

# Toxic effects of ketamine on reproductive system via disrupting hypothalamic-pituitary-testicular axis

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**Abstract. – OBJECTIVE:** In this paper, we focused on the toxic effect of ketamine on the reproductive system in male rats and its underlying mechanisms.

**MATERIALS AND METHODS:** Rats were randomly allocated into four groups (n=10), i.e. a control group and 3 ketamine groups (high-dose, mid-dose, low-dose). Animals in the ketamine groups received an intraperitoneal injection of ketamine (20, 40 or 60 mg/kg) every 3 days for 7 times. Control rats were injected with normal saline instead. To investigate the disruption potential on the hypothalamic-pituitary-testicular (HPG) axis, the relative hormone levels in serum and mRNA expressions for some reproduction-related genes in reproductive organs were evaluated.

**RESULTS:** Ketamine significantly decreased the serum concentrations of testosterone (T), inhibin B, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Meanwhile, the mRNA expressions of GnRH in the hypothalamus, GnRH receptor, LH- $\beta$  and FSH- $\beta$  in the pituitary, and LH receptor and FSH receptor in testes were also significantly inhibited by ketamine compared with the control ( $p < 0.01$ ).

**CONCLUSIONS:** These results demonstrated that the ketamine had a toxic effect on the reproductive system via breaking the HPG equilibrium.

Key Words:

HPG axis, Reproductive system, Ketamine, GnRH.

## Introduction

Ketamine, a receptor complex antagonist of N-methyl-D-aspartic acid, evokes profound analgesia, loss of consciousness, amnesia, and

immobility<sup>1</sup>. Neurotoxicity of ketamine in the neonatal nervous system has made it controversial for pediatric use<sup>2,3</sup>. Even so, ketamine remains an important medicine in both specialist anaesthesia and aspects of pain management. At the same time, it produces hallucinatory effects on human<sup>3</sup> and has spread in many parts of the world during the past few years<sup>4</sup>, which used as a recreational drug ('K', 'ket', 'Special K'). In 2009, China reported the seizure from two illicit laboratories of 8.5 million tons of the immediate chemical precursor for ketamine<sup>5</sup>.

Increasing ketamine abuse has triggered growing concern about its toxic effects. Inhaling ketamine causes distortion of time and space, hallucinations, and mild dissociative effects<sup>6</sup>. Ketamine use can have a severe and potentially long-lasting impact on the individual, describing symptoms such as urinary frequency, nycturia, dysuria and severe bladder pain<sup>7</sup>. Another emergent physical health problem associated with frequent, high-dose ketamine use appears to be hydronephrosis (water on the kidney) secondary to urinary tract problems<sup>8</sup>.

A third of 90 ketamine users in one study spontaneously reported 'K-cramps'-intense abdominal pain-as a result of prolonged, heavy ketamine use<sup>9</sup>. Besides, frequent ketamine users exhibit profound impairments in both short- and long-term memory<sup>10</sup>.

Furthermore, Tan et al<sup>11</sup> demonstrated that chronic administration of ketamine affected the genital system. However, the potential mechanism is still indeterminable. A major goal of this pa-

per is to investigate the underlying mechanism of ketamine on the reproductive system of male rats.

## Materials and Methods

### Animals and Treatment

Forty male Sprague-Dawley (SD) rats (5-6 week-of-age) were obtained from the experimental Animal Center of Suzhou Aiermaite Technology Co. Ltd. (Suzhou, Jiangsu, China). Animals were maintained in a controlled environment at  $21 \pm 2^\circ\text{C}$  with a relative humidity of 50-60% and a 12-hr light/dark cycle. All the experiments were performed in accordance with protocols and International Guidelines for Care and Use of Laboratory Animals and approved by the local Experimental Ethics Committees.

After 10 days of acclimatization, animals were randomly allocated into four groups ( $n=10$ ), i.e. a control group and 3 ketamine groups (high-dose, mid-dose, low-dose). In the anesthesia groups, rats received an intraperitoneal injection of ketamine (20 mg/kg, 40 mg/kg, or 60 mg/kg) every 3 days for 7 times. Rats in the control group (group C;  $n=10$ ) were injected intraperitoneally with nothing but normal saline in place of ketamine, in an equivalent injection volume. Afterwards, animals were sacrificed by cervical dislocation. The hypothalamus, hypophysis, and testis were immediately excised *en bloc*.

### Body and Reproductive Organs Analysis

The body weights were recorded before sacrifice. The isolated median eminence from the hypothalamus and pituitary gland were also weighed. Both testes were excised and dissected free of epididymides. The weight (as a pair), length, and width were measured. Testis volume was calculated using the formula:  $V = 4\pi (\text{width}/2)^2 (\text{length}/2)^3$ . One testis was frozen immediately in liquid nitrogen and stored at  $-70^\circ\text{C}$  for gene expression analysis and the other was used for testicular histological analysis.

### Serum Analysis

Before the decapitation, blood samples were assembled from the caudal artery and then centrifuged at  $\times 2000\text{ g}$  for 20 min at  $4^\circ\text{C}$ . Serum was stored at  $-20^\circ\text{C}$  for subsequent hormone evaluation.

Serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were analyzed by rat-specific RIA kits (DRG International, Marburg, Germany) using immunofluorometric assays (Delfia; PerkinElmer Wallac, Wellesley,

MA, USA). For both assays, sensitivities were 1.0 mIU/mL and the intra- and inter-assay CVs were 10% and 15%, respectively.

Serum testosterone level was measured by electrochemiluminescence using Elecsys Testosterone II kit (Roche Diagnostics, Indianapolis, IN, USA). The sensitivity of the assay was 0.025 ng/mL, with intra- and inter-assay CVs of 10% and 15%, respectively.

Commercially available and for rats validated ELISAs were used for the determination of the serum concentration of inhibin B (Creative Diagnostics, Shirley, NY, USA). The sensitivity of the assay was 34.6 pg/mL, with intra- and inter-assay CVs of 10% and 15%, respectively.

### Quantitative Analysis of mRNA Expressions

Total RNA was isolated from the hypothalamus, pituitary, and testis using the TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA), according to the instructions. First-stand cDNA was reverse-transcribed using PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Bio, Co. Ltd, Dalian, Liaoning, China). Quantitative Real-time polymerase chain reaction (PCR) was analyzed in triplicated on CFX96 Real Time PCR detection system (BioRad, Hercules, CA, USA) with SYBR green II.

The PCR contained 40 ng cDNA, 500 nmol/l each of forward and reverse primers, and  $2 \times$  SYBR Premix Taq (TaKaRa Bio, Co. Ltd, Dalian, Liaoning, China). The primer sequences of the target and reference genes are listed in Table I.

### Testicular Histological Analysis

Testes were immersed in neutral-buffered formalin fixation fluid for 12 h and washed with 70 % alcohol. Afterwards, testes specimens were dehydrated and embedded in paraffin. 5  $\mu\text{m}$  thick sections were obtained from paraffin blocks using a rotator microtome and stained with hematoxylin-eosin (H&E). The testes sections were observed with an optical microscope (AX70, Olympus, Tokyo, Japan) in a blind-manner.

### Statistical Analysis

Data statistic analysis was performed by SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and all data were expressed as mean values  $\pm$  standard deviation. Changes between samples were compared by Student's *t*-test, and differences between groups were compared by the method of one-way ANOVA. The LSD test was used as a post-hoc test for ANOVA.  $p < 0.05$  was considered statistically significant.

**Table I.** Primer sequences of body tissue genes.

| Genes   | Genebank accession No. | Primer sequence (5'-3')                              | Product annealing size (bp) temperature (°C) |      |
|---------|------------------------|------------------------------------------------------|----------------------------------------------|------|
| GnRH    | NM_012767              | F: GGCAAGGAGGAGGATCAAA<br>R: CCAGTGCATTACATCTTCTTCTG | 142                                          | 60   |
| FSH-β   | NM_001007597           | F: CATCCTACTCTGGTGCTT<br>R: CACTCTTCCTTCTCTACTGA     | 84                                           | 60.5 |
| LH-β    | NM_012858              | F: CTTCTCCTCTTCTGATGC<br>R: TTTATTGGGAGGGATGGT       | 80                                           | 60.5 |
| AR      | NM_012502              | F: GGCAGTCATTCACTATTCC<br>R: AGTAGAGCATCCTAGAGTTG    | 89                                           | 60.5 |
| GnRH-R  | NM_031038              | F: TCTGCAATGCCAAAATCATC<br>R: GTAGGGAGTCCAGCAGATGAC  | 164                                          | 60.5 |
| FSH-R   | NM_199237              | F:GAATGATGTCTTGGAAGTAATAG<br>R:CTTAATGCCTGTGTTGGA    | 163                                          | 59.5 |
| LH-R    | NM_012978              | F:TTACACATAACCACCATACC<br>R:TCCAGCGAGATTAGAGTC       | 134                                          | 60   |
| β-actin | NM_031144              | F:CACAGCTGAGAGGGA AAT<br>R:TCAGCA ATGCCTGGGTAC       | 155                                          | 60.5 |

## Results

### **Effects of Ketamine on Testes Weight, Volume and Pituitary Weight**

In the adult rats experiment, testes weight, volume, and pituitary weight were detected in control and treatment groups. Compared with the control rats, the administration of ketamine significantly decreased the testes weight and volume, and the pituitary weight, in a dose manner (Table II). No mortality was observed. The testes weight, volume and pituitary weight were severally decreased by 52.28% ( $p<0.01$ ), 46.28% ( $p<0.01$ ) and 23.78% ( $p<0.05$ ).

### **Effects of Ketamine on Serum Hormones**

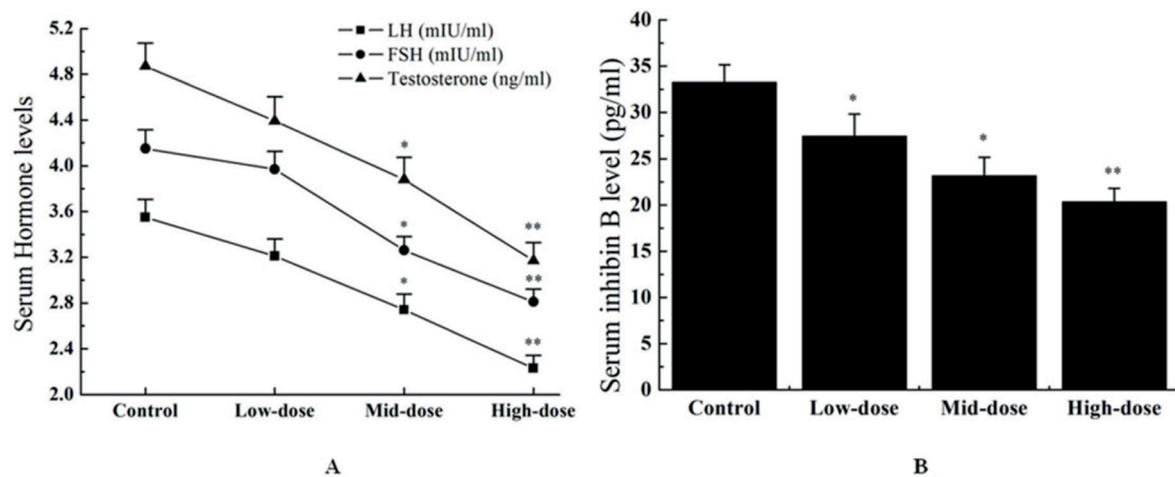
As shown in Figure 1A, evident decreasing trends of serum levels of LH, FSH, and testosterone were

observed between control and treatment rats in a dose manner. The serum LH level was significantly decreased in the mid-dose and high-dose groups, respectively from  $3.55\pm0.25$  mIU/ml in the control group to  $2.74\pm0.23$  ( $p<0.05$ ) and  $2.23\pm0.21$  ( $p<0.01$ ) mIU/ml. Serum FSH level was also significantly impaired from  $4.15 \pm 0.26$  mIU/ml in the control group to  $3.26\pm0.22$  ( $p<0.05$ ) and  $2.81\pm0.21$  mIU/ml ( $p<0.01$ ) after administration of mid-dose and high-dose ketamine, in several. Moreover, significant changes of serum were observed in the mid-dose and high-dose groups, separately from  $4.87 \pm 0.41$  ng/ml to  $3.88\pm0.29$  ( $p<0.05$ ) and  $3.17\pm0.31$  ng/ml ( $p<0.01$ ). Figure 1B depicted the effect of ketamine on the serum inhibin B level. Inhibin B was significantly suppressed in the ketamine groups, especially in the high-dose rats ( $p<0.01$ ).

**Table II.** Effects of ketamine on testes weight, volume and pituitary weight.

| Items                            | Contro group   | Low-dose ketamine group | Mid-dose ketamine group | High-dose ketamine group |
|----------------------------------|----------------|-------------------------|-------------------------|--------------------------|
| Testes weight (g)                | $3.29\pm0.23$  | $2.91\pm0.25$           | $2.07\pm0.19^*$         | $1.57\pm0.08^{**}$       |
| Testes volume (mm <sup>3</sup> ) | $1.21\pm0.07$  | $1.05\pm0.05$           | $0.83\pm0.04^*$         | $0.65\pm0.04^{**}$       |
| Pituitary weight (mg)            | $11.27\pm0.51$ | $10.78\pm0.43$          | $9.72\pm0.67$           | $8.59\pm0.40^*$          |

Data were presented as value  $\pm$  SD. \*Statistically significant difference from the control ( $p<0.05$ ); \*\*Statistically significant difference from the control ( $p<0.01$ ).



**Figure 1.** Effect of ketamine on serum hormone levels. Data were presented as value  $\pm$  SD. \*Statistically significant difference from the control ( $p < 0.05$ ); \*\*Statistically significant difference from the control ( $p < 0.01$ ).

### Effects of Ketamine on mRNA Expressions in Hypothalamus, Hypophysis, and Testis

The mRNA expressions for reproduction-related genes are shown in Figure 2. Compared with the controls, mid-dose and high-dose ketamine significantly suppressed the mRNA expressions of AR and GnRH in rat hypothalamic (Figure 2A). After the administration of mid-dose and high-dose ketamine, the expression of AR was reduced by  $32.77 \pm 4.00\%$  ( $p < 0.05$ ) and  $43.29 \pm 3.27\%$  ( $p < 0.01$ ), respectively; GnRH was reduced by  $36.85 \pm 3.42\%$  ( $p < 0.05$ ) and  $49.63 \pm 3.59\%$  ( $p < 0.01$ ), respectively.

In the pituitary, the mRNA expressions of GnRH-R, LH- $\beta$ , and FSH- $\beta$  were determined (Figure 2B). GnRH-R and FSH- $\beta$  were significantly decreased in the low-dose ketamine group, relative to the control, respectively by  $21.88 \pm 1.97\%$  ( $p < 0.05$ ) and  $23.67 \pm 2.77\%$  ( $p < 0.05$ ); In the mid-dose group, GnRH-R, LH- $\beta$ , and FSH- $\beta$  were all significantly diminished, relative to the control, severally by  $33.17 \pm 3.09\%$  ( $p < 0.01$ ),  $38.07 \pm 3.47\%$  ( $p < 0.01$ ) and  $40.95 \pm 2.65\%$  ( $p < 0.01$ ).

In the testes, the expression levels of LH-R and FSH-R were all significantly down-regulated in the ketamine groups (Figure 2C,  $p < 0.01$ ).

### Effects of Ketamine on Testicular Histological

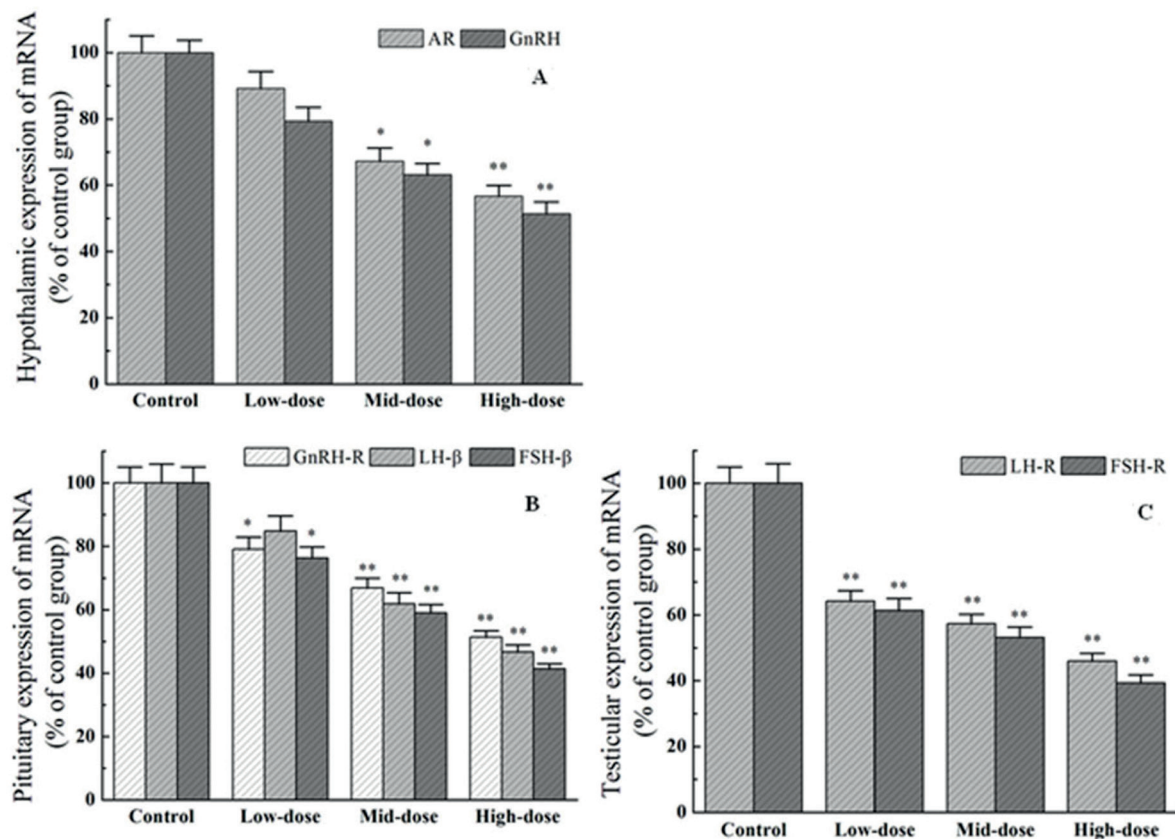
The testicular histological analysis was conducted to investigate the effects of ketamine on testes. Figure 3 depicted the photomicrographs of testes from the control group and ketamine

groups. Testes in the control rats (Figure 3A) had normal seminiferous tubules, nearly all stages of spermatogenic cells (i.e. spermatogonia, primary spermatocyte, spermatid, and spermatozoa) and complete interstitial tissues with Leydig cells. Histological changes in the testes in all of the ketamine rats were observed (Figure 3B, 3C, 3D). Ketamine groups showed a disruption of normal spermatogenic cell organization in the tubules, and the total number of germ cells inside the tubules decreased markedly, and the spermatocytes were connected to the lumen, indicating cell disorganization.

## Discussion

This study is the first report that demonstrated the mechanism of toxic effects of ketamine on the reproductive system. After administration of ketamine, significant changes were observed in serum testosterone, inhibin B, FSH, and LH levels, reproductive organs, HPG axis genes expression, and testicular histopathology. Based on these data, we inferred that toxic effects of ketamine on the genital system might be through breaking the HPG equilibrium.

In both male and female, gametogenesis is regulated by HPG axis that corresponds to the hormonal axis, gonadotropin-releasing hormone (GnRH)-gonadotropins-steroids. The main target of GnRH is the gonadotrope cells, located in the adenohypophysis. These in return, release two gonadotropin hormones, i.e. FSH and LH, through the main circula-

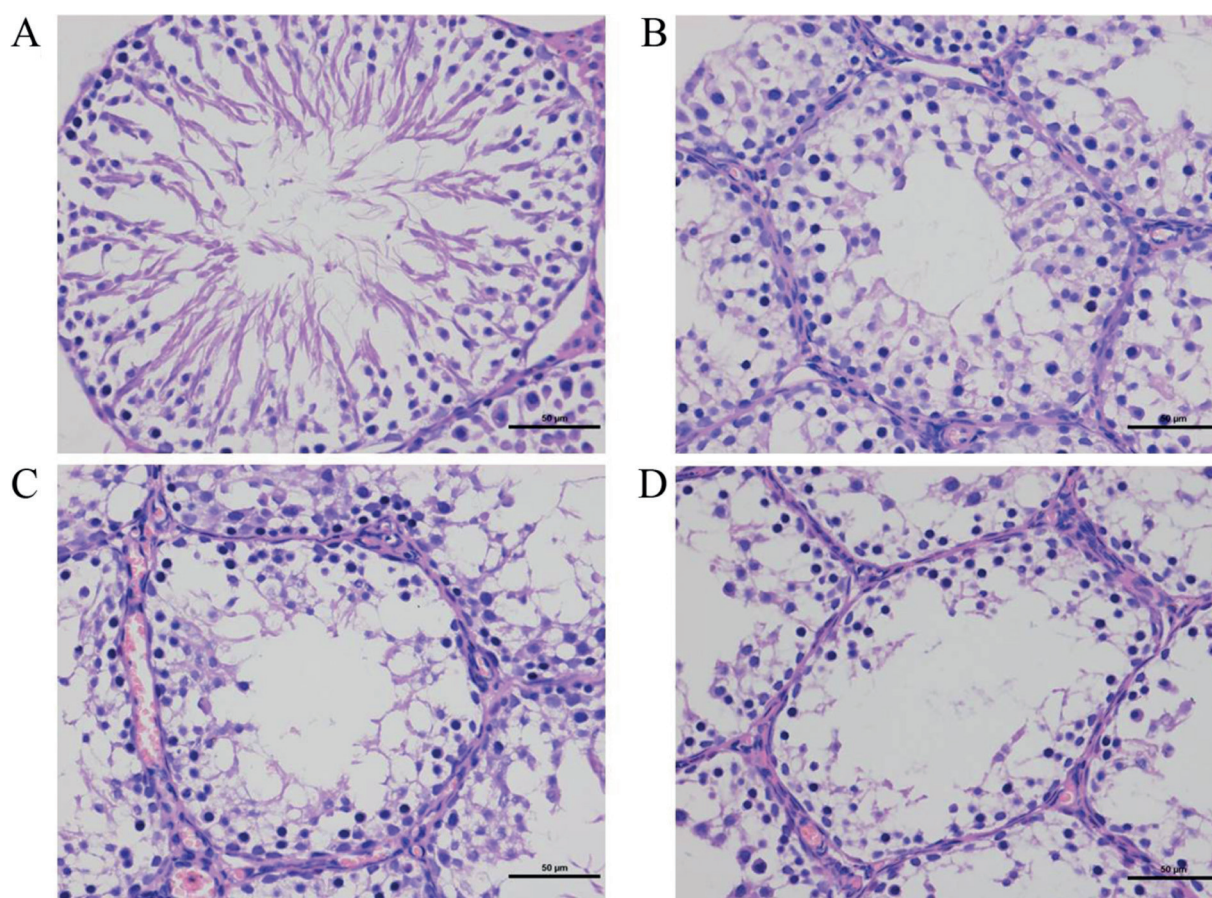


**Figure 2.** Data were presented as value  $\pm$  SD. \*Statistically significant difference from the control ( $p < 0.05$ ); \*\*Statistically significant difference from the control ( $p < 0.01$ ).

tion reach gonads to regulate gametogenesis via the synthesis of steroid hormones. In the male, LH stimulates the production of testosterone by testicular Leydig cells. FSH binds to receptors on the surface of Sertoli cells and functions in concert with testosterone to promote the proliferation of spermatogonia as well as the meiosis and postmeiotic development of germ cells<sup>12</sup>. In the present work, mRNA expression of GnRH was significantly decreased in the ketamine group compared with the control, which inferred that ketamine could inhibit the synthesis of GnRH. Once the GnRH decreased, serum LH and FSH level were significantly decreased relative to the control in this study, which confirmed the effects of ketamine on the synthesis and secretion of GnRH. At the same time, ketamine significantly reduced mRNA expression of GnRH receptor in the pituitary, suggesting the necessity of GnRH stimulation in sustaining pituitary GnRH receptor expression. In that regard, reduced expressions of GnRH receptor reduce the responsiveness of the pituitary to GnRH stimulation<sup>13</sup>.

Sex steroids is an important physiologic signal to regulate hypothalamic GnRH physiology<sup>14</sup>. Their actions are mediated through receptors located in the hypothalamus. In the current study, ketamine significantly suppressed mRNA expression of androgen receptor (AR) in the hypothalamus. We inferred that the expression reduction in the hypothalamus was owing to the deficiency of testicular steroids, consistent with a previous study that AR expression was auto-regulated by androgens<sup>15</sup>.

Furthermore, the spermatogenesis and the physiological functions of Sertoli cells in mammals depend largely on T production by Leydig cells in response to stimulation by FSH and LH<sup>16-18</sup>. The serum T level decreased significantly in the ketamine groups, which indicated that ketamine administration could lead to Leydig cells' degeneration and reduction, and the decrease of their capacity of T synthesis. A previous study<sup>18</sup> demonstrated that any decrease in T level could cause a severe reduction in inhibin-B synthesis of Sertoli cells.



**Figure 3.** Photomicrographs of testes ( $\times 20$  magnification) from control (*A*) and low-dose (*B*), mid-dose (*C*) and high-dose (*D*) ketamine groups.

Inhibin B is an important marker of the competence of Sertoli cells and spermatogenesis<sup>19</sup>, and secretion of FSH and LH is regulated by T and inhibin B through a negative feed-back mechanism of HPG axis<sup>18</sup>. In the current study, inhibin B production was significantly suppressed by ketamine, which indicated that ketamine could firstly damage Leydig cells and Sertoli cells and subsequently inhibit the T secretion of Leydig cells and the inhibin-B secretion of Sertoli cells, and the decrease of T and inhibin-B secretions result in the decrease of FSH and LH secretion in a feed-back way. Moreover, the HPG axis is regulated by complex androgenic negative feedback mechanisms like decreased GnRH synthesis in the hypothalamus and decreased gonadotropin secretion in the pituitary gland<sup>20</sup>. However, the feedback regulations didn't work. Thus, we inferred that the normal feedback regulations need minimum concentrations which is coincident with Ding et al<sup>12</sup>.

Consistent with serum concentrations of LH and FSH, the mRNA expressions of LH- $\beta$  and

FSH- $\beta$  in the pituitary gland were both significantly suppressed after ketamine administration. Moreover, pituitary gland weight was also significantly decreased by ketamine administration, reflecting reduced pituitary storage capacity of gonadotropins. At the same time, mRNA expression of testicular LH receptor was significantly suppressed, suggesting that testicular responsiveness to LH stimulation was reduced or abolished. The mRNA for FSH receptor was also significantly reduced by ketamine, indicating that the reduction of FSH-R affected Sertoli cell function, which supported the result that toxic effect of ketamine was primarily owing to dysfunction of both Leydig and Sertoli cells.

## Conclusions

After the administration of ketamine, significant changes were observed in serum testosterone, inhibin B, FSH and LH levels, reproductive

organs, HPG axis genes expression, and testicular histopathology. Based on these data, we inferred that toxic effects of ketamine on the genital system might be through breaking the HPG equilibrium.

### Conflict of interest

The authors declare no conflicts of interest.

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