence of the NADH oxidation respiratory chain. Previous studies have shown that phoxim exposure increases the activity of the ETC complex in the chondriosomes and leads to ROS accumulation in the midgut in silkworms (Li et al., 2017, 2018). We speculate that the abnormal changes in the activity of ETC complexes might increase electron leakage, which may in turn lead to increased H_2O_2 and $O_2^{\cdot-}$ production in the testis, and the accumulation of H_2O_2 and $O_2^{\cdot-}$ (ROS) in the testis may be the main cause of testicular injury.

A cyclic mechanism may exist between oxidative damage and oxidative phosphorylation. Oxidative phosphorylation produces free radicals, which may damage DNA, proteins and membrane lipids. This damage blocks oxidative phosphorylation and produces more free radicals, which may further cause oxidative damage to mitochondria (Pradesh et al., 2011; Fukai et al., 2016). Our previous study has confirmed that treatment with different concentrations of NaF can lead to oxidative damage of the testis in silkworms (Tang et al., 2016, 2018a). We further explored the influence of NaF treatment at different times on the histopathology of silkworm testis tissue and found that the degree of damage was more severe with increasing NaF exposure time. The damage manifested as swollen and ruptured or tubular mitochondrial structures; dilatation of the endoplasmic reticulum, and increased

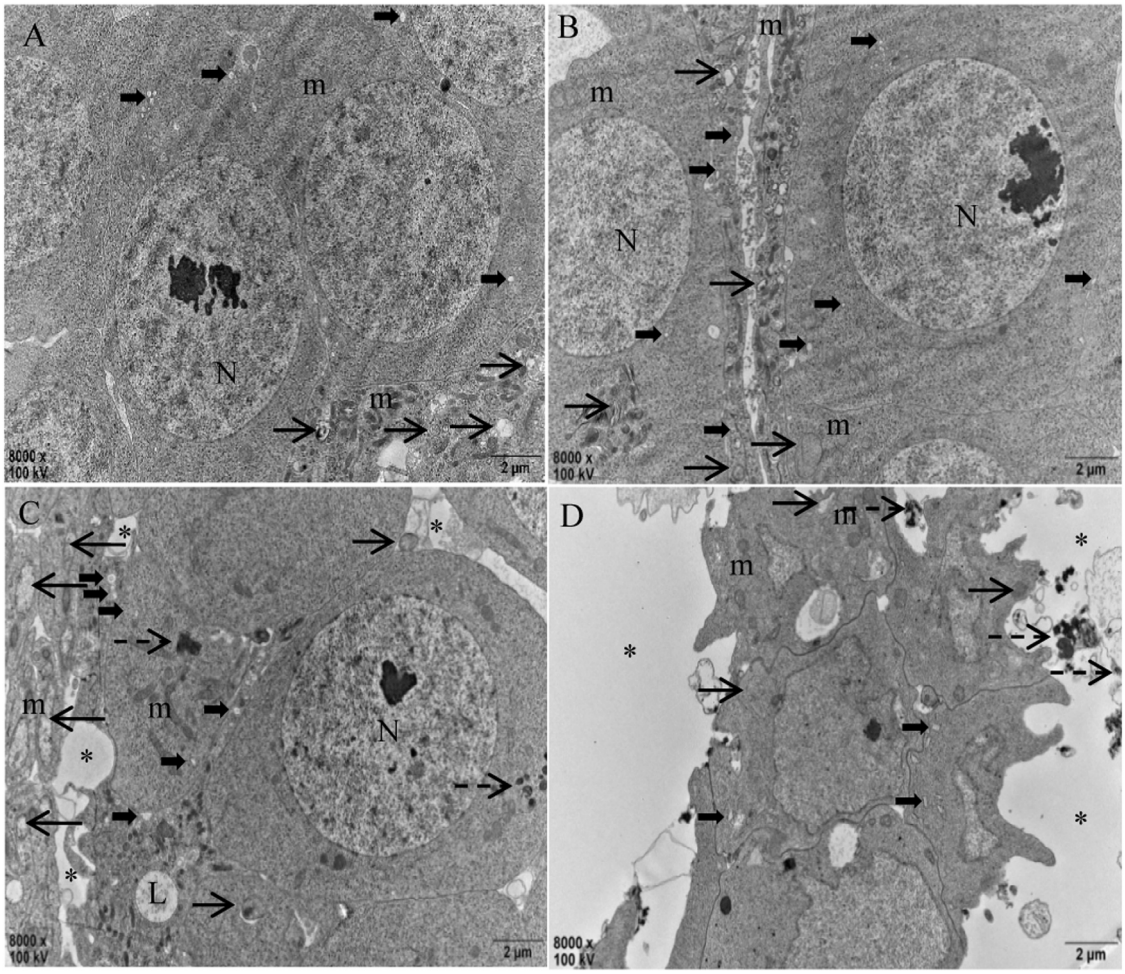

Fig. 6. Effect of NaF exposure on testis ultrastructure at different time. (A) control groups; (B) 200 mg/L NaF-treatment group at 24 h; (C) 200 mg/L NaF-treatment group at 72 h; (D) 200 mg/L NaF-treatment group at 120 h. The thin arrows indicate swelling of mitochondria, the bold arrows indicate expansion of endoplasmic reticulum, the dashed arrows indicate apoptosis and necrosis of cell, asterisk: vacuoles, N: nucleus, m: mitochondria, L: lysosome.

numbers of vacuoles, and apoptosis and necrosis of cells. These results were in line with those from previous studies in vertebrates (Wang et al., 2004; Ngoc et al., 2012; Zhang et al., 2013; Ribeiro et al., 2017), fluoride induces necrosis and apoptosis of testicular cells. Notably, the damage to testicular cells is positively correlated with the accumulation of ROS (H_2O_2 and O_2^-) in silkworms. Therefore, fluoride induced reproductive damage in silkworms is caused by the influence of the oxidative phosphorylation pathway.

5. Conclusions

The molecular mechanisms underlying fluoride induced reproductive damage in *Bombyx mori* may involve the NADH respiratory chain of the mitochondrial ETC, thus increasing the enzymatic activity of related complexes and destroying the balance of the ETC. The resulting electrical leakage significantly increases the content of ROS and causes oxidative stress reactions in cells.

CRediT authorship contribution statement

Wenchao Tang: Data curation, Writing - original draft preparation, Formal analysis. **Yuanyuan Xiao:** Conceptualization, Methodology, Investigation. **Yaohang Long:** Visualization, Investigation. **Yaofeng Li:** Software, Project administration. **Fang Peng:** Supervision, Validation. **Can Zhu:** Supervision, Validation. **Tinggui He:** Formal analysis. **Didong Lou:** Writing - review & editing, Project administration. **Yong Zhu:** Writing - review r& editing.

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.

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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.2021.112229.

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