Effects of flavonoids from semen cuscutae on the hippocampal-hypothalamic-pituitary-ovarian sex hormone receptors in female rats exposed to psychological stress

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Summary

Objective: To investigate the effects of flavonoids from semen cuscutae (FSCs) on the hippocampal-hypothalamic-pituitary-ovarian sex hormone receptors in female rats exposed to psychological stress and to explore the related mechanism. *Materials and Methods:* Flavonoids were obtained from semen cuscutae using solvent extraction and polyamide column chromatography. Sound, light, and electricity were combined into psychological stress for endocrine dysfunction model establishment in female rats. The effects of FSCs on estrogen receptor (ER) in the hippocampus, hypothalamus, and pituitaries, as well as on follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) in the ovaries of the psychologically stressed rats were quantitatively analyzed using immunohistochemistry and image analysis. *Results:* FSCs increased ER expression in the hippocampus, hypothalamus, and pituitaries, as well as LHR expression in the ovaries, but had no effect on FSHR expression in the ovaries. *Conclusion:* FSCs are an effective medicine in the treatment of ovarian endocrine dysfunction in psychologically stressed rats.

Key words: Flavonoids from semen cuscutae; Psychological stress; Hippocampus; Hypothalamic-pituitary-ovarian (HPO) axis; Sex hormone receptors.

Introduction

The reproductive endocrine system is a system which is susceptible to stress-induced injuries. Psychological stress can induce reproductive endocrine disorders, which lead to reproductive endocrine disease. To date, an effective regulatory measure for this type of disease in Western medicine has not been found, and hormone replacement therapy is the primarily-adopted current treatment method in Western medicine practice. The administration of hormones however, has only an uncertain curative effect, and even worse, long-term use of them can result in obvious adverse effects. Chinese medicine has the characteristics of an integrative adjustment and multi-target and manner regulation. These characteristics give it advantages in the prevention and treatment of female ovarian functional disorders. Modern integrated traditional Chinese and Western medicine studies indicate that kidneys are correlated with the hypothalamic-pituitary-ovarian (HPO) axis and reproductive endocrine system. The occurrence of ovarian dysfunction is closely correlated with kidneys, liver, spleen, and hemostasis, among which kidney deficiency performs a basic role; kidney deficiency is caused by abnormal gene expression of neuroendocrine-immune network balance function-mediating related substances, which breaks the functional balance of the neuroendocrine-immune system [1]. Therefore, in the treatment of reproductive endocrine disorders, attention should be placed on kidney reinforcement, considering kidneys being the foundation of prenatal life, which store congenital essence, and govern reproduction. Kidney-reinforcing Chinese medicine has pluralistic and bi-directional regulatory effects on hypothalamus, pituitaries, and ovaries.

Semen cuscutae are the mature seeds of the convolvulaceae plant Cuscuta chinensis Lam. They are widely applied in Chinese medicine practice, especially in the treatment of reproductive endocrine disorders [2-4]. The authors of the current study have performed some studies of the regulatory effect of semen cuscutae on the reproductive endocrine system, and have discovered that semen cuscutae have multiple effects on the hypothalamic-pituitary-gonadal (HPG) axis: They improve the promotive effect of hypothalamus and pituitaries on gonads, enhance the reactivity of pituitaries to gonadotropin-releasing hormone (GnRH), promote follicular development, and increase the numbers of human chorionic gonadotropin (hCG)/luteinizing hormone receptors (LHR), and reinforce their functions, but without obvious influence on the LH level in plasma [5]; semen cuscutae, as well as the flavonoids from them (FSCs), obviously increase the testicular and epididymal weights of young male rats [6] and have estrogen-like activity.

Semen cuscutae are a kind of Chinese medicine which can reinforce and benefit liver and kidneys; FSCs, the efficacious component of semen custutae, have an obvious regulatory effect on ovarian endocrine disorders caused by psychological stress. It significantly increases estradiol (E2) and progesterone (P) levels, pituitary LH level, and hypothalamic beta-estradiol progesterone (β-EP) content, but does not affect the follicle-stimulating hormone (FSH) content in psychologically stressed rats

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[7, 8]. Usually, hormones exert physiological actions through their binding with their corresponded receptors. Then, what changes occur in hippocampal-HPO sex hormone receptors when female rats are exposed to psychological stress and how do FSCs regulate these changes? These questions remain to be explored.

In the current study, the effects of FSCs on the hippocampal, hypothalamic, and pituitary estrogen receptor (ER), as well as on the ovarian FSHR and LHR in psychologically stressed rats were investigated.

Materials and Methods

Investigational drug

Flavonoid extracts were obtained from semen custutae using solvent extraction and polyamide column chromatography. The semen custutae were identified as the mature seeds of Cuscuta chinensis Lam by the Chinese Traditional Medicine Identification Division of the Children's Hospital of Jiangxi Province. Thin-layer chromatography demonstrated that four major components were contained in the extracts, including quercetin, hyperoside, astragulin, and quercetin-3-0-β-galactosyl-7-0-β-glucose, which was consistent to that reported in literature [9]. Colorimetry showed that the flavonoid content was between 40% and 50%, taking rutin as the standard reference. Suspensions at concentrations of 10 mg/ml and 5 mg/ml were prepared during the experiment, which were equivalent to two and one gram of drug substance, respectively.

Animals

Female Sprague-Dawley (SD) rats, weighing 180-220 g, were supplied by the Laboratory Animal Center of Jiangxi. Experimental procedures were performed strictly following the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the animal use protocol had been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Children's Hospital of Jiangxi province.

These animals were bred under natural light exposure at room temperature (20 ± 2°C) and had liberal access to food and water. After acclimation for 2 d, 50 animals were subjected to a daily vaginal smearing examination for possible changes in estrous cycle. They were then divided randomly into the normal control, model, high-dose FSC, low-dose FSC, and positive control (given menstruation-regulating and pregnancy-promoting pills) groups. Of the animals, those with two continuous normal estrous cycles were selected for experimental model establishment [10] while others were discarded.

Drug administration

The abovementioned five groups were given physiological saline (0.9%), physiological saline (0.9%), high-dose FSC (10 mg/ml), low-dose FSC (5 mg/ml), and menstruation-regulating and pregnancy-promoting pills (90 mg/ml; Beijing Tongrentang, China), respectively, according to 1.0 ml/100 g after lavage. In the meantime, they, except for the normal control group, underwent psychological stress for 20-d model establishment. After modelling, all groups received five more days of drug administration (once/d).

ER, FSHR, and LHR determination

Hippocampus, hypothalamus, pituitaries, and ovaries were taken rapidly, fixed in 10% formalin and embedded with paraffin. Pathological sections were made for immunohistochemistry. ER was observed after nickel ammonium sulfate-diaminobenzidin (N-DAB) coloration (positive substances are blue-black rather than buffy stained, which are mainly located in neural nuclei). FSHR was observed after DAB coloration (positive substances are buffy-stained, which are mainly located on the membranes of ovarian granular cells). LHR content was determined using an indirect method, based on the reactions between anti-LH antibody and LH-combing sites (LHR content and the reactions are in a positive correlation) [11, 12]. Then, LHR was observed after DAB coloration (positive substances are buffy-stained and are mainly located on the membranes of follicular endomembranous, interstitial, and luteal granular cells). ER, FSHR, and LHR were analyzed quantitatively by a DVPM image analyzer (Nanjing Great Wall, China). Ten sections were taken from each type of tissue, and ten visual fields (× 40) were randomly selected for each section for integral optical density (IOD) determination. The obtained IOD values were used to represent the relative intensity of the expression of ER, FSHR, and LHR in the corresponding tissues.

Statistical analysis

Data were presented as means ± standard error ($\bar{x} \pm s$) and analyzed by the SPSS10.0 software. Paired t-test was also performed.

Results

ER in the hippocampus

As shown in Table 1, the ER content in the model group significantly decreased, compared with the normal control group ($p < 0.05$); ER in the high-dose FSC, low-dose FSC, and positive control groups was significantly higher than the model group ($p < 0.01$); and ER in the high-dose and low-dose FSC groups was significantly lower than that in the positive control group ($p < 0.05$ and $p < 0.01$). These results indicate that high-dose FSCs have a better effect on ER than low-dose FSCs, though neither of them can achieve an effect as good as menstruation regulating pills.

ER in the hypothalamus

As shown in Table 2, the ER content in the model group was significantly lower than that in the normal control group ($p < 0.05$); ER in the high-dose FSC, low-dose FSC, and positive control groups was significantly higher than that in the model group ($p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively); the high-dose FSC and positive control groups did not show a significant difference ($p > 0.05$), but the low-dose FSC group did show a significant difference as compared with the positive control group ($p < 0.01$). These results indicate that high-dose FSCs can have a good effect on ER in the hypothalamus, which is very close to that achieved by menstruation regulating pills.

ER in the pituitaries

As show in Table 3, the ER content in the model group was significantly lower than that in the normal control group ($p < 0.01$); ER in the high-dose FSC and positive control groups was significantly higher when compared with the model group ($p < 0.01$), but the low-dose FSC

Table 1. — *Changes in hippocampal ER content in different groups ($\bar{x} \pm s$).*

Group	Animal number (n)	ER (IOD)
Normal control	9	22.33 ± 6.97
Model	8	13.45 ± 2.87▲
Low dose	8	27.03 ± 2.63**□□
High dose	9	37.84 ± 6.99**□
Positive control	8	49.70 ± 8.47**

▲ $p < 0.05$, compared with the normal control group; ** $p < 0.01$, compared with the model group; and □ $p < 0.05$ and □□ $p < 0.01$, compared with the positive control group.

Table 2. — *Changes in hypothalamic ER content in different groups ($\bar{x} \pm s$).*

Group	Animal number (n)	ER (IOD)
Normal control	9	32.65 ± 4.9
Model	8	18.19 ± 1.12▲
Low dose	8	34.18 ± 6.79**□□
High dose	9	47.65 ± 15.95*
Positive control	8	56.48 ± 19.60**

▲ $p < 0.05$, compared with the normal control group; * $p < 0.05$ and ** $p < 0.01$, compared with the model group; and □□ $p < 0.01$, compared with the positive control group.

Table 3. — *Changes in pituitary ER content in different groups ($\bar{x} \pm s$).*

Group	Animal number (n)	ER (IOD)
Normal control	9	42.25 ± 7.86
Model	8	24.44 ± 6.86▲▲
Low dose	8	38.43 ± 1.66□□
High dose	9	50.24 ± 2.5**
Positive control	8	45.42 ± 4.64**

▲▲ $p < 0.01$, compared with the normal control group; ** $p < 0.01$, compared with the model group; and □□ $p < 0.01$, compared with the positive control group.

Table 4. — *Changes in the ovarian content of FSHR and LHR in different groups ($\bar{x} \pm s$).*

Group	Animal number (n)	FSHR (IOD)	LHR (IOD)
Normal control	9	68.97 ± 16.21	99.16 ± 12.93
Model	9	102.44 ± 8.28▲▲	72.03 ± 10.47▲▲
Low dose	8	101.90 ± 13.6□	84.74 ± 15.07*□
High dose	9	97.89 ± 15.98	104.31 ± 16.7**
Positive control	8	86.68 ± 16.71*	101.60 ± 15.40**

▲▲ $p < 0.01$, compared with the normal control group; * $p < 0.05$ and ** $p < 0.01$, compared with the model group; and □ $p < 0.05$, compared with the positive control group; IOD = integrated otpical density.

group did not show such a significant difference ($p > 0.05$), which indicate that low-dose FSCs cannot prevent an increase in pituitary ER; the high-dose FSC and positive control groups did not show a significant difference ($p > 0.05$), but a significant difference was presented between the low-dose FSC and positive control group ($p < 0.01$). These results indicate that high-dose FSCs have a better effect on ER in the pituitaries than a low dose.

FSHR and LHR in the ovaries

As shown in Table 4, the FSHR in the model group significantly increased, but LHR significantly decreased when compared with the normal control group ($p < 0.01$); FSHR in the high-dose and low-dose FSC group did not show significant differences when compared to the model group ($p > 0.05$), but LHR in these two groups did ($p < 0.01$ and $p < 0.05$), which indicates that high-dose FSCs can better down-regulate LHR expression in the ovaries than a low dose, although they cannot down-regulate FSHR expression in the ovaries; the positive control group showed significant differences in both FSHR and LHR as compared with the model group ($p < 0.05$ and $p < 0.01$); the high-dose FSC group did not show significant differences in FSHR and LHR as compared with the positive control group ($p > 0.05$), but the low-dose FSC group did ($p < 0.05$).

Discussion

The present study aimed to solve the questions of whether there are changes in hippocampal-HPO sex hormone receptors when rats are exposed to psychological stress and whether FSCs have a regulatory effect on these changes. The results in this study gave affirmative answers: hippocampal-HPO sex hormone receptors do change when rats are exposed to psychological stress and FSCs have a regulatory effect on these changes.

The hippocampus is a structure which contains different messenger receptors; when there is psychological stress, it does not only serve as a high-position center of accommodation, but becomes the most sensitive region to the stress [13, 14]. Evidence has shown that the hippocampus contains alpha and beta types of estrogen receptors (ERα and ERβ). ER protein in the rat brain is basically located in neuronal nuclei, and it is not positively expressed in cytoplasm or process; its high expression in female rats is mainly located in hippocampal fissures, lateral amygdaloid nuclei, and the horizontal part of the diagonal band; it is extensively expressed in the basal forebrain in rats, suggesting that estrogens may participate in the regulation of the neural structure and function in this area; estrogens can up-regulate ERβ expression, which, in turn, regulates reproductive endocrine [15]. The present study showed that decreased hippocampal ER expression in psychological stressed rats influenced the reproductive endocrine, but the study failed to demonstrate how this decrease influenced the hypothalamus. Presumably, this process is mediated by ER, through which the synthesis of the neurotransmitters in neuronal cytoplasm is influenced first and this condition further influences the hypothalamus through neurotransmitter pathways (i.e., nerve fiber bundles, like the projective region of noradrenergic nerves); or, psychological stress causing changes in the content and proportion of neurotransmitters first and then such a condition is mediated by ER to influence the hypothalamus (i.e., through the feedback action of ER). Nevertheless, how a change in ER influences the hypothalamus still needs to be explored in the future.

ER and P receptor coexist in some hypothalamic regions [16]. The binding of estrogens and progesterone

with their receptors can affect the secretion of GnRH through regulation on neurotransmitters; a decreased level of E2 causes reduced GnRH, which results in an inhibitory effect on the HPO axis [17, 18]. Therefore, psychological stress causes decreases in E2 and ER in rats, which affect the synthesis and secretion of hypothalamic neurotransmitters. This condition may have an inhibitory effect on the HPO axis, which further affects the reproductive endocrine.

Since the first application of immunohistochemistry in the observation of the pituitary ERα and ERβ distributions in rats [19], more and more studies have focused on pituitary ER. ERα mRNA expression in rats decreases after ovariectomy, but such a decrease can be inversed after estrogen replacement treatment [20]. Surgical stress can induce the appearance of noticeable particles and Golgi vesicles in rat anterior pituitary prolactin (PRL) cells, increase PRL, and E2 in plasma and decrease ER in hepatic cells [21]. The present study showed that a decrease in hippocampal-hypothalamic-pituitary ER as well as ovarian LHR, and an increase in ovarian FSHR in psychologically stressed rats affected the secretion of reproductive hormones and neurotransmitters by the pituitaries, which further influenced the reproductive endocrine function of the ovaries.

This study also showed that FSCs can up-regulate hippocampal- hypothalamic-pituitary ER, and ovarian LHR in psychologically stressed rats, but cannot bring about an effect on ovarian FSHR. Presumably, FSHR and LHR are distributed in different regions of the ovaries (e.g., FSHR is mainly distributed on the surface of granular cells while LHR is mainly distributed on the surface of follicular endomembranous cells), which results in different sensitive degrees of FSHR and LHR to FSCs. In addition, this study showed that the effects of FSCs on hippocampal-hypothalamic-pituitary ER and ovarian LHR in psychologically stressed rats displayed a certain dose-dependent manner.

To draw a conclusion, FSCs can up-regulate hippocampal-hypothalamic-pituitary ER and ovarian LHR expression in psychologically stressed rats, which is, at least, the partial mechanism of the regulatory effects of FSCs on HPO functions, and FSCs can serve as effective medicine in the treatment of ovarian endocrine dysfunction in rats exposed to psychological stress.

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