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Fluoride-Induced Autophagy via the Regulation of Phosphorylation of Mammalian Targets of Rapamycin in Mice Leydig Cells

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S *Supporting Information*

ABSTRACT: Fluoride is known to impair testicular function and decrease testosterone levels, yet the underlying mechanisms remain inconclusive. The objective of this study is to investigate the roles of autophagy in fluoride-induced male reproductive toxicity using both *in vivo* and *in vitro* Leydig cell models. Using transmission electron microscopy and monodansylcadaverine staining, we observed increasing numbers of autophagosomes in testicular tissue, especially in Leydig cells of fluoride-exposed mice. Further study revealed that fluoride increased the levels of mRNA and protein expression of autophagy markers LC3, Beclin1, and Atg 5 in primary Leydig cells. Furthermore, fluoride inhibited the phosphorylation of mammalian targets of rapamycin and 4EBP1, which in turn resulted in a decrease in the levels of *AKT* and *PI3K* mRNA expression, as well as an elevation of the level of *AMPK* expression in both testes and primary Leydig cells. Additionally, fluoride exposure significantly changed the mRNA expression of the *PDK1*, *TSC*, and *Atg13* regulator genes in primary Leydig cells but not in testicular cells. Taken together, our findings highlight the roles of autophagy in fluoride-induced testicular and Leydig cell damage and contribute to the elucidation of the underlying mechanisms of fluoride-induced male reproductive toxicity.

KEYWORDS: *fluoride, autophagy, testis, Leydig cell, mTOR phosphorylation*

INTRODUCTION

Fluoride is ubiquitously present in the environment and has pronounced reactivity in many physiological processes. Fluoride is one of the most significant inorganic pollutants in groundwater that affects human health globally,[1] especially in densely populated regions. For example, in Asia nearly 35 million people in China and 26 million people in India are at risk of fluoride toxicity.[2−4] In addition to groundwater, other natural sources of fluoride include food, dental products, and pesticides, which lead to added exposure for many people.[5] For decades, fluoride attracted the interest of toxicologists because of its adverse effects in humans and experimental animals. In these populations, fluoride can negatively impact various tissues, including brain,[6] liver,[7] kidney,[5] thyroid,[8] aorta,[9] ovary,[10] the skeletal system,[11,12] and the male reproductive system.[13]

The toxicological effects of fluoride on the male reproductive system are usually evaluated on the basis of the deterioration of sperm quality and the increase in infertility. Previous studies found that the reproductive toxicity of fluoride was involved in lowering sperm quality and serum testosterone,[14] as well as altering testicular structure,[15−17] multiple reproductive hormone levels,[18] the blood−testis barrier,[19] and spermatogenic proteins.[20] Fluoride also plays a role in capacitation,[21] apoptosis,[22] and hyperactivation and chemotaxis[23] in male reproductive cells. However, the molecular mechanisms underlying these effects are still not fully understood.

Autophagy is a process in which the subcellular membrane structures undergo dynamic morphological changes that lead to the degradation of cellular proteins and organelles by lysosomes.[24] Autophagy plays key roles in cellular homeostasis during embryonic development, postnatal cell survival, and death.[25,26] In the male reproductive system, autophagy has been associated with spermatogenesis,[27] germ cell death,[28] spermatid differentiation,[29] sperm function,[30] Sertoli cell apoptosis,[31] and testosterone secretion.[20,32] Moreover, autophagy can be induced by various stress stimuli, such as oxidative stress[33] and environmental factors.[26] Fluoride, as an environmental factor, was reported to induce oxidative stress[15] and endoplasmic reticulum stress[34] in testes. Several studies have shown that fluoride could induce autophagy in the LS8 ameloblast cell line.[35−37] A recent study indicated that fluoride exposure was associated with the increasing number of autophagosomes in the testes of rats.[38] However, the molecular mechanism of autophagy induced by fluoride in testes is still poorly understood.

In this study, we demonstrated that fluoride exposure induced an increase in the number of autophagosomes and changed expressions of genes and proteins described as markers of autophagy, including LC3, Beclin1, and Atg5, both in both *in vivo* and *in vitro* Leydig cell models. Furthermore, the phosphorylations of mammalian targets of rapamycin (mTOR) and target protein phospho-4EBP1 and phospho-p70-S6 kinase

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were detected by using the mTOR inhibition model. The mRNA expression of the critical molecular regulator genes, including *PI3K*, *AKT*, *AMPK*, *PDK1*, *TSC*, *ULK1*, and *Atg13*, in both testis and Leydig cells was investigated to elucidate the association between fluoride-induced autophagy and the regulation of mTOR phosphorylation.

MATERIALS AND METHODS

Materials. Sodium fluoride (NaF), dimethyl sulfoxide (DMSO), monodansylcadaverine (MDC), rapamycin (mTOR inhibitor), and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich. Dulbecco's modified Eagle's medium/F12 (DMEM/F12), 10% fetal bovine serum (FBS), and 0.25% trypsin were purchased from Gibco. Myllicin was purchased from Solarbio (Beijing, China). The PrimeScript RT Master Mix kit and the SYBR Premix Ex Taq II kit were purchased from Takara (Dalian, China). TRIZOL and primers were purchased from Invitrogen. RIPA lysis buffer and BCA protein assay kits were purchased from Beyotime Biotechnology (Shanghai, China). The anti-LC3 rabbit polyclonal antibody, the anti-Beclin1 rabbit polyclonal antibody, the anti-Atg5 rabbit polyclonal antibody, the anti-β-actin rabbit polyclonal antibody, and the HRP-conjugated goat anti-rabbit secondary antibody were purchased from Proteintech (Wuhan, China). Phospho-mTOR (Ser2448), phospho-4E-EP1 (Thr37/46), and phospho-p70 S6 kinase (Thr389) monoclonal antibodies were purchased from Cell Signaling Technology. All other chemicals used in this study not specifically mentioned above were analytical grade.

Treatment of Mice. Forty-eight healthy male Kunming mice (each weighing approximately 20−25 g), 8 weeks of age, were obtained from the Experimental Animal Center of Shanxi Medical University. Mice were maintained at a standard temperature (22−25 °C) and a 12 h light/dark cycle, with water and food *ad libitum*. After being acclimated for 1 week, all mice were randomly divided into four groups of 12: one control group (drinking deionized water) and three fluoride-administered groups (exposed to 25, 50, and 100 mg/L NaF in drinking water). After being exposed to fluoride for 60 days, mice were euthanized, and testes were immediately isolated for further study. All animals were treated humanely, and all handling procedures were approved by the Institutional Animal Care and Use Committee of Shanxi Agricultural University.

Primary Leydig Cell Culture and Determination of Purity. Leydig cells were isolated from the testes of 4-week-old Kunming mice. Mice were sacrificed and soaked in 75% ethanol for 5 min, and then testes were isolated and placed in a sterile plate containing phosphate-buffered saline (PBS). Leydig cells were detached and cultured according to the procedure described previously.[32,39] The tunica albuginea was nipped with tweezers and gently removed from the testis to expose the seminiferous tubule. The testis was gently shaken until the PBS became turbid. Thus, the Leydig cells were gradually separated from the testis. Then, the PBS was collected and centrifuged at 1200 rpm for 5 min to pellet the cells. The supernatant was discarded, and after the Leydig cells had been washed three times in PBS, the cells were collected, resuspended with DMEM/F12 supplemented with 10% FBS and myllicin, seeded in culture flasks, and cultured at 37 °C in an incubator with a humidified atmosphere containing 5% CO_2.

The purity of Leydig cells was assessed using the modified method described by Wang et al.[40] Then, the Leydig cell suspension was smeared on slides, dried at room temperature, incubated at 22 °C for 90 min, and then washed with deionized water. The positive cells were stained with a dehydroepiandrosterone solution. The purity was defined as the percentage of positive cells to total cells. Leydig cells that were >90% pure were used for further experimentation.

Leydig Cell Viability Assay and Treatments. The effects of NaF on cell viability were determined using the MTT assay. The Leydig cells were plated at a density of 5×10^4 cells per well in 96-well culture plates. The cells were treated with different concentrations of NaF for 24 h. The cells were treated with 10 μL of 10 mg/mL MTT, and the resulting formazan crystals were dissolved in dimethyl sulfoxide. The absorbance was measured with a microplate spectrophotometer at 490 nm. Results were expressed as percentages of the controls, which were assigned with 100% viability. The half-maximal inhibitory concentration (IC_{50}) and 95% confidence intervals were calculated.

When Leydig cells reached a confluence of 80−90%, they were detached from flasks using 0.25% trypsin and subcultured in six-well plates for fluoride treatment. According to the MTT result, Leydig cells were treated for 24 h with 0, 0.125, 0.25, and 0.5 mM NaF.

Table 1. Target Genes, Primer Sequences, and Product Sizes for Quantitative RT-PCR

gene	GenBank accession number	primer sequence (5′−3′)	product size (bp)
β-actin	NM_007393.5	forward, AGACTTCGAGCAGGAGATGG	233
		reverse, GCACTGTGTTGGCATAGAGG	
LC3	NM_026160.4	forward, GATAATCAGACGGCGCTTGC	99
		reverse, ACTTCGGAGATGGGAGTGGA	
Beclin1	NM_019584.3	forward, CTCTGAAACTGGACACGAGC	124
		reverse, CCTGAGTTAGCCTCTTCCTCC	
Atg5	NM_001314013.1	forward, GGACCTTCTACACTGTCCATCC	152
		reverse, TGTCATTCTGCAGTCCCATC	
PI3k	NM_001024955.2	forward, GGCAGAAGAAGCTGAACGAG	147
		reverse, GCAATAGGTTCTCCGCTTTG	
AKT	NM_001165894.1	forward, ACTCATTCCAGACCCACGAC	144
		reverse, AGTCCAGGGCAGACACAATC	
AMPK	NM_001013367.3	forward, ACCTGAGAACGTCCTGCTTTG	135
		reverse, GAAATGACTTCTGGTGCGGC	
mTOR	NM_020009.2	forward, TGTGAACGGAACATACGACC	117
		reverse, TTGCTTGCCCATCAGAGTCAG	
PDK1	NM_172665.5	forward, TGCTACTCAACCAGCACTCC	105
		reverse: TAATGACCTCCACCACGTCG	
TSC2	NM_011647.3	forward, AGCTCCAAAGACCCTTGAGC	135
		reverse, TCACGCTGTCTGGTCTTGTC	
ULK1	NM_009469.3	forward, AGTTCTGGAGATTGCAGCCC	81
		reverse, ACCACACTTTCCTGGAGCTG	
Atg13	NM_145528.3	forward, TGGCGGAAGATTTGGACTCC	84
		reverse, GGGTTTCCACAAAGGCATCG	

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mTOR Inhibition Model and Fluoride Treatment. Leydig cells were equally divided into six groups (subcultured in six-well plates, each well labeled as one group) for mTOR inhibition and fluoride treatment. Leydig cells without any treatment serve as the control; Leydig cells treated with only DMSO [0.01‰ (v/v)] were used as the solvent control. The other groups are the 0.5 mM NaF treatment group, the DMSO/0.5 mM NaF group, the rapamycin treatment group, and the rapamycin/0.5 mM NaF treatment group. In some experiments, rapamycin was dissolved in DMSO and prepared for the stock solution, and Leydig cells were pretreated with rapamycin (20 nM, diluted with medium) for 1 h before being stimulated with 0.5 mM NaF.[41] After treatment for 24 h, phosphorylated proteins from Leydig cells in all groups were extracted for phospho-4EBP1 and phospho-p70-S6 kinase expression analysis.

MDC Staining for Autophagic Vacuoles. The fluorescent dye MDC was used to stain the autophagic vacuoles as a marker of autophagy in testicular tissue and primary Leydig cells *in vivo*.[42,43] The testes were fixed in Bouin's solution, dehydrated in a gradient of ethanol (30% → 50% → 70% → 90% → 100%), cleared in xylol, and embedded in paraffin. The resulting samples were then sectioned serially into 4 μm slices using a Leica slicer (Leica) and mounted on glass slides. The sections were deparaffinized with xylene, rehydrated in a gradient of ethanol solutions, and incubated with a 50 μM solution of MDC dye (dissolved with DMSO) at 37 °C for 1 h. *In vitro*, Leydig cells attached to the coverslip were washed three times with PBS (5 min each time), fixed with 4% paraformaldehyde for 15 min, washed again, and incubated with a 50 μM solution of MDC dye at 37 °C for 30 min. All sections were washed three times in PBS after being incubated (5 min each time). MDC fluorescence levels were measured with a Leica inverted microscope (excitation wavelength of 380 nm, emission wavelength of 525 nm).

Transmission Electron Microscopy (TEM). The testes were rapidly cut into ~1 mm × ~1 mm × ~1 mm pieces, fixed in a solution of 2% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) at room temperature for 2 h, and then postfixed in 2.5% osmium tetroxide in 0.1 M PB. The ultrathin sections were prepared, mounted on copper grids after dehydration and embedding, and stained with uranyl acetate and citrate. The sections were then examined and photographed with a transmission electron microscope (JEM 1011).

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from testes and primary Leydig cells with TRIZOL reagent (Invitrogen). The quality of total RNA was analyzed via 1% agarose gel electrophoresis and a NanoDrop 2000 instrument (Thermo Fisher). RNA was reverse-transcribed using a PrimeScript reverse transcription Master Mix kit. Quantitative RT-PCR was performed using the QuantStudio 7 Flex quantitative RT-PCR system (Life Technologies) and SYBR@ Premix Ex TaqTM II kit. The quantitative RT-PCR primers were designed with Primer 3.0 plus Software (Applied Biosystems) according to the complete cDNA sequences deposited in GenBank (Table 1). The β-actin gene was used as an internal reference. The PCR amplification conditions were as follows: predegeneration at 95 °C for 30 s, 50 cycles of polymerase chain reaction (PCR) at 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s, and dissociation at 95 °C for 15 s, 60 °C for 1 s, and 95 °C for 15 s. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting Analysis. Forty milligrams of testis tissue was homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF). The homogenization solution was centrifuged at 12000*g* for 10 min at 4 °C, and the supernatant was collected. The protein concentration was determined by a bicinchoninic acid (BCA) protein assay kit with bovine serum albumin (BSA) as the standard. Total protein equivalents for each sample were mixed with loading buffer followed by denaturation at 100 °C for 10 min, separated on a 10 to 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis gel, and subsequently transferred to nitrocellulose (NC) membranes. The membranes were first blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 for 2 h at room temperature. They were then incubated overnight at 4 °C with the primary antibodies against LC3 (1:300 dilution), Beclin1 (1:1000 dilution), Atg5 (1:1000 dilution), phospho-mTOR (1:1000 dilution), phospho-4E-BP1 (1:1000 dilution), phospho-p70-S6 kinase (1:1000 dilution), and β-actin (1:1000 dilution). The membranes were washed three times with Tris-buffered saline Tween (TBST) for 5 min each, followed by incubation with a secondary antibody at room temperature for 2 h. Protein bands on membranes were detected by enhanced chemiluminescence (ECL). A Fluor Chem Q Imaging System and its analysis software system (Cell & Bioscience) were used to acquire, quantify, and analyze the intensity of the protein bands.

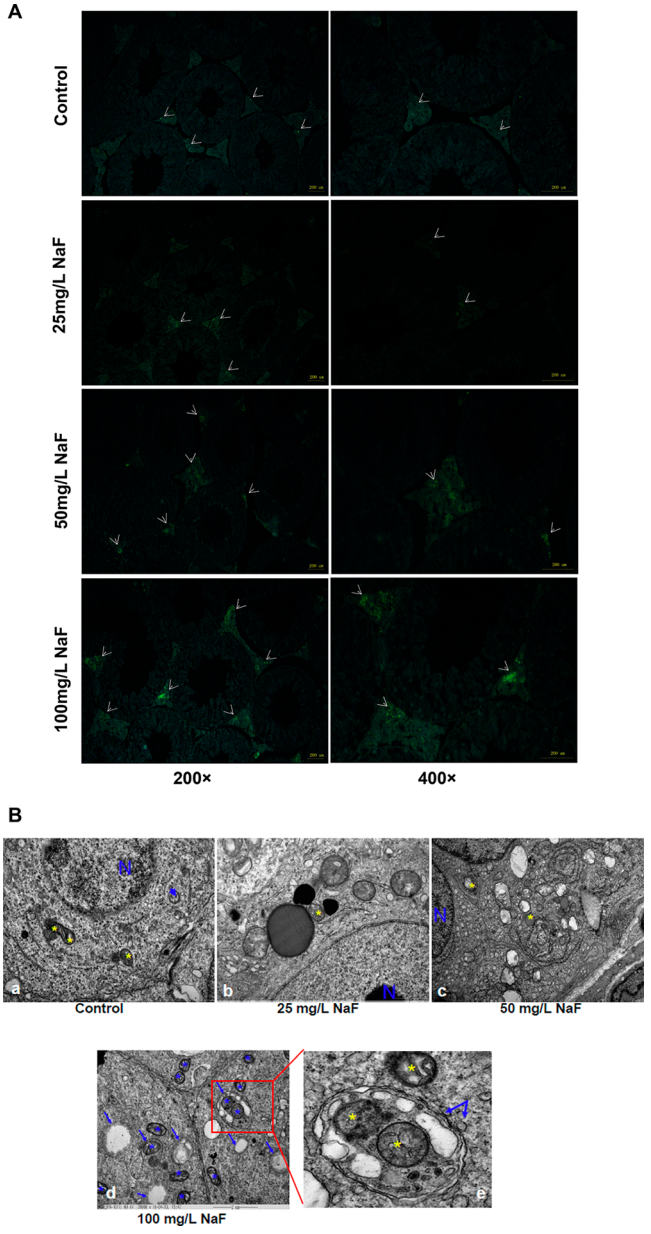

Figure 1. Representative images of autophagosomes in testicular tissues of mice exposed to fluoride. (A) Fluorescent images of MDC staining of mice testis sections treated with 25, 50, and 100 mg/L NaF. Arrows indicate the regions stained positive for MDC; green areas are autophagic vacuoles in testicular tissue. Many autophagic vacuoles appeared in the Leydig cells of testicular tissues. (B) Transmission electron microscopy revealed the testicular ultrastructure in control (Ba) and fluoride-treated mice (Bb−d). In contrast, a large amount of typical autophagosome (Be) caused by fluoride is presented in testicular tissue, especially in Leydig cells. The asterisk indicates mitochondria. The autophagosomes are denoted by blue arrows. N denotes the cell nucleus.

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Statistical Analysis. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The results were evaluated with one-way analysis of variance followed by Dunnett's multiple-comparison test. The means and standard errors (SE) of the data were analyzed and compared. A P value of <0.05 was considered statistically significant.

RESULTS

Detection of Autophagic Vacuoles in Mice Testicular Tissues. MDC was preferentially used to label the autophagosomes as a stain via its integration into lipids in autophagic vacuoles.[42] The number of autophagosomes increased significantly in testicular tissues, especially in Leydig cells in NaF-treated groups with increasing NaF concentrations (Figure 1A). The ultrastructure of testicular tissue determined by TEM (Figure 1B) further demonstrated that the amount of autophagosomes was distinctly increased in mice testes exposed to 25, 50, and 100 mg/L fluoride. A typical example of a single Leydig cell with many autophagosome caused by 100 mg/L fluoride is presented at higher magnification (Figure 1Be). These findings indicate that fluoride increased the number of autophagic vacuoles in Leydig cells in mice testes.

Effects of Fluoride on Autophagy Marker Protein Expression in Testes. The mRNA levels of autophagy marker proteins LC3, Beclin1, and Atg5 were examined by real-time RT-PCR. A significant increase in the level of LC3 mRNA expression was noted in testes of mice exposed to 50 and 100 mg/L NaF ($P < 0.05$, and $P < 0.01$) (Figure 2A), while no significant changes were observed in either Beclin1 or Atg5 mRNA expression (panel B or C, respectively, of Figure 2). The levels of LC3 II, Beclin1, and Atg5 protein expression in testes were further validated by Western blot. The results showed that the level of expression of LC3 II (Figure 2E) but not that of Beclin1 or Atg5 (Figure 2F and G) increased significantly in NaF-treated groups ($P < 0.05$, and $P < 0.001$) when compared with the controls. The results of protein expression are consistent with mRNA expression from quantitative RT-PCR. Because LC3 II serves as a reliable marker for mature autophagosomes, the results described above further suggest that

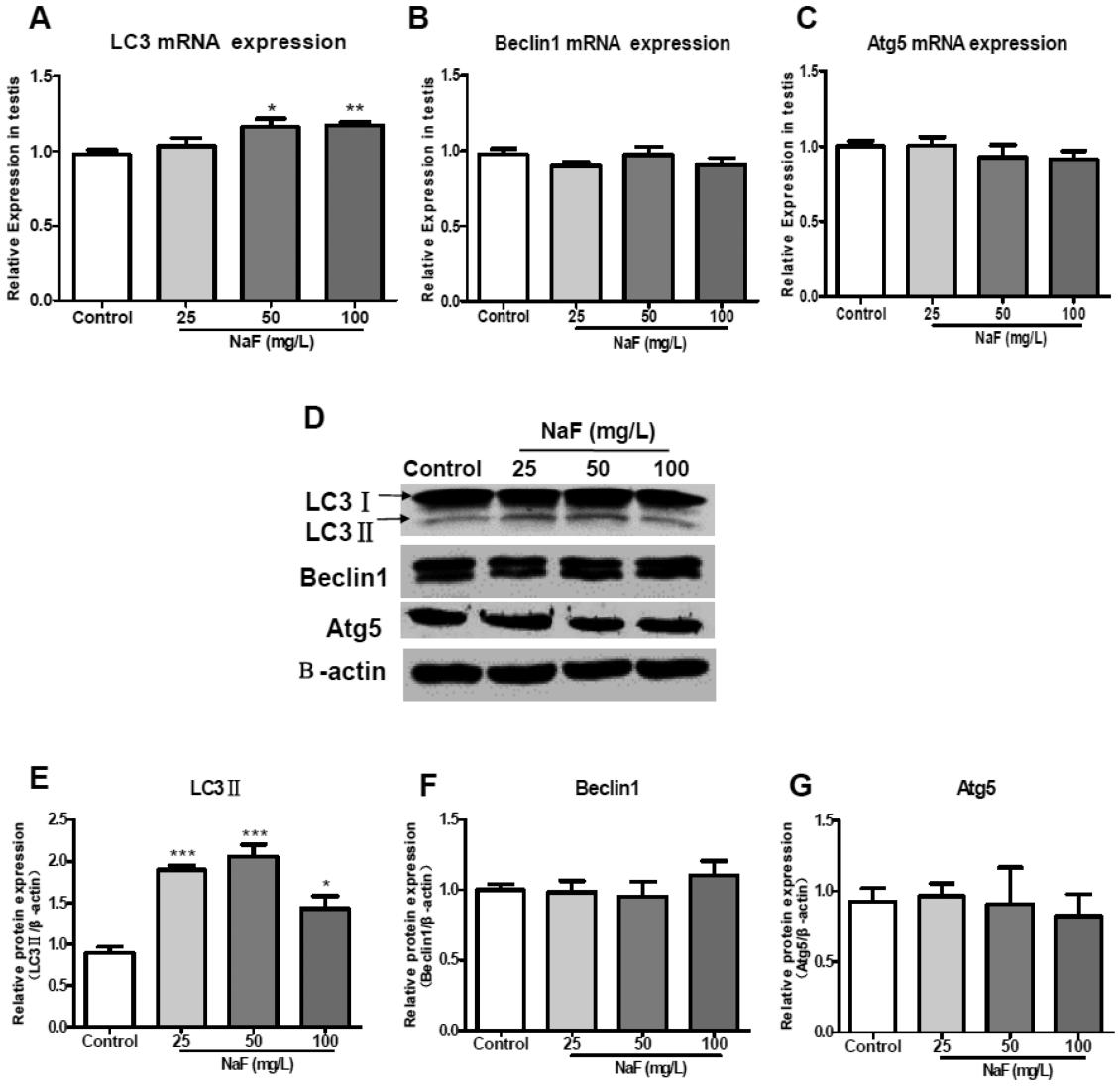

Figure 2. Effects of fluoride on the expression of autophagy markers in mRNA and protein levels in mice testes. (A–C) Levels of *LC3*, *Beclin1*, and *Atg5* mRNA expression, respectively, were detected by quantitative RT-PCR. The data are expressed as relative values measured against the control group. (D–G) Levels of protein expressions of LC3 II, Beclin1, and Atg5, respectively, were examined using Western blot. β-Actin was used as a control. The values are presented as means ± SEM ($n = 5$). ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ indicate significant differences compared to the control.

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fluoride exposure increased the level of formation of mature autophagosomes in mice testes.

Effects of Fluoride on Autophagy in Leydig Cells. To further confirm the results from both MDC staining and autophagy marker protein in testes, we also performed experiments with the primary cultured mice Leydig cell model. Leydig cells with validated purity and a passing MTT assay were employed for further analysis with MTT results shown in Figures S1 and S2. The IC_{50} of fluoride with primary cultured mouse Leydig cells was 1.29 mM. The results of MDC staining and autophagy marker LC3, Beclin1, and Atg5 expression are shown in Figure 3. The fluorescence intensity of MDC indicates that the autophagic vacuoles in autophagosomes were greatly increased in Leydig cells exposed to 0.25 and 0.5 mM NaF for 24 h (Figure 3A). When compared with that of the control group, the levels of mRNA expression of all autophagy marker proteins LC3, Beclin1, and Atg5 increased significantly in the 0.5 mM NaF group (Figure 3B−D), while levels of LC3 and Atg5 mRNA expression also increased significantly with an even lower NaF concentration in the 0.25 mM NaF group (Figure 3B,D). The levels of protein expression of all autophagy marker proteins LC3 II, Beclin1, and Atg 5 were upregulated markedly in all treatment groups (Figure 3F−H) except for Beclin1 in the 0.125 mM NaF groups. The results as evaluated by autophagy marker proteins indicated that Leydig cells are sensitive to autophagy caused by autophagy.

Changes in mTOR Phosphorylation Induced by Fluoride. As the activity of mammalian targets of rapamycin (mTOR) plays a key role in driving autophagy, we determined the expression of phosphorylated mTOR (Ser2448) both in mice testes and in Leydig cells. When compared with that of the corresponding control group, the level of protein expression of Ser 2448 p-mTOR was reduced significantly in mice testes of the 50 and 100 mg/L NaF groups ($P < 0.05$, and $P < 0.01$) and in Leydig cells treated with the 0.5 mM NaF group (Figure 4A,B). Furthermore, mRNA expression of mTOR was examined in both mice testes and Leydig cells by quantitative RT-PCR. The result showed that the mRNA expression of mTOR in mice testes was not significantly different across treatment groups (Figure 4C), while the level of mRNA expression of mTOR in Leydig cells treated with 0.25 and 0.5 mM NaF was markedly reduced (Figure 4D).

To confirm the results that mTOR signaling was suppressed by fluoride, rapamycin, which is a specific inhibitor, was used in Leydig cells for the mTOR inhibition model and effective fluoride treatment; two marked proteins of 4EBP1 and p70S6 K phosphorylation levels were detected as shown in Figure 5. The results indicate that the 0.5 mM fluoride treatment significantly decreased the level of phospho-4EBP1 expression in Leydig cells. Moreover, after mTOR signaling inhibited by rapamycin, the levels of phospho-4EBP1 expression are not significantly different between the fluoride treatment and the controls (Figure 5A,B). Nevertheless, p70S6 K phosphorylation levels in Leydig cells were affected by fluoride under this experimental condition (Figure 5A,C). Taken together, these results indicate that inhibition of mTOR phosphorylation could be associated with fluoride-induced autophagy in testes, especially in Leydig cells.

mRNA Expression of mTOR Regulation-Related Genes. We then investigated a range of seven genes associated with mTOR regulation, including PI3K, AKT, AMPK, PDK1, TSC, ULK1, and Atg13. Our *in vivo* experiments showed that levels of PI3K and AKT mRNA expression decreased

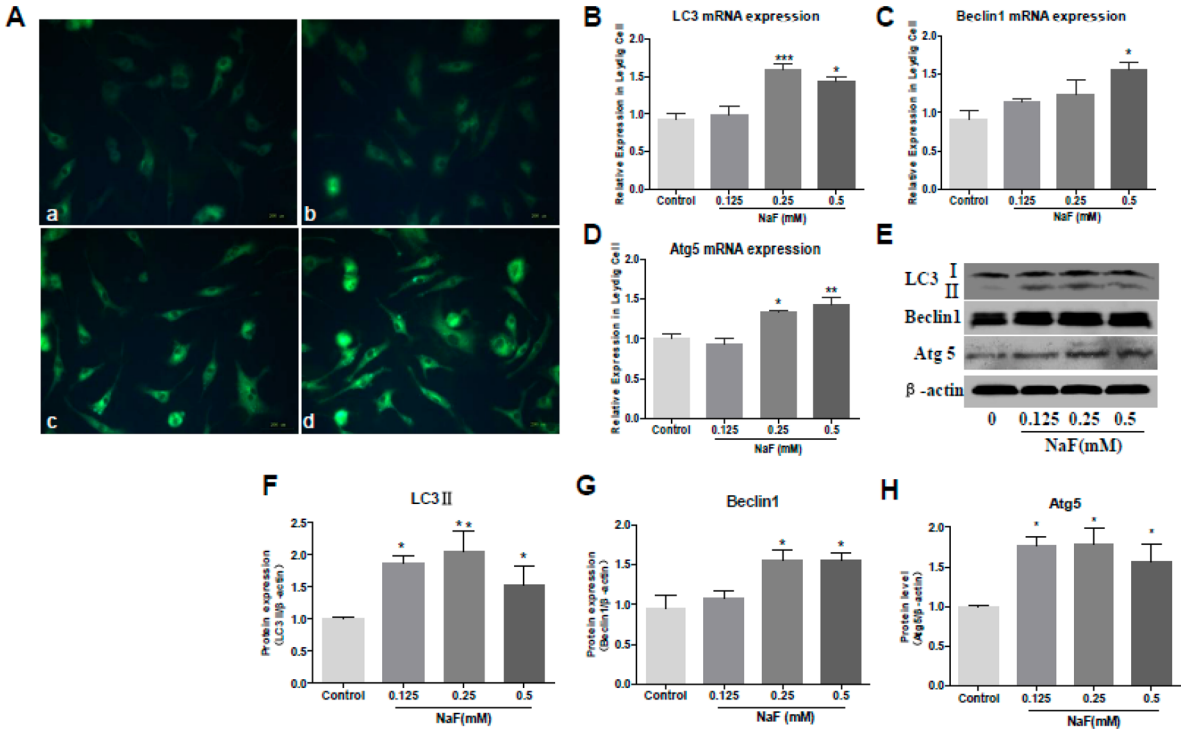

Figure 3. Effects of fluoride on autophagy in primary cultured mice Leydig cells. (A) Autophagic vesicles shown in the primary cultured Leydig cells treated with (a) 0, (b) 0.125, (c) 0.25, and (d) 0.5 mM NaF for 24 h by MDC staining. (B−D) Changes in levels of *LC3*, *Beclin1*, and *Atg5* mRNA expression induced by fluoride by quantitative RT-PCR, respectively. (E−H) Levels of protein expression of LC3, Beclin1, and Atg5, respectively, in Leydig cells detected by Western blot. The data are presented as means ± SEM ($n = 5$). ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ indicate significant differences compared to the control.

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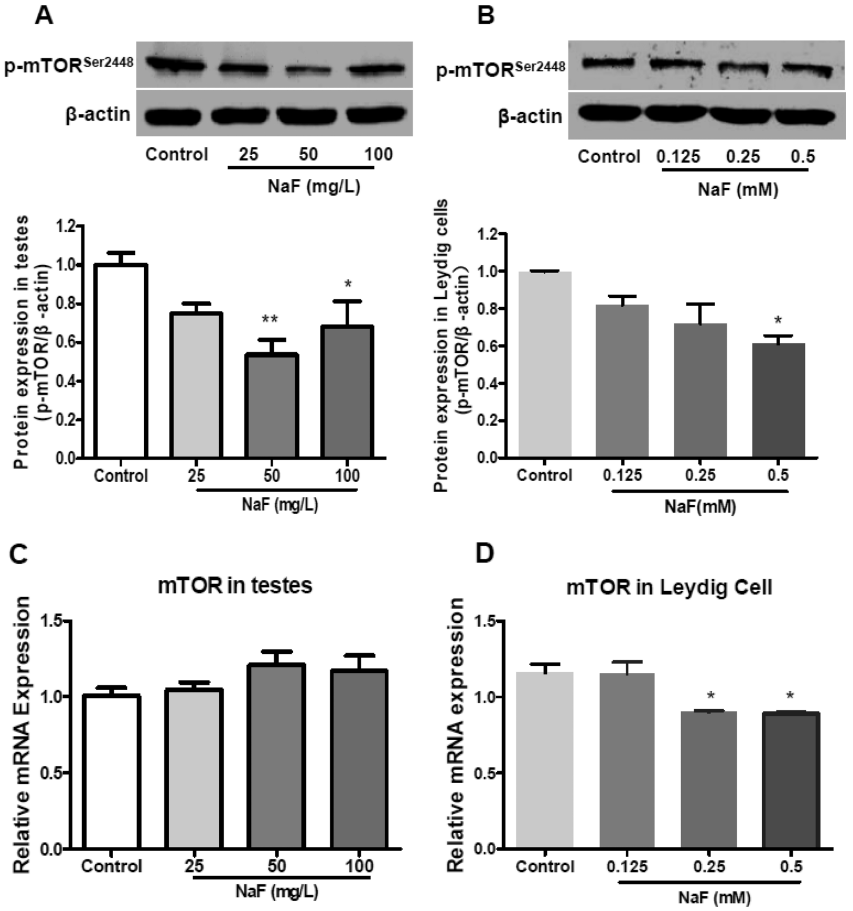

Figure 4. Effects of different doses of fluoride on phospho-mTOR protein and mRNA levels in testes and Leydig cells of mice. (A and B) Expression of phospho-mTOR in testes and Leydig cells, respectively, detected by Western blot. (C and D) mTOR mRNA expression in testes and Leydig cells detected by quantitative RT-PCR. The values are presented as means ± SEM (n = 5). $^{**}P < 0.01$ and $^{*}P < 0.05$ indicate significant differences compared to the control.

significantly in the 50 and 100 mg/L NaF groups ($P < 0.05$, and $P < 0.01$), while the level of AMPK mRNA expression increased significantly in the 100 mg/L NaF group ($P < 0.01$) (Figure 6A). Our *in vitro* experiments with Leydig cells showed that the level of PI3K mRNA expression was significantly decreased in the 0.25 mM NaF group ($P < 0.05$), while the level of AKT mRNA expression was significantly decreased in the 0.25 and 0.5 mM NaF groups ($P < 0.05$, and $P < 0.01$), and the level of AMPK mRNA expression was increased significantly in the 0.5 mM NaF group ($P < 0.01$) (Figure 6B).

Despite the significant changes in PI3K, AKT, and AMPK mRNA expression, the mRNA expression of other genes associated with mTOR phosphorylation (PDK1, TSC, ULK1, and Atg13) was not significantly different between the control and fluoride exposure groups in mice testes (Figure 6C). In Leydig cells, only ULK1 mRNA expression remained unchanged in fluoride-exposed groups. The level of PDK1 mRNA expression was markedly decreased in the 0.5 mM NaF group ($P < 0.05$); the level of TSC mRNA expression was significantly increased in all NaF exposure groups ($P < 0.05$, and $P < 0.01$), and the level of Atg13 mRNA expression was significantly increased in the 0.5 mM NaF group ($P < 0.05$) (Figure 6D). These results suggested that most genes associated with mTOR regulation participated in the fluoride-induced formation of the autophagosome to various degrees in testes, especially in Leydig cells.

DISCUSSION

Autophagy, one of the degradation systems in eukaryotic cells, plays key roles in maintaining intracellular homeostasis by degrading and recycling damaged organelles and macromolecules.[44,45] The aim of this study was to explore the roles of autophagy in the testicular damage induced by fluoride, which could contribute to mechanisms of fluoride-induced male reproductive toxicity.

We first investigated whether fluoride influenced autophagy in mice testes. In this study, we found a large amount of typical autophagosomes in mice testes exposed to fluoride using TEM, which is consistent with a previous study.[34] We further confirmed by MDC staining, a specific autophagosome marker,[42,43] that fluoride exposure increased the number of autophagosomes in mice testes. We also found that fluoride-induced autophagy mainly occurred in Leydig cells of mouse testicular tissue as verified by an *in vitro* primary culture mice Leydig cell model. These data agree with the report that autophagy is induced in Leydig cells after zearalenone exposure.[40]

Autophagy-related proteins that play important roles in the induction of autophagy include LC3, Beclin1, and Atg5. LC3 is one of the most important proteins and currently the only reliable marker of autophagosomes.[46] Under the promotion of an Atg5−Atg12−Atg16L complex, LC3-I (the cytoplasmic form) covalently conjugates with phosphatidylethanolamine (PE) and forms LC3 II, which is bound to the autophagosome

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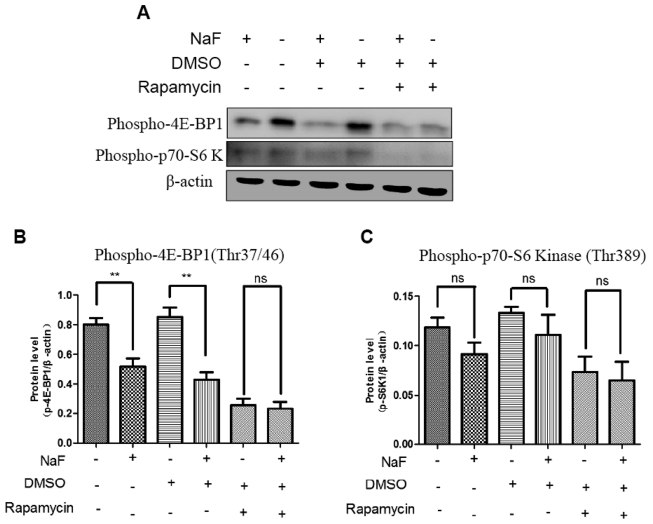

Figure 5. Effects of fluoride on phospho-4EBP1 and -p70S6 K expression in Leydig cells after mTOR signaling inhibition. Leydig cells without any treatment serve as the control; Leydig cells treated with only DMSO [0.01‰ (v/v)] were used as the solvent control. Leydig cells were pretreated with rapamycin (20 nM, diluted with medium) for 1 h before the 0.5 mM NaF treatment. After 24 h, the phosphorylated proteins were extracted for Western blotting. Panel A shows the representative images of the two marker proteins. Panels B and C demonstrate the changes in phospho-4EBP1 and phospho-p70S6 K expression in Leydig cells. The values are presented as means ± SEM (n = 5). $^{**}P < 0.01$ indicates significant differences compared to the paired control. ns indicates that the difference between the groups is not statistically significant.

membrane.[47] LC3 II promotes autophagosome formation likely by facilitating membrane elongation.[33] Beclin1 is an essential autophagy protein that regulates the initiation of autophagy and autophagosome−lysosome fusion by interacting with two protein complexes.[37] Atg5 is a gene product required for the formation of autophagosomes.[48] Fluoride has been reported to increase the level of LC3II but not Beclin1 in rat testes.[38] In the study presented here, fluoride treatment enhanced the protein expression of LC3 in mice testes, while the changes in mRNA and protein expression of both Beclin1 and Atg5 were not detected in mice testes. However, using an *in vitro* primary Leydig cell model, the levels of mRNA and protein expression increased significantly for not only LC3 but also Beclin1 and Atg5 upon exposure to fluoride. The results suggest that Beclin1 and Atg5 also play roles in fluoride-induced autophagy. Moreover, the changes in autophagy marker levels are consistent with the results from MDC staining and TEM that fluoride induces autophagy more distinctly in Leydig cells than in the whole testicular tissue of mice.

The regulatory role of the mTOR pathway in autophagy was first demonstrated in yeast[49] and later in *Drosophila*.[50] As a central checkpoint that negatively regulates autophagy, the suppression of mTOR stimulates autophagy.[51,52] In this study, fluoride significantly dephosphorylated mTOR and 4EBP1 in Leydig cells by using the mTOR inhibitor model, indicating that mTOR signaling was suppressed by fluoride. Therefore, the study suggests that fluoride induces autophagy through mTOR dephosphorylation and suppression in mice Leydig cells.

The key regulators of mTOR are AKT and AMPK.[53,54] mTOR integrates signals that either inhibit autophagy via the PI3K/Akt pathway[55] or trigger autophagy via activation of AMPK.[56] On the other hand, activation of AKT can be regulated by PDK1 (phosphoinositide-dependent kinase 1), which is phosphorylated by PI3K.[57] The activation of AMPK and the inhibition of AKT activity led to the suppression of TSC2 (tuberous sclerosis complex 2)−mTOR signaling, which promoted autophagy.[58,59] Therefore, the mRNA expression of all of these key genes upstream and downstream of the mTOR signal pathway involved in autophagy was detected in this study, to further verify the regulation of mTOR phosphorylation in the autophagy induced by fluoride in Leydig cells. The results showed that most genes associated with mTOR regulation participated in the fluoride-induced formation of autophagosomes to various degrees in testes, especially in Leydig cells. For instance, fluoride downregulated the mRNA expression of PI3K, AKT, and PDK1 and upregulated the mRNA expression of AMPK, TSC2, and Atg13 in Leydig cells.

According to the background presented above, it is easy to understand that the downregulation of PI3K and AKT mRNA expression may be associated with low levels of active mTOR, whereas an increased level of AMPK may be associated with the suppression of mTOR. In either case, the suppression of mTOR would induce autophagy. Upon inactivation of mTOR, both ULK1 and Atg13 were dephosphorylated, which relieved the inhibition of autophagy.[60] The relationship between autophagy and mTOR regulation-related genes is shown in Figure 7. In addition, our data suggest that the cellular response of fluoride-induced autophagy may be associated with the AMPK and AKT signaling pathway, and the precise regulation needs to be studied further.

This specific investigation of Leydig cells as opposed to whole testes is well grounded because the weight of Leydig cells is only 2% of that of a whole testis. Consequently, it is understandable that the levels of mRNA and protein expression in Leydig cells induced by fluoride are not always consistent with that in testicular tissues, such as Beclin1, Atg 5, mTOR, PDK1, TSC, and Atg13. The expression of several genes changed significantly only in Leydig cells but not in testes perhaps because of the obviously autophagy-induced effect of fluoride on Leydig cells. A possible explanation for the greater level of fluoride-induced damage to the Leydig cells is that Leydig cells are more sensitive to fluoride than other cells in the testis, because of their presence in the connective tissue of the seminiferous tubes. Because Leydig cells are important endocrine cells and are responsible for androgen production, previous studies that report the fluoride-induced decrease in serum testosterone levels also provided indirect evidence for our results on fluoride-induced autophagosome formation in Leydig cells.[19,20,61]

The World Health Organization gives guideline values for naturally occurring chemicals of health significance in drinking water that normally represent the concentration of the constituent that does not result in any significant risk to health over a lifetime of consumption. The guideline value given for fluoride is 1.5 mg/L.[62] In April 2015, the U.S. Department of Health and Human Services Federal Panel of Community Water Fluoridation replaced a 1962 recommendation that the optimal range for fluoride in drinking water, based on the outdoor air temperature, was 0.7−1.2 mg/L with a new recommendation of 0.7 mg/L. Fluoride ion concentrations in groundwater have been reported to be as high as 48 mg/L.[63] Previous studies also indicate that mice have special tolerances to fluoride that are 10−20 times higher than that of humans.[64,65] According to environmental levels, mice were

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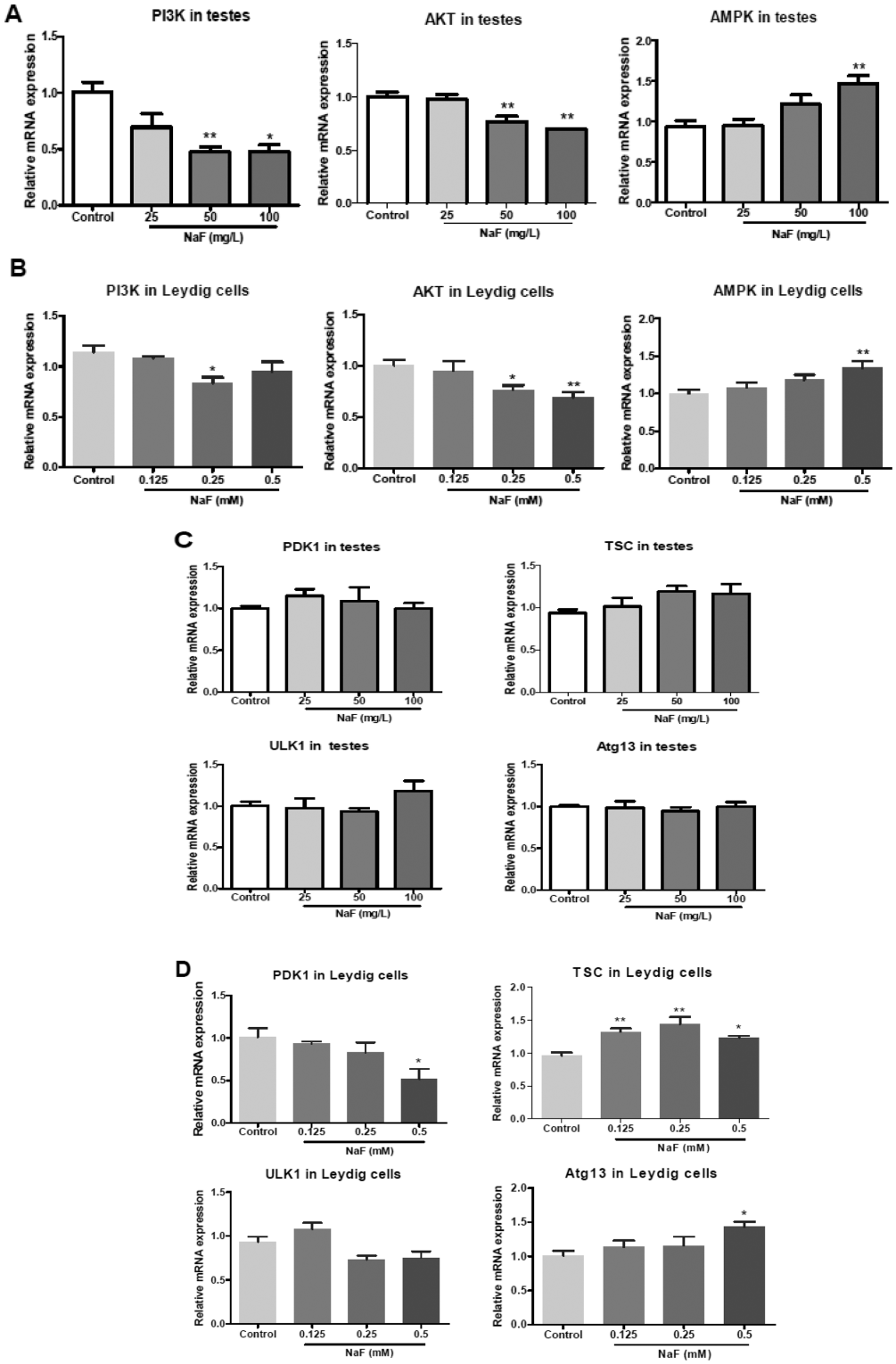

Figure 6. Changes in mRNA expression of genes associated with mTOR phosphorylation induced by fluoride in testes and Leydig cells by quantitative RT-PCR. (A and B) *PI3K*, *AKT*, and *AMPK* mRNA expression in testes and Leydig cells, respectively. (C and D) Levels of expression of PI3K/AKT/AMPK-regulated genes of *PDK1*, *TSC*, *ULK1*, and *Atg13* induced by fluoride in testes and Leydig cells. The values are presented as means ± SEM (n = 5). $^{**}P < 0.01$ and $^{*}P < 0.05$ indicate significant differences as compared to the control.

maximally treated with 100 mg/L NaF, corresponding to 45.2 mg/L fluoride ion in our study.

The various concentrations of fluoride exposure *in vivo* and *in vitro* used in our study were within the effective ranges of fluoride-induced autophagy in mice testes and Leydig cells. The NaF doses determined by the MTT assay in the *in vitro* primary Leydig cell model indicated that the IC_{50} was 1.29 mM, with a 95% confidence interval of 0.81−2.07 mM (Figure S2). Therefore, we chose 0.125, 0.25, and 0.5 mM NaF, corresponding to 5, 10, and 20 mg/L NaF, respectively, as fluoride exposure dosages. The chosen fluoride exposure levels were also administered in a recent study.[66]

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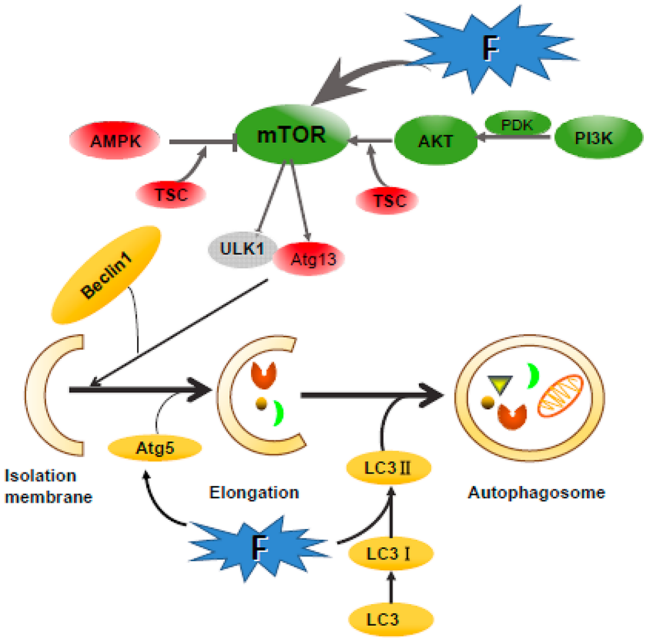

Figure 7. Suggested regulated mechanisms of autophagy induced by fluoride (F) in testes and Leydig cells. Fluoride can promote the formation of autophagy via mTOR phosphorylation. Green ellipses indicate downregulated genes or proteins; red ellipses indicate upregulated genes, and yellow denotes the proteins of the autophagosome.

Taken together, our study provides evidence that fluoride could induce autophagy in both mice testes and Leydig cells, with a stronger tendency in Leydig cells, by both *in vivo* and *in vitro* experiments. Several lines of evidence indicate that mTOR is involved in fluoride-induced autophagosome formation and contribute to the elucidation of the underlying mechanisms of fluoride-induced male reproductive toxicity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b03822.

Representative images of primary culture mice Leydig cells (Figure S1) and cell viability results of exposure of primary Leydig cells to fluoride from the MTT assay (Figure S2) (PDF)

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Notes

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