Original Research Article

Effect of *Lycii fructus* polysaccharides on ovulation failure in rats

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Abstract

Purpose: *To investigate the effect of Lycii Fructus polysaccharides (LFPS) on ovulation failure.*
Methods: *A rat model of ovulation failure was established by intragastric administration of hydroxyurea (300 mg/kg). Rats with ovulation failure then received LFPS via oral administration at doses of 100, 200, or 400 mg/kg. The body, uterus and ovary of each rat were weighed using electronic scales. The hypothalamic-pituitary-ovarian (HPO) axis hormones, including estradiol (E_2) level, follicle-stimulating hormone (FSH) activity, and luteinizing hormone (LH) activity in the serum of each rat were determined by enzyme-linked immunosorbent assay (ELISA). The levels of pro-apoptotic proteins (Fas, FasL, FADD, c-caspase-8, c-caspase-10, c-caspase-3, c-caspase-6, and c-caspase-7) in the ovarian tissue of each rat were detected by western blot.*
Results: *Hydroxyurea reduced significantly (p < 0.01) uterus and ovary indices (uterus or ovary weight/body weight) (0.119 and 0.026 %), E_2 level (3.42 pmol/L), and FSH and LH activities (2.28 and 2.76 U/L), compared with those in the normal group (0.169 and 0.039 %; 6.72 pmol/L; 2.76 and 3.75 U/L). Hydroxyurea increased significantly (p < 0.01) the levels of the above-mentioned pro-apoptotic proteins relative to those in the normal group. LFPS (100, 200, or 400 mg/kg) reversed significantly (p < 0.05 or 0.01) the effect of hydroxyurea on all of the above indices.*
Conclusion: *LFPS exhibits a protective effect on hydroxyurea-induced ovulation failure by regulating the HPO axis hormones and death receptor-mediated apoptotic pathway.*

Keywords: *Lycii Fructus polysaccharides, Ovulation failure, Hypothalamic-pituitary-ovarian axis, Death receptor-mediated apoptotic pathway*

INTRODUCTION

Infertility is a common gynecological disease and its incidence has gradually increased in recent years [1,2]. Infertility, a worldwide social and medical problem, is caused by many etiologies, including ovulation failure [3-5]. Infertility caused by ovulation failure accounts for roughly 25 - 30 % of infertility cases [6].

Hormonal ovulation-stimulating drugs are usually used to treat ovulation failure in Western medicine [7,8]. While the ovulation rate of patients increases after treatment with hormonal ovulation-stimulating drugs, their pregnancy rate is low and there are many adverse reactions associated with this type of treatment, such as follicular mal-development, ovarian hyperstimulation syndrome, internal secretion dysfunction, and ovarian cancer [9-11]. By

contrast, natural herbal medicines are typically used to treat ovulation failure in Chinese medicine [12,13] and few adverse reactions induced by Chinese medicine have been reported [10]. Therefore, Chinese medicine may be a possible source of new and effective agents for treating ovulation failure.

Lycii Fructus, the fruit of *Lycium barbarum* L. (Solanaceae), is generally used by Chinese physicians to treat male infertility and previous reports on the effects of *Lycii Fructus* polysaccharides (LFPS) on infertility have focused primarily on males [14,15]. The effects of LFPS on female infertility caused by ovulation failure have thus far remained unknown. Therefore, this work was initiated to investigate the effects of LFPS on ovulation failure in rats by calculating the uterus and ovary indices, determining the hypothalamic-pituitary-ovarian (HPO) axis hormones, including estradiol (E_2) level, follicle-stimulating hormone (FSH) activity and luteinizing hormone (LH) activity in serum, and detecting the levels of pro-apoptotic proteins, including Fas, FasL, FADD, cleaved (c)-caspase-8, c-caspase-10, c-caspase-3, c-caspase-6, and c-caspase-7 in ovary tissue.

EXPERIMENTAL

Chemicals and reagents

LFPS was obtained from Ningxia Wolfberry Biological and Food Engineering Co., Ltd. (Yinchuan, China). LFPS was dissolved in normal saline to obtain 10, 20, and 40 g/L of LFPS for experiments. Hydroxyurea tablets were provided by Anter Biopharmaceutical Co., Ltd. (Jinzhong, China). Chloral hydrate was obtained from Sigma-Aldrich (Shanghai, China). E_2, FSH, and LH ELISA kits were purchased from Shanghai Baili Biotechnology Co., Ltd. (Shanghai, China). Enhanced BCA protein assay reagent was obtained from Beyotime (Haimen, China). Primary antibodies for β-actin, Fas, FasL, FADD, c-caspase-8, c-caspase-10, c-caspase-3, c-caspase-6, and c-caspase-7, along with HRP-conjugated goat anti-rabbit antibody were obtained from Abcam (Cambridge, UK), Cell Signaling Technology (Beverly, MA, USA), or Jackson Immuno Research Laboratories (West Grove, Pennsylvania, USA). An enhanced chemiluminescence detection kit for HRP was purchased from Biological Industries (Kibbutz Beit Haemek, Israel).

Animals

Hebetic female SD rats (200 ± 20 g) were obtained from the Experimental Animal Center of Henan Province (Zhengzhou, China). All animal treatments were performed in strict compliance with the National Institutes of Health Guide concerning the care and use of laboratory animals [16]. All experiments in this study were approved by Henan Hospital of Traditional Chinese Medicine Hospital (Protocol No. HNHTCM2011049).

Establishment of ovulation failure model and treatment

Hydroxyurea was used to induce ovulation failure in rats according to a previous method [17]. Hydroxyurea tablet (30 g) was dissolved in normal saline (1 L) to obtain a 30 g/L turbid hydroxyurea liquid. Rats were administrated orally with hydroxyurea at a dose of 300 mg/kg once a day for 10 days to establish the ovulation failure model. Next, rats with ovulation failure were randomly divided into 4 groups: one model group and three groups that were administrated with different doses of LFPS (n = 10). Rats without ovulation failure served as normal group (n = 10). Rats in the normal and model groups were administrated orally with normal saline once a day for 14 days. Rats in the LFPS groups were administrated orally with LFPS at doses of 100, 200, or 400 mg/kg once a day for 14 days, respectively. All rat received an intragastric volume of 10 mL/kg.

Determination of uterus and ovary indices

After 2 h of drug treatment on 14th day, the rats were anesthetized with 10 % chloral hydrate (3 mL/kg) by intraperitoneal injection. The 5 - 6 mL blood from the abdominal aorta of each rat was collected using a vacuum blood collection tube. After the blood was collected, the rats were immediately sacrificed by decapitation. The uterus and ovary were then obtained, and their surrounding connective and adipose tissues were peeled off. Subsequently, the weights of the uterus (A) and ovary (B) were measured using electronic scales. Based on the body weight of the corresponding rat (C), the uterus and ovary indices were calculated as Eqs 1 and 2.

Uterus index (%) = (A/C)100………………….. (1)
Ovary index (%) = (B/C)100…………………... (2)

Enzyme-linked immunosorbent assay

The collected blood was immediately centrifuged at 3000 rpm for 10 min at 4 °C to obtain the

serum. Subsequently, the E_2 level and the FSH and LH activities in the serum were determined by using the corresponding ELISA kit according to the manufacturers' instructions for each. After the reactions were completed, the absorbance was determined using a 680Microplate Reader (Bio-Rad, USA). The absorbance for these indices was used to calculate the level or activity according to the corresponding standard curves.

Western blot assay

The total proteins from the ovary tissue were extracted, and their concentration was determined using enhanced BCA protein assay reagent. Equal amounts of proteins (40 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5 % skim milk, the PVDF membranes were incubated overnight with the corresponding primary antibodies for β-actin, Fas, FasL, FADD, c-caspase-8, c-caspase-10, c-caspase-3, c-caspase-6, and c-caspase-7 at 4 °C. Subsequently, the PVDF membranes were washed with Tris-buffered saline-Tween (TBS-T) and incubated with HRP-conjugated goat anti-rabbit antibody in TBS-T at room temperature for 2 h. Next, the PVDF membranes were washed with TBS-T and incubated with an enhanced chemiluminescence detection kit for HRP. The bands were detected on x-ray film. β-actin was used as an internal control for equal protein loading.

Statistical analysis

All data are presented as mean ± standard deviation (SD). The difference between two groups was analyzed by one-way ANOVA (Dunnett's test) on SPSS 19.0 (IBM Corporation, Armonk, NY, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of LFPS on uterus and ovary indices in rats with ovulation failure

As shown in Table 1, the uterus and ovary indices in the model group were reduced significantly ($p < 0.01$), compared with those in the normal group. LFPS (100, 200, or 400 mg/kg) increased significantly ($p < 0.05$ or 0.01) the uterus and ovary indices relative to those in the model group.

Effect of LFPS on E_2 level, FSH activity, and LH activity in the serum of rats with ovulation failure

As shown in Table 2, the E_2 level and the FSH and LH activities in the model group were reduced significantly ($p < 0.01$) relative to those in the normal group. LFPS (100, 200, or 400 mg/kg) increased significantly ($p < 0.05$ or 0.01) the E_2 level and the FSH and LH activities, compared with those in the model group.

Effect of LFPS on pro-apoptotic proteins levels in the ovary tissue of rats with ovulation failure

As shown in Figures 1 and 2, the levels of pro-apoptotic proteins (Fas, FasL, FADD, c-caspase-8, c-caspase-10, c-caspase-3, c-caspase-6 and c-caspase-7) in the model group were up-regulated significantly ($p < 0.01$), compared with

Table 1: LFPS up-regulated uterus and ovary indices in rats with ovulation failure

Group	Uterus index (%)	Ovary index (%)
Normal	0.169 ± 0.026	0.039 ± 0.006
Model	0.119 ± 0.021[##]	0.026 ± 0.005[##]
LFPS 100 mg/kg	0.138 ± 0.019[*]	0.032 ± 0.006[*]
LFPS 200 mg/kg	0.142 ± 0.024[*]	0.034 ± 0.007[**]
LFPS 400 mg/kg	0.147 ± 0.022[**]	0.037 ± 0.009[**]

[##]$p < 0.01$, compared with those in the normal group; [*]$p < 0.05$, [**]$p < 0.01$, compared with those in the model group

Table 2: LFPS increased E_2 level, FSH activity and LH activity in the serum of rats with ovulation failure

Group	E_2 (pmol/L)	FSH (U/L)	LH (U/L)
Normal	6.72 ± 1.95	2.76 ± 0.25	3.75 ± 0.49
Model	3.42 ± 1.02[##]	2.28 ± 0.23[##]	2.76 ± 0.34[##]
LFPS 100 mg/kg	4.93 ± 1.34[*]	2.54 ± 0.21[*]	3.08 ± 0.29[*]
LFPS 200 mg/kg	5.46 ± 1.47[**]	2.62 ± 0.23[**]	3.21 ± 0.36[*]
LFPS 400 mg/kg	6.04 ± 1.78[**]	2.71 ± 0.22[**]	3.51 ± 0.45[**]

[##]$p < 0.01$, compared with those in the normal group; [*]$p < 0.05$, [**]$p < 0.01$, compared with those in the model group
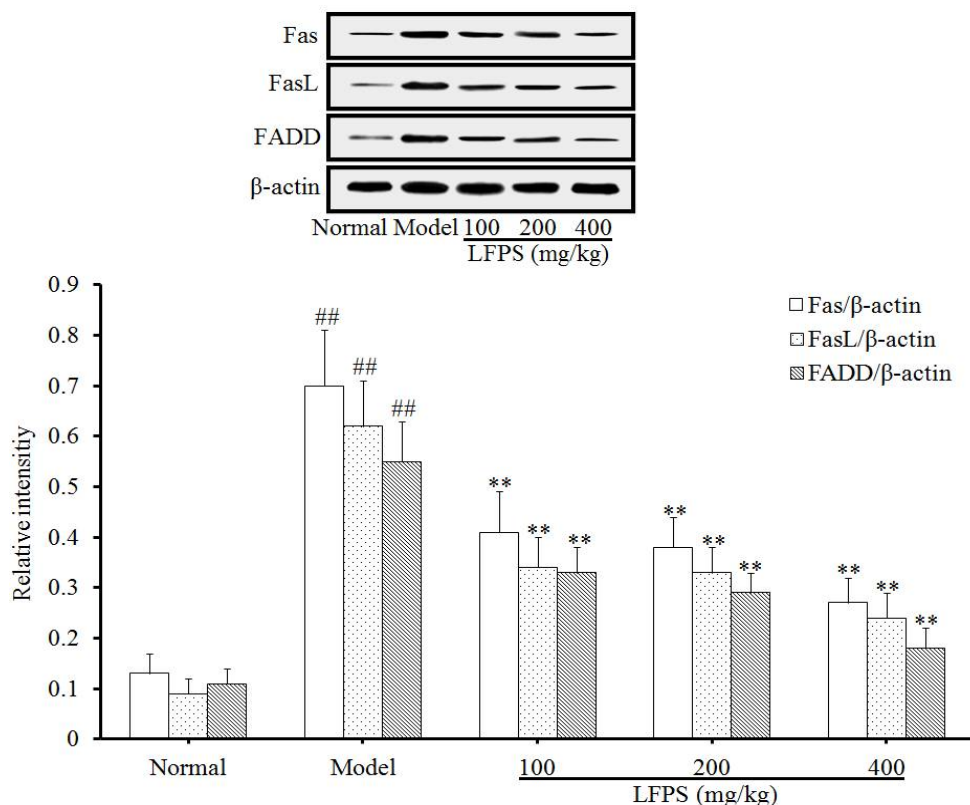
Figure 1: Inhibitory effect of LFPS on Fas, FasL and FADD proteins levels in the ovary tissue of rats with ovulation failure; $^{\#\#}p < 0.01$, compared with those in the normal group; $^{**}p < 0.01$, compared with those in the model group
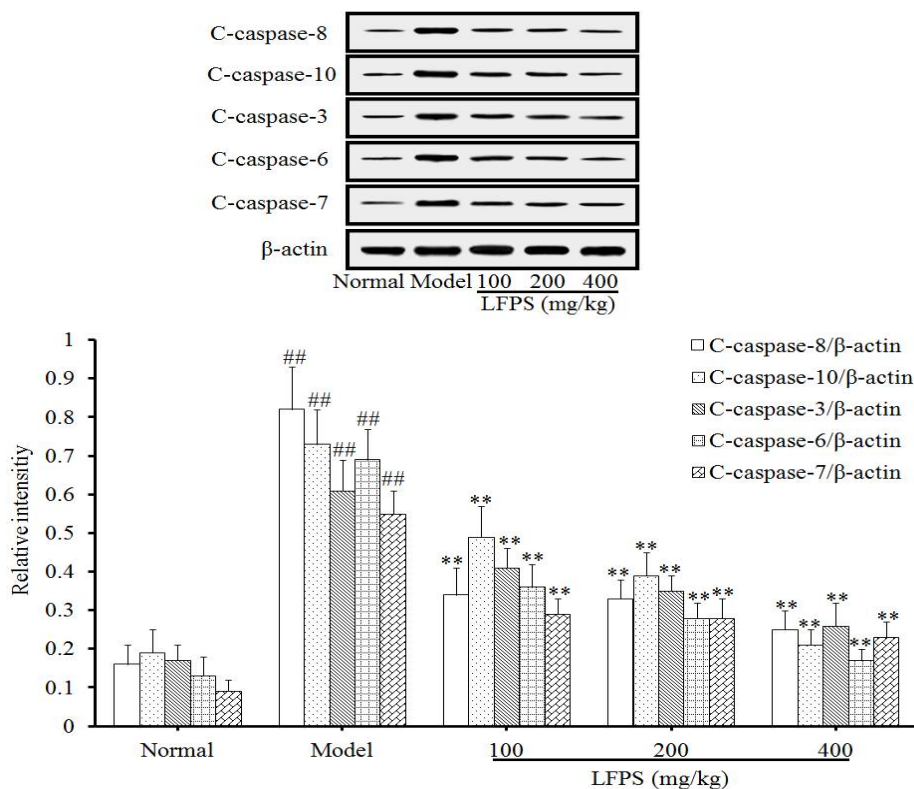

Figure 2: Inhibitory effect of LFPS on c-caspase-8, c-caspase-10, c-caspase-3, c-caspase-6 and c-caspase-7 proteins levels in the ovary tissue of rats with ovulation failure; $^{\#\#}p < 0.01$, compared with those in the normal group; $^{**}p < 0.01$, compared with those in the model group

those in the normal group. LFPS (100, 200, or 400 mg/kg) down-regulated significantly ($p < 0.01$) these pro-apoptotic proteins levels relative to those in the model group.

DISCUSSION

It has been reported that LFPS exhibits a protective effect on the male reproductive system [14,15,18]. However, the effect of LFPS on the female reproductive system is unknown. Infertility, a common gynecological disease, can be caused by dysfunctions of the fallopian tube, ovary, uterus, internal secretions, etc. [19,20], and ovulation failure is a leading cause of infertility [5]. Hydroxyurea is an accepted drug for establishing an ovulation failure model in rats [21,22]. It has been reported that hydroxyurea reduced significantly the uterus and ovary indices and down-regulated the levels and activities of HPO axis hormones such as E_2, FSH and LH [13,17]. The ovary was pale following treatment with hydroxyurea; hydroxyurea may therefore induce ovary tissue apoptosis.

Based on these previous studies, the aim of this study was to investigate the effect of LFPS on hydroxyurea-induced ovulation failure in rats by calculating the uterus and ovary indices, determining the E_2 level and the FSH and LH activities in serum, and detecting the levels of pro-apoptotic proteins (Fas, FasL, FADD, c-caspase-8, c-caspase-10, c-caspase-3, c-caspase-6, and c-caspase-7) in ovary tissue.

Uterus and ovary indices can visually reflect whether the reproductive system is healthy [23]. In the present study, we have found that hydroxyurea damaged the reproductive system of rats by reducing uterus and ovary indices (Table 1). However, LFPS maintained the health of the reproductive system by inhibiting the reduction of uterus and ovary indices.

Dysfunction of the HPO axis and ovary tissue apoptosis can result in ovulation failure [17,24]. E_2, FSH, and LH play important roles in maintaining the functions of the HPO axis [25]. The main function of E_2 is to promote the transition of the endometrium from its normal condition into its proliferative phase, which creates favorable conditions for ovulation. The main functions of FSH and LH are to promote follicle development and maturation and ovulation, respectively [26]. In the present study, we have found that hydroxyurea inhibited ovulation in rat by down-regulating the E_2 level and the FSH and LH activities, which were reversed by treatment with LFPS (Table 2).

The death receptor-mediated apoptotic pathway plays an important role in cell apoptosis [27]. Fas, FasL, FADD, c-caspase-8, c-caspase-10, c-caspase-3, c-caspase-6, and c-caspase-7 are the main regulatory proteins in the death receptor-mediated apoptotic pathway [27,28]. Up-regulations of these proteins levels may promote cell apoptosis. In the present study, we have found that hydroxyurea induced the apoptosis of ovary tissue by up-regulating the levels of the above-mentioned pro-apoptotic proteins, which were reversed by treatment with LFPS.

CONCLUSION

LFPS exhibits a protective effect on hydroxyurea-induced ovulation failure by regulating HPO axis hormones and death receptor-mediated apoptotic pathway. Therefore, LFPS may be a useful drug candidate to treat ovulation failure. However, further studies on the mechanisms by which LFPS protects against ovulation failure are required.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest associated with this work.

AUTHORS' CONTRIBUTION

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Chun-Xia Wang and Yong-Wei Li conceived and designed the study while Chun-Xia Wang, Jian-She Chen, Zi-Xue Sun, Hui Li, Bo Men, Xiao-Qian Zhang performed the experiments, collected and analyzed the data.

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