Fluorochloridone Induces Autophagy in TM4 Sertoli Cells:Involvement of ROS-mediated AKT-mTOR Signaling Pathway

Zhijing Ni
Fudan University

Weiqi Sun
Fudan University

Rui Li
Shanghai Institute for Food and Drug Control

Mingjun Yang
Shanghai Institute of Planned Parenthood Research

Fen Zhang
Fudan University

Xiuli Chang
Fudan University

Weihua Li
Shanghai Institute of Planned Parenthood Research

Zhijun Zhou (✉ zjzhou@fudan.edu.cn)
Fudan University   https://orcid.org/0000-0002-2407-2071

Research

Abstract

Background

Fluorochloridone (FLC), a selective pyrrolidone herbicide, had medium persistence in soil and groundwater, indicating that its environmental fate was highly correlated with mammals and human health. FLC has been recognized as a potential endocrine disruptor and reported to induce male reproductive toxicity, but the underlying mechanism is largely unclear.

Methods

Adult C57BL/6 mice were raised to divided into one control group (0.5% sodium carboxymethyl cellulose), and four FLC-treated groups (3,15,75,375 mg/kg). The animals (ten mice per group) received gavage for a period of 28 days. After treatment, histological analysis, sperm parameters, the microstructure of autophagy and the expression of autophagy-associated proteins in testis were evaluated. Furthermore, to explore the autophagy mechanism, TM4 Sertoli cells were treated with FLC (0,40,80,160μM) in vitro for 24 h. Cell activity and cytoskeletal changes were measured by MTT assay and F-actin immunofluorescence staining. The formation of autophagosome, accumulation of reactive oxygen species and expression of AKT, mTOR were detected.

Results

In vivo, it showed that FLC exposure caused testicular injuries, abnormality in epididymal sperm. Moreover, FLC increased the formation of autophagosomes, the accumulation of LC3, Beclin-1 and the expression of P62 protein, which is related to the degradation of autophagy. In vitro, the upregulation of TM4 cells autophagy was confirmed by FLC increased the formation of autophagosomes and upregulation of autophagy marker proteins (LC3, Beclin-1 and P62) levels. In addition, FLC induced ROS production and inhibited the activities of AKT and mTOR kinases. The Inhibition of AKT/mTOR signaling pathways and the activation of autophagy induced by FLC could be efficiently reversed by pretreatment of ROS generation by N-acetylcysteine. SC79, AKT agonist, could restore the autophagy induced by FLC in TM4 cells. Intriguingly, FLC-induced autophagy could be inhibited through AKT agonists, which indicated that FLC-induced autophagy may be pro-death.

Conclusion

Taken together, our study provided the evidence that FLC promoted autophagy in TM4 Sertoli cells and that this process may involve ROS-mediated AKT/mTOR signaling pathways.

Introduction

Fluorochloridone (FLC), a selective pyrrolidone herbicide, is used for the control of various broad-leaved weeds. Our previous experiments revealed that the target cell was Sertoli cell with pathological characteristic of vacuolation, which may induce adverse effects in the reproductive functions and

hormonal systems of male rats (Zhang, S, Cheng, X and Wang, Y, et al.,2015). Notably, we also confirmed that FLC induced apoptosis of Sertoli cells and the loss in BTB integrity, which cause testis abnormally and affect fertility(Liu, L, Zhang, Y and Chang, X, et al.,2018). FLC induced testis injuries in rats, however, there still exist data gaps to mouse.

Sertoli cell lines comprise a good model for evaluating the mechanism of toxin-induced testis damages. Sertoli cells orchestrate the processes of spermatogenesis by providing nutrition for developing germ cells, and are absolutely crucial for germ cell development and viability(Murphy, C J and Richburg, J H,2014). Impairment of Sertoli cells function by many environmental chemical toxicants may compromise spermatogenesis and hence male fertility(Viswanatha, S A, Wangikar, U and Koti, B C, et al.,2011).

Autophagy, a process of programmed cell death, is a highly conserved metabolic process that provides nutrients and energy cell repair and reconstruction by degrading abnormal macromolecular proteins and organelles(Eskelinen, E L,2008). Autophagy begins with the formation of autophagosome precursors and then is degraded by fusion with lysosomes. This process enables cells to cope with various stresses, such as oxidative stress, nutrient deprivation or endoplasmic reticulum (ER) stress, which is considered as a survival mechanism(Glick, D, Barth, S and Macleod, K F,2010). The autophagy could be detected by transmission electron microscopy (TEM) for cell structural changes as well as expression of light chain3 (LC3), Beclin-1 and P62, a marker for autophagy (Mizushima, 2004). However, many studies suggest that autophagy promotes adverse effects in the body and cell death by excessive self-digestion and degradation of essential cellular constituents(Codogno, P and Meijer, A J,2005). Therefore, it is necessary to clarify the exact role of autophagy.

Oxidative stress, including ROS production, is considered to be the initiating factor of autophagy. Under normal physiological conditions, the production and clearance of ROS are maintained at a stable level and regulated by oxidation and antioxidant systems in the organism, while excessive amounts of ROS induce cell autophagy(Simon, H U, Haj-Yehia, A and Levi-Schaffer, F,2000). In addition, ROS was shown to play a regulatory role in autophagy modulating the AKT-mTOR signaling pathway(Wang, X, Fu, Y F and Liu, X, et al.,2018). The protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, a classic autophagy pathway(Hou, X, Hu, Z and Xu, H, et al.,2014), acts as a crucial part in cell growth, proliferation and autophagy.

Our previous studies have shown that FLC exposure induces apoptosis of Sertoli cells, but whether FLC can induce autophagy, and the underlying mechanism of autophagy in Sertoli cells, remain unclear. In the present study, we investigated the effects of FLC on reproduction and explored the active states of ROS-mediated AKT/mTOR signaling pathways and relationship with autophagy. The results will provide novel clues to reveal the reproductive toxicity mechanism of FLC.

Methods

2.1 Experiment animals and treatment

Adult Six-week-old male C57BL/6 mice was obtained from the Shanghai Lingchang Biotechnology Co., LTD (Shanghai, China). The use of animals was in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by the Ministry of Health of the People's Republic of China. The animals were housed under standard laboratory conditions at constant temperature (21-24°C) and humidity (40–70%) on 12-h light/dark cycles. Standardized granular diet and water were available.

2.2 Cell Culture and treatment

Mouse TM4 Sertoli cells were obtained by Chinese Academy of Sciences. Cells were cultured at 37°C in 5% CO2 in Dulbecco's Modified Eagle Medium/F12 supplemented with 10% fetal bovine serum and antibiotics (0.5% penicillin G -streptomycin). FLC was dissolved in DMSO and diluted with DMEM medium to final concentrations (40, 80 and 160μM). For intervention experiments, cells were pretreated with NAC (5mM for 2 hours), SC79 (20μM for 2 hours) followed by treatment with 160μM FLC.

2.3 Epididymis sperm analysis

The epididymal fluid was obtained by mincing the cauda epididymis in warm phosphate-buffered saline(pH = 7.4). Sperm motility and viability determined by computer assisted semen analysis (CASA).

2.4 Testicular histopathology measurement

After fixation with 4% paraformaldehyde for 24 h, the fixed testis was embedded in paraffin blocks cut into 10μm sections. The sections were stained with HE according to a standard protocol. The stained sections were mounted and examined under a light microscopy.

2.5 Measurement of cell viability by MTT assay

Cells were seeded into 96-well plates and adhered overnight. Cells were treated with allicin (40, 80, 120, 160 and 200μM) for 24 h. Afterwards, cells were added with 10μl MTT solution and incubated for 4 h at 37°C. After the medium was removed, 150μl of DMSO was added to each well to dissolve the formed formazan crystals. Cell viability was measured at 490 nm using a spectrometer.

2.6 F-actin staining

Cells were fixed in 4% paraformaldehyde and incubated with FITC-conjugated phalloidin at 1:50 dilution for 1h, to visualize F-actin. Mounted with DAPI to visualize cell nuclei. The fluorescence images were

recorded using Inverted Fluorescence Microscope.

2.7 Determination of reactive oxygen species (ROS)

Intracellular ROS levels were measured by using a reactive oxygen species assay kit according to the manufacturer's protocol. Cells were incubated with the fluorescent probe 2, 7-dichlorodihydrofluorescindiacetate (DCFH-DA) (1: 1000) at 37°C in the dark for 30 min, and then fluorescence microscopy was observed and photographs were taken.

2.8 Detection of autophagosome formation

For ultrastructural studies, testis and TM4 Sertoli cells were pelleted and fixed in 2.5% phosphoric acid buffer solution of glutaraldehyde, subsequent procedures were performed using standard methods. The ultrastructure of testis and cells were observed with a transmission electron microscope.

2.9 Measurement of autophagy vesicles by MDC staining

For quantitative assessment of the late-stage autophagosomes, autophagic vacuoles were stained with MDC for autophagy analysis as previously described(Ma, K G, Shao, Z W and Yang, S H, et al.,2013). Briefly, after treatment, cells were incubated with MDC for 45 min and collected by centrifugation, and finally MDC-positive cells were quantified by flow cytometry.

2.10 Western blot analysis

Briefly, the proteins of testis and cells were extracted using the RIPA lysis buffer and mixed with loading buffer were collected and boiled for 5 min. The protein was extracted and electrophoresed on SDS-polyacrylamide gel, and then transferred onto a PVDF membrane. The PVDF membrane was incubated with primary antibodies against, p-AKT, AKT, p-mTOR, mTOR, LC3, Beclin1, P62 and GADPH (Cell Signaling Technology, Inc.) at 1: 1000 dilutions overnight at 4°C. The membranes were incubated with 5 % non-fat milk for 2 h at room temperature and then incubated with primary antibodies overnight, followed by peroxidase-conjugated secondary antibody for 1 h at room temperature. The protein signals were detected by enhanced chemiluminescence (ECL) detection system and analyzed by using ImageJ.

2.11 Statistical analysis

All experiments were repeated independently at least in triplicate. Each experiment was performed at least in triplicate. The values were expressed as the mean ± SD (standard deviation). The data from

multiple groups were performed using one-way ANOVA, whereas pairwise comparisons between two groups were compared using t-tests. *P < 0.05, **P < 0.01 were considered statistically significant.

Results

3.1 Effects of FLC exposure on mice testis

There was a significant decrease in organ coefficient from 375mg/kg/day FLC exposure, though no significant decrease in body weights was observed. The findings revealed the sperm motility decreased after 75mg/kg/day FLC exposure. Notably, the sperm motility and concentration decreased after 375mg/kg/day FLC exposure. Testicular pathological examination showed that FLC exposure injured the testicular structure by reducing spermatogenic cells, loosening the connection of Sertoli cells, and causing fibrotic Changes in the interstitium.

TEM analysis found that autophagosomes with membranous structures was presented clearly in the 375 mg/kg/day FLC exposure group, which suggested that FLC could promote Sertoli cell autophagy in testis. After 28 days of FLC exposure, compared with in the control, the expression levels of LC3II/I and Beclin-1 significantly increased in the 75 mg/kg/day FLC exposure groups. The expression level of LC3II/I、Beclin-1 and P62 in the 375 mg/kg/day FLC exposure groups was also increased, which indicated that FLC might induce occurrence of autophagy in testis.

3.2 FLC-induced changes in TM4 Sertoli cell viability, morphology and autophagy

MTT assay was applied to test the inhibitory effect of FLC on changing cell viability of cells in vitro. The cells were, respectively, treated with different concentrations (0, 40, 80, 120, 160, 200μM) of curcumin for 24h.The results of an MTT assay also revealed that FLC exhibited an anti-proliferative effect on cells and significantly suppressed the growth of cells in a dose-dependent manner. In order to carry out follow-up studies, the control group and FLC exposure group (FLC concentrations of 40, 80 and 160) were set respectively in this study.

In order to visualize the changes in the cytoskeleton, F-actin was stained with FITC-conjugated phalloidin. Compared to the control group, 160μM FLC treatment not only reduced the length of microfilament but also inhibited the microfilament branching. Normal Sertoli cells are Conical and narrow, FLC treatment at 160μM for 24h caused abnormal morphological changes, including cell shrinkage and appearance of floating cells.

MDC immunostaining was used to confirm autophagy induction by FLC. Compared with the control group, FLC treatment at 160μM increased the fluorescence intensity of cell indicating extensive MDC-positive autophagy. As illustrated in Fig.3C, we noted that FLC stimulated the formation of lysosomes as well as autophagosomes. We observed more membrane whorls and vacuoles containing degraded

organelles, and swollen mitochondria devoid of cristae. we next investigated the potential effects on the expression of autophagy-activating proteins. Compared to the control group, 160μM FLC treatment significantly increased the expression of LC3-II/I, Beclin-1 and P62.

3.3 FLC triggered Reactive Oxygen Species(ROS) accumulation and inhibited AKT Signaling Pathway

DCFH-DA immunostaining was conducted to detect the level of ROS in TM4 Sertoli cells. FLC dose-enhanced diffuse cytosolic labeling. The result further indicating that FLC triggered ROS accumulation. To further investigate the mechanism of FLC-induced TM4 Sertoli cells autophagic cell death, we examined the expression level of AKT, p-AKT, mTOR, and p-mTOR, As showed in Fig3B, the expression levels of p-AKT/AKT, p-mTOR/ mTOR were decreased in a concentration-dependent manner.

3.4 NAC effectively reversed the inhibition of AKT signaling pathway and autophagy activation

To determine the role of ROS in FLC-induced TM4 Sertoli cell autophagy,5 mM NAC was used to pretreat the cell samples for 2h prior to FLC treatment. NAC reduced the FLC-induced ROS level elevation and the expression of LC3II/LC3I, Beclin-1 and P62. Importantly, NAC increased the expression of p-AKT/AKT, which suggested that ROS was likely to be the upstream signal molecule of AKT signaling. The results showed that NAC pretreatment reversed TM4 Sertoli cells autophagy induced by FLC.

3.5 SC79 inhibits FLC-induced autophagy and increased cell viability

To determine whether FLC-induced autophagy is related to AKT/mTOR signaling pathway, we used 20μM SC79（the AKT agonist）to pretreat the cell samples for 2h prior to FLC treatment. The overexpression of AKT increased expression of p-AKT/AKT and reduced expression of LC3II/LC3I, Beclin-1 and P62.The date indicated that the overexpression of AKT reversed FLC-induced autophagy. Moreover, the MTT assay result showed that the inhibited cell growth effects were decreased when treatment with FLC was combined with SC79. Our study showed that combined treatment with an established AKT agonist increased FLC-induced cell viability.

Discussion

In our mice model, we administered FLC daily at 0, 3, 15, 75, 375mg/kg for 28 days. The dosages were determined considering species differences data: The acute oral lethal dose 50 (LD50) was 4000 mg/kg in male rats and the sensitivity of mouse to FLC is decreased. FLC exposure compromised testis structure

and caused deterioration in sperm quality in this study. Accumulating evidence indicated that autophagy plays a regulatory role in the damage of spermatogenesis(Feng, N, Wang, B and Cai, P, et al.,2020). During autophagy induction, the conversion of LC3-I to LC3-II and the formation of autophagosomes are hallmark features of autophagy(Mizushima, N, Yoshimori, T and Levine, B,2010). In the current study，we found that FLC triggered autophagy and elevated the expression of Beclin-1 and LC3 in testis. Interestingly, the expression of P62 also increased, as an indication of autophagic flux, which indicated that autophagy degradation is impaired. Based on this, we performed electron microscopy and found the accumulation of autophagy vesicles in the Sertoli cells. Furthermore, we suspect that the autophagy in Sertoli cells was partly responsible for the sperm count reduction, however, the underlying mechanism was not clear.

To investigate the mechanism of autophagy, we established an in vitro model of the TM4 Sertoli cells. In the current study, we found that autophagy was induced by FLC as evidenced by the accumulation of autophagic vesicles and upregulation of the protein expression of LC-3, Beclin-1 and P62. Recent studies have revealed that the biogenesis and trafficking of autophagy depend on the activities of cytoskeletal components (Zhuo, C, Ji, Y and Chen, Z, et al.,2013).Our results also revealed that FLC could suppress cell proliferation and inhibited the microfilament branching, which indicated the possibility of FLC cytotoxicity in TM4 Sertoli cells.

One of the striking features of the autophagy in Sertoli cells is the generation of ROS. ROS forms as natural byproduct of the normal metabolism of oxygen and plays a critical role in inducing both cell apoptosis and autophagy(Trachootham, D, Alexandre, J and Huang, P,2009). Our present data showed that FLC induced a significant increase in ROS generation. NAC, the ROS scavenger, remarkably activated the signaling of AKT, and decreased the expression level of LC-3, Beclin1 and P62. All these results indicate that FLC may inhibited the signaling of AKT and triggered autophagy by induction of ROS. Duan, P(Duan, P, Hu, C and Quan, C, et al.,2016) found NP promotes autophagy in TM4 Sertoli cells and that this process may involve ROS-dependent AKT /mTOR pathways, which is consistent with our study.

Recent studies have shown that multiple signaling molecules, such as MAPKs, AMPK, and class III PI3K, have been related to apoptosis and autophagy(Wang, R, Zhang, Q and Peng, X, et al.,2016; Kumar, D, Shankar, S and Srivastava, R K,2014). PI3K/AKT signaling pathway can negatively regulate autophagy through mediating the phosphorylation of mTOR.

Our current results showed that FLC triggered the inhibition of AKT and mTOR. In this work, we further researched the effects of ROS and AKT/mTOR signaling pathways on the crosstalk autophagy induced by FLC. In addition, SC79, AKT agonist, significantly decreased the expression of LC-3, Beclin-1 and P62, which could restore the autophagy induced by FLC in TM4 Sertoli cell. Combined treatment with an established AKT agonist increased FLC-induced cell viability, which indicated that FLC-induced autophagy may be pro-death. Zhang, Y found that the autophagy activator Rapa alleviated intracellular ROS and BPA-driven cell viability(Zhang, Y, Han, L and Yang, H, et al.,2017), which similar to our study. Taken together, all the above results suggested that FLC triggered autophagy which could be mediated by the activation of ROS and the inhibition of AKT/mTOR signaling pathways.

Conclusion

In conclusion, we demonstrated that FLC induced Sertoli cells autophagy in vivo and vitro. FLC induced autophagy through the activation of ROS and the inhibition of AKT/mTOR signaling pathways. Moreover, AKT agonist restore the autophagy and increased FLC-induced cell viability, indicating that FLC-induced autophagy is a pro-death process in cells. Our findings not only put further insights into the potential mechanisms of FLC, but also provide a strategy for therapy.

Abbreviations

FLC Fluorochloridone

CMC-Na Sodium carboxymethyl cellulose CMC-Na

TEM Transmission electron microscopy

CASA Computer assisted semen analysis

DMSO Dimethyl sulfoxide

NAC N-acetyl-l-cysteine

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MDC Monodansylcadaverine

ROS Reactive oxygen species

RIPA Radio Immunoprecipitation Assay

NP Nonyl Phenol

BPA Bisphenol A

Declarations

Acknowledgments

Authors contributions

ZN designed the study under direction of ZZ; WS, RL, FZ and MY participated in the study; XC, WL participated in study design and gave advice in the paper. All authors read and approved the final

manuscript.

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Availability of date and materials

All data supported the conclusions during this study are included within the article.

Ethics approval and consent to participate

All animal care and experimental protocols were approved by the institutional animal care.

Competing interests

The authors declare that they have no competing interests.

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References

1. Zhang S, Cheng X, Wang Y, et al. Ninety day toxicity and toxicokinetics of fluorochloridone after oral administration in rats[J]. Int J Environ Res Public Health, 2015,12(5):4942-4966.
2. Liu L, Zhang Y, Chang X, et al. Fluorochloridone perturbs blood-testis barrier/Sertoli cell barrier function through Arp3-mediated F-actin disruption[J]. Toxicol Lett, 2018,295:277-287.
3. Murphy C J, Richburg J H. Implications of Sertoli cell induced germ cell apoptosis to testicular pathology[J]. Spermatogenesis, 2014,4(2):e979110.
4. Viswanatha S A, Wangikar U, Koti B C, et al. Cardioprotective effect of ascorbic acid on doxorubicin-induced myocardial toxicity in rats[J]. Indian J Pharmacol, 2011,43(5):507-511.
5. Eskelinen E L. New insights into the mechanisms of macroautophagy in mammalian cells[J]. Int Rev Cell Mol Biol, 2008,266:207-247.
6. Glick D, Barth S, Macleod K F. Autophagy: cellular and molecular mechanisms[J]. J Pathol, 2010,221(1):3-12.

7. Codogno P, Meijer A J. Autophagy and signaling: their role in cell survival and cell death[J]. Cell Death Differ, 2005,12 Suppl 2:1509-1518.

8. Simon H U, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction[J]. Apoptosis, 2000,5(5):415-418.

9. Wang X, Fu Y F, Liu X, et al. ROS Promote Ox-LDL-Induced Platelet Activation by Up-Regulating Autophagy Through the Inhibition of the PI3K/AKT/mTOR Pathway[J]. Cell Physiol Biochem, 2018,50(5):1779-1793.

10. Hou X, Hu Z, Xu H, et al. Advanced glycation endproducts trigger autophagy in cadiomyocyte via RAGE/PI3K/AKT/mTOR pathway[J]. Cardiovasc Diabetol, 2014,13:78.

11. Ma K G, Shao Z W, Yang S H, et al. Autophagy is activated in compression-induced cell degeneration and is mediated by reactive oxygen species in nucleus pulposus cells exposed to compression[J]. Osteoarthritis Cartilage, 2013,21(12):2030-2038.

12. Feng N, Wang B, Cai P, et al. ZEA-induced autophagy in TM4 cells was mediated by the release of Ca(2+) activates CaMKKbeta-AMPK signaling pathway in the endoplasmic reticulum[J]. Toxicol Lett, 2020,323:1-9.

13. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research[J]. Cell, 2010,140(3):313-326.

14. Zhuo C, Ji Y, Chen Z, et al. Proteomics analysis of autophagy-deficient Atg7-/- MEFs reveals a close relationship between F-actin and autophagy[J]. Biochem Biophys Res Commun, 2013,437(3):482-488.

15. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?[J]. Nat Rev Drug Discov, 2009,8(7):579-591.

16. Duan P, Hu C, Quan C, et al. 4-Nonylphenol induces apoptosis, autophagy and necrosis in Sertoli cells: Involvement of ROS-mediated AMPK/AKT-mTOR and JNK pathways[J]. Toxicology, 2016,341-343:28-40.

17. Kumar D, Shankar S, Srivastava R K. Rottlerin induces autophagy and apoptosis in prostate cancer stem cells via PI3K/Akt/mTOR signaling pathway[J]. Cancer Lett, 2014,343(2):179-189.

18. Wang R, Zhang Q, Peng X, et al. Stellettin B Induces G1 Arrest, Apoptosis and Autophagy in Human Non-small Cell Lung Cancer A549 Cells via Blocking PI3K/Akt/mTOR Pathway[J]. Sci Rep, 2016,6:27071.

19. Zhang Y, Han L, Yang H, et al. Bisphenol A affects cell viability involved in autophagy and apoptosis in goat testis sertoli cell[J]. Environ Toxicol Pharmacol, 2017,55:137-147.
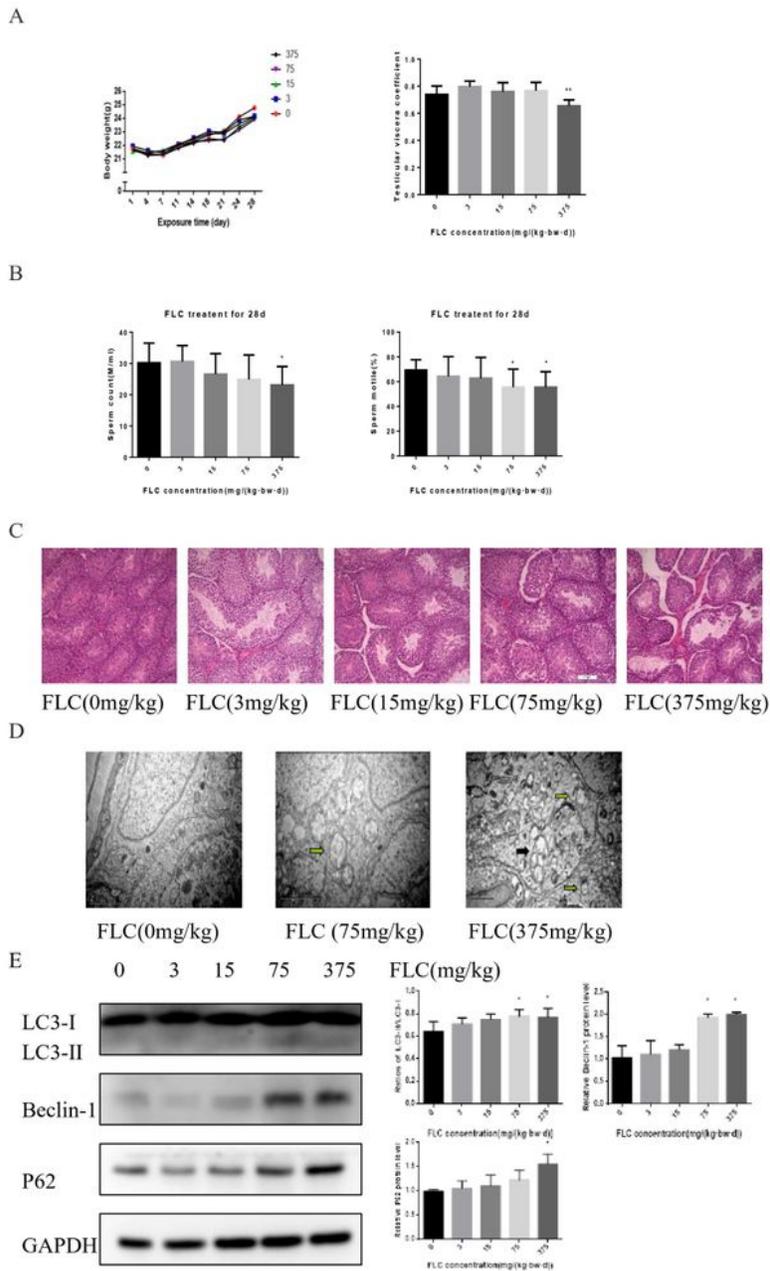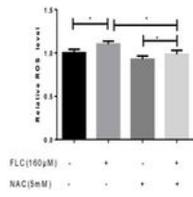
Figures

Figure 1

Effects of FLC exposure on mouse testis in vivo. A, Body weights and testicular weights relative to body weight. B, Effects of sperm concentration and motility. C, Pathological injuries after 28d FLC exposure in testis (200×). D, Testicle tissue sections were analyzed by TEM. Green arrowheads represent autophagy precursors; black arrows point at mature autophagic vesicles with mitochondria. E, The protein levels of LC3, Beclin-1, and P62 were determined by western blotting. *P < 0.05; **P < 0.01.
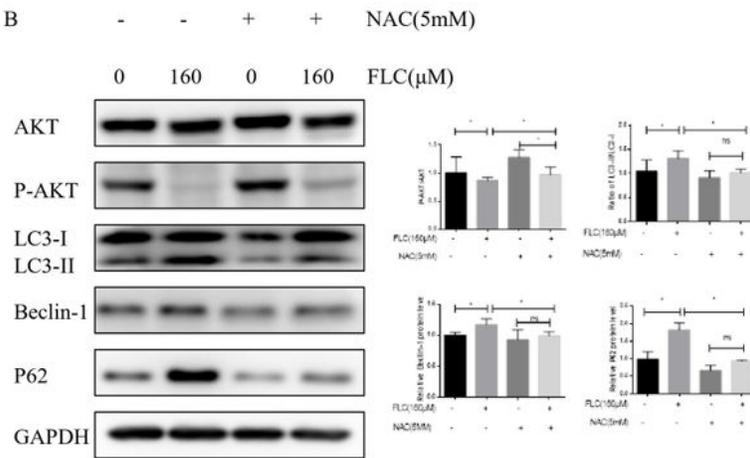
Figure 4

The role of NAC in FLC-induced ROS levels, AKT activation and autophagy levels. Sertoli cells were pretreated with 5 mM NAC for 2 h and then exposed to FLC. A, The effect of FLC-induced ROS level was observed. B, Western blot detected AKT signaling pathway proteins (AKT, p-AKT) and autophagy-activating proteins (LC3, Beclin-1, P62).
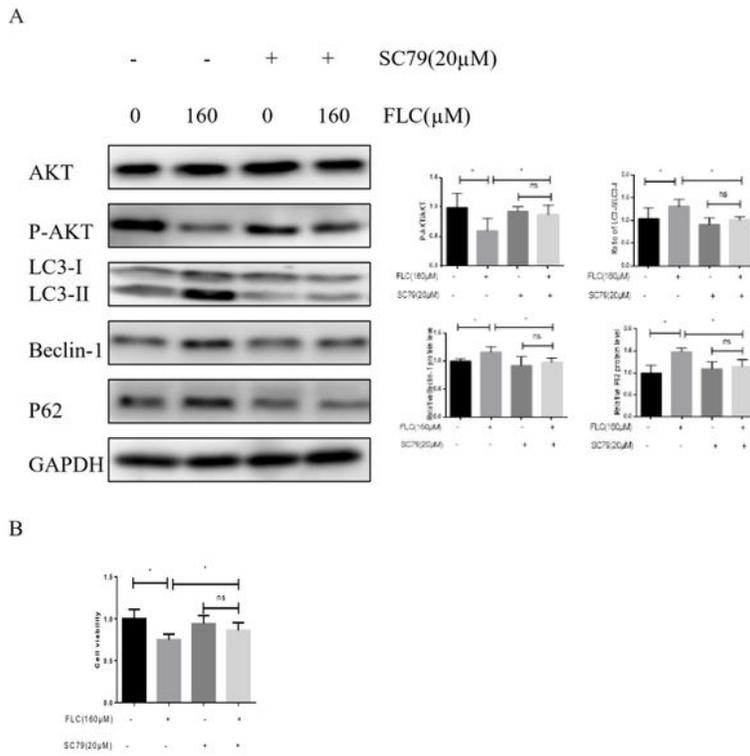
Figure 5

The role of SC79 in FLC-induced AKT activation, autophagy levels and cell viability. TM4 Sertoli cells were pretreated with 20 μM SC79 for 2h and then exposed to FLC. A, Western blot detected AKT signaling pathway proteins (AKT, p-AKT) and autophagy-activating proteins (LC3, Beclin-1, P62). B, Cell viability as determined by using MTT assay.