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Effect of Hyperin and Icariin on steroid hormone secretion in rat ovarian granulosa cells

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ABSTRACT

Aim of the study: This study was designed to investigate the effect of different concentrations of Hyperin and Icariin (ICA)on proliferation and the secretion of estrogen (E2), and progesterone (P) in granulosa cells, and to explore the effect of Hyperin and Icariin on the expression of *CYP17* and *CYP19*.

Materials and methods: Rat ovary granulosa cells were cultured *in vitro* and treated with different concentrations of Hyperin and Icariin. The proliferation of ovarian granulosa cells was measured with the MTT assay. The concentration of estradiol was measured with a magnetic particle-based enzyme-linked immunosorbent assay (ELISA) kit. The *CYP17* and *CYP19* mRNA expression was detected by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). The CYP17 and CYP19 protein expression was determined with Western blotting.

Results: Hyperin (50 μg/l) and Icariin (10 μg/l) significantly increased proliferation of ovarian granulosa cells and secretion of estrogen and progesterone. Hyperin and Icariin stimulated the mRNA and protein expression of CYP17 and CYP19.

Conclusions: These results showed that Hyperin and Icariin can promote the secretion of E2 and P through up-regulation of CYP17 and CYP19. Frequently used Chinese herbs like Cuscuta Chinensis Lam and Epimedium Brevicornu maxim, which contain Hyperin and Icariin, could improve the ovarian endocrine function through these effects.

1. Introduction

Cuscuta Chinensis Lam and Epimedium Brevicornu maxim are the most widely used kidney-tonifying herbs in traditional Chinese medicine [1]. Cuscuta Chinensis Lam (Cuscuta Chinensis) is a parasitic plant which is also known as Chinese Dodder or Tu-Si-Zi in Chinese [2,3]. Hyperin is the main flavonoid in Cuscuta Chinensis seed and is considered the major bioactive component of this plant [4]. Hyperin from Cuscuta Chinensis seeds (FSC) could improve the ovarian endocrine function [5]. Epimedium Brevicornu Maxim (Epimedium Brevicornum) has been widely used in treatment of infertility for thousands of years in China [6]. Icariin is the main flavonoid in Epimedium Brevicornum and is considered the major bioactive component of this plant [7,8]. Previous studies have indicated that Icariin can enhance estrogen biosynthesis in human ovarian granulosa-like KGN (a human ovarian granulosa tumor cell line) cells by increasing the expression of aromatase [9].

Estrogen (E2) and progesterone (P) are necessary for menstruation cycle and other reproductive processes. In the menstrual cycle, the production of estrogen and progesterone is closely interconnected [10]. Estrogen is mostly produced by the granulosa cells of the developing follicle and exerts negative feedback on LH production in the early part of the menstrual cycle [11–13]. Progesterone (P4) is synthesized and secreted from both the follicular and luteal components of the mammalian ovary [14]. The corpora lutea secretes progesterone and the expression of progesterone gradually increases as the follicles develop [15]. Progesterone can influence granulosa cell function in developing follicles prior to ovulation. Granulosa cells of developing follicles do not express progesterone receptor in rat, mouse, monkeys, and human follicles [16–19]. Progesterone can regulate the function of granulosa cells through activating P4PGRMC1(progesterone progesterone receptor membrane component 1) [20].

Aromatase encoded by genes CYP17 and CYP19 is the key enzyme that catalyzes the formation of estrogen and progesterone [21,29]. Cytochrome P-450 17A1, also called 17α-hydroxylase (*CYP19*) is a single chain protein of 419 amino acids that catalyzes three different generic reactions at the same active site. Three androgenic steroids, androstenedione, testosterone and 16α-hydroxyandrostenedione are

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the substrates of CYP19 [21]. The multifunctional enzyme, cytochrome P-450 17α-hydroxylase-17,20-lyase (*CYP17*) lies at the crossroads of corticoid and androgen biosynthesis. It catalyzes the conventional cytochrome P-450 hydroxylation reaction required for androgen formation [22]. In addition, the enzyme promotes the formation of two other steroids (20a and 20b), the former being a precursor to a pheromone in the pig and postulated to have an analogous role in man [23,24]. The 17α-hydroxysteroid (20b) is the probable precursor to epitestosterone, whose physiological function is uncertain [25]. The study on the mechanism of their formation has provided key points to understanding the detailed mechanism of *CYP17* and *CYP19* [26–28]. It has been observed that many bioactive compounds exert their function through regulating the expression of *CYP17* and *CYP19*, which leads to estrogenic and progestational effects [29,30].

The aim of the study is to investigate the effects of different concentrations of Hyperin and Icariin on the proliferation of granulosa cells, the secretion of estrogen and progesterone (P) by granulosa cells and to explore the effect of Hyperin and Icariin on the expression of CYP17 and CYP19. Such studies will help us to better understand the bioactivity of these frequently used herbs.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS, heat-inactivated), DPBS, Trypsin (0.25%)-EDTA, antibiotic/antimycotic solution (100×) and sodium dodecyl sulfate (SDS) were purchased from Gibco-Invitrogen (Grand Island, USA). Pregnant mare serum gonadotropin (PMSG), Bovine serum albumin (BSA), Tris and DMSO were obtained from Sigma Chemical Co (Saint Louis, USA). Hyperin and Icariin were purchased from National Institutes for Food and Drug Control (Beijing, China). RNAprep Pure Tissue Kit and HiScript® Q RT SuperMix for qPCR were products of TIANGEN (Beijing, China).

2.2. Isolation of granulosa cells

Immature (21–25 days old) female Sprague-Dawley rats were injected subcutaneously with 40 IU PMSG. 48 h after PMSG injection, the rats were anesthetized with 10% hydrated chlorine aldehyde. Ovaries were dissected and granulosa cells in follicles were isolated and maintained in DMEM containing antibiotics and 10% FBS.

2.3. Cell culture

Cells were cultured in DMEM supplemented with 10% FBS, 10,000 IU/ml penicillin and 10,000 mg/ml streptomycin, at 37 °C and 5% CO2.

2.4. Evaluation of cell proliferation with MTT assay

Granulosa cells were seeded in 24-well plates at 1×10^6 cells/well in 2 ml of DMEM containing 10% FBS. Cells were allowed to attach for 24 h before a medium change with fresh one. After 72 h of culture, cell proliferation was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as described previously [31]. MTT is cleaved to a blue-colored product (formazan), which is indicative of mitochondrial succinate-dehydrogenase activity in viable cells and detected by a spectrophotometer. Absorbance was measured at 570 nm in a plate reader.

2.5. Histological staining of the cells

When cell culture grew to 90–95% confluence (approx. 72 h), medium was removed and cells were fixed in 4% formaldehyde for 25 min at 4 °C. HE (haematoxylin and eosin) staining was performed for morphological observation on a light microscope (Nikon Eclipse TS100; Nikon, Tokyo, Japan). Cell images were taken with a digital camera (ELWD 0.3 T1-SNCP; Nikon).

Table 1
Primer used in detecting the expression of CYP17 and CYP19.

GAPDH	F5′-CACAATGCTGGGACACAAAC-3′
	R5′-TGGCGTGAGCAGTTTATCAG-3′
CYP17	F5′-ATCAGGCCGGTGGCTCCCAT-3′
	R5′-TCGGGGACCAGCTCCGAAGG-3′
CYP19	F5′-CCATCTGGTCTCCTGCTAG-3′
	R5′-CCACTTACCCTCAACACACA-3′

2.6. Measurement of steroids

Granulosa cells were cultured for 72 h before estrogen and progesterone secretion was determined. Hyperin and ICA were measured by magnetic particle-based ELISA according to the manufacturer's instructions (Elabscience). All analyses were performed in triplicates.

2.7. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagents following manufacturer's instructions (TIANGEN, Beijing, China). Quantitative RT-PCR was performed as previously describe [31] using CYP17 and CYP19 primers listed in Table 1. The thermal cycling conditions were: Initial denaturation at 95 °C for 300 s; 45 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. Standard curves were established for each primer set and both reference and target gene reactions were performed for each sample. The relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method.

2.8. Western blotting

The aromatase protein (CYP17 and CYP19) expression levels were determined by Western blotting. Aliquots of total cell lysates (40 μg protein) were mixed with loading buffer, boiled for 5 min, and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following protein transfer to polyvinylidene difluoride membrane (Millipore Corporation, USA), primary antibodies (CYP17, 1:2000; CYP19, 1: 2000, or GAPDH, 1:2000, Santa Cruz, USA) were added and incubation continued at 4 °C overnight. The blots were washed with Tris-buffered saline (pH 7.2) containing 1% Tween 20 (Sigma) before horse-radish peroxidase-conjugated secondary antibody (1:2000, Santa Cruz, USA) was applied at room temperature for 2 h. Signals were detected with a chemiluminescent system. GAPDH levels was determined and the results were used as protein loading controls.

2.9. Statistical analyses

Statistical analyses were performed with the GraphPad Prism 7.0 software (GraphPad, USA). The results are expressed as the mean ± standard deviation of individual values from three experiments. Data from different groups was compared by one-way ANOVA. P values smaller than 0.05 were considered statistically significant.

3. Results

3.1. Effects of Hyperin and ICA on the viability of ovary granulosa cells

Hyperin (50 μg/l) induced a significant increase in cell proliferation compared to control ($P < 0.05$; Fig. 1A). ICA (10 μg/l) induced a significant increase in cell proliferation compared with control ($P < 0.05$;

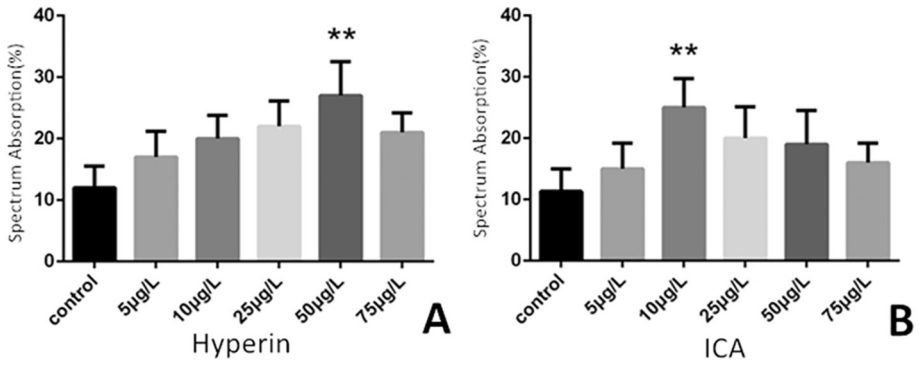

Fig. 1. Granulosa cells viability in different concentration of Hyperin and Icariin tested using MTT assay. Granulosa cells showed a significantly higher viability in Hyperin (50 μg/l) and Icariin (10 μg/l). A) granulosa cells viability at different concentration of Hyperin; B) granulosa cells viability at different concentration of Icariin. **P = 0.01.

Fig. 1B). Higher doses of Hyperin (75 μg/l) and ICA (75 μg/l) demonstrated a compromised cell proliferate compared with Hyperin (50 μg/l) and ICA (10 μg/l) at lower concentrations, indicating the presence of cytotoxic effect.

3.2. Morphology of cultured granulosa cells

The morphology of granulosa cells displayed some fibroblast features in culture. The cells were star- or spindle-shaped, with intercellular connection through the filamentous protrusions. The cells displayed round nucleus with multiple particles and vacuoles in the cytoplasm. Treatment with Hyperin group or ICA led to increased particles and vacuoles in the cytoplasm (Fig. 2).

3.3. Effect of Hyperin and ICA on steroid hormone secretion

The results showed that Hyperin and ICA were able to increase the production of both estrogen and progesterone by granulosa cells compared with control group. As shown in Table 2, combined treatment with Hyperin (50 μg/l) and ICA (10 μg/l) led to the highest level of estrogen and progesterone production by granulosa cells.

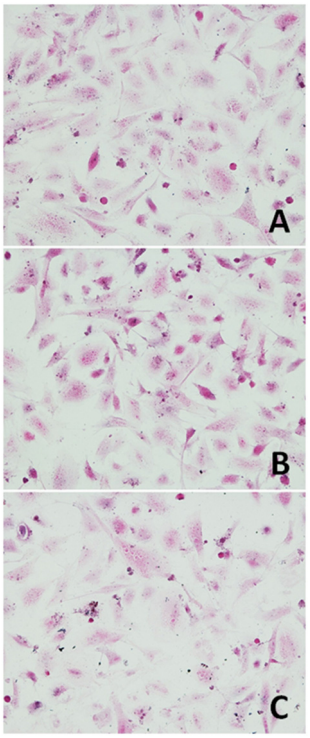

Fig. 2. Morphology of cultured granulosa cells. A) granulosa cells cultured in Hyperin (50 μg/l); B) granulosa cells cultured in Icariin (10 μg/l) C) granulosa cells cultured in culture medium without treatment. Granulosa cells in Fig. 2A and B have higher number of cells and higher percentage of round shaped cells than control. Pictures captured at 200× magnification.

Table 2
Production of estrogen and progesterone by granulosa cells treated with different concentration of Hyperin and Icariin.

Group	Drug concentration (μg/l)	E_2 (pg/ml)	P (ng/ml)
Control		11.07 ± 2.38	1.68 ± 0.09
Hyperin	5	11.27 ± 2.83	1.97 ± 0.33
	10	11.96 ± 2.19	2.12 ± 0.21
	25	13.96 ± 1.16*	2.41 ± 0.39*
	50	14.93 ± 1.22**	2.64 ± 0.18**
	75	12.45 ± 2.03	2.51 ± 0.30*
ICA	5	15.61 ± 2.00**	3.03 ± 0.41**
	10	18.85 ± 1.64**	4.44 ± 0.37**
	25	17.09 ± 1.56**	2.95 ± 0.25**
	50	16.32 ± 0.84**	2.80 ± 0.23**
	75	12.81 ± 2.06	2.78 ± 0.17**

Hyperin and icariin added to the culture medium could increase the secretion of estrogen and progesterone by granulosa cells.
We performed two biological replicates of the experiments for each group.
* $P \leq 0.05$.
** $P \leq 0.01$.

3.4. Effect of Hyperin and ICA on the expression of CYP17 and CYP19 in granulosa cells

The expression of CYP17 and CYP19 mRNA levels in the cultured granulosa cells were significantly increased after treatment with Hyperin (50 μg/l) compared with control group (Fig. 3 A, B). A positive dose-response effect can be seen in a wide range of concentrations. The decline of CYP17 and CYP19 mRNA levels at 75 μg/l of Hyperin likely represent a cytotoxic effect. Treatment with ICA also led to a significant increase in the expression of CYP17 and CYP19 (Fig. 3 C, D) at 10 μg/l concentration. Further increase in ICA concentration also led to a reduction of CYP17 and CYP19 mRNA levels.

3.5. Effect of Hyperin and ICA on protein expression of CYP17 and CYP19 in granulosa cells

RT-PCR analysis showed that CYP17 and CYP19 expression increased in granulosa cells exposed to Hyperin and ICA. Consistent with their effects on aromatase mRNA levels, Hyperin and ICA treatment increased aromatase protein expression (Fig. 4). These results indicated that Hyperin and ICA enhances estrogen biosynthesis in granulosa cells by increasing the expression of aromatases.

4. Discussion

Estrogen and progesterone act on the central nervous system, ovary, and uterus, and are important for ovulation, fertilization, implantation

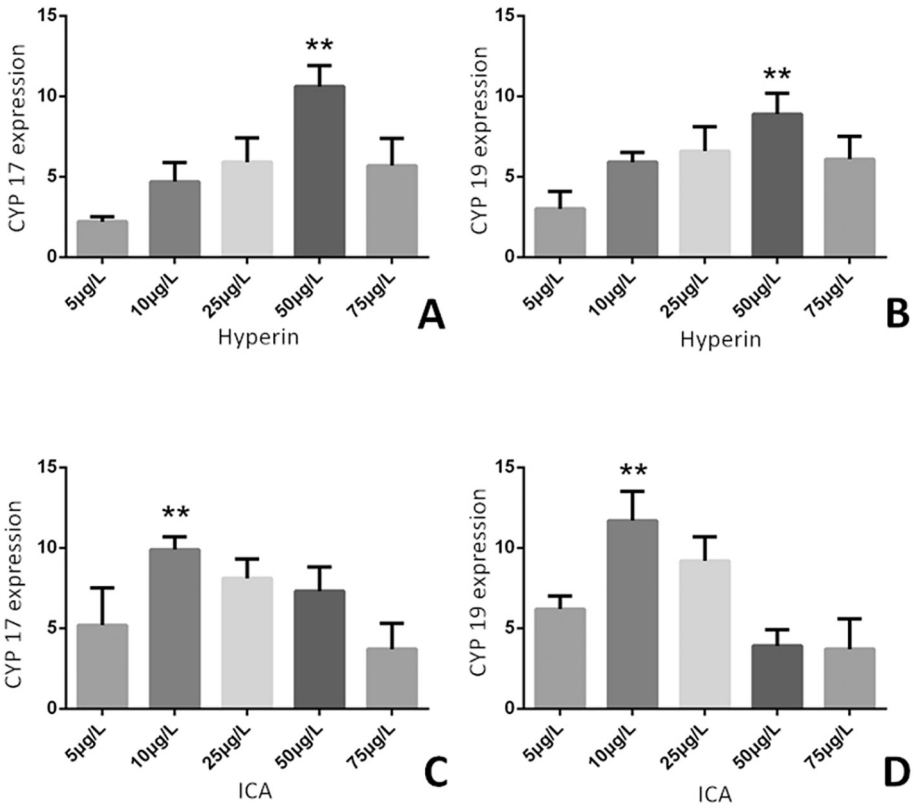

Fig. 3. mRNA expression of *CYP17* and *CYP19* of granulosa cells treated by different concentrations of Hyperin and Icariin. A) mRNA expression of *CYP17* of granulosa cells treated with different concentration of Hyperin; B) mRNA expression of *CYP19* of granulosa cells treated with different concentration of Hyperin; C) mRNA expression of *CYP17* of granulosa cells treated with different concentration of Icariin; D) mRNA expression of *CYP19* of granulosa cells treated with different concentration of Icariin. The mRNA expression of *CYP17* and *CYP19* was significantly higher after treatment with Hyperin (50 μg/l) or Icariin (10 μg/l) than control. **P ≤ 0.01.

of embryo, maintenance of pregnancy, mammary gland development and lactation [32]. Increased progesterone plus estrogen in luteal phase is required for normal fertility potential [33]. Oxidative stress affects female and male gametes and the developmental capacity of embryos [34]. Long-term moderate oxidative stress decreased the fertility and fecundity potential of mice. This effect may be due to poor follicle quality and decreased progesterone levels [35]. Since ROS increase can be neutralized by antioxidant, the antioxidant properties of estrogens were investigated in pig luteal and follicular tissues exposed to *in vitro* H_2O_2. High doses of estrogen (≥ 40 pg/ml) protected against apoptosis [36]. Vitamin D may also play a physiological role in reproduction including ovarian follicular development and luteinization *via* altering anti-müllerian hormone (AMH) signaling and increased progesterone production by granulosa cells [37]. In human ovarian tissue, Vitamin D was found to stimulate progesterone production by 13%, estradiol production by 9%, and estrone production by 21% [38]. It was demonstrated in a choriocarcinoma cell line that P450 aromatase expression and activity were stimulated by calcitriol and an atypical vitamin D response element is located in the CYP19 (CYP19A1) promoter [39]. Thus, expression of aromatases in ovary is regulated by multiple hormones as well as exogenous compounds.

Chinese medicinal plants can regulate the antiapoptotic pathway (Bcl2 and Bax expression) to protect the growing follicles and to enhance antioxidant-mediated defense against the mitochondrial damage in the follicles, preserving the endocrine and reproductive ability of the aging ovary [40]. Cuscuta Chinensis and Epimedium Brevicornum are most commonly used to nourish and improve the kidney conditions in China and other Asian countries. Cuscuta Chinensis and Epimedium

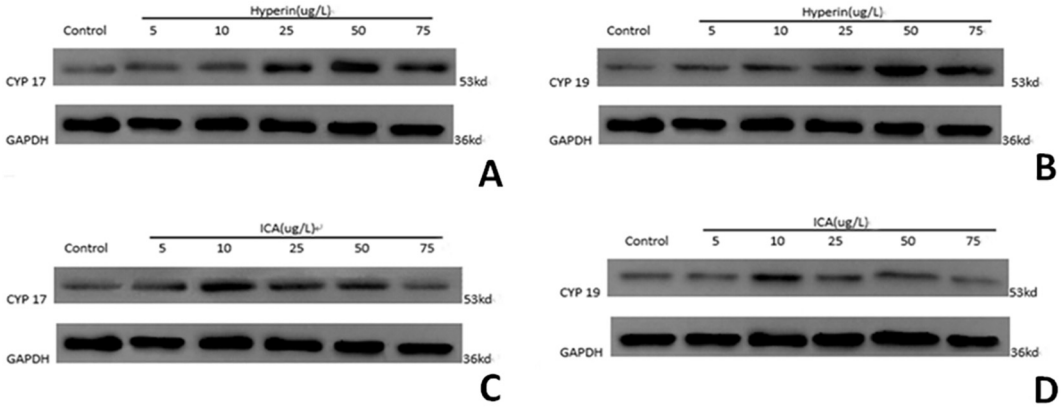

Fig. 4. CYP17 and CYP19 protein levels in granulosa cells treated with different concentrations of Hyperin and Icariin. CYP17 protein levels in granulosa cells treated with different concentrations of Hyperin; B) CYP17 protein levels in granulosa cells treated with different concentrations of Hyperin; C) CYP17 levels following treatment with different concentrations of Hyperin; D) CYP17 levels following treatment with different concentrations of Hyperin. GAPDH was detected and the results were used as protein loading controls. The protein expression of *CYP17* and *CYP19* is significantly higher by treatment with Hyperin (50 μg/l) and Icariin (10 μg/l).

Brevicornum showed antioxidant activities in various diseases [41,42]. The ethanol extracts of Cuscuta Chinensis can prevent hepatic injuries from APAP-induced hepatotoxicity in rats and this is likely mediated by its antioxidant activities [43]. Extracts of Epimedium Brevicornum was found to significantly promote estrogen biosynthesis in human ovarian granulosa-like KGN cells. Icariin, the bioactive compound in Epimedium Brevicornum, was also found to promote estrogen biosynthesis in KGN cells in a concentration-dependent and time-dependent manner, while additional estrogen biosynthesis-promoting compounds from the extracts of this medicinal plant is being isolated [9].

In this study we found that Hyperin and Icariin Hyperin and Icariin promoted estrogen and progesterone biosynthesis in granulosa cells by enhancing the production of aromatases. Forskolin was known to activate adenylate cyclase to increase intracellular cAMP production, resulting in the activation of PKA/CREB for aromatase transcription [44]. It is unclear if and how PKA/CREB pathway might be involved in Hyperin and ICA mediated regulation of aromatase. Phosphodiesterase type 5 (PDE5) inhibitors have been found to stimulate aromatase expression in human adipocytes [45]. Icariin was found to be a potent inhibitor of PDE5 [46]. Thus, Hyperin and Icariin may inhibit the expression of PDE5, increase intracellular cAMP and activate the PKA/CREB pathway [47]. Further studies is required to delineate the underneath molecular mechanism.

Plant herbs were Often observed to carry higher efficacy and less side effects than chemical drugs due to their combined effects *in vivo*. Hyperin and Icariin are easy to extract from Cuscuta Chinensis, Saint John's Wort and Epimedium Brevicornum. ftble Our data indicated that Hyperin and Icariin can be used as antioxidants to improve ovarian function and improve the pregnancy outcome. Hyperin and Icariin are valuable herb components that deserve further investigative efforts for the treatment of estrogen and progesterone deficiency-related diseases.

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