Heshouwu decoction, a Chinese herb for tonifying kidney, ameliorates hypothalamic-pituitary-testicular axis secretion in aging rats

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Abstract
An increasing amount of evidence demonstrates the anti-aging effect of Heshouwu in pill form. In this study, a subacute aging rat model was established by continuous intraperitoneal injection of D-galactose and treated with Heshouwu decoction (a Chinese herb for tonifying the kidney, comprising Heshouwu pill, Herba Epimedii, Radix Salviae Miltiorrhiae, and Poria). Heshouwu pill treated rats were the positive control group. Radioimmunoassay, immunohistochemical staining, and western blot assay showed hypothalamic gonadotropin-releasing hormone, hypothalamic substance P, and serum gonadotropin levels to be significantly increased in the model rats; the concentrations of hypothalamic β-endorphin, and serum levels of insulin-like growth factor 1 and testosterone were significantly decreased. 17β- and 3β-hydroxysteroid dehydrogenase expression in testicular tissue was also decreased. Intragastric administration of Heshouwu decoction at high (9.6 g/mL/100 g), medium (4.8 g/mL/100 g), and low (2.4 g/mL/100 g) doses, Heshouwu decoction pretreatment at a medium dose (4.8 g/mL/100 g), and Heshouwu pill (2.06 g/mL/100 g) significantly reversed these changes. Heshouwu decoction pretreatment and high-dose Heshouwu decoction had the greatest anti-aging effects. These experimental findings indicate that Heshouwu decoction can improve hypothalamic-pituitary-testicular axis secretion in a subacute aging rat model, and prevent and delay gonadal axis aging, with an effect superior to that of Heshouwu pill.

Key Words
Heshouwu decoction; pituitary gonadal axis; aging; gonadotropin-releasing hormone; gonadotropin; hydroxysteroid dehydrogenase; hypothalamic-pituitary-testicular axis; neural regeneration

Research Highlights
(1) Gonadotropin-releasing hormone, substance P, and gonadotropic hormone are significantly increased in the hypothalamic-pituitary-testicular axis of aging rats, whereas β-endorphin, insulin-like growth factor-1, androgen, and testosterone were significantly decreased. 17β- and 3β-hydroxysteroid dehydrogenase expression in testicular tissue was significantly increased. Intragastric administration of Heshouwu decoction at high (9.6 g/mL/100 g), medium (4.8 g/mL/100 g), and low (2.4 g/mL/100 g) doses, Heshouwu decoction pretreatment at a medium dose (4.8 g/mL/100 g), and Heshouwu pill (2.06 g/mL/100 g) significantly reversed these changes. Heshouwu decoction pretreatment and high-dose Heshouwu decoction had the greatest anti-aging effects. These experimental findings indicate that Heshouwu decoction can improve hypothalamic-pituitary-testicular axis secretion in a subacute aging rat model, and prevent and delay gonadal axis aging, with an effect superior to that of Heshouwu pill.

Abbreviations
GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; IGF-1, insulin-like growth factor 1; HSD, hydroxysteroid dehydrogenase
INTRODUCTION

Aging is an irreversible, natural phenomenon, and gonadal hormones may directly or indirectly regulate the aging process[1]. This study aimed to improve gonadal hormone levels, which are closely related to human aging. An increasing amount of evidence has demonstrated the anti-aging effect of Heshouwu decoction, and the underlying mechanism may depend on the following factors[2-3]: (1) improved antioxidant capacity and regulation of blood lipids; and (2) influences on p53/pRb related protein expression in the aging pathway in testicular cells. However, the influence of Heshouwu decoction on hypothalamic-pituitary-testicular axis secretion and on the activity of the key enzymes in testosterone biosynthesis remains unclear.

In the present study, Heshouwu decoction pretreatment and treatment was given to a D-galactose-induced rat aging model and hypothalamic-pituitary-testicular axis secretion was investigated in an attempt to understand the regulatory effect on gonadal axis secretion.

RESULTS

Quantitative analysis of experimental animals

Eighty Sprague-Dawley rats, after 1 week of adaptive feeding, were divided randomly into seven groups. Normal group (n = 10) rats were injected intraperitoneally with normal saline. For the model group (n = 10), subacute aging was induced by intraperitoneal injection of D-galactose. In the medium-dose Heshouwu decoction pretreatment group (n = 12), rats were pretreated with 4.8 g/mL/100 g Heshouwu decoction to observe the protective effect of this drug against D-galactose-induced subacute aging. In the Heshouwu decoction treatment groups (n = 12), following intraperitoneal injection of D-galactose, rats were injected with Heshouwu decoction at high (9.6 g/mL/100 g), medium (4.8 g/mL/100 g), or low (2.4 g/mL/100 g) dose, or given Heshouwu in pill form (2.06 g/mL/100 g) suspension, to observe the therapeutic effects on D-galactose-induced subacute aging. In the natural recovery group, model rats were allowed to recover spontaneously, to determine whether recovery from D-galactose-induced subacute aging can occur naturally over time. All 80 rats were involved in the final analysis.

17β-hydroxysteroid dehydrogenase (HSD) and 3β-HSD expression in rat testicular tissue

Immunohistochemical staining showed that 17β-HSD and 3β-HSD were expressed in a dispersed pattern in the cytoplasm of rat Leydig cells. Semi-quantitative analysis by western blot assay demonstrated that 17β-HSD and 3β-HSD protein expression in the model rats was significantly lower than that in the normal group (P < 0.01). Expression was increased to varying degrees in the Heshouwu decoction pretreatment group, the Heshouwu decoction treatment groups, and the Heshouwu pill group (P < 0.05). Levels in the Heshouwu decoction pretreatment group and the high-dose Heshouwu decoction treatment group were higher than those in the other groups (P < 0.05; Table 1, Figures 1 and 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>17β-HSD (mean ± SD)</th>
<th>3β-HSD (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.83±0.04ab</td>
<td>0.71±0.01ab</td>
</tr>
<tr>
<td>Model</td>
<td>0.12±0.01</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>Spontaneous recovery</td>
<td>0.19±0.02h</td>
<td>0.18±0.02h</td>
</tr>
<tr>
<td>Heshouwu decoction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium-dose pretreatment</td>
<td>0.72±0.03ab</td>
<td>0.63±0.01ab</td>
</tr>
<tr>
<td>High dose treated</td>
<td>0.68±0.02ab</td>
<td>0.65±0.02ab</td>
</tr>
<tr>
<td>Medium dose treated</td>
<td>0.39±0.01ab</td>
<td>0.36±0.02ab</td>
</tr>
<tr>
<td>Low dose treated</td>
<td>0.44±0.03*</td>
<td>0.39±0.01*</td>
</tr>
<tr>
<td>Pill treated</td>
<td>0.52±0.02*</td>
<td>0.48±0.03*</td>
</tr>
</tbody>
</table>

*P < 0.01, vs. model group; **P < 0.05, vs. pill treated group. The absorbance ratio of 17β-HSD and 3β-HSD to β-actin is expressed as mean ± SD, n = 8 for each group, using one-way analysis of variance and the Student-Newman-Keuls test. HSD: Hydroxysteroid dehydrogenase.

These symptoms were apparently reversed after intragastric administration of Heshouwu decoction at high (9.6 g/mL/100 g), medium (4.8 g/mL/100 g), and low (2.4 g/mL/100 g) doses, by Heshouwu decoction pretreatment (4.8 g/mL/100 g), and by Heshouwu pill (2.06 g/mL/100 g).

Gonadotropin-releasing hormone (GnRH), substance P and β-endorphin changes in rat hypothalamus

Radioimmunoassay showed that hypothalamic GnRH and substance P levels were significantly increased in the model rats compared with the normal group (P < 0.01), whereas β-endorphin was significantly decreased (P < 0.01). High-dose Heshouwu decoction and Heshouwu decoction pretreatment were superior to the other treatments (P < 0.05; Table 2).

Serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and insulin-like growth factor 1 (IGF-1)

Radioimmunoassay showed that serum LH and FSH levels in the model rats were significantly higher than those in the normal group (P < 0.01), whereas testosterone levels were significantly decreased (P < 0.01).
Enzyme-linked immunosorbent assay demonstrated that the IGF-1 level in aging rats was significantly lower than that in the normal rats ($P < 0.01$). Serum LH and FSH levels were decreased to varying degrees in the Heshouwu decoction pretreatment group, the Heshouwu decoction treated groups, and the Heshouwu pill treated group, but IGF-1 and testosterone levels were increased ($P < 0.01$). Heshouwu decoction pretreatment and high-and medium-dose Heshouwu decoction were superior to low-dose Heshouwu decoction and Heshouwu pill ($P < 0.05$; Table 3).

Table 2  The effect of Heshouwu decoction on GnRH, SP, and β-EP levels in rat hypothalamus

<table>
<thead>
<tr>
<th>Group</th>
<th>GnRH (pg/mg)</th>
<th>SP (μg/mL)</th>
<th>β-EP (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>126.3±7.33</td>
<td>2.35±0.92</td>
<td>168.7±9.01</td>
</tr>
<tr>
<td>Model</td>
<td>268.9±11.2</td>
<td>8.01±0.68</td>
<td>94.8±6.33</td>
</tr>
<tr>
<td>Spontaneous recovery</td>
<td>258.7±12.3</td>
<td>7.81±0.66</td>
<td>103.7±11.4</td>
</tr>
<tr>
<td>Heshouwu decoction</td>
<td>168.9±10.3</td>
<td>3.69±0.12</td>
<td>152.5±6.33</td>
</tr>
<tr>
<td>Medium-dose pretreatment (4.8 g/mL/100 g)</td>
<td>156.7±9.63</td>
<td>3.45±0.55</td>
<td>154.1±1.51</td>
</tr>
<tr>
<td>High dose treated (9.6 g/mL/100 g)</td>
<td>173.1±8.24</td>
<td>4.69±0.12</td>
<td>136.2±6.22</td>
</tr>
<tr>
<td>Medium dose treated (4.8 g/mL/100 g)</td>
<td>201.4±10.6</td>
<td>5.32±0.34</td>
<td>121.9±9.03</td>
</tr>
<tr>
<td>Low dose treated (2.4 g/mL/100 g)</td>
<td>198.3±9.01</td>
<td>6.92±0.12</td>
<td>109.4±3.69</td>
</tr>
<tr>
<td>Pill treated (2.06 g/mL/100 g)</td>
<td>198.3±9.01</td>
<td>6.92±0.12</td>
<td>109.4±3.69</td>
</tr>
</tbody>
</table>

*P < 0.01, vs. model group; *P < 0.05, vs. pill treated group. Data are expressed as the mean ± SD, n = 8 for each group, using one-way analysis of variance and the Student-Newman-Keuls test. GnRH: Gonadotropin-releasing hormone; SP: substance P; β-EP: beta-endorphin.

Table 3  The effect of Heshouwu decoction on serum levels of FSH, LH, IGF-1, and T in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>FSH (mIU/mL)</th>
<th>LH (mIU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.1±0.01</td>
<td>2.5±0.36</td>
</tr>
<tr>
<td>Model</td>
<td>11.9±1.02</td>
<td>10.6±0.33</td>
</tr>
<tr>
<td>Spontaneous recovery</td>
<td>12.3±1.11</td>
<td>9.2±0.22</td>
</tr>
<tr>
<td>Medium-dose pretreatment (4.8 g/mL/100 g)</td>
<td>4.6±0.12</td>
<td>4.9±0.19</td>
</tr>
<tr>
<td>High dose treated (4.8 g/mL/100 g)</td>
<td>3.2±0.16</td>
<td>4.2±0.39</td>
</tr>
<tr>
<td>Medium dose treated (4.8 g/mL/100 g)</td>
<td>5.9±0.38</td>
<td>6.1±0.54</td>
</tr>
<tr>
<td>Low dose treated (2.4 g/mL/100 g)</td>
<td>8.9±0.44</td>
<td>8.8±0.33</td>
</tr>
<tr>
<td>Pill treated (2.06 g/mL/100 g)</td>
<td>7.2±0.31</td>
<td>7.1±0.12</td>
</tr>
<tr>
<td>Heshouwu decoction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium-dose pretreatment (4.8 g/mL/100 g)</td>
<td>275.8±8.01</td>
<td>4.3±0.53</td>
</tr>
<tr>
<td>High dose treated (9.6 g/mL/100 g)</td>
<td>289.7±6.09</td>
<td>3.9±0.15</td>
</tr>
<tr>
<td>Medium dose treated (4.8 g/mL/100 g)</td>
<td>256.9±11.6</td>
<td>3.1±0.09</td>
</tr>
<tr>
<td>Low dose treated (2.4 g/mL/100 g)</td>
<td>201.3±6.33</td>
<td>1.9±0.14</td>
</tr>
<tr>
<td>Pill treated (2.06 g/mL/100 g)</td>
<td>220.3±10.3</td>
<td>2.1±0.09</td>
</tr>
</tbody>
</table>

*P < 0.01, vs. model group; *P < 0.05, vs. pill treated group. Data are expressed as the mean ± SD, n = 8 for each group, using one-way analysis of variance and the Student-Newman-Keuls test. FSH: Follicle-stimulating hormone; LH: luteinizing hormone; IGF-1: insulin-like growth factor 1; T: testosterone.
DISCUSSION

The D-galactose-induced subacute aging model is based on Senescence Metabolism Theory. The aging phenomena induced by D-galactose reflect the natural state of senescence within a short period of time, so this model is commonly used\[4-5\]. The drug is administered via intraperitoneal injection at a dose of 40–500 mg/kg per day for 20–60 days\[6\]. This study used intraperitoneal injection of D-galactose to produce a subacute aging model in the rat.

Increasing evidence indicates the anti-aging effects of various single herbs and herbal compounds. Wang et al\[7\] demonstrated that saponins in Radix Ginseng, American Ginseng, and Radix Notoginseng can scavenge free radicals. Barbarum polysaccharide also exhibits an antioxidant capacity, with effects such as improving superoxide dismutase activity in the serum, heart, liver, and brain tissue in a D-galactose-induced aging mouse model, reducing malondialdehyde, and increasing serum and cardiac telomerase activity\[8\].

Weng et al\[9\] studied the influence of Ganoderma tinctures A and B (two monomer compounds) in Ganoderma on the replication and survival of Saccharomyces cerevisiae, and found an anti-aging effect. Many Chinese herbal compounds, such as Apozem of Tremella and Wolfberry Fruit, Yangzhen Oral Liquid, and Yishuotiaozi Tablet, increase the activity of antioxidant enzymes, reduce malondialdehyde accumulation, and improve immunity. Vitality Reinforcing Prescription downregulates expression of the tumor suppressor gene p53 in spleen and liver cells of aging male mice at the transcription and translation levels. Jinguishenqi Pills are well known as antagonists of the DNA damage caused by cyclophosphamide. Liuwei Dihuang decoction can prolong the survival of Drosophila and increase telomerase activity in the brain and gonadal tissue of aging mice\[10\]. However, there is little evidence available on pituitary-testicular axis secretion, and the present study is the first demonstration of the action of Heshouwu decoction on pituitary-testicular axis secretion.

Heshouwu decoction can regulate serum antioxidant capacity and lipid metabolic disorders\[2\], through regulation of Rb/p16 and p53/p19/p21, this decoction also relieves cell conduction blockade, promotes the proliferation of ovarian and testicular cells, and inhibits apoptosis\[3, 11-13\]. Less attention had been paid to the regulation by Heshouwu decoction of testosterone. Our study showed that serum testosterone levels were significantly lower in aging rats than in the normal group, and Heshouwu decoction apparently improved serum testosterone levels in a dose-dependent manner.

Pretreatment with Heshouwu decoction achieved optimal anti-aging effects. Testosterone synthesis and secretion are modulated by hypothalamic GnRH, peptide neurotransmitters, gonadotropins, and growth hormones secreted from the pituitary gland. Pituitary endocrine dysfunction is closely related to hypothalamic regulatory function\[14\], and the pulse and wave coordination of pituitary gonadotropic hormone and sex hormone secretion is decreased or absent in older males and females\[15-16\]. The hypothalamus-generated neuropeptides substance P and β-endorphin are also involved in GnRH synthesis and secretion\[17\]. By a feedback mechanism, testosterone can inhibit hypothalamic GnRH, pituitary FSH, LH, and IGF-1 levels. The role of Heshouwu decoction in this process has not yet been elucidated.

In the present study, increased levels of hypothalamic substance P and GnRH, significantly decreased β-endorphin, and higher levels of serum FSH, LH, and IGF-1 were observed in aging rats and normal rats, and reversed by Heshouwu decoction. There is evidence that kidney-tonifying Chinese herbs regulate testosterone through hypothalamic GnRH secretion and release of the neurotransmitters β-endorphin and substance P, thereby affecting the secretion of FSH, LH, and IGF-1, or through the regulation of testosterone secretion by Leydig cells, providing negative feedback regulation of hypothalamic and pituitary function when gonadal axis secretion becomes abnormal as aging proceeds. Our findings support the conclusion that kidney-tonifying Chinese herbs regulate testosterone levels through the hypothalamic-pituitary axis.

Leydig cell-secreted steroid dehydrogenases play an important regulatory role in testosterone synthesis and secretion. Various factors contribute to the influence on testosterone synthesis of 3β-HSD and 17β-HSD activity\[18-20\], but the role of Heshouwu decoction on 3β-HSD and 17β-HSD synthesis and secretion in Leydig cells is unclear. This study observed 3β-HSD and 17β-HSD expression in testis tissue from aging rats and explored the regulatory effect of Heshouwu decoction.

We found that 3β-HSD and 17β-HSD protein expression was significantly lower in aging rats than in normal rats, and Heshouwu decoction upregulated their expression in a dose-dependent manner. These experimental findings indicate that Heshouwu decoction can increase testosterone secretion in Leydig cells and regulate 3β-HSD and 17β-HSD levels. In summary, Heshouwu decoction can ameliorate hypothalamic-pituitary-testicular axis secretion in a subacute aging rat model, and prevent and delay gonadal axis aging, with an effect greater than that of Heshouwu pills.
MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
Experiments were performed from November 2009 to September 2010 at the Cell Biology Laboratory, School of Basic Medical Science, Hebei University, China.

Materials

Animals
Ninety clean, healthy male Sprague-Dawley rats, weighing 180–220 g, were provided by the Experimental Animal Center of Hebei Medical University, China (license No. 1005050). All experimental use of animals complied with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China.[21]

Chinese medicine compounds
Heshouwu decoction prescription: The decoction comprised Radix Polygoni Multiflori, Herba Cistanches, Achyranthes bidentata Blume, epimedium, Salvia miltiorrhiza, and tuckahoe (Traditional Chinese Medicine Hospital of Hebei Province, China) according to the ratio 3:2:3:2:5:3, immersed in eight times the volume of distilled water for 1 hour. The herbs were then decocted twice in simmer water, for 30 minutes each time. The decoction was condensed to high-, medium-, and low-dose liquids containing 2.4, 4.8, and 9.6 g/mL crude drug, respectively. All decoctions were stored at 4°C and rewarmed to 25–30°C before administration.

Heshouwu pill prescription: The pill comprised Radix Polygoni Multiflori, Herba Cistanches, and Achyranthes bidentata Blume (Traditional Chinese Medicine Hospital of Hebei Province, China) according to the ratio 3:2:3, immersed in eight times the volume of distilled water for 1 hour. The herbs were then decocted twice in simmer water, for 30 minutes each time. The decoction was condensed to liquid containing 2.06 g/mL crude drug, which was stored at 4°C and rewarmed to 25–30°C before administration.

Methods

Establishment of subacute aging rat model
Eighty 8-week-old, clean grade, healthy male Sprague-Dawley rats, weighing 180–220 g, were used in this study to produce the subacute aging model. Under aseptic conditions, D-galactose (Beijing Chemical Reagent Company, Beijing, China) was soaked in normal saline to prepare a 6% solution, which was given to the rats via intraperitoneal injection at a dose of 300 mg/kg per day for 60 consecutive days.[3-6] Other groups of rats were injected with normal saline solution for 60 days, once per day.

Rat testis tissue and brain tissue samples
Rats were killed under 10% chloral hydrate anesthesia, and blood samples of 2 mL were collected using a capillary pipette inserted into the medial orbital venous plexus and centrifuged at 800 × g for 20 minutes. The supernatant was discarded and stored at -80°C. Following thoracotomy, cannulation was performed from the left ventricle to the ascending aorta for rapid infusion of cold saline (approximately 200 mL), then bilateral testicular tissue and brain tissue were rapidly removed. The left testis and the brain tissue were preserved in liquid nitrogen for 30 minutes and stored at -80°C. The right testis was fixed in paraformaldehyde, embedded in paraffin, and stained immunohistochemically.

Radioimmunoassay for serum LH, FSH, and testosterone levels
A radioimmunoassay kit (Beijing Northern Biotechnology Research Institute, Beijing, China) was used to determine the serum levels of LH, FSH, and testosterone in strict accordance with the manufacturer’s instructions. Levels were calculated using a standard curve method.

Enzyme-labeled immunosorbent assay for serum IGF-1 levels
An enzyme-labeled immunosorbent assay kit (Wuhan Boster Biological Engineering Co., Ltd., Wuhan, Hubei Province, China) was employed to measure serum IGF-1 (rabbit anti-IGF-1 monoclonal antibody, goat anti-rabbit IgG; 78–5 000 pg/mL) in strict accordance with the manufacturer’s instructions. Levels were calculated using a standard curve method.

Radioimmunoassay for hypothalamic GnRH, substance P, and β-endorphin levels
Five rats in each group were selected for harvesting of brain tissue. The hypothalamus was removed to the rear of the optic chiasma at the ventral side and weighed (approximately 30 mg). The specimens were then homogenized with 400 μL cell lysate, ground in an ice bath, and centrifuged at 4°C; the supernatant was
discarded. GnRH, substance P, and β-endorphin were measured using a radioimmunoassay kit (PLA Navy Radioimmunoassay Center, China) according to the manufacturer's instructions. Serum levels were calculated using a standard curve method.

**Immunohistochemical staining for 17β-HSD and 3β-HSD expression in testicular tissue**

Slices were dewaxed to water and rinsed with PBS (0.01 M, pH 7.4) three times for 5 minutes each time, then incubated with 3% hydrogen peroxide-methanol solution at room temperature for 30 minutes to remove endogenous peroxidase activity. The specimens were incubated with normal goat serum at 37°C for 30 minutes, after which the serum was absorbed with filter paper and the specimens were incubated with rabbit anti-17β-HSD (1:100) and anti-3β-HSD monoclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight (20 hours), biotin-labeled secondary antibody (goal anti-rabbit IgG; Santa Cruz) at 37°C for 30 minutes and horseradish peroxidase-conjugated streptavidin (Beijing Zhongshann Jinqiao Biological Co., Ltd., Beijing, China) at 37°C for 30 minutes, and subjected to 3,3'-diaminobenzidine coloration (Beijing Zhongshann Jinqiao) under optical microscopy (Leica DM6000M, Wetzlar, Germany). Between each culture step, the specimens were rinsed with PBS three times for 5 minutes each time. After termination of the coloration, the specimens were counterstained with hematoxylin, dehydrated in an alcohol gradient, rendered transparent in xylene, and mounted with neutral gum. For negative controls, PBS was used instead of antibody, with the other steps being the same as those described above. The appearance of brownish yellow particles or fine particles in a diffuse distribution was considered a positive response.

**Western blot assay for 17β-HSD and 3β-HSD expression in testicular tissue**

Total protein was extracted with a cell lysis kit and the total protein content of the specimens was determined with a bicinchoninic acid quantitative protein detection kit. Four per cent stacking gel and 10% separation gel were prepared, and 60 μg of testicular tissue protein was mixed with sampling buffer solution, boiled in a water bath for 5–10 minutes, and placed in the gel sample holes after natural cooling. Electrophoresis was performed under 80 V on the stacking gel and 100 V on the separation gel. The extracted proteins were transferred to polyvinylidene fluoride membrane using a water-bath electric transfer device at 4°C, at 30 V constant pressure overnight. The membrane was blocked with 5% skimmed milk powder and shaken gently for 1 hour at room temperature. The blocked polyvinylidene fluoride membrane was dried, placed into the hybridization bag, and incubated with rabbit anti-17β-HSD, 3β-HSD, and β-actin monoclonal antibodies (1:200; Santa Cruz) for 2 hours. The membrane was rinsed with TBST membrane three times for 10 minutes each time and oscillated gently, then dried and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG antibody, 1:2 000; Santa Cruz) at 37°C for 1 hour and rinsed in TBST three times for 10 minutes each time. Following polyvinylidene fluoride membrane coloration, Pierce chemiluminescent substrate solutions A and B were mixed for 15 minutes at room temperature and the scanned image was analyzed with a FUJI Mini-4000 (Fuji, Tokyo, Japan).

**Statistical analysis**

Measurement data were expressed as mean ± SD using SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences between groups were compared with one-way analysis of variance, and paired comparisons were made using the Student-Newman-Keuls test. P values less than 0.05 were considered significant.

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**Author contributions:** Siyun Niu had full access to the study concept and design, wrote the manuscript and managed the funds. Suru Kou was responsible for data collection and integration. Xiaochun Zhou performed statistical processing and data analysis. Liang Ding validated the study.

**Conflicts of interest:** None declared.

**Ethical approval:** This pilot was approved by the Experimental Animal Ethics Committee of Hebei University in China.

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