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Original Article

The treatment effect of novel hGHRH homodimer to male infertility hamster

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ABSTRACT Extra-hypothalamic growth hormone-releasing hormone (GHRH) plays an important role in reproduction. To study the treatment effect of *Grin* (a novel hGHRH homodimer), the infertility models of 85 male Chinese hamsters were established by intraperitoneally injecting 20 mg/kg of cyclophosphamide once in a week for 5 weeks and the treatment with *Grin* or human menopausal gonadotropin (hMG) as positive control was evaluated by performing a 3-week mating experiment. 2-8 mg/kg of *Grin* and 200 U/kg of hMG showed similar effect and different pathological characteristics. Compared to the single cyclophosphamide group (0%), the pregnancy rates (H-, M-, L-*Grin* 26.7, 30.8, 31.3%, and hMG 31.3%) showed significant difference, but there was no difference between the hMG and *Grin* groups. The single cyclophosphamide group presented loose tubules with pathologic vacuoles and significant TUNEL positive cells. *Grin* induced less weight of body or testis, compactly aligned tubules with little intra-lumens, whereas hMG caused more weight of body or testis, enlarging tubules with annular clearance. *Grin* presented a dose-dependent manner or cell differentiation-dependentincrease in testicular GHRH receptor, and did not impact the levels of blood and testicular GH, testosterone. *Grin* promotes fertility by proliferating and differentiating primitive cells through up-regulating testicular GHRH receptor without triggering GH secretion, which might solve the etiology of oligoasthenozoospermia.

Author contributions: S.S.T. took responsibilities of all the designs, method applications, and grant application in the study. X.D.Z. conceived of the study, supported in finance, participated in its design and coordination, carried out ELISA assay, and checked the English writing of the manuscript. X.Y.G. raised the hamsters, participated in the preparation of hamster model, participated in the ELISA, fluorescent staining, and Western blot assays, and searched for the references. J.X.T. participated in the primary design and coordination of the manuscript and ELISA assays, and checked the English writing of the manuscript. J.H.Z. participated in the preparation of tissue section and the design of the study. L.N.Y. performed fluorescent staining, and Western blot assays. Y.X.D. participated in the protein sequence alignment and searched for the references. T.L. participated in the writing of the primary grant in the research. All the authors read and approved the final manuscript.

INTRODUCTION

Infertility affects approximate 15% of couples and the male factors covers 20-50% of cases with the reduction of sperm quantity or/and quality [1].

The medical treatment of infertility is divided into two main categories: specific and non-specific. The specific treatments are used for certain etiologies such as hypogonadotropic hypogonadism, male accessory gland infection, retrograde ejaculation, and positive anti-sperm antibody. These endocrine therapies include gonadotropins, androgens, anti-estrogens, and aromatase inhibitors [2]. Non-specific treatment, also known as empirical medical treatment (EMT), divided into two categories, hormonal treatment [3] and antioxidant supplementation [4].

Fertility has been related with the central GHRH-growth hormone (GH)-IGFs endocrinal axis. Pituitary GH is involved in a wide array of reproductive functions in mammals such as sexual differentiation, pubertal maturation, gonad steroidogenesis, gametogenesis, and ovulation as well as pregnancy and lactation [5]. Chubb [6] reported that pituitary GH deficiency leads to a significant reduction of male sexual behavior or fertility. Bartke et al. [7] reported that there were markedly reproductive deficits in GH receptor-knock-out or GH transgenic mice. Although most of the GH transgenic male mice are fertile, their fertility tends to be quantitatively reduced and plasma testosterone levels were not altered [8]. Pituitary GH promotes sperm motility and longevity [9]. Debeljuk et al. [10] reported that the plasma testosterone and luteinizing hormone (LH) levels are normal in transgenic metallothionein-I/hGHRH mice, but the response to gonadotropin-releasing hormone (GnRH) was significantly less in the transgenic mice.

Some publications concerned the autocrine/paracrine GHRH and GH signals in the reproductive system. Berry et al. [11] reported that extra-hypothalamic GHRH-like mRNA and immune-reactive peptide presented in rat testis and placenta, suggesting that testis and placenta are extra-hypothalamic sites of expression of GHRH gene. Martínez-Moreno et al. [12] reported that GHRH co-localized with GH in the germinal epithelium and in interstitial zones within the chicken testes. GH affects the proliferation and differentiation functions of chicken reproductive tissues [13].

These reports clearly reflected the relationship of the development of reproductive tract with pituitary GH. Although testicular GHRH, GHRH receptor, and GH signal molecules were discovered, their roles in fertility and the relationship with central GHRH-GH axis are not clearly known.

In our previous publication [14], *Grin* peptide (also known as 2F) showed the strongest and long-lasting *in vitro* effect on rat GH release and similar species-specificity compared to natural hGHRH(1-44)NH_2. Up to now no publication about hGHRH agonist was reported in the pharmacodynamics of infertility. The treatment effect of *Grin* on the infertility models of male hamsters were reported in the paper.

METHODS

Synthesis and activity of *Grin*

Grin monomer and FITC-labeled hGHRH(1-44)NH_2 peptides were synthesized in the solid phase polypeptidesynthesis (*China Peptides Co., Ltd,* China). Grinwas synthesized in a special dimerization protocol [14]. The amino acid sequences of these hGHRH peptides were referred to Table 1.

Animals

Kunming mice (female, 18-20 g) were purchased from the Animal Center of Guangzhou University of Traditional Chinese Medicine (China) for the maximal tolerated dose assay. Chinese hamsters (5-week old male and female) were purchased from Sichuan Dasuo Animal Center (China) for male infertility models. All experiments were performed in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals. The animals were acclimated to a light-dark cycle of 12:12 h by housing them in individual cages in the temperature of 26±1°C. The studies were approved by the Animal Centers on animal care. After all hamsters used were raised for 2 days, they were randomly grouped.

Infertility model of male Chinese hamster

The infertility model of male hamster was established by referring to the Tripathi DN method [15]. Briefly, according to an indifferent body weight, 85 male Chinese hamsters were divided into five groups [*Grin* groups (high, middle, and low dose), hMG

Table 1. *Grin* and hGHRH peptide with or without *FITC*-labeled K

Peptide	Amino acid sequence
hGHRH(1-44)NH_2	(H)YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL-NH_2(OH)
FITC-labeled hGHRH(1-44)NH_2	(H)YADAIFTNSYRKVLGQLSARK-(FITC)LLQDIMSRQQGESNQERGARARL-NH_2(OH)
Grin Mononer	(H)PADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARLGGC-OH(OH)
Grin	(H)PADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARLGGC(OH)-C(OH)GGLRARAGREQNSEGQQ RSMIDQLLKRASLQGLVKRYSNTFIADAP(H)

group, and single cyclophosphamide (CPA) group] (n=17). The hamster infertility models were established by injecting (*ip*) CPA (20 mg/kg, Ratification No.12032925, *Jiangsu Henrui Pharmaceutical Co.* China) once a week for 5 weeks.

Experimental design

The detailed design was explained in Fig. 1. The hamster infertility models were established in the experimental 1-5th week. After the fourth CPA injection, *Grin* (2, 4, 8 mg/kg) or hMG (200 U/kg or 46.2 mg/kg, FSH:LH=1:1, Ratification No.120506, *Livzon Pharmaceutical Group Co., Ltd.* China) as positive drug was injected (*im*) twice in a week in the hind leg muscle until the end of the ten-week experiment. In the experimental 6-8th week, all the male hamster models mated with normal female hamsters (10 weeks age) for 3 weeks (1:1 mating ratio). In the last two weeks, the male models and female hamsters were separated alone to feed. All the hamsters were sacrificed after the experiment was finished. The blood, testes, and livers from the male hamsters were collected and the organs were weighed. The pregnant female hamsters were recorded by anatomizing the ovarian ducts. The daily activities of the hamster models were observed and they were weighed per week. The pregnancy rate was calculated according to obvious feta and new hamster baby.

Blood biochemical assay

GH and testosterone were measured in the sera of the hamster models. Serum GH was measured in a rat GH ELISA kit (*Millipore Co.*, USA) and Thermo Multiskan MK3microplate reader (*Thermo Fisher Scientific*, USA). Serum testosteronewas measured in the Elecsys Testosterone II kit (*Roche Diagnostics GmbH*, Germany) and Cobase 411 Automatic Electrochemiluminescence Immunoassay System (*Roche Diagnostics GmbH*, Germany), respectively.

Morphology analysis of testis tissue

Five micrometer-thick and formalin-fixed hamster testis tissue sections were performed in H-E staining. The morphology of seminiferous tubules was observed. The average size of seminiferous tubule was obtained by measuring the longitudinal and horizontal axes of 45 tubules and calculating their areas (μm^2, ellipsoid area=π×longitudinal axis×horizontal axis/4) in the H-E staining section. For the H-E staining, three unilateral testis tissues per group were used.

Fluorescent staining analysis of testis tissue

For the fluorescent staining, three unilateral testis samples per group were used.

FITC-labeled hGHRH(1-44)NH_2fluorescent staining: The fluorescent staining protocol was referred to the method of Zhou et al. [15]. Briefly the slides were soaked in a 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer 3 times for 15 min. 200 μl of HEPES buffer containing 76±1 fluorescent intensity of FITC-labeled hGHRH(1-44)NH_2 peptide was dropped on each tissue section to incubate for 1 h at 30°C. After the slides were washed twice for 10 min in the HEPES buffer, DAPI water solution (0.01 g/ml) was dropped in tissue section for 15 min at room temperature. The staining was finished after the sections were washed twice for 10 min in PBS and then mounted with a fluorescent mounting medium (*Shanghai Biaoben Model Co.*, China). The staining was performed in a dark environment. The location and distribution of membrane GHRH receptor in the testicular cellswas examined and documented with an epifluorescence microscope (Carlzeiss micro-imaging Gmbh 37081, *Carl Zeiss Microscopy GmbH Inc.*, Germany).

Fluorescentimmunohistochemistry (*IHC*) staining: The testicular GHRH receptor or GH fluorescent *IHC* was performed in our previous protocol [16]. Briefly the slides were soaked in a

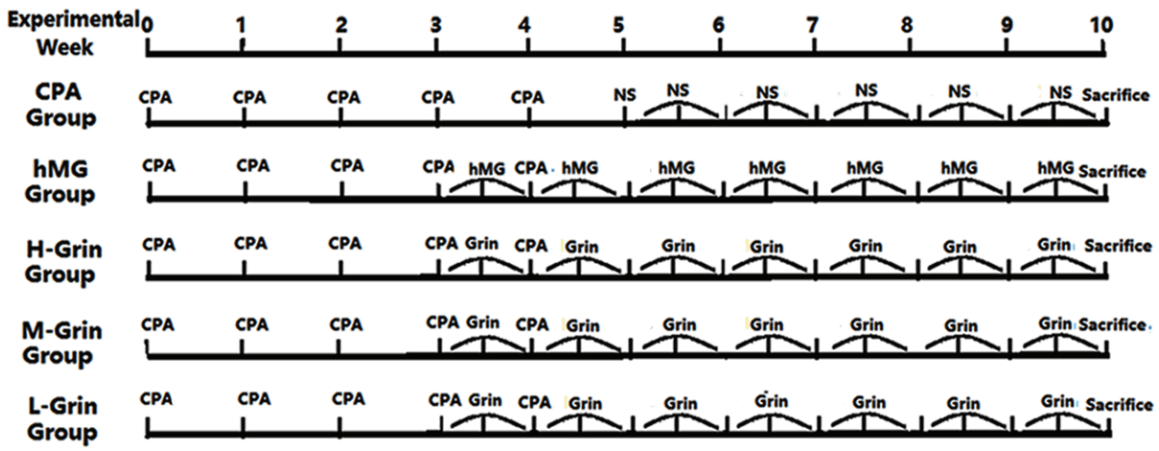

Fig. 1. Experimental design schema. The single CPA group was administered CPA *i.p.* once in a week in the former 5 weeks and saline *i.m.* twice in a week in the latter 5 weeks. The hMG or *Grin* groups were administered CPA *i.p.* once in a week in the former 5 weeks and added hMG or *Grin i.m.* twice in a week from the fourth week to the tenth one. All the animals were sacrificed after the tenth week.

cool PBS buffer 2 times for 20 min. A 30-min pre-incubation was completed in a blocking solution containing 2% horse serum, 0.1% BSA, and PBS. A rabbit anti-GHRH receptor polyclonal antibody (1:500 diluted, Cat No.ab76263, *Abcam Co.* USA) or anti-GHpolyclonal antibody (1:500 diluted, Cat No.ab200726, *Abcam Co.*) were incubated at 4°C overnight. The next day, after washed three times with PBS for 15 min, they were incubated for 2 h with an appropriate secondary antibody (1:380 diluted in the blocking solution, *Vector Co.*, USA). After the slides were washed twice for 10 min in PBS buffer, DAPI water solution was dropped in tissue section for 15 min at room temperature. The staining was finished after the sections were washed twice for 10 min and then mounted with the fluorescent mounting medium.

TUNEL assay of testicular tissue: The terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay was used to study the DNA fragmentation in the testicular tissue sections (*Biotool LLC*, USA) according to the manufacturer's instructions. Briefly, the testicular tissue sections were added 50 μl of the enzyme-substrate mixture (1:9). After wrapped with tinfoil to keep in darkness and subsequently incubated for 1 h at 37°C in an incubator, the slides were washed three times with PBS for 15 min. Total cells were observed under epifluorescence microscope and images were documented in the software IPP (image-pro-plus). The total cell population and TUNEL positive cells in image were counted manually. In the cell counting, 5 pictures each section were randomly taken and three sections (n=3) each group were used. TUNEL positive cells were expressed as percentage of total cells.

Western blot analysis

Tissue protein extraction was referred to our previous publication [16]. Protein (30 μg per gel lane) was in turn fractionated by SDS-PAGE (12%), transferred to a PVDF membrane (*Millipore Co.*) (175 mA for 2.5 h), and probed with an appropriate antibody. The antibody bound to the membrane was detected with the enzyme-catalyzed chemiluminescent (ECL) method (*Beyotime Institute of Biotechnology*). A rabbit anti-GHRH receptor polyclonal antibody (1:1000 diluted, Cat No.ab76263, *Abcam Co.*), GH polyclonal antibody (1:1000 diluted, Cat No.ab200726, *Abcam Co.*), and mouse anti-β-Actin antibody ($IgG_{2\alpha}$, *Beyotime Institute of Biotechnology*) as control were used in the Western blot experiments. All appropriate secondary antibodies were purchased from *Beyotime Institute of Biotechnology*.

Evaluation of maximum tolerated dose

According to the *Chinese Pharmacopoeia* 2015, the maximum tolerated dose (MTD) assay was performed in 12 Kunming mice by intravenous injecting a 0.5 ml volume containing maximum dose of *Grin* once and following 14-day observation.

Statistical analysis

These data are present as mean±SD. Statistical evaluationwas performed by the Student's T test for weight and TUNEL results, or X^2 test (Fisher's exact probabilities) for pregnancy rates. Significant p value (≤0.05 or 0.01) is shown *vs*. control group.

RESULTS

Change of body weight

All the hamsters which were injected CPA grew slower during the modeling period and there was no statistical significance in body weight between experimental groups. From the 6th week on, the hamsters in the single CPA group grew faster. Compared to

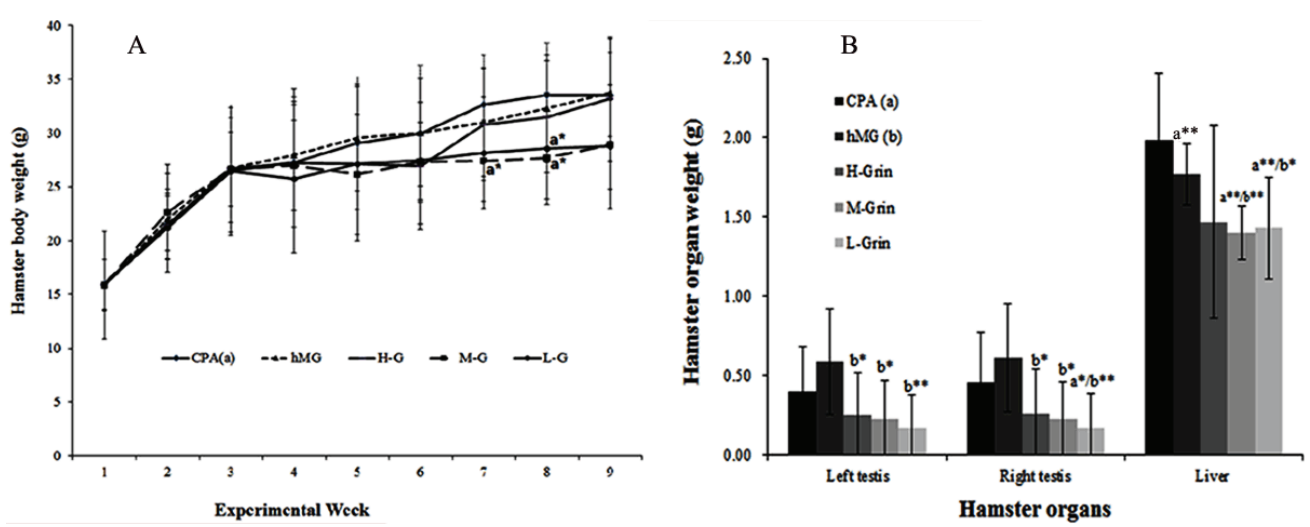

Fig. 2. Changes of body weights or organ weights of the hamster models. (A) Body weight analysis, *p<0.05 *vs.* single CPA group, *T* test; (B) Organ weight analysis, *p<0.05, **p<0.01, *a vs.* single CPA, *b vs.* hMG group, *T* test.

the single CPAgroup, the seventh- and eighth-week of hamsters in the M-*Grin* group or the eighth-week of ones in the L-*Grin* group grew slower (p<0.05) (Fig. 2A). After CPA injection, the hamster models occurred in certain mortality rate because of toxicity, so 15, 16, 15, 13, or 16 animals in the single CPA, hMG, H-, M-, or L-*Grin* group survived.

Change of organ weight

The *Grin* groups showed lighter testes than the hMG group, or the L-*Grin* group did lighter right testes than the single CPA group (p<0.05). The average liver weight in the M- or L-*Grin* group was less than that in the single CPA or hMG group (p<0.01 or 0.05). Compared to the single CPA group, the average liver weight in the hMG group obviously decreased (p<0.05) (Fig. 2B).

Pregnancy rate of the male hamster models

Compared to the single CPA group (0%), the pregnancy rates (H-, M-, L-*Grin* 26.7, 30.8, 31.3%, and hMG 31.3%) showed significant differences (p<0.05) (Table 2), but there was no difference between hMG and each *Grin* group (p>0.05). The hMG or H-*Grin* group had 12.5% or 13.3% of birth rates.

Morphology of hamster testicular tissue

Pathological morphology: From the H-E staining (Fig. 3), the loose tubules in the single CPA group presented obvious pathological vacuoles and swelling. 5-8 layers of chaotically aligned epithelial cells and obvious annular clearances occurred in the enlarging tubules of the hMG group. Some tubule walls were broken and cells moved out. In the *Grin* groups the compactly aligned tubules with 8-12 layers of epithelial cells showed obvious proliferation of cells. The proliferating epithelial cells spread to the center and lots of germs fill in the intra-lumens of tubules. The pathological vacuoles were not observed in the *Grin* groups.

Size of seminiferous tubule: From the H-E staining, the ellipsoid tubules in the *Grin* groups showed a dose-dependent enlargement (Table 3). The H-*Grin* or hMG significantly induced larger tubules than the M- and L-*Grin* (p<0.05 or 0.01).

TUNEL results: The single CPA, *Grin*, or hMG group showed obviously positive staining to primitive germinal cells, specially spermatogonia and spermatocytes, because of CPA toxicity (Fig. 4). Compared to the single CPA group, the hMG or *Grin* groups showed far less TUNEL positive rate (p<0.001). Compared to the hMG group, the H- or L-*Grin* group showed less TUNEL positive rate (p<0.05).

Analysis of testicular GHRH receptor

By using FITC-labeled hGHRH(1-44)NH_2 peptide or an anti-GHRH receptor antibody to stain testicular tissue section, the location and distribution of GHRH receptor in the testicular cells of the hamster were observed (Figs. 5A, 6A). Compared to that of the single CPA or hMG group, more GHRH receptor expression presented in the testicular cells of the *Grin* groups on dose-dependent manner (p<0.05 or 0.01) (Figs. 5B, 6B). GHRH receptor

Table 2. Pregnancy rate of the male hamster models

Groups	N	Pregnancy rate (%)	Birth rate (%)	Total pregnant rate (%)
Single CPA	15	0.0	0.0	0
hMG	16	18.8	12.5	31.3*
H-*Grin*	15	13.4	13.3	26.7*
M-*Grin*	13	30.8	0.0	30.8*
L-*Grin*	16	31.3	0.0	31.3*

*p<0.05 *vs.* single CPA group, X^2 test.

Table 3. Sizes of the seminiferous tubules in the testes of the male hamsters (X±SD)

Groups	Seminiferous tubule		
	Shape	Area (μm^2)	*N*
Single CPA	Ellipsoid	111506± 28802	45
hMG	Ellipsoid	121328±30069a*, *b***	45
H-*Grin*	Ellipsoid	121789±24366a*, *b***	45
M-*Grin*(a)	Ellipsoid	108942±20777	45
L-*Grin*(b)	Ellipsoid	103239±30761	45

*p<0.05, **p<0.01; *a vs.* M-*Grin* group, *b vs.* L-*Grin* group; *T* test.

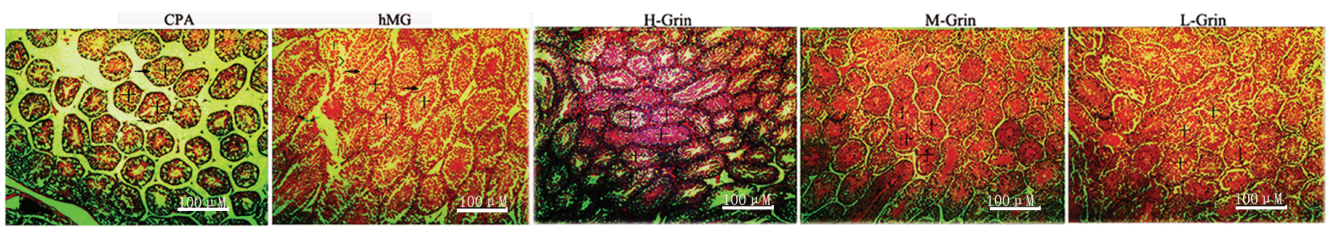

Fig. 3. H-E staining of the testicular tissues [(Mag.4×10 times, bar 100 μm, eosin (pink cytoplasm) and hematoxylin (blue nucleus)]. "十" refers to the intralumen in seminiferous tubule. CPA: cyclophosphamide group, "→" points at pathological vacuole; hMG: human menopausal gonadotropin group, "→" points at the annular clearance, ">" points at the broken wall of seminiferous tubule; H-*Grin*: high *Grin* dose; M-*Grin*: middle *Grin* dose. L-*Grin*: low *Grin* dose.

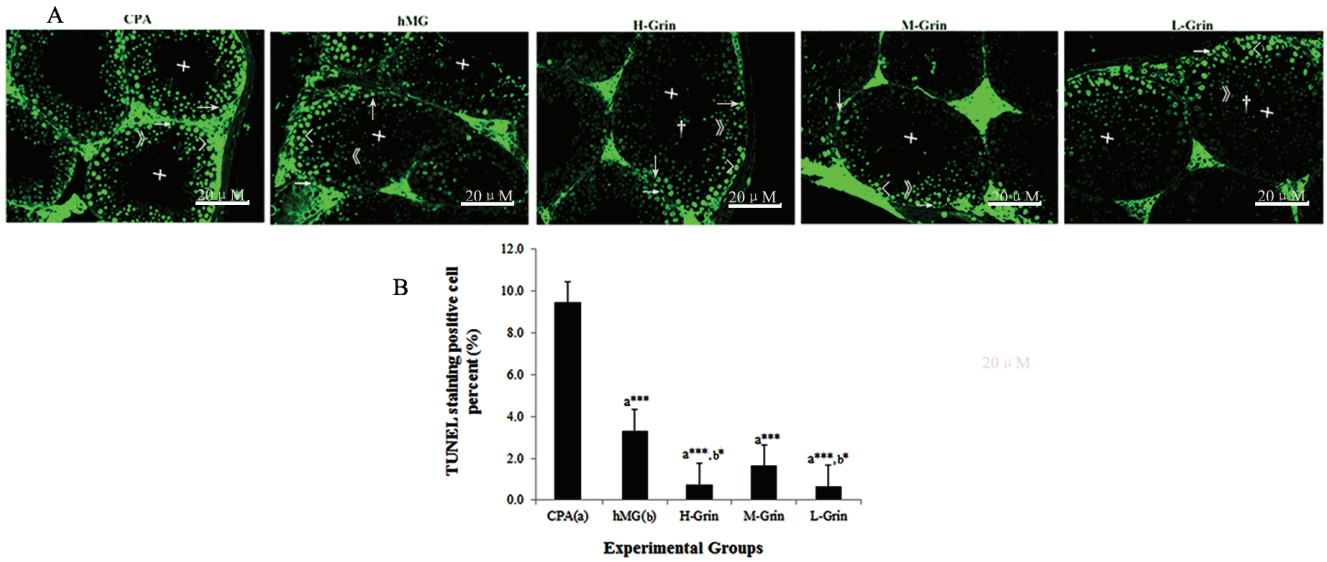

Fig. 4. Effect of CPA-induced DNA damage in testicular cells. (A) TUNEL staining (Mag.20×10 times, bar 20 μm, yellow-green TUNEL positive cells). "┼" refers to the intra-lumen, "→" points at spermatogonium, ">" points at spermatocyte, ">>" points at spermoblast, "†"points at sperm nuclei, and "‡" refers to sperm body and tail; (B) Quantitative estimation of TUNEL-positive cells. p<0.05* or 0.01**, 0.001*** *vs.* the single CPA (a) or hMG (b) group.

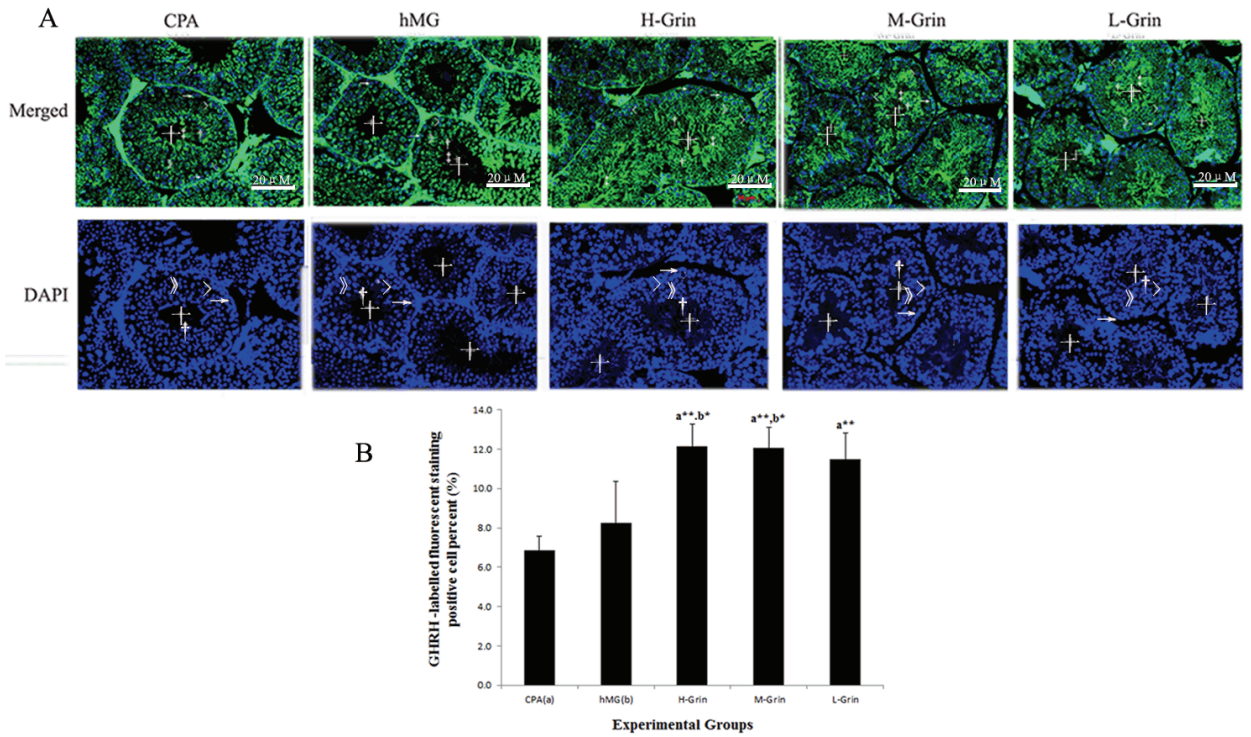

Fig. 5. Fluorescent staining of FITC-hGHRH(1-44)NH_2 peptide in the testis tissues. (A) Picture merged (Mag.20×10 times, bar 20 μm, GHRH receptor (yellow green) and DAPI (blue nucleus). Symbols are the same as those in Fig. 4; (B) Quantitative estimation of fluorescent staining positive cells of FITC-hGHRH(1-44)NH_2 peptide. p<0.05* or 0.01** *vs.* the single CPA (a) or hMG (b) group.

obviously distributes on the cell membranes of spermatogonia, spermatocytes, spermoblasts, and sperm heads. With the differential maturation of the spermatogonia and spermatocytes, the expression of GHRH receptor gradually increased. The staining of the fluorescent peptide and anti-GHRH receptor antibody showed similar results.

Western blot analysis showed that the *Grin* groups presented more testicular GHRH receptor expression on dose-dependent manner compared to the single CPA group (p<0.01 or 0.05) (Fig. 8A). The results are similar with those of the fluorescent staining.

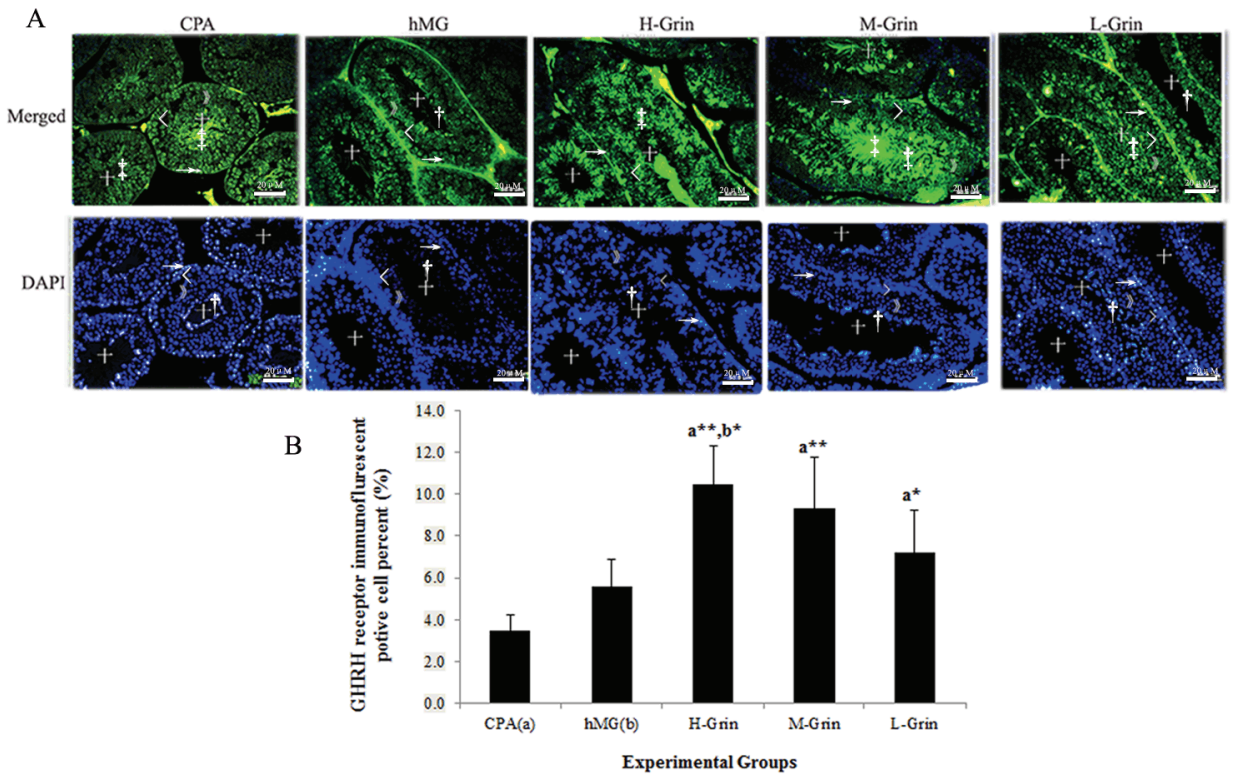

Fig. 6. Immunofluorescent staining of GHRH receptor protein in testicular tissues. (A) Picture merged [Mag.20×10 times, bar 20 μm, FITC (yellow GHRH receptor) and DAPI (blue nucleus)]. Symbols are the same as those in Fig. 4; (B) Quantitative estimation of positive cells of GHRH receptor immunofluorescent staining. p<0.05* or 0.01** *vs.* the single CPA (a), or hMG (b) group.

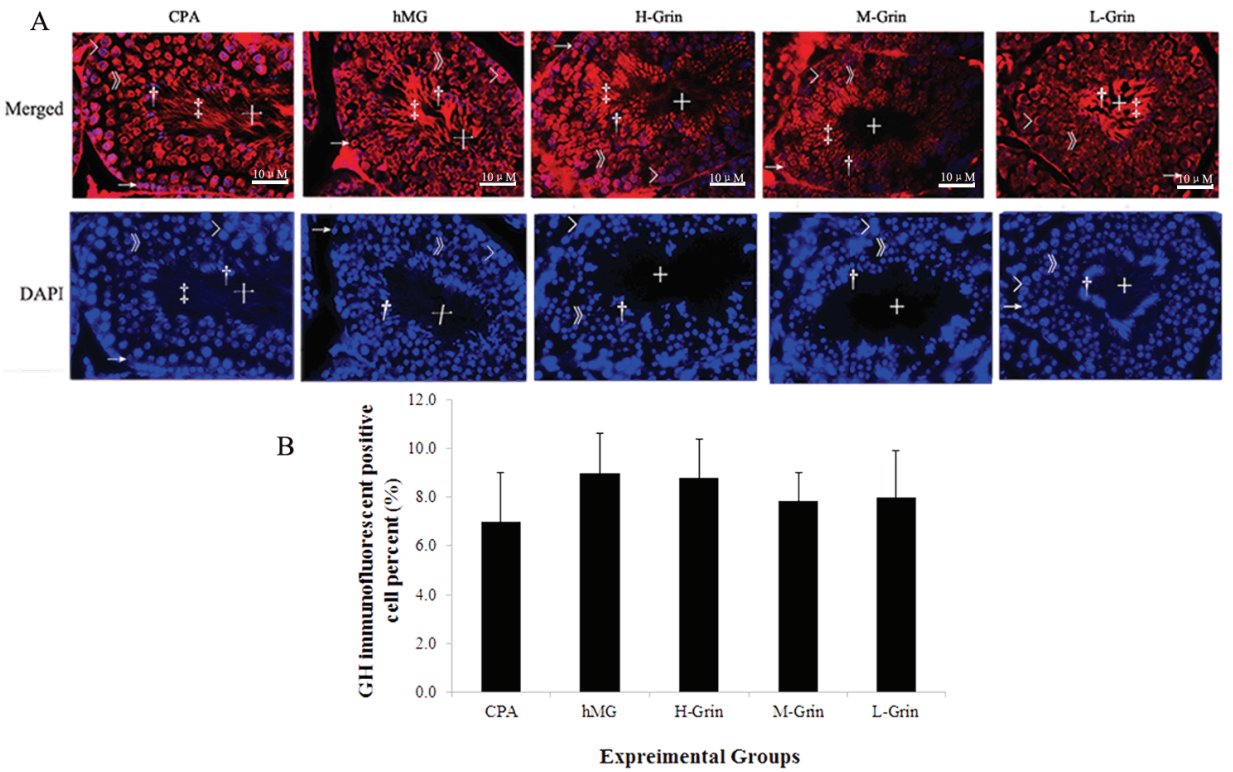

Fig. 7. Immunofluorescent staining of GH protein in testicular tissues. (A) Picture merged [Mag.40×10 times, bar 10 μm, Cy3 (red GH) and DAPI (blue nucleus)]. Symbols are the same as those in Fig. 4; (B) Quantitative estimation of positive cells of GH immunofluorescent staining. *p<0.05 or **p<0.01 *vs.* the single CPA (a) or hMG (b) group.

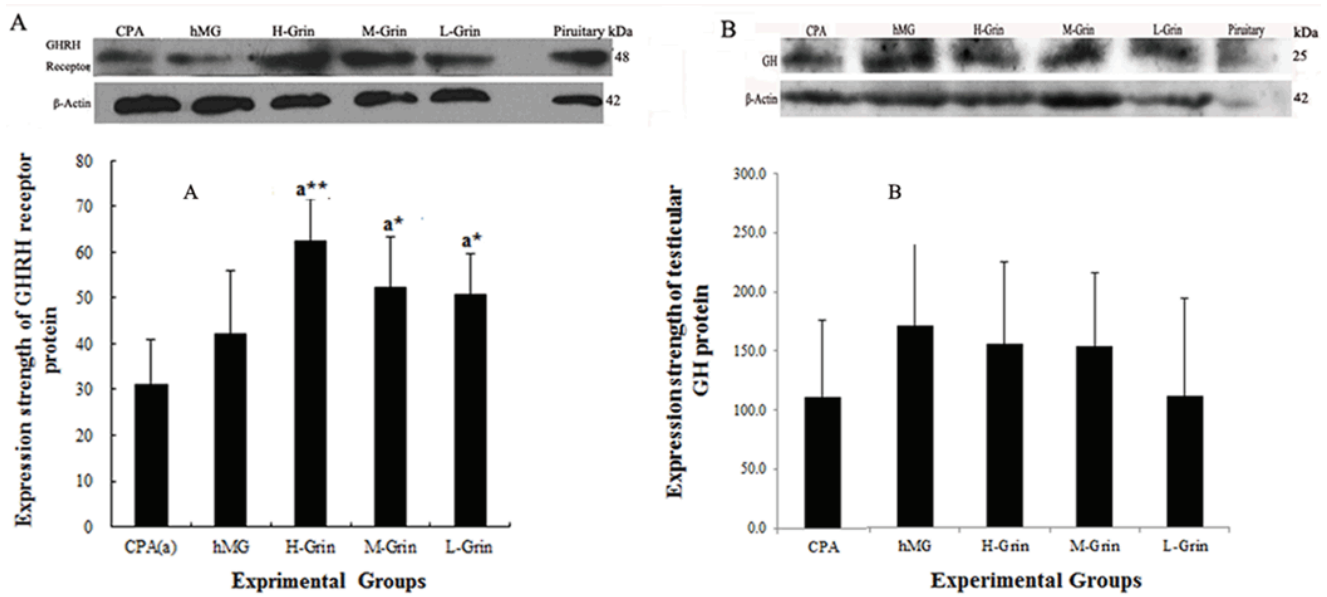

Fig. 8. Western blotting analysis of testicular GHRH receptor or GH protein. (A) GHRH receptor (47 kDa) and β-Actin (42 kDa) protein were marked. *p<0.05 or **p<0.01 *vs*. the single CPA group. (B) GH (24 kDa) and β-Actin (42 kDa) protein were marked.

Analysis of GH protein

By using GH ELISA kit, the serum GH in the hamster models was analyzed (data not shown). By using fluorescent *IHC* staining (Figs. 7A and B) or Western blot method (Fig. 8B), the GH expression in the hamster testis was analyzed. All the results showed that there was not significant in GH protein between experimental groups.

Toxicity result

2.8, 4.0, or 4.8 g/kg dose of *Grin* in 0.5 ml volume was injected *iv* in 12 mice, respectively. The doses of 2.8 and 4.0 g/kg did not cause death in 24 h. During the observation period, no abnormal observations were discovered regarding the behavior, diet, fur color, and death of the mice. 4.8 g/kg of *Grin* leaded to 90% death rates. Hence, the maximum tolerated dose of *Grin* was determined to be 4.0 g/kg/0.5 ml (Table 4).

Table 4. Maximal tolerance dose analysis of *Grin* for mice

Sample	Administered approach	Dose (g/kg)	Death rate (%)	N
Grin	i.v.	2.8	0	12
Grin	i.v.	4.0	0	12
Grin	i.v.	4.8	90	12

DISCUSSION

The effect of extra-hypothalamic GHRH might reflect the autocrine/paracrine GHRH receptor mediated functions.

Human hypothalamic GHRH has 93%, 71%, or 61% of identity with that from the Chinese hamster, rat, or mouse species (Table 5). The difference of the C-terminal four amino acids in GHRH between Chinese hamster and human lead to less than 2% of the activity difference. Although GHRH species specificity is not too strict [17], we still chose Chinese hamster (Cetulus griseus) as infertile animal models so that the species difference of GHRH molecule was overcome. Moreover Chinese hamster has an identity of 97, 97, or 67% in GH with mouse, rat, or human, so rat GH ELISA kit is easy to detect the hamster GH level. Chinese hamster is small like mouse, but its testis weight nearly covers 2% of body weight, so Chinese hamster is very suitable to be prepared as model of male infertility.

Cyclophosphamide is a regular clinic drug against some tumors. It is transformed into aldehyde phosphoramide by hepatic microsomal oxidase. Aldehyde phosphoramide is further degraded into amide nitrogen mustard and acrolein with alkylating function. By combining with loose DNA molecules in chromosome, DNA synthesis is inhibited in mitotic cells, *i.e.* spermatogonia and apermatocytes etc. But cyclophosphamide has weaker toxicity to quiescent cells, *i.e.* Leydig and Sertoli cells because the tightly wound chromatins in these cells are not combined easily by the alkylating agents. The infertility animal models with oligospermia or azoospermia are prepared to be based on the CPA toxicity to mitotic cells. Our preliminary research showed when CPA dose were no more than 200 mg/kg, Kunming mice kept 100% of survival rates, whereas the Chinese hamsters had 88.2% survival rates at 20 mg/kg.

20 mg/kg-CPA-induced hamster models did not significantly alter in androgen level both in the 5-week modeling period and in

Table 5. Identities of GHRH protein sequences from different species

Species	Amino acid sequence	Identity (%; vs hGHRH)
Homo sapiens	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGAR	100
Cricetulus griseus (hamster)	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEPGARVRL	93
Sus crofa (porcine)	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGARVRL	93
Mesocricetus auratus	YADAIFTSSYRKVLGQLSARKLLQDIMSRQQGERNQEQGPRVRL	89
Bos taurus (bovine)	YADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGAKVRL	89
Capra hircus (caprine)	YADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGAKVRL	86
Cavia porcellus	YADAIFTNSYRRVLGQLFARKVVQDIANRPQEEGNQEQEARMRL	73
Rattus norvegicus (rat)	HADAIFTSSYRRILGQLYARKLLHEIMNRQQGERNQEQRSR	71
Mus musculus (mouse)	HVDAIFTTNYRKLLSQLYARKVIQDIMNK-QGERIQEQRAR	61

the 5-week treatment period (data not shown), suggesting that the CPA dose did not impact the function of Leydig cells. In our preliminary research, when 10 hamsters (n=10) in each group were set (the minimal 6 hamsters were survived), the pregnancy rates (*Grin* 12.5-22.2% or hMG 22.2%) showed no significant difference compared to that (0%) of the single CPA group, suggesting that the animal models (n=10) was insufficient, so 17 hamsters in each group were used in the research. Because all the hamsters had been grouped before the animal experiment started, the final animals in each group were not uniformly distributed due to the different CPA tolerance. The final survivals of 13-16 hamsters suggest that the CPA dose was moderate to male hamsters. The higher androgen levels and more epithelial cells and clearance in the hMG group suggest that the CPA dose did not impact the functions of Sertoli and Leydig cells. In the TUNEL pictures, most of the TUNEL-positive cells are spermatogonia and spermatocytes, suggesting that the mitotic cells are more sensitive to CPA than the quiescent cells. In one word, the CPA dose only temporarily inhibited the proliferation of testicular mitotic cells to lead to infertility.

Before CPA *ip*, there was no significant difference in body weight between each group. After CPA injection, there was no statistical significance in body weight between experimental groups during the modeling period. In the treatment period,except that the seventh- and eighth-week hamsters in the M-*Grin* group or the eighth-week ones in the L-*Grin* group statistically showed less body weights than those in the single CPA or hMG group, there was not significant in body weight between the other time points in the experimental groups, which suggests that the parent hamster models may not impact the development of fetus and the growth of baby.

The more liver weight in the single CPA or hMG group suggests that CPA or hMG was metabolized in liver. Compared to the single CPA and hMGgroup, the less liver, testis, and body weights in the *Grin* groups might result from the active fat degradation of GHRH-like peptide [18], because we observed that there was less fat tissue distribution around testes and in abdomen in the *Grin* groups. We will deeply search this in the future. The less liver weights also reflected that the *Grin* hardly had stimulation to liver because *Grin* had extremely low onset dose of 2 mg/kg (*i.m.*) and maximal tolerated dose (MTD) of 4 g/kg (*i.v.*). The H-*Grin* more obviously protected testis weight from reduction than the M- or L-*Grin*, because the H-*Grin* promoted stronger proliferation of epithelial cells in the seminiferous tubules. Compared to the single CPA or *Grin* groups, the more weights of hibateral testes in the hMG group suggest that hMG promotes growth of testis by releasing more androgen.

Our preliminary research showed when the hMG dose was less than 200 U/kg, the effect was less than that of the *Grin* groups in pregnancy rate. The pregnancy rate of H-, M-, L-*Grin*, or hMG group was 26.7, 30.8, 31.3, or 31.3%, respectively, showing significant differences compared to that (0%) of the single CPA group, but there was no difference between hMG and each *Grin* group. The hMG or H-*Grin* group had 12.5% or 13.3% of birth rates, suggesting that the H-*Grin* or hMG promotes early pregnancy. Because the hamster numbers (13-16) of each experimental group were not uniformly distributed, the calculated pregnancy rates of the H-, M-, L-*Grin* groups show a dose-inconsistent alteration. 2-8 mg/kg of *Grin* and 200 U/kg (46.2 mg/kg, 120 times equivalent to the clinical dose) of hMG showed similar therapeutic effect and different pathologic characteristics. The effect of *Grin* is at least 5.81 times stronger than that of hMG in a dose-effect relationship. In the single CPA group the loosely aligned tubules showed obvious intra-lumens, toxic swelling, and pathological vacuoles. The compactly arranged tubules in the *Grin* groups obviously showed the proliferation of germinal epithelial cells. All the results suggest that *Grin* promotes the proliferation and differentiation of primitive epithelial cells.

The ellipsoid seminiferous tubules in the *Grin* groups showed a dose-dependent enlargement, suggesting that the H-*Grin* strongly protects testis with reduction by promoting the proliferation of testicular cells, whereas hMG promotes growth of testis in efficacy.

The results of GHRH receptor in the testis tissues confirm that there is obvious GHRH receptor distribution in primitive germinal cells. With the increasing of differential maturation, the expression of GHRH receptor gradually increased in the primitive germinal cells, suggesting that expression of GHRH recep-

tor has a positive relationship with spermatogenesis. The more GHRH receptor expression in the *Grin* groups reflects the unique mechanism of *Grin* peptide.

There was no significance in blood or testicular GH protein between the experimental groups, suggesting that pituitary or testicular GH did not follow the *Grin* administration. The inconsistent result of GHRH receptor and GH suggests that the testicular GHRH receptor-mediated function may differ from GH in fertility. Some publications [19] reported that GH directly provides a gonadotropin-dependent effect.

The distributions of GHRH receptor and GH proteins in the testicular tissue show similarity, *i.e.* wide distribution in the testicular epithelial cells. But some characteristics were obvious. The testicular GHRH receptor presented a cell differentiation-dependent distribution, whereas the testicular GH uniformly distributes in all epithelial cells. Moreover the GH has stronger expression than the GHRH receptor. Although *Grin* has the significant *in vitro* pituitary GH release [14], *in vivo* the *Grin* doses did not induce pituitary or testicular GH release, suggesting that the fertility effects of *Grin* on the models were not mediated by GH. Also, the insignificant alteration in serum and testicular GHs may reflect that *Grin* had an insufficient dose which did not trigger pituitary GH secretion, or more affinity to injury testes instead of pituitary.

Although some publications reported the reduced fertilities or the reproductive abnormalities of GH transgenic male mice [20], or the treatment with hGH produced controversial results [21-23], the treatment effect of *Grin* on male infertility hamsters was significant. Our research answers the relationship of the testicular GHRH receptor with fertility: an appropriate GHRH receptor level in testis may be necessary for fertility, which may be that testis contains a subset of GHRH target cells that have the capacity to respond to multiple releasing hormones and support fertility like anterior pituitary [24], because we discovered that testicular GH cells are more than testicular GHRH receptor cells, indicating that the subset of cells may be multifunctional. Or, GHRH molecule has multiple signaling pathways to produce diverse functions, such as Adenylatecyclase→cAMP→PKA [25], the voltage-gated Ca^{2+} channels→Ca^{2+} influx/intracellular Ca^{2+} mobilization→Ca^{2+}↑ [26], an alternative RNA processing mechanism of GHRH receptor gene [27], phospholipase C→inositol phosphate-dependent pathway [28], and mitogen-activated protein kinase pathway [29]. *Grin* will be deeply studied to answer the difference with GH treatment in the future.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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