

Full Length Research Paper

# Therapeutic effect of petroleum ether extract from *Semen cuscutae* against $\beta$ -estradiol 3-benzoate induced kidney-yang deficiency in mice

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This study aimed to investigate the ameliorative effect of the petroleum ether extract of *Semen cuscutae* (SCPEE) on  $\beta$ -estradiol 3-benzoate (EB) induced kidney-yang deficiency (KD) by using an animal model. This model was established by daily intraperitoneal injection of EB (4 mg/kg) to ICR male mice for 15 days. To evaluate the SCPEE effect, after the first EB injection, the mice were orally administrated daily by SCPEE (10 ml/kg), and their physiological, kinematic, histological, and biochemical parameters were assessed at the beginning (Day 0) and the end (Day 15) of the experiment. The results showed that the EB injection could induce typical KD symptoms including physiological, kinematic and biochemical disorders of the mice. After SCPEE administration, a positive response against these symptoms was observed, and most of the disorders were recovered completely. This is the first report to demonstrate the significant anti-KD effect of SCPEE, indicating the ether-soluble components of *S. cuscutae* may have a potential clinical value for KD treatment.

**Key words:** *Semen cuscutae*, kidney-yang deficiency, traditional Chinese medicine,  $\beta$ -estradiol 3-benzoate, petroleum ether.

## INTRODUCTION

Kidney-yang deficiency (KD) is a typical pattern of kidney-disorder induced syndrome, which was defined by traditional Chinese medicine (TCM) theory in clinical practice. It was originally described by the *Medical Classic of the Yellow Emperor* about two thousand years ago and was characterized by metabolic disorder of body fluid and physiological dysfunctions presenting as aversion to cold, cold limbs, loin soreness, knee weakness, leg and pedal flaccidity, urine incontinence, tinnitus, hearing impairment and teeth looseness which are caused by intrinsic factors (example, aging and inherent defects) or extrinsic factors (example, over-fatigue and sexual overstrain) (the National Technology Bureau, 1997; Chen et al., 2008; Ding et al., 2009).

Likewise, in animal model of KD, it can also lead to a series of pathological signs, including weight loss, temperature decrease, activity reduction, organ shrinkage, metabolic dysfunction, etc. Accordingly, the physiological, kinematic, histological and biochemical parameters in correspondence with such pathological signs are suitable for the evaluation of therapeutic effect of medicinal materials against KD.

*Semen cuscutae* (SC), the dried ripe seed of *Cuscuta chinensis* Lam. (Convolvulaceae family), is a well-known Chinese herb recorded firstly in the famous book *Shen Nong's Herbal* as an upper grade drug. For thousands of years, it has been widely used in China as a traditional tonic and aphrodisiac to invigorate the kidneys (National Commission of Chinese Pharmacopoeia, 2010). A number of investigations indicate that SC possesses many pharmacological activities such as curing reproductive diseases, improving immune responses, and protecting against organ injury (Xiong and Zhou, 1994; Miyahara et

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al., 1996; Du et al., 1998; Qin et al., 2000; Quan, 2000; Wang et al., 2000; Pan et al., 2005; Kim et al., 2007; Yen et al., 2007; Yang et al., 2008). It was also found that the main constituents of SC are flavonoids, saccharide, lignans, organic acids, etc. (Shoji et al., 1994; Miyahara et al., 1996; Du et al., 1998; Wang et al., 2000; Li et al., 2002; Ye et al., 2002; 2005; Kim et al., 2007). Although SC has been extensively studied for its pharmaceutical application, little attention was paid to its potential for anti-KD therapy and only one relevant report was available (Yang et al., 2008). This single report showed that only the SC ethanol extract containing total flavones are responsible for its therapeutic effect, whereas no other extract with different constituents of SC has been studied.

Guifu Dihuang Pills (GD), a known standardized Chinese herbal medicine in Chinese Pharmacopoeia, is commonly used in the treatment of KD symptoms. This pill is made from eight medicinal herbs, such as *Cortex Cinnamomi (Rougui)*, *Radix Aconiti Lateralis Preparata (Fuzi)*, and *Cortex Paeonia (Mudanpi)* (National Commission of Chinese Pharmacopoeia, 2010). Preclinical and clinical studies have demonstrated its significant effect against KD (Sun, 2001; Huang et al., 2004; Peng et al., 2004; Gao et al., 2009). Therefore, GD is being used as a standard agent for anti-KD study in animal models.

In the present study, to evaluate the beneficial effect of the petroleum ether extract of SC (SCPEE) on KD symptoms, we assessed many relevant parameters of the mentioned pathological signs by using an animal model developed by intraperitoneal (ip) injection of  $\beta$ -estradiol 3-benzoate (EB) to mice.

## MATERIALS AND METHODS

### Samples preparation

SC was purchased from Zhejiang Traditional Chinese Medical University Electuary Factory (Hangzhou, China) and identified as the seed of *C. chinensis* Lam. (Family Convolvulaceae) by the author at Zhejiang Chinese Medical University. Powdered sample was boiled three times in a ten-time volume of 65% ethanol, followed by filtration and evaporation. The concentrated filtrates were treated with petroleum ether and again evaporated. This final concentrates were diluted to 150 mg/ml with aqueous 0.4% Tween 80 and termed as SCPEE.

### Animals

Male ICR mice (Grade II, 5-weeks-old) weighing 17 to 20 g were obtained from Animal Supply Center of Zhejiang Academy of Medical Science (certificate no. SCXK2003-0001, Hangzhou, China) and acclimatized for at least one week. During this period, the mice were supplied with tap water and rodent laboratory chow *ad libitum*, as well as a daily health inspection under a controlled room with a temperature of 25  $^{\circ}$ C, humidity of 55%, and a 12/12 h light/dark cycle. All the procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals and with the Animal Management Rules of the Health Ministry of PR China (document No 55, 2001).

### Animal treatments

The 40 mice were equally partitioned into four groups. All groups except the normal one were treated with 4 mg/kg ip injection of 0.4 mg/ml EB (purchased from General Pharmaceutical Co. Ltd., Shanghai, China) as KD model. After ip injection, mice in treated groups were administrated by oral gavage as follows: negative control (control-) group was treated with 0.4% Tween 80; positive control (control+) group was treated with 150 mg/ml GD (purchased from Taibao Pharmaceutical Co. Ltd., Lanzhou, China) solution; and SCPEE group was treated with SCPEE. All the aforementioned procedures were daily conducted at a dose of 10 ml/kg for 15 days. During this period, the physiological and kinematic tests were performed followed by a biochemical analysis using the mice blood. Then, all mice were sacrificed with ether anesthesia and their kidneys, testicles and seminal vesicles were removed immediately for organ-body index (OBI) measurement.

### Physiological measurements

Body weight (BW) and rectal temperature (RT) were applied for the physiological tests of the mice. The parameters were respectively recorded with an electronic balance (LP123, Changshu Weighing Apparatus Factory, Changshu, China) and a digital thermometer (DT-1TB, Huachen Medical Instruments Co. Ltd., Shanghai, China) at the beginning (Day 0) and the end (Day 15) of the experiment. Data from BW and RT were expressed as averaged values.

### Kinematic assessments

Grip strength (GS) and swimming time (ST) as the kinematic parameters of mice were both assessed on Days 0 and 15. The GS data as averaged values were measured twice per day using a rat/mice grip-strength meter (YLS-13A, Yiyuan Science & Technology Development Co. Ltd., Jinan, China). The ST data were obtained according to the method of Yu et al. (2002).

### Histological diagnose

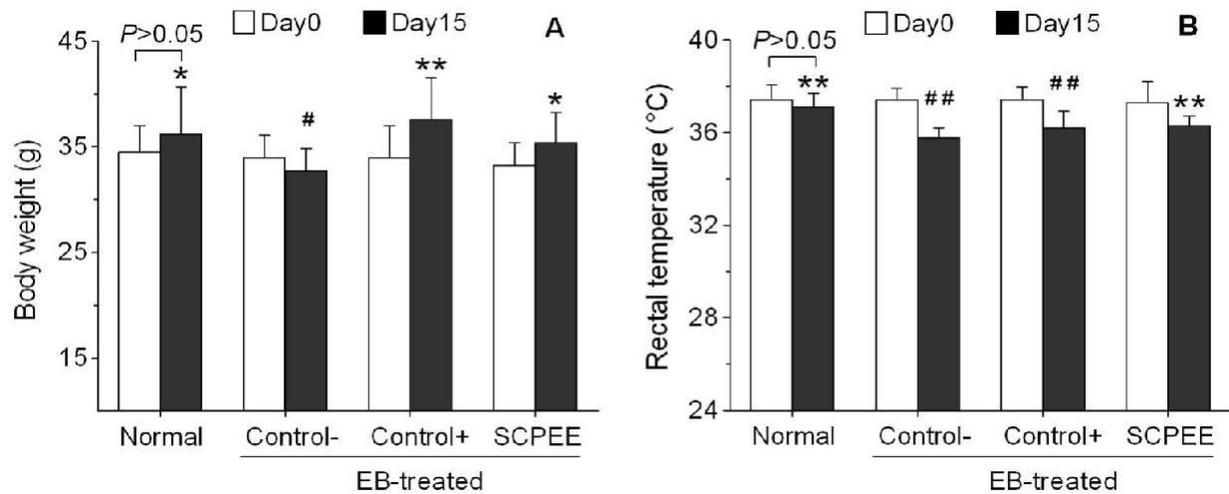
Kidneys, testicles and seminal vesicles of the mice were excised quickly, rinsed in 0.89% saline, blotted and weighed. The OBI of each organ was determined by the ratio of organ weight  $\times$ 100 to the animal-body weight.

### Biochemical assays

Freshly collected blood sample were obtained from the mice ophthalmic venous plexus and were immediately analyzed with the Vitalab Selectra E Chemistry Analyzer (Selectra E, Vital Scientific N. V., Dieren, the Netherlands) and reagent kits (Fuxing Changzheng Medical Science Co. Ltd., Shanghai, China). Blood biochemical assessments were conducted for two items: UR (mmol/L) and CR ( $\mu$ mol/L).

### Statistical analysis

All measurements were expressed as the mean  $\pm$  standard deviation and subjected to one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) comparison. *P* value of  $<0.05$  was considered statistically significant. All analyses were performed using an updated version of DPS software (Tang and Feng, 2007).



**Figure 1.** Effect of petroleum ether extract of *S.cuscutae* (SCPEE) on the physiological parameters of  $\beta$ -estradiol 3-benzoate (EB)-treated mice. Body weight (A) and rectal temperature (B) were measured on Day 0 (white bar) and Day15 (black bar) with the values expressed as means $\pm$ S.D (n=10).  $P < 0.05$  and  $**P < 0.01$  vs. control- group;  $#P < 0.05$  and  $##P < 0.01$  vs. normal group.

## RESULTS

### Effect of SCPEE on KD-altered animal physiological parameters

BW and RT regarded as the primary physiological parameters of mice were both measured at Days 0 and 15 (Figure 1). No significant difference was found among all groups at Day0 ( $P > 0.05$ ), and the data in the normal group between Days 0 and 15 were also not significantly different ( $P > 0.05$ ). These findings indicated that no other factor but only the EB was able to alter these physiological parameters after 15-day treatment. Therefore, we used the Day15 data to evaluate the effect of SCPEE on KD-induced physiological changes.

As shown in Figure 1A, a significant difference of mice BW was observed between the normal group and the control- group on Day15 ( $P < 0.05$ ), indicating an obvious mice hypogravity resulted from KD treatment. When compared with the normal group and the control- group respectively, the SCPEE group showed complete recovery of BW from the hypogravity level ( $P > 0.05$  vs normal group;  $P < 0.05$  vs. control- group). A similar result was seen in the control+ group ( $P > 0.05$  vs. normal group;  $P < 0.01$  vs. control- group).

As shown in Figure 1B, a significant decrease of mice RT on Day15 was found in the control- group compared to the normal ( $P < 0.05$ ), suggesting a severe mice hypothermia in the control- group. As compared with the normal group and the control- group respectively, RT in the SCPEE group was found to be almost fully recovered to the normal level ( $P > 0.05$  vs. normal group;  $P < 0.01$  vs. control- group), whereas that in the control+ group was still kept abnormal ( $P < 0.01$  vs. normal group;  $P >$

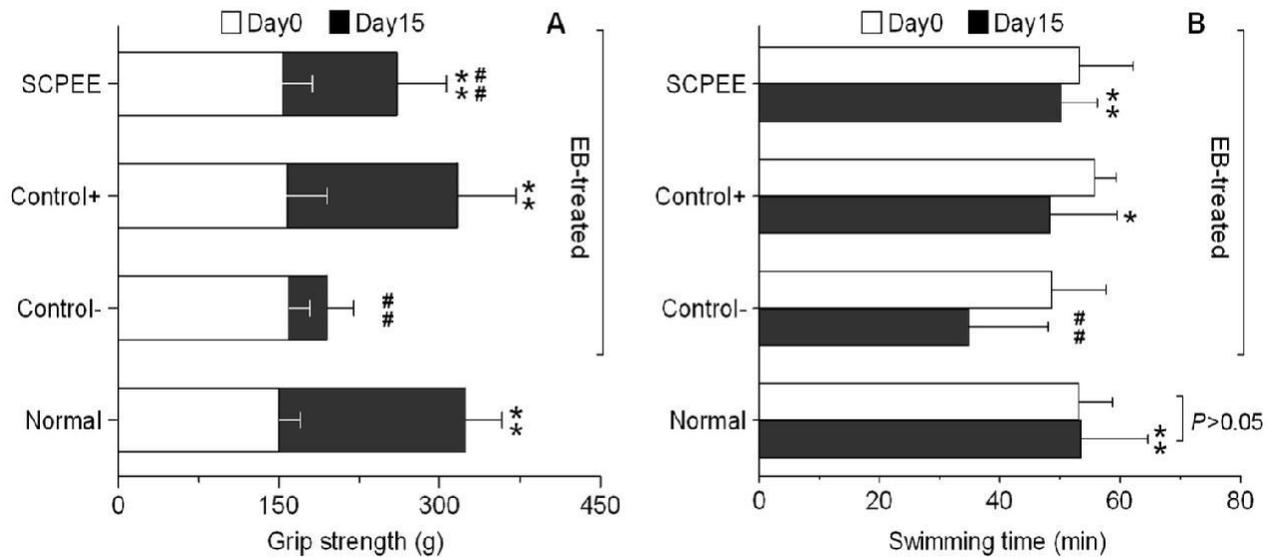
0.05 vs. control- group).

### Effect of SCPEE on KD-altered animal kinematic parameters

Columns in Figure 2 displayed mice GS and ST on Days 0 and 15. Before any administration, no significant differences were observed among all groups on Day 0 ( $P > 0.05$ ). Further, unlike GS as a time-dependent variable, ST in the normal group showed no significant difference between Days 0 and 15 ( $P > 0.05$ ). These suggested that any significant change of the kinematic parameters during the experimental period were only due to our treatment. We employed these parameters on Day15 to evaluate the SCPEE effect on KD-induced kinematic abnormalities.

As illustrated in Figure 2A, on Day15, mice GS had a significant difference between the normal group and the control- group ( $P < 0.01$ ), demonstrating a grip-strength deficit in the KD treated group. When compared with the control- group and the normal group respectively, GS in the SCPEE group was significantly increased from the abnormal level although not up to the normal ( $P < 0.01$  vs. control- group;  $P < 0.01$  vs. normal group), while that in the control+ group was fully recovered ( $P < 0.01$  vs. control- group;  $P > 0.05$  vs. normal group).

As illustrated in Figure 2B, a significant difference of mice ST was present on Day15 between the normal group and the control- group ( $P < 0.01$ ), indicating a markedly decline of mice exercise capability in the control- group. As compared with the control- group and the normal group respectively, ST in the SCPEE group was significantly prolonged and then completely restored



**Figure 2.** Effect of petroleum ether extract of *S. cuscutae* (SCPEE) on the kinematic parameters of  $\beta$ -estradiol 3-benzoate (EB)-treated mice. Grip strength (A) and Swimming time (B) were measured at Day 0 (white bar) and Day15 (black bar) as means $\pm$ S.D ( $n=10$ ). \* $P < 0.05$  and \*\* $P < 0.01$  vs. control- group; # $P < 0.05$  and ## $P < 0.01$  vs. normal group;  $P > 0.05$  vs. normal group).

to the normal state ( $P < 0.01$  vs. control- group;  $P > 0.05$  vs. normal group), which is similar to that of the control+ group ( $P < 0.05$  vs. control- group;  $P > 0.05$  vs. normal group).

### OBI analyses of mice kidneys, testicles and seminal vesicles

After the target organs of mice were excised, no histological difference was seen between the normal group and any other groups. As shown in Figure 3, OBIs of the tested organs in the control- groups were significantly lower than the normal levels ( $P < 0.05$  for kidneys;  $P < 0.01$  for others), indicating obvious organ-weight losses as KD-induced pathological changes of the organs. By comparing with each normal group and each control-group respectively, the abnormal OBIs of kidneys and seminal vesicles in their SCPEE groups were found to be completely recovered (both  $P > 0.05$  vs. normal group;  $P < 0.05$  vs. control- group for kidneys and  $P < 0.01$  vs. control- group for seminal vesicles), and that of testicles was also significantly increased but not fully recovered from the abnormal level ( $P < 0.01$  vs. normal group;  $P < 0.05$  vs. control- group). However, only the control+ group of kidneys exhibited a full recovery of OBI from its previously abnormal level ( $P > 0.05$  vs. normal group;  $P < 0.01$  vs. control- group).

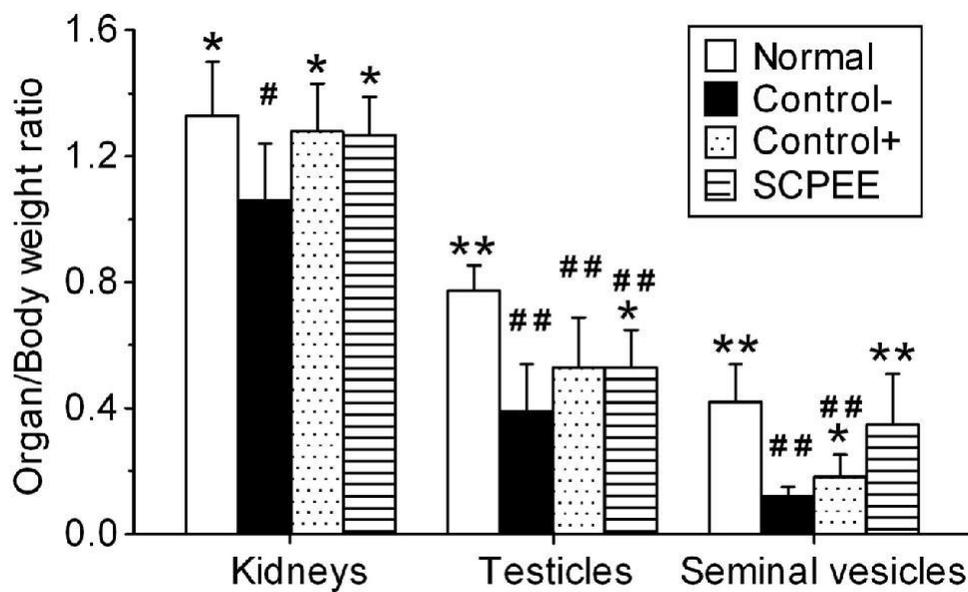
### Biochemical analysis of all groups

As shown in Table 1, it is evident that the mice UR and

CR concentrations in the control- groups were significantly increased as compared with the normal levels ( $P < 0.01$  for UR;  $P < 0.05$  for CR), indicating that the KD treatment could give rise to abnormal alterations of such biochemical parameters. Significant decreases were observed in the SCPEE groups of both UR and CR compared to their control- groups (both  $P < 0.01$ ), while no significant difference was seen between each SCPEE group and each normal group (both  $P > 0.05$ ), resulting in a complete recovery of these abnormal parameters.

### DISCUSSION

In terms of the TCM theory, a certain insufficiency of kidney essence is regarded as the pathogenesis of KD, which may trigger a chain of systematic abnormalities of human body because the kidneys are of importance in supporting the major physiological functions. Modern medicine study indicates that the functional disorder with different degree of hypothalamic-pituitary-target gland (adrenal, thyroid and gonad) axis is the significant mechanism for forming KD syndrome (Lu et al., 2011). To mimic such syndrome, the exogenous estrogen EB was always used to inject animals for establishing the animal model of KD (Chen et al., 2008; Lv et al., 2008), by which the hormone homeostasis of adrenal cortex as well as the function of reproductive system were disrupted (Gallagher, 2001; Hunt et al., 2001; Toyama et al., 2001). In this study, we also applied EB to establish the KD model of mice and then proved its success since a series of KD syndrome signs involving hypogravity, hypothermia, strength reduction, exercise-capability decrease,



**Figure 3.** Effect of petroleum ether extract of *Semen cuscutae* (SCPEE) on the organ/body weight ratios of the three organs in  $\beta$ -estradiol 3-benzoate (EB)-treated mice. Data are means  $\pm$  S.D. \*  $P < 0.05$  and \*\*  $P < 0.01$  vs. control- group; #  $P < 0.05$  and ##  $P < 0.01$  vs. normal group.

**Table 1.** Effect of SCPEE on blood biochemical parameters of EB-treated mice. Values are means  $\pm$  SD.  $n=10$  in each group.

Groups	EB (ml/kg)	SCPEE (ml/kg)	Biochemical parameters	
			Urea (mmol/L)	Creatinine ( $\mu$ mol/L)
Normal	—	—	6.26 $\pm$ 1.1**	35.6 $\pm$ 3.3*
Control-	10.0	10.0	8.47 $\pm$ 1.4###	40.2 $\pm$ 4.7#
Control+	10.0	10.0	7.20 $\pm$ 1.5**	38.0 $\pm$ 3.7*
SCPEE	10.0	10.0	6.86 $\pm$ 0.9**	35.6 $\pm$ 4.4*

\*  $P < 0.05$  and \*\*  $P < 0.01$  vs. control- group; #  $P < 0.05$  and ##  $P < 0.01$  vs. normal group.

organ shrinkage, and an abnormality of biochemical parameters were presented due to the EB treatment only. Moreover, those symptoms seemed approximated to that found in the clinic (Chen et al., 2008, 2010). Therefore, the methodology used in our study is feasible and the evaluation of SCPEE on KD based on such model is considered to be scientifically reliable.

In this study, the aforementioned parameters were chosen to assess the SCPEE effect on KD as a result of the following reasons:

- 1) BW and RT as the physiological parameters are regarded as primary physical characteristics for diagnosing the disease status and can directly reflect somewhat KD syndrome such as aversion to cold and cold limbs.
- 2) GS and ST, the typical kinematic parameters representing body strength and exercise capability, are thought to be the indicators of the clinical KD symptoms

(example, loin soreness, knee weakness, leg and pedal flaccidity).

3) OBIs of the reproductive organs as histological parameters are sensitive to organ shrinkages and organ weight losses which lead to a decline of reproductive capacity. (4) UR and CR as the biochemical parameters are susceptible to kidney failure and excretion dysfunction which is also defined as the KD symptoms. Accordingly, those parameters are good indicators of the KD syndrome and are thereby suitable for anti-KD activity assessment of SCPEE.

In this study, SCPEE was able to successfully counteract all EB-induced KD symptoms in mice. It showed a similar profile of activity as the standard agent GD which is one of the most popular anti-KD drugs in clinic and has been used to cure kidney-related disorders in China for a long time. The only published study regarding our topic has shown that SC possesses anti-KD effect only depending

on its ether-insoluble flavones (Yang et al., 2008). Moreover, our unpublished data have suggested that SC total flavones are mostly involved in the water extract and ethanol extract, far more than in the petroleum ether extract which mainly contained lignans and sterols without flavones. Therefore, this is the first report evaluating the therapeutic effect of SC ether extract on KD using such animal model. The 'new' extracted bioactive components need to be further investigated and developed as a new candidate for KD therapy.

## Conclusion

In combination of all obtained findings, we can conclude that our discovery of the anti-KD effect of SCPEE were to complement the previous study and to extend the knowledge of SC activity. Further works are required to elucidate the qualitative and quantitative composition as well as the mechanism of action of the bioactive components of SCPEE for its future application.

## ACKNOWLEDGEMENT

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