Flurochloridone Induced Abnormal Spermatogenesis by Damaging Testicular Sertoli Cells in Mice

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Research

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Abstract

Background

Flurochloridone (FLC), a selective herbicide used on a global scale, has been reported to have male reproductive toxicity which evidence is limited and the mechanism is still unclear. The present study was conducted to systematically explore the male reproductive toxicity of FLC, including sperm quality, spermatogenesis process, toxicity targets and possible mechanisms.

Methods

Male C57BL/6 mice aged 6-7 weeks received gavage administration of FLC (365/730 mg/kg body weight) for 28 consecutive days. Then the tissue and sperm of mice were collected for analysis. We measured the coefficient of male reproductive organs, and analyzed sperm concentration, motility, malformation rate and mitochondrial membrane potential. Spermatocyte immunofluorescence staining was performed to analyze meiosis processes. At the same time, we performed pathological staining on the testis and epididymis tissue, and performed TUNEL staining, immunohistochemical analysis and ultrastructural observation on the testicular tissue.

Results

The results showed that FLC caused mice testicular weight reduction, dysfunction and architectural damage, but no significant adverse effect was found in epididymis. The exposure interfered with the proliferation of spermatogonia and the process of meiosis, affecting sperm concentration, motility, kinematic parameters, morphology and mitochondrial membrane potential, leading to sperm quality decline. Furthermore, mitochondrial damage and apoptosis of testicular Sertoli cells were observed in mice treated with FLC.

Conclusion

We found that FLC has significant adverse effects on spermatogonia proliferation and meiosis. Meanwhile, apoptosis and mitochondrial damage may be the potential mechanism of Sertoli cell damage. Our study demonstrated that FLC could induce testicular Sertoli cell damage, leading to abnormal spermatogenesis which resulted in sperm quality decline and provided a methodological reference for related studies.

1. Introduction

The usage of herbicides is constantly increasing worldwide (Benbrook, 2012; De et al., 2017; Kim et al., 2017) and their residual components and by-products may enter the soil and water have become important environmental pollutants (Choung et al., 2013; Ulber et al., 2013). Flurochloridone (FLC), (3RS, 4RS; 3RS, 4SR)-3-chloro-4-chloromethyl-1-(α, α, α-trifluoro-m-tolyl)-2-pyrrolidone, is widely used as a selective herbicide in the planting of winter wheat, sunflowers, cotton and potatoes to control a wide

range of broadleaf weeds and annual grasses (Klicova et al., 2002; Theodoridis, 2006). In recent years, FLC has been increasingly used in agriculture due to its efficient removal of weeds and high safety for crops, but it also leads to the occurrence of residues in the environment (Milanova and Grigorov, 2010; EFSA, 2018). According to the report of the European Food Safety Authority (EFSA), after oral or intravenous exposure, FLC is mainly distributed in liver, kidney, testis, epididymis and blood, causing damage to male reproductive function when it acts on rats (EFSA, 2010; 2013).

Spermatogenesis is a highly coordinated complex process of proliferation and differentiation of spermatogenic cells. In mammals, spermatogenesis mainly includes spermatogonia proliferation, spermatocyte meiosis and sperm metamorphosis, which requires a specific environment and the participation of multiple factors including cells and hormones (Zhou et al., 2019; Ibtisham and Honaramooz, 2020). The proliferation of spermatogonia is the basis for the production of a large number of sperm. As a proliferation-related nuclear antigen, Ki67 is closely related to the synthesis of ribosomes during cell mitosis and can reflect cell proliferative activity, which can be used to evaluate the proliferation of spermatogonia (MacCallum and Hall, 2000; Yerushalmi et al., 2010; Sun and Kaufman, 2018). Meiosis is a strictly regulated process which is divided into two stages and essential for spermatogenesis. During the process, DNA double-strand breaks (DSBs) formation and repair are related to ensure the correct separation of homologous chromosomes (Gray and Cohen, 2016). These events are vulnerable to the interference of both internal and external factors, ensuring their correct and orderly progress is essential for spermatogenesis (Kleckner, 1996). Synaptic complex is a special structure that connects and supports homologous chromosomes during meiosis. It is composed of lateral elements and central elements, which are mainly responsible for providing a stable scaffold structure for various functional proteins in the prophase of meiosis Ⅰ. The lateral elements are composed of several lateral proteins, and its assembly is one of the markers of meiosis initiation (Petronczki et al., 2003). In mice, known components of lateral elements mainly include synaptic complex proteins SYCP3 and SYCP2 (Heyting, 1996), which can be labeled to distinguish different stages of the prophase of meiosis Ⅰ through different states of lateral elements. DSBs are the most serious form of DNA damage, and there are two main repair methods: non-homologous end joining and homologous recombination (Okayasu, 2012). Between them, homologous recombination repair, an accurate repair realized by copying sister chromatids as template (Wright et al., 2018), mainly occurs in the S/G2 phase, which is the way that carried out in programmed DSBs repair of meiosis. γH2AX protein is an important component of chromatin nucleosome histones and is crucial for the transduction of DNA damage signals, which is considered to be one of the biomarkers indicating that chromosomes are in the repair stage (Rogakou et al., 1998; Marchetti et al., 2006). Abnormal expression of γH2AX can lead to stagnation of spermatogenesis. Normally, during pachytene, autosomal repair is almost complete, while γH2AX accumulates due to the incomplete pairing of the XY chromosomes (Celeste et al., 2002; Fernandez-Capetillo et al., 2003). DMC1, first discovered in yeast, is a protein expressed during meiosis and closely related to homologous chromosome pairing and recombination (Bishop, 1994). In mice, DMC1 is expressed specifically from leptotene to zygotene, and its deletion causes the meiosis process to stop (Pittman et al., 1998; Yoshida et al., 1998). In the late stage of spermatogenesis, the morphology of round

sperm cells will undergo significant changes (Yan, 2009), and abnormal sperm morphology is an important factor in the occurrence of infertility (WHO, 2010).

Testicular Sertoli cells are generally irregularly conical, extending from the base to the top of the seminiferous epithelium, which are an important part of the testicular microenvironment, providing nutrients and cytokines for germ cells. The secretion function of Sertoli cells plays an important role in the self-renewal and differentiation of spermatogonial stem cells (Meng et al., 2000; Busada et al., 2015; Griswold, 2016). At the same time, Sertoli cells have the function of phagocytosis and can phagocyte apoptotic germ cells and residual bodies, which are formed by the cytoplasm shed by differentiated sperm when they are crushed into the lumen of seminiferous tubules (Maeda et al., 2002; Nakagawa et al., 2005). When Sertoli cells are damaged, a decrease in their phagocytic capacity leads to accumulation of residual bodies in the lumen (Yefimova et al., 2008). Sertoli cells and the blood-testis barrier (BTB) formed by them are closely related to spermatogenesis and can affect meiosis (Cheng and Mruk, 2010; 2012; Chen and Liu, 2015; Gao et al., 2017). Meanwhile, they are often the targets for testicular damage caused by various environmental chemicals and result in male reproductive damage (Gao et al., 2015). GATA4, which can be used to specifically label Sertoli cells, is a key transcription factor for Sertoli cell function, regulating lactic acid metabolism and BTB function in Sertoli cells (Kyrönlahti et al., 2011; Schrade et al., 2016).

Previous research have shown that FLC has male reproductive toxicity, but the evidence is limited (Zhang et al., 2015; Liu et al., 2018). This study will be carried out from four aspects including sperm quality, spermatogenesis, toxic targets and mechanisms, aims to systematically explore the specific characteristics of FLC-induced male reproductive toxicity through a male mouse model and attends to find out the possible mechanism of its toxic effects, so as to provide more comprehensive evidence and directions of subsequent research.

2. Materials And Methods

2.1. Animals and treatment

Specific pathogen free male C57BL/6 mice aged 6-7 weeks were purchased from Shanghai Experimental Animal Research Center (Shanghai, China) and fed adaptively for one week before exposure to FLC. Mice were housed in controlled environment (12 h light/dark cycle, 21-24°C, 40%-70% relative humidity) and had free access to drinking water and a standard rodent chow diet. FLC (purity: 95.1%) was purchased from Shanghai DuMa Biotechnology Co. Ltd (Shanghai, China) and prepared with 0.5% CMC-Na (Abmole, Shanghai, China) aqueous solution to the desired concentration and prepared fresh weekly. The dose was determined according to general toxicological principles and the LD_{50} (median lethal dose) of rats (NCBI, 2021). The mice were randomly divided into 3 groups with 10 mice in each group and received 365mg/kg bw FLC, 730mg/kg bw FLC and 0.5% CMC-Na aqueous solution, respectively. Gavage performed once a day with a capacity of 20 mL/kg bw for 28 consecutive days, and the body weight was measured weekly. After the end of exposure, the mice were sacrificed and the materials were quickly collected for follow-up

experiments according to the requirements. All procedures of this study were approved by the Institutional Animal Care and Use Committee at Fudan University. The use of animals was in accordance with the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health of the People's Republic of China.

2.2. Sperm analysis

After the epididymis was removed from mice, the caudate of epididymis was immediately placed into 1mL HTF-HEPES sperm culture solution (Sigma-Aldrich, St. Louis, MO, USA), and a deep hole was cut in each caudate nucleus with micro scissors. The sperm were placed into an incubator at 37℃ for 10 min to spread out, and then 3 μL of the suspension was sucked by a capillary tube and dropped into a 37°C preheated sperm counting slide chamber. Sperm analysis was performed using computer assistant sperm analysis (CASA) system (IVOS II, HamiltonThorne, Beverly, MA,USA), 10 visual fields were randomly selected, 30 frames were recorded in each visual field, and a minimum of 400 sperm were recorded in each sample.

2.3. Sperm malformation rate detection

Sperm malformation rate was detected by Papanicolaou staining using Papanicolaou Staining Kit (Biolab, Beijing, China). Briefly, the semen was centrifuged and smeared to slides, fixed with 95% ethanol for 10 min, and washed with distilled water twice. After staining with hematoxylin for 5 min and washing with warm water for 15 min, 95% ethanol was used for 10 min. The slides were then treated with orange G dye for 2 min and washed with 95% ethanol, followed by EA50 staining for 15 min and washed with 95% ethanol. Subsequently, the slides were dehydrated with ethanol and transparent with xylene, then mounted with neutral gum. The slides were observed and photographed under a microscope (BX43, Olympus, Tokyo, Japan) and the sperm malformation rates were calculated. Each slide counted 200 intact sperm.

2.4. Sperm mitochondrial membrane potential (MMP) detection

Sperm MMP was detected by MMP Assay Kit with JC-1 (Beyotime, Shanghai, China). According to the instructions, appropriate amount of JC-1 working solution and JC-1 staining buffer were prepared and placed in ice bath for reserve. JC-1 working solution was added to the sperm suspension, mixed upside down and incubated at 37℃ for 30 min. Then the supernatant was discarded by centrifugation, and the sperm were re-suspended and washed with JC-1 staining buffer. Sperm suspension was smeared, observed and photographed under fluorescence microscope (IX53, Olympus, Tokyo, Japan).

2.5. Immunofluorescence staining of spermatocytes

Testes of mice were removed and placed into hypotonic extracts [50mM sucrose, 17mM sodium citrate, 30mM Tris (pH 8.2), 2.5mM DTT, 1mM PMSF (pH8.3) and 5mM EDTA] after the tunica albuginea was removed, then incubated on ice for 20 min and minced in 100mM sucrose. The spermatocyte spreads were prepared on a slide and solidified in 1% paraformaldehyde containing 0.1% Triton X-100. The slides

were incubated for 6 h in a damp room and washed with 0.04% Photo-Flo (Kodak, NY, USA). After that, the slides were dried and blocked 30 min using 10% donkey serum with 3% bovine albumin and incubated overnight with the primary antibody [γH2AX (1:500), SYCP3 (1:100) or DMC1 (1:100)] (Abcam, Cambridge, MA, USA) at 37℃, followed by incubated with secondary antibody in darkness for 1 h at 37℃. After washing, drying and mounting with Vecta shield cover slips (Vector Labs, Burlingame, CA, USA), the slides were observed and photographed using super-resolution microscope (Nikon N-STORM, Tokyo, Japan).

2.6. Histopathologic staining

Freshly isolated testes and epididymides were fixed with 4% paraformaldehyde, then embedded in paraffin and prepared into 5μm thick sections. Before staining, slides were baked at 60℃ for 30 min and then dewaxed with xylene.

Histopathological examination of the testes was performed by Periodic Acid Schiff Staining Assay Kit (BaSO, Zhuhai, Guangdong, China). The slides were treated with periodic acid solution, schiff and hematoxylin for 10, 15 and 3 min respectively, the excess water was shaken off after each treatment. Histopathological examination of the epididymides was performed by hematoxylin-eosin staining. The slides were treated with ethanol in gradient (100%, 95%, 85%, 75%) and washed twice with distilled water. After staining with hematoxylin (ZSGB-BIO, Beijing, China) for 3 min, they were rinsed with warm water for 10 min and treated with 95% ethanol for 1 min. After that, the slides were stained with eosin (Sinopharm Group, Beijing, China) for 30 s, and then treated with ethanol in a gradient (75%, 85%, 95%).

The dyed slides were dehydrated with ethanol and transparent with xylene, then mounted with neutral gum. The slides were observed and photographed by microscope and the residual bodies were counted. Each slide counted 100 seminiferous tubules.

2.7. Cell apoptosis detection

Cell apoptosis detection was performed using TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China). Briefly, slides were baked at 60℃ for 30 min and then dewaxed with xylene. After treated with ethanol in gradient (100%, 95%, 85%, 75%), the slides were incubated at 37°C for 20 min with protease K, followed by incubated at 37°C for 1 h with TUNEL working solution. At the end of each incubation, the slides were washed with PBS for 3 times. Finally, the slides were mounted with Antifade Mounting Medium with DAPI (Beyotime, Shanghai, China) after all treatment. The cell apoptosis was observed and photographed using confocal microscope (TCS SPE, Leica, Wetzlar, Germany).

2.8. Immunohistochemical analysis

After baking, dewaxing, and ethanol treatment, the slides were immersed in Citrate Antigen Retrieval Solution (Beyotime, Shanghai, China), and then bathed in boiling water for 30 min. The slides were blocked in Endogenous Peroxidase Blocking Buffer (Beyotime, Shanghai, China) for 10 min, treated with 0.3% Triton-X for 10 min, and then blocked in goat serum (Gibco, Carlsbad, CA, USA) at 37°C for 30 min. After that, the slides were incubated overnight with primary antibodies [GATA4 (1:200) or Ki67 (1:100)]

(Abcam, Cambridge, MA, USA) at 4°C and then with secondary antibody for 30 min at 37°C. After treatment with freshly prepared diaminobenzidine working solution (ZSGB-BIO, Beijing, China) and redyeing with hematoxylin, the dyed slides were dehydrated with ethanol and transparent with xylene, followed by mounted with neutral gum. The slides were observed and photographed by microscope. Sertoli cells in 20 spermatogenic tubules were counted for each slide.

2.9. Ultrastructural observation

Ultrastructural observation of Sertoli cells was performed by transmission electron microscopy. Small blocks of testis were trimmed into 1 mm^3 and were fixed in 2.5% (v/v) glutaraldehyde in PBS (pH 7.0, 0.1M) at 4°C for 24 h. The samples were then rinsed in PBS and fixed with 1% (w/v) osmium tetroxide for 1 h. After being washed with PBS, samples were dehydrated in ascending concentrations of ethanol, embedded in araldite and hardened at room temperature for 16 h. Ultra-thin sections were mounted on formvar-coated grids and stained with uranyl acetate and then lead citrate. Finally, sections were examined, and electron micrographs were obtained using a transmission electron microscope (Tecnai G2 Spirit, Thermo Fisher Scientific, Waltham, MA, USA).

2.10. Data processing and statistical analysis

All quantitative data for diverse assays were presented as the means ± standard deviations. Statistical comparison was performed with Student's t-test in the GraphPad Prism software (La Jolla, CA, USA) and the probability value (*P*-value) less than or equal to 0.05 was considered significant.

3. Results

3.1. Effects of FLC on body weight and organ coefficient in male mice

During the FLC exposure period, the body weight of male mice in each group showed an increasing trend, but there were no significant difference among them (Fig. 1A). After exposure, testicular weight and organ coefficient were significantly decreased in a dose-dependent manner, while there were no significant changes in epididymis, seminal vesicle and prostate (Fig. 1B-C). These results displayed that FLC exposure could reduce testicular weight and organ coefficient in male mice.

3.2. Effects of FLC on sperm quality in male mice

In order to investigate the effects of FLC exposure on sperm quality, the concentration, motility, kinematic parameters, morphology and MMP of sperm were detected. Compared with the control group, sperm concentration and motility decreased in a dose-dependent manner in the FLC exposed groups and progressive motility were significant decreased in high-dose group (Fig. 2A).

The analysis of sperm kinematic parameters showed that the average path velocity (VAP) and curvilinear velocity (VCL) were decreased in the high-dose group while the amplitude of lateral head displacement

(ALH) was decreased in both exposure groups (Table 1).

Table 1

Effects of FLC exposure on sperm kinematic parameters in mice ($\bar{x}$±SD,, =10)

Group	Sperm Kinematic Parameters				
	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm/s)	BCF (HZ)
Control	72.70±6.36	57.15±4.75	135.50±11.01	6.71±0.34	28.19±1.87
365mg/kg bw	69.69±4.55	56.60±3.00	125.00±8.85	6.11±0.31**	27.99±1.61
730mg/kg bw	63.74±1.34**	53.90±2.26	110.60±5.92**	5.46±0.47**	29.24±1.28

The morphological analysis of sperm showed that the head of normal sperm was oval and the tail was long, like tadpole. Abnormal sperm mainly showed head and tail deformities (Fig. 2B). Compared with the control group (53.6%), the sperm abnormality rate in the low-dose group and high-dose group was increased to 68.8% and 80.2%, respectively (Fig. 2C).

Mitochondria are the most important organelle for intracellular energy supply. Reduced sperm MMP leads to blocked production of Adenosine triphosphate (ATP), which in turn leads to sperm motility dysfunction. After JC-1 staining, compared with the control group, the JC-1 aggregates (red fluorescence) of sperm in the FLC exposed group decreased while the JC-1 monomers (green fluorescence) increased and showed a dose-dependent trend (Fig. 2D), indicating that the FLC exposure reduced the sperm MMP.

These results indicated that FLC exposure could affect sperm concentration, motility, kinematic parameters, morphology and MMP of male mice, leading to sperm quality decline.

3.3. Effects of FLC on spermatogenesis in male mice

Based on the obvious effects of FLC on sperm quality, we further studied the proliferation and meiosis of germ cells using immunohistochemistry and immunofluorescence. The results showed that FLC treatment significantly reduced the proportion of Ki67-positive cells in the testicular tissue (Fig. 3A), indicating that FLC exposure affected the proliferation of spermatogonia.

SYCP3 is involved in the formation of synaptic complexes. The immunofluorescence results showed that FLC exposure significantly increased the proportion of spermatocytes in the leptotene and zygotene, while that in the pachytene and diplotene has decreased correspondingly (Fig. 3B), suggesting that FLC exposure disturbed the meiosis process.

The presence of γH2AX indicates that the chromosomes are in the repair phase. The results showed that FLC exposure significantly increased the abnormal proportion of spermatocytes homologous chromosome repair (Fig. 3C), indicating that FLC exposure interfered with the DSBs repair. DMC1 expressed during meiosis, which deletion led to spermatogenesis to stagnate (VAN DER LAAN, et al., 2005) The results showed that FLC exposure significantly reduced the recruitment of DMC1 to

axial/lateral elements (SYCP3) in spermatocytes compared with the control group (Fig. 3D), indicating that FLC exposure had adverse effects on spermatocyte meiosis.

Taken together, these results revealed that FLC exposure interfered with the proliferation of spermatogonia and the process of meiosis.

3.4. Effects of FLC on histological structure of testis and epididymis in male mice

Testis and epididymis are closely related to spermatogenesis and sperm storage. We performed histopathological analysis on the testis and epididymis of mice to evaluate the effects of FLC exposure.

In the control group, the testicular seminiferous tubules were regularly distributed, round or elliptical, and seminiferous epitheliums had an intact structure with multiple layers of spermatogenic cells at various stages. Sertoli cells in the testicular tissue were tightly bound to the basement membrane, with normal number and compact arrangement. In the low-dose group, the testis was damaged, the spermatogenic epithelial layers were reduced and the spermatogenic cells were reduced and exfoliated into the lumen, accompanied by the vacuolation of Sertoli cells and the presence of residual bodies. In the high-dose group, the testis damage was intensified, with irregular changes in the shape of the convoluted seminiferous tubules, disorderly arrangement of germ cells, significantly reduced spermatogenic epithelial layers, accompanied by a large number of vacuolated Sertoli cells and residual bodies. Meanwhile, partial lumen collapse and obviously abnormal seminiferous tubules were also observed (Fig. 4A). The presence of residual bodies was associated with decreased phagocytosis of Sertoli cells. The analysis of residual bodies showed that FLC exposure significantly increased the proportion of seminiferous tubules containing residual bodies and the total number of residual bodies, with a dose-dependent relationship (Fig. 4B).

The epididymis structure of the control group was normal, the cells of the tube wall were tightly arranged with complete structure, and spermatogenic cells at all stages were distinct in the lumen. In the FLC-exposed group, decreased sperm density was observed in the lumen, accompanied by the presence of cell debris (Fig. 4C).

These results indicated that FLC exposure resulted in decreased sperm density, pathological changes in testis, vacuolization of Sertoli cells and an increase in residual bodies, suggesting that testis and Sertoli cells were the targets of FLC toxicity.

3.5. Effects of FLC on Sertoli cells in testis of male mice

TUNEL staining results showed that there were only a small proportion of TUNEL-positive cells in testicular tissue of the control group, while the positive cells were significantly increased in the FLC-exposed groups in a dose-dependent manner and were mostly located near the basement membrane (Fig. 5A). This result suggested that FLC exposure induced cell apoptosis in testis of male mice, and most cell apoptosis occurred near the basement membrane.

GATA4-labeled immunohistochemical analysis showed that Sertoli cells in the control group were evenly distributed on the basement membrane of seminiferous tubules, and the number was normal, while FLC exposure significantly reduced the number of Sertoli cells in a dose-dependent manner (Fig. 5A, 5B). The ultrastructural observation results showed that the Sertoli cells in the control group had regular nuclei, uniform chromatin distribution and normal mitochondria. In the low-dose group, mitochondrial swelling and cristae structure disappearing occurred, while mitochondria were obviously twisted and Sertoli cells suffered secondary necrosis after apoptosis in the high-dose group (Fig. 5D).

Collectively, these results indicated that FLC exposure induced the decrease of Sertoli cells in testis of male mice, and it was closely related to mitochondrial damage and cell apoptosis.

4. Discussion

Although FLC has been considered as an environment-friendly herbicide, recent studies have shown that FLC exposure can cause reproductive toxicity. The underlying mechanism has not yet been fully understood, while previous studies have limited evidence and lack experimental data in mice (EFSA, 2010; 2013; Zhang et al., 2015; Liu et al., 2018; Zhu et al., 2019). The EFSA reported that the target organs of male reproductive toxicity of FLC in rats were testis and epididymis. In the present study, FLC was found to cause male reproductive damage in mice at relatively higher doses than in rats. The results showed that FLC exposure caused the weight reduction and pathological changes of the testis, but not epididymis in mice. While the occurrence of cell debris and sperm loss in the epididymal were likely to be secondary to the damage of testis.

Sertoli cells are considered to be the target cells for testicular injury caused by a variety of environmental chemicals and apoptosis is the main form of damage (Choi et al., 2014; Gao et al., 2015). We found a dose-dependent trend of Sertoli cell number decrease, vacuolation and the appearance of residual bodies in the testicular tissue, which means that Sertoli cells have been damaged and their phagocytic ability has decreased (Yefimova et al., 2008). At the same time, the TUNEL assay showed that FLC increased the cell apoptosis in testicular tissue and mainly neared the basement membrane which were considered to be the Sertoli cells apoptosis. It was further confirmed by the ultrastructural observation of testicular tissue, which also found mitochondrial damage in Sertoli cells. All these revealed that the target cell for male reproductive injury caused by FLC exposure was testicular Sertoli cell, while apoptosis and mitochondrial damage played an important role in this toxic process.

Spermatogonia proliferation and spermatocyte meiosis are the basis of the spermatogenesis, which respectively ensure the mass production of sperm and the correct transmission of genetic material. The supporting and secretory functions of Sertoli cells play a key role in the proliferation and differentiation of spermatogonial stem cells and their damage may cause the decrease of spermatogonial proliferation (Meng et al., 2000; Hara et al., 2014). Our results on proliferation level detection showed that FLC exposure dose-dependently reduced spermatogonia proliferation, which may have contributed to subsequent sperm concentration declines. Meanwhile, spermatocyte meiosis is also regulated by Sertoli

cells on the luminal side of the blood-testis barrier through paracrine signaling (Chen and Liu, 2015). We further demonstrated that FLC treatment induced spermatogenesis disorder through targeting the process of meiosis. Compared with the control group, the proportion of spermatocytes in the leptotene and zygotene increased significantly, while in the pachytene and diplotene showed a decreasing trend, indicating that FLC hindered the process of the meiosis □, leading to the delay of meiosis. γH2AX can be used to mark DNA damage sites (Shibata et al., 2014). With the progress of synapsis and homologous recombination repair, DNA damage signals will gradually shrink from the entire chromosome region to the chromosome axis, and finally gather at the sex chromosome in pachytene. The results that FLC exposure significantly increased the proportion of homologous chromosome repair abnormalities in pachytene, suggested that FLC exposure interfered with the repair of programmed DSBs. DMC1 is specifically expressed in meiosis and co-participates in the repair of DSBs with Rad51 (Da et al., 2013; Liu et al., 2014). Male mice with DMC1 deficiency had stagnant spermatogenesis, leading to infertility (Pittman et al., 1998). FLC exposure affected the expression of DMC1, which in turn interfered with the meiosis of spermatocytes.

Spermatogenesis abnormalities usually result in a decline in sperm quality, which in turn affects male reproductive function. Concentration and morphology play an important role in sperm fertilizing capacity. In the present study, we found that exposure of mice to FLC decreased the concentration and motility of sperm, increased sperm malformation percentage. In addition, sperm kinematic parameters are important indicators to evaluate whether sperm are likely to reach the fallopian tube and complete the fertilization process. In general, sperm with high speed and large ALH have better fertilization potential (Ji et al., 2018). Our results showed that compared with the control group, sperm motility, VAP, VCL and ALH decreased in the FLC-exposed group. Mitochondria play an important role in spermatogenesis and fertilization, and their damage will lead to lack of energy supply for sperm movement, leading to asthenospermia (Durairajanayagam et al., 2021). Sperm MMP is of great significance for ensuring sperm motility and predicting fertilization ability, which decline indicates abnormal mitochondrial function of sperm (Paoli et al., 2011; Zhang et al., 2019). FLC treatment also induced MMP disruption, which suggested that mitochondrial dysfunction, leading to the decline of sperm motility and fertilization ability.

In view of the above mentioned results, we demonstrated that FLC could cause a series of reproductive injuries in male mice, including testicular injury and Sertoli cells damage, spermatogenesis abnormalities and sperm quality decline. Moreover, such damage of Sertoli cells was probably related to mitochondrial damage and apoptosis. Our study complemented the data on male reproductive toxicity of FLC in mice and elucidated its toxicity characteristics and possible mechanisms. However, the toxic mechanism of FLC and the implication of the hypothalamic-pituitary-gonadal axis in the toxicity needs further exploration. At the same time, further studies based on the results, such as in vitro fertilization and natural breeding experiments, will help further confirm the male reproductive toxicity of FLC, which may become the focus of our follow-up work.

5. Conclusion

Overall, our results showed that FLC could cause a series of reproductive impairment in male mice, including testicular injury and Sertoli cells damage, spermatogenesis abnormalities and sperm quality decline, but did not affect epididymis under the current condition. We found that FLC has significant adverse effects on spermatogonia proliferation and meiosis. Meanwhile, apoptosis and mitochondrial damage may be the potential mechanism of Sertoli cell damage. These results demonstrated that FLC could induce testicular Sertoli cell damage, leading to abnormal spermatogenesis which resulted in sperm quality decline (Fig. 6).

Declarations

Ethics approval and consent to participate

All procedures of this study were approved by the Institutional Animal Care and Use Committee at Fudan University. The use of animals was in accordance with the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health of the People's Republic of China.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this article.

Competing interests

The authors declare that there are no conflicts of interest.

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Authors' contributions

Weiqi Sun undertook the main design and experimental conduct of the study and was a major contributor to the manuscript. Fang Tian and Hongjie Pan made major contributions to experiments and participated in the revision of the manuscript. Weihua Li, Mingjun Yang and Zhijun Zhou participated in the study design, carried out the whole quality control of the research and provided funding support. Other authors participated in related experiments. All authors read and approved the final manuscript.

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Figures

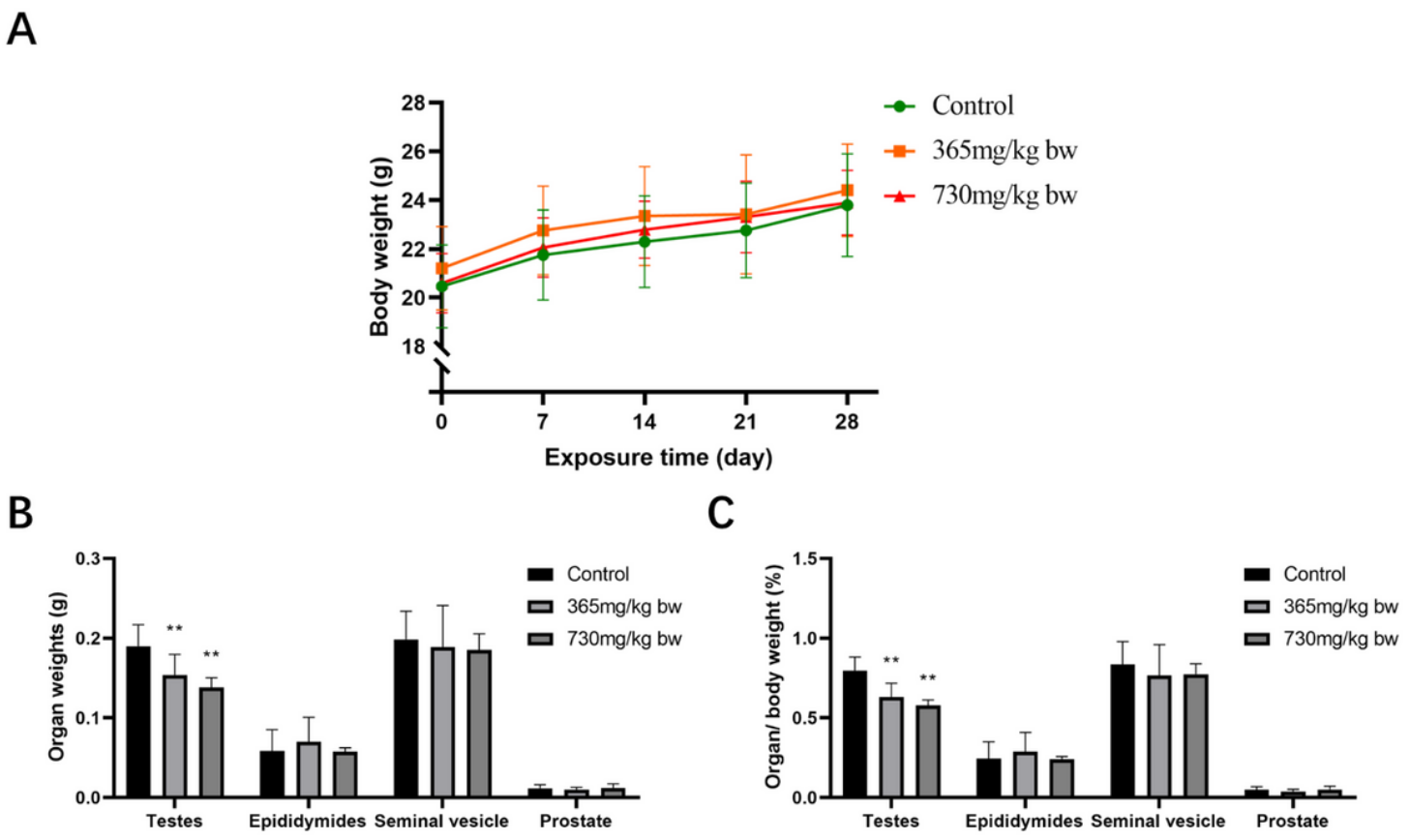

Figure 1

Effects of FLC on body weight and organ coefficient in male mice (A) Changes in body weight (n=10). (B) Changes in organ weight (n=10). (C) Changes in organ coefficient (n=10). * P<0.05, ** P<0.01 vs. Control group.

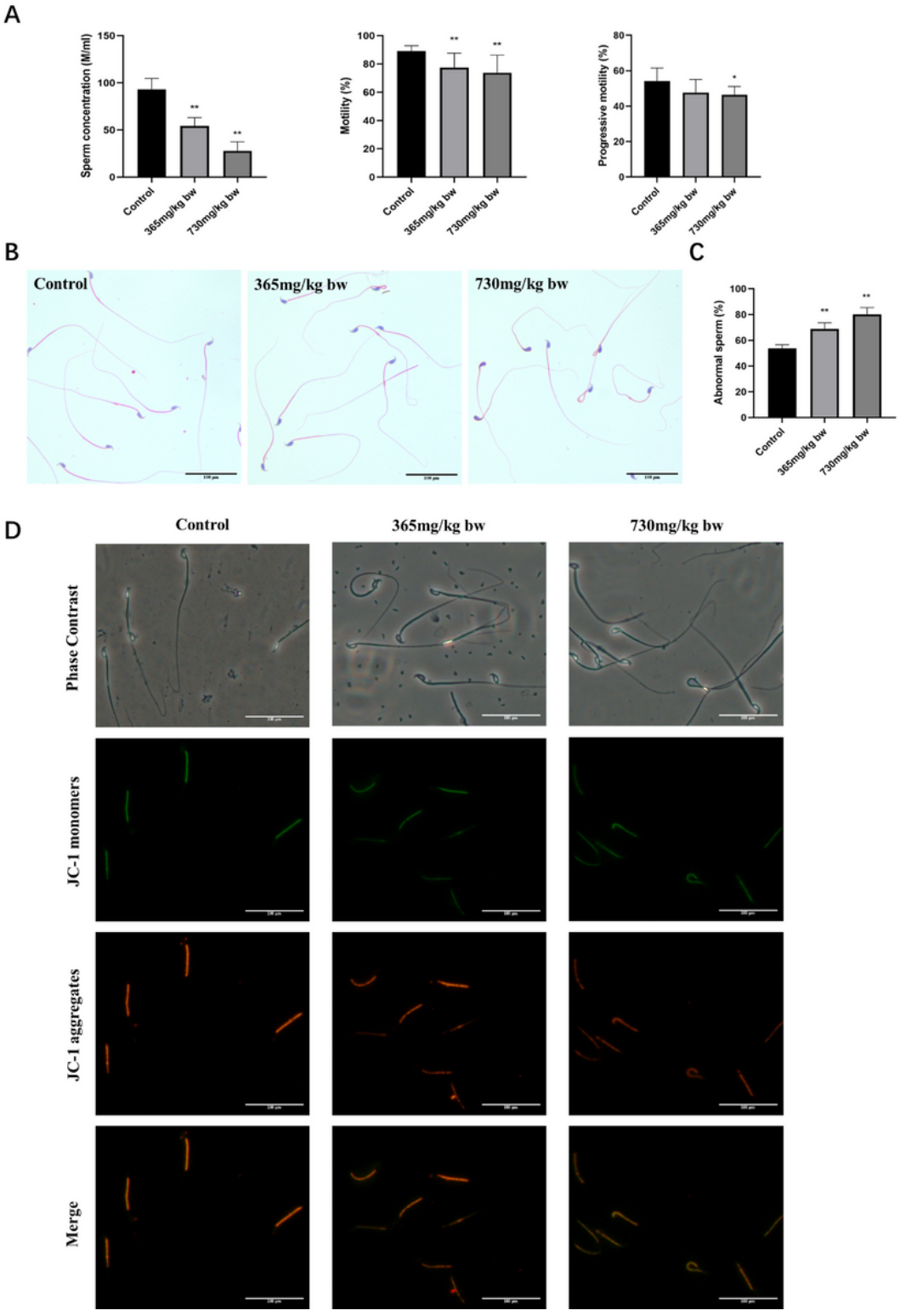

Figure 2

Effects of FLC on sperm quality in male mice (A) Sperm analysis by CASA (n=10). (B) Effects of FLC exposure on sperm morphology detected by Papanicolaou staining. Scale bar=100μm. (C) Abnormal sperm statistics (n=10). (D) MMP of sperm detected by JC-1 fluorescence staining. Scale bar=100μm. Three independent fields were acquired for each experimental condition. Representative samples from one field of view were shown. * P<0.05, ** P<0.01 vs. Control group.

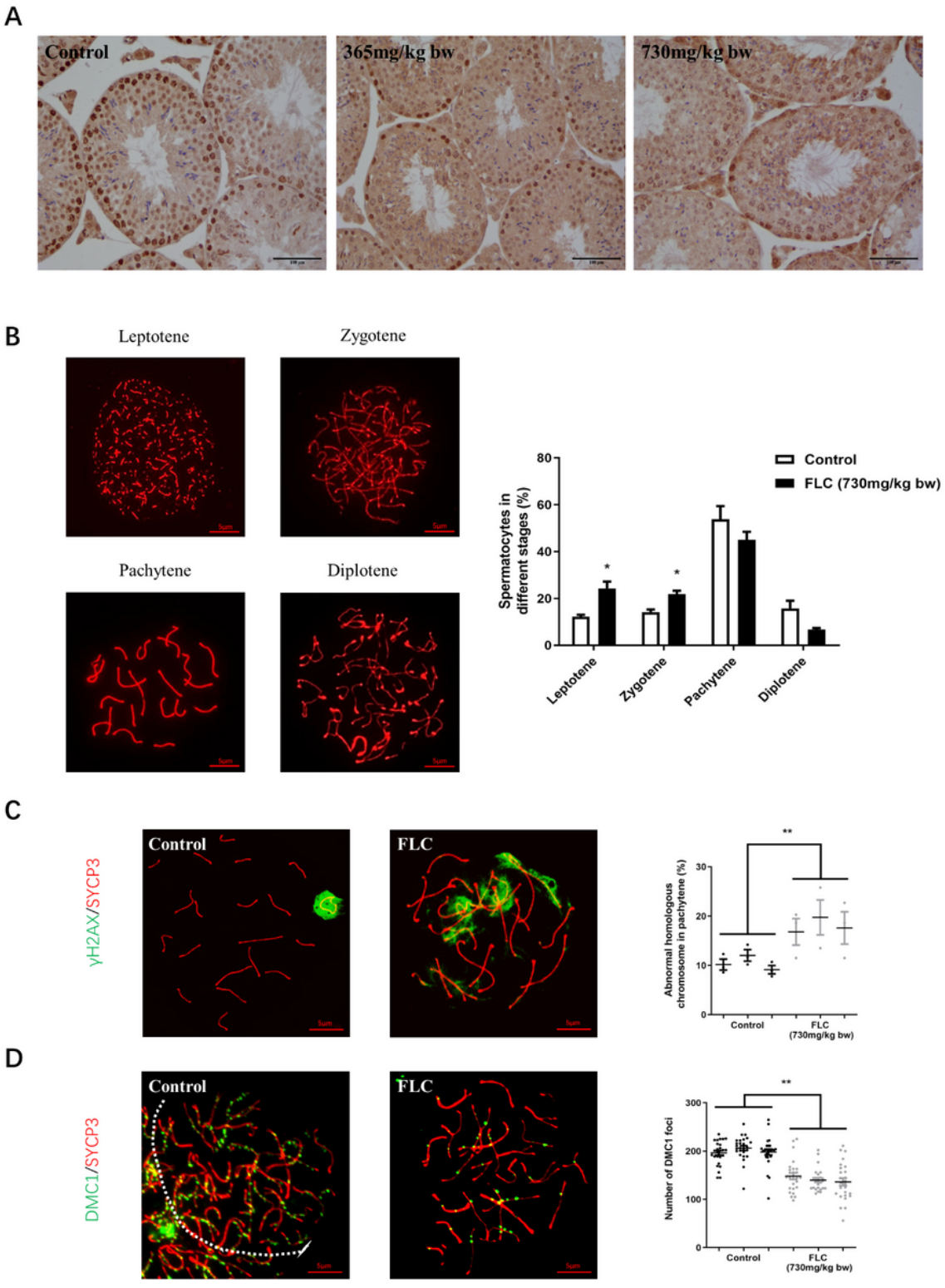

Figure 3

Effects of FLC on spermatogenesis in male mice (A) Immunohistochemical assay with Ki67 antibody. Scale bar=100μm. (B) Immunofluorescence assay with SYCP3 antibody (n=3). Scale bar=5μm. (C) Immunofluorescence assay with γH2AX/SYCP3 antibody (n=3). Scale bar=5μm. (D) Immunofluorescence assay with DMC1/SYCP3 antibody (n=3). Scale bar=5μm. Three independent fields were acquired for

each experimental condition. Representative samples from one field of view were shown. * P<0.05, ** P <0.01 vs. Control group.

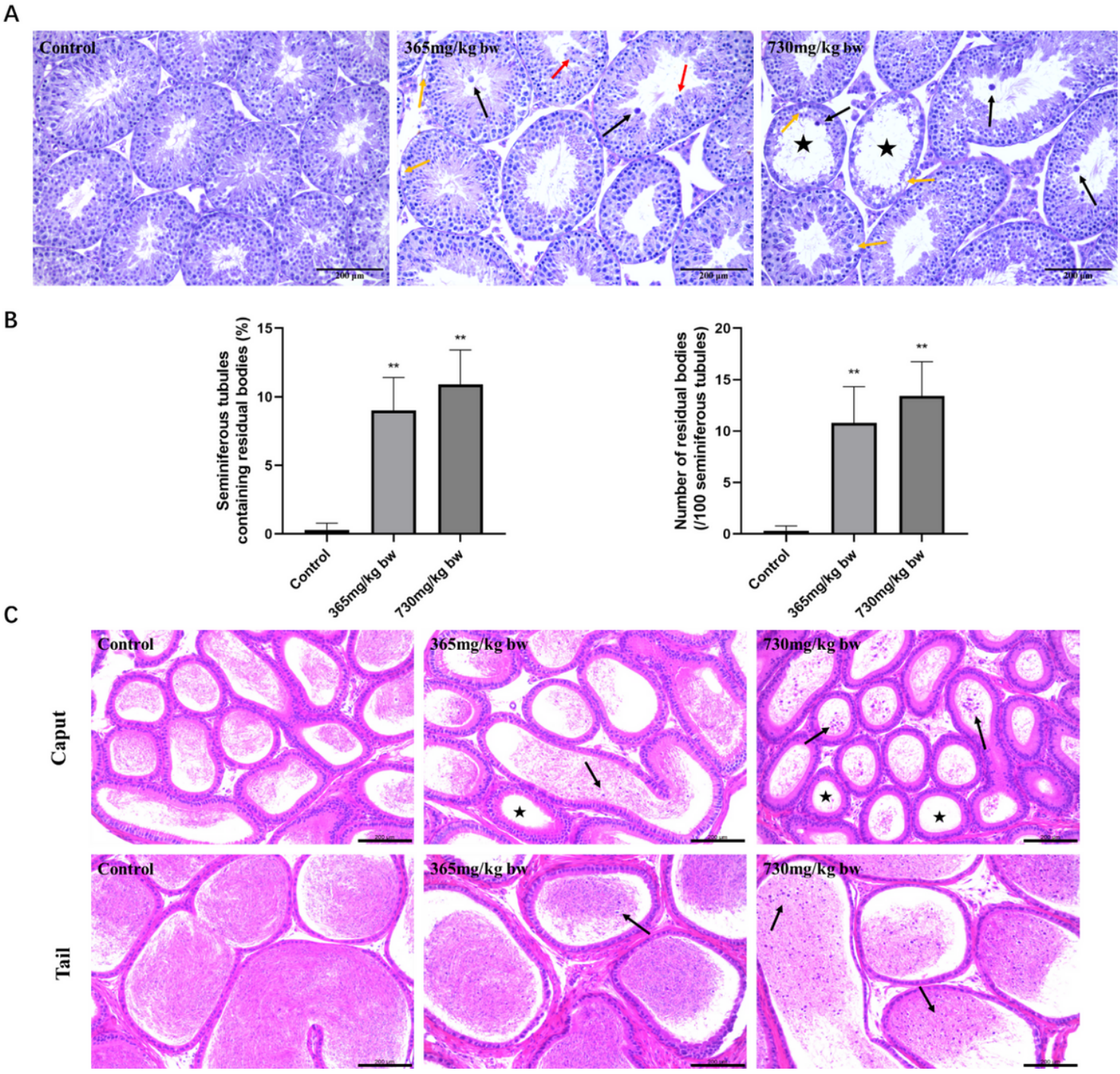

Figure 4

Effects of FLC on the histological structure of testis and epididymis in male mice (A) Changes in testicular tissue structure detected by Periodic Acid-Schiff staining. Black arrows: residual bodies. Yellow arrows: vacuolation of Sertoli cells. Red arrows: Shedding of spermatogenic cells. Pentagrams: spermatogenic tubules severely damaged. Scale bar=200μm. (B) Counting of residual bodies (n=10). * P <0.05, ** P<0.01 vs. Control group. (C) Changes in epididymis tissue structure detected by hematoxylin-

eosin staining. Arrows: cell debris. Pentagrams: sperm loss in the epididymis. Scale bar=200μm. Three independent fields were acquired for each experimental condition. Representative samples from one field of view were shown.

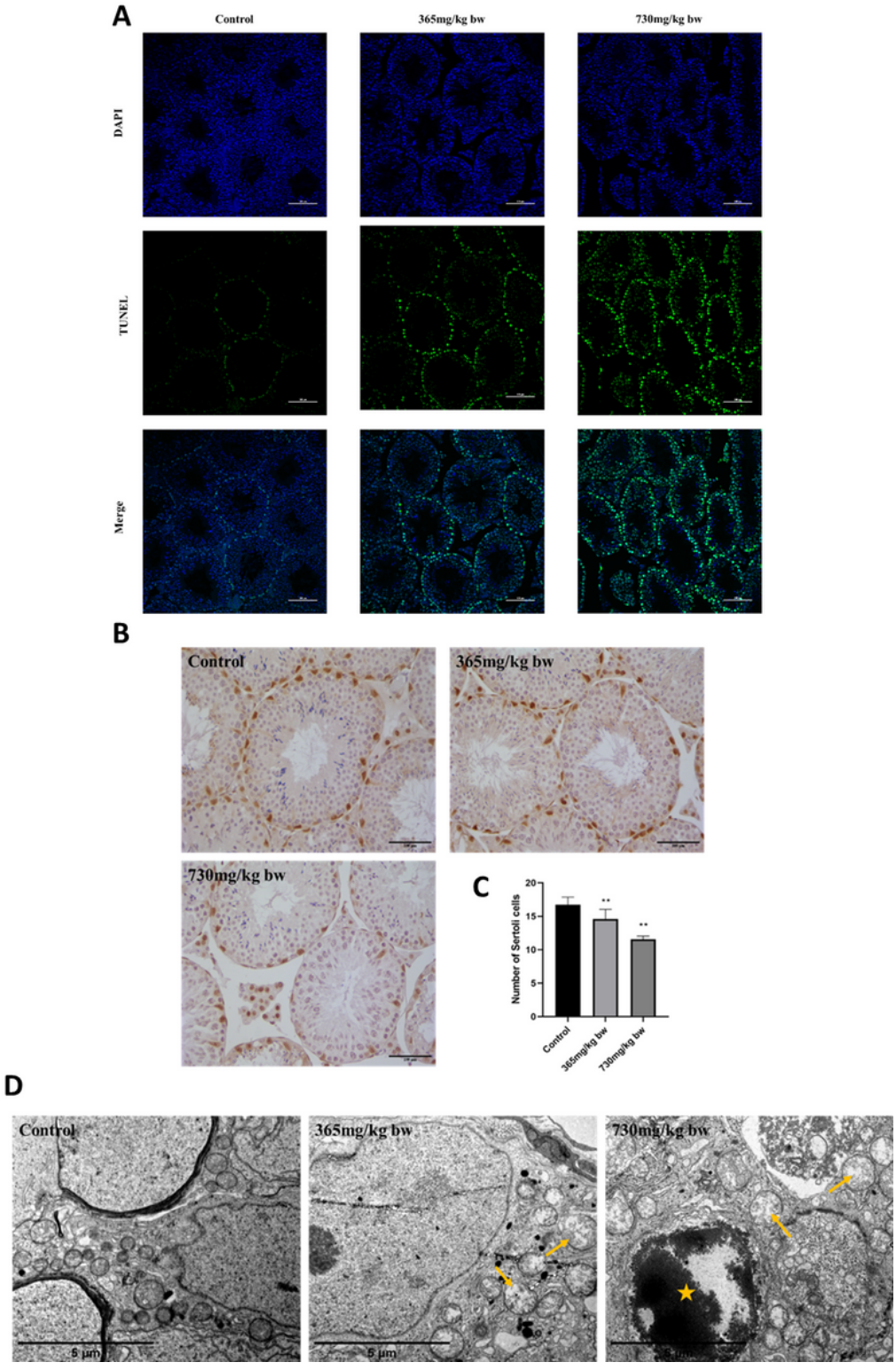

Figure 5

Effects of FLC on Sertoli cells in testis of male mice. (A) TUNEL staining of testicular tissue. Scale bar=100μm. (B) Immunohistochemical assay with GATA4 antibody. Scale bar=100μm. (C) Counting of Sertoli cells (n=10). * P<0.05, ** P<0.01 vs. Control group. (D) Ultrastructure observation of testicular tissue. Arrows: mitochondria with swelling and missing cristae. Pentagrams: Sertoli cell with apoptotic secondary necrosis. Scale bar=5μm. Three independent fields were acquired for each experimental condition. Representative samples from one field of view were shown.

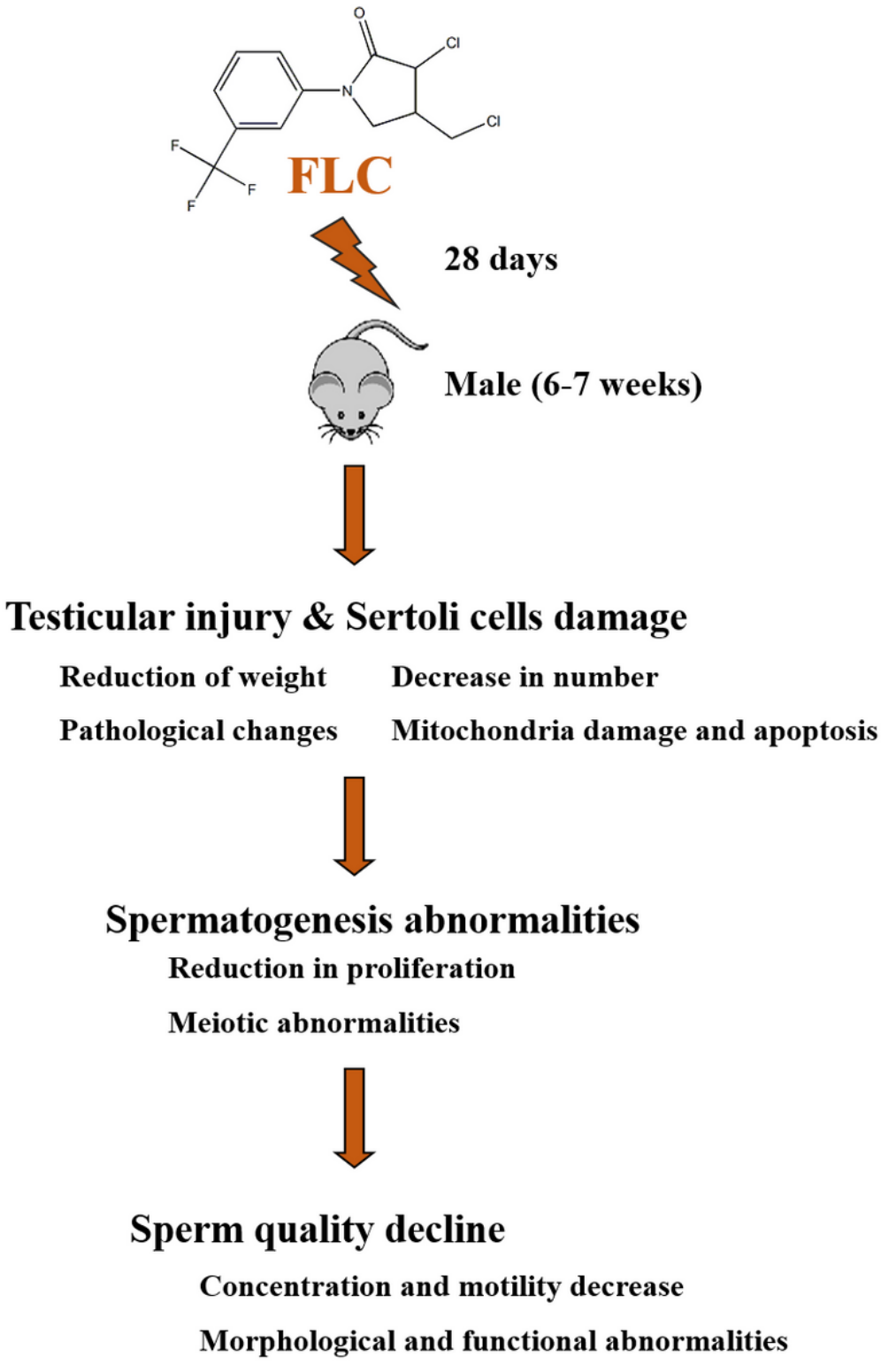

Figure 6

Flurochloridone induced abnormal spermatogenesis by damaging testicular Sertoli cells in mice.