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The total flavones from *Semen cuscutae* reverse the reduction of testosterone level and the expression of androgen receptor gene in kidney-yang deficient mice

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ABSTRACT

Objective: To discover the effect of the total flavones from *Semen cuscutae* (TFSC) on the kidney-yang deficiency male mouse, especially on hormone levels and the androgen receptor (AR) mRNA and protein level in the kidney and testicle.
Methods: ICR male mouse were separated into six groups, and, except for the normal group, hydrocortisone was injected intraperitoneally to make the kidney-yang deficiency. The groups were then treated with TFSC, methyltestosterone and Jinkui Shenqi Wan, except for the normal group and control group. 14 days after administering, testosterone levels in the total serum were analysed by radioimmunoassay, AR mRNA levels measured by real time RT-PCR and protein levels by immunohistochemistry.
Results: The control group had the lowest testosterone level, AR mRNA level and protein level, and the TFSC can reverse the reduction of testosterone level, AR mRNA level and protein level induced by the hydrocortisone.
Conclusions: This is the first study to demonstrate that TFSC treatment may reverse kidney-yang deficiency symptoms by restoring the levels of testosterone and AR mRNA and protein expression in the kidney and testicle.

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1. Introduction

Semen cuscutae is a well-known Chinese medicine first recorded in the famous book "Shen Nong's Herbal" as an upper grade drug. It has been used in China to treat impotence and seminal emission as a tonic for thousands of years. Modern pharmacological experiments suggest that it has many biological activities, such as improving sexual function, regulating the body's endocrine and immune system, and antioxidation (Ye et al., 2002). The Pharmacopoeia of People's Republic of China (Version 2005) specifies *Semen cuscutae* as the dried ripe seeds of *Cuscuta chinensis* Lam. (Family *Convolvulaceae*) (Xiong and Zhou, 1994; National Commission of Chinese Pharmacopoeia, 2005).

Semen cuscutae is widely used as a tonic, as an aphrodisiac, to nourish the liver and kidneys and treat impotence and seminal emission (Du et al., 1998; Pan et al., 2005). It has also been described that the ethanol extract of *Cuscuta chinensis* significantly enhanced splenocyte proliferation stimulated with Con A and release of IL-2 from mice splenocytes in vitro (Xiao et al., 1990; Li et al., 1997). In addition, preparations of the crude drug have been reported to have a positive inotropic effect on frog heart specimens, and lower the blood pressure of anesthetized dogs (Bensky and Gamble, 1986). Flavonoids are the main biologically active constituents in *Semen cuscutae*. In addition, quercetin, kaempferol and hyperoside have been reported to exhibit various pharmacological activities, which to some extent might elucidate the mechanism of clinical effects of this commonly used Chinese medicine.

Androgens play key roles in male character development of vertebrates. They act through the binding and activation of the androgen receptor (AR), a ligand-dependent transcription factor that belongs to the steroid nuclear receptor superfamily. AR cDNAs have been cloned from several vertebrate species. The AR gene is located at Xq11-12 and is 90 kb long with eight exons (Richard and Liao, 1998). The AR itself plays a role in a number of tissues, most obviously in the development of male traits in animals. These AR sequences show a common structural organization and high homology in DNA-binding domains (DBD) and ligand-binding domains (LBD) among species (Jenster et al., 1993; Gobinet et al., 2002; Hironori et al., 2006). Upon androgen binding, AR dissociates from heat-shock proteins, dimerizes, and binds to androgen responsive elements (AREs) in the DNA, leading to the regulation of androgen responsive genes.

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Mice with kidney-yang deficient always have some symptoms such as weakness and soreness of the loins and the knees, the pedal flaccidity, beriberi and diabetes. Modern studies showed that damages and functional disorders of hypothalamic cells were main pathological elements of kidney-yang deficiency, and to some extent they resulted in functional disorders of hypothalamic–pituitary–gonadal (HPG) axis and pathological changes of metabolism organs and immune system. There are several ways to replicate the animal model of kidney-yang deficiency. The common methods are injected intraperitoneally with hydrocortisone acetate or adenine to mice, excess sexual activity and tired excessiveness and subtotal ectomy of adrenal gland and gonad to establish the mouse model with kidney-yang deficiency. Hydrocortisone belonging to glucocorticoid came from adrenal gland cortex, which can destroy the function of HPG axis and has been used to simulate the kidney-yang deficiency syndrome from 1960s. This model made with hydrocortisone has almost the same symptoms with the essential of kidney-yang deficiency syndrome based on study production in recent years, so it becomes a typical way to simulate kidney-yang deficiency syndrome and is widely used in China for investigator to study the mechanism of androgen and other hormone-related disease. So we choose hydrocortisone to make the model of kidney-yang deficiency to study whether the TFSC can alleviate the kidney-yang deficiency symptom.

Jin Kui Shen Qi Wan is among the most regarded ancient Chinese herbal formula. It is indicated in Chinese Pharmacopoeia (2005) for Yang insufficiency of kidney; weakness and soreness of the loins and the knees; cold feeling in the limbs; frequent urination. Published and unpublished human clinical studies have supported that Jinkui Shenqi Wan exert a certain therapeutic effect on the kidney-yang deficiency. And methyltestosterone is currently widely used in the therapy of impotence, seminal emission and kidney-yang deficiency. For all above, we use Jinkui Shenqi Wan and methyltestosterone as positive control.

In this paper, we chose TFSC to ameliorate the kidney-yang deficiency syndrome. The aim of this study was to clarify the mechanism of clinical effects of TFSC, and accelerate the clinical application of TFSC as a tonic and aphrodisiac medicament.

2. Materials and methods

2.1. Materials

Testosterone radioimmunoassay kit was purchased from Eiken Kagaku Co., Ltd., (Tokyo, Japan). Taq DNA polymerase, dNTP, oligo d(T)18 primers, moloney murine leukemia virus reverse transcriptase (M-MLV RT; 200 units/μl) and molecular weight markers for DNA were purchased from Promega Corporation (Madison, WI). SYBR® Premix Ex Taq™ (Perfect Real Time) purchased from TaKaRa Biotechnology Co., Ltd. (TaKaRa Japan). The monoclonal mouse anti-AR antibody was purchased from Santacruz Biotechnology Inc. (USA). The biotinylated goat anti-rabbit IgG were purchased from Boster Biotechnology Inc. (Wuhan, China). The SABC (Strept Avidin Biotin Complex) kit was purchased from Boster Biotechnology Inc. (Wuhan, China). The DAB kit was purchased from Beijing Zhongshan golden bridge Biotechnology Co., Ltd. (Beijing, China).

2.2. Preparation of extract

Semen cuscutae was purchased from a drugstore in Xi'an (China) and identified as the seed of *Cuscuta chinensis* by Professor Xianhua Tian at College of Life Sciences, Shaanxi Normal University according to the China Pharmacopoeia (CP). The powdered samples (100 g) were extracted with 95% ethanol (EtOH) at 85 °C (3 × 1000 ml). After filtration, the samples were concentrated by evaporation (80 °C) to evaporate the solvent to give a small volume. After extracting with mineral ether (3 × 200 ml), the ethanol layer was separated by a polyamide column chromatography. After eluting by water to remove the polysaccharide and proteins, the ethanol (EtOH) elution was collected. The elution was concentrated and dried in vacuum (60 °C) to make an ethanol extract of *Semen cuscutae* (EESC, yellowish brown powder, yield 2.6%). The total flavones in the ethanol extracts (TFSC) were measured by a spectrophotometer, and the contents of total flavones in the ethanol extracts were 57.3%.

2.3. Animal experiments

2.3.1. Animals and housing

Male ICR mouse (were purchased from Shaanxi Institute of Chinese Medicine), weighing 25 ± 2 g (age of 8 weeks) were separated into different groups by randomized procedure and acclimatized for 1 week prior to treatment. They received tap water and laboratory standard mouse diet (in pellet form). Groups of 10 animals were kept in Makrolon cages with sniff bedding (3/4 Faser) at 25 ± 2 °C a relative humidity of 30–70%. All animal experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Health, and Government of China.

2.3.2. Treatment of animals

The mice were partitioned into six groups (n = 10 in each group), they were given an intraperitoneal injection of 25 mg/kg hydrocortisone (purchased from Lijun Pharmaceutical Co., Ltd., Xi'an, China) for 10 days except for group VI. After this treatment, we could consider the mice as a model for kidney-yang deficiency. The animals of group VI were injected with an equal volume physiological saline. After 10 days, six groups were treated as follows: group I (high dose) were treated with 67.6 mg/kg TFSC; group II (low dose) with 33.8 mg/kg TFSC; group III with 1.3 g/kg methyltestosterone; group IV with 6 g/kg Jinkui Shenqi Wan (purchased from Beijing Tongrentang Group Co., Ltd., Beijing, China), groups V and VI with an equal volume of distilled water. All the samples are administered by gastric perfusion. Groups III and IV were designed as positive control groups, group V was control group, and group VI was normal group. Twenty-four hours after the last treatment (on day 15), blood samples (500 μl) were collected by puncture of the ophthalmic venous plexus with micro-hematocrit tubes in Eppendorf tubes, and then centrifuged at 3000 rpm for 15 min collect the serum then stored at −20 °C until hormone determination. After the experiment, mice were decapitated and one kidney and testicle were rapidly removed and frozen in liquid nitrogen for mRNA studies. The other kidney and testicle were fixed in 4% paraformaldehyde for 24 h at 4 °C, and subsequently embedded in paraffin using standard procedures.

2.4. Weight analyses

During testing mice were visually inspected for health effects twice a day and weighed on the last day.

2.5. Hormone analyses

Before the mice were executed, their serum was collected. Total serum testosterone levels were determined by radioimmunoassay using the Testosterone radioimmunoassay kit (Eiken Kagaku Co., Ltd., Tokyo, Japan). The lower limit of detection and the highest calibrator value are 10 and 400 ng/ml, respectively. Testosterone concentration was measured in duplicate using a double antibody

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