



# Effects of Recombinant Human Growth Hormone on the Onset of Puberty, Leydig Cell Differentiation, Spermatogenesis and Hypothalamic KISS1 Expression in Immature Male Rats

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**Purpose:** Recombinant human growth hormone (rhGH) has been used to treat short stature and rhGH-related syndromes. However, there are concerns that rhGH-treatment may cause precocious puberty. We investigated the effects of rhGH-treatment on the puberty onset, sexual maturation, androgen production, and hypothalamic gene expression in prepubertal male rats.

**Materials and Methods:** Sprague-Dawley male rats were injected subcutaneously daily with 1 or 2 IU/kg/d rhGH or 0.1 mL saline from postnatal day (PND) 21 to 30. At PND 31 bodyweight, reproductive organs weight, preputial separation, testis histology, circulating testosterone, and expression of testicular steroidogenic pathway genes and hypothalamic *Kiss1* were examined.

**Results:** By day 4 of injection bodyweights of rhGH groups were significantly higher than those of controls. rhGH 2 IU group showed earlier preputial separation compared to the control group. At PND 31, the weights of testes, epididymides, seminal vesicles, prostates, and preputial glands of the 2 IU-rhGH group were significantly higher than control group. Serum testosterone levels of the 2 IU-rhGH group were significantly higher than control group. Testicular steroidogenic pathway gene *Hsd17b3* and *Nr5a1* mRNA and cell counts and areas of Leydig cells in rhGH groups were significantly higher than control group, suggesting functional differentiation of Leydig cells. Hypothalamic *Kiss1* mRNA levels of the 1 IU-rhGH group were significantly lower than control group, suggesting negative feedback of *Kiss1* by elevated testosterone.

**Conclusions:** Prepubertal rhGH-treatment in male rats may induce early onset of puberty, sexual maturation, elevation of testosterone, and spermatogenesis, and accompanies downregulation of hypothalamic KISS1.

**Keywords:** Growth hormone; Kisspeptin 1; Rats; Sexual maturation; Testosterone

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## INTRODUCTION

The clinical use of recombinant human growth hormone (rhGH) has increased in the treatment of short stature, growth hormone deficiency, Prader-Willi syndrome, Turner syndrome, renal transplantation for chronic renal failure, small for gestational age infant, and human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) [1,2]. In children, however, there are concerns about rhGH-induced skeletal maturation and an early onset of puberty [3-5]. In GH-deficient and GH receptor-knockout (GHR-KO) mice, puberty and sexual maturation were delayed [6]. In GH-deficient mice, rhGH therapy normalizes the progression of puberty [7]. The physiological effects of GH on puberty have been primarily explained by action of insulin-like growth factor-1 (IGF-1) [5,8]. In humans, patients with Laron syndrome characterized by an insensitivity to GH are infertile, and administration of IGF-1 restored sexual maturation [9,10]. In mammals, circulating IGF-1 levels increase during puberty. In immature female rats, intravenous administration of IGF-1 induced advanced puberty [11]. IGF-1 elicits gonadotropin-releasing hormone (GnRH) release from the median eminence of immature female rats *in vitro* [12]. Furthermore, chronic intraventricular infusion of IGF-1 antibody decreased serum gonadotropin and testosterone levels [13]. Recently, the implication of kisspeptin, a hypothalamic neuropeptide in the onset of puberty, in regulating sexual maturation and adult reproductive activity has been highlighted [14]. In rats, the initiation of puberty is accompanied by increased levels of hypothalamic *Kiss-1 metastasis-suppressor (KISS1)* and *KISS1 receptor (KISS1R)* mRNA [15]. In rats, kisspeptin stimulates GnRH neurons that express KISS1R [15], increasing the release of GnRH at puberty [15]. In mice, KISS1 neurons are targets for the action of sex steroids in both sexes [16]. In rats, IGF-1 increased *Kiss1* mRNA levels in kisspeptin neurons [17]. To date, however, a limited number of studies have shown that prepubertal rhGH can modulate the onset of puberty *via* alteration of hypothalamic KISS1. In an effort to elucidate the neuroendocrine control of the puberty-promoting effects of prepubertal rhGH-treatment, we examined the onset of puberty, sexual maturation, androgen production, and hypothalamic KISS1 expression in male rats given prepubertal injections of rhGH.

## MATERIALS AND METHODS

### 1. Animals and recombinant human growth hormone-treatments and ethics statement

The rhGH dosages were set according to previous studies in peripubertal rat model [18,19]. Male Sprague-Dawley rats (Daehan Biolink, Deajeon, Korea) were maintained in the 12 h/12 h light/dark cycle with food and water provided *ad libitum*. At postnatal day (PND) 14, immature male rats (30 heads) were randomized into 3 groups and subcutaneously injected with rhGH 1 or 2 IU/kg/d bodyweight or with 0.1 mL saline from PND 21 to 30 at 11 A.M. every day. There were 10 male rats in each group. All animal experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals in the Hanyang University (HY IACUC 10-052A).

### 2. Monitoring of body weights and preputial separation

The bodyweights of the rats were measured at 10 A.M. from PND 21 to 30. The preputial separation is referring to the separation of prepuce from the glans penis, which indicates the commencement of puberty [20]. Preputial separation was examined daily from PND 21 to 31, and the first date of preputial separation for each rat were recorded.

### 3. Sampling for reproductive organs and brain

Rats were sacrificed at PND 31 by following CO<sub>2</sub> asphyxiation. Male reproductive organs including testes, epididymides, ventral prostates, seminal vesicles, and preputial gland were washed in phosphate buffer saline to clear blood, and wet weights of organs were measured after dissected. Brains were rapidly removed from the skull. Hypothalamus and pituitary were collected according to anatomical landmarks were based on the Swanson rat brain atlas [21].

### 4. Histological analysis of testes

Testes were fixed in Bouin's solution (Sigma-Aldrich Korea, Seoul, Korea) for 24 hours. After fixation, samples were dehydrated, cleared by xylene (DAEJUNG Chemicals & Metals Co., Siheung, Korea) and embedded in paraffin (Leica Biosystems, DeMeen, Netherlands). Paraffin blocks were sectioned as 5 μm and mounted onto poly-L-lysine-coated slides. After rehy-

dration, hematoxylin-eosin (H&E) staining was carried out. Histological observation was conducted using the DM2000 microscope system (Leica, Heerbrugg, Switzerland). The surface areas and numbers of Leydig cells per unit of interstitial region were measured by image analysis program (IMT iSolution Lite, Version 7.8; IMT i-Solution Inc., Vancouver, BC, Canada).

### 5. Enzyme-linked immunosorbent assay for testosterone

The concentration of circulating testosterone of male rats at PND 31 was measured using the testosterone enzyme-linked immunosorbent assay (ELISA) kit (Demeditec Diagnostics, Kiel-Wellsee, Germany) according to the manufacturer's instructions.

### 6. Real-time reverse transcription-polymerase chain reaction

In order to confirm the effects of rhGH on steroidogenesis in Leydig cells, the mRNA levels of steroidogenesis pathway genes *hydroxysteroid (17β) dehydrogenase 3 (Hsd17b3)*, and *nuclear receptor subfamily 5, group A (Nr5a1)* were analyzed by reverse transcription-polymerase chain reaction (RT-qPCR). As a spermatogenic marker, *protamine 2 (Prm2)* levels were examined. To address regulation of puberty by KISS hypothalamic *Kiss1* mRNA levels were analyzed. *Ribosomal protein L7 (Rpl7)* mRNA levels were used as an internal control. Total testicular RNA was extracted

from frozen testes by TRI reagent (Molecular Research Center, Cincinnati, OH, USA). RNA samples (1 μg) were reverse-transcribed using the murine leukemia virus reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Primers used RT-qPCR are presented in Supplement Table. RT-qPCR was performed using the SYBR Premix EX Taq kit (Takara, Shiga, Japan) in MyiQ iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA).

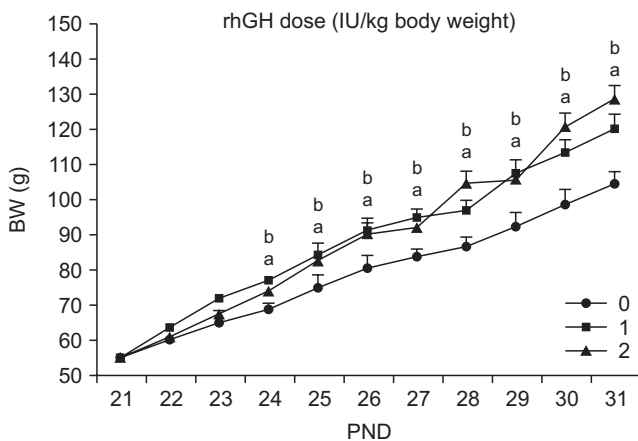
### 7. Statistical analysis

Data were expressed as mean±standard deviation or standard error of the mean. Statistical calculations were performed using the Student's t-test (IBM SPSS Statistics 21.0; IBM Corp., Armonk, NY, USA), and the results were accepted as significant when the p-values were lower than 0.05.

## RESULTS

### 1. Effects of recombinant human growth hormone on body and reproductive organ weights

From PND 24 to 31, the bodyweights of the rhGH injection groups (1 and 2 IU) were significantly higher than those of the control group (Fig. 1). At PND 31, the weights of the testes, epididymides, prostates, and seminal vesicles of the 2 IU/kg/d rhGH group were significantly higher than those of the control group. No differences were observed in the pituitary or brain



**Fig. 1.** Daily increase in bodyweights (BW) of prepubertal male rats given recombinant human growth hormone (rhGH). Immature male rats were given 1 or 2 IU of rhGH from postnatal day (PND) 21 to 30 and sacrificed at PND 31. Bodyweight was measured every day from PND 21 to 31 and daily increases in BW were presented. Values are mean±standard deviation (n=10). <sup>a</sup>1 IU/kg rhGH, <sup>b</sup>2 IU/kg rhGH; significantly different from control group by Student's t-test at p<0.05.

**Table 1.** Effects of rhGH-treatments on various organ weights on PND 31

Organ weight	rhGH dose (IU/kg bodyweight)		
	0	1	2
Testes (mg)	446.80±20.50	442.70±34.90	537.90±37.90*
Epididymides (mg)	46.10±2.60	46.70±2.80	54.70±2.30*
Prostates (mg)	62.80±9.20	61.60±12.90	77.20±11.00*
Seminal vesicles (mg)	28.50±4.20	28.10±8.20	45.70±3.50*
Preputial glands (mg)	33.70±4.10	29.80±4.20	43.00±9.60*
Brain (g)	1.70±0.03	1.70±0.05	1.70±0.04
Pituitary gland (mg)	4.70±0.40	4.50±0.40	4.80±0.80

The paired weights of testes, epididymides, seminal vesicles and preputial glands are presented. Values are presented as mean±standard error of mean (n=10).

rhGH: recombinant human growth hormone, PND: postnatal day.

\*Significantly different from control group by Student's t-test at p<0.05.

weights among the three groups (Table 1).

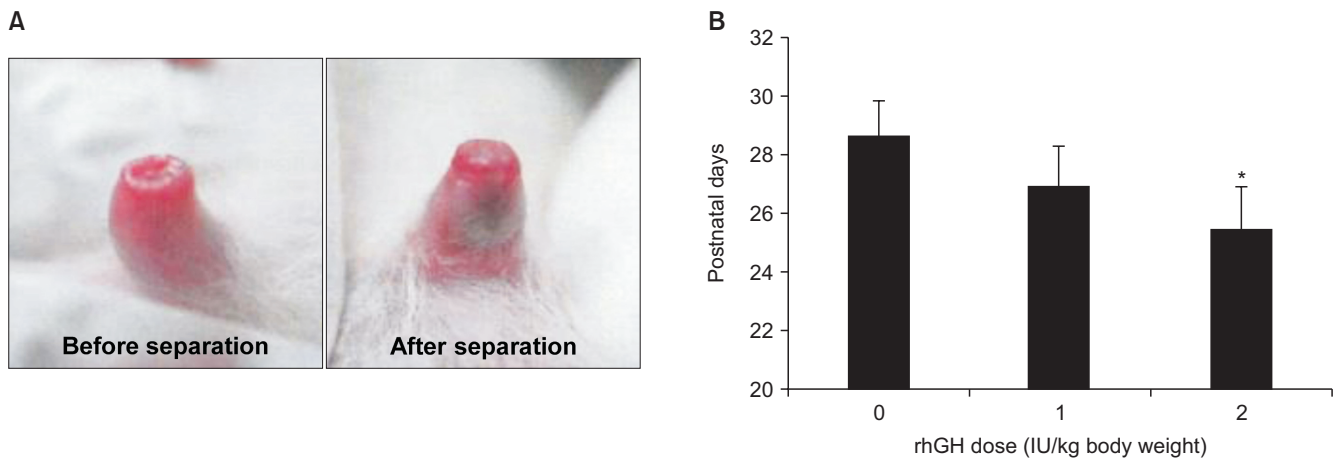
## 2. Effects of recombinant human growth hormone on preputial separation

From PND 24 to 31 the day of preputial separation from the glans penis was significantly advanced in rhGH 2 IU/kg/d group (PND 25.3±1.6) compared with control group (PND 28.9±1.2). However, rhGH 1 IU/kg/d group (PND 27.0±1.4) did not show any significant dif-

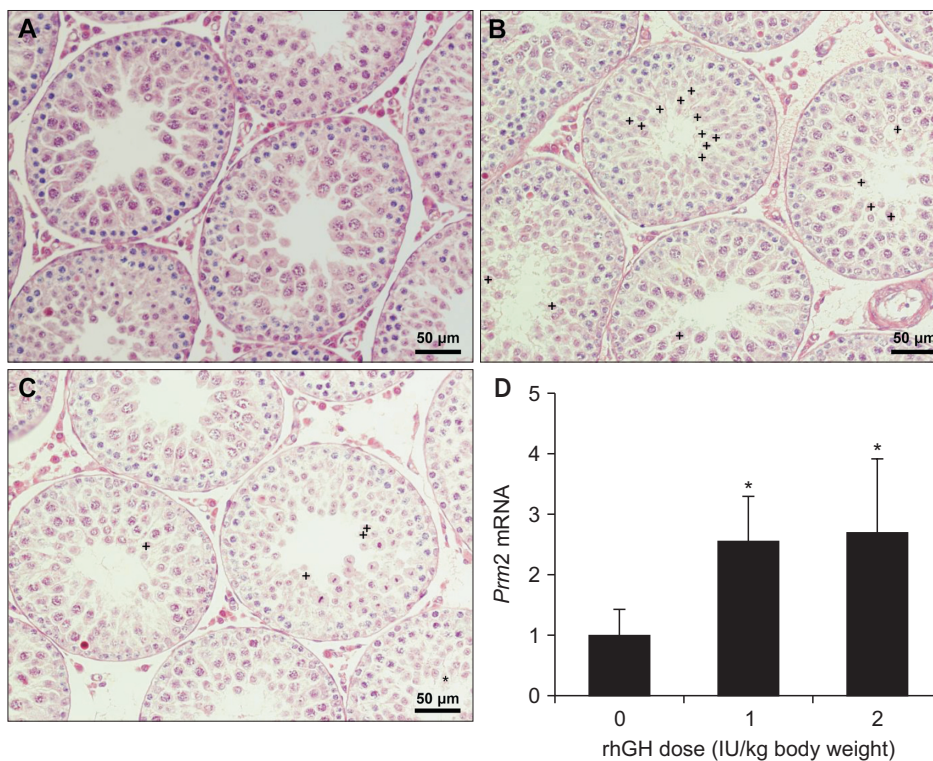
ference from control (Fig. 2).

## 3. Effects of recombinant human growth hormone on testis histology, *Prm2* mRNA, and counts and size of Leydig cells

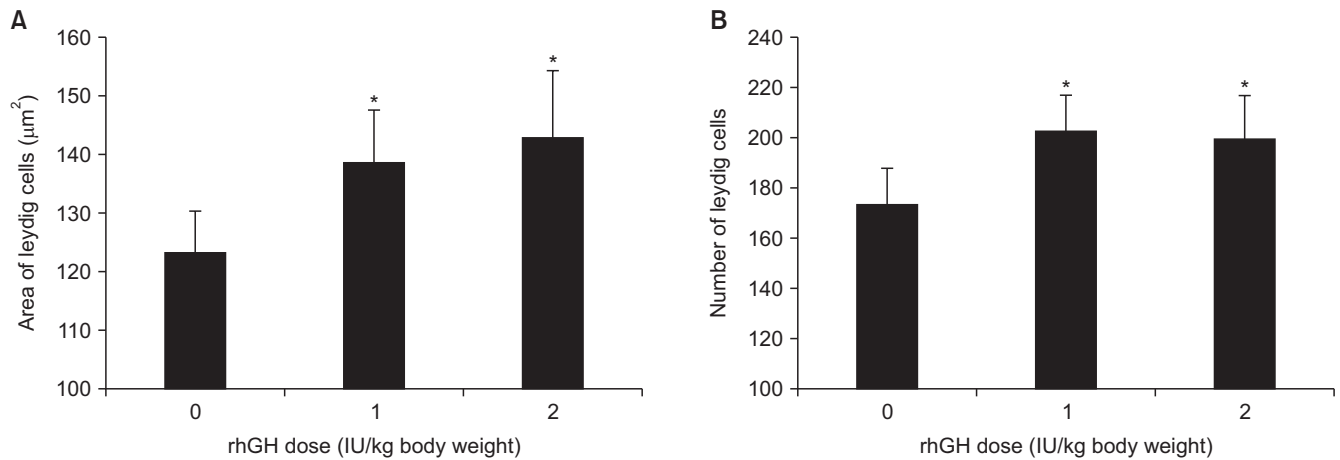
In H&E-stained testis sections, elongating spermatids were observed from the 1 and 2 IU/kg/d rhGH groups seminiferous tubules but not from the control seminiferous tubules. Testicular *Prm2* mRNA levels of the



**Fig. 2.** Day of preputial separation in recombinant human growth hormone (rhGH)-treated male rats. (A) Penile morphology for examination of preputial separation. (B) The first day of preputial separation. Values are presented as mean±standard deviation (n=10). \*Significantly different from control group by Student's t-test at p<0.05.



**Fig. 3.** Spermatogenesis of rat testes given recombinant human growth hormone (rhGH). (A-C) H&E staining of postnatal day (PND) 31 testes of rats given 0, 1, and 2 IU rhGH from PND 21 to 30, respectively (+: elongating spermatids). (D) Expression of *Prm2* mRNA in rhGH-treated rat testes. Values are presented as mean±standard deviation (n=10). \*Significantly different from control group by Student's t-test at p<0.05.



**Fig. 4.** Histological assays of Leydig cells of rat testes given recombinant human growth hormone (rhGH). (A) Average area of Leydig cells. (B) Average number of Leydig cells. Values are presented as mean±standard deviation (n=10). \*Significantly different from control group by Student's t-test at p<0.05.

**Table 2.** Effects of rhGH-treatment on serum testosterone levels in male rats

	rhGH dose (IU/kg bodyweight)		
	0	1	2
Testosterone (ng/mL)	0.24±0.06	0.32±0.13	0.58±0.20*

Serum testosterone concentration at postnatal day 31 were measured by ELISA. Values are presented as mean±standard error of mean (n=10).

rhGH: recombinant human growth hormone.

\*Significantly different from control group by Student's t-test at p<0.05.

1 IU/kg/d (2.56±0.74) and 2 IU/kg/d (2.70±1.23) rhGH groups were significantly higher than those of control (1.00±0.43) (Fig. 3, Supplement Fig). The numbers and sizes of Leydig cells in the rhGH groups were significantly higher than those of the controls (Fig. 4, Supplement Fig).

#### 4. Effects of recombinant human growth hormone on serum testosterone levels and steroidogenesis pathway genes expression

Serum testosterone levels of the 2 IU/kg/d rhGH group were significantly higher than those of the control group (0.58±0.20 versus 0.24±0.06). Serum testosterone levels of the 1 IU/kg/d rhGH group were not different from those of the control group (0.32±0.13 versus 0.24±0.06) (Table 2). In real-time RT-PCR of testicular steroidogenic genes, *Hsd17b3* and *Nr5a1* mRNA levels of the 1 and 2 IU/kg/d rhGH groups were significantly higher than those of the control group (1 IU/kg/d rhGH group [*Hsd17b3*: 1.48±0.25 versus 1.01±0.25; *Nr5a1*:

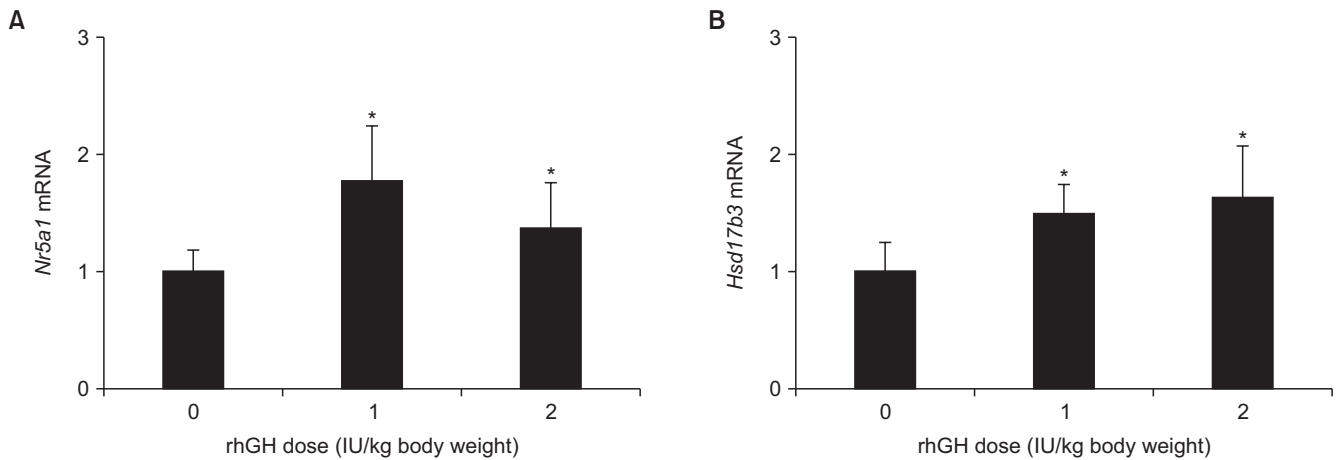
1.78±0.46 versus 1.01±0.19]; 2 IU/kg/d rhGH group [*Hsd17b3*: 1.63±0.45 versus 1.01±0.25; *Nr5a1*: 1.37±0.38 versus 1.01±0.19]) (Fig. 5).

#### 5. Effects of recombinant human growth hormone on hypothalamic *Kiss1* mRNA

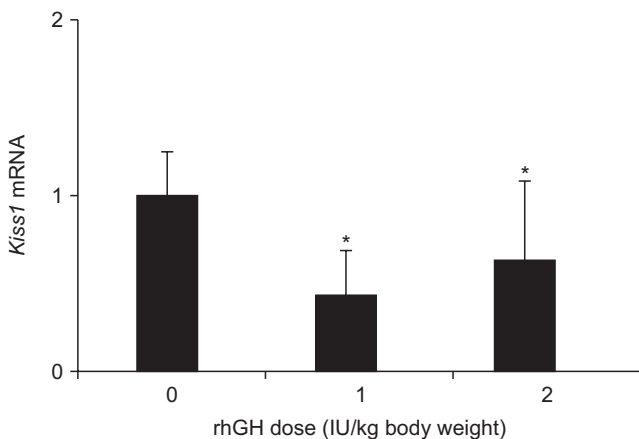
Hypothalamic *Kiss1* mRNA levels of the 1 IU/kg/d rhGH group were significantly lower than those of control (0.44±0.11 versus 1.00±0.33), but those of the 2 IU/kg/d rhGH group were not (0.63±0.30 versus 1.00±0.33) (Fig. 6).

## DISCUSSION

GH regulates all levels of the hypothalamic pituitary-gonadal (HPG) axis, either directly or indirectly via hepatic or locally produced IGF-1 [22]. The endocrine actions of hypophyseal GH are complemented by GH produced locally within the reproductive tissues [22]. In mice, the HPG axis becomes active during puberty and the increased GH is believed to promote the onset of puberty by activating the hypothalamic GnRH pulse generator [23]. In prepubertal male rats, rhGH-treatment significantly increased body weights, suggesting growth-promoting effects of rhGH. Importantly, there was a precocious preputial separation as well as an increase in reproductive organ weights, indicative of an early onset of puberty. Similarly, in children with idiopathic short stature, high-dose rhGH-treatment before puberty induces an early onset of puberty [3]. At the light microscopic level, elongating spermatids were



**Fig. 5.** Expression of steroidogenic genes *Nr5a1* and *Hsd17b3* in recombinant human growth hormone (rhGH)-treated rat testes. (A) Expression of *Nr5a1* mRNA in rhGH-treated rat testes. (B) Expression of *Hsd17b3* mRNA in rhGH-treated rat testes. Melting curve and amplification graphs. Values are presented as mean±standard deviation (n=10). \*Significantly different from control group by Student's t-test at p<0.05.



**Fig. 6.** Expression of *Kiss1* mRNA in the recombinant human growth hormone (rhGH)-treated rat hypothalamus. Values are presented as mean±standard deviation (n=10). \*Significantly different from control group by Student's t-test at p<0.05.

observed in the testes of GH-treated groups at PND 31. *Prm2* mRNA levels, a spermatids biomarker, in the testes of rhGH groups were significantly higher than those of control group, suggesting the activation of the late steps of spermatogenesis in the rhGH rat testes. Furthermore, in the testes of rhGH groups *Nr5a1* and *Hsd17b3* mRNA were significantly higher than those of control along with significantly increased number and size of Leydig cells compared to the control group. Importantly, serum testosterone levels of the rhGH-rats were significantly higher than those of the control. These results demonstrate that in prepubertal male rats, rhGH accelerated the functional maturation of Leydig cells and increased testosterone synthesis,

contributing to an early onset of puberty. In rhGH therapy for human children with short stature, effective dosages of rhGH are 0.08–0.13 IU/kg/d [24,25]. In peripubertal rat model study, administration of rhGH at 2–11 IU/kg/d increased body growth [18,19]. In the present study, rhGH at much lower dosage (1 IU/kg/d) activated spermatogenesis and steroidogenesis, indicating that the minimum effective dosage of rhGH for precocious puberty and sexual maturation by gonadal activation is much lower than previous reports. Therefore, in clinical trial, reduction of rhGH dosage can be considered to avoid precocious puberty.

There are possible explanations for the effects of prepubertal rhGH-treatment on the early onset of puberty. In immature hypophysectomized rats, rhGH injection increased testicular IGF-1, which promotes maturation and steroidogenesis of Leydig cells by paracrine and autocrine actions [26]. Therefore, at the gonad level, rhGH can activate steroidogenesis by induction of IGF-1, resulting in an early onset of puberty. In GHR-KO male mice that displayed a reduced plasma luteinizing hormone (LH) response to GnRH treatment, administration of rhGH elevated plasma LH levels [27]. In monkeys, rhGH-treatment potentiated an initial rise in serum LH levels at puberty [28]. At the stage when precursor Leydig cells mature to immature Leydig cells, LH and IGF-1 have been shown to stimulate cell proliferation in humans, rats and mice [29]. Therefore, prepubertal rhGH-treatment may stimulate the early onset of puberty *via* potentiation of pituitary LH production, which stimulates the functional differentia-

tion of immature Leydig cells to produce testosterone. At the brain level, rhGH can indirectly activate GnRH neurons *via* increased IGF-1 produced in the liver. Infusion of IGF-1 stimulated GnRH release from the median eminence and accelerated the onset of puberty in female rats [11,12]. Hypothalamic *KISS1* has been known to stimulate the onset of puberty. In the brain of the prepubertal female rat, cerebroventricular injection of IGF-1 can activate *Kiss1* mRNA in kisspeptin neurons [17]. Nonetheless, hypothalamic *Kiss1* mRNA levels of the rhGH groups were significantly lower than those of the control group. Steroid hormone receptors were detected in kisspeptin neurons [30], and expression of *KISS1* is subjected to negative feedback of sex steroids [31]. In the adult male rhesus monkey, down-regulation of hypothalamic *KISS1* is involved in the negative feedback of testosterone to regulate LH secretion [32]. Therefore, decreased hypothalamic *Kiss1* mRNA by rhGH might be attributable to the negative feedback of elevated testosterone. Further studies are needed to determine whether IGF-1 induced by rhGH is the prime mediator of the early onset of puberty and sexual maturation while decreasing the hypothalamic *KISS1*.

## CONCLUSIONS

In summary, in prepubertal male rats, rhGH-treatment resulted in increase in body weight, early onset of puberty, activation of spermatogenesis, Leydig cells differentiation, and testosterone production. The negative feedback action of elevated testosterone may be responsible for the decrease in hypothalamic *KISS1*.

## Conflict of Interest

The authors have nothing to disclose.

## Author Contribution

Conceptualization: MJP, MCG. Data curation: MJP, MCG. Formal analysis: WHN, MCG. Funding acquisition: MJP. Investigation: KH, WHN. Methodology: WHN. Project administration: MJP, MCG. Resources: WHN, MCG. Software: YX. Supervision: MJP, MCG. Validation: MJP, MCG. Visualization: WHN, YX. Writing – original draft: KH, WHN. Writing – review & editing: MJP, MCG, YX.

## Supplementary Materials

Supplementary materials can be found *via* <https://doi.org/10.5534/wjmh.200152>.

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