Estrogenic effect of the extract of Renshen (Radix Ginseng) on reproductive tissues in immature mice

Ding Jie, Xu Ying, Ma Xiaoping, An Jinna, Yang Xiudong, Liu Zhiqiang, Lin Na

OBJECTIVE: To evaluate the estrogenic efficacy of Renshen (Radix Ginseng) (GS) on reproductive target tissues in immature mice.

METHODS: One hundred and ten female immature Kunming (KM) mice, 21-day-old, were randomly assigned to eleven groups, 10 for each; one served as control group treated with 0.154 mg/kg estradiol valerate (EV, n = 10), the rest were treated respectively with GS intragastrically at a daily dose of 0.5, 1.0, 1.5, 3.0, 6.0, 12.0, 18.0, 24.0 and 30.0 g/kg (n = 10 in per group) for 7 days. The estrous cycle, uterine weight, hormone levels in circulation and histomorphology changes of uterus and vagina were scrupulously examined. The estrogen receptor (ER) α and ERβ expressions in the uterus and vagina were detected by immunohistochemistry and western blotting.

RESULTS: Treatment with GS at the dose of 12.0, 18.0 and 24.0 g/kg resulted significant estrogenic activity in the mice, as indicated by advanced and prolonged estrous stage and increased uterine weight (all P < 0.05). GS treatment substantially promoted development of reproductive tissue by thickening the uterine endometrium and increasing vaginal epithelial layers. In addition, treatment with GS induced significant up-regulation of ERα and ERβ expressions in reproductive tissues, and ERα up-regulation was stronger than that of ERβ. GS could raise levels of circulating estrogen, simultaneously decrease levels of luteinizing hormone and follicle-stimulating hormone (all P < 0.001) compared with the control group.

CONCLUSION: Our findings suggest that GS had estrogenic effect on reproductive tissues in immature mice by stimulating biosynthesis of estrogen in circulation and up-regulating ERs.

Key words: Panax; Uterus; Vagina; Receptors, estrogen; Follicle stimulating hormone; Luteinizing hormone

INTRODUCTION

Renshen (Radix Ginseng) (GS) is an herbal medicine
that has been used for over 2000 years in oriental countries. It has been reported that GS has a wide range of pharmacological activities in cardiovascular, endocrine, immune, and central nervous systems. Studies also showed that GS could relieve menopausal symptoms, bleeding disorders, sleeping disorders, depression and anxiety, which indicates that some components of GS act as phytoestrogens and/or involve activation of the estrogen receptor (ER). In vitro studies revealed GS extracts were able to stimulate the growth of ER-positive cells. Ginsenoside-Rg1, Rb1, and -Rh2 were the major estrogen active compounds of ginsenoside. was shown to be a potent phytoestrogen that preferentially activated ERα via phosphorylation of the activation function-1 domain in the absence of receptor binding. Nowadays, most studies focus on mechanism of GS in vitro. However, the estrogenic actions of GS on reproductive tissues and the underlying mechanism are not fully addressed, besides Wang et al. reported that administration of Rb1 caused an increase uterine weight of the normal female mouse. In the present study, we aimed to investigate the estrogenic effects of GS on reproductive target tissues of immature mice by monitoring estrous cycle, uterine weight gain, hormone levels, observing the histological structure changes and ERα and ERβ expressions, as part of an ongoing effort to provide scientific data and identify potent estrogen-like activity of GS.

MATERIALS AND METHODS

Animals and experimental design

Totally 110 of 21-day-old female immature mice (12 ± 2) g were purchased from Experimental Animal Center of Academy of Military Medical Sciences (Certificate of quality No. SCXK [jun] 2007-004). The mice were randomly divided into eleven groups (10 for each) by random number table method; one was the control group treated with 0.154 mg/kg estradiol valerate (EV); the rest were treated respectively with GS intra-peritoneal injection (EV); the rest were treated respectively with GS intra-peritoneal injection (EV); the rest were treated respectively with GS intra-peritoneal injection (EV). Untreated control mice received distilled water only. All animals were maintained in a 12-h light/dark cycle under constant temperature (24 ± 2 °C) and humidity (55% ± 5%), and were allowed free access to food and water. All procedures for consideration of animal welfare were reviewed and approved by the ethical committee of China Academy of Traditional Chinese Medicine.

Chemicals and reagents

Estradiol valerate (EV) was purchased from Bayer Schering Pharma (Berlin, Germany). ERα antibody (MC-20) was purchased from Abcam Biotechnology (Cambridge, UK). ERβ antibody (ab3577) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (14C10) was purchased from Cell Signaling (Boston, MA, USA). All other chemicals were of analytical grade.

The preparation of GS extract

GS was purchased from Changchun Medicinal Herbs Co., Ltd., (Jilin, China) and identified and authenticated by an expert at Changchun Institute of Applied Chemistry Chinese Academy of Sciences. GS was pulverized to a fine powder and boiled twice with distilled water for 1 hour under reflux. The aqueous extracts were collected and filtered. The filtrates were then concentrated under reduced pressure at 50°C and to a concentration of 0.6 g/mL. As previous described, the representative chemical compositions of Rb1 (0.228%), Rf (0.252%), Rc (0.252%), Rg1 (0.636%), Rd (0.06%), Rg2, and Re (0.56%) in the GS extract were determined by high performance liquid chromatography (HPLC) analysis. The total ginsenosides, calculated as the sum of the above individual ginsenosides, represented 1.884% in the GS extract.

Monitoring estrus cycle

All mice were monitored by daily vaginal epithelium cells smear testing during the 7 days administration period. The vaginal lavage was fixed with 95% ethanol for 10 min and stained with methylene blue for 10 min. Vaginal epithelial cells were observed by Olympus BX41 microscope (Olympus, Tokyo, Japan), and keratinized vaginal cells were taken as being indicative of estrus.

Analysis of tissue and serum

Blood was collected from the eye venous plexus and animals were sacrificed by decapitation after 7 days of treatment. The serum were analyzed for estradiol (E2), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by enzyme-linked immunosorbent assay (ELISA). The uterus and vagina were removed and weighed. The left horns of the uterus and the upper portion of vagina were stored at -80°C. The right horns of the uterus and the under portion of vagina were fixed with 4% polyoxymethylene for 24 h. All samples were embedded in paraffin and prepared for cross sections; sections 4 mm thick were cut, mounted, and stained with Hematoxylin & Eosin (HE) for microscopy (Olympus, Tokyo, Japan).

Immunohistochemistry

Tissue sections 4 µm thick of uterus and vagina were mounted on polylysine-coated slides, deparaffinized, rehydrated, and then heated with 10 mM citrate buffer, pH 6.0. After two washes with phosphate-buffered saline (PBS), slides were incubated with 3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity. After washing with PBS, tissues were incubated with blocking serum (Boster
RESULTS

Effect of GS on the estrus cycle

The estrous cycle of all mice was monitored by daily inspection of vaginal epithelium cell smears. Smears of the vaginal epithelium cells of the untreated immature mice consisted of leukocytes, which is indicative of constant diestrus. In contrast, the vaginal cells from the immature mice treated with GS (12.0, 18.0, 24.0 or 30.0 g/kg) or EV became keratinized after treatment for about 4 days, indicating that immature mice treated entered into the status of estrus earlier than the immature mice untreated. Moreover treatment with GS prolonged the estrous stage of the immature mice, suggesting very potent estrogenic activity.

Effect of GS on uterine weight

Uterine weight was significantly increased in immature mice treated with EV (P < 0.01). Treatment of immature mice with GS had modest stimulatory effects on uterine weight, the higher dose 12.0, 18.0, 24.0 or 30.0 g/kg treatment resulted significant differences compared with untreated controls (all P < 0.05) (Figure 1), suggesting that GS has significant estrogenic activity at the four doses. Notably, the largest changes were found at the 18.0 g/kg dose. These data prompted further studies to elucidate the activity and mechanism of GS at the three doses of 12.0, 18.0 and 24.0 g/kg.

Effect of GS on levels of serum $E_2$, LH and FSH

Treatment of immature mice with GS 12.0, 18.0, 24.0 g/kg or with EV, significantly raised levels of circulating $E_2$ compared to those of untreated immature mice (all $P < 0.001$). GS 18.0 g/kg treatment of immature mice induced 95% increase in $E_2$ level compared to those of untreated immature mice. The GS treatment had significant stimulatory effects on LH and FSH, the higher dose, 18.0 g/kg, resulted 30% decrease in LH and FSH (both $P < 0.001$) compared with the control group respectively (Figure 2).

Effect of GS on histopathology of uterus and vagina

Histological analysis of uterine sections revealed treatment of immature mice with EV or GS 12.0, 18.0 or 24.0 g/kg substantially changed uterine morphology (Figure 3A-3E), as indicated by thickening of the uterine endometrium, increased number of glands, and more extended glandular cavities compared with untreated controls. Thus, GS had potent estrogenic activity, similar ability to EV. Figure 3F-3J shows microscopic preparations of representative vagina from one animal per treatment group, compared with untreated immature mice, the EV animals (Figure 3G) displayed a typical squamous multilayered epithelium layers with cornification. Treatment with GS at the three doses increased epithelial thickness and also the number of cell layers. Taken together, these studies provide evidences that GS has significant estrogenic activity, comparable
to that of the synthetic estrogen EV. These data prompted further studies to elucidate the molecular basis of GS activity.

**Effect of GS on ERs expression by immunohistochemistry in uterus and vagina**

The expressions of ERα and ERβ in the uterus and vagina from each group and quantitative analysis are shown in Figure 4. Treatment with either EV or GS at the three doses induced clear and comparable up-regulation of ERα and ERβ in the uterus and vagina, and the largest increase of them were found in the three groups treated with dose of 18.0 g/kg and higher (both

**Figure 1** effect of GS on uterine weight

The uterine weights were measured at the end of the 7 days treatment. Con group: immature mice without treatment (n = 10); EV group: immature mice treated with EV at a daily dose of 0.154 mg/kg (n = 10); GS group: immature mice treated with GS at a daily dose of 0.5, 1.0, 1.5, 3.0, 6.0, 12.0, 18.0, 24.0 and 30.0 g/kg respectively (n = 10). Con: control; EV: estradiol valerate; GS: Renshen (Radix Ginseng). Compared with untreated Con group, *P < 0.01 and *P < 0.05.

**Figure 2** effect of GS on serum E2, LH and FSH in immature mice

A-C: the levels of E2, LH and FSH in serum. Con group: immature mice without treatment (n = 10); EV group: immature mice treated with EV at a daily dose of 0.154 mg/kg (n = 10); GS group: immature mice treated with GS at a daily dose of 12.0, 18.0 and 24.0 g/kg respectively (n = 10). Con: control; EV: estradiol valerate; GS: Renshen (Radix Ginseng). Compared with untreated Con group, *P < 0.001.

**Figure 3** effect of GS treatment on the histology of uterus and vagina in the immature mice

A-E: uterine histopathologic changes of Con, EV and GS group (HE staining, × 100); F-J: vaginal histopathologic changes of Con, EV and GS group (HE staining, × 400). A: uterine histological features of Congroup; B: uterine histological features of EV group; C: uterine histological features of GS 12.0 g/kg group; D: uterine histological features of GS 18.0 g/kg group; E: uterine histological features of GS 24.0 g/kg group; F: vaginal histological features of Con group; G: vaginal histological features of EV group; H: vaginal histological features of GS 12.0 g/kg group; I: vaginal histological features of GS 18.0 g/kg group; J: vaginal histological features of GS 24.0 g/kg group. Con group: immature mice without treatment (n = 10); EV group: immature mice treated with EV at a daily dose of 0.154 mg/kg (n = 10); GS group: immature mice treated with GS at a daily dose of 12.0, 18.0 and 24.0 g/kg respectively (n = 10). Con: control; EV: estradiol valerate; GS: Renshen (Radix Ginseng); HE: hematoxylin and eosin.
$P < 0.001$). ERs in uterus were expressed in similar cell types in the GS treated or EV treated groups, namely in the epithelial cells of the endometrium, interstitial cells and smooth muscle cells. ERs in vagina were expressed in vaginal epithelium cells of squamous cell and smooth muscle cells (Figure 4). These data further support the indication that GS had very potent estrogenic activity.

**Figure 4** The effects of GS treatment on the expressions of ERα and ERβ in the uterus and vagina. A1-E1: the expression of ERα of Con, EV, GS 12.0, 18.0 and 24.0 g/kg in the uterus; A2-E2: the expression of ERβ of Con, EV, GS 12.0, 18.0 and 24.0 g/kg in the uterus. F1-J1: the expression of ERα of Con, EV, GS 12.0, 18.0 and 24.0 g/kg in the vagina; F2-J2: the expression of ERβ of Con, EV, GS 12.0, 18.0 and 24.0 g/kg in the vagina. Immunohistochemistry was used in uterine frames (× 100) and vaginal frames (× 400) respectively. K and L: IOD of ERs in uterus and vagina. Con group: immature mice without treatment ($n = 10$); EV group: immature mice treated with EV at a daily dose of 0.154 mg/kg ($n = 10$); GS group: immature mice treated with GS at a daily dose of 12.0, 18.0 and 24.0 g/kg respectively ($n = 10$). GS: Renshen (Radix Ginseng); ER: estrogen receptor; Con: control; EV: estradiol valerate. Compared with untreated Con group, $^aP < 0.001$ and $^bP < 0.01$. 

*Experimental Study*
Effect of GS on ERs expressions by western blot in uterus and vagina

Further evidence for the interaction of the GS with the ERs system was sought by determining the effects on ER subtype expressions on protein in target tissues by western blot. The results as shown in Figure 5, compared with the control group, treatment with either EV or GS at the three doses induced significant up-regulation of ERα and ERβ expressions in target tissues. The higher dose, 18.0 g/kg, resulted in a largest up-regulation of ERα (P < 0.001) and 1.96-fold ERβ (P < 0.001) in uterus compared with untreated immature mice respectively. GS at the dose of 18.0 g/kg also up-regulated 1.88-fold ERα (P < 0.001) and 1.88-fold ERβ expressions (P < 0.001) in vagina.

DISCUSSION

In present study, we investigated the estrogenic effects of GS on reproductive target tissues in immature mice. The results showed treatment with GS at the dose of 12.0, 18.0 and 24.0 g/kg resulted significant estrogenic activity in immature mice, as indicated by advanced and prolonged estrous stage and increased uterine weight. GS treatment substantially promoted development of uterus and vagina, and induced significant up-regulation of ERα and ERβ expressions in reproductive target tissues. In addition, GS could raise levels of circulating E2, simultaneously decrease levels of LH and FSH. GS’s estrogenic activity may be mediated by stimulating biosynthesis of estrogen and increasing the quantity of ERs in the reproductive target tissues. Studies showed that treatment with estrogen can increase uterine wet weight, epithelial cell height and the numbers of glands. In our study, treatment with GS promoted sexual maturation as indicated by increasing uterine weight, thickening the uterine endometrium and vaginal epithelial layers, thus, GS had potent estrogenic activity on reproductive tissues in immature mice. Chen et al. reported that chronic administration of ginsenoside Rg1 did not result any estrogenic effects on reproductive tissues in an ovariectomized animal model, which suggest other components in GS exert activity on reproductive target tissues.

Figure 5 effect of GS on the expressions of ER
A,C: protein expression levels of ERs in the uterus; B,D: protein expression levels of ERs in the vagina. 1: Con group; 2: EV group; 3: GS 12 g/kg group; 4: GS 18 g/kg group; 5: GS 24 g/kg group. Con group: immature mice without treatment (n = 10); EV group: immature mice treated with EV at a daily dose of 0.154 mg/kg (n = 10); GS group: immature mice treated with GS at a daily dose of 12.0, 18.0 and 24.0 g/kg respectively (n = 10); GS: Renshen (Radix Ginseng); ER: estrogen receptor; Con: control; EV: estradiol valerate. Compared with untreated Con group, *P < 0.001 and **P < 0.01.

---

**Table 1**

<table>
<thead>
<tr>
<th>Dose (g/kg)</th>
<th>ERα</th>
<th>ERβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS 24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5**

A,C: protein expression levels of ERs in the uterus; B,D: protein expression levels of ERs in the vagina. 1: Con group; 2: EV group; 3: GS 12 g/kg group; 4: GS 18 g/kg group; 5: GS 24 g/kg group. Con group: immature mice without treatment (n = 10); EV group: immature mice treated with EV at a daily dose of 0.154 mg/kg (n = 10); GS group: immature mice treated with GS at a daily dose of 12.0, 18.0 and 24.0 g/kg respectively (n = 10); GS: Renshen (Radix Ginseng); ER: estrogen receptor; Con: control; EV: estradiol valerate. Compared with untreated Con group, *P < 0.001 and **P < 0.01.
Estrogen plays an important role in the growth, development and reproduction. Estrogens are synthesized by ovary in immature age. The increased serum estrogen concentration after treatment with GS suggests that GS might take it action by mediating the hypothalamus-pituitary-ovary axis and stimulate biosynthesis of estrogen in the ovary. In addition, using FSH and LH levels as markers of estrogenic effect of GS on hypothalamic-pituitary axis where the gonadotropin-releasing hormone pulse generator resides in the hypothalamus. The gonadotropin-releasing hormone pulse generator is overactive in the absence of estrogens, which results in high serum FSH and LH release from the in pituitary of postmenopausal woman. Treatment with GS at higher doses promoted E2 release and diminished the ascending FSH and LH levels in immature mice, which suggest the short-loop effect of GS directly on hypothalamus. Under physiological conditions, the biological effects of the estrogen depend on not only the level of estrogen but also on the distribution and expression levels of the corresponding ERs in the target cell, ERα, and ERβ. Estrogen and ERs are involved in the physiological function regulation of the female reproductive system. Estrogens carry out their action by binding to a high affinity nuclear receptor, the ERs. In a recent report, ginsenosides showed two types of regulatory effect on ERs: Rg1 acted as ERα-selective agonists and only at high concentration activated ERβ. Whereas Rb, activated both ERα and ERβ and the activation was inhibited by the ER antagonist ICI 182,780. It is likely that the ability of GS to up-regulate both ERs can be explained by the presence of multiple active components contained in the ingredient herbs, that together exhibit polyvalent activities on ER regulation in target tissue. In our study, GS significantly upregulated the expressions of ERα and ERβ in uterus and vagina respectively. It is worthy of mentioning that ERα up-regulation induced by GS extract was stronger than that of ERβ, which suggests that GS might bind to ERα with higher selectivity than ERβ in reproductive target tissues.

In conclusion, GS may be capable of promoting development of reproductive tissues of immature mice, and GS’s estrogenic activity may take it action by stimulating biosynthesis of estrogen and increasing the quantity of ERs in the target organs.

REFERENCES

10. Chen WF, Gao QG, Dai ZJ, Kung AW, Guo DA, Wong MS. Estrogenic effects of ginsenoside Rg1 in endometrial cells in vitro were not observed in immature CD-1 mice or ovariectomized mice model. Menopause 2012; 19(9): 1052-1061.


22 Padilla-Banks E, Jefferson WN, Newbold RR. The immature mouse is a suitable model for detection of estrogenicity in the uterotropic bioassay. Environ Health Perspect 2001; 109(8): 821.


