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Original Article

Expression of kisspeptin-GnRH system is down-regulated in hypothalamic arcuate nucleus of male rats with high-fat-diet

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Abstract: This study is to investigate the potential effect of obesity on Kisspeptin/Kiss1 r-GnRH/GnIH system in arcuate nucleus (ARC) of obesity rat model. Forty-two male rats were randomly divided into control group and test group (obesity model). Body parameters, serum metabolic parameters, and neuroendocrine hormones were assayed. Immunohistochemistry was used to determine the localization of Kisspeptin, Kiss1 r, GnRH, and GnIH. The protein levels of Kisspeptin, Kiss1 r, GnRH, and GnIH were measured by Western blot. QRT-PCR was performed to determine Kiss1-, Kiss1 r, GnRH, and GnIH-mRNA levels. Our results showed that the obesity model was successfully established. Lee index, fat coefficient, serum Leptin and TG levels of the nutritional obese rats were significantly increased ($P<0.05$), while serum GnRH and the ratio of GnRH/GnIH was significantly decreased ($P<0.05$). Both protein and mRNA levels of Kisspeptin and GnRH were decreased significantly in the test group than that of the controls ($P<0.05$). There was no obvious difference protein and mRNA expression levels of Kiss1 r and GnIH between the two groups ($P>0.05$). This study reveals that nutritional obesity can decrease serum GnRH levels and the ratio of GnRH/GnIH, which might be associated with down-regulation of kisspeptin and GnRH in Kisspeptin/Kiss1 r-GnRH/GnIH system in ARC of male rats.

Keywords: Obesity rats, kisspeptin, GnRH, GnIH, arcuate nucleus

Introduction

Modern society and economic development have dramatically altered the diet and lifestyle of individuals worldwide. There has been a sharp increase in the incidence of overweight and obesity [1]. One of the main features in obesity is that there is too much body fat which plays an important role in endocrine regulation. Obesity is associated with a variety of diseases, such as cardiovascular disease (CVD), type 2 diabetes mellitus (DM), and sleep apnea syndrome. Among the various effects of obesity, its impact on fertility has attracted attention recently [2]. Severe obesity affects the normal development of puberty, which could further lead to gonadal disjunction, menstrual disorders, and even infertility. Studies have shown that obesity is one of the important causes of male reproductive dysfunction [3], and the long period of disorders in energy metabolism regulation may lead to the declination of reproductive function in obese men [4].

It is well documented that Kisspeptin/Kiss1 r-GnRH/GnIH system play a significant role in central regulation of reproduction in the mammalian. The related research demonstrated that loss of function or mutations in Kisspeptin or Kiss1 r genes could cause infertility due to the lack of pubertal maturation and hypogonadotropic hypogonadism in mice and humans [5]. Kisspeptin neurons, located in the arcuate nucleus of the hypothalamus (ARC), secrete kisspeptin proteins that bind to Kiss1 r on GnRH neurons and stimulate GnRH secretion [6]. GnRH stimulates the anterior pituitary to release the gonadotropins, such as luteinizing

hormone (LH) and follicle stimulating hormone (FSH). LH and FSH act on gonads and promote synthesis and secretion of steroid hormones to adjust reproductive endocrine functions.

However, the recent findings indicated that GnRH was not the sole hypothalamic regulatory neuropeptide of vertebrate reproduction, and gonadotropin-inhibitory hormone (GnIH) is also an important neuroendocrine regulator of the HPT axis. GnIH-R is expressed on GnRH 1 neurons, and GnIH inhibits reproductive function by directly acting on GnRH 1 neurons [7] which playing a key role in the suppression of reproduction through inhibiting GnRH and gonadotropin secretion [8, 9].

Furthermore, it has been reported that obesity might affect male fertility through HPG axis, however concrete details have not yet been elucidated. It is found that under different state of energy metabolism in the body, there is difference in the hypothalamus Kisspeptin expression level, additionally, Kisspeptin/Kiss1 r system also plays a significant role in body energy metabolism regulation [10]. Sánchez demonstrates that nutritional stress, especially HFD, has a profound deleterious impact on metabolic and gonadotropic function, and HFD-induced obesity decreases hypothalamic Kiss1/kisspeptin expression [11]. Zhai et al. [12] also obtained similar results, of which the obese male mice hypothalamic Kiss1 mRNA expression level is significantly lower than that of the control group. However, Brown study indicated that hypothalamic Kiss1 mRNA level increased significantly in male SD rat that are fed a high-fat diet for 12 weeks [13]. In male mice after high-fat diet fed for 16 weeks, although the body weight is significantly higher than the control group, there was no statistically significant difference between obesity group and control group in hypothalamic Kiss1 and Kiss1 r mRNA levels [14].

Recently, emerging data support the concept that central Kisspeptin could be a link between metabolism and reproduction [15], and Kisspeptin has been advocated as a major signal for transferring body metabolism-related information to the neuroendocrine reproductive axis [15, 16]. However, few studies have involved changes of Kisspeptin/Kiss1 r-GnRH/GnIH as a whole under the background of obesity, especially in male rats. Hence, in the current study we established an obesity rat model, in order to determine the changes of Kisspeptin/Kiss1 r-GnRH/GnIH system and the potential roles of nutrition on Kisspeptin/Kiss1 r system in hypothalamic ARC of male rats.

Materials and methods

Animals modeling

Specific-pathogen-free (SPF) male SD rats at 6 weeks of age (body weight: 130.43±7.15 g) were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were maintained on a 12 h day/12 h night schedule (light on from 19:00 to 07:00 h) under a constant temperature (22±1°C) and humidity (60%). The rats were housed five per cage, and food and filtered tap water were provided ad libitum. Experimental procedures were in accordance with the Ethical Principles in Animal Research and approved by the National Research Institute for Family Planning Ethics Committee for Animal Research. All efforts were made to minimize animal suffering, and euthanasia was performed by CO_2 inhalation.

For modeling, forty-two male rats were randomly divided into control group (n=18 normal diet) and test group (n=24 high-fat diet). The normal control group and the test group were fed with a normal diet and a high-fat diet, respectively. The high-fat formula was as follows: 10% lard oil, 10% sucrose, 1.5% cholesterol, 0.5% bile salt, 5% yolk powder and 73% normal feed. Body weight and length (from the tip of the nose to the anus) of the rats were determined once a week for 16 weeks. The rats in the lower quartiles for weight gain (n=6) at the 16th week were defined as obesity resistant and excluded from the test group. Thus, 18 obese rats were enrolled.

General metabolic parameters and neuroendocrine hormones

After 16 weeks, all rats were anesthetized with CO2. Analytical balance was employed to weigh rats and visceral fat that surrounded kidney, testicles, and greater omentum. For relevant parameters calculation, the following formulas were applied: Lee index = [weight (g) × 10^3/ Body length (cm)] $^{1/3}$; fat coefficient = [visceral fat weight (g) * 100%/body weight (g)]. Blood samples were harvested from the abdominal

aorta, centrifuged at 2400 rpm for 20 min at 4°C, and frozen at -70°C. Then, the samples were used for the measurement of metabolic parameters including insulin (INS), leptin, triglycerides (TG), and glucose (GLU). In addition, serum hormone levels of Gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibitory hormone (GnIH) were determined, followed by calculating the ratio of GnRH/GnIH.

Serum GnRH and GnIH were tested by an enzyme-linked immunosorbent assay (ELISA) kit obtained from Beijing Northern Biological Technology Research Institute (Beijing, China). Serum TG, TC and GLU were measured with assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu Province, China). Serum Leptin and INS were detected with radioimmunoassays (RIAs) using a kit provided by Beijing Northern Biological Technology Research Institute (Beijing, China).

Brain tissue preparation

Rat brains were used for Western blot (n=6), qRT-PCR (n=6), and Immunohistochemistry (n=6) analysis for both groups. The brains were placed in a matrix with the ventral surface facing up. Three 1.0 mm coronal sections were made with the middle optic chiasma as the anterior boundary. The sections were placed on a glass slide and ARC (Bregma 2.6 mm to 3.3 mm) according to the atlas of Paxinos and Watson [17] and was rapidly micro-dissected under a microscope using the fornix and the third ventricle as landmarks. For the ARC, the area adjacent to the bottom of the third ventricle was dissected parallel to the border of the ventricle, with the width of 0.1 mm at the top gradually widening to 0.3 mm at the bottom. These dissections were immediately frozen in liquid nitrogen and stored at -80°C until processed.

Immunohistochemistry

Rats were perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer for brain collection and immunohistochemical staining. Coronal sections (4.0 mm thick) throughout the ARC were cut from each brain on a sliding microtome to provide three sets of consecutive sections. One set of sections was labeled for Kisspeptin by incubating with polyclonal rabbit anti-kisspeptin/kiss1 primary antibody (PLaboratories, CAN, 1:400), followed by biotinylated goat anti-rabbit IgG secondary antibody (Beijing Golden Bridge Biotechnology Company Ltd., Beijing, China). The signal was amplified using avidin-biotin peroxidase (Beijing Golden Bridge Biotechnology Company Ltd., Beijing, China) and then visualized with diaminobenzidine solution to assess the level of Kisspeptin (Abcam, ab19028, 1:1000), Kiss1 r (Alomone, AKR-001, 1:500), GnRH (Santa, sc-20941, 1:500), and GnIH (Santa, sc-32380, 1:200) immunoreactivity. Three sections of the ARC from each rat were counted. The other three sections were used for analysis of Kiss1 r, GnRH, and GnIH in the similar procedures.

Western blot analysis

For western blot analysis, brains were rapidly removed (n=6 rats in each group), and hippocampus were separated on ice and then stored at -80°C in plastic bags for further use. Thereafter, the brain tissue was homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease/phosphatase inhibitor mixture (Beyotime Institute of Biotechnology, China). Soluble protein was obtained by centrifugation at 14,000 g for 15 min at 4°C. The protein concentration of each sample was measured by bicinchoninic acid (BCA) protein assay (Beyotime Institute of Biotechnology, China). For immunoblotting, equal volume of brain tissue lysates were subjected to electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE Gel Preparation Kit, Beyotime Institute of Biotechnology, China). Separated proteins were then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were blocked in 5% nonfat milk (Sigma-Aldrich) in PBS at room temperature for an hour and then incubated with different primary antibodies at 4°C overnight. The primary antibodies used were as follows: Kiss1 (Abcam, ab19028, 1:1000), Kiss1 r (Alomone, AKR-001, 1:500), GnRH (Santa, sc-20941, 1:500), GnIH (Santa, sc-32380, 1:200), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:2000, Immunoway, YM3029, 1:20000). After washing with TBS-T for three times, membranes were incubated at room temperature for 1 h with HRP-conjugated relevant secondary antibodies (1:2000, Sangon Biotech, Shanghai, China), and then washed for another three times with TBS-T. At last, the antigen-antibody complexes were visualized with the ECL chemi-

Table 1. Sequences of primers for real time PCR

Name	Sequences of primers (5' to 3')	Product (bp)
Kiss1 forward	ACCCCAGGAACTCGTTAATGC	240
Kiss1 reverse	TAGCGCAGGCCAAAGGAGT	
Kiss1 probe	FAM-AGGGCCCGCGGTATGCAG-BHQ1	
Kiss1 r forward	TCGGGAACTCACTGGTCATCTT	83
Kiss1 r reverse	CGCCAGGTTAGCGATGTAGAAAT	
Kiss1 r probe	FAM-CCGCCACAAGCACATGCAGAC-BHQ1	
GnRH forward	GCCGCTGTTGTTCTGTTGACT	154
GnRH reverse	TCTGGGGTTCTGCCATTTGAT	
GnRH probe	FAM-CCAGCACTGGTCCTATGGGTTG-BHQ1	
GnIH forward	GGAATCCCAAAAGGGGTAAAG	168
GnIH reverse	GGGGCTTCTTCTGTCTTCTATGTT	
GnIH probe	FAM-AAAGATTGGGGGGCAAAGAAAGAT-BHQ1	
GAPDH forward	TTCCTACCCCCAATGTATCCG	87
GAPDH reverse	GCTTCACCACCTTCTTGATGTCAT	
GAPDH probe	FAM-ACATGCCGCCTGGAGAAACCT-BHQ1	

Table 2. General metabolic parameters and neuroendocrine hormones

	Control Group (n=18)	Obesity Group (n=18)	*P* Value
Body weight (g)	531.33±25.98	598.33±47.98	0.000**
Body length (cm)	26.46±1.03	26.75±0.58	0.406
Lee index	0.306±0.011	0.315±0.007	0.048*
Fat (g)	24.54±5.86	37.17±8.95	0.000**
Fat coefficient (%)	4.64±1.16	6.15±1.18	0.005**
Insulin (μIU/ml)	22.44±8.39	32.41±21.97	0.156
Leptin (ng/ml)	2.21±0.76	3.12±0.82	0.009**
Glucose (mmol/L)	7.77±1.19	8.19±1.62	0.475
TG (mmol/L)	0.38±0.18	0.57±0.16	0.010*
TC (mmol/L)	1.16±0.24	1.26±0.38	0.459
GnRH (pg/ml)	48.57±6.04	38.57±4.64	0.000**
GnIH (pg/ml)	37.10±3.59	34.52±3.45	0.086
GnRH/GnIH	1.314±0.159	1.125±0.147	0.006**

Note: Data are presented as mean ± SD *P<0.05, **P<0.01, compared with normal group. Lee index = [weight (g) × 10^3/body length (cm)] $^{1/3}$. Fat coefficient = [visceral fat weight (g) × 100%/body weight (g)]; TG, triglycerides; TC, total cholesterol; GnRH, Gonadotropin-releasing hormone; GnIH, Gonadotropin-inhibitory hormone; GnRH/GnIH, the ratio of serum GnRH/GnIH; s.e.m, standard error of mean.

luminescence system (Amersham) and exposed to Kodak X-OMAT film. The relative densities of bands were analyzed with NIH Image J.

Reverse transcriptase-polymerase chain reaction (qRT-PCR)

Transcription levels of Kiss1-, Kiss1 r-, LepR- and GnRH-genes were determined by qRT-PCR in the hypothalamic ARC from both groups. Total RNA was isolated from ARC using a TRIzol reagent kit (Invitrogen Co., Carlsbad, CA, USA). cDNA was synthesized with oligo (deoxythymidine) primers at 50°C, using the SuperScript III First-Standard Synthesis System for RT-PCR (Invitrogen Co.). Quantitative real-time PCR was run in triplicate using TaqMan PCR Master Mix (Roche). Real-time quantitative primers were designed and purchased from Applied Biosystems (Life Technologies Corporation). The selected forward and reverse primers are shown in **Table 1**.

The PCR cycling conditions were as follows: initial denaturation and enzyme activation at 95°C, for 30 s, followed by 40 cycles of denaturation at 95°C, for 5 s, annealing at 60°C, for 20 s, and extension at 72°C, for 1 min. The copy number of the transcripts was normalized against that of the GAPDH transcripts for each sample. Each sample was run in triplicate and quantified as the number of cycles (Ct) after which the fluorescence exceeded the background threshold minus the Ct for the GAPDH. Relative expression levels were calculated by formula $2^{-\Delta\Delta CT}$ method comparing obesity group and control group. The identity and purity of the amplified product were checked through analysis of the melting curve carried out at the end of amplification.

Statistical analysis

Statistical analysis was carried out with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). All data in this study were presented as mean ± SD. Data were analyzed with Student's t-test and analysis of variance. P<0.05 indicated significant difference.

Results

General metabolic parameters and neuroendocrine hormones

To testify the efficiency of modelling, the general metabolic parameters and neuroendocrine

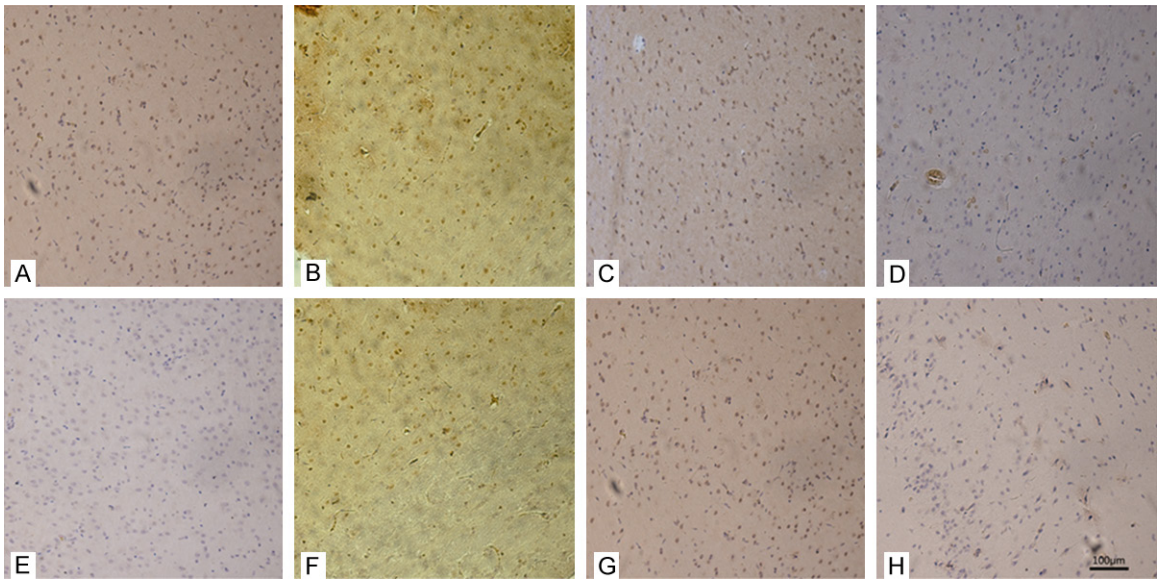

Figure 1. Effects of HFD on kisspeptin-, kiss1 r-, GnRH- and GnIH-IR expression in the ARC of rats. A, E: Kisspeptin; B, F: Kiss1 r; C, D: GnRH; E, F: GnIH. A-D: In the control group; E-H: In the obese group. Kisspeptin-(brown cytoplasmic staining), kiss1 r- (brown cytoplasmic staining), and GnRH (brown cytoplasmic staining), GnIH-IR (brown nuclear staining) cell bodies were detected. No immunoreactive fibers were observed in both groups.

hormones of the two groups were compared at the end of the 16th week. The results showed that the body weight, Lee index, fat, fat coefficient, serum Leptin and TG levels of the nutritional obese rats were significantly increased ($P<0.05$), while serum GnRH and the ratio of GnRH/GnIH were significantly decreased ($P<0.05$). No obvious differences were found in body length, serum INS, GLU, and GnIH levels between the two groups ($P>0.05$). The results argued that animal modeling of obesity was successfully performed (**Table 2**).

Detection of the localization of kisspeptin, kiss1 r, GnRH and GnIH proteins in the hypothalamic ARC of rats

To investigate the localization of these hypothalamus reproductive regulation genes coded proteins, immunohistochemistry was applied, Kisspeptin-, kiss1 r-, GnRH-, and GnIH-IR cell bodies could be detected in the ARC of male rats in the both groups (**Figure 1**).

Western-blot analysis on protein expressions of kisspeptin, kiss1 r, GnRH, and GnIH in hypothalamic ARC of both groups

To further determine the expression level of these hypothalamus reproductive regulation related proteins, Western-blot analysis was undertaken. Kisspeptin and GnRH protein expressions were significantly decreased in hypothalamic ARC of obese rats compared with the controls ($P<0.05$), while Kiss1 r and GnIH protein expressions were not significantly changed in hypothalamic ARC of obese rats compared to the controls ($P>0.05$). In conclusion, the results showed that HFD-induced obesity could decrease the protein levels of Kisspeptin and GnRH in the hypothalamic ARC of rats (**Figure 2**).

qRT-PCR analysis on mRNA expression levels of kisspeptin, kiss1 r, GnRH, and GnIH in hypothalamic ARC of the two groups

In order to detect the transcription of the hypothalamus reproductive regulation genes, qRT-PCR analysis was performed. Real-time RT-PCR results demonstrated that the mRNA levels of Kiss1 and GnRH genes were significantly decreased ($P<0.05$) in hypothalamic ARC of obese rats compared with that of the controls. However, expressions of kiss1 r and GnIH at the mRNA level were not significantly changed ($P>0.05$) in hypothalamic ARC of obese rats compared with the normal rats (**Figure 3**). Together, the results demonstrated that HFD-induced obesity could decrease the expression levels of Kiss1 and GnRH genes in hypothalamic ARC of rats.

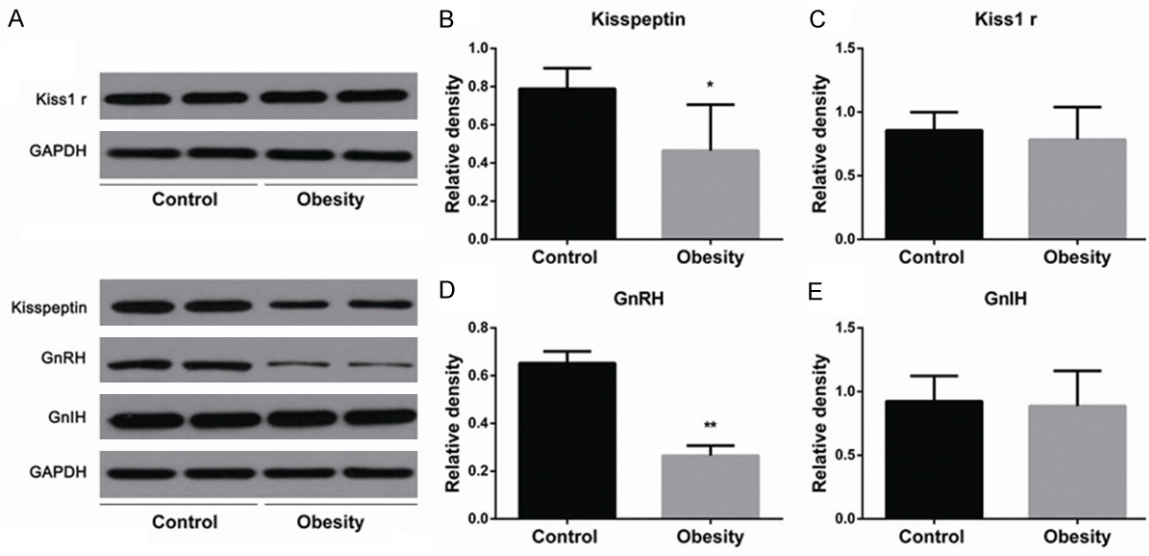

Figure 2. Image J quantitative analysis on Western blot bands of kisspeptin, kiss1 r, GnRH, and GnIH in hypothalamic ARC of obese rats compared to the controls. *indicates P<0.05, and **indicates P<0.01.

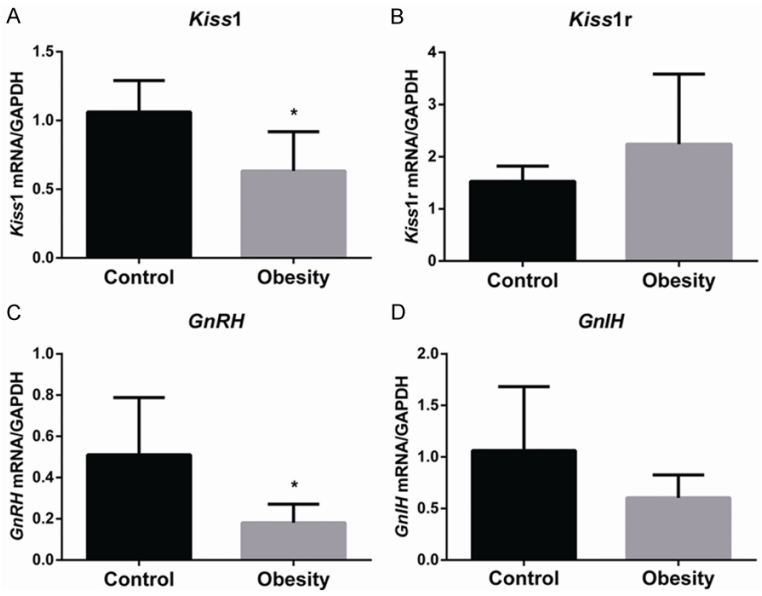

Figure 3. Quantification of Kiss1, Kiss1 r, GnRH, and GnIH mRNA expression, assessed by real-time RT-PCR. *indicates P<0.05.

Discussions

There is no unified standard for evaluation of obesity rats. MO Lee [18] proposed in 1929 using Lee's index to evaluate obesity of rats, which is currently and the most commonly used for evaluation of obese rats. In diet-induced model of obese rats reported by Levin BE, rats became obesity resistant under high-fat diet [19], and differences existed in their energy metabolism, endocrine, fat and sugar metabolism. Based on relevant literature and Lee index, one fourth rats in high-fat diet group were excluded in our study design since they were obesity resistant. The rest three-quarters rats were classified as the obesity group [20].

Human obesity share common features including polygenic inheritance, hyperleptinemia, hypercholesterolemia, hipertrigliceridemia, insulin resistance, and lipid metabolism disorder. Adipose tissue plays a crucial role in energy homeostasis, not only by storing triglycerides, but also in response to neural, nutrient, and hormonal signals mediated through adipokine secretion [1]. In this experiment, results showing that obese rats have excessive and abnormal accumulation of body weight, and increase in Lee index, body fat and fat coefficient. Besides, serum TG and Leptin of obese rats were also increased, indicating that the obese rats were developed into lipid metabolic disorder. The above results attest successful construction model of obese rats in our study.

Interestingly, in the present study, it showed that with increased accumulation of body mass, adipose tissue, serum Leptin, and TG. There is a significant decline in serum GnRH of obese rats as compared with the control. At the same time, serum INS and Glu manifested a trend of increase, while serum GnIH witnessed a trend of decrease significantly. It is well known that Leptin plays a role in the regulation of energy homeostasis and reproduction, and acts as a mediator in the crosstalk between adipose tissue [21]. Our study suggests that accumulation of body mass, adipose tissue, serum Leptin, and TG have a negative effect on serum levels of neuroendocrine hormones (lower GnRH). Thus, the mechanism through which adipose tissue and Leptin regulates the serum GnRH requires further investigation.

Serum GnRH promotes gonadotropin release whereas serum GnIH inhibits gonadotropin release. The normal ratio of serum GnRH/GnIH plays an important physiological role in reproductive hormone homeostasis. The higher the ratio of serum GnRH/GnIH, the stronger effect of promoting gonadotropin release, and vice versa. Interestingly, our previous experiment also found that the ratio of serum GnRH/GnIH in obese rats was significantly lower than that in the control group, indicating that effect of promoting the release of gonadotropin in obese rats was reduced. Ultimately, the metabolic activity of the HPT axis was inhibited. We proposed that the ratio of serum GnRH/GnIH might reflect the metabolic activity of HPT axis precisely compared with the individual serum concentration of GnRH or GnIH, Obesity genes express Leptin which is the main mediator of nutritional signals to reproduction through activating Leptin receptors, by combining Leptin with Lep R. This could affect a variety of neuroendocrine hormone secretion and adjust the feeding and control of energy balance. Lep R are highly expressed in arcuate nucleus (ARC) neurons, where they partially colocalize kisspeptin, one of the most potent regulators of the reproductive axis [6]. Our current data suggested that obesity rats kiss1 gene expression decreased, and Kiss1 r mRNA expression tended to be improved but without statistically significant. In this context, an appealing hypothesis is that HFD-induced obesity can decrease kisspeptin, but in order to maintain a delicate balance between stimulatory (Kisspeptin/GnRH) and inhibitory (GnIH) signals acting at central levels of the HPG axis, which exert a negative feedback effect and may cause augment the Kiss1 gene expression. However, due to the different levels of existence in obesity degree and observation stage of the experiment, there may exist different situation between the level of Kiss1 gene expression. This also precisely explains why in the state of obesity, hypothalamic Kiss1 mRNA expression level can show higher, lower or unchanged. We assume that, because of the existence of the negative feedback effect, HFD-induced obesity may decreases or increases for a certain time period, but it might reduce hypothalamic Kiss1/kisspeptin expression in the long term HFD fed.

Leptin related neuroscience research has shown that hypothalamic arcuate nucleus GnRH neurons do not exist in LepR without expression of ob-mRNA, and Leptin could not directly act on GnRH neurons [22]. But hypothalamic neuropeptide Y (neusopeptide Y, NPY) neurons exist on ob-R distribution and Leptin may be indirectly by NPY neurons to GnRH neurons that regulate reproductive function [23]. The investigators confirmed that obesity can lead to GnRH secretion declination and GnRH secretion of obesity mice was decreased obviously in their studies [12, 24, 25]. In our study, the serum concentration of GnRH, amount of protein and mRNA expression of the hypothalamus GnRH were reduced, suggesting that obesity developed an adverse impact on rat hypothalamus GnRH metabolism eventually.

In this study, serum GnIH concentration, protein and mRNA expression of hypothalamic GnIH showed a downward trend though there was no significant difference between the two groups. Indeed, experimental studies have verified that hypothalamic GnIH protein expression was significantly decreased after the injection of Leptin in rat lateral ventricle [26]. The possible explanation for decrease of GnIH is due to the existence of negative feedback mechanism. Kisspeptin and GnRH are stimulators for the HPG axis, whereas GnIH is the inhibitory opponent. In case Kisspeptin and GnRH levels in obese rats were decreased significantly, the body made an adjustment accordingly in order to maintain the balance between promotion and inhibition of gonadal hormone secretion.

In conclusion, we believe that obesity leads to a series of changes in the body metabolism, in which serum Leptin concentration will increase and Leptin resistance as core characteristics of metabolic disorder will occur. Furthermore, increase of serum Leptin will affect the senior nerve center of HPT axis, leading to decreased Kisspeptin levels and GnRH mRNA expression, serum GnRH and the ratio of serum GnRH/GnIH, which finally brings inhibitory action to the metabolic activity of HPT axis, yet Kisspeptin, GnRH, GnIH may present different changes due to the differences exists in extent and negative feedback effects.

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Disclosure of conflict of interest

None.

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