Chinese herbal medicine enhances sexual function and c-Fos/nNOS expression in the nucleus accumbens of orchidectomized rats

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Abstract

OBJECTIVE: To determine whether the central nervous system is involved in the effect of Chinese herbal medicine on sexual function recovery in orchidectomized rats.

METHODS: Orchidectomized rats were administered intragastrically with a decoction of “kidney-nourishing” Chinese herbal medicine once per day for 28 days. Accessory genital organ weight, plasma testosterone, and mating behavior were investigated. The expression of c-Fos and neuronal nitric oxide synthase (nNOS) in neuronal cells in the nucleus accumbens (NAc) was analyzed by immunohistochemistry.

RESULTS: There was a decrease in accessory genital organ weight, plasma testosterone, and sexual behavior, as well as a low number of c-Fos-positive cells and a large nNOS-positive cell area in orchidectomized rats. Administration of the herbal medicine increased accessory genital organ weight, testosterone level, mating behavior, and c-Fos-positive cell number, while it decreased the nNOS-positive cell area in orchidectomized rats.

CONCLUSION: An increase of plasma testosterone after administration of “kidney-nourishing” herbal medicine might contribute to the elevated sexual function and activity in orchidectomized rats. In addition, a central nervous system mechanism, such as the functional alteration of NAc, might be involved.

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Key words: Medicine, Chinese traditional; Reinforcing kidney; Orchidectomy; Sexual function, physiological; Nucleus accumbens; c-Fos; Nitric oxide synthase type I

INTRODUCTION

In Traditional Chinese Medicine, sexual dysfunction is usually treated by using herbs described as “kidney-nourishing” such as Shechuangzi (Fructus Cnidii), Yinyanghuo (Herba Epimedii Brevicornus) and Tusizi (Semen Cuscutae). However, their pharmacological mechanisms of action are not completely understood. The nucleus accumbens (NAc) is involved in the regulation of sexual arousal and sexual behavior. Moreover, sexual motivation and mating behavior are associated with dopamine release from the NAc in male rats.
and sex-related environmental cues can also activate NAc. A study using Chinese herbal medicines found that improvement of sexual behavior was associated with high production of the dopamine metabolite 3, 4-dihydroxyphenylacetic acid in NAc, indicating that NAc may be involved in the mechanism of action of “kidney-nourishing” herbal medicines. Our previous studies demonstrated that a “kidney-nourishing” herbal formula (Xuefuruon capsule) increased testosterone levels in orchidectomized rats. In this study, this formula was administered to orchidectomized rats to investigate the involvement of NAc in mechanism of action of “kidney-nourishing” herbal medicine by determining changes in c-Fos and neuronal nitric oxide synthase (nNOS) expression.

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats, weighing 200-250 g, were provided by the Animal Center of Shanxi Medical University [ Permit No. SCXK (Jin) 2009-0001]. Thirty male and 15 female rats, which had never mated, were housed 3 per cage in a controlled temperature [20 ± 2 °C] and humidity [45% ± 10%] environment and allowed free access to water and chow. The experimental protocol was approved by the Institutional Animal Care Committee of the North University of China and was in accordance with the guidelines of the National Institutes of Health (USA) regarding the care and use of animals for experimental procedures.

Drugs and reagents

The herbal medicine was prepared from commercially-available Xuefuruong capsules (Batch No. 20040611), mainly composed of Tusizi (Semen Cuscutae), Heshouwu (Radix Polygoni Multiflori), Huangjing (Rhizoma Polygonati Sibirici) and Fuling (Poria), provided by Shanxi Caozetang Bioengineering Co., Ltd. (Taiyuan, China). The capsule was dissolved in water at 1.5 g/L, and administered intragastrically (i.g.) to rats at 5 mg/kg dose. The kits for neuronal c-Fos detection (Lot No. SA2053) and neuronal nitric oxide synthase (nNOS) detection (Lot No. BA0360) were purchased from Boster Bioengineering Co. (Wuhan, China). The testosterone radioimmunoassay kit (Lot No. S10940108) was from Tianjin Depu Biotechnology and Medical Products Co., Ltd. (Tianjin, China).

Experimental design

Thirty male rats were randomly divided into 3 groups of 10 animals: Control (without orchidectomy, water i.g.), Model (orchidectomized, water i.g.), and Treatment (orchidectomized, herbal medicine i.g.). Three days after the surgery, herbal medicine solution or water was administered i.g with the same dose (0.66-0.83 mL every time) to rats in each group daily for 28 days. The weight index of accessory genital organs, plasma testosterone, locomotion, mating behavior, c-Fos-positive neurons, and nNOS-positive neurons were assessed. Five ovariectomized female rats were used in the sexual function test of male rats in each group.

Surgery

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) (Shanghai Chemistry Preparation Co., Ltd., Shanghai, China). Male and female rats were subjected to a bilateral orchidectomy and ovariectomy, respectively. After suturing the wound, bacitracin ointment (North Pharmaceutical Co., Ltd., Shijiazhuang, China) was applied to prevent infection.

Analysis of accessory genital organs

The weight index of accessory genital organs, which is a common index for sexual function, is defined as the wet weight of the genital organs relative to the whole body weight (mg/100 g). In this experiment, the preputial gland and levator ani muscle of rats were separated and measured.

Open field test

The open field test apparatus was a square tank (100 cm width × 100 cm length × 50 cm height) with white, opaque walls. The floor was divided into 25 equal square areas. A top-view video recording was taken, and each rat was tracked for 5 min in a quiet room. Then, the results were scored by a specialized observer blinded to the procedure. Locomotion was defined as the number of crossings of the square areas.

Assay of plasma testosterone

Twenty hours after the last administration of the herbal medicine solution, blood samples were collected from the tail vein and centrifuged to separate the plasma. The levels of plasma testosterone were measured by using a radioimmunoassay kit.

Test of mating behavior

All rats were adapted to the experimental conditions for three days before the test. Tests on male rats were performed 12 h after the last administration of the herbal solution. To induce estrus, females were administered 30 μg of estradiol benzoate and 50 μg of progesterone subcutaneously 48 h and 4 h before the test, respectively. Each receptive female rat underwent two mating tests.

Mating behavior tests were performed during the dark phase (between 20:00 and 22:00) in a dim and soundproof room. A male rat was placed in the observation cage first and allowed 5 min for adaptation before a receptive female rat was introduced. The behavioral parameters were recorded for 30 min according to a published procedure: (a) capture latency (time from introduction of the female until first capture); (b) capture frequency (number of captures); and (c) mounting frequency.
**Immunohistochemical staining and image analysis**

Two days after the sexual function test, all male rats were deeply anesthetized by intraperitoneal injection of 2% sodium pentobarbital (40 mg/kg), and then perfused transcardially with phosphate-buffered saline (PBS, made by Shanghai Chemistry Preparation Co., Ltd., Shanghai, China) and 300 mL of 4% paraformaldehyde (Shanghai Chemistry Preparation Co., Ltd., Shanghai, China). The brain was removed immediately and kept in a 4% paraformaldehyde/PBS solution for 3 h at 4 °C and was then transferred to a 20% sucrose solution for cryoprotection. Serial frozen coronal 40 μm-sections were cut and every third section was analyzed.13,14

The c-Fos- and nNOS-positive neurons in the NAc were immunohistochemically labeled using a commercially available detection kit according to the provided protocol. Briefly, after incubation with 0.5% Triton X-100 PBS and 10% normal goat serum at 37 °C for 1 h, the sections were incubated with the primary antibody (rabbit anti-c-Fos; 1:100) at 37 °C for 3 h, and then at 4 °C for 36 h. The sections were incubated with the secondary antibody (biotin-conjugated goat anti-rabbit; 1:100) and avidin-biotin (1:100) at 37 °C for 1 h, 3, 3’-Diaminobenzidine (DAB) was used to develop the color. Finally, the sections were dehydrated in a gradient of alcohols, cleared in xylene, and covered with neutral gum. For control sections, the primary antibody was replaced with normal rabbit serum and PBS. The sections were incubated with the secondary antibody (1:100) followed by DAB to develop the color.

The numbers and area of immuno-positive neurons were determined by using a light microscope and a morphologic image analysis system (JD-801, Jiangsu JEDA, China).13-c-fos expression kit was from Boster Bioengineering Co., Wuhan, China; nNOS from Zhongshan Biotechnical Co., Beijing, China.

**Statistical analysis**

The weight index of accessory genital organs were presented by mg/100g, plasma testosterone levels by concentration (ng/dL), behavior tests by time, times or frequency, and the data were presented as mean ± standard deviation (x ± s). The measurement of c-fos and nNOS-positive cells was recorded by numbers and area, and the data were presented as mean ± standard error. Differences among the groups were assessed by using one-way analysis of variance followed by a Bonferroni test. All analyses were performed by using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistically significant level was defined as P-values less than 0.05.

**RESULTS**

**Accessory genital organ weight and plasma testosterone level**

There were significant differences in plasma testosterone levels (F(2,26) = 556.78, P < 0.001), levator ani muscle weight index (F(2,25) = 12.77, P < 0.001), and preputial gland weight index (F(2,27) = 11.14, P < 0.001) between the different groups. Post hoc tests revealed that orchidectomized rats in the model and treatment groups had significantly lower preputial gland and levator ani muscle weight indices and plasma testosterone levels than those of rats in the control group. However, after treatment with herbal medicine, the weight indices of the preputial gland and levator ani muscle, and plasma testosterone levels of rats in the treatment group were significantly higher than those of rats in the model group (Table 1).

**Locomotion and mating behavior**

The behavioral effects of herbal medicine treatment on orchidectomized rats are shown in Figure 1. There were significant differences in locomotion (F(2,27) = 20.34, P < 0.001, Figure 1A), capture latency (F(2,26) = 19.88, P = 0.001, Figure 1B), capture frequency (F(2,26) = 10.11, P = 0.001, Figure 1C), and mounting frequency (F(2,26) = 11.25, P < 0.001, Figure 1D) between the groups. Post hoc tests revealed that orchidectomized rats in the model and treatment groups had significantly lower locomotion, longer capture latency, and lower capture and mounting frequencies as compared to rats in the control group. However, following treatment with herbal medicine, the locomotion, capture latency, and capture and mounting frequencies of rats in the treatment group significantly increased as compared with those of rats in the model group.

**Image analysis of c-Fos-positive neurons**

Image analysis showed that c-Fos-positive cells were distributed throughout the NAc as indicated by the brown staining (Figure 2A-2C). There were differences in the cell number [F(2,27) = 12.39, P < 0.001, Figure 2B].

<table>
<thead>
<tr>
<th>Groups</th>
<th>Testosterone (ng/dL)</th>
<th>Levator ani muscle (mg/100 g)</th>
<th>Preputial gland (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>309.3±26.0</td>
<td>50.5±16.7</td>
<td>58.6±10.5</td>
</tr>
<tr>
<td>Model</td>
<td>3.6±2.5</td>
<td>28.3±7.7</td>
<td>38.7±15.1</td>
</tr>
<tr>
<td>Treatment</td>
<td>10.1±5.4*</td>
<td>37.5±18.5</td>
<td>49.8±30.2*</td>
</tr>
</tbody>
</table>

Notes: control group was treated without orchidectomy, water intragastrically; model group was treated with orchidectomized, water intragastrically; treatment group was treated with orchidectomized, herbal medicine (Xuefurong capsules) intragastrically 0.66-0.83 mL every time, daily for 28 days. *P < 0.01, †P < 0.05, ‡control group; †P < 0.01, ‡P < 0.05 vs model group.
Image analysis of nNOS-positive neurons
nNOS-positive cells were distributed throughout the NAc as indicated by the brown staining (Figure 3A-3C). There were differences in the cell number \( F_{(2,27)} = 6.01, P = 0.007 \), Figure 3D) and cell area \( F_{(2,27)} = 12.40, P < 0.001 \), Figure 3E) of nNOS-positive neurons between the groups. Compared with the control group, the cell numbers and cell area of nNOS-positive neurons were significantly higher in the model and treatment groups. However, there was a decrease in the cell area in the treatment group as compared with the model group.

DISCUSSION
In this study, the effect of a "kidney-nourishing" prescription of Chinese herbs on sexual function of orchidectomized rats was investigated by assessing accessory genital organ weight, mating behavior, and c-Fos / nNOS expression in NAc. The results indicated that orchidectomy in male rats resulted in decreased accessory genital organ weight index, plasma testosterone level, locomotion, mating behavior, and c-Fos expression, while nNOS expression was increased. Herbal medicine treatment partially reversed the effects of orchidectomy. The therapeutic effects were manifested as follows: (a) physical improvement including increased preputial gland and levator ani muscle weight and plasma testosterone level, (b) improvement of sexual behavior including capturing and mating, and (c) restoration of c-Fos/nNOS expression in NAc.

Plasma testosterone originates mainly in the testicles and orchidectomized rats had very low testosterone levels. However, the adrenal gland can also produce testosterone, which might have contributed to the herbal medicine-induced increase in plasma testosterone. Accessory genital organs, such as the preputial gland, are regulated by testosterone. Thus, it is not surprising that herbal medicine treatment increased the weight of the preputial gland and levator ani muscle in this study. The mating test, a direct and credible method used to evaluate the sexual activity of rats, is indexed by capture latency, mounting rate, insertion, and ejaculation. In this study, the capturing and mounting behaviors were selected as the indices of sexual ability. The results indicated that the herbal medicine treat-
ment decreased the capture latency and increased the frequencies of capturing and mounting. However, the underlying mechanisms remain unresolved. Plasma testosterone plays an important role in maintaining sexuality, sexual behavior, and penile erection in male mammals. After orchidectomy, testosterone decreased sharply.

Figure 2 Effect of herbal medicine on neuronal c-Fos expression in NAc of orchidectomized rats
A-C: immunohistochemical staining, ×200 (×200 for insets). A: control group; B: model group; C: treatment group. Immunohistochemical analysis showed that the number and area of c-Fos-immunoreactive neurons were significantly lower, arrow in the NAc core of orchidectomized rats (B) prior to treatment with herbal medicine (C). Control group was treated without orchidectomy, water intragastrically; Model group was treated with orchidectomy, water intragastrically; Treatment group was treated with orchidectomy, herbal medicine (Xuefurong capsules) intragastrically 0.66-0.83 mL every time, daily for 28 days. D: quantification of c-Fos-positive cell number; E: quantification of c-Fos-positive cell area. ac: anterior commissure; NAc: nucleus accumbens; c-Fos: c-proto-oncogene. *P < 0.01, vs control group; bP < 0.01, vs model group.

Figure 3 Effect of herbal medicine on neuronal nNOS expression in NAc of orchidectomized rats
A-C: immunohistochemical staining, ×200 (×400 for insets). A: control group; B: model group; C: treatment group. Immunohistochemical analysis showed that the number and area of nNOS-immunoreactive neurons were significantly higher, arrow in the NAc core of orchidectomized rats (B) prior to treatment with herbal medicine (C). Control group was treated without orchidectomy, water intragastrically; Model group was treated with orchidectomy, water intragastrically; Treatment group was treated with orchidectomy, herbal medicine (Xuefurong capsules) intragastrically 0.66-0.83 mL every time, daily for 28 days. D: quantification of nNOS-positive cell number; E: quantification of nNOS-positive cell area. ac: anterior commissure; NAc: nucleus accumbens; nNOS: neuronal nitric oxide synthase. *P < 0.01, vs control group; bP < 0.01, vs model group.
and mating ability was gradually lost in rats.\textsuperscript{17} Therefore, it is possible that herbal medicine treatment improved sexual function by increasing plasma testosterone in orchidectomized rats via activation of the adrenocortical zona reticularis.\textsuperscript{6,18} However, the contrast between the small increase in plasma testosterone and the major improvement in mating behavior indicate that mechanisms other than testosterone modulation might be involved.

c-Fos expression is an indicator of neuronal activation.\textsuperscript{19} It has been shown that sexual behavior can induce c-Fos expression in many brain areas including NAc.\textsuperscript{20} The results of this study indicated that there were fewer c-Fos-positive neurons in orchidectomized rats than in control animals, which implies that decreased c-Fos expression is associated with decreased sexual function resulting from orchidectomy. After the 28-day administration of the herbal medicine solution, c-Fos expression in the NAc of orchidectomized rats was upregulated. In the brain, testosterone regulates nNOS,\textsuperscript{21} while NO modulates behavioral motivation.\textsuperscript{22} The decrease in nNOS expression induced by herbal medicine treatment indicated changes in NO signaling in NAc. These findings suggest that NAc function may be involved in the effect of the "kidney-nourishing" herbal medicine on sexual behavior in orchidectomized rats.

In brief, this study provides preclinical evidence that the "kidney-nourishing" Chinese herbal medicine can improve sexual function. The underlying mechanism might involve NAc and an enhancement of sexual motivation associated with the physical improvement of accessory genital organs. This study is significant because it indicates that research on herbal medicines should focus on central neuro- or psycho-pharmacological mechanisms.

REFERENCES