

Original Article

Effect of letrozole on interstitial cell proliferation and spermatogenic function regulation of mouse testicle

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Abstract: Objective: To explore the influence of P450 aromatase inhibitor letrozole on endogenous androgen synthesis, testicular interstitial cell proliferation and spermatogenic function. Methods: Forty SPF-grade male Kunming mice were randomly divided into control group and experimental group. Clysion of 0.01 mg/kg letrozole was conducted on all rats for three days in the experimental group and eyeball vein blood collection was conducted 24 h, 4 W and 8 W after withdrawal. ELISA method was adopted to test the levels of testosterone (T) and estradiol (E₂) and then testicular histological change was observed through light microscope and electron microscope; epididymal development was observed and testis coefficient and epididymis coefficient (testicular or epididymal weight on both sides/body weight) of mice were calculated; sperm density was calculated and apoptosis of testicular interstitial cells was tested with the method of flow cytometry. Proliferation capacity of testicular interstitial cells was tested through CCK8. Results: Compared with the control group, the levels of testosterone (T) and estradiol (E₂) in serum of mice was obviously reduced by letrozole 8 W upon medication (both P<0.05) and there was no obvious difference between 24 h and 4 W upon medication (both P>0.05). Compared with the control group, testicular and epididymal coefficients were both obviously reduced by letrozole 4 W and 8 W upon medication (both P<0.05) and there was damage to testicular histomorphology to different degrees below electron microscope in the experimental group; sperm density of testicular tissue was significantly reduced by letrozole 4 W and 8 W upon medication (both P<0.05). Compared with the control group, obvious decrease of sperm density and significant increase of cell apoptosis in testicular tissue were observed by applying letrozole for 4 W and 8 W of medication (all P<0.05). Conclusion: Letrozole could inhibit proliferation of testicular interstitial cells, reduce testis coefficient and epididymis coefficient, decrease sperm density and testicular interstitial cells apoptosis through reducing synthesis of endogenous estrogen in the mouse and making testosterone and estradiol level lower.

Keywords: Testicular interstitial cell, proliferation, apoptosis, letrozole, P450 aromatase inhibitor

Introduction

Starting and maintaining of sperm generation rely on the regulation of endocrine system and the regulation center is hypothalamus-pituitary gland-testicular axis [1]. Follicle-stimulating hormone and luteinizing hormone, which are two kinds of glycoprotein hormone secreted by anterior pituitary, are indispensable to sperm generation in normal physiological condition. With the effect of cytochrome P450 (CYP450) aromatase, estrogen can promote the C19 position of testosterone or androstenedione to form phenol ring chain, which is the marker of estrogen, so as to transform androgen into estrogen

[2]. P450 aromatase is a terminal oxidase in CYP450 enzyme system, and it is not only the key component of the whole enzyme system, but also the vital catalyzing enzyme in the biosynthesis of estrogen [3, 4]. Researches indicate that P450 aromatase is expressed in all kind of cells in testicles in adulthood of mammals, such as interstitial cell, supporting cell, spermatocyte and spermatid, etc. [5]. Sustentacular cell can play a role of providing nutrition for spermatogenic cells, secretion of various bio-active proteins, protective isolation and phagocytosis; while testicular interstitial cell is mainly aimed at generating and secreting testosterone to adjust sperm generation and main-

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tain secondary sex characteristics of males. Related literatures indicate that P450 aromatase is mainly expressed in early development phase of spermatogenic cells and the estrogen synthesis capacity is gradually increased in the maturing process of spermatogenic cells [6].

Stocco et al. found that aromatase was essential to proliferation of testicular interstitial cells and sperm generation [7]. Briefly, there is no significant abnormality of testicular development upon birth for mice lacking P450 aromatizing enzyme gene. However, it gradually changes with age increase: there is sperm obstacle at the age of four or five months and complete male infertility occurs at the age of 12 months. Moreover, there is plenty of apoptosis and coenocyte and the number of round and long and narrow-type spermatids is significantly reduced in early phase of spermatogonium. In addition, normal reproductive endocrinology is disturbed by high concentration of estrogen, thereby the sperm generation is inhibited. Estrogen level increase, cryptorchidism, incomplete orchioecatastasis, spermatogenesis retention and proliferation of interstitial cells can be caused by over expression of P450 aromatase [8, 9]. However, affecting sperm proliferation and production via regulating activity of endogenous P450 aromatase has not yet been reported so far. Therefore, effect of P450 aromatase specific inhibitor letrozole on the proliferation of testicular interstitial cells and spermatogenesis of male mice was observed.

Materials and methods

Materials

Sixty wild type male Kunming mice (age: five to six weeks, weight: 20-25 g) were purchased from experimental animal center of Second Military Medical University of the Chinese People's Liberation Army. Adaptive breeding was conducted on the mice in different cages with five or six mice in a cage and breeding temperature and humidity as 20-25°C and 20-30%; and they were raised in periodical condition of light and darkness of 12 h respectively without outside stresses such as noise, etc. All the mice were allowed to move free and eat and drink on time in ration. Our experiments were approved by experimental Ethics Committee of Second Military Medical Uni-

versity and experimental animal nursing and user guide both conformed to provisions of Second Military Medical University.

Preparation of letrozole: the drug was diluted with distilled water to 83 µg/mL.

Grouping and treatment

Sixty male Kunming mice were randomly divided into two groups: 30 in control group and thirty in experimental group. Continual clysis with letrozole (10 µg/kg) for three days was conducted on mice in the experimental group and isovolumetric distilled water was performed clysis in the control group. Mice in the experimental group were further sub-grouped into three groups (24 h group, 4 W group and 8 W group) respectively according to execute time: 24 h, four weeks and eight weeks upon clysis (10 mice at each time point). Mice in the control group were all executed on the these three time points as well and existing research shows that involved indexes of the control group in the experiment within three months are stable and do not vary with time [10].

Determination of testosterone and estradiol

Serum was collected from above treated and executed mice in the experimental group and control group via removing eyeball blood method. Then, the concentrations of testosterone and estradiol were tested through Quantikin ELISA testosterone and estradiol kits (R&D Company). The optical density (OD) values was detected at 450 nm and 570 nm; standard curve was drawn with step dilution concentration and OD value of standards to calculate the sample concentration.

Determination of testicles and epididymis development

Determination of testicles and epididymis development: Weighted mice were executed through cervical dislocation method and dissected to obtain complete testicles and epididymis. The form was observed before weighing. Development coefficients of testicles/epididymis = weight of testicles or epididymis on both sides/body weight.

Sperm density

Above treated epididymis on either side was put in a sterile six-well plate respectively and 1

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mL of 1 × PBS solution was added in each well, then the sample was very carefully and gently grinded and stood for ten minutes. Take 0.2 mL of supernatant and add sterile PBS solution to 2.0 mL, then fully blend and take 10 µL of mixture to cell counter, finally, observe sperm morphology and count sperm cell number under microscope.

Extraction of testicular interstitial cell

The extraction method was adopted percoll density gradient centrifugation that came from Klinefelter et al.: 1) a testicle on either side of the executed mice according to above method was put in F10 buffer solution (4°C) and then testicular dorsalis was penetrated by a remaining needle; then 0.5 mL of type II collagenase (1 mg/mL) was injected until the testicle was whitened and it was cultivated in F10 buffer solution (2.5 mL/testis) at 35°C for 10 minutes after stripping the testicular albuginea; later isovolumetric type II collagenase (0.5 mg/mL) was added and the mixture was incubated at 35°C for 10 minutes. Then the reaction was interrupted. Finally, it was filtered by nylon nets with 100 meshes and 200 meshes respectively and the filtered cell suspension was centrifuged at 250 g for ten minutes; the supernatant was abandoned and F10 buffer solution was added to blend it into cell suspension; 2) respective 2 mL of percoll solution with three densities (30%, 58% and 70%) was prepared and then above cell suspension was added to prepare percoll liquid for centrifugation at 4°C and 3,000 rpm for 45 minutes; then the second cell band was collected, diluted and centrifuged to obtain purified testicular interstitial cells for cultivation [11].

Testing testicular interstitial apoptosis by flow cytometry

Testicular interstitial cells obtained according to above method were added to RNase for water bath of 30 minutes at 37°C and then washed with sterile PBS; it was centrifuged at 1,000 rpm for five minutes and the supernatant was discarded. A total of 800 µL PI and Annexin was added to dye for 30 minutes at 4°C in dark surrounding; then it was tested. Cells showed PI-negative and Annexin-negative were normal live cells and cells with PI-negative and Annexin-positive were early-stage apoptotic cells; cells with PI-positive and Annexin-

positive were late-stage apoptotic cells and ratio of apoptotic cells was the sum of early-stage apoptosis cell ratio and late-stage apoptosis cell ratio.

Cell proliferation experiment

Extracted testicular interstitial cells in four groups of mice were prepared into suspension and then inoculated in a 96-well plate. The culture plate was pre-cultivated in the incubator for 24 hours (37°C, 5% CO₂). Then the culture plate was incubated for a certain period of time (6, 12, 24 or 48 hours). 10 µL CCK8 solution was added to each well which had 200 µL culture medium and the culture plate was incubated for two hours (37°C, 5% CO₂). The OD value was determined with Microplate Reader at 450 nm and the OD value is in linear relation to cell number.

Data analysis

All data was analyzed with SPSS17.0 software and GraphPad Prism 6.01 software. Measurement data was expressed as mean ± standard deviation and all data conforms to normal distribution and homogeneity test of variance; one-way analysis of variance was adopted for comparison among groups and LSD-t test was adopted for pair-wise comparison among groups. Inspection level was 0.05 and there was statistical meaning of the difference if P<0.05.

Results

The control group was untreated and the mice in control group were sacrificed 24 h, 4 W and 8 W upon medication with letrozole and testicular coefficients at the three time points, while the result showed that there was no statistical difference (P>0.05), which was consistent with research results of Chu et al. [10].

Serological level of testosterone (T) and estradiol (E₂) of mice 8 W upon medication with letrozole was significantly reduced

Serological level of testosterone (T) and estradiol (E₂) of experiment group mice executed 8 W after three days clysis with letrozole that tested with ELISA method was obviously reduced compared with the control group (P = 0.036, P = 0.028); there was no statistical difference for

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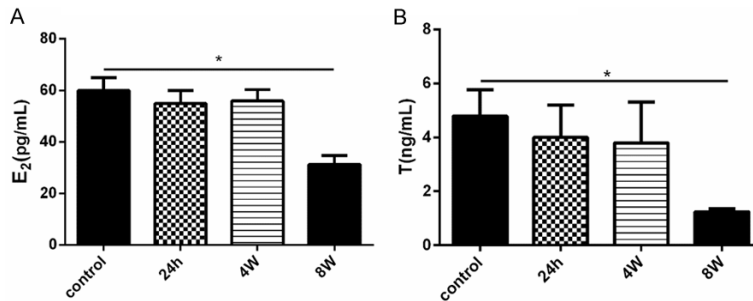


Figure 1. Serological level of serological testosterone (T) (A) and estradiol (E₂) (B) of mice in the four groups. Compared with the control group, the levels of testosterone (T) and estradiol (E₂) in serum of mice was obviously reduced by letrozole 8 W upon medication, *P<0.05.

Testicular and epididymis coefficients were reduced and testicular histomorphology was damaged 24 h, 4 W and 8 W upon medication with letrozole

There was no obvious change of testicular and epididymis coefficients in experiment group mice 24 h after three days of clysis with letrozole compared with the control group (P>0.05). Testicular and epididymis coefficients were obviously reduced in the experiment group compared with control group at 4 W and 8 W after three days of clysis with letrozole (P = 0.012, P = 0.007). After 3 days of letrozole clysis, the testis coefficient and epididymis coefficient of mouse executed at 8 W were obviously reduced than the control group (P = 0.010, P = 0.005, Figure 2).

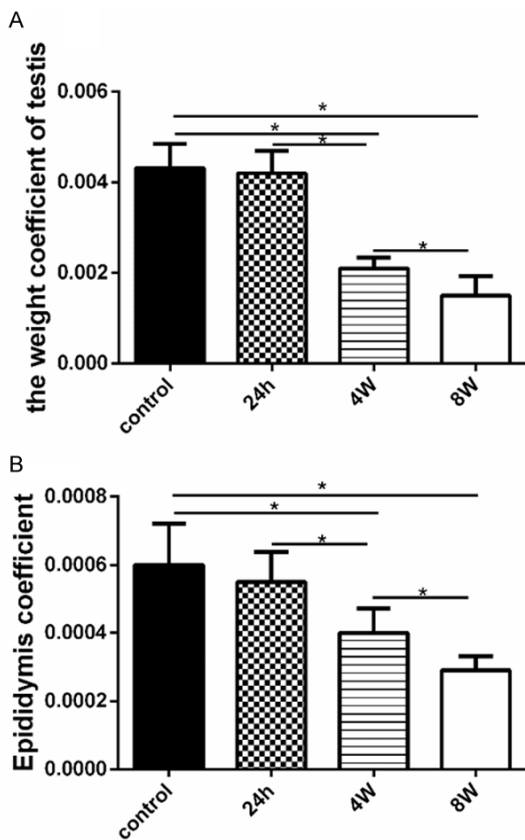


Figure 2. Testicular and epididymis coefficients of mice in the four groups. Control: control group; 24 h: executed 10 mice 24 h upon three days of clysis with letrozole; 4 W: executed 10 mice 4 weeks upon three days of clysis with letrozole; 8 W: executed 10 mice 8 weeks upon three days of clysis with letrozole; *P<0.05.

HE staining result of testicular for mice in the control group: Structure of convoluted tubule was complete and basement membrane was closed to the underside of supporting cells; round spermatogonium with big and round cell nucleus and deep dyeing was close to the basement membrane.

HE staining result of the experiment groups: There was no obvious change of testicular HE dyeing for mice of 24 h group as testicular interstitial cells were arranged well and there was no obvious degenerative necrosis; for samples from 4 W and 8 W groups, testicular HE dyeing showed that structure of convoluted tubule was damaged and cellular level was disordered and there was no spermatogonium with complete structure and convoluted tubule was degenerated, as shown in Figure 3.

Significant reduction of sperm density in testicular tissue and cell proliferation of testicular interstitial cells caused by medication with letrozole

There was no obvious change of sperm density and proliferation of testicular interstitial cells of 24 h group compared with the control group. However, these two were obviously reduced in experiment group 4 W and 8 W after three days

the difference between the control group 24 h and 4 W after three days administration. See Figure 1.

There was no obvious change of sperm density and proliferation of testicular interstitial cells of 24 h group compared with the control group. However, these two were obviously reduced in experiment group 4 W and 8 W after three days

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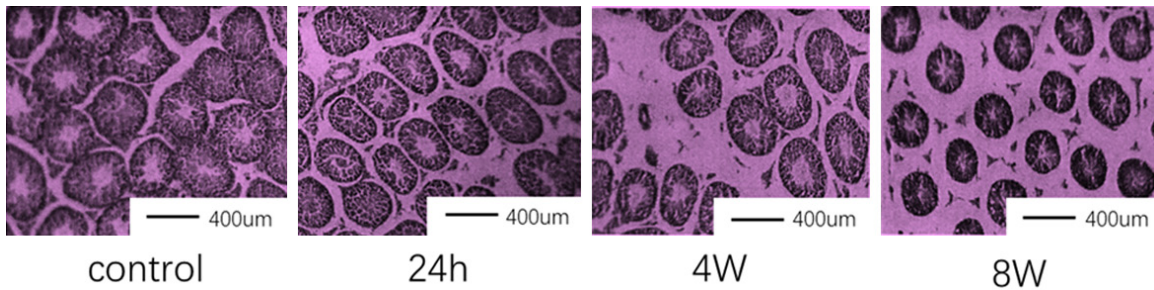


Figure 3. Testicular HE dyeing for mice in four groups (400 ×). Control: control group; 24 h: executed 10 mice 24 h upon three days of clysis with letrozole; 4 W: executed 10 mice 4 weeks upon three days of clysis with letrozole; 8 W: executed 10 mice 8 weeks upon three days of clysis with letrozole. *P<0.05.

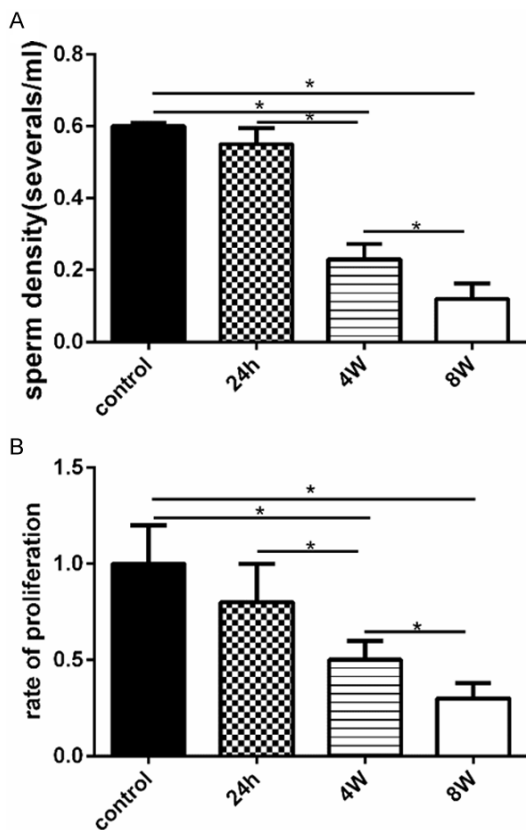


Figure 4. Testicular sperm density and proliferation ratio of testicular interstitial cells for mice in four groups. Control: control group; 24 h: executed 10 mice 24 h upon three days of clysis with letrozole; 4 W: executed 10 mice 4 weeks upon three days of clysis with letrozole; 8 W: executed 10 mice 8 weeks upon three days of clysis with letrozole; *P<0.05.

of clysis with letrozole ($P = 0.025$, $P = 0.018$), as shown in **Figure 4**.

Obvious increase of testicular interstitial apoptosis was caused by letrozole

There was no obvious change of testicular interstitial apoptosis in 24 h group compared

with the control group. While testicular interstitial apoptosis in experiment group 4 W and 8 W after three days of medication with letrozole was obviously increased ($P = 0.031$, $P = 0.001$); meanwhile, interstitial cellular apoptosis rate of 4 W group was obviously increased compared with that in 24 h group ($P = 0.04$); interstitial cellular apoptosis rate of mice of 8 W group was obviously increased compared with that of 4 W group ($P = 0.047$). See **Figure 5**.

Discussion

CYP450 enzyme system is a kind of metabolism system, widely spread in animals, plants and microorganisms, etc. There are several steps for estrogenic synthesis in male animal organisms; the last step was that testosterone or C19 hydroxylated androstatrione is transformed to estradiol or estrone under the catalysis of aromatase [9, 12]. Wickman and Raymond et al. found that contents of testosterone and estradiol in surrounding circulation to the testicles and epididymis could be reduced by aromatase inhibitor [13, 14]. Namely endogenous estrogen level can be reduced by aromatase inhibitor, so as to inhibit E level maximally. In our study, we found that serological testosterone and estradiol contents of mice are obviously reduced by applying P450 aromatase inhibitor letrozole and the results are consistent with the above researches.

It was proved in the 1930s that estrogen could be synthesized in male bodies [15]. Then estrogen synthesis was reported to occur in certain somatic cells of the testicles, such as testicular interstitial cells in 1970s. But the function of estrogen in male animals was not recognized then, which was still unclear until 1990s that influence of estrogen on reproductive capacity was noticed [16]. Endogenous estrogenic syn-

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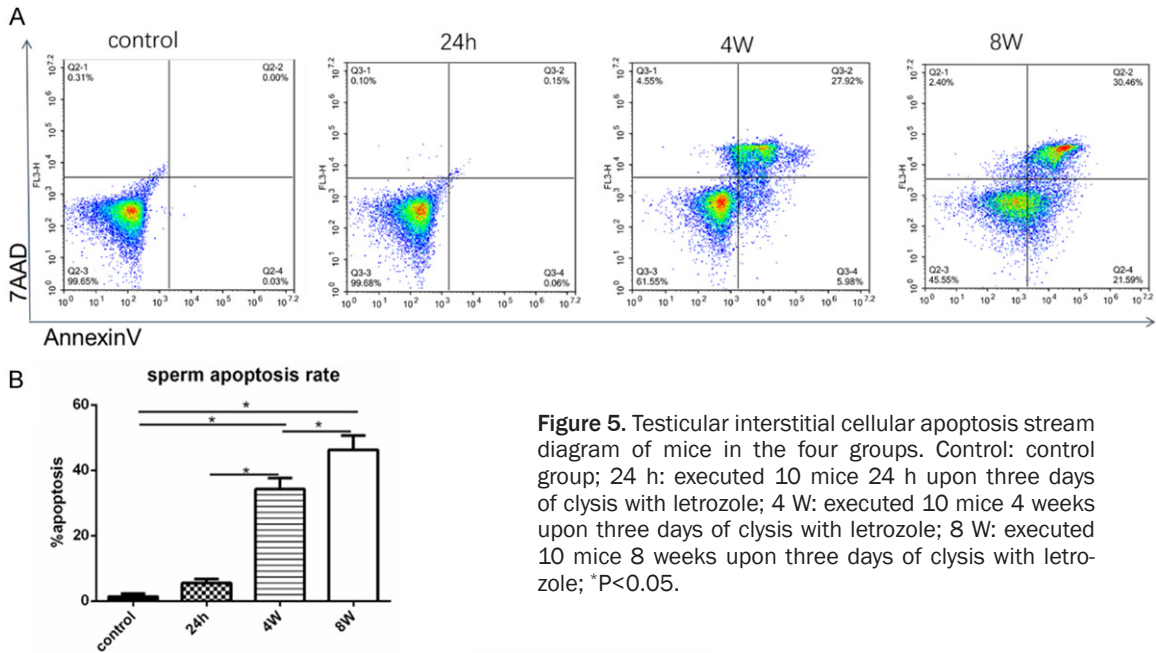


Figure 5. Testicular interstitial cellular apoptosis stream diagram of mice in the four groups. Control: control group; 24 h: executed 10 mice 24 h upon three days of clysis with letrozole; 4 W: executed 10 mice 4 weeks upon three days of clysis with letrozole; 8 W: executed 10 mice 8 weeks upon three days of clysis with letrozole; *P<0.05.

thesis and mediation systems in various cells in the testicle tissues and influence of endogenous and exogenous estrogen on spermatogenesis were included in these researches [1, 17]. These researches indicate that CYP450 aromatase can affect estrogenic receptor α (ER α) on efferent ductules of testis and epididymis epithelial cells and estrogenic receptor 13 on spermatogenic cells and testicular interstitial cells through adjusting estrogenic concentration in synthetic process of endogenous estrogen to play its physiological role, as P450 aromatase is the only key enzyme in male bodies that can transform androgen to estrogen [18, 19]. It has been manifested that spermatogonium apoptosis occurs and spermatid quantity is reduced when P450 aromatase gene of mice is knocked out to block endogenous estrogenic synthesis; while spermatogenetic malfunction of mice and humans can be caused by over expression of P450 aromatase and estrogenic level increase, moreover, testicular interstitial cellular development can also be inhibited by estrogen *in vitro* [20, 21]. It is also found in our research that testicular interstitial edema occurred, convoluted tubule was degenerated and its development is slow, the luminal epithelium is thinned, its cellular level is disordered in the HE staining when the P450 aromatase was inhibited; but the inhibition function is only worked for certain period, namely corre-

sponding change of testicular interstitial cells is caused in the 4 W and 8 W after administration; in addition, testicular interstitial apoptosis is obviously increased and proliferation capacity is obviously reduced when P450 aromatase activity is inhibited.

Only indexes such as proliferation and apoptosis of testicular interstitial cells 24 h, 4 W and 8 W after three days of clysis with letrozole were observed in our research and we found that there is no difference at 24 h time point between the two groups; while there are obvious differences of all indexes at 4 W between two groups. However, specific data with statistical difference cannot be determined because spanning time is too long. Testicular interstitial cellular change on time points such as 24 h, 48 h and 72 h, etc. upon three days of clysis will be further observed to determine the exact role of letrozole and provide more data for clinical medication time points.

In conclusion, letrozole at the dosage of 10 $\mu\text{g}/\text{kg}$ could decrease endogenous estrogenic synthesis of mice to reduce testosterone and estradiol to a relative low level, thereby, proliferation of testicular interstitial cells was inhibited which was showed as the reduction of testicular and epididymis coefficients, reduction of sperm density and apoptosis of testicular interstitial cells.

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Disclosure of conflict of interest

None.

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