Increased Testicular Insulin-like Growth Factor 1 is Associated with Gonadal Activation by Recombinant Growth Hormone in Immature Rats

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Research Article

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Abstract

To understand the mechanism of precocious sexual maturation following prepubertal growth hormone (GH) therapy, the effects of recombinant human GH (rhGH) on the kisspeptin-gonadotropin-releasing hormone-luteinizing hormone (GnRH-LH) system in the hypothalamus-pituitary axis, systemic and testicular insulin-like growth factor-1 (IGF1), spermatogenesis and Leydig cell steroidogenesis, and circulating testosterone levels were examined in immature rats. Following daily injection of rhGH (1 or 2 IU/kg) from postnatal day (PND) 21 to PND 23 or 30, testicular steroidogenic pathway genes and spermatogenesis marker genes mRNA levels, the number and size of HSD17B(+) Leydig cells, and blood testosterone levels in the rhGH rats were significantly higher than those of controls on PNDs 24 and 31. Hypothalamic *Kiss1* and *Gnrh1* mRNA in the rhGH rats were significantly higher than those in the controls on PND 24, indicating early activation of hypothalamic kisspeptin-GnRH neurons by rhGH. Hypothalamic *Igf1* mRNA levels in rhGH rats were significantly higher than those in the controls on PND 24 but significantly lower than those in controls on PND 31. Testicular *Igf1* mRNA levels were significantly higher in rhGH rats than in the controls on PNDs 24 and 31 whereas liver *Igf1* mRNA levels and circulating IGF1 levels were not. In progenitor Leydig cells, rhGH significantly increased the *Igf1* and steroidogenic pathway genes mRNA levels and the testosterone production. Therefore, local increases in testicular IGF1 might be an important mediator of gonadal activation via steroidogenic activation of Leydig cells in immature rats given rhGH.

1. Introduction

Puberty is initiated by the activation of the hypothalamus-pituitary-gonad axis (HPG axis). In the hypothalamus, kisspeptin activates gonadotropin-releasing hormone (GnRH) secretion, resulting in luteinizing hormone (LH) secretion in the pituitary gland, steroidogenesis and gametogenesis in the gonads, and the onset of puberty (Navarro *et al.*, 2004; Tena-Sempere, 2006; Huh *et al.*, 2021). As a neuromodulator, kisspeptin not only conveys the modulatory actions of sex steroids to GnRH neurons but also directly stimulates LH secretion in the pituitary gland (Smith, 2008; Skorupskaite *et al.*, 2014). Growth hormone (GH) has been shown to play a role in the onset of puberty, and it regulates the expression of steroidogenic acute regulatory protein and steroidogenesis in progenitor Leydig cells (Kanzaki & Morris, 1999). GH-receptor knockout mice and GH-deficient mice show delayed puberty (Chandrashekar *et al.*, 2001; Keene *et al.*, 2002). GH therapy normalizes the progression of puberty in GH-deficient mice (Darendeliev *et al.*, 1990; Stanhope *et al.*, 1992). Most of the physiologic effects of GH on puberty have been explained by the actions of insulin-like growth factor-1 (IGF1) (Roith *et al.*, 2001; Christoforidis *et al.*, 2005; Ipsa *et al.*, 2019). IGF1 is primarily produced in the liver, but it is also synthesized by almost all tissues, including the testes (Rosen, 1999; Cannarella *et al.*, 2018). In mammals, including humans, circulating IGF1 levels increase at the onset of puberty, and the administration of IGF1 advances the process of puberty (Hiney *et al.*, 1996; Chang *et al.*, 2018). In Laron syndrome patients, who have congenital IGF1 deficiency, the administration of IGF1 restores sexual maturation (Laron & Klinger, 1998). In immature animals, IGF1 can activate the brain-gonadal axis and

contributes to the timing of puberty in males and females by affecting GnRH neurons and possibly kisspeptin neurons in the hypothalamus (Hiney *et al.*, 1991; Wolfe *et al.*, 2014).

Recombinant human growth hormone (rhGH) therapy has been introduced as a treatment for a variety of conditions associated with short stature, such as GH deficiency, Turner syndrome, chronic renal failure, short-stature homeobox gene, and Noonan syndrome (Blum *et al.*, 2007; Romano *et al.*, 2009). However, there are concerns that it can induce skeletal maturation and the early onset of puberty, though some studies have reported that rhGH therapy did not correlate with early puberty onset (Kamp *et al.*, 2002; Crowe *et al.*, 2006; Huh *et al.*, 2021). To understand the mechanism by which rhGH could stimulate the early onset of puberty in males, changes in hypothalamic kisspeptin, GnRH and IGF1 levels, pituitary and circulating LH, spermatogenesis, testicular steroidogenesis, and IGF1 in the circulation, liver, and testes were examined in immature rats following prepubertal rhGH treatment.

2. Materials And Methods

2.1. Animals and tissue sampling

Immature male rats (Sprague Dawley) were purchased on their postnatal day (PND) 14 from Daehan Biolink (Deajeon, Korea) and maintained on a 12:12-hrs light/dark cycle with food and water available *ad libitum* (lights on at 8:00 a.m.). The animals were randomized into three groups and subcutaneously injected with rhGH (1 or 2 IU/kg) or 0.1 mL of saline every morning at 11 a.m. from PND 21 to PND 23 or 30. The rats were sacrificed by CO₂ asphyxiation on PND 24 or 31. Blood was collected by cardiac puncture and prepared for serum. The testes, epididymis, prostate, preputial glands, seminal vesicle, and brain were dissected and weighed. The hypothalamus and pituitary gland were further dissected from the brain. Organs were subjected to histological and gene-expression analyses.

2.2. Real-time reverse transcription-polymerase chain reaction (RT-qPCR)

All tissues were frozen in liquid nitrogen and then subjected to total RNA extraction using TRI reagent (Molecular Research Center, Cincinnati, OH). RNA samples (1 µg) were reverse transcribed for 60 min at 42°C in a 20 µL reaction with ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). In the testes, steroidogenic pathway genes, spermatogenesis marker genes (*Transition protein 1*, *Tnp1*; *Transition protein 2*, *Tnp2*; *Protamine*, *Pm*), and *Igf1* were analyzed by RT-qPCR. In the hypothalamus and pituitary gland, *Igf1*, *Kiss-1 Metastasis Suppressor (Kiss1)*, *gonadotropin Releasing Hormone 1 (Gnrh1)*, and *luteinizing hormone beta polypeptide (Lhb)* mRNA levels were analyzed. *Ribosomal protein L7 (Rpl7)* was used as an endogenous control. The primer sequences and PCR conditions are summarized in Table 1. RT-qPCR was performed using an SYBR Premix EX Taq kit according to the manufacturer's instructions (Takara, Shiga, Japan) in a My-iQ real-time PCR detection system (Bio-rad Laboratories, Hercules, CA). Relative mRNA levels were calculated using the comparative 2^{-ΔΔCt} method (Livak & Schmittgen, 2001).

Table 1
Primer sequences used in RT-qPCR analyses

Genes		Primer sequences (5'-3')	Size	Ann. temp.	GenBank ID
<i>Igf1</i>	F	tacctggcactctgcttgct	194	64	NM_001082477
	R	cggaagcaacactcatccac			
<i>Lhcgr</i>	F	ctcacctatctccctgtcaa	365	64	NM_012978
	R	acagactcgttattcatccc			
<i>Sf1</i>	F	cgccaggagtttgctgtct	185	67	NM_001191099
	R	acctccaccaggcacaatag			
<i>Star</i>	F	aaccaggaaggctggaagaa	123	64	NM_031558.3
	R	tctgtccatgggctgggtcta			
<i>Cyp11a1</i>	F	ttgcctttgagtccatcacc	187	64	NM_017286.2
	R	gcatggtccttccaggctctt			
<i>Cyp17a1</i>	F	aacgttgactccagcattgg	163	60	NM_012753.2
	R	gcgtgggtgtaatgagatgg			
<i>Hsd3b1</i>	F	gcattaaccccactcccact	146	60	NM_001007719.3
	R	ggaccctgacctccttcaga			
<i>Hsd17b3</i>	F	gtccctggcctctttacagc	191	64	NM_008291
	R	tttaacaaactcatcggcgg			
<i>Cyp19a1</i>	F	tatccggagggtggaacagc	181	60	NM_017085
	R	cgtcaatcacgtcatcctcc			
<i>Tnp1</i>	F	gatgcaagtcgcaattaccg	184	67	NM_017056.2
	R	ccgaatttcgtcacaactgg			
<i>Tnp2</i>	F	gaagaccttgaagggaaagtg	150	64	NM_017057.2
	R	tggctatctcctttgggat			
<i>Prm2</i>	F	tatgggaggacagaaagggg	157	58	NM_012873
	R	tcctccttcgggatcttctg			
<i>Kiss1</i>	F	agctgctgcttctcctctgt	152	64	NM_181692
	R	aggcttgctctctgcatacc			
<i>Gnrh1</i>	F	ccgctgttggtctgttgact	150	64	NM_012767

Genes	Primer sequences (5'–3')	Size	Ann. temp.	GenBank ID
	R ggggttctgccatttgatcc			
<i>Lhb</i>	F tcccaggactcaaccaatga	112	60	NM_001082477
	R tggtagaacacctgctggc			
<i>Rpl7</i>	F tcaatggagtaagcccaaag	246	60	NM_011291
	R caagagaccgagcaatcaag			

2.3. Histological analysis of the testes

Dissected testes were fixed in Bouin's solution (HT10132, Sigma-Aldrich, St. Louis, MO) for 12 hrs. After dehydration, the tissues were cleared with xylene and embedded in Paraplast (Leica Biosystems, Wetzlar, Germany). Sections (5 µm thick) were placed on slides coated with poly-L-lysine (P2658, Sigma-Aldrich). After deparaffinization and rehydration, hematoxylin and eosin staining were conducted. Microscopic observation and photography were conducted using a microscope equipped with a digital camera system (IX71; Olympus, Tokyo, Japan). Image analyses of the seminiferous tubule diameter and luminal epithelial area were conducted using an image-analysis program (IMT iSolution Lite, version 7.8; iMTechnology, Bucheon-si, Korea). At least eight testes at each rhGH dosage and more than 10 seminiferous tubules per testis were measured to determine the seminiferous tubule diameter and luminal area.

2.4. Immunohistochemical analysis of HSD17B in testes from PND 24

Immunohistochemical labeling of 17β-hydroxysteroid dehydrogenase (HSD17B) was conducted in the testes of rhGH-injected rats. Briefly, Bouin's solution-fixed testes slides were coated with poly-L-lysine. After deparaffinization and rehydration, the slides were blocked by incubation with 1% rabbit serum in phosphate buffered saline (PBS) and incubated with rabbit anti-HSD17B antibody (sc-31620, Santa Cruz Biotechnology, Dallas, TX) diluted to 4 µg/mL in 1% rabbit serum in PBS in a humidified chamber overnight at 4°C. In the negative control, normal rabbit IgG (ab172730, Abcam, Cambridge, UK) diluted to 4 µg/mL in 1% rabbit serum in PBS was used instead of the primary antibodies. After washing in PBS, horseradish peroxidase–conjugated goat anti-rabbit IgG (ab175470, Abcam) diluted 1:200 in 1% rabbit serum in PBS was applied, and the slides were incubated for 30 min at room temperature. After being washed in PBS, the slides were stained with hematoxylin and eosin, mounted with Canada balsam (03984, Sigma-Aldrich), and observed with an epifluorescence microscope (IX71, Olympus, Tokyo, Japan) equipped with a digital imaging system (DP71, Olympus).

2.5. Immunofluorescence of HSD3B in rhGH-treated rats' testes

Paraformaldehyde (PFA, 4%)-fixed testis sections were subjected to immunofluorescence staining with 3 β -hydroxysteroid dehydrogenase (HSD3B). Briefly, sections were placed on slides coated with poly-L-lysine. After deparaffination with xylene, the sections were washed 3 times with PBS, incubated for 30 minutes with blocking solution (1.5% normal donkey serum in PBS), and then incubated overnight at 4°C with rabbit anti-HSD3B antibody (sc-30820; Santa Cruz Biotechnology) at 1:100 in blocking solution. As a negative control, normal rabbit IgG (ab172730, Abcam) replaced the primary antibody. After washed in PBS, slides were incubated in a 1:200 dilution of donkey polyclonal antibody to rabbit IgG Alexa Fluor (ab175692, Abcam) in blocking solution for 30 minutes. After 4',6-diamidino-2-phenylindole (DAPI; 32670, Sigma-Aldrich) staining, observation and photography were conducted using a microscope system (IX71).

2.6. *In vitro* rhGH treatment of Leydig cells from rat testes

immature Sprague-Dawley rats (PND 21) were killed by CO₂ asphyxiation, and the testes were removed. The decapsulated testes were washed with PBS and incubated with collagenase (0.25 mg/mL)-RPMI medium (11875093, Gibco, Dublin, Ireland) for 20 min. The interstitial cells from the supernatant were washed 2 times with RPMI medium. To obtain purified Leydig cells, 1 mL of the interstitial cells was loaded at the top of a 36–60% (v/v; 2 mL each) Percoll (P1644, Sigma-Aldrich) gradient. After centrifugation at 800 X g for 25 min, four visible bands of testicular cells were obtained, with highly purified Leydig cells found in the third band from the top, corresponding to a 38–52% (v/v) Percoll concentration. The Leydig cells were collected, washed twice with RPMI and resuspended in RPMI with 10% fetal bovine serum (10270106, Gibco) and 1 X Antibiotic-Antimycotic (15240062, Gibco). The purity of the Leydig cells was assessed using HSD3B immunofluorescence staining. Briefly, isolated Leydig cells were plated in 35 mm culture dishes at 10⁵ cells/mL and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 6 hrs. And then, Leydig cells were fixed with 4% PFA for 5 min and washed 3 times with PBS. After PBS washing, Leydig cells were incubated overnight at 4°C with rabbit anti-HSD3B antibody (sc-30820; Santa Cruz Biotechnology) at 1:100 in blocking solution. As a negative control, normal rabbit IgG (ab172730, Abcam) replaced the primary antibody. After washed in PBS, cells were incubated in a 1:200 dilution of donkey polyclonal antibody to rabbit IgG Alexa Fluor (ab150073, Abcam) in blocking solution for 30 minutes. After DAPI (32670, Sigma-Aldrich) staining, observation and photography were conducted using a microscope system (IX71). The HSD3B(+) cells were identified as Leydig cells. For rhGH treatment, purified Leydig cells were plated in 35-mm culture dishes at 10⁵ cells/mL and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After 24 hrs, the medium was removed and replaced with rhGH (0, 10, or 50 μ g/L) and LH (10 μ g/L) in RPMI medium. After 48 hrs of that treatment, the spent media were collected and subjected to enzyme-linked immunosorbent assay (ELISA) (Demeditec Diagnostics) to determine the testosterone concentration. The mRNA expression of the rhGH-treated Leydig cells was analyzed by RT-qPCR.

2.7. ELISA for circulating testosterone, LH, and IGF1 levels

On PNDs 24 and 31, samples of rat blood collected via heart puncture were centrifuged at 14,000 rpm for 10 min. The serum supernatants were stored at -80°C . Total serum testosterone (T), IGF1, and LH concentrations in male rats were measured on PNDs 24 and 31 by ELISA using testosterone and IGF1 ELISA kits (Demeditec Diagnostics, Kiel-Wellsee, Germany) and LH kit (Shibayagi, Shibukawa, Japan) according to the manufacturers' instructions.

2.8. Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD) values. Statistical calculations were performed using two-tailed Student's *t*-test (IBM Corporation, Armonk, NY). A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Changes in testis weight, seminiferous tubule histology, and mRNA levels of spermatogenic marker genes

On PND 24, following three daily administrations of rhGH, the testis weights of the 2 IU/kg rhGH and 1 IU/kg rhGH rats were significantly lower than those of the control rats. However, on PND 31, following 10 daily administrations of rhGH, the testis weights of the 2 IU/kg rhGH and 1 IU/kg rhGH rats were significantly higher than those of the control rats. In the testis histology examination, the seminiferous tubule diameter and luminal area were significantly increased in the 2 IU/kg rhGH rats on PND 31 but not in the 1 IU/kg rhGH rats. No significant difference in seminiferous tubule diameter or luminal area was observed between the control and 1 or 2 IU/kg rhGH rats on PND 24 (Fig. 1; Supplemental Fig. 1). The testicular *Tnp1*, *Tnp2*, and *Prm2* mRNA levels of the rhGH rats were significantly higher than those of the control rats on PND 31 but not on PND 24 (Fig. 2).

3.2. Changes in the number and size of Leydig cells, testicular steroidogenic pathway gene mRNA levels, and blood testosterone levels

Quantitative image analysis of HSD17B(+) immunoreactivity in testes revealed a significant increase in the number and size of Leydig cells in rhGH rat testes on PNDs 24 and 31 (Fig. 3; Supplemental Fig. 2). In the HSD3B immunofluorescence labeling of testes from rhGH rats on PND 24, both the number of HSD3B(+) Leydig cells and their size was significantly increased compared with the control (Fig. 3; Supplemental Fig. 3). However, the total number of interstitial cells from rhGH rat testes was not different from control on PND 24 or 31. In the RT-qPCR analysis, the mRNA levels of testicular steroidogenic pathway genes, *luteinizing hormone/choriogonadotropin receptor (Lhcgr)*, *steroidogenic factor 1 (Sf1)*, *steroidogenic acute regulatory protein (Star)*, *cytochrome P450 family 17 subfamily A member 1 (Cyp17a1)*, and *hydroxysteroid 17 β dehydrogenase 3 (Hsd17b3)*, in the rhGH rats were significantly

higher than those in the control rats on PNDs 24 and 31 (Fig. 4). Serum testosterone levels were significantly higher in the 2 IU/kg rhGH rats than in the control rats on PNDs 24 and 31 (Fig. 5).

3.3. Changes in hypothalamic *Kiss1* and *Gnrh1* and pituitary *Lhb* mRNA and circulating LH levels

Hypothalamic *Kiss1* and *Gnrh1* mRNA levels in the rhGH rats were significantly higher than those in the control rats on PND 24, but they were significantly lower than those in the controls on PND 31. Pituitary *Lhb* mRNA levels in the rhGH rats were significantly lower than those in the control rats on PNDs 24 and 31. No significant differences were found in circulating LH levels between the rhGH rats and control animals on PND 24 or 31 (Fig. 6).

3.4. Changes in circulating IGF1 and mRNA levels in the hypothalamus, liver, and testes

The hypothalamic *Igf1* mRNA levels in the rhGH rats were significantly higher than those in the control animals on PND 24, but they were significantly lower on PND 31. The liver *Igf1* mRNA levels and blood IGF1 levels of the rhGH rats did not differ significantly from those of the control rats on PND 24 or 31. The testicular *Igf1* mRNA levels of the rhGH rats were significantly higher than those of the control rats on PNDs 24 and 31 (Fig. 7).

3.5. Changes in *Igf1* expression and steroidogenesis in Leydig cells *in vitro*

In the immunocytochemical staining, 96.3% of the isolated interstitial cells were positive for HSD3B. The *Igf1*, *cytochrome P450 family 11 subfamily A member 1 (Cyp11a1)*, *Cyp17a1*, *cytochrome P450 family 19 subfamily A member 1 (Cyp19a1)*, and *Hsd17b3* mRNA levels in Leydig cells treated with 10 µg/L of rhGH were significantly higher than those in the control cells, but the levels in cells treated with 50 µg/L of rhGH did not differ from the control cells. Compared with the control, *Sf1* mRNA levels were significantly higher in Leydig cells treated with 10 or 50 µg/L of rhGH. *hydroxy-delta-5-steroid dehydrogenase, 3 beta-and steroid delta-isomerase 1 (Hsd3b1)* mRNA levels, on the other hand, were significantly higher in Leydig cells treated with 10 µg/L of rhGH but lower in cells treated with 50 µg/L of rhGH, compared with the control. *Lhcgr* and *Star* mRNA levels did not differ significantly between the rhGH-treated cells and the control. In the spent media, testosterone levels were significantly higher than the control in Leydig cells treated with 10 µg/L of rhGH but not in those treated with 50 µg/L of rhGH (Fig. 8).

4. Discussion

4.1. Effect of rhGH on spermatogenesis and steroidogenesis in immature rats

Correlations between GH and sexual maturation have been reported in both sexes (Zaczek et al., 2002; Sanchez-Cardenas *et al.*, 2010). In immature male rats, puberty was significantly advanced, together with an increase in total body weight and reproductive organ weight, following rhGH administration (Ronis et al., 1998). In this study, the testis weights of the 2 IU/kg rhGH rats were significantly lower than those of the control rats on PND 24, although that difference was mitigated on PND 31. Similarly, in immature male dogs, a high dose of rhGH (25 IU/kg/day) induced atrophy of the testes and accessory organs (Sjögren *et al.*, 1998). GH can induce apoptosis and atretic changes in the ovaries and testes of mammals, including humans, by activating the PI3K-Akt pathway (Santos *et al.*, 1999; Sirotkin, 2005). Though no explanation for the reduced testis weights in rhGH rats during the early prepubertal period has been confirmed, high doses of rhGH in the early prepubertal period might transiently reduce testis development in a way that is impermanent and recoverable. In histology, the diameter and luminal areas of the seminiferous tubules were visibly increased in the 2 IU/kg rhGH rats on PND 31. In parallel, testicular mRNA levels of spermatogenic marker genes in the rhGH rats were significantly higher than those in the control rats on PND 31, indicating that rhGH activated spermatogenesis in the immature rats. Similarly, in immature rats, GH treatment for four weeks induced testicular growth and germ cell differentiation (Ipsa *et al.*, 2019). In the 2 IU/kg rhGH rats, blood testosterone levels were significantly higher than in the control rats on PNDs 24 and 31. Similarly, rhGH injection stimulated testosterone synthesis in both immature and adult rodents (Colón et al., 2005; Sriraman et al., 2005). Given that circulating LH levels did not differ significantly between the control and rhGH rats, the activation of androgen production might indicate that rhGH has a direct effect on Leydig cells. In Leydig cell cultures, rhGH directly activated steroidogenesis (Wang & Hardy, 2004; Ipsa *et al.*, 2019). In rat testes, HSD3B(+) progenitor Leydig cells are typically observed on PND 21 before they increase in number and become HSD17B(+) immature Leydig cells that go on to differentiate into functional adult Leydig cells (Hu *et al.*, 2010). In light of the observed mRNA levels of steroidogenic pathway genes, increases in the number and mean size of HSD17B(+) Leydig cells in rhGH rat testes on PNDs 24 and 31, and the increased number and mean size of HSD3B(+) Leydig cells in rhGH rat testes on PND 24, prepubertal rhGH administration might have accelerated the functional differentiation of progenitor Leydig cells (PLCs) into immature, testosterone-producing Leydig cells. In the testes, GH and IGF1 modulate the proliferation and steroidogenesis of Leydig cells via the MAPK-ERK, JAK-STAT, and PI3K-AKT pathways (Rotwein, 2012; Ipsa *et al.*, 2019). In the HSD3B(+) progenitor Leydig cells isolated on PND 21, 10 µg/L of rhGH treatment increased the expression of steroidogenic pathway gene mRNA (*Igf1*, *Sf1*, *Cyp11a1*, *Cyp17a1*, *Cyp19a1*, *Hsd3b1*, and *Hsd17b3*) and testosterone secretion. This suggests that rhGH-treatment directly activates steroidogenesis in progenitor Leydig cells, which is consistent with the results of previous studies (Dufau, 1988; Maran *et al.*, 2000). The increase in circulating testosterone found in rhGH rats on PNDs 24 and 31 might be attributable to greater steroidogenic differentiation of PLCs to testosterone-producing Leydig cells, activating the spermatogenesis.

4.2. rhGH triggered changes in kisspeptin, GnRH, and LH in immature male rats

During the prepubertal period, kisspeptin activates the HPG axis and increases sex steroids (Soliman *et al.*, 2014; Devesa & Caicedo, 2019). In male rats, hypothalamic kisspeptin and GnRH concentrations were elevated after PND 7 (Semaan *et al.*, 2013; Luo *et al.*, 2016). In this study, hypothalamic *Kiss1* and *Gnrh1* mRNA levels in the rhGH rats were significantly higher than those in the control rats on PND 24, when kisspeptin is elevated in male rats. Thus, prepubertal rhGH administration might potentiate hypothalamic kisspeptin and GnRH production. In adult rodents, hypothalamic *Kiss1* and *Gnrh1* mRNA are downregulated by sex steroids (Smith, 2008; Ng *et al.*, 2009). In this study, circulating testosterone levels in the rhGH rats were significantly higher than those in the control rats on PNDs 24 and 31. Although the circulating LH levels of the rhGH rats did not differ from those of the control rats on PND 24 or 31, the pituitary *Lhb* mRNA levels in the rhGH rats were significantly lower than those in the control rats on PNDs 24 and 31, suggesting that elevated testosterone levels provide negative feedback for the expression of pituitary *Lhb*.

4.3. rhGH triggered IGF1 changes in the hypothalamus, liver, and testes of immature rats

IGF1 mediates GH-dependent and GH-independent anabolism and growth (Cheng & Chen, 1995; Kolodziejczyk *et al.*, 2003). In rodents and primates, hypothalamic IGF1 expression increases at puberty, which activates kisspeptin-GnRH neurons (Hiney *et al.*, 1996; Wolfe *et al.*, 2014; Huh *et al.*, 2021). In human and mouse brains, hypothalamic GnRH neurons express the IGF1 receptor (D'Ercole *et al.*, 1996; Divall *et al.*, 2010). In prepubertal female rats, an intracerebroventricular infusion of IGF1 stimulated the secretion of GnRH and might advance the onset of puberty (Hiney *et al.*, 1991 and 1996). In prepubertal male rats, a central infusion of IGF1 antiserum delayed pubertal development (Pazos *et al.*, 1999). Altogether, brain IGF1 is an important factor in the initiation of puberty. In this study, hypothalamic *Igf1* mRNA was increased in the rhGH rats on PND 24, suggesting that the activation of kisspeptin-GnRH expression can be attributed to the activation of hypothalamic IGF1. In addition, in the hypothalamus, sex steroids activate the GH-IGF1 axis, accelerating the growth and maturation of reproductive organs for puberty (Veldhuis, 1997). Together, increased hypothalamic *Igf1* mRNA in the rhGH rats on PND 24 might be attributable to the elevated testosterone levels, as well as the direct action of rhGH on hypothalamic *Igf1* expression. In contrast, on PND 31, hypothalamic *Igf1* mRNA levels in the rhGH rats were significantly lower than those in the control rats, which could be a result of negative feedback from elevated androgens at puberty in the rhGH rats. Stimulation of IGF1 production in the liver has been considered to be a major effect of GH (LeRoith & Roberts Jr, 2003; Bielohuby *et al.*, 2009). Systemic IGF1 plays a major modulatory role in testicular endocrine function. In the rhGH rats in this study, however, liver *Igf1* mRNA and blood IGF1 levels tended to increase, though not to a statistically significant degree, by PNDs 24 and 31. Similarly, in immature mice given rhGH, circulating IGF1 and hepatic cell *Igf1* mRNA levels did not increase (Mireuta *et al.*, 2014). Of note, in the rhGH rats in this study, testicular *Igf1* mRNA levels were significantly higher than those in the control rats on both PND 24 and 31. Similarly, in immature hypophysectomized rats, rhGH increased testicular IGF1 levels (Grizard, 1994; Laron & Klinger, 1998; Diez-Caballero *et al.*, 2006). Given that IGF1 and cognate receptor are expressed in germ cells, Leydig cells and Sertoli cells (Grizard, 1994; Wang & Hardy, 2004; Diez-Caballero *et al.*, 2006; Griffeth *et*

al., 2014), intratesticular IGF1 could regulate various aspects of testicular function in both autocrine and paracrine manners. In rodents, IGF1 can promote the proliferation, maturation, and steroidogenesis of Leydig cells by means of para- and autocrine action (Khan et al., 1992; LeRoith & Roberts Jr, 2003; Diez-Caballero *et al.*, 2006). In mouse Leydig cells, the expression of the LH receptor and response to LH are potentiated by IGF1 (Lin et al., 1986; Kasson & Hsueh, 1987). In the rhGH rat testes in this study, the number and size of HSD3B(+) and HSD17B(+) Leydig cells increased significantly. In addition, in the HSD3B(+) progenitor Leydig cells isolated on PND 21, rhGH treatment increased the expression of *Igf1* mRNA. Therefore, testicular increases in IGF1 could mediate functional differentiation of testosterone-producing Leydig cells in rhGH rats, and the increased circulating testosterone in rhGH rats may be due to the increased LH response in Leydig cells by the testicular IGF1. In the rhGH rats in this study, the elevated testicular IGF1 levels might be responsible for the activation of testosterone production and spermatogenesis (Fig. 9). In gonadotropin-independent precocious puberty (GIPP), sexual maturation is induced by sex steroids that increase through a gonadotropin-independent mechanism such as testotoxicosis, tumors, or environmental hormones (Kremer *et al.*, 1993; Traggiai and Stanhope, 2003). The elevation of testicular IGF1 through prepubertal administration of rhGH could thus evoke the early onset of sexual maturation without increasing circulating LH levels, resembling GIPP.

5. Conclusion

In prepubertal male rats, rhGH administration increased testicular but not systemic IGF1 levels, which potentiated testosterone production in Leydig cells and spermatogenesis.

Abbreviations

% (v/v) percent (volume per volume)

°C degree Celsius

µm micrometer

a.m. ante meridiem

CO₂ carbon dioxide

Cyp17a1 cytochrome P450 family 17 subfamily A member 1

ELISA enzyme-linked immunosorbent assay

GH growth hormone

GIPP gonadotropin-independent precocious puberty

GnRH gonadotropin-releasing hormone

Gnrh1 gonadotropin Releasing Hormone 1

HPG axis hypothalamus-pituitary-gonad axis

hr hour

HSD17b 17 β -hydroxysteroid dehydrogenase

Hsd17b3 hydroxysteroid 17 β dehydrogenase 3

Hsd3b 3 β -hydroxysteroid dehydrogenase

Hsd3b1 hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1

Igf1 insulin-like growth factor-1

IgG immunoglobulin G

IU international units

kg kilogram

Kiss1 KiSS-1 Metastasis Suppressor

L liter

LH luteinizing hormone

Lhb luteinizing hormone beta polypeptide

Lhcgr luteinizing hormone/choriogonadotropin receptor

mL milliliter

mRNA messenger RNA

PBS phosphate buffered saline

PCR polymerase chain reaction

PFA paraformaldehyde

PLC progenitor Leydig cell

PND postnatal day

Prm protamine

rhGH recombinant human growth hormone

RNA ribonucleic acid

Rpl7 ribosomal protein L7

rpm revolutions per minute

RPMI Roswell Park Memorial Institute medium

RT-qPCR real-time reverse transcription-polymerase chain reaction

SD standard deviation

Sf1 Steroidogenic factor 1

Star steroidogenic acute regulatory protein

T testosterone

Tnp1 transition protein 1

Tnp2 transition protein 2

µg microgram

Declarations

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics declarations

This study was approved by the Hanyang University Institutional Animal Care and Use Committee (HY-IACUC-11-021).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Contributions

YX and CYH contributed to the experiment performance. YX contributed to the manuscript writing and submission. MCG, MJP, YX, and CYH contributed to the experiment performance and manuscript revision. All have read this final version and have approved this submission.

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Figures

Figure 1

The effects of rhGH on testis weight, histology, seminiferous tubule diameter, and size of the luminal area in immature rats. (A) The testis weights in immature rats given rhGH. (B) Hematoxylin and eosin staining of testes from immature rats. (C) Image analysis of seminiferous tubule diameter (blue line) and luminal area (dotted line). (D) Diameter of seminiferous tubules from immature rats. (E) Size of the luminal area

of seminiferous tubules from immature rats. Values presented are the mean $\pm$ SD. *, significantly different from control rats by Student t-test at $p \leq 0.05$.

Figure 2

The effect of rhGH on spermatogenic marker mRNA in immature rats. Testicular Tnp1, Tnp2, and Prm2 mRNA levels were examined using RT-qPCR. Values are presented as the mean $\pm$ SD. * and **, significantly different from control rats by Student t-test at $p \leq 0.05$ and 0.01 , respectively.

Figure 3

The effects of rhGH on HSD3B(+) and HSD17B(+) Leydig cells in immature rat testes. (A) Immunohistochemistry of HSD17B in immature rat testes on PNDs 24 and 31. (B) Image analysis of HSD17B(+) Leydig cells. Red cross, nuclei of Leydig cells; black dotted line, boundary of HSD17B(+) Leydig cells; red dotted line, boundary of blood vessels. (C) Number of HSD17B(+) Leydig cells in each unit area (1 mm²) of the testes. (D) Mean size of HSD17B(+) Leydig cells. (E) Total number of

HSD17B(+) interstitial cells in the testes of immature rats on PNDs 24 and 31. Values are presented as the mean $\pm$ SD. * and **, significantly different from control rats by Student t-test at $p \leq 0.05$ and 0.01 , respectively. (F) Immunofluorescence of HSD3B in immature rat testes on PND 24. White cross, nuclei of Leydig cells; white dotted line, boundary of HSD3B(+) Leydig cells; white box, area shown in the high magnification photograph. (G) Number of HSD3B(+) Leydig cells in each unit area (1 mm^2) of the testes. (H) Mean size of HSD3B(+) Leydig cells. (I) Total number of HSD3B(+) interstitial cells in the testes of immature rats on PND 24. Values are presented as the mean $\pm$ SD. * and **, significantly different from control rats by Student t-test at $p \leq 0.05$ and 0.01 , respectively.

Figure 4

Effects of rhGH on blood testosterone levels in immature male rats. ELISA for blood testosterone levels on PNDs 24 and 31. Values are presented as the mean $\pm$ SD. *, significantly different from control rats by Student t-test at $p \leq 0.05$.

Figure 5

Effects of rhGH on the expression of steroidogenic pathway gene mRNA in immature male rats. RT-qPCR analysis of *Lhcgr*, *Sf1*, *Star*, *Cyp17a1*, *Hsd3b1*, and *Hsd17b3* mRNA levels in the testes on PNDs 24 and 31. Values are presented as the mean ± SD. * and **, significantly different from control rats by Student t-test at $p \leq 0.05$ and 0.01, respectively.

Figure 6

The effects of rhGH on hypothalamic Kiss1 and GnRH1 and pituitary Lhb mRNA and blood LH levels. (A–C) RT-qPCR results for Kiss1 and GnRH1 mRNA in the hypothalamus and pituitary Lhb mRNA levels on PNDs 24 and 31. Values are presented as the mean $\pm$ SD. (D) ELISA for blood LH levels on PNDs 24 and 31. Values are presented as the mean $\pm$ SD. *, significantly different from control rats by Student t-testing at $p \leq 0.05$.

Figure 7

The effects of rhGH on blood IGF1 levels and Igf1 mRNA levels in the hypothalamus, liver, and testes of immature rats on PNDs 24 and 31. (A) Igf1 mRNA levels in the hypothalamus, liver, and testes on PNDs 24 and 31. (B) ELISA for blood IGF1 on PNDs 24 and 31. Values are presented as the mean $\pm$ SD. *, significantly different from control rats by Student t-test at $p \leq 0.05$.

Figure 8

Changes in steroidogenesis in Leydig cells in vitro. (A) Purity of Leydig cells as shown by immunocytochemistry for HSD3B. (B–J) RT-qPCR results for *Igf1*, *Lhcgr*, *Sf1*, *Star*, *Cyp11a1*, *Cyp17a1*, *Cyp19a1*, *Hsd3b1*, and *Hsd17b3* mRNA in rhGH-treated Leydig cells in vitro ($n = 4$). (K) ELISA for testosterone in the culture medium of Leydig cells after 48 hrs of rhGH treatment ($n = 4$). Values are presented as the mean $\pm$ SD. *, significantly different from control rats by Student t-test at $p \leq 0.05$.

Figure 9

Local increase in IGF1 levels in the testes might be an important mediator for gonadal activation triggered by rhGH in immature rats. Changes in kisspeptin, GnRH, LH, IGF1, and testosterone in the hypothalamus-pituitary-testis axis following daily injections of rhGH from PND 21 to 23 or PND 21 to 30 are summarized. Testicular steroidogenesis, circulating testosterone, and spermatogenesis were higher than control levels in the rhGH rats on PNDs 24 and 31. Hypothalamic Kiss1, Gnrh1, and Igf1 mRNA levels were also higher than control levels in rhGH rats on PND 24, but they became lower than the control levels by PND 31. No significant change in circulating LH levels was observed on PND 24 or 31 in the rhGH rats. In prepubertal male rats, rhGH administration increased testicular but not systemic IGF1 levels, which potentiated testosterone production in Leydig cells and thus increased circulating testosterone, stimulating sexual maturation and spermatogenesis.

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