Icariin impacts molecular changes in estrogen receptors but not other reproductive parameters in female mice

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ABSTRACT

This study was conducted to assess the effects of icariin (ICA) an ingredient of Epimedium species, on reproductive parameters in mice. Serum, uterus and ovarian samples were collected after treatment. We determined the reproductive index (RI) and in vitro maturation (IVM) rates of oocytes. The serum was tested for 17β-estradiol (17β-E2) and progesterone (P4) using radioimmunoassay (RIA) and ovaries were analyzed for mRNA expression of Steroidogenic acute regulatory protein (Star), Cytochrome p450 family 19 subfamilies a polypeptide 1 (Cyp19a1) and 17β-hydroxysteroid dehydrogenase (Hsd17b1), genes using Real-time Quantitative RT-PCR (RT-qPCR). Protein expression of estrogen receptors (ER) α, β, and progesterone receptor A (PRA) were detected using western blot (WB). ERα from treatment group was increased (p<0.05) while ERβ and PRA were decreased (p<0.05). ICA regulated ovarian development through the estrogen receptor pathway. ERα expression was evidently increased and this may be the pathway that is utilized for its mode of action.

Key words: Estrogen, Estrogen receptors, Gene expression, Mice, Progesterone.

INTRODUCTION

Epimedium, a traditional herbal medicine, has been widely used to treat a variety of medical conditions in China De Naeyer et al. (2005). This herb is known as “ying yang huo” in Chinese, which means “horny goat weed” in English. Normally, the dried leaves of several Epimedium species, alone or in complex formulations with other herbs, are commonly used Yap et al. (2007). This herb has been used to treat many ailments and disorders, including as an aphrodisiac, for hormonal regulation, to treat “coldness”, infertility, senile functional diseases, gonadal dysfunction, and male impotence; as well as to improve female health Ye and Lou (2005), Kang et al. (2012a) Yang et al. (2013) and Kang et al. (2012b). Icariin (ICA,C21H22O16; molecular weight: 676.67) is a flavonoid present in Epimedium species and a major bioactive component of Epimedium. ICA is therefore used for Epimedium’s herbal quality control De Naeyer et al. (2005) and Yang et al. (2013).

Although Epimedium is reputed to improve reproduction, the role of its ingredient icariin in reproduction is unknown. Female reproduction including ovary development is influenced by estrogen and progesterone hormones, the genes that regulate their production and the estrogen and progesterone receptors that mediate their action Kang et al. (2012a) Naikoo et al. (2016) and Dhami et al. (2017). The purpose of the study was therefore, to determine the role of ICA in reproduction by assessing the reproductive index (RI), In Vitro Maturation (IVM) rates of oocytes seen as extrusion of polar body 1 (pb1), steroidogenesis of 17β-estradiol (17β-E2) and progesterone (P4), gene expression of steroidogenic (Star, Cyp19a1 and Hsd17b1) genes, important in estrogen synthesis and protein expression of estrogen receptors (ER) α, β and progesterone receptor A (PRA).

MATERIALS AND METHODS

Animals and experimental design

Animals: All procedures and protocols involving animals were approved by the Animal Research Committee (ARC) of Nanjing Agricultural University (NJAU) according to the guidelines for the care and use of laboratory animals of the National Research Council (ILAR). The experiment was carried out in Nanjing Agricultural University in the year 2015 and 2016.

Healthy Kunming female mice weighing approximately 25 g (4 weeks old) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Permit No. SCXK-Hu2012-0002) and used in this 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.

Experimental design

Icariin (ICA) with 98 % purity was purchased from Nanjing Zelang Company (Catalogue No. 489327). Thirty
mice were divided into two groups. One group administered with 0.1 mL of 0.5 mg ICA and the control group was orally administered 0.1 mL of 0.9% physiological saline for seven days. Parameters assessed were reproductive index, in vitro maturation and hormonal assay of 17β-E2 and P4 mRNA expression of ovaries through real time RT-qPCR and protein receptor expression through western blot.

**Reproductive index, in vitro maturation, hormonal analysis, real time RT-qPCR and western blot methods**

**Reproductive index (RI) determination:** The reproductive index was determined as described in Afedo et al. (2015). RI = [Ovary, Uterus and fallopian tubes weight (g) / mouse live weight (g)] × 100.

**Oocyte collection and incubation for in vitro maturation (IVM) of mice oocytes:** We prepared M16 medium according to Behringer et al. (2014). All reagents were sourced from sigma Aldrich, unless stated otherwise. After the experiment ovaries harvested from mice were placed in M16 medium and punctured to release oocytes. Each group of 30 oocytes was transferred into a microdrop of IVM medium in a culture dish pre-equilibrated for at least 12 hours, covered in oil and then cultured in a 5% CO2 incubator at 37°C for 24 hours. Results were recorded as oocytes that reached MII stage seen as extrusion of polar body 1 (pbl1), under stereomicroscope (SMZ140, Nikon).

**Serum sample collection for 17β-E2 and P4 hormone analysis:** After 7 days, blood sample was collected from the orbit, after applying topical anesthesia (tetracaine drops) to relieve pain and the mice were immediately euthanized through cervical dislocation. Blood samples were centrifuged at 12000 rpm for 15 min. Serum samples were pooled and then stored at 70°C until assayed for 17β-E2 and P4. Radioimmunoassay (RIA) was undertaken. Serum samples were analyzed for 17β-E2 and P4 using RIA kits Iodine [125I] Estradiol RIA kit, coefficients of variation 1.6 - 0.3% for a concentration of 50 and 1000 pg/mL respectively (Catalogue No. 140620), Lower limit of detection was 5 pg/mL; Iodine [125I] Progesterone RIA kit, sensitivity was 0.1ng/mL, coefficients of intra assay variation 14.8% and 2.1% for 1 and 100 ng/mL respectively (Catalogue Number 140702) [125I] Beijing North Institute of Biological Technology.

**Ovarian tissue preparation for RT-qPCR experiment:** Ovarian tissue samples were frozen in liquid nitrogen within 10 min of euthanizing the mice and stored at 70°C until real-time quantitative PCR (RT-qPCR) was performed. The RT-qPCR was run using a 7300 Fast Real-Time PCR system (Applied Biosystems). Total RNA was extracted from three ovaries in each group using Trizol reagent (Invitrogen), according to the manufacturer’s instructions. RNA from each sample was converted to cDNA by using PrimeScript RT Master Mix (Takara, catalog number RR036A) as per the manufacturer’s instructions. Total RNA purity and yield were determined using a NanoDrop 2000 (Thermo Fisher). Real-time PCR was performed using the SYBR Premix Ex Taq (Takara, catalog number RR420A) as previously described Ju et al. (2010). The following parameters were used: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 31 s. For each cDNA sample, both target and reference genes –β-actin were amplified independently on the same plate and each experiment was performed in triplicate. Primer sequences and PCR amplicons are shown in Table 1.

**Ovarian tissue preparation for ERα, ERβ, and PRA analysis using WB:** Western Blot experiment was performed according to Kipp et al. (2007). Briefly, total proteins were extracted from pooled ovarian (five) samples in radio immuno-precipitation assay (RIPA) buffer (Beyotime, P0013B) and their concentration was determined using SDS-PAGE gels. The samples were pretreated and electrophoresed in SDS-PAGE gels and then transferred onto nitrocellulose membranes. After transfer, the membrane was blocked with skimmed milk. Primary antibodies (Anti-Estrogen Alpha, (Abcam Catalogue number ab37438, RRID: AB_732246), Anti-Estrogen Beta, (Abcam Catalogue number ab3576, RRID: AB_303922), Anti progesterone A, (Abcam Catalogue number ab313486, RRID: AB_11156044) and reference antibody (GAPDH, AP0063, Abcam), dilution 1:1000 were applied and incubated overnight. Secondary antibody (Goat anti-rabbit Catalogue number P0013B) and their concentration was determined using SDS-PAGE gels. The samples were pretreated and electrophoresed in SDS-PAGE gels and then transferred onto nitrocellulose membranes. After transfer, the membrane was blocked with skimmed milk. Primary antibodies (Anti-Estrogen Alpha, (Abcam Catalogue number ab37438, RRID: AB_732246), Anti-Estrogen Beta, (Abcam Catalogue number ab3576, RRID: AB_303922), Anti progesterone A, (Abcam Catalogue number ab313486, RRID: AB_11156044) and reference antibody (GAPDH, AP0063, Abcam), dilution 1:1000 were applied and incubated overnight. Secondary antibody (Goat anti-rabbit Catalogue number BS10950- HRP conjugate), 1:5000 dilution; Bioworld were applied for three hours. The proteins were viewed using a luminescence imager (Fujifilm LAS-4000) after applying the luminescent kit and the intensities of each band were analyzed.

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**Table 1:** List of primer sequences used for real time qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’ to 3’)</th>
<th>Product size (bp)</th>
<th>Gen Bank accession no</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>F: AAATCTGGTCGGTGACATCAA R: ATGCCACAGGATTCCTACCC</td>
<td>19 bp</td>
<td>X03765.1</td>
<td>Kawao et al. (2014)</td>
</tr>
<tr>
<td>Star</td>
<td>F: TGGGGAGGTGGAACCCAAA R: ACCTCTGCTCCTGGTACAG</td>
<td>20 bp</td>
<td>AC_000030.1</td>
<td>Novansa et al. (2011)</td>
</tr>
<tr>
<td>Hsd17b1</td>
<td>F: TCATTGTGCTCCTGGAATG T: AGGCAGTTGGTCCTTTATG</td>
<td>21 bp</td>
<td>NT_096135</td>
<td>Ruiz et al. (2016)</td>
</tr>
<tr>
<td>Cyp19a1</td>
<td>F: AAATGAGGACAGOCACCTTG R: GTTCAGCCACCTACTCGAT</td>
<td>19 bp</td>
<td>NM_007810.3</td>
<td>Zhang et al. (2014)</td>
</tr>
</tbody>
</table>

Beta-Actin (Actb), Steroidogenic acute regulatory protein (Star), Cytochrome p450 family 19 subfamilies a polypeptide 1 (Cyp19a1) and 17α-hydroxysteroid dehydrogenase (Hsd17b1)
Statistical analysis: Statistical analysis was conducted using Graphpad prism software version 6.0 (Graphpad La, Jolla, CA, USA). The results are expressed as the mean ± Standard Deviation from three different experiments. One way ANOVA was used followed by post hoc analysis of the least significant difference (LSD), and the p value was set at 0.05. The RT-qPCR Ct values were obtained. The relative gene expression was calculated as per the 2^(-ΔΔCt) method Livak and Schmittgen (2001) to obtain the fold differences between control and treatment. The intensities of the western blot protein bands were analyzed using Image J 1.48v software NIH.

RESULTS AND DISCUSSION

Reproductive index: The results are as shown in (Fig. 1). There was however no significant differences, when control and treatment groups were compared. Our study found that the reproductive index was not significantly different (p>0.05) from that of controls. This result is similar to a study by Kang and colleagues where the reproductive index in prepubertal rats was not significantly increased after Icariin treatments Kang et al. (2012a). Our study therefore demonstrates a similar effect of icariin in mice reproductive index.

In vitro maturation rates: The in vitro maturation rates of oocytes were assessed by germinal vesicle breakdown and extrusion of polar body 1 (pb1) are as shown in Table 2. Our study did not show any significant differences in maturation rates after ICA treatment (p>0.05). This is despite other ingredients of Epimedium spp Epimedium polysaccharide showing increase in the percentage of oocytes reaching the GVBD stage Aledo et al. (2015).

Effects of ICA on 17β-E₂ and P₄ hormones: The 17β-E₂ results are as shown in (Fig 2). There were no significant differences between the control and treatment groups (p>0.05). Similarly when Kang et al. (2012a) and Kang et al. (2012b) evaluated the serum estradiol levels in rats after treatment with ICA was insignificant. However other studies have described a reduction in estradiol levels when ovariectomized rats were treated with ICA Xue et al. (2012). The Xue and colleagues study used ovariectomised rats while we used mice, therefore explain the difference in results. The P₄ hormone results (Fig 3) showed a lack of significant differences (p>0.05) between control and treatment group. In this study, we report for the first time the effects levels of progesterone after ICA treatment. Therefore no literature available on ICAs effects on progesterone. There are however other plant extracts such as Cimicifuga racemosa (Klimadynon, Bionorica, and Neumarkt) which have been shown to increase serum progesterone levels and therefore used to treat women with polycystic ovarian syndrome Kamel et al. (2013).

Fig 1: Reproductive index after treatment with Icariin (ICA)

Fig 2: Serum concentrations of 17-b estradiol in control and treatment groups after icariin treatment.

Fig 3: Serum concentrations of progesterone of control and treatment groups after icariin treatment.
Gene expression of Steroidogenic acute regulatory protein (Star), Cytochrome p450 family 19 subfamilies a polypeptide 1 (Cyp19a1) and 17α-hydroxysteroid dehydrogenase (Hsd17b1) after icariin treatment in mice ovary

Effects of ICA on mRNA expression of steroidogenic enzyme genes: The qRT-PCR results are outlined in Fig 4. Genes analyzed, were, Star, Cyp19a1 and Hsd17b1. The formation of estrogens from C19 steroids is catalyzed by the Cyp19 gene. Estrogens are important in the development of the follicle and eventually ovulation Fisher et al. (1998). Hsd17b1 codes for the enzyme Hsd17b1 that mediates the conversion of estrone to estradiol in ovarian granulosa cells Miller and Auchus (2010). The gene expressions varied especially in Hsd17b1 (3.2-fold) and Cyp19a1 (34-fold). There were however no significant differences (p>0.05). In contrast, Yang et al. (2013) reported an increase in both estrogen and aromatase (Cyp19a1) mRNA expressions after KGN cells were treated with ICA. The different results could be explained due to difference in the cell line cultures while Yang et al used pure cell lines, in this we study used a mouse model. In another study when prepubertal rats were subjected to ICA treatment, aromatase (Cyp19a1) levels were reported to decrease Kang et al. (2012a). This means further studies are needed to conclusively demonstrate the role of ICA in these genes.

Effects of ICA on ERα, ERβ, and PRA expression: When the protein expression of ERα were measured using WB, (Fig 5). The treatment showed significantly higher (p<0.05) level of protein compared to the control. The ERβ protein concentration for ICA showed significantly lower level (p<0.05) compared to control. The ICA PRA protein concentration was significantly lower compared to the control (p<0.05). ERα and ERβ are nuclear receptors that act as transcription factors for estrogen-regulated genes Beck et al. (2005). Estrogens and estrogen-like compounds effect their action based on these receptors. Thus ICA having the potential to change the expression of these receptors means that the action of estrogens is changed. The treatment group showed a significant increase in ERα expression (p<0.05), compared to control. While ERα was highly expressed, ERβ was significantly decreased (p<0.05). It is well known, that when ERα and ERβ are co-expressed they have antagonistic tendencies, with ERβ opposing the effects of ERα Mathews et al. (2003). In this case, ICA treatment resulted in the high expression of ERα and the decrease in ERβ and PRA. Estrogen action is mediated through a subtle balance between the two receptors (ERα and ERβ) Mathews et al. (2003). The significant increase of ERα, while ERβ is significantly decreased, may result in the actions of 17β estradiol that are mediated specifically through the ERα being increased Hewitt et al. (2003) such as the proliferation of ovarian follicles. In addition the antagonistic effects of ERβ on ERα gene expression would be reduced Mathews et al. (2003). This study demonstrates that ICA significantly increases the expression of ERα, and this may be the mechanism that results in a proliferation of ovarian follicles.

Effects of ICA on ERα, ERβ, and PRA expression: The in vitro maturation rates of oocytes were tabulated in Table 2. The treatment resulted in a significant increase (p<0.05) in the extrusion rate of first polar body (pb1) compared to control. The significant increase of extrusion rate was observed for ICA treatment compared to control (p<0.05).

![Fig 4: Gene expression of Steroidogenic acute regulatory protein (Star), Cytochrome p450 family 19 subfamilies a polypeptide 1 (Cyp19a1) and 17α-hydroxysteroid dehydrogenase (Hsd17b1) after icariin treatment in mice ovary](image)

![Fig 5: The protein expression of Estrogen Receptor α (ERα), Estrogen Receptor β (ERβ) and Progesterone Receptor (PRA) A. Demonstrating the protein expression of ERα, ERβ and PRA after treatment (p<0.05*); B. Expression of ERα, (ERA), ERβ (ERB) PRA and GAPDH after treatment with icariin (From left to right, Lane 1-Control Lane 2- Treatment)](image)
After treatment with ICA, PRA was significantly decreased (p<0.05) (Fig 5). These findings have been corroborated by others, who reported no increase in progesterone receptors Shen et al. (2007). Our study however shows a significant decrease in PRA expression. Since PRA opposes the action of the ERs Hewitt et al. (2000). Its reduction in this study could potentiate the ER action.

CONCLUSION

In conclusion, we report that ICA could possibly regulate ovarian follicle development through the estrogen receptor pathway by increasing ERα expression and this may be the pathway that is utilized for its mode of action.

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