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Fluoride induced mitochondrial impairment and PINK1-mediated mitophagy in Leydig cells of mice: *In vivo* and *in vitro* studies☆

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ABSTRACT

It is very important to explore the potential harm and underlying mechanism of fluoride due to the extensive distribution and the significant health risks of fluoride in environment. The objective of this study to investigate whether fluoride can induce mitochondrial impairment and mitophagy in testicular cells. For this, 40 male mice were randomly divided into four groups treated with 0, 0.6, 1.2, 2.4 mM NaF deionized water, respectively, for 90 days continuously. The results showed that mitophagy was triggered by F in testicular tissues, especially in the Leydig cells by transmission electron microscopy and mitophagy receptor PHB2 locations by immunofluorescence. Furthermore, TM3 Leydig cells line was employed and treated with 0, 0.125, 0.25, and 0.5 mM NaF for 24 h. The mitochondrial function indicators and mitophagy maker PHB2, COX IV and regulator PINK1 in transcript and protein levels in Leydig cells were examined by the methods of qRT-PCR, western blotting, and immunofluorescence co-localization. The results showed that fluoride decreased the mitochondrial membrane potential with a concomitant increase in the number of lysosomes. Meanwhile, fluoride exposure also increased the expressions of PINK1 and PHB2 in TM3 Leydig cells. These results revealed that fluoride could induce mitochondrial impairment and excessive PINK1/Parkin-mediated mitophagy in testicular cells, especially in Leydig cells, which could contribute to the elucidation of the mechanisms of F-induced male reproductive toxicity.

1. Introduction

Fluoride (F) is one of the major food-borne and ground water contaminants, which is associated with several human diseases (Fawell et al., 2006). F through industrial emission and food consumption like seafood, tea, toothpaste, and pesticides contributes a lot in the human daily activity (Choubisa and Choubisa, 2016; Steele et al., 2014; Xu et al., 2017). More than 260 million people in 20 countries suffer from fluorosis caused by various sources of fluoride (Herath et al., 2018). So it is very important to explore the potential harm and mechanism of fluoride.

It has been reported that fluoride could damage various organs or systems such as ovary (Jhala et al., 2008), liver (Liang et al., 2018; Zhao et al., 2018b), kidney (Gao et al., 2018), brain (Niu et al., 2018; Wang et al., 2018a), skeletal system (Everett, 2011), and reproductive system (Jiang et al., 2019). Nevertheless, the adverse effects of fluoride on male reproductive are attracting more attention due to the overall fertility decline worldwide (Barbier et al., 2010). Numerous studies from our group and other teams focused on the changes in blood-testis barrier (Zhang et al., 2016a; He et al., 2018), immune-privileged function of testis (Huo et al., 2016; Sun et al., 2017), spermatogenesis process (Zhao et al., 2018a; Han et al., 2019), and fertilizing ability (Liu et al., 2019). However, the exact mechanism by which fluoride interferes with male reproduction is still remain unclear.

Mitochondrion is the most important energy metabolism organelle, which is closely related to the regulation of physiological functions in eukaryotic cells (Harbauer et al., 2014; Nunnari and Suomalainen, 2012). The dysfunction of mitochondria can reduce the mitochondrial membrane potential (MMP) and stabilize PINK1, resulting in the recruitment of the Parkin E3 ligase to ubiquitinated several mitochondrial outer membrane (MOM) proteins, followed by mitochondria membrane rupture and exposure of prohibitin 2 (PHB2). While PHB2 who is an important inner mitochondrial

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membrane protein and regulates mitochondrial assembly and function (Bavelloni et al., 2015; Osman et al., 2009), can binds directly to LC3 on the phagophore and trigger mitophagy (Pickrell and Youle, 2015; Wei et al., 2017).

Mitophagy is a highly complex and dynamic cellular process that regulates both mitochondrial quality and quantity. As a well-studied type of selective autophagy, mitophagy is also known to play a crucial role in protecting eukaryotic cells against deleterious damage or excessive mitochondria accumulations (Green and Levine, 2014; Lemasters, 2005). Moreover, PINK1/Parkin-mediated mitophagy was also involved in many disease process, such as neurodegenerative diseases (Amadoro et al., 2014; Holmström et al., 2010; Song et al., 2014b·b), immunological diseases (Parikh et al., 2015; Tsai et al., 2016), and cancers (Cho et al., 2017; Jin et al., 2018; Tsai et al., 2016). Although it was mentioned that autophagosomes-like structures containing mitochondria were observed in NaF-treated testes (Zhang et al., 2016b), with a stronger tendency in Leydig cells (Song et al., 2014; Zhang et al., 2017), there is no definite evidence that F can induce mitophagy in this target cells and its mechanism.

Therefore, the present study was aimed to investigate whether F can induce mitochondrial impairment and mitophagy in testicular cells. Further, PINK1-mediated mitophagy caused by fluoride in testes and particularly in Ledig cells were explored by in vivo and *in vitro* approaches, which will contribute to the mechanisms of F-induced male reproductive toxicity.

2. Materials and methods

2.1. Mice treatment

40 male 8-weeks old C57BL-6 male mice and their standard diets were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. of China. After one week's environmental adaptation, the mice were randomly allocated into four groups of 10 mice each: group-I (control, drinking distilled water), group-II (drinking 0.6 mM NaF water), group-III (drinking 1.2 mM NaF water), and group-IV (drinking 2.4 mM NaF water). All mice were housed under environmental conditions of 22 °C, 40–70% humidity, and a 12:12hr dark: light cycle. After 90 consecutive days, the mice were anesthetized with 20% Urethane solution and the testes were collected and frozen in liquid nitrogen quickly. Three of testicular tissues from each groups were fixed in Bouin's solution and 2.5% glutaraldehyde solution for immunofluorescence and transmission electron microscope assays, respectively. All experiments were performed in compliance with the guidelines of the committee of Animal Welfare of Shanxi Agricultural University.

2.2. Cell culture

Mouse testicular Leydig cell lines (TM3 Leydig cells) were purchased from Zhong Qiao Xin Zhou Biotechnology Co. Ltd (Shanghai, China). The culture medium contains 91.5% Dulbecco's modified Eagle's medium (DMEM/F12) (BI, Kibbutz Beit Haemek, Israel), 5% horse serum (Gibco, Grand Island, USA), 2.5% fetal bovine serum (AusGeneX, Brisbane, Australia) and 1% Penicillin-Streptomycin Liquid (100X) (Solarbio, Beijing, China). TM3 Leydig cells were cultured in an incubator at 37 °C with a humidified atmosphere containing 5% CO_2.

2.3. Cells treatment and viability assay

The effects of NaF on TM3 Leydig cells viability were determined by MTT assay (KeyGEN Biotech, Jiangsu, China). Briefly, the cells were seeded into 96-well culture plates at a density of 1×10^4/well and cultured at 37 °C and 5% CO_2 in an incubator. After 24 h, the cells were treated with 0, 0.025, 0.25, 0.5, 1, 1.5, 2, 2.5 and 3 mM NaF for 24 h and 48 h, respectively. Subsequently, 50 μL 1 × MTT solution was added into each well and incubated under the same condition. After 4 h, the absorbance was measured with a microplate spectrophotometer at 508 nm. The half-maximal inhibitory concentration (IC_{50}) and 95% confidence intervals were calculated. According to MTT result, 0.125, 0.25, and 0.5 mM NaF were used as fluoride exposure dosages for further study.

2.4. Transmission electron microscope (TEM)

The testes tissues were cut into about 1 cubic millimeter pieces rapidly upon the ice and then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) at 4 °C for 2 h. Then, fixed in 1% osmic acid and dehydrated in gradient series of ethanol. Subsequently, the ultrathin sections were prepared and mounted on copper grids, after being dehydrated, embedded, and stained with uranyl acetate and citrate. Finally, the sections were examined and photographed under transmission electron microscope (JEM 1011, Japan).

2.5. Mitochondrial membrane potentials (MMP) assay

JC-1(5, 5′, 6, 6′-Tetrachloro-1, 1′, 3, 3′-tetraethyl-imidacarbocyanine iodide, KeyGEN Biotech, China) was employed to detect mitochondrial depolarization of TM3 Leydig cells treated with different concentrations of NaF. The cells were seeded into six-well plates with a density of 3×10^5/well and treated with 0, 0.125, 0.25, and 0.5 mM NaF for 24 h, and then incubated with an equal volume of JC-1 dye (5 μg/mL) at 37 °C keep in dark place for 20 min. After washed twice with PBS, the fluorescence of JC-1 monomer and aggregates were detected by fluorescence microscope (Olympus DP80, Japan) at 490/530 nm and 525/590 nm (excitation/emission wavelength), respectively. The fields of the stained cells were randomly selected and imaged. Fluorescence intensity of those images was counted by Image-ProPlus Version 5.1 (Media Cybernetics Inc, America). The normal and depolarized mitochondria were indicated by J-aggregates (Red channel) and JC-1 monomers (Green channel), respectively. The higher ratio of green/red fluorescence intensity represents the lower the MMP.

2.6. Co-localization and quantification of mitochondrial and lysosomal content

After treatment with NaF for 24 h, the cells were replaced with the fresh mediums and added into 50 nM Mito Tracker Green (MTG, KeyGEN Biotech, Jiangsu, China) to incubate for 30 min within incubator. Subsequently, the cells were washed twice with fresh medium and incubated with 50 nM LysoTracker Red (LTR, Beyotime, Shanghai, China) for 40 min, and then washed and covered the cells with the fresh medium again. The green fluorescence of MTG and red fluorescence of LTR were detected at different channels of 577/590 nm and 490/516 nm wavelengths respectively by fluorescence microscope. The visual fields of the cells were randomly selected for image, and the fluorescence intensity was analyzed by the software of Image-Pro Plus Version 5.1.

2.7. Immunofluorescence

For testes tissues, the paraffin-embedded testes samples were cut into 5 μm thickness transverse slices, and were deparaffinized with xylene and rehydrated in graded ethanol solutions.

Subsequently, the slices were treated with pre-heated 0.01 M citrate buffer at 95 °C for 16 min, and cooled to the room temperature (RT), rinsed once with phosphate-buffered saline (PBS) for 5 min, and blocked with 5% bull serum albumin (BSA) for 1 h at RT. Subsequently, the slices were incubated with anti-mouse PHB2 antibody (1:200, Proteintech, Wuhan, China) overnight at 4 °C. After rinsed three times (10 min each) with PBST, Fluorescein (FITC)-conjugated Affinipure Goat Anti-Mouse IgG (H+L) (1:400, Proteintech, Wuhan, China) were incubated for 2 h in dark place at RT. After rinsed three times with PBST, The slices were sealed with inhibit fluorescence decay (Solarbio, Beijing, China) for 5 min and observed by fluorescence microscope. Ten visual fields per slice were selected randomly for analyzing fluorescence intensity by Image-Pro Plus Version 5.1 software.

For TM3 Leydig cells, the cells sub-cultured on the coverslips were fixed in 4% paraformaldehyde in PBS for 15 min. After that, the cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min, and washed with PBS three times (5 min each). And then the cells were blocked with 0.5% goat serum for 30 min at RT and incubated with the first antibodies (PHB2, PINK1, and COX IV, 1:200 dilution) overnight at 4 °C. After washing, fluorescein (FITC)-conjugated affinipure goat anti-mouse IgG (H+L) (1:400, Proteintech, Wuhan, China) were added on the cells for 1 h at RT. Subsequently, washed with PBST solution for 3 times (3 min each), and sealed with antifade fluorescence mounting medium containing DAPI for 5 min. Finally, the coverslips were mounted on glass slides for fluorescence microscopic examination and analysis as the above mention.

2.8. Quantitative real-time PCR

The total RNA was extracted with RNAiso kit (Takara, Dalian, China), and then the quality and concentration were analyzed by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was reverse transcribed into cDNA by using a Reverse Transcriptase kit (Promega, Madison, WI, USA). QRT-PCR was performed with the GoTaq® qPCR Master Mix kit (Promega, Madison, WI, USA) by the QuantStudio 7 Flex qRT-PCR system (Life Technologies Corporation, Gaithersburg, MD, USA). All primers were designed by the Primer 3 Plus online program in accordance to the sequences obtained from NCBI. The information was shown in Table 1. The PCR amplification conditions were maintained as follows: predegeneration at 95 °C for 120 s, 45 cycles at 95 °C for 15 s, 60 °C for 30 s, and dissociation at 95 °C for 15 s, 65 °C for 60 s, and 95 °C for 15 s. β-actin was used as the housekeeping gene. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression levels of target genes.

2.9. Western blotting

To extract the total protein of the testis tissues or/and TM3 Leydig cells, the samples were homogenized in RIPA lysis buffer (KeyGEN Biotech, Jiangsu, China) with 1% PMSF (Beyotime, Shanghai, China) and centrifuged at 12,000 g for 10 min at 4 °C. The protein concentration was determined by Bicinchoninic acid kit (BCA, Beyotime, Shanghai, China). The 40 μg protein samples were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis. Then, target proteins were transferred onto nitrocellulose (NC) membranes (Biosharp, Hefei, China). After that, the membranes were blocked with 5% nonfat dry milk (Boster Biological Technology, Wuhan, China) for 2 h at room temperature. Next, the membranes were incubated with the primary antibodies of PINK1, PHB2 and β-actin (1:1000, Proteintech, Wuhan, China) for overnight at 4 °C. After washing 3 times (10 min each) with TBST, the membranes were incubated with HRP-conjugated secondary antibodies (1:5000, TransGen Biotech, Beijing, China) for 2 h at room temperature. Protein bands on membranes were detected by Enhanced Chemiluminescence (ECL) Kit which were purchased from Affinity BioReagents (Golden, Colorado, USA). The Fluor Chem Q System and its software (Cell & Bioscience, USA) were applied to acquire, quantify and analyze the images. β-actin was used as a housekeeping protein.

Table 1
Primer sequences for qRT-PCR.

Genes	Primer sequences	Accession no.	Product sizes (bp)
PINK1	F:GCTTTGGCTGGAGAGTATGG R:CGGATGATGTTAGGGTGTGG	NM_026880.2	86
PHB2	F:TGAGGTGCTCAAGAGTGTGG R:GCTCTCTTCGGATCAACAGG	NM_007531.2	89
B-actin	F:CTGGGTATGGAATCCTGTGG R:GCACTGTGTTGGCATAGAGG	NM_007393.5	97

2.10. Statistical analysis

Experimental data were analyzed with Graph Pad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) and were presented as mean ± SD. One-way analysis of variance (ANOVA) and Dunnett's multiple comparison were employed for the differences comparison. $p < 0.05$ or 0.01 were considered as statistically significant.

3. Results

3.1. Fluoride induced mitochondrial structural impairment and mitophagy in mice testes

The ultra-structure of testicular tissues of mice in the fluoride-treated groups and the control group were examined by Transmission Electron Microscope (TEM). The results showed that the altered mitochondrial structures in various degrees were observed either in germ cells or Sertoli cells, as well as Leydig cells in NaF-treated groups in contrast to the control group (Fig. 1). In spermatogenic cells, the mitochondrial cristae partly or disintegrated, fully disintegrated, and the membranes of mitochondrion in all NaF-treated groups were ruptured and demonstrated vacuolation (Fig. 1B–D, B1-D1). Meanwhile, a few mitophaosomes with the typical double-membrane structure appeared in germ cells in the 2.4 mM NaF groups (Fig. 1D, D1). However, few mitophaosomes were found in Sertoli cells from all fluoride treatment groups, even if the similar mitochondrial structural impairments were observed (Fig. 1F–H, F1-H1) when compared with the control group (Fig. 1E, E1). In Leydig cells, severe mitochondrial structural damage and more mitophaosomes occurred in both 1.2 and 2.4 mM NaF exposed groups (Fig. 1K or K1, L or L1). From this, we found that fluoride induced mitochondrial structural impairment in germ cells, Sertoli cells and Leydig cells in mice testes, but most of all, fluoride caused more mitochondrial changes and mitophagosomes occurrence in Leydig cells.

3.2. Fluoride changed on the expressions of mitophagy key proteins PHB2 and PINK1 in mice testes

To further confirm if fluoride cause mitophagy in testis, we examined the expressions of the important conserved inner mitophagy receptor PHB2 and regulated protein PINK1 in testicular tissue of mice exposed to fluoride. The results demonstrated that the expressions of PHB2, both in mRNA and protein levels, were increased significantly in testes, especially in the Leydig cells from fluoride-treated groups compared to the controls ($p < 0.01$,

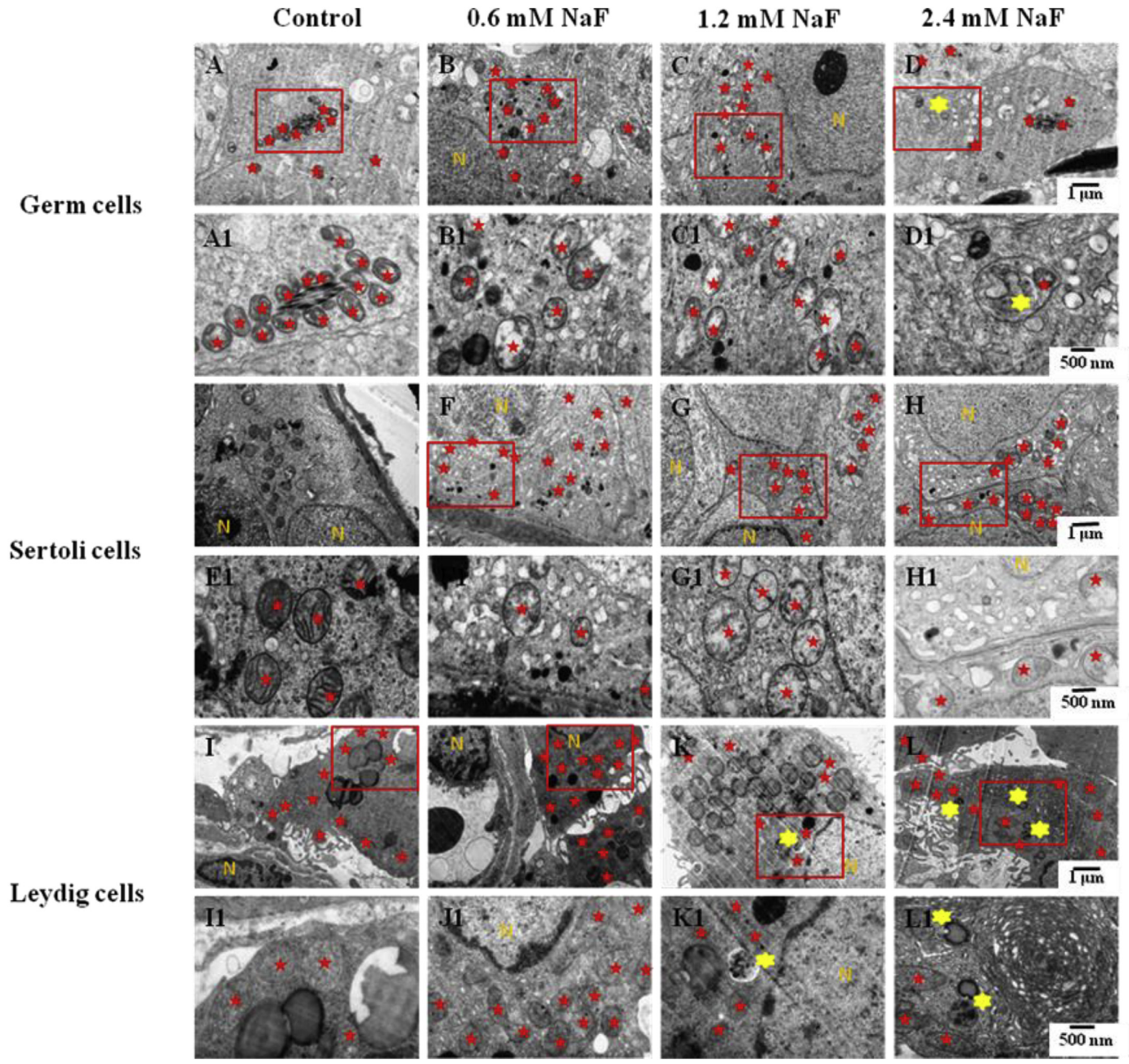

Fig. 1. Mitochondrial impairment and mitophagy occurrence induced by fluoride in mice testis tissues by Transmission Electron Microscopy observation. The red asterisk indicates mitochondrion, yellow asterisk pointing the mitophagosome, and N is logogram of cell nucleus. (A–L) The typical images of mitochondrial ultrastructure and mitophagosomes in spermatogenic cells, Sertoli cells, and Leydig cells of 0, 0.6, 1.2, 2.4 mM NaF-treated mice testes, respectively (15,000 ×), A1-L1 is their corresponding partial enlargements at 40,000 ×. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2A–D). Meanwhile, the mRNA expressions of PINK1 increased significantly in the 2.4 mM NaF group in vivo ($p < 0.05$) with a significant increase in its protein levels in the 1.2 and 2.4 mM NaF ($p < 0.01$) groups (Fig. 2E and F) with a dose-dependent manner. All these suggest that F-induced mitophagy mainly focus on the Leydig cells of testicular tissues, and PINK1 may be involve in this process in vivo study.

3.3. Fluoride weakened mitochondrial function in TM3 Leydig cells

To explore if the Leydig cells is the target cell of fluoride F-induced mitochondrial impairment and mitophagy, the TM3 Leydig cell line was employed to evaluate the mitochondrial membrane potentials (MMP) and the mitochondrial & lysosomal numbers in this study. According the results of cell viability analysis (MTT), the IC50 values of the TM3 cells exposure to sodium fluoride for 24 h and 48 h are 2.94 mM and 1.99 mM, respectively (Fig. S1). So 0.125, 0.25, and 0.5 mM NaF were used to treat TM3 cells for MMP detection. The results showed that the green fluorescent monomers were increased and the red fluorescent J-aggregates were decreased gradually in NaF treated TM3 Leydig cells in a dose-dependent manner compared to the control group (Fig. 3A). The merged green and red fluorescence intensity ratio was significantly increased in 0.25 mM and 0.5 mM NaF exposed groups ($p < 0.01$)

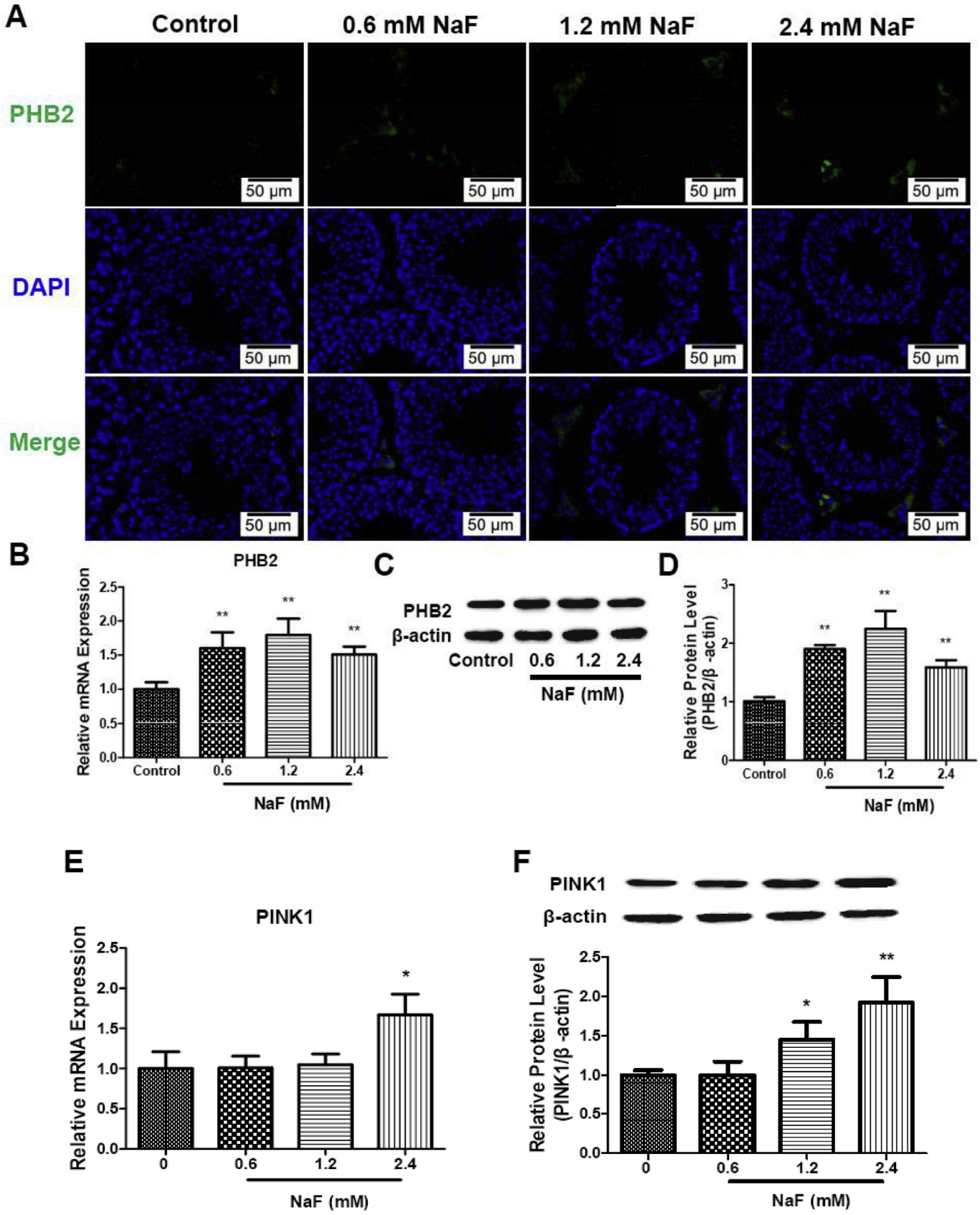

Fig. 2. Effects of fluoride on the expression of mitophay key proteins PHB2 and PINK1 in mRNA and protein levels in mice testes. (A) Representative images of PHB2 expression localizations in testes of mice by immunofluorescence. The green color fluorescence is PHB2 positive cells and cell nuclei were stained with DAPI (blue). (B) Results of mRNA expression of PHB2 in mice testes by qRT-PCR. (C, D) Western blotting analyses results of PHB2 in mice testes. (E, F) Levels of mRNA and protein expressions of PINK1 in mice testes were examined using qRT-PCR and Western blot, respectively. The values are presented as mean ± SD (n = 6), $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant differences compared to the control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

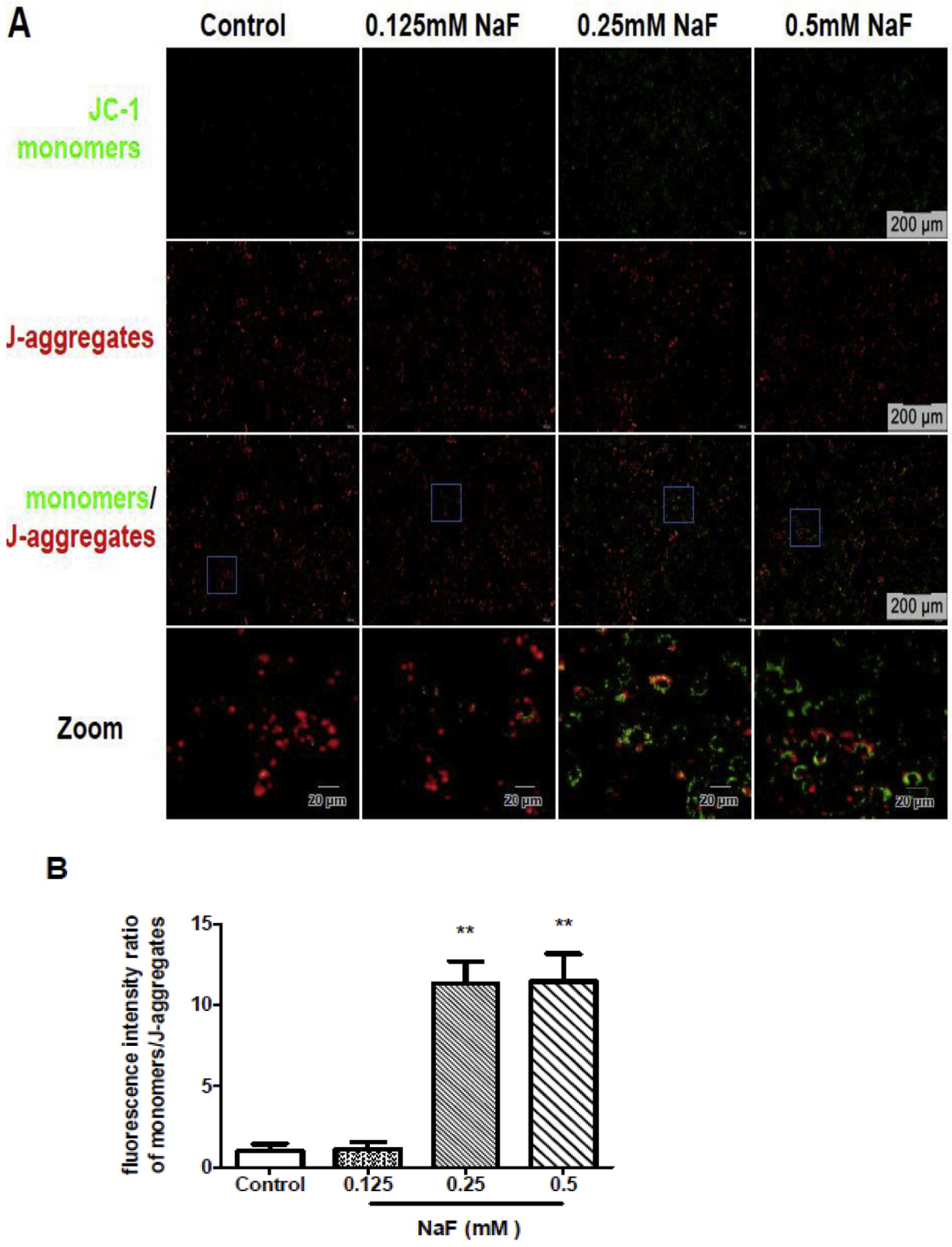

Fig. 3. Changes in mitochondrial membrane potential (MMP) induced by fluoride in TM3 Leydig cells. (A) Representative JC-1 staining images of TM3 Leydig cells treated with 0, 0.125, 0.25, and 0.5 mM NaF for 24 h. JC-1 monomer (Green fluorescence) shows the damaged mitochondria and J-aggregates (Red fluorescence) represent the normal mitochondria. (B) Quantification of the fluorescence intensity ratio of the monomers and J-aggregates which is indicates the MMP. The values are represented as mean ± SD (n = 6), $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant differences, compared with the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

while no significant changes were observed in 0.125 mM NaF exposed group (Fig. 3B). These results suggested that fluoride exposure (0.25 or 0.5 mM NaF) significantly decreased the MMP, and this depleted MMP is subjected to damage mitochondrial function in TM3 Leydig cells.

Furthermore, the results of co-localization and quantification of mitochondrial and lysosome demonstrated that the mitochondrial number were reduced and the lysosomes numbers ($p < 0.01$) were increased significantly with a dose-dependent manner in TM3 Leydig cells from all NaF-treated groups compared with the control

group (Fig. 4). Meanwhile, it was observed that much more mitochondria were encapsulated by lysosomes which are a characteristic of mitophagy occurrence in fluoride treatment groups by the staining of Mito Tracker Green and LysoTracker Red. From these results, it implies that under fluoride can weaken mitochondrial function and then induce mitochondrial autophagy in Leydig cells.

3.4. Fluoride enhanced PHB2 expression levels in TM3 Leydig cells

To support mitophagy appearance after fluoride exposure, the expression levels of the inner mitophagy receptor PHB2, and immunofluorescence co-localization of PHB2 and mitochondria marker protein COX IV in Leydig cells were further analyzed in this study. The results showed that fluoride treatment markedly increased PHB2 expression with a concomitant decrease in the number of mitochondria (Fig. 5). Analogously, in comparison with the control group, the mRNA expression levels of PHB2 increased significantly in all the treated groups ($p < 0.01$), and its proteins expression levels significantly increased in 0.125 mM ($p < 0.05$), 0.25 mM ($p < 0.01$) and 0.5 mM ($p < 0.05$) NaF treated groups as

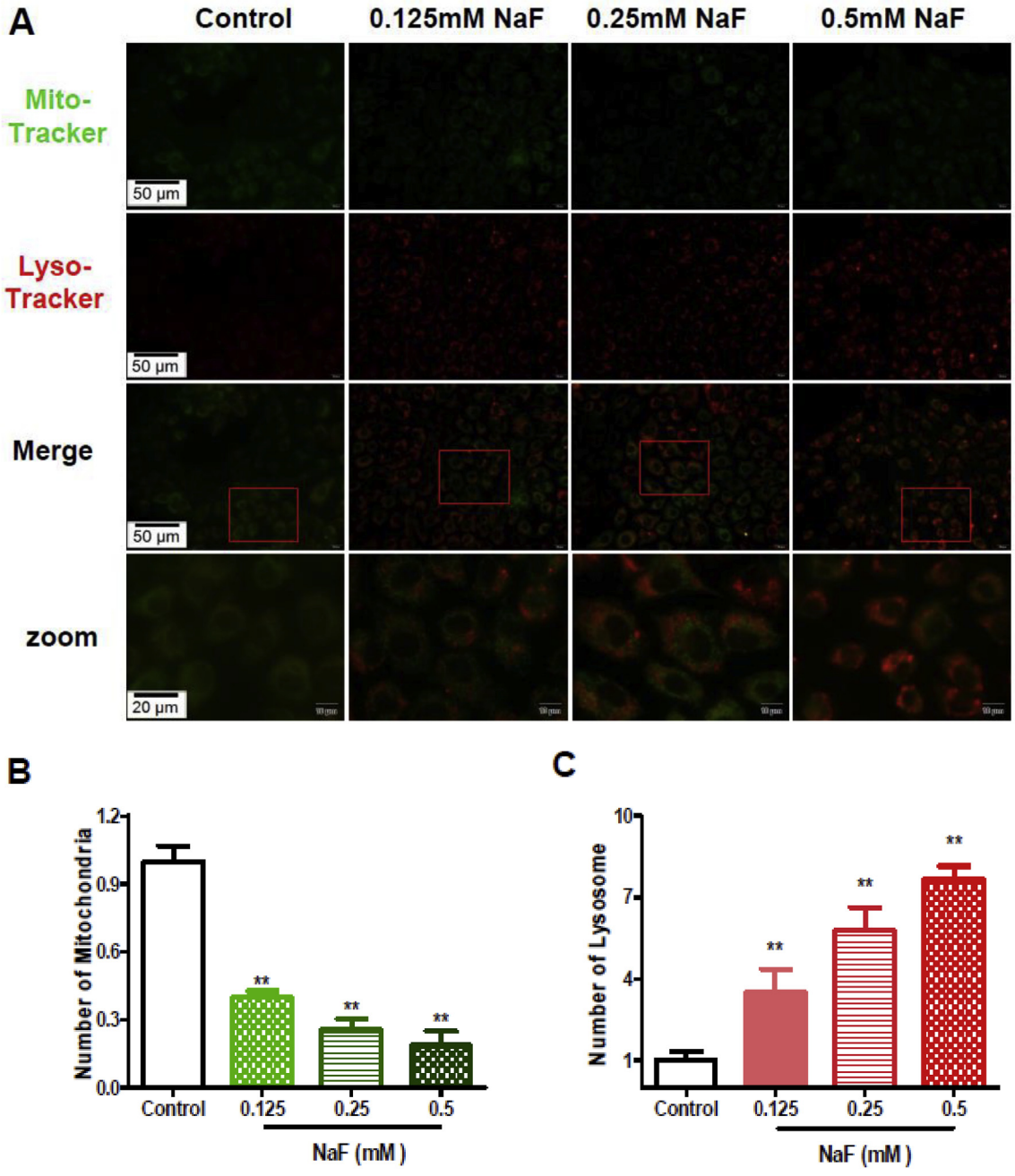

Fig. 4. Co-localization and quantification of mitochondrial and lysosome in NaF treated TM3 Leydig cells by MTG and LTR staining. (A) Representative images of the distribution of mitochondria and lysosomes in TM3 Leydig cells by MitoTracker Green (MTG) and LysoTracker Red (LTR) staining. Green fluorescence indicates mitochondria and the red fluorescence indicates lysosomes. Room shows the enlargements fields of marked with the red rectangular frame. (B) The numbers of mitochondria in TM3 Leydig cells obtained by the quantification of green fluorescence. (C) The numbers of lysosomes in TM3 Leydig cells obtained by the quantification of red fluorescence. The values are presented as mean ± SD (n = 8), $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant differences, compared with the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

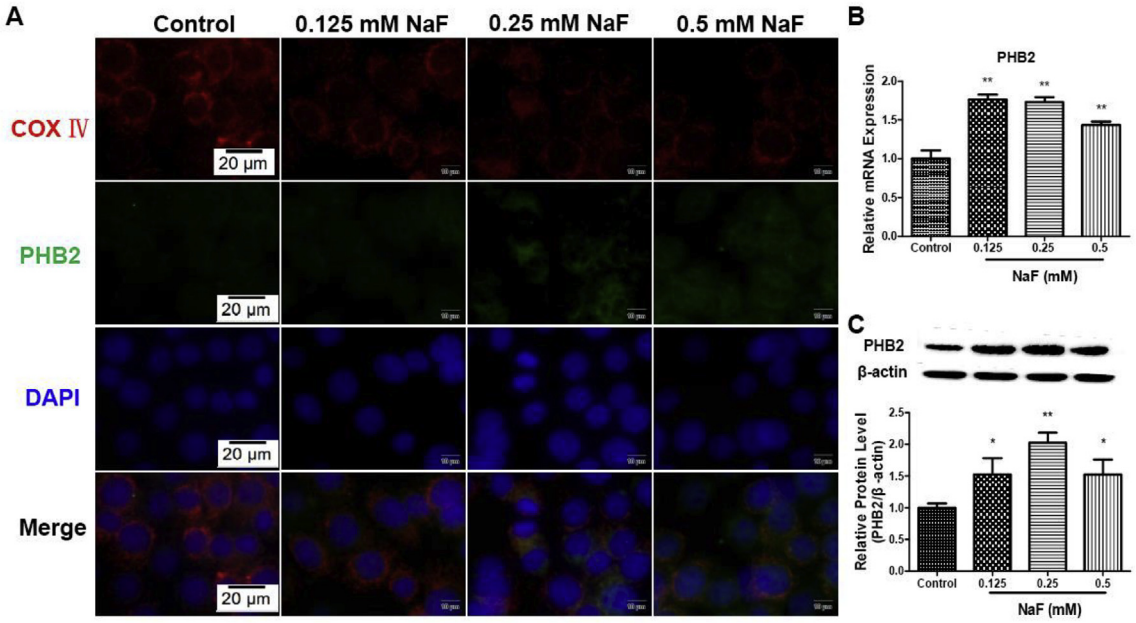

Fig. 5. Effects of fluoride on PHB2 expressions in mRNA and protein levels in TM3 Leydig cells. (A) Representative immunofluorescence images of PHB2 and COX IV co-localizations in TM3 Leydig cells treated with 0, 0.125, 0.25, 0.5 mM NaF for 24 h, respectively. The green color fluorescence indicates PHB2 expression, the red color fluorescence shows COX IV which indicates mitochondria, and cell nuclei were stained with DAPI (blue). (B, C) mRNA and protein expression levels of PHB2 in TM3 leydig cells by qRT-PCR and Western blot analyses. The values are presented as mean ± SD (n = 6), $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant differences compared with the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

well. These results provide a new evidence of mitophagy occurrence in Leydig cell caused by fluoride.

3.5. *PINK1 participates in the regulation of mitophagy induced by fluoride in Leydig cells*

As a key protein of PINK1/Parkin pathway, PINK1 plays a pivotal role in the regulation of mitophagy. To further confirm if PINK1 participates in the regulation of mitophagy induced by fluoride in Leydig cells *in vitro*, PINK1 expression levels in Leydig cells were examined by immunofluorescence, western blot, and QRT-PCR. The results indicated that PINK1 expressions were increased, and were accompanied by a decreased expression of mitochondrial marker protein COX IV in fluoride-treated Leydig cells in a dose-dependent manner (Fig. 6A). Correspondingly, the mRNA and protein expressions levels of PINK1 were also significantly increased in 0.5 mM NaF group *in vitro* ($p < 0.05$; Fig. 6B and C) compared with the control group.

4. Discussions

Mitophagy is a highly complex and dynamic cellular process that regulates both mitochondrial quality and quantity (Ashrafi and Schwarz, 2013). Mitophagy is also a peculiar form of autophagy that culminates in the selective autophagic clearance of damaged mitochondria in response to various mitochondrial stressors leading to the depolarization and/or malfunctioning of the organelle (Song et al., 2014b). In this study, we firstly reveal that fluoride can induce mitochondrial impairment and PINK1-mediated mitophagy in testicular cells, especially in Leydig cells by in vivo *and in vitro* approaches. On account of the extensive distribution and the significant health risks of fluoride in both food science and agriculture fields, the present study not only imply a novel toxic mechanism of fluoride-induced reproductive outcomes, but also provides examples for the potential risk study of other chemicals.

It is well known that the use of combinatory approaches including morphological and biochemical hallmarks to measure mitophagy is strongly recommended (Reichert et al., 2009). In the present study, we firstly observed more mitophagosomes formed in Leydig cells compared to other types cells in testes of NaF-exposed mice by using transmission electron microscopy (TEM). In order to clarify the major cells in which mitophagy occurred, immunofluorescence was applied to locate the mitophagy receptor protein PHB2 expressions in mice testes. Meanwhile, the model of Leydig cell line TM3 treated with fluoride were employed to investigate the expressions of mitophagy makers PHB2 and PINK1 in transcript and protein levels by qRT-PCR and western blotting. The immunofluorescence co-localization of PHB2 and mitochondria marker protein COX IV in Leydig cells were further analyzed in this study. All these results strongly supported that mitophagy was triggered by fluoride in mouse Leydig cells. The results are consistent with our previous study on autophagy, and it concludes that Leydig cells is one of the most sensitive cells in testicular tissues response to fluoride exposure (Zhang et al., 2017).

The fluoride leads to function or dysfunction of liver and cardiomyocyte via inducing mitochondrial injury. (Lu et al., 2017; Wang et al., 2018a,b). The studies from mice, rat and human lymphocytes found that fluoride exposure could cause ROS accumulation, mitochondria damage (Zhou et al., 2015), JNK signaling excitation (Suzuki et al., 2015) and apoptosis (Jothiramajayam et al., 2014). Ca^{2+} metabolism disorder induced by NaF was also looked as one of the promote mitochondrial damages (Wang et al., 2018a,b). In the present study, we observed that fluoride exposure caused mitochondrial structural impairment in germ cells, Sertoli cells and Leydig cells in mice testes. Most of all, mitochondrial changes in Leydig cells are most obvious. Moreover, the mitochondrial membrane potential (MMP) reduction, decrease of mitochondrial mass and increase of lysosomes were also detected in NaF-treated TM3 Leydig cells. This results showed that fluoride could induced mitochondrial impairment in Leydig cells, which maybe one of the

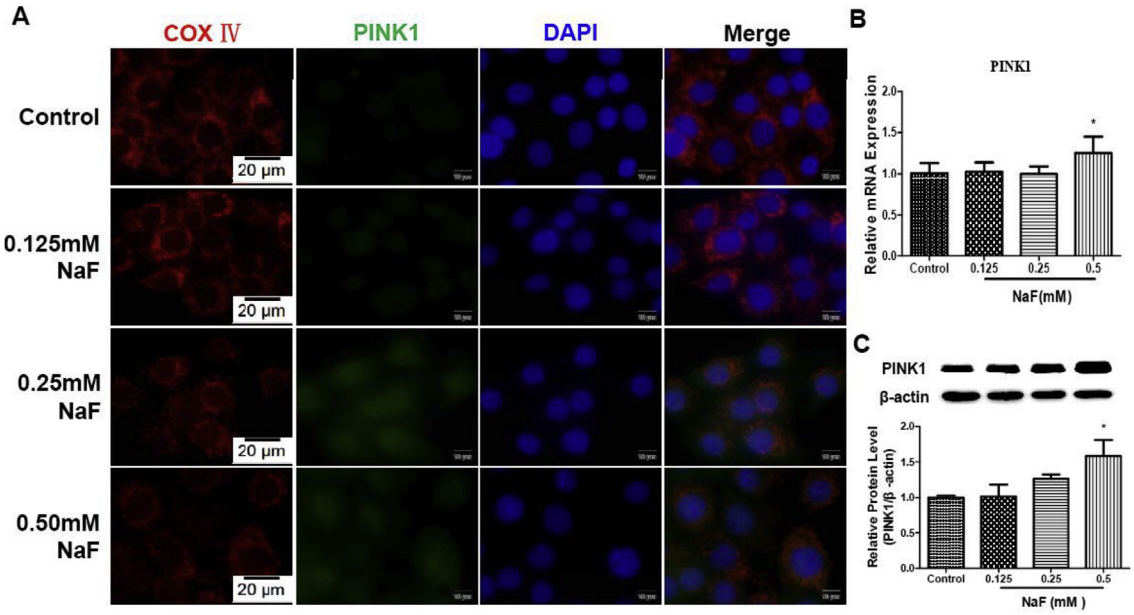

Fig. 6. The accumulation of mitophagy key proteins PINK1 induced by fluoride in Leydig cells. (A) Representative images of PINK1 immunofluorescence staining in TM3 Leydig treated with the different doses of NaF. The green fluorescence shows the PINK1 expression locations, COX IV indicates mitochondria (Red color fluorescence). (B, C) The mRNA and protein expression levels of PINK1 in TM3 leydig cells by qRT-PCR and Western blot, respectively. The values are presented as mean ± SD (n = 6), $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant differences compared with the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

potential mechanisms of fluoride reproductive toxicity.

It has been proved that mitochondrial damage accompanied by MMP reduction is early events of mitophagy evoked (Rodriguez-Enriquez et al., 2006). Our results from TEM and mitophagy makers expressions confirmed that fluoride exposure triggered mitophagy in Leydig cells. So future studies should address whether there are common or converging pathways to mediate mitophagy in response to distinct mitochondrial stresses.

Mitophagy in mammals can be mediated through various pathways like PINK1/Parkin pathway (Yamaguchi et al., 2016), Nix/BNIP3L (Ney, 2015), BNIP3 (Gonzalez et al., 2008) and FUNDC1 (Liu et al., 2012). PINK1/Parkin pathway which is mainly involved in the elimination of damaged mitochondria was reported as one of important pathways to mediate mitophagy (Chen and Dorn, 2013; Eiyama and Okamoto, 2015; Matsuda et al., 2010). For instance, PINK1 is known to be stabilized and accumulated at the mitochondrial membranes when mitochondria are damaged. PHB2, as an important inner mitochondrial membrane protein, regulates mitochondrial assembly and function (Bavelloni et al., 2015; Osman et al., 2009), also be proved as a receptor and marker for PINK1/

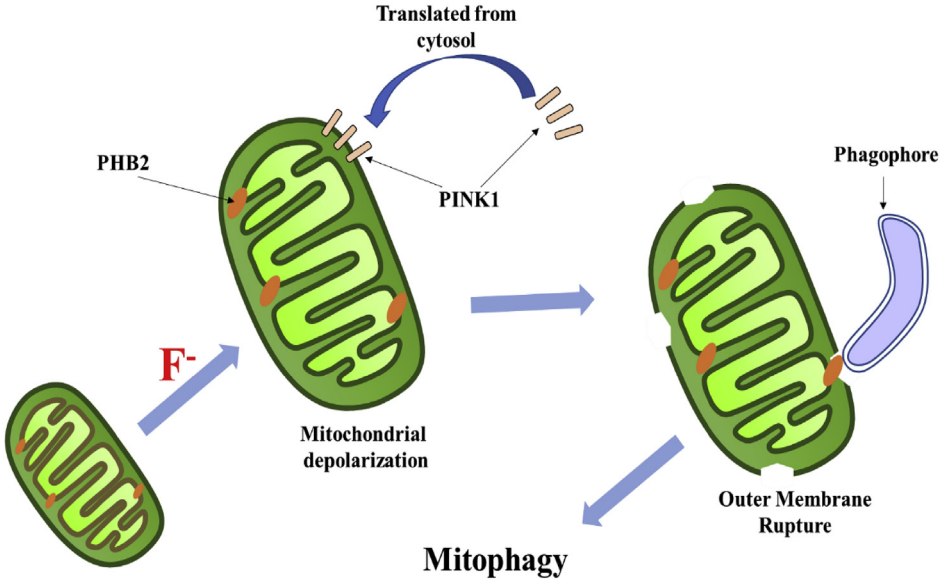

Fig. 7. Suggested mechanism of mitophagy formation in Leydig cells of testis induced by fluoride. Mitochondrial depolarization induced by F^{-} attacking, which causes the accumulation of PINK1 on the outer membrane, followed by subsequent ubiquitination and degradation of mitochondrial outer membrane proteins. The resulting rupture of the outer membrane allows the phagophore to bind the mitochondrial inner membrane protein PHB2, leading to mitophagy. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Parkin-mediated mitophagy in mitochondria (Wei et al., 2017; Xiao et al., 2018). Thus, we directly detected the expressions of PINK1 and PHB2 in both testicular tissues and TM3 Leydig cells. Results showed that transcripts and protein levels of PINK1 and PHB2 were increased by fluoride exposure both in vivo and *in vitro*. These results illustrated that PINK1/Parkin pathway participated in F-induced mitophagy in Leydig cells (Fig. 7).

Take together, our present in vivo and *in vitro* results revealed that F can induce mitochondrial impairment and mitophagy in testicular cells, especially in Leydig cells, and PINK1/Parkin-mediated mitophagy participants in this process, which will contribute to the mechanisms of F-induced male reproductive toxicity.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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