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Effects of *Epimedium* polysaccharide on female mouse (*Mus musculus*) ovarian and uterine development

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Abstract: This study was conducted to investigate the effects of *Epimedium* polysaccharide (EPS) on ovarian and uterine development in female mice and assess the in vitro maturation (IVM) rate. Healthy Kunming female mice were divided into 4 groups and administered 2, 4, 6, and 8 mg/0.1 mL of EPS, respectively; the control group was given 0.1 mL of 0.9% physiological saline solution. After 7 and 14 days, the mice were sacrificed and their reproductive tracts (uterus, oviduct, and ovaries) were removed. The reproductive tract was weighed; some ovaries were punctured to harvest oocytes for IVM culture and the others were used for histological analysis. EPS had virtually no effect on the body weight of the female mice. EPS at 4 mg/0.1 mL for 14 days of treatment had a significant effect ($P < 0.05$) on ovarian and uterine development when compared with 2, 6, and 8 mg/mL but no significant effect ($P > 0.05$) when compared with the control group. The number of follicles increased in the experimental groups and the arrangements of ovarian follicles were not negatively affected during histological observation. In conclusion, EPS at appreciable dosages has a positive effect on mouse ovarian development.

Key words: *Epimedium* polysaccharide, ovaries, development, in vitro maturation, reproductive index

1. Introduction

The genus *Epimedium* has been used as a tonic drug in traditional Chinese medicine for centuries and is officially listed in the Chinese Pharmacopoeia. There are more than 60 species of this herbaceous flowering plant belonging to the family Berberidaceae (1,2). It has several common names, such as horny goat weed, bishop's hat, fairy wings, rowdy lamb herb, and barrenwort, due to its wide usage. In addition, the various species of the plant genus *Epimedium* have been used in the treatment of a variety of human illnesses. Colloquially known as "yin yang huo" in Chinese, these plants have, as their names imply, been of particular interest for their perceived efficacy in the management of sexual concerns (3). Many of these Chinese herbs and their constituents have been reported to have the effect of immune enhancement (4,5) and they have great potential in practical applications in China, Korea, and Japan. For instance, many studies have demonstrated that *Epimedium* plants have extensive pharmacological efficacy for treating infertility, impotence, spermatorrhea, amenorrhea, rheumatic arthritis, chronic bronchitis (6,7), and the genus has been proven to have estrogen-like and antiosteoporotic activity and is used for the treatment of osteoporosis, menopausal syndrome, and age-associated diseases (8). These traditional Chinese medicines containing multiple interactive compounds have attracted the attention of researchers for their effects in the management of menopausal and related medical conditions (9). The polyphenolic extract of the leaves of *Epimedium brevicornum* was also reported to exhibit significant estrogenic activity in a recombinant yeast cell assay and the Ishikawa Var-I assay (10). In another in vivo study, the ethanolic *E. brevicornum* extracts increased estrogen receptor α activity in rats after oral administration (11).

Research has shown that ovarian follicular growth and development begins when primordial follicles emerge from their quiescent state. The initiation of follicular growth is characterized by morphologic changes, including a change in granulosa cell shape from flattened to cuboidal, proliferation of granulosa cells, enlargement of the oocyte, and formation of the zona pellucida. Granulosa cell proliferation and the change to a cuboidal shape precede increases in oocyte diameter. In humans, the first substantial increase in oocyte diameter occurs when there are 15 granulosa cells in the largest follicle cross-section

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(12). In the present study, we set out to examine how *Epimedium* polysaccharide EPS affects the growth and development of mouse ovarian follicles and uterus.

2. Materials and Methods

2.1. Animals

Healthy Kunming female mice weighing approximately 23 g (4 weeks old) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Permit No. SCXK - Hu 2012 - 0002) and used in this experimental study. All mice were routinely raised in a clean area with normal room temperature and fed with standard mouse feed and ordinary water ad libitum. The treated groups received EPS at 2, 4, 6, and 8 mg/0.1 mL for 7 or 14 days. The control group was orally administered 0.9% physiological saline (sodium chloride) solution. All procedures and protocols involving animals were approved by the Animal Research Committee of Nanjing Agricultural University (NJAU) with reference to the Guide for the Care and Use of Laboratory Animals (13).

2.2. Experimental design

The type of study employed in this experiment was a randomized controlled clinical trial. Sixty Kunming female mice were randomly divided into 10 groups (n = 6) and treatment started 1 week after acclimatization to the new environment. The EPS was kindly provided by Dr Deyun Wang of the Laboratory of Traditional Chinese Medicine of NJAU and dissolved as well as diluted with ordinary water to prepare the medicine for the experiment. The mice were orally administered EPS (for the treatment group) and 0.9% saline solution (for the control group) through a cannula fitted to a 1.0-mL syringe and euthanasia was performed 7 or 14 days after treatment.

The mice were weighed before treatment and at the time of sacrifice to determine the difference in weight from the time of treatment to sacrifice. After the 7 or 14 days of EPS treatment, the mice were humanely sacrificed by cervical dislocation and the female reproductive system (uterus, oviduct, and ovaries) was removed after the abdomen was opened by a small lateral incision at the midline.

2.3. Preparation of fixatives and tissue culture media

Bouin's solution (for ovary fixation) and tissue culture medium M16 (for oocyte maturation) and human tubal fertilization (HTF) medium (for in vitro fertilization) were prepared before the mice were sacrificed. These media were prepared according to the guidelines and protocols from the textbook *Manipulating the Mouse Embryo: A Laboratory Manual* (14).

2.3.1. Bouin's solution

Bouin's solution was prepared as follows: 75 mL of saturated picric acid aqueous solution, 25 mL of 40% formalin aqueous solution, and 5 mL of acetic acid.

2.3.2. M16 medium and HTF medium

The M16 medium and HTF medium were prepared according to Behringer et al. (14).

2.4. Ovarian tissue preparation

Ovaries and uteri were carefully examined, removed from the mice, and collected onto filter papers for weighing and fixation in Bouin's solution or tissue culture.

2.4.1. For weighing

Paired uterine horns and ovaries were weighed on an analytical electronic balance. They were then distributed into Bouin's solution bottles and petri dishes containing PBS for various experiments.

2.4.2. For Bouin's fixation

The ovaries from each group of mice were placed in Bouin's solution for 24 h to preserve normal morphology and facilitate further processing into paraffin blocks.

2.5. Oocyte culture in vitro

On the day of sacrifice, ovaries from each group [control (0 mg/0.1 mL) and 2 mg/0.1 mL, 4 mg/0.1 mL, 6 mg/0.1 mL, and 8 mg/0.1 mL of EPS for 7 or 14 days] were punctured in separate petri dishes containing PBS under a microscope to collect oocytes. The oocytes were then aspirated with a mouth-controlled pipette into petri dishes containing PBS for washing. Next, 5 microdrops of M16 were added to different tissue culture dishes representing the groups and covered with mineral oil. The media microdrops were prepared and equilibrated at 37 °C in a CO_2 incubator overnight. The oocytes were carefully dropped in each microdrop and cultured in the incubator for 20–24 h. After in vitro maturation, oocytes were checked for the extrusion of their first polar bodies (pbI) under a stereomicroscope (SMZ140, Nikon). With the maturated oocytes, in vitro fertilization was conducted. Semen were obtained from the epididymis of healthy male mice of the same strain and capacitated in HTF medium for 90 min at 37 °C in a CO_2 incubator. Fertilization results were read after 6 h as pronuclei formation.

2.6. Determination of reproductive index

The reproductive index (RI) was determined using the recorded weight values, live weight of mice before sacrifice and weight of reproductive tract (the uterus, oviduct, and ovaries). The RI was calculated from the ratio of the weight of the female reproductive tract to the live weight of the mice and expressed as a percentage:

$$RI = [\text{FRT weight (g)} / \text{live weight (g)}] \times 100,$$

where FRT = female reproductive tract, FRT weight = weight (ovary + uterus + oviduct), and live weight = weight of the mouse before it was sacrificed.

2.7. Preparation of histological sections of mice ovaries

Isolated mice ovaries were fixed in Bouin's fixative for 24 h, washed in PBS, and dehydrated through a graded series of ethanol baths (70% for 1 h, 80% for 1 h, and 90% 2 h, to 100% for 3 h). The sections were cleared in a dimethylbenzene and ethanol mixture (1:1, v/v) for 60 min and then put into dimethylbenzene until transparent and embedded in a dimethylbenzene and paraffin mixture (1:1, v/v) for 1 h, followed by paraffin for 2 h. Sections (5 mm) were deparaffinized in dimethylbenzene, rehydrated through a graded series of ethanol solutions (from 100% to 70%), and finally stained with H&E.

2.8. Statistical analysis

Data were collated and analyzed as mean and standard deviation (SD). Statistical analysis was performed using SPSS 20.0 (IBM Corporation, Armonk, NY, USA). The significant differences were determined using one-way ANOVA. The level of significance was set at $P < 0.05$.

3. Results

3.1. Effects of EPS on body weight

Healthy female mice were exposed to different doses of EPS, and body weight was measured after 7 or 14 days. The body weight did not change significantly in any group ($P > 0.05$), suggesting that EPS has no obvious effects on female mouse growth, which is in line with other similar reports (15,16).

3.2. Effects of EPS on reproductive index

When the female mice were administered with different doses of EPS continuously for 7 and 14 days, there were no negative effects on the body weight of the mice. There was a marginal increase in the body weight of the mice. The RI value of the 4 mg/0.1 mL group for 14 days was significantly higher than that of other treatment groups (2, 6, and 8 mg/0.1 mL), but not that of the control (Table 1).

3.3. Oocyte culture evaluation

Table 2 summarizes the pbI extrusion rates of the oocytes. The percentage of oocytes that extruded pbI after culture was higher in the treated groups as compared to the control. However, there were no differences between experimental groups (except the 8 mg/0.1 mL group for 7 days) and the control ($P > 0.05$). The 8 mg/0.1 mL group had the highest pbI rates at 7 days and the 4 mg/0.1 mL group had the highest pbI rate at 14 days.

3.4. Histological evaluation

EPS was given orally to adult female mice for 7 and 14 days and then animals were sacrificed respectively. No histopathological alterations in the ovaries were observed in the control or EPS-treated mice. Ovarian follicles appeared normal morphologically and the theca cells and basal laminas of developing follicles were continuous and complete. There seemed to be no significant differences for the number and size of antral follicles among groups. Morphological findings suggested that the EPS did not adversely affect ovarian histology. Other histological observations include the following: in the experimental groups, ovarian follicles were developing normally, as in the control groups, with primordial follicles being the least developed. A careful observation under the microscope revealed that the EPS experimental groups had higher numbers of primordial, primary, and secondary follicles, but none of the follicles had reached the Graafian follicle stage of development. The control group had more primordial and primary follicles. However, there were many more secondary follicles in the experimental group (2, 4, 6, and 8 mg/0.1 mL) than the control group, an indication that EPS enhances follicular development.

4. Discussion

This study was designed to investigate the effects of EPS on mouse ovarian and uterine development. The evaluation of the treatments was done with RI, IVM, and histologic analyses, in addition to weight measurements.

EPS, an active ingredient of *Epimedium*, has been reported to possess mitogenic-like function and promote the incorporation of ^{3}H-TdR in mice thymocytes (17). However, little is known about its effect on mouse ovarian

Table 1. Reproductive indexes among groups.

Group (EPS dosage)	Reproductive indexes in % ± SD	
	EPS, 7 days	EPS, 14 days
Control (0 mg/0.1 mL)	0.460 ± 0.041	1.088 ± 0.001
2 mg/0.1 mL	0.755 ± 0.004	0.906 ± 0.004
4 mg/0.1 mL	0.728 ± 0.007	1.419 ± 0.007*
6 mg/0.1 mL	0.623 ± 0.004	0.890 ± 0.004
8 mg/0.1 mL	0.475 ± 0.001	0.708 ± 0.002

*: $P < 0.05$.

Table 2. Effects of EPS for 7 days and 14 days on IVM of mice oocytes.

EPS dosage	EPS, 7 days		EPS, 14 days	
	No. of oocytes cultured	pbI extrusion (%)	No. of oocytes cultured	pbI extrusion (%)
Control (0 mg/0.1 mL)	20	45.0	47	42.5
2 mg/0.1 mL	18	63.6	62	46.8
4 mg/0.1 mL	19	62.0	44	60.6
6 mg/0.1 mL	14	64.3	51	51.3
8 mg/0.1 mL	15	80.0*	48	41.7

*: $P < 0.05$.

and uterine development. As an experimental tool, we showed that EPS had some significant effect on mouse ovarian and uterine growth and development.

The RI calculations and statistical analysis showed that values in the 4 mg/0.1 mL group at 14 days were significantly higher than in other treatment groups (2, 6, 8 mg/0.1 mL), but not higher than those of the control.

The IVM experiments also showed that there were no differences between experimental groups (except 8 mg/0.1 mL for 7 days) and the control ($P < 0.05$), and that the 8 mg/0.1 mL group had the highest pbI rates at 7 days and the 4 mg/0.1 mL group had the highest pbI rate at 14 days. However, histologic analyses revealed that ovarian follicles in the treatment groups developed normally when compared with the control. The investigators also concede that EPS may not be entirely responsible for the development of the ovaries as strong evidence exists that genes expressed in oocytes play a role in their growth and development (18–21). Histologic observations showed normal secondary follicle development with the formation of the antrum, multiple granulosa cells (leading to the formation of the membrane granulosa, cumulus oophorus, and corona radiata), and oocyte nucleus. Ovarian follicular developments were also seen in primary follicles as these follicles were at various stages of development. There were more follicles in the treatment groups than the control groups. Faddy et al. reported that more than half of the primordial follicles present in the mouse ovary at birth degenerate before 3–5 weeks of age, but little is known about the hormonal and local factors controlling this loss (22). Eppig et al. also reported that the female mouse reaches maturity at approximately 6 weeks of age, depending on the strain and environmental conditions. By this time, each ovary contains approximately 10^4 oocytes at different stages of maturity. Techniques have been established for isolating and culturing both immature oocytes from preantral follicles and mature oocytes from antral follicles (23,24). Maturation of oocytes from antral follicles occurs spontaneously under these conditions; they can be fertilized in vitro and develop normally thereafter. Oocytes from preantral follicles, on the other hand, must be cultured for several days with their surrounding follicle cells before they can be fertilized in vitro.

The findings suggest that EPS has an effect on mouse ovarian and uterine development and could be useful for reproductive medicine. We think that at the end of the comprehensive research on EPS we will be able to tell how EPS actually works on the ovaries and uteri of mice to enhance their development. This research revealed that EPS as a constituent of herbal *Epimedium* enhances female reproductive system development when administered at appreciable doses to matured female mice; however, its effect on reproductive system development may not be seen if the dosage is very low. The effect may also be harmful if the dosage is very high. This is the first report in this regard, and further research is being conducted to determine how EPS actually works, because the results of our preliminary study did not show how EPS works on the reproductive system development of female mice.

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