Original Research

Effect of Icariin on the Number of Follicles and Endometrium Thickness in Mice with Premature Ovarian Failure

Qifeng Li1,†, Zhonglin Xiao2,3,†, Wen Wang1,†, Aiwen Le4,‡

1Department of Pathology, Huazhong University of Science and Technology Union Shenzhen Hospital, Shenzhen Nanshan People’s Hospital, the Affiliated Nanshan Hospital of Shenzhen University Health Science Center, 518052 Shenzhen, Guangdong, China
2Faculty of Data Science, City University of Macau, 999078 Macau, China
3Center for Energy Metabolism and Reproduction, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, 518055 Shenzhen, Guangdong, China
4Department of Gynecology, Huazhong University of Science and Technology Union Shenzhen Hospital, Shenzhen Nanshan People’s Hospital, the Affiliated Hospital of Shenzhen University Health Science Center, 518052 Shenzhen, Guangdong, China

*Correspondence: leaiwen362531@126.com (Aiwen Le)
†These authors contributed equally.
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Abstract

Background: This study explored icariin’s effects on endometrial thickness and follicle numbers in mouse models of premature ovarian failure (POF). Methods: We divided 8-week-old C57BL/6J female mice into five groups: NC (control), CTX (cyclophosphamide-induced POF), CTX-ICA (CTX-induced POF with icariin), OVX (ovariectomy-induced POF), and OVX-ICA (ovariectomy-induced POF with icariin). We monitored estrous cycles and assessed endometrial thickness and follicle counts using Hematoxylin & Eosin (H&E) staining. Results: CTX and OVX reduced estrous activity, and the CTX-ICA group exhibited higher activity. Compared with NC, the primary follicles were decreased in the CTX group (p = 0.005) but not in the CTX-ICA group (p = 0.272). Endometrial thickness was respectively thinner in the OVX and OVX-ICA vs. CTX-ICA, CTX, and NC groups (p < 0.001); the CTX vs. NC (p < 0.001) groups; and the CTX vs. CTX-ICA groups (p < 0.001). No significant differences were found between the CTX-ICA and NC groups (p = 0.972). Conclusions: In conclusion, CTX and OVX induce POF in mice, causing endometrial thinning and decreased follicles (CTX). Icariin may partly restore endometrial thickness.

Keywords: premature ovarian failure; icariin; endometrium thickness; follicles

1. Introduction

Premature ovarian failure (POF) is ovarian failure occurring before age 40 years, mainly manifesting as high follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels and declining estradiol (E2) levels; severe cases display amenorrhea and even infertility [1]. The etiology of POF is complex, with the most common cause being physicochemical, followed by genetic, metabolic, factors, drugs, and infection, autoimmunity [2]. The management of POF includes psychological and lifestyle interventions, hormonal therapy, and fertility consultation for women desiring pregnancy [3–5]. The mainstay of treatment is either hormone replacement therapy or a combined estrogen/progestogen contraceptive pill until the average age of natural menopause (50–51 years). Hormonal therapy can eliminate menopausal symptoms such as hot flashes, night sweats, and dyspareunia and prevent accelerated bone loss. In addition, a physiological sex steroid replacement regimen was reported to improve bone mineral density in women with primary ovarian insufficiency [3–5]. Still, hormonal treatment mainly deals with symptoms and improves the quality of life but fails to address fertility issues significantly [5,6]. Assisted reproductive techniques can achieve pregnancy in women with POF, but the success rates are relatively low [7,8]. Therefore, novel modalities are necessary to improve the prognosis of such women.

Cyclophosphamide (CTX)-induced aging model is similar to the human ovarian aging process, and the ovarioctomy (OVX) POF model is also a widely used ovarian aging modeling method. These two models are widely used to explore the mechanisms of POF [9,10]. Icariin (ICA) is a flavonoid compound from the Epimedium genus of Berberaceae and has a variety of pharmacologically active components that enhance body immunity, regulate the endocrine system, improve the cardiovascular and cerebrovascular system functions, and fight tumors (Chinese National Medical Products Administration National Drug Standards (YBZ-PFKL-2021144)) [11].

Icariin could have beneficial effects on POF. Indeed, icariin activates the estrogen receptor and effectively prevents osteoporosis resulting from estrogen deficiency in OVX rats [12]. Icariin has an anti-aging effect in protecting ovarian granulosa cells against D-galactose-induced aging by promoting DNA damage repair [13]. Yang et al. [14] suggested that icariin, quercetin, and resveratrol can protect against ovarian aging. Still, the effects of icariin on fertility parameters remain unknown.
Therefore, this study aimed to examine the effects of icariin on endometrial thickness and follicle number in POF CTX and OVX mouse models. The results could help provide a scientific basis for the management of POF using icariin.

2. Materials and Methods

2.1 Experimental Animals

The study was approved by the Animal Ethics Committee of Shenzhen Nanshan People’s Hospital (#072652). Fifty 8-week-old specific pathogen-free (SPF)-grade C57BL/6J wild-type female mice weighing 16–20 g was purchased from Guangdong Pharmaceutical Kang Biotechnology Co., Ltd. (Shenzhen, Guangdong, China) (mouse production license #91440605MA52EJ7N96) and maintained five mice/cage in a 12-h dark/light cycle under controlled temperature (20–26 °C) and humidity (40%–70%). Food and water were provided at will throughout the study.

2.2 Establishment of the Premature Ovarian Failure Model

The mice were randomly divided into five groups (10 mice/group): control group (NC), CTX-induced POF model group (CTX), CTX-induced POF with ICA intervention group (CTX-ICA), OVX-induced POF model group (OVX), and OVX-induced POF with ICA treatment group (OVX-ICA). The strategies for establishing the POF models in mice are as follows: The CTX-induced POF state was simulated by intraperitoneal injection (i.p.) of CTX (Sigma, St. Louis, MO, USA, 97% purity; CTX and CTX-ICA groups). The injection regimen consisted of an initial dose of 70 mg/kg on day one, followed by 30 mg/kg every two days for a total of 21 days, as per established protocols [15]. In contrast, POF was directly induced in the OVX and OVX-ICA groups through ovariectomy. Simultaneously, NC group received an equivalent volume of saline treatment for 21 days to eliminate non-specific effects.

Subsequently, all groups received treatment with icariin (McLean, Shanghai Yunguan Electromechanical Equipment Co., Ltd., Shanghai, China, 97% purity) was at a dose of 200 mg/kg/day for 16 days. Similarly, the other three groups underwent intragastric administration of an equal volume of saline during the same period. After completion of the treatment period, all mice underwent euthanasia through cervical dislocation.

OVX was performed as previously published [16]. The mice were fasted 1 day before surgery. Anesthesia was induced with 4 vol% isoflurane (Shanghai Yuquan Scientific Instrument Co., Ltd., Shanghai, China) and subsequently maintained at a concentration of 1.0–1.5 vol% with a continuous flow rate of 192 ml/min. Following the successful induction of anesthesia, the skin was meticulously prepared for the upcoming surgical procedure. After surgery, the mice were given penicillin (400,000 U/mL, 0.1 mL/kg, thigh muscle injection, and incision; Shanghai Zeye Biotechnology Co., Ltd., Shanghai, China). A double incision was performed on the back to open the peritoneal membrane and expose the abdominal cavity, which showed the adipose tissue located directly beneath the kidney. A slight tissue pull could reveal the pale pink ovary wrapped in the adipose tissue and the closely connected fallopian tube. The ovary and fallopian tubes were ligated using a #2 surgical suture and removed. The remaining tissue was returned to the abdominal cavity, and the peritoneal membrane was closed layer by layer using a 5/0 ribbon suture needle. The skin incision was closed using a triangular needle and a 2nd surgical suture gauge.

2.3 Hematoxylin & Eosin Staining (H&E)

Hematoxylin & Eosin staining was used to observe the endometrium thickness and the number of follicles. The mouse ovaries were harvested, and the specimens were fixed in 4% paraformaldehyde solution, dehydrated, embedded, and stained with H&E. The ovarian morphology was observed under light microscopy (DM3000LED2.5.0.143918, Leica Microsystems, Wetzlar, Germany). For the follicular counts, ovaries were fixed with 4% paraformaldehyde solution for 24 h, dehydrated, and made transparent in paraffin, sectioned (5 µm thick) at 20-µm intervals. Three sections were evaluated from each mouse. After conventional H&E staining, five random non-overlapping fields were used to observe the ovarian histological changes and the total number of follicles. Follicular count: under light microscope, starting from the maximum cross section of the center of the ovary, the follicles of each developmental stage were counted in 6 consecutive sections, and the average value was calculated. The average number of different follicles in each slice of each mouse was calculated. The whole process was counted three times by three pathologists.

The sections were incubated at 50 °C for 2 h, de-waxed with xylene three times for 10 min, rehydrated with 100%, 90%, 80%, and 70% alcohol for 2 min each, and rinsed with tap water for 3 min. The sections were stained with hematoxylin for 10 min, rinsed in tap water for 3 min, incubated with 1% hydrochloride in 70% alcohol for 6 s, rinsed with tap water for 3 min, incubated in saturated lithium carbonate blue for 10 s, rinsed with tap water for 3 min, and stained with eosin for 5 s. The sections were dehydrated with 70%, 70%, 80%, 90%, and 100% alcohol, 2 min each, made transparent with xylene two times, each for 5 min, and sealed in neutral tree gum. Endometrial thickness and follicle count were measured using the CaseViewer 2.5.0.143918 software (3DHISTECH, Budapest, Hungary).

2.4 Statistical Analysis

SPSS 26.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The continuous data were tested for normality and homogeneity of variance, and continuous data meeting a normal distribution were expressed as means ±
standard deviation and analyzed using analysis of variance (ANOVA) and Tukey’s post hoc test. Non-normally distributed continuous data were analyzed using the rank-sum test. Categorical data were expressed as n (%) and analyzed using the chi-squared test. Spearman correlation analyses were performed to examine the relationship between endometrial thickness and follicle number. \( p \)-values < 0.05 were considered statistically significant.

3. Results

3.1 Icariin could Increase the Primary Follicular Counts in Mouse Models of POF

The effects of icariin on follicular counts were examined in mice. In the CTX, CTX-ICA, and NC groups, primordial follicles, primary follicles, secondary follicles, antral follicles, and atresia follicles were visible (Fig. 1). The distribution of the total number of follicles was normal in the three groups, and the difference among the three groups was statistically significant \((p = 0.006)\). Compared with the NC group (20.0 ± 5.2 follicles), the numbers of follicles were lower in the CTX-ICA (14.4 ± 3.9 follicles, \( p = 0.014 \)) and CTX (12.6 ± 3.0 follicles, \( p = 0.002 \)) groups, while the differences between the CTX-ICA and CTX groups were not significantly significant \((p = 0.398)\) (Fig. 2). Compared with the NC group, the numbers of primary follicles were lower in the CTX group \((p = 0.005)\) but not in the CTX-ICA group \((p = 0.272)\), the numbers of secondary follicle were lower in the CTX \((p = 0.002)\) and CTX-ICA \((p = 0.016)\) groups, the antral follicle numbers were lower in the CTX \((p = 0.024)\) and CTX-ICA \((p = 0.015)\) groups, and there were no differences regarding the atresia follicles. Therefore, POF leads to decreased follicular counts, but icariin did not reverse this effect.

3.2 Icariin Could Increase the Endometrial Thickness in Mice with POF

Endometrial thickness is associated with endometrial receptivity and embryo implantation \([17]\). Endometrial thickness was normally distributed in the five groups and significantly different among the five groups \((p < 0.001)\).
Fig. 2. Analysis of the differences in mouse follicle numbers in CTX, and CTX-ICA groups compared with control. *p < 0.05, n = 7–9, compared with the control group.

Table 1. Comparison of endometrial thickness (µm) (mean ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Uteri (n)</th>
<th>Endometrium thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>7</td>
<td>538.94 ± 87.64 (CTX, OVX, OVX-ICA)</td>
</tr>
<tr>
<td>CTX</td>
<td>8</td>
<td>283.57 ± 46.38 (CTX-ICA, NC, OVX, OVX-ICA)</td>
</tr>
<tr>
<td>CTX-ICA</td>
<td>8</td>
<td>538.04 ± 26.00 (CTX, OVX, OVX-ICA)</td>
</tr>
<tr>
<td>OVX</td>
<td>9</td>
<td>217.31 ± 26.56 (CTX-ICA, NC, CTX)</td>
</tr>
<tr>
<td>OVX-ICA</td>
<td>9</td>
<td>232.51 ± 27.06 (CTX, CTX-ICA, NC)</td>
</tr>
</tbody>
</table>

Statistics is ANOVA, CTX-ICA vs. CTX, OVX, OVX-ICA, all p < 0.001; OVX-ICA vs. OVX groups, p = 0.512, and the CTX-ICA vs. NC groups, p = 0.972, the CTX and OVX groups (p = 0.136), the CTX and OVX-ICA groups (p = 0.748).

Endometrial thickness was thinner in the OVX and OVX-ICA groups compared with the CTX-ICA, and NC groups (p < 0.001), thinner in the CTX group than in the NC group (p < 0.001), and thinner in the CTX group compared with the CTX-ICA group (p < 0.001). There were no significant differences between the OVX-ICA and OVX groups (p = 0.512), the CTX and OVX groups (p = 0.136), the CTX and OVX-ICA groups (p = 0.748), and the CTX-ICA and NC groups (p = 0.972) (Fig. 3 and Table 1). Therefore, these results suggest that icariin could protect the mice with POF from endometrial thinning.

3.3 Correlations Between the Endometrial Thickness and the Number of Ovarian Follicles

Table 2 showed no correlations between endometrial thickness and follicle number within the NC, CTX, and CTX-ICA groups (all p > 0.05).

4. Discussion

Currently, the management of POF mainly includes symptom control and improving quality of life, and assisted reproduction techniques for women seeking pregnancy. Still, the physiological impact of such management of POF on reproductive parameters remains uncertain. Novel modalities directly influencing endometrial thickness and follicular counts are necessary for managing infertility due to POF. Hence, this study aimed to explore the effects of icariin on endometrium thickness and follicle number in the CTX and OVX mouse models of POF. The results suggest that CTX and OVX induce POF in mice, leading to endometrial thinning and decreased follicle numbers (for CTX). Icariin improves mouse POF symptoms and restores endometrial thickness in the CTX models.
Table 2. Correlation between endometrial thickness and follicle number.

<table>
<thead>
<tr>
<th>Group</th>
<th>Endometrium thickness (µm)</th>
<th>Follicle number</th>
<th>p</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>538.94 ± 87.64</td>
<td>20.00 ± 5.16</td>
<td>0.904</td>
<td>0.788</td>
</tr>
<tr>
<td>CTX</td>
<td>283.57 ± 46.38</td>
<td>12.63 ± 3.02</td>
<td>0.265</td>
<td>0.814</td>
</tr>
<tr>
<td>CTX-ICA</td>
<td>538.04 ± 26.00</td>
<td>14.38 ± 3.85</td>
<td>0.081</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Fig. 3. Hematoxylin and eosin staining of the mouse uterus. (A) Control group. (B) CTX group. (C) CTX-ICA group. (D) OVX group. (E) OVX-ICA group. Magnification: 10×. OVX, ovariectomy-induced POF; OVX-ICA, ovariectomy-induced POF with icariin.

The clinical symptoms of POF include hot flashes, night sweats, facial flushing, vulvovaginal atrophy, urinary frequency changes, and mood disorders, among others [18]. POF can also cause other serious health consequences, such as autoimmune diseases, ischemic heart disease, osteoporosis, Parkinson’s disease, and infertility [19]. CTX is an alkylating agent that seriously damages the ovaries, resulting in ovarian envelope thickening, ovarian granulosa cell apoptosis, vascular damage, and stagnant development of large follicles, leading to amenorrhea and infertility, as seen in patients with POF [20,21] and the present study. Hence, the CTX model is an appropriate model for the study of POF [9]. Since there is no gold standard for the diagnosis of POF, only hormonal indicators and clinical symptoms can be used for diagnosis, and the observation of clinical symptoms is particularly important for determining the POF condition [22]. In the present study, the mice in the CTX and OVX groups showed decreased estrous activity compared with the NC group, indicating that both CTX and OVX achieve POF models. Icariin can have beneficial effects on POF [12–14]. In the present study, icariin increased the estrous activity of the POF-ICA group, suggesting that icariin could improve the symptoms of POF. Icariin also decreased the size of the follicles, indicating a partial reversal of that sign of POF [20,21].

The follicle count can be used as a marker POF [23, 24]. Accordingly, the number of follicles was smaller in the CTX group than in the NC group, indicating that ovarian function was decreased after CTX modeling, as previously described [25,26]. Although the number of follicles in the CTX-ICA group was slightly higher than in the CTX group, the difference was not statistically significant, and the numbers were lower than in the NC group (p = 0.014), indicating that the CTX ovarian function was improved by icariin treatment but did not fully return to normal. Nevertheless, the CTX-ICA group showed a higher number of primary follicles than the CTX group, suggesting some effect of icariin on follicle counts, which could be consistent with Zuo et al. [13] and Yang et al. [14] who showed that icariin could protect against ovarian aging. Additional studies will be necessary to examine the exact effects of icariin on the ovaries.

Endometrial thickness is associated with endometrial receptivity and embryo implantation and can be measured as a marker of fertility [17]. The endometrium is thinner in POF [27]. Accordingly, the endometrial thickness in the CTX and OVX groups was thinner than in the NC group, indicating that either CTX or OVX modeling resulted in endometrial thickness thinning. The endometrial thickness of the OVX groups was even thinner than in the CTX group, consistent with the fact that CTX damages the ovarian function while OVX suppresses any ovarian functions. Indeed, only a few organs, such as the adrenal gland, can secrete a small amount of estrogen, but it is insufficient to maintain endometrial thickness [28]. There were no significant differences in endometrial thickness between the CTX-ICA and NC groups, suggesting that the endometrial thickness could be restored after treatment with icariin. Zhou et al. [12] showed that icariin could activate the estrogen receptor, which could explain the thicker endometrium in the CTX mouse models after icariin treatment. The effect of icariin in the OVX model was more modest, probably be-
cause the complete absence of ovaries induces too important hormonal changes to compensate with icariin. Of note, only one icariin dosage was used in the present study. A higher dosage might show effects but must be examined in future studies. Furthermore, Liu et al. [29] showed that icariin has estradiol-like effects, reducing the proportion of Tc cells and increasing the proportion of Tregs in the peripheral blood and spleen of the castrated female rats. Future studies should examine these factors in rat models since the immune system is involved in embryo implantation.

All three groups had no correlations between endometrial thickness and follicle numbers. It could be related to a lack of statistical power to detect these correlations, if any, but it could also be related to the fact that the mature follicle secretes estrogen. Additional studies are necessary to examine that relationship in POF models.

The limitations of this study were the relatively small sample size, the lack of measurements of estrous cycle observation and the levels of sex hormones such as estrogen. Only one dosage of icariin was used. The immune cells in the endometrium were not examined. Finally, the mechanisms of icariin were not explored.

5. Conclusions

CTX and OVX can induce POF in mice, leading to endometrial thinning and decreased follicle numbers (for CTX). Icariin improves mouse POF symptoms and restores endometrial thickness.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

AL carried out the studies and drafted the manuscript. AL performed the statistical analysis and participated in its design. QL, ZX and WW collected the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. Each author believes that the manuscript represents honest work.

Ethics Approval and Consent to Participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Shenzhen Nanshan People’s Hospital (#072652).

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Conflict of Interest

The authors declare no conflict of interest.

References


