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Full Length Research Paper

Epimedium herb extract intervening in hypothalamic-pituitary-testicular axis of male rats in delayed puberty caused by diet-induced obesity

DongFang Zhang[1]*, Kun Li[2] and MeiHuan Zhou[3]

[1]College of Pharmaceutical Science, China Medical University, Shenyang, 110001, PR China.
[2]College of Chemistry and Chemical Engineering, Liaoning Normal University, Dalian 116029, PR China.
[3]Northeast Pharmaceutical Group Company Limited, Shenyang, 110023, PR China.

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Epimedium **Herb (Berberidaceae) is one of the traditional Chinese medicines (TCM) with unique effect on reproductive system in China for thousands of years. We aim to evaluate the potential activity in starting hypothalamic–pituitary–testicular (HPT) axis of male rats in delayed puberty. Delayed puberty model of male Sprague–Dawley(SD) rats were established with high-fat diet and were treated by** ***Epimedium*** **Herb Extract(EHE) or Testosterone Undecanoate (TU) for 8 weeks. Body weight, body length, Lee's index, testis weight, serum testosterone (T) and estradiol (E_2) were determined. Expresses of GnRH mRNA and LH mRNA were measured by RT-PCR. Body weight, body length, testes weigh and testosterone of the EHE groups and the TU group were significantly higher than those of high-fat model group. Lee's index of the EHE groups and the TU group was significantly lower that of the model group. E_2 of all groups were non-significance of difference. Expresses of GnRH mRNA and LH mRNA were increased significantly, however, TU had no work in this way. The results of the present research indicate that EHE is effective to start the HPT axis in puberty, and can significantly improve sexual developmental inhibition caused by diet-induced obesity.**

Key words: *Epimedium,* Yin yang huo, delayed puberty, obesity, traditional Chinese medicine TCM, testicular function.

INTRODUCTION

Among the 60 known species of genus *Epimedium* (Berberidaceae), some are used for medicinal purposes in traditional Chinese-system of medicine (TCM) and else-where as a cure for different diseases of reproductive system. *E. grandiflorum, E. sagittatum*, *E. koreanum*, *E. pubescens*, *E. brevicornum*, and *E. seittatum*, have a history of hundreds of year to combat erectile dysfunction, fatigue, kidney disorders, joint pain, and as hepatoprotective agent (Shindel et al., 2010). It was also used to protect old age memory impairment (May et al., 2008). *Epimedium* species are herbaceous flowering plants endemic to southern China, Europe and central, southern and eastern Asia. These plant species are known with their popular names as: Yin Yang Huo, barrenwort, and horny goat weed (Tillotson, 2009). In spring season, majority of these flowering plant species bear four-petaled spinder-shaped flowers, which are a part of recognized herbal aphrodisiacs (Chen and Chiu, 2006).

Various known and new chemical constituents were isolated and characterized in *Epimedium* species including flavonoids: chrysoeriol, quercetin, apigenin, kaempferol, and luteolin, epimedin A, epimedin B,

*Corresponding author. E-mail: lnsyzdf@sohu.com. Tel: +86-24-2325-6666-5271.

epimedin C, icariin, and baohuoside, however, icariin was reported to be the primary active component (Du et al., 2002; Luk et al., 2007**;** May et al., 2008; Chen et al., 2009). Prenylated flavonol glycosides, sagittasine A-C, ikarisoside A, icarisid II, yinyanghuo A-E, and epimedokoreanoside were also isolated. In addition, *Epimedium* species contained: acylated flavonol glycosides, together with flavonolignans, 2-phenoxychromones, lignin, and aromatic acid derivatives. The five major flavonoids of *Epidedium* and their variation in such plant species were analyzed (Jin et al., 2009; Xie et al., 2010). The leaves contained a variety of sterols, flavonoids, and an alkaloid called magnaflorine (Shen et al., 2007a, b). The presence of inhibitors for phosphodiesterase 5 and compounds with anti-osteoporosis potential were also confirmed in *Epimedium* species (Chen et al., 2009).

Epimedium herb is one of the oldest remedy in TCM, with unique effect on reproductive system (Chen et al., 2006). Usually, delayed puberty is defined as lack of secondary sexual characteristics from the age of 13 years in females and 14 years in males. It is one of the most common causes of constitutional delay in growth and puberty (CDGP), occurring mainly in boys. Delayed puberty is a chronic disease induced by hypernutrition, hypomotility, environment, estrogen, etc. (Wang et al., 2007). The puberty is a specific period for sexual development of male. Delayed puberty can induce poor sexual development, leading to sterility in males, which has serious social consequences. Therefore, it is suggested to be treated timely in puberty for its particularity (Hammoud et al., 2008; Pauli et al., 2008).

Diet-induced obesity is one of the major inducement. Diet composition of children is making great economic changes in developing countries. There is common trend of using excessive high-fat and high-protein foods. In return, the proportion of obese children is increasing, especially, the ratio of children in adolescence has now reached to 13% in China (Wang et al., 2007). Nevertheless, research on influence of obesity on male pubertal development remained relatively less, as compared to the work done on females. The treatment of male delayed puberty is an important problem which needs more attention to be resolved.

It is well established that in rodents, the intake of high-fat diet generally caused obesity (Warwick and Schiffman, 1992). In different experiments, rats fed with high fat diet showed increase in their body weight which seemed to be similar to human obesity. It was demonstrated and understood that increased free fatty acid or saturated fatty acid influx from high-fat diet might induce obese syndrome in rodents and humans (Woods et al., 2003). Pubertal obese model of animals was designed to scientifically demonstrate the effect of high-fat-diet (Wang et al., 2007).

Epimedium Herb is a unique drug in TCM which could clearly induce effects on nourishing kidney-yang. It could improve the hypothalamic–pituitary–testicular (HPT) axis in adults (Wang et al., 2009). Furthermore, it was also found to accelerate secretion of semen and elicit penile erection in the treated rats (Chen et al., 2006). But, the condition of puberty in children is different from adults. The current study was designed with the aim to evaluate the potential activity of triggering HPT axis of diet-induced obesity in male mice in puberty and to investigate any acceleration in sexual development.

MATERIALS AND METHODS

Chemicals and plant materials

Testosterone undecanoate (TU) were purchased from Nanjiang Ojianong Medicine Company. Sucrose, sodium tauroglycocholate and cholesterol were purchased from Sinopharm Chemical Reagent Company. *Epimedium* Herb was purchased from: Supply and Marketing Company, Northeast Pharmaceutical Group Company Limited.

Preparation of *Epimedium* Herb extract (EHE) and HPLC analyses

Epimedium Herb *was* ground into powder and refluxed in 70% alcohol (V/V) (100 g/800 mL, 1.5 h×2). The filtrate was concentrated under reduced pressure, and processed with HPD-100 macro-reticular resins (purchased from Cangzhou Baoen Chemical Industry Company). The resulting powder was used. Icariin in the powder were determined by high performance liquid chromatography (HPLC). The content of icariin in EHE was found to be 20.8%.

Grouping of animals and obesity model

Seventy three-week-old male Sprague–Dawley (SD) mice were supplied by the Experimental Animal Centre, Liaoning University of Traditional Chinese Medicine. The animals were housed in an air-conditioned room at an ambient temperature of 24℃ and 50 to 65% relative humidity with automatic 12-h light/12-h dark cycles. In normal control group (NC), ten rats fed with standard rodent chow, and the rest rats fed with high-fat chow (high-fat food including normal chow (74.5%), lard (12%), cholesterol (1.5%), Sucrose (5%), yolk powder (5%) and sodium tauroglycocholate (2%) for 3 weeks, weighted, and then, 40 rats were chosen from the rest 60 rats according to body weight, and were randomly subdivided into 4 groups (10 rats each): high-fat diet (HFD), high-fat diet and testosterone undecanoate (10 $mg \cdot kg^{-1}$) (HFD+TU), high-fat diet and EHE - 0.2 $g \cdot kg^{-1}$ (HFD + EHE - 0.2 $g \cdot kg^{-1}$), high-fat diet and EHE - 0.4 $g \cdot kg^{-1}$ (HFD + EHE - 0.4$g \cdot kg^{-1}$). The EHE dry powder of water extract was dissolved in water (20 $ml \cdot kg^{-1}$), and orally administered to the rats for 8 weeks. The normal control and high-fat diet groups received only water. The other groups were given high-fat chow. All animals received humane care during the study with unlimited access to chow and water (Wang et al., 2007).

Sampling harvesting

At the end of 14th week (treated for 8 weeks), the body weight and body length of all rats were measured and Lee's index (Lee's index = body weight (g) $^{1/3} \times 10^3$/body length (cm) was calculated (Stephens, 1980). The rats were anesthetized with 40 mg/kg sodium pentobarbital. Blood sample was collected from abdominal

Table 1. Effect of the EHE on Body weight, Body length, Lee's index, Testes weight and Testicular organ coefficient in rats.

Groups	Body weight (g)	Body length (cm)	Lee's index	Testes weight (g)	Testicular organ coefficient (g/100 g)
Control	371.9 ± 33.2	23.8 ± 0.95	299.6 ± 5.2	1.71 ± 0.08	0.46 ± 0.02
HFD	336.3 ± 26.2[a]	22.5 ± 0.84[a]	305.6 ± 2.6[a]	1.55 ± 0.10[a]	0.44 ± 0.02[a]
HFD+TU-10 mg·kg^{-1}	387.2 ± 21.8[b]	22.9 ± 0.80[a,b]	303.1 ± 3.1[a]	1.72 ± 0.12[b]	0.45 ± 0.02
HFD+EHE-0.2 g·kg^{-1}	382.6 ± 17.4[b]	23.1 ± 0.70[a,b]	301.0 ± 2.1[a]	1.68 ± 0.10[b]	0.45 ± 0.02[a]
HFD+EHE-0.4 g·kg^{-1}	391.4 ± 21.3[a,b]	23.3 ± 0.68[a,b]	316.5 ± 6.5[a,b]	1.72 ± 0.10[b]	0.45 ± 0.02[a]

All values are expressed as mean ± SE, [a] $p<0.05$, when compared with control rats, [b] $p<0.05$, when compared with high-fat model rats.

aorta, and then centrifuged at 3000 r/min×30 min, the serum was kept at -70℃.

The testes specimens were immediately collected, dried by filter paper, and weighted in the wet state, and then testicular organ coefficient (testis weight (g)/body weight (100 g) was calculated. The testes specimens were fixed in 10% phosphate-buffered formalin. The rats were inflicted into death; and hypothalamus and hypothesis were collected and snap-frozen in liquid nitrogen, and then the tissues were stored at -70℃.

Testis histological analysis

Formalin-fixed testis tissue was processed in 5 μm thick paraffin sections, which were stained with hematoxylin (HE) and eosin. The tissue sections were subjected to microscopic examination.

Biochemical analysis

The concentrations of T and E_2 were determined by enzyme linked immunosorbent assay (ELISA), and test kits were purchased from Adlitteram Diagnostic Laboratories, Ins., USA.

Expression of GnRH and LH mRNA

The tissues of hypothalamus and hypophysis were homogenated. The total RNA was isolated and purified by olig (dT), and then the RNA was amplified by the verse transcriptase polymerase chain reaction (RT-PCR)。The primers used for RT-PCR were as follows:

GnRH: forward 5'-CGAGAATTGTTGGAATGAAAGCC-3';
reverse 3'-GCGGAGAATGATTGATTGGGACACCGAA-5'

LH: forward 5'-CGTCTAGAGGCCTGTGTTGTGTCC-3'
reverse 3'-AAGGACTCCCCGACCGAAGGTA-5'

The express levels of GnRH and LH mRNA were detected (Cai et al., 2002).

Statistical analysis

All results were expressed as mean ± SE. The data were analyzed by using one-way analysis of variance (ANOVA) followed by Student's *t*-test using SPSS computer software version 16.0, Level of significance was fixed at 0.05.

RESULTS

Effect of EHE on body weight and body length

High-fat chow could suppress body development of rat. Body weight and body length of the high-fat diet group were significantly decreased, compared with those of the control group, while, Lee's index of the high-fat model rats was higher than that of normal control group. EHE and TU could accelerate body development. Body weight and body length of the EHE groups and the TU group were significantly higher than those of high-fat diet group, however, Lee's index of all high-fat diet groups was higher than that of control rats (Table 1).

Effect of EHE extract on testis weight and testicular organ coefficient

High-fat diet could restrain testicular development. Testes weight and testicular organ coefficient of the high-fat model rats were significantly lower than those of the control group, however, EHE and TU could enhance Testes weight significantly, compared with that of the high-fat model group, and made them reach normal level, compared with that of the control group (Table 1).

Effect of EHE on serum biochemical parameters

Testosterone secret was suppressed by high-fat chow. Testosterone of the high-fat model group was significantly lower than that of control group. EHE and TU could enhance level of testosterone and made it reach normal level, compared with that of the control group, and compared with that of high-fat model group. E_2 of all groups were non-significance of difference (Table 2).

Testes histological examination

Testis seminiferous tubule of the control rats developed normally. Spermatogenic cells were arranged tightly and the structure of the cells was clear. Spermatogium,

Table 2. Effect of the EHE on serum biochemical parameters and express of GnRH and LH.

Groups	T(ng)	E_2(ng)	GnRH mRNA	LH mRNA
Control	14.15 ± 1.76	202.4 ± 16.3	0.491 ± 0.011	0.454 ± 0.016
HFD	11.82 ± 2.68[a]	198.0 ± 26.0	0.430 ± 0.016[a]	0.411 ± 0.009[a]
HFD+TU-10 mg·kg^{-1}	18.32 ± 2.34[a,b]	204.6 ± 32.2	0.431 ± 0.021[a]	0.409 ± 0.021[a]
HFD+EHE-0.2 g·kg^{-1}	14.80 ± 3.68	199.6 ± 43.1	0.465 ± 0.025[a,b]	0.444 ± 0.021[b]
HFD+EHE-0.4 g·kg^{-1}	16.78 ± 2.34[a,b]	195.5 ± 14.8	0.511 ± 0.020[b]	0.445 ± 0.019[b]

All values are expressed as mean ± SE, [a] $p<0.05$, when compared with control rats, [b] $p<0.05$, when compared with high-fat model rats.

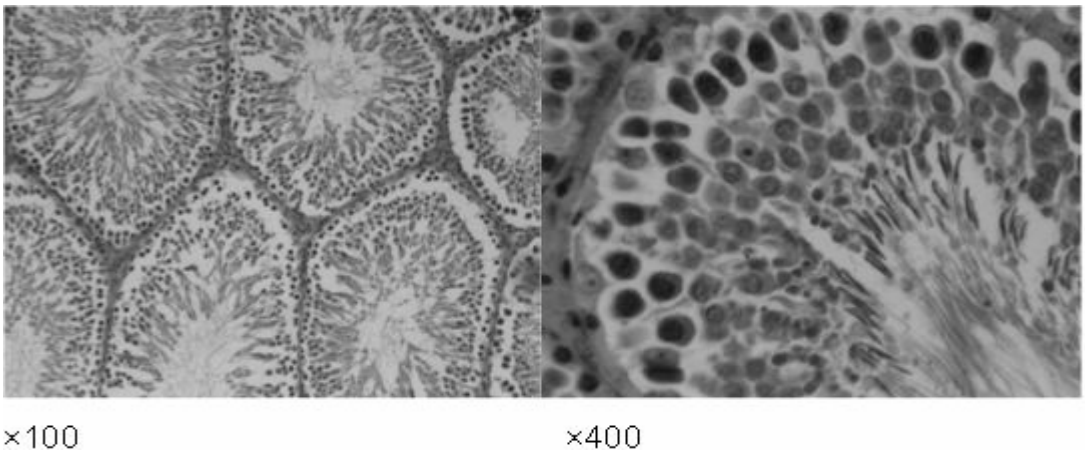

Figure 1. Photomicrograph of male rat testis orally administered with normal chow and distilled water (control). It shows seminiferous tubules develop normally. The seminiferous tubules contain sperm cells in various stages of maturation.

primary spermatocyte and spermatid in tuber could be all examined. Seminiferous tubule had been full-maturely developed (Figure 1). Wall of seminiferous tubule of high-fat model rats was thinner and number of spermatid was less. Cells falling off could be observed and individual spermatogenic cells separated from basement membrane and the clustered cells fallen in the seminiferous tuber (Figure 2); seminiferous tubule of the EHE groups and the TU group was the same as that of control group (Figures 3, 4 and 5). Drug treatment can make testis seminiferous tubule develop normally, avoiding suppression of fatness.

Effect of the EHE on expresses of GnRH and LH

The results showed that fatness could suppress the express of GnRH mRNA and LH mRNA. The expresses of the high-fat model group were significantly lower than those of the control group. The EHE could improve the levels of GnRH mRNA and LH mRNA, and made the expresses reach normal levels, but, TU had no work in this way (Table 2).

DISCUSSION

The puberty of male rat is from 6 to 10 weeks (Korenbrot et al., 1977). Three-week-old male rat in prepuberty was fed with High-fat diet, and diet-induced obesity model come into being in three weeks. The EHE was fed since 6th week. The rats were treated and put to death at 14th weekend (treated for 8 weeks), when the rat is adult. Body weight, body length, Lee's index can reflect body growth condition, T, E_2. The expression of GnRH mRNA and LH mRNA can reflect HPT axis function, testes weight and testis organ coefficient can reflect sexual development condition. The results show that the EHE can enhance T concentration, express of GnRH mRNA and express of LH mRNA. It can trigger the HPT axis. Body weight, body length, Lee's index, testes weight and testis organ coefficient data are just the results of start-up of the HPT axis (Clarke et al., 1982).

Researches show that Herba Epimedii and Fructus Ligustrilucidi can adjust the hypothalamic-pituitary-adrenal (HPA) axis (Liu et al., 2010), while, there is no any report about Herba Epimedii in the side of pubertal HPT axis. That the HPT axis is triggered timely is

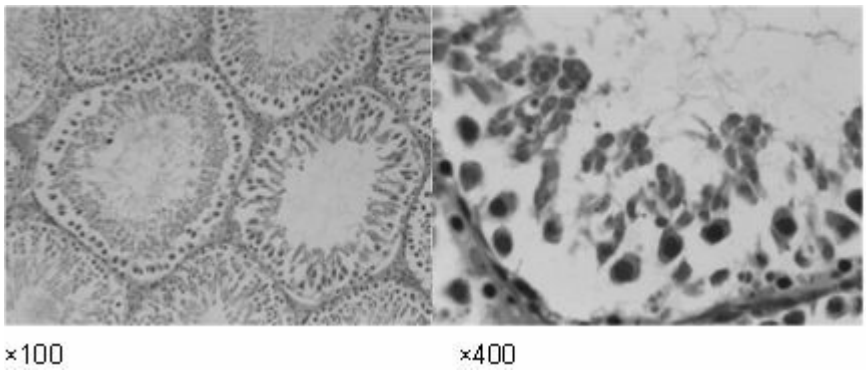

Figure 2. Photomicrograph of male rat testis orally administered with high-fat diet and distilled water (model). It shows wall of seminiferous tubule of model rats was thinner and number of spermatid was less. Cells falling off could be observed and individual spermatogenic cells separated from basement membrane and the clustered cells fallen in the seminiferous tuber.

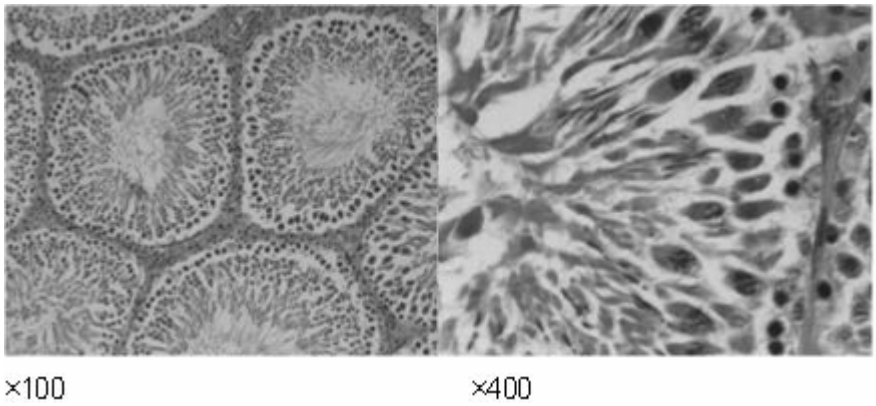

Figure 3. Photomicrograph of male rat testis orally administered with high-fat diet and 10mg·kg^{-1} body weight of TU. It shows seminiferous tubules develop normally. The seminiferous tubules contain sperm cells in various stages of maturation, and just like that of the control rat.

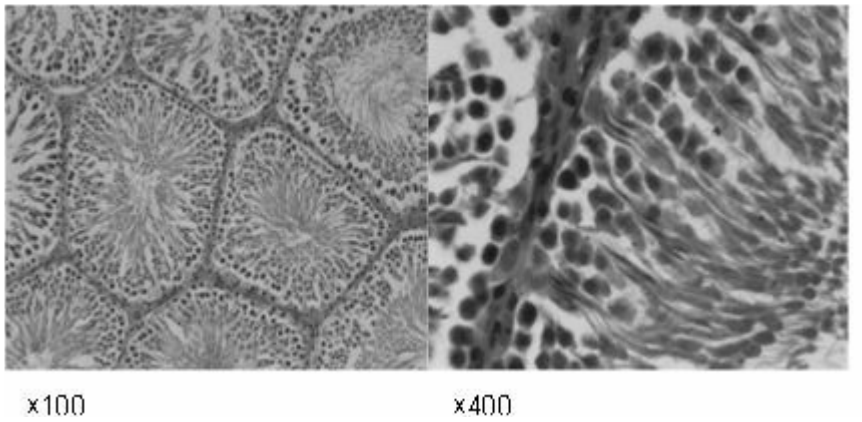

Figure 4. Photomicrograph of male rat testis orally administered with high-fat diet and 1.0 g·kg^{-1} body weight of EXD extract. It shows seminiferous tubules develop normally. The seminiferous tubules contain sperm cells in various stages of maturation, and just like that of the control rat.

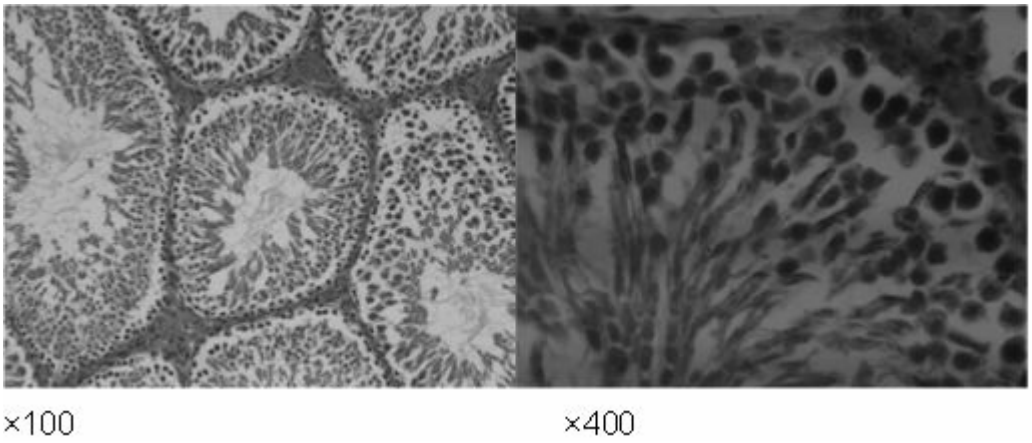

Figure 5. Photomicrograph of male rat testis orally administered with high-fat diet and 2.0 g·kg^{-1} body weight of EXD extract. It shows seminiferous tubules develop normally. The seminiferous tubules contain sperm cells in various stages of maturation.

important to growth and development of male children in puberty. The results of experiment show that the diet-induced obesity can suppress start-up of the HPT axis and the EHE can improve the inhibition action of obesity. The express of GnRH and LH mRNA of the high-fat model rats is significantly lower than that of control rats. It expresses that obesity can suppress the release of GnRH and LH in hypothalamus or hypothesis, and suppress the run-up of the HPT axis. The data obtained on GnRH and LH mRNA of the EHE treated rats, were significantly higher than that of the high-fat model rats and comparable to the control rats. It well demonstrated that EHE can elevate the express levels of GnRH and LH mRNA and startup the axis. Testosterone of the high-fat model rats was significantly lower than that of control rats, while, EHE treatment was found to enhance the level of testosterone, and facilitate sexual development, accelerate body growth, and testis development in this paper.

Start-up of puberty is also start-up of the hypothalamic–pituitary–testicular (HPT) axis. It needs adequate body weight and fatness, but, hyperadiposity can suppress the HPT axis and delay development of testis and sperm production. Clinical and pharmacological researches express that many hormones are correlated with functions of the HPT axis and influence directly and indirectly sexual development in puberty, especially, GnRH, LH and testosterone play very important roles in pubertal development of male (Kimihiko et al., 2010).

Common treatment drugs for delayed puberty including testosterone undecanoate, testosterone intramuscular and oxandrolone (Stanhope et al., 1985; Mushtaq et al., 2007), but, the side effect of these drugs were obvious. Natural testosterone and its esters rarely produce side-effects, but exogenous testosterone suppresses the production of lutenizing hormone and follicle stimulating hormone, and leads to reduced testicular sperm production, and, consequently, reduced testicular volume (Rolf et al., 1998). Androgen is considered normal in adult men and is inappropriate in children (Rolf et al., 1998). The results of the current study showed that TU has effect in start-up of puberty, but not in start-up of the HPT axis. *Epimedium*, the traditional Chinese medicine (TCM) is advantageous in this side.

Conclusions

The results of the present study indicated that EHE is effective to trigger the HPT axis in puberty, and can significantly improve the inhibition of the growth and development of rats caused by diet-induced obesity. The results provide an experimental evidence in treating male delayed puberty. It is, therefore, suggested to develop EHE as a drug to intervene early in pubertal development.

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