

## The effect of kisspeptin on spermatogenesis and apoptosis in rats

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**Background/aim:** To study the effect of kisspeptin, a gonadotropin release stimulator, on the testicular tissue of the rat.

**Materials and methods:** Four groups were formed as follows: control, Kiss-10 50 nmol administration for 1 day, Kiss-10 administration for 13 days, and one last group kept for 7 days following Kiss-10 applied for 13 days. Testicular tissues were stained with hematoxylin-eosin, periodic acid Schiff, Masson trichrome staining, terminal deoxynucleotidyl transferase UTP nick-end labeling, and Ki-67 immune staining. Serum testosterone levels were determined.

**Results:** Serum testosterone level increased following acute application, while it was reduced by chronic treatment. Spermatogenic cells as stained by Ki-67 and TUNEL increased in the treated groups compared to the controls. Following a 7-day rest after treatment, a decrease in testosterone levels and Ki-67-stained cell numbers and an increase in TUNEL-stained cells were observed. Leydig cells showed increased vacuolization in the Kiss-1 group. Leydig cell vacuolization continued in the Kiss (13) group and was reduced in the Kiss (13 + 7) group.

**Conclusion:** Kiss-10 increased spermatogenic cell proliferation, while testosterone level and proliferation decreased and apoptosis increased during the waiting period.

**Key words:** Kisspeptin, spermatogenesis, apoptosis, Leydig cells

### 1. Introduction

Kisspeptins, the peptide products of the KiSS-1 gene, bind to the G protein-coupled receptor 54 (GPR54), a critical regulator of GnRH secretion. The N-terminally truncated peptide metastin 45-54 exhibits 10-fold higher receptor-binding affinity than full-length metastin; it also shows agonistic KISS-1R activity (1).

Kisspeptins are known to play a role in puberty, cancer metastasis, and vasoconstriction (2,3-7). They are synthesized in the arcuate and anteroventral periventricular hypothalamic neurons and in the perioptic area (8). Kisspeptin expression is variable according to sex in rats; its expression is more marked in female animals (9). Its known derivatives are kisspeptin-10, kisspeptin-13, kisspeptin-14, and kisspeptin-54 (10-12). All kisspeptins seem to have the same interactions under *in vitro* conditions. Kiss-10 is well characterized

in mammals, in which it is found in large concentrations (10,11). It has been suggested, however, that Kiss-54 is the most effective form (12). Kisspeptin has been reported to also be synthesized in the testes, ovaries, pancreas, gut, liver, lung, muscle tissue, kidney, nervous system, and most densely in the placenta (13,14). As for its receptor, G protein-coupled receptor-54 (GPR54) is mostly expressed in the hypophysis, placenta, and pancreas (10,15,16). Kisspeptins are highly potent neuropeptides that stimulate the secretion of LH and FSH from the hypophysis, an effect exerted through the release of GnRH (7,17). While this pathway has been satisfactorily defined in several mammal species, the molecular and cellular events at the proencephalic origin of this process are incompletely elucidated. In recent years, studies have indicated that kisspeptin plays a role in the transition to puberty (18,19). Kiss-1 knockout mice showed hypogonadism and low

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levels of circulating gonadotropin (20,21). Kisspeptin has been shown to control seasonal fertility in sheep (22) and hamsters (23,24). A literature search revealed only a limited number of studies on the effects of kisspeptin.

The aim of the present study was to study the structural and biochemical effects of acute and chronic kisspeptin administration on seminiferous tubular cells, spermatogenesis, apoptosis, and Leydig cells and the reversibility of such effects.

## 2. Materials and methods

### 2.1. Experimental animals

All experimental protocols were performed according to the guidelines for the ethical treatment of experimental animals and were approved by the Animal Care and Use Local Ethics Committee. Eight-week-old male Wistar albino rats (200–230 g) were housed at a constant room temperature ( $21 \pm 2$  °C) under a 12-h light/dark cycle. They were fed standard rat chow (210 kcal/100 g per day) and drank tap water.

Twenty-four male Wistar rats weighing 200–300 g were used in the study. Four groups were constructed:

Control group (n = 6): Received intraperitoneal normal saline only.

Kiss (1) (n = 6): Kiss-10 [metastin (45-54) amide, M2816-1MG, SIGMA], 50 nmol, intraperitoneally was given once to these animals.

Kiss (13) (n = 6): These animals received Kiss-10, 50 nmol, intraperitoneally daily for 13 days.

Kiss (13 + 7) (n = 6): After receiving Kiss-10, 50 nmol, intraperitoneally daily for 13 days, these animals were kept for 7 days without any treatment. Kisspeptin-10 was used because it is the minimal sequence required for full receptor binding and activation (10,11). The single injection treatment was defined as acute and the 13-day treatment as chronic. The rats were weighed before the study and at its termination. Testes were removed and weighed. Gonadosomatic index percent (GSI %) was determined using the following formula (25):

$$\text{GSI (\%)} = \frac{\text{Testes weight}}{\text{Body weight}} \times 100$$

### 2.2. Biochemistry

Blood was drawn under anesthesia from the inferior vena cava, centrifuged at  $1000 \times g$  for 20 min, and kept at  $-20$  °C. Total testosterone level was measured using an ELISA kit (USCN Life Science Inc., Wuhan, E90458Ra, L101101379, P.R. China). The measurement was performed following the instructions provided with the kit.

### 2.3. Light microscopy

The left testes were fixated in a 10% solution of neutral formalin. The right testes were frozen at  $-96$  °C to examine with immunofluorescence microscopy. Blocks

were prepared after routine preparation. Sections were stained with hematoxylin-eosin, periodic acid Schiff (PAS), and Masson's trichrome methods; the diameters of the seminiferous tubules were measured. Staining with Ki-67 was performed (9106S1003B, Thermo Scientific, USA) for marking proliferating cells and with terminal deoxynucleotidyl transferase UTP nick-end labeling (TUNEL) (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit, S7101, Chemicon International; Lot PSO1736498) for apoptotic cells. The numbers of apoptotic and proliferating cells were determined by counting stained dark-brown nuclei in 10 cross-sectional fields of seminiferous tubules per testis section. Tissues were frozen at  $-96$  °C for immune fluorescence determination of Kiss-10 expression. Sections 15- $\mu\text{m}$  thick obtained by a freezing microtome were stained using a Kiss-1 antibody [(C-20), goat polyclonal IgG, #H1910, Santa Cruz Biotechnology, Inc., Dallas, TX, USA] and examined under an immune fluorescence microscope.

### 2.4. Electron microscopy

Tissues were taken and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h. After rinsing with phosphate buffer, tissues were postfixed with 2% osmium tetroxide in sodium phosphate buffer. Dehydration was accomplished by gradual ethanol series and embedded in epoxy resin. Semithin sections (1  $\mu\text{m}$ ) were stained toluidine blue. Sections were then viewed and photographed with light microscopy.

### 2.5. Statistical evaluation

The study data were evaluated using GraphPad Prism software (version 3.00, GraphPad Software, La Jolla, CA, USA). The Kruskal–Wallis test was used for between-group comparison of apoptotic and Ki-67(+) cell counts and Dunn's multiple comparison test for within-group evaluations. Subject weights, testosterone levels, testis weights, gonadosomatic index (GSI %), and tubular diameters were evaluated by the Mann–Whitney U test. Biochemical measurements were compared using one-way ANOVA, while within-group comparisons used Tukey's test. P-values smaller than 0.05 were accepted as significant.

## 3. Results

There was no difference in body weight, testicular weight, GSI %, or diameter of seminiferous tubules among the groups. Kiss-1 immune positive cells were not detected in any group (data not shown).

### 3.1. Serum testosterone levels

While serum testosterone levels were significantly elevated after a single administration of kisspeptin ( $P < 0.03$ ) they decreased in the Kiss (13) and Kiss (13 + 7) groups, although with no significant difference from the controls (Figure 1).

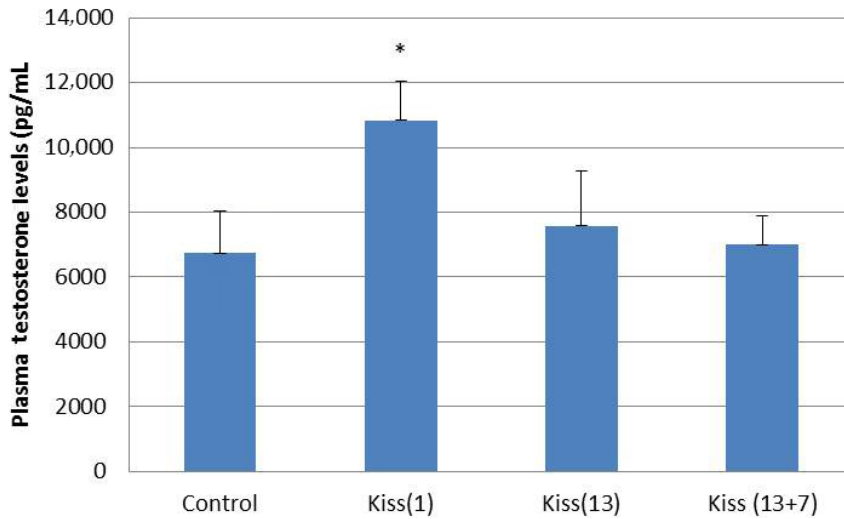


Figure 1. Plasma testosterone levels. \*P < 0.03 compared to other groups.

### 3.2. Proliferating cell count

Ki-67 immune staining was applied to determine the number of proliferative cells in the seminiferous tubules. The Ki-67–stained spermatogenic cells were counted for all groups. Ki-67–stained cell counts were higher in the Kiss (13) group compared to both Kiss (1) and the controls (P < 0.001). As for the (13 + 7) group, after a 7-day rest following 13 consecutive days of treatment, the stained cell count was higher than that in both the controls and Kiss (1) (P < 0.001 and 0.01, respectively) but lower than that of Kiss (13). No statistical significance was detected between the Kiss (13) and Kiss (13 + 7) groups (Figure 2).

### 3.3. Apoptotic cell count

TUNEL staining was applied to mark apoptotic cells in the seminiferous tubules. TUNEL (+)-stained spermatogenic cells were counted in all groups (Figure 3).

A lower number of TUNEL (+) cells were detected in the Kiss (1) group, but the difference was not significant. Apoptotic TUNEL (+) cell counts were significantly higher in the Kiss (13) and Kiss (13 + 7) groups compared to the controls (P < 0.05) and Kiss (1) (P < 0.001).

### 3.4. Light microscopy findings

Kisspeptin expression was not observed in either the control or the treatment groups

(data not shown). Seminiferous tubules in treated animals were largely similar to those of the control group (Figures 4a–4d). Cell shedding was present but not widespread in the lumens of some tubules in the treatment groups. Tissues from all groups were stained with PAS. No disruption or thickening of the basal membrane was seen with PAS staining in the treatment groups. Staining intensity was comparable throughout all animal groups. Connective tissue proliferation among seminiferous

tubules was not observed following Masson trichrome staining (data not shown).

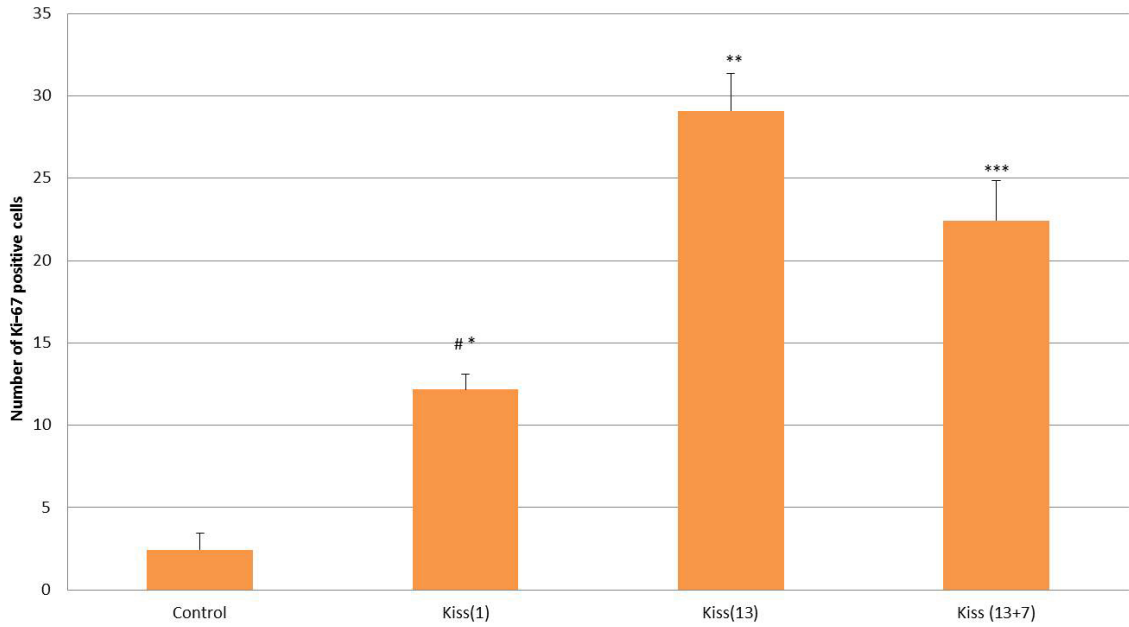
The appearance of Sertoli and Leydig cells, spermatogonia, primary spermatocytes, spermatids, developing acrosomes, and spermatozoa was by definition normal for the control group (Figure 5a). The seminiferous tubule basal membrane was markedly corrugated in the Kiss (1) group, where vacuolization in the Sertoli cells and spermatocytes, increased intercellular intervals, and numerous vacuoles in Leydig cells were observed (Figure 5b). Leydig cell vacuolization continued in the Kiss (13) group (Figure 5c). In the Kiss (13 + 7) group, such vacuoles in the Leydig cells were fewer; excessive intercellular distances remained (Figure 5d).

The cells that stained positive with Ki-67 were generally identified as spermatogonia. No immune staining was found in Leydig or Sertoli cells. Mitotic activity was least in the controls and highest in the Kiss (13) group (Figures 6a–6d).

TUNEL (+)-stained cells, the spermatogonia, were very few in the controls and more abundant in the treatment groups. TUNEL (+) immune staining was not observed in the Sertoli or Leydig cells (Figures 7a–7d).

## 4. Discussion

Since 2003, research has revealed the important role of kisspeptins in initiation and timing of puberty and in regulating fertility in adulthood. Continuous administration of kisspeptins downregulates the hypothalamic–gonadal–pituitary (HPG) axis (26). Kisspeptin is a potent peptide that initiates luteinizing hormone (LH) release in male and female mice (27), in both prepubertal and adult rats (28) and in prepubertal donkeys (29). Plasma LH and testosterone levels were reported to



**Figure 2.** Number of Ki-67(+) cells. Ki-67 staining cells in the acute treatment, Kiss (1) (\*), chronic treatment Kiss (13) (\*\*)

and additional waiting period Kiss (13 + 7) (\*\*\*) groups were statistically significantly higher than in the control group (P < 0.001)

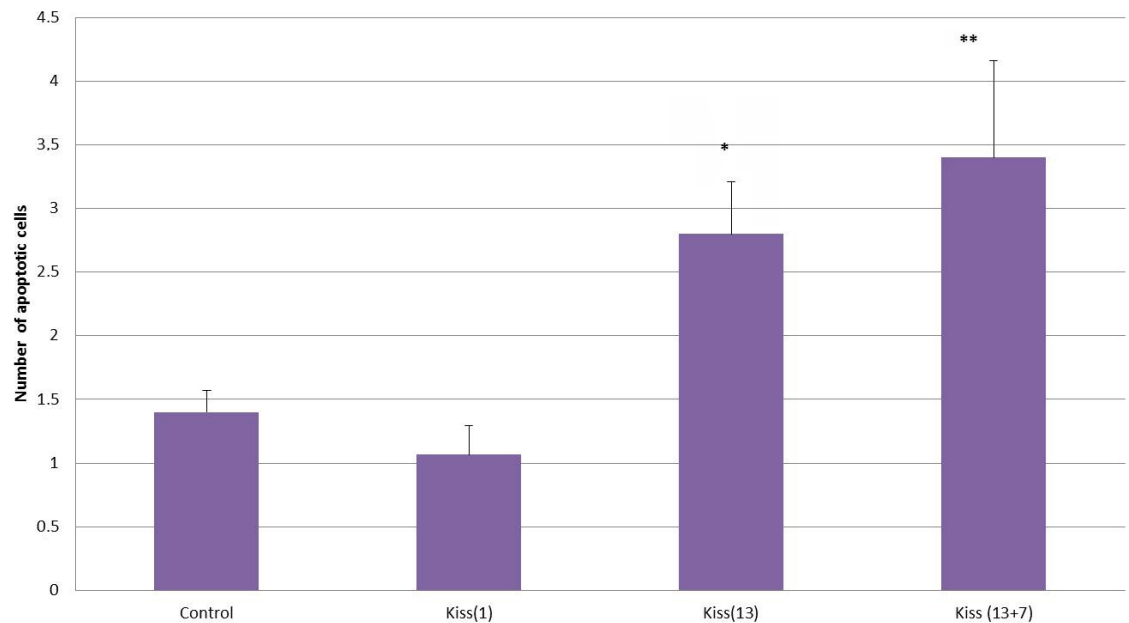
\*Control vs. Kiss (1) group, P < 0.001

\*\*Control vs. Kiss (13) group, P < 0.001

\*\*\*Control vs. Kiss (13 + 7) group, P < 0.001

#Kiss (1) vs. Kiss (13) group, P < 0.001

##Control vs. Kiss (13 + 7) group, P < 0.01



**Figure 3.** Apoptotic TUNEL (+) cell counts were significantly higher in the Kiss (13) and Kiss (13 + 7) groups compared to the controls (\*P < 0.05) and Kiss (1) (\*\*P < 0.001).

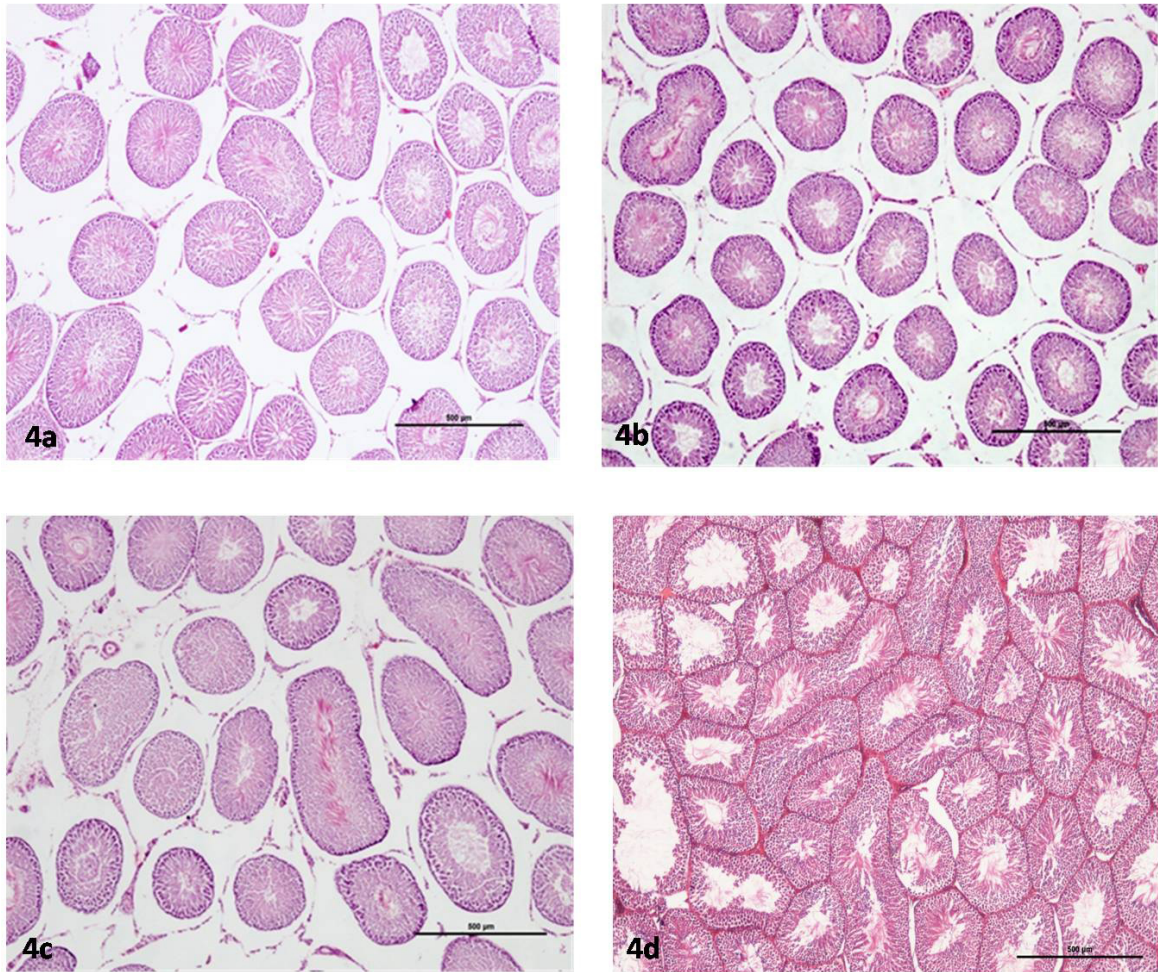
\*Control vs. Kiss (13) group, P < 0.05

\*\*\*Control vs. Kiss (13 + 7) group, P < 0.05

\*\*Kiss (1) vs. Kiss (13) group, P < 0.001

\*\*Kiss (1) vs. Kiss (13 + 7) group, P < 0.001





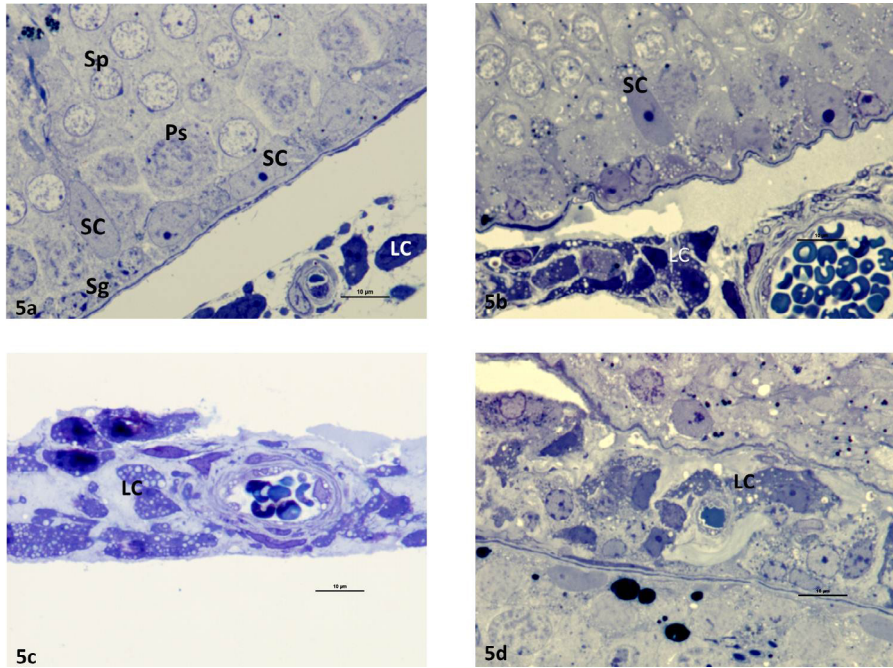
**Figure 4.** Light microscopic view of seminiferous tubules in experimental groups. The seminiferous tubules structure can be seen to resemble each other. Control (4a), Kiss (1) (4b), Kiss (13) (4c), and Kiss (13 + 7) (4d) groups. Hematoxylin and eosin stain.

be increased on the day following the first administration of Kisspeptin-54 (50 nmol/day), an effect that disappeared after the second day of treatment, when hormone levels returned to normal. This result was interpreted as being due to desensitization of the HPG axis by the continuous use of kisspeptin (12). A single dose of Kiss-54 was reported to cause ovulation in rats (30) and to significantly increase sex steroid levels in prepubertal fish (31). Gonadotropin increases were registered following a single IV or IM dose of Kiss-10 in prepubertal cats (32), sheep (33), and goats (34). It was reported that Kiss-10 given at varying doses reduces testosterone levels, a reduction that was attributed to testicular degeneration rather than a fall in LH secretion (25). However, another published study indicated that different doses of Kiss-10 were all followed by a testosterone increase within 20 min of administration (35). We observed that Kiss-10 significantly increased total testosterone plasma levels in the acute phase while

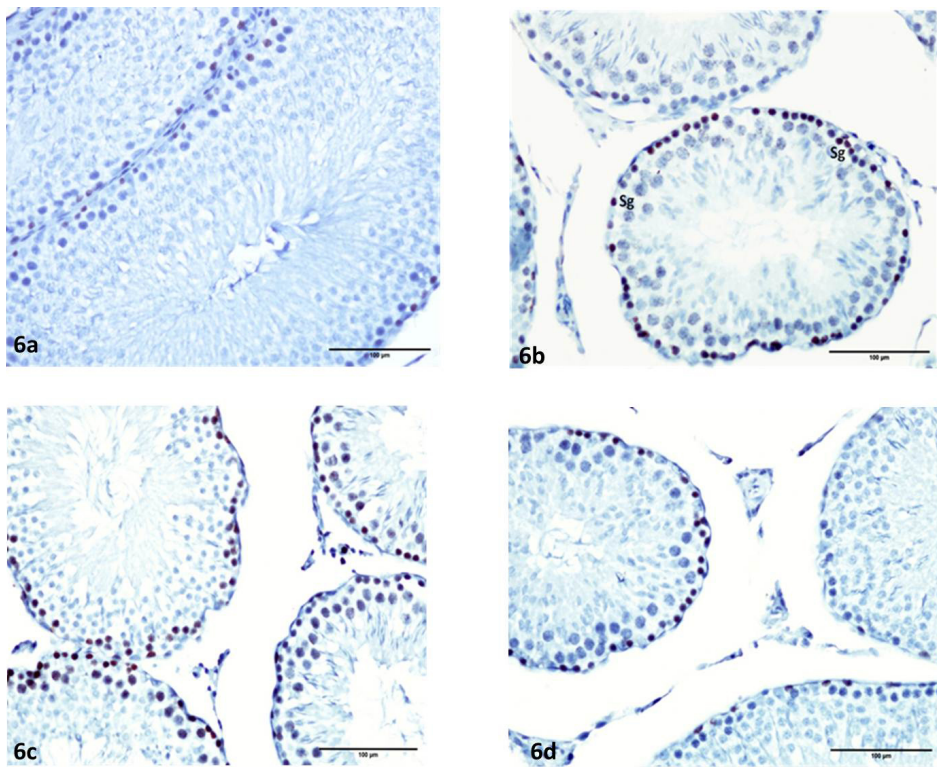
chronic administration was associated with a decrease, which continued in the additional waiting period without reaching the control levels. The increased vacuolization in the Leydig cells was interpreted as a possible enlargement of endoplasmic reticulum, consistent with active secretion. Kisspeptin mRNA, characterized in human testicular tissue (11), was not identified in rat testes (36). Wang et al. reported that Kiss-10 expression is strong in Leydig cells of 5-week- and 15-week-old mice, while it is weak in 2-week-old mice (37). Salehi et al., while observing Kisspeptin expression in Leydig cells, found no development in germ cells or Sertoli cells at any stage (38). We did not detect any positive immune staining for Kiss-1 in any group in our study.

Kisspeptins were reported to have a predominately autocrine and paracrine action in the testes (39,40). Our results are similar to those reported by Thompson et al. in that testosterone levels decreased over a 13-day

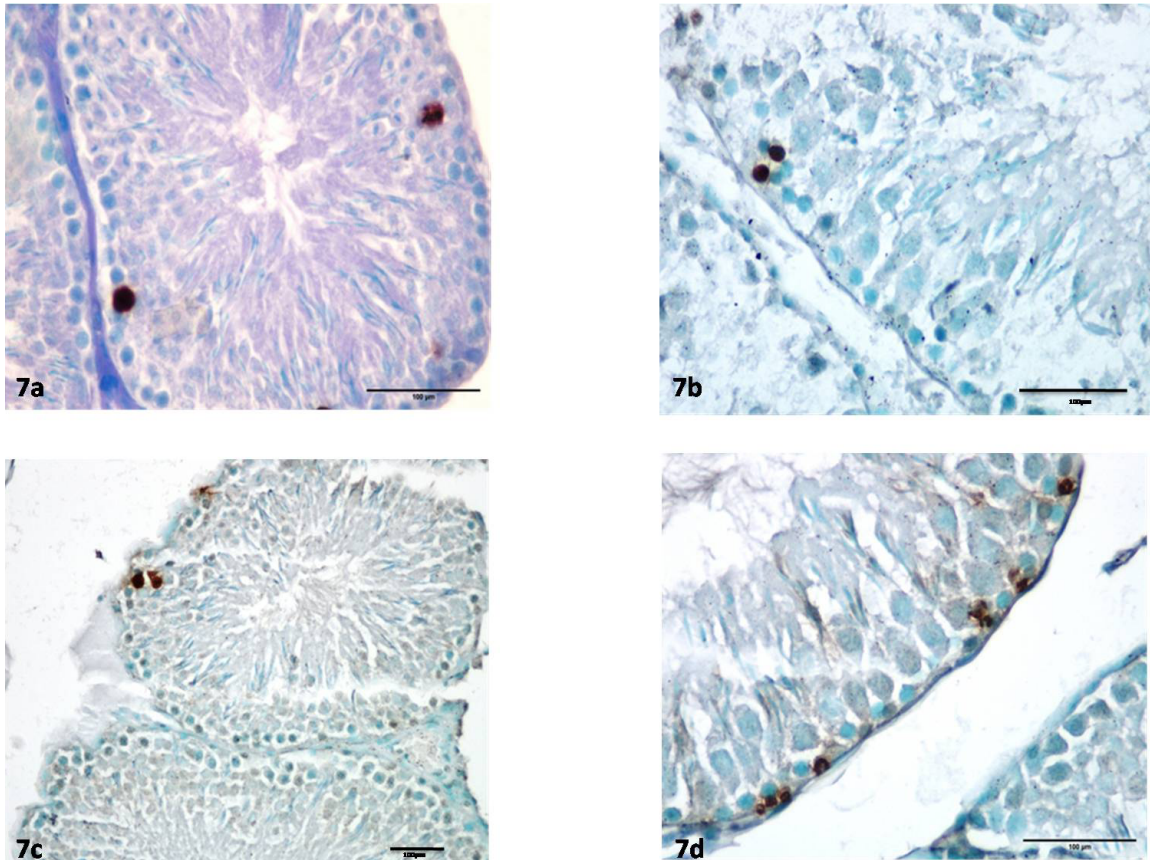




**Figure 5.** Semithin section view of experimental groups. Seen intact spermatogenic and Sertoli cells in the control group (5a). More folding basement membrane, many vacuoles in the Sertoli and Leydig cell cytoplasm are seen in the Kiss (1) group (5b). Increased vacuoles in the Leydig cells cytoplasm of the Kiss (13) group (5c) and decreased vacuoles in the Leydig cells cytoplasm of the Kiss (13 + 7) group (5d) are seen. Sp: Spermatid, Ps: Primary spermatocytes, SC: Sertoli cells, LC: Leydig cells. Toluidine blue stain.



**Figure 6.** Ki-67 immunohistochemistry stained view of groups. Control (6a), Kiss (1) (6b), Kiss (13) (6c), and Kiss (13 + 7) (6d) groups. Ki-67-positive-stained spermatogenic cells of Kiss (1) and Kiss (13) groups are increased compared to the control and Kiss (13 + 7) groups.



**Figure 7.** TUNEL-positive staining in spermatogenic cells in the Kiss (13 + 7) group is higher than the other groups. Control (7a), Kiss (1) (7b), Kiss (13) (7c), Kiss (13 + 7) (7d) groups.

treatment period and adult male rats were used (12,39). The increase seen in Ki-67-stained spermatogonia within the seminiferous tubules in our study was interpreted as related to the increase in testosterone levels; their proliferation increased with both acute and chronic treatment and decreased in the additional waiting period without, however, reaching control levels. This finding was attributed to the reversibility of Kiss-10 action.

A twice-weekly high-dose (250 ng/g) Kiss-10 treatment for 7 weeks increased the GSI of prepubertal white bass, while no significant change could be detected in striped bass (41). Another report indicated that Kiss-15 increases GSI in prepubertal fish (31). Reports vary according to subject type and physiological development stages. Varying stimuli have been determined for different modes of administration (central or systemic) and different physiological stages (42,43). Kisspeptin-54, 50 nmol daily for 13 days, led to testicular degeneration in adult rats (12,39). Application of Kiss-10 led to seminiferous tubule degeneration in prepubertal rats; cellular degeneration was seen to increase along with increasing dose. Cytoplasmic vacuolization was demonstrated by light and electron

microscopy in Sertoli cells and spermatogonia (25). In our study, no obvious degeneration was observed in seminiferous tubule cells even though the dose administered amounted to 50 nmol; in contrast, the increased testosterone level increased spermatogenesis. While apoptotic spermatogonia were identified in our study, no apoptosis was seen in the Sertoli or Leydig cells. Semithin sections showed a large degree of cytoplasmic vacuolization in the Sertoli cells and spermatogonia. Vacuolization decreased in the cells of animals in the groups subjected to chronic treatment and in those with the additional waiting period. Apoptotic cell counts were low in the acute treatment animals and increased in the two other treatment groups. The increase in Ki-67 staining cells in acute treatment animals paralleled the increase in testosterone level. However, proliferation increased in chronic treatment animals despite the testosterone decrease. The increased apoptotic cell count was possibly offsetting the increase in total cell count that occurred over the same period. Testosterone levels continued to decrease, apoptosis counts increased, and proliferation of spermatogenic cells decreased over the 7-day waiting period following chronic administration.



To summarize, acute or chronic administration of 50 nmol/kg kisspeptin-10 was not observed to cause degeneration in the rat seminiferous tubules, while it increased cellular proliferation; the interruption of kisspeptin was associated with a decrease in proliferation and testosterone levels and with an increase in apoptosis. Kiss-10 was seen to exert its effects through testosterone

secretion; these effects were reversible. The increased proliferation in spermatogenic cells may lead to new developments in the treatment of infertility.

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