Peripubertal Administration of Icariin and Icaritin Advances Pubertal Development in Female Rats

Hyun Ku Kang1,2, Sang-Bum Lee1,2, Hyosuk Kwon1, Chung Ki Sung3, Young In Park1,2,* and Mi-Sook Dong1,*

1School of Life Sciences and Biotechnology, Korea University, Seoul 136-701,  
2College of Pharmacy, Korea University, Jochiwon 339-700,  
3College of Pharmacy, Chonnam National University, Gwangju 500-757, Republic of Korea

Abstract
Epimedi Herba is a traditional medicinal herb used in Korea and China and exerts estrogenic activity. In this study, we investigated the effect of peripubertal administration of Epimedi Herba on pubertal development in female rats using a modified protocol of the rodent 20-day pubertal female assay. Female Sprague-Dawley rats (21 days old after weaning, 10 rats per group) were divided into five groups: saline (Con), ethinyl estradiol (E2), Epimedi Herba ext (Ext), icariin (ICI), and icaritin (ICT), which were administered by oral gavage (E2 by subcutaneous injection) from postnatal day (PND) 21 through PND40. The time to vaginal opening (VO) was shorter for the Epimedi groups, particularly for the ICT group (p<0.05). Treatment with ICI and ICT significantly increased the duration of the estrus cycle (ICI, 2.78 days; ICT, 4.0 days; control, 1.78 days). Ovary weight was reduced by E2 treatment and increased by the Ext, ICI, and ICT treatments while the weight of the uterus and pituitary glands increased significantly only in the E2 and ICT groups. Although Epimedi Herba displayed relatively weak estrogenic activity, its repeated administration could affect pubertal development in female rats.

Key Words: Epimedi Herba, Icariin, Icaritin, Female pubertal development

INTRODUCTION
Epimedi Herba, the aerial parts of Epimedium species (Berberidaceae), has been traditionally used in Korea and China to treat coronary heart disease, male impotence, improve female health, and strengthen bones (Yap et al., 2007; Ma et al., 2011). More than 130 compounds have been isolated from Epimedium species, including prenylated flavonoids and their glycosides. Among these compounds, icarin (ICI), epimedin A, B, C, and hyperin are the main components in Epimedi Herba. Among these compounds, icarin (ICI), epimedin A, B, C, and hyperin are the main components in Epimedi Herba (Chen et al., 2008; Islam et al., 2008). ICI, which is the principal Epimedium prenylflavonoid, significantly inhibits human phosphodiesterase-5 and induces nitric oxide synthase expression in corpus cavernosum smooth muscle (Liu et al., 2005; Chiu et al., 2006). In animal studies, ICI and Epimedium flavonoid administration inhibits bone resorption, stimulates bone formation, prevents osteoporosis in ovariectomized rats (Zhang et al., 2009), and improves erectile function in aged male rats (Makarova et al., 2007). Icaritin (ICT), which is the aglycone of ICI, stimulates estrogen-driven cell proliferation and estrogen responsive element (ERE)-dependent reporter genes (Wang and Lou, 2004; Dong et al., 2012).

Phytoestrogens are plant-derived polyphenolic compounds that produce estrogenic or antiestrogenic-like biological effects in the body. These compounds are found in a wide variety of foods and have been widely marketed as a natural alternative to estrogen replacement therapy. Previous studies have shown that the estrogenic activity of phytoestrogens has beneficial health effects, including a lowered risk for osteoporosis, heart disease, breast cancer, and menopausal symptoms. However, phytoestrogens may interfere with the role of E2 by acting as an endocrine disruptor and causing adverse health effects (Patisaul and Jefferson, 2010). Clinical and experimental studies examining the impact of soy or soy phytoestrogen consumption on human health have produced mixed and often conflicting results. Phytoestrogens may interfere with endogenous estrogen action either by acting as agonists when endogenous estrogen levels are low or by acting as antagonists when endogenous estrogen levels are high (Dong et al., 2012). Emerging evidence suggests that exposure to dietary phytoestrogens may pose a risk to some groups, particularly infants and the unborn (Strom et al., 2001; Cassidy, 2003).
Consequently, the question of whether or not phytoestrogens are beneficial or harmful to human health remains controversial (Patisaul and Jefferson, 2010).

Recently, we assessed the estrogenic and antiestrogenic activity of Epimedi Herba extract (Ext), ICI, and ICT (Fig. 1) using an *in vitro* estrogen receptor (ER) α or β-mediated ERE-driven reporter gene assay and an *in vivo* uterotrophic assay. ICI and ICT were used as a representative glycoside and aglycone of prenylflavonoids from Ext, respectively. We found that although ICI did not have any *in vitro* estrogenic or antiestrogenic activity mediated by ER α or β, it displayed the highest estrogen activity among the three groups in the *in vivo* uterotrophic assay using adult rat. The dried extract contains approximately 2.56 ± 0.25% ICI and has *in vitro* and *in vivo* estrogenic activity (Dong et al., 2012).

To examine any potential hormone-like effects of Epimedi Herba and its compounds ICI and ICT on the neuroendocrine axis during the sensitive period of puberty, the effects of Ext, ICI and ICT on female sexual development was evaluated after peripubertal administration using a modified protocol of the rodent 20-day pubertal female assay (Goldman et al., 2000).

**MATERIALS AND METHODS**

**Preparation and identification of major compounds from *Epimedium koreanum***

Epimedi Herba was purchased from a farmer in Chulwon (Kangwon, Korea) and authenticated by Prof. Je-Hyun Lee at the College of Oriental Medicine at Dongguk University (Gyeongju, Korea).

Preparation and identification of Ext, ICI, and ICT were described by Dong et al. (in submission). ICI was determined to be the most abundant flavonoid in Ext (2.56 ± 0.25%); however, ICT was not detected.

**Animals**

Animal studies were conducted in accordance with the institutional guidelines for care and use of laboratory animals, and the experimental protocol was approved by the Animal Ethics Committee at Korea University, Seoul, Korea. Preparations of 17β-estradiol, icariin, and icaritin.

Female Sprague-Dawley rats (14-day timed pregnant) were purchased from Orient Bio Inc. (Sungnam, Korea). Pregnant rats were housed individually in polycarbonate cages and maintained under controlled temperature (23 ± 1°C), humidity (35 ± 5%), and light (12 h light/12 h dark) conditions. Food and water were freely available. Pregnant dams were allowed to deliver their pups naturally. The day of birth was recorded as postnatal day (PND) 0. Upon weaning on PND 21, females were ranked by body weight (BW) and allocated into five groups based on BW. Littermates were equally distributed among treatment groups.

**Experimental design for female rat sex maturation**

Females in the five treatment groups received either saline (control group, Con) or estrogen (E2 group: 1 μg/3 ml/kg/day), and Ext (200 mg/3 ml/kg/day), ICI (20 mg/3 ml/kg/day) or ICT (20 mg/3 ml/kg/day). All test samples, except E2, were administered by oral gavage from PND21 through PND40. E2 was administered by subcutaneous injection. BWs were recorded daily, and the dose administered each day was adjusted for BW. Females were killed on PND 41, and blood was collected to determine the levels of E2 and testosterone. The weights of the kidney, liver, adrenal glands, ovary, uterus, and pituitary gland were recorded.

**Assessment of puberty and estrous cyclicity**

All females were checked daily for vaginal opening (VO) starting at PND22. The day of complete VO and the BW on that day were recorded. Vaginal lavage fluid was collected once per day by repeatedly pipetting 0.9% saline into the vagina until VO occurred. The lavage fluid was placed on a glass slide, sprayed with Spraycyte fixative (Fisher Scientific, Pittsburgh, PA, USA), and stained with hematoxylin and eosin (Sigma, St. Louis, MO, USA) to determine the estrous cycle stage, as previously described (Champlin et al., 1973). The smear was observed immediately under low magnification (100× or 200×) using a light microscope. The vaginal smears were classified as diestrus (presence of leukocytes), proestrus (presence of nucleated epithelial cells), or estrus (presence of cornified epithelial cells) as described by Everett (1989). Extended estrus was defined as samples containing cornified cells with no leukocytes for 3 or more days, and extended diestrus was defined as samples containing the presence of leukocytes for 4 or more days (Goldman et al., 2007).

**Fig. 1.** Chemical structures of 17β-estradiol, icariin, and icaritin.

**Table 1.** Primer sequences used in the PCR reactions

| Genes   | Primer sequences | Amplicon (bp) |
|---------|------------------|---------------|
| ER alpha | (F) TCA CAC CAA AGC CTC GGG AA | 879 |
|         | (R) GGC CAA AGG TTG GCA GCT CT | |
| ER beta  | (F) GGC ACC CAT TGC CAA TCA TC | 801 |
|         | (R) GAA AAT GAG CTT GCC GGG GT | |
| C3      | (F) TGG TGC GCA ATG AAC AGG TG | 805 |
|         | (R) AGC CAT TTG ACA GCC CCA CA | |
| CaBP9K  | (F) AGC TGG GCA CAG TGG CAA AA | 839 |
|         | (R) CAC ATG CAG GCA AAA TGC CA | |
| PR      | (F) TTC AGC TGC CCA TTC TGC CT | 862 |
|         | (R) TTA TGC TGC CCT TGC ATC GC | |
| IGFBP1  | (F) TGC CGG AGT TCC TAA CTG TTG TTT | 802 |
|         | (R) TGC CGG AGT TCC TAA CTG TTG TTT | |
Clinical signs and BWs
Each animal was observed at least once daily throughout the study period for clinical signs of toxicity related to chemical treatment. On working days, all cages were checked for dead or moribund animals in the morning and afternoon. The BW of each rat was measured daily just prior to treatment.

Hormonal measurements
Blood was collected from the abdominal aorta approximately 24 h after the last test compound treatment. Serum was prepared immediately and stored at -80°C until analyzed for serum hormone concentrations. Commercially available radio-immunoassay kits were used to measure serum concentrations of thyroid stimulating hormone (TSH) and E2 (Amersham Corp., Piscataway, NJ, USA).

RNA preparation and reverse transcription polymerase chain reaction (RT-PCR) analysis of estrogen-related mRNA expression in ovary and liver
Levels of estrogen responsive protein mRNA were analyzed by RT-PCR. Total RNA was extracted from rat liver and ovarian tissue using Easyblue (Intron Biotechnology Co., Dajeon, South Korea) following the manufacturer’s procedure. Total RNA (5 μg) was reversed transcribed in a final volume of 50 μl using M-MLV Reverse Transcriptase in the presence of oligo-dT primer, deoxy-NTP and Rnasin (Promega, Madison, WI, USA) for 60 min at 42°C, after denaturation for 5 min at 95°C. The primers used and the PCR conditions are listed in Table 1.

Statistical analysis
Data are reported as mean ± SD. Data for the VO day, BW, and organ weight of the rats were evaluated using a one-way analysis of variance with a level of significance set at p<0.05.

RESULTS

Clinical signs and body weights
No mortality or clinical signs of toxicity were observed in any of the treatment groups. A statistically significant decrease in mean necropsy BW was observed at PDN 41 for rats treated with E2 (p<0.05). Ext, ICI, and ICT had no significant effects on mean necropsy BW or BW gains during the treatment period from PND 21 to PND 41 (Fig. 2).

Vaginal opening
VO was used as an indicator of puberty. The mean age and body weight at the time of vaginal opening in female rats treated with ethinyl estradiol, Epimedii Herba extract, icariin, or icaritin from postnatal day (PND) 21 for 20 days are shown in Table 2.

Table 2. Mean age and body weights at the time of vaginal opening in female rats treated with ethinyl estradiol, Epimedii Herba extract, icariin, or icaritin from postnatal day (PND) 21 for 20 days

| Treatment  | Mean age  | Mean body weight |
|------------|-----------|------------------|
| Control    | 36.6 ± 1.01 | 130.0 ± 9.21 |
| E2         | 26.6 ± 0.53** | 68.7 ± 5.59** |
| Ext        | 35.6 ± 0.88  | 120.2 ± 12.19 |
| Icariin    | 35.0 ± 1.32  | 120.3 ± 10.91* |
| Icaritin   | 33.9 ± 1.83*  | 117.6 ± 11.41* |

Mean ± SD (n=9 animals per treatment group). *Significantly different from vehicle control (ANOVA), p<0.05.

Table 3. Effects of Epimedii Herba extract, icariin, and icaritin on the estrus cycle in female rats

| Treatment  | Proestrus  | Estrus + Metestrus | Diestrus  |
|------------|------------|--------------------|----------|
| Control    | 0.89 ± 0.33 | 1.78 ± 0.44         | 3.00 ± 0.50 |
| E2         | 1.00 ± 0.53 | 11.67 ± 1.50*       | 3.33 ± 1.22 |
| Ext        | 0.89 ± 0.33 | 1.78 ± 0.97         | 3.89 ± 0.93 |
| ICI        | 0.78 ± 0.44 | 2.78 ± 0.67*        | 3.44 ± 0.88 |
| ICT        | 0.67 ± 0.50 | 4.00 ± 0.71*        | 3.44 ± 1.51 |

Mean ± SD (n=9 animals per treatment group). *Significantly different from the control group (p<0.05).
BW at VO in the control animals were 36.6 ± 1.01 days (range, 35-38 days) and 130.0 ± 9.21 g, respectively. E2 treatment significantly accelerated the time of VO to 26.6 days and decreased mean BW to 68.7 ± 5.59 g (Fig. 3, Table 2). The day of VO decreased in each Epimedii group (Table 2), particularly in the ICT group (p<0.05).

Estrous cycle
The estrus cycles of individual animals were observed starting the day following VO until the end of the study. The number of days in the complete estrus cycle increased dramatically with E2 treatment. This increase in estrus cycle duration in the E2-treated rats was due to an extended estrus cycle of 8-11 days when compared to that of the control (1-3 days) (Table 3). Treatment with ICI and ICT significantly increased the duration of the estrus cycle (ICI, 2.78 days; ICT, 4.0 days; control, 1.78 days), whereas proestrus and diestrus did not change significantly in the Epimedii Herba groups.

Organ weights
No signs of apparent organ toxicity were observed in any of the treatment groups. Rats in the E2 group had significantly lower ovarian weights and higher uterus and pituitary gland weights than those of the other groups (Fig. 4). However, rats in the Ext, ICI, and ICT groups had significantly higher ovarian and uterus weights than those in the Con group. Pituitary gland weight increased significantly in the E2 and ICT groups but not in the Ext and ICI groups. No significant difference in the weights of the liver, kidney, or adrenal glands was observed (data not shown).

Serum gonadal sex hormones
Serum hormone concentrations were evaluated individually in all rats regardless of estrus cycle stage. Mean serum estradiol level decreased significantly in the E2 group. However, Ext, ICI, and ICT tended to have higher blood estradiol levels, but the increase was not significant except ICT (Fig. 5). Serum levels of TSH were below the detection limit in all groups (data not shown).

Estrogen response gene expression in the liver, uterus, and ovaries
The expression of well-known estrogen related genes was evaluated in the liver, uterus, and ovary to further access the estrogenic activity of Epimedii Herba in rats. Estrogen effects in the liver were investigated by measuring the expression of calcium binding protein 9kDa (CaBP9K) and insulin like growth factor binding protein 1 (IGFBP1), which are reasonably good markers for the effect of estrogen on the liver (Geis et al., 2005).

Both the E2 and ICT groups showed significantly up-regulated expression of the IGFBP-1 gene, and the Ext and ICI groups had slightly up-regulated levels of these proteins in liver tissue (Fig. 6A). CaBP9k expression was up-regulated in the E2 and ICT groups. Both the Ext and ICI treatments elevated CaBP9k and IGFBP-1 gene expression in the liver (Fig. 6A). ER-α, C3, and progesterone receptor (PR) mRNA
lows for only a partial evaluation of the estrogenic potency of all treatment groups. An analysis of uterine wet weight also permitted a more detailed and mode of action. Additional endpoints such as changes in levels in the uterus were also assessed (Fig. 6B). The levels of ER-α were down-regulated following treatment with E2 and ICT, whereas C3 and PR mRNA was up-regulated (Fig. 6B). E2 treatment caused a significant reduction in the level of ER-α mRNA (Fig. 6C). ER-β mRNA was expressed in rats of all treatment groups. An analysis of uterine wet weight allows for only a partial evaluation of the estrogenic potency and mode of action. Additional endpoints such as changes in estrogen sensitive gene expression permit a more detailed analysis of responses.

**DISCUSSION**

Epimedi Herba not only stimulates sexual activity in males by tonifying the kidney and strengthening yang (Chiu et al., 2006) but also functions as a phytoestrogen, which can have potential health benefits in females by countering menopausal symptoms and lowering the incidence of hormone-dependent diseases including breast cancer (Shen et al., 2007). Epimedi Herba has been used as a traditional herbal medicine and a tea to improve health in rural areas of Korea. We previously reported that although Ext and ICT, but not ICI, exhibits in vitro estrogen receptor α and β-mediated estrogenic activity, ICI displays higher estrogenic anti-estrogenic activities than those of Ext or ICT in ovariectomized adult rats (Dong et al., 2012). In this study, we found that Epimedi Herba affected maturation of the female reproductive system by acting as an exogenous estrogen in weanling rats.

The onset of puberty in female rats includes VO and subsequent estrous cycles. VO in the rat commonly takes place between PND 33 and 42, although variations occur between strains and different colonies of the same strain (Goldman et al., 2000; Kim et al., 2002). In this study, rats had a mean VO age of 36.5 ± 1.61 days (range, 35-38 days), which was consistent with previous studies. E2 (1.0 µg/kg/day) treatment significantly advanced the day of VO (10 days) and decreased BW at VO as expected. Although ICT only decreased the time to VO by 2.7 days and significantly reduced BW at the VO day, all groups tended to develop precocious VO (Table 2, Fig. 3). A normal cycle is generally defined as 4-5 days and contains 1-2 days of estrus (Goldman et al., 2000; Kim et al., 2002). The E2 group produced a persistent estrus state, and ICI and ICT increased significantly by 1.0 and 2.22 days, respectively, in estrus days when compared to those of the control (Table 3). Taken together, these results indicate that although Ext possessed very weak estrogenic activity, these treatments advanced pubertal timing in the order of ICT, ICI, and Ext.

Most of the compounds identified from Ext including the major component of ICI were prenyllavonoid glycosides, which possess very weak or non-estrogenic activity in vitro. The potency of the in vitro ER α or ER β-mediated estrogenic activity of these compounds is as follows: ICT > Ext > ICI (Dong et al., 2012). However, ICI displayed higher in vivo estrogenic activity than that of ICT in the adult female and ICT produced the strongest estrogenic activity in the pre-pubertal onset assay. Interestingly, Ext displayed significant estrogenic activity in adult female rats but not in young rats. The discrepancies between adult and pre-pubertal rats with regard to the potency of estrogenic activity in Ext, ICI, and ICT might be due to the rates of absorption, metabolism, and clearance. Absorption of intact glycosides is much slower than the corresponding aglycones, and intestinal and microbial glycosidases are a critical first step in the intestinal and microbial disposition of flavonoid glycosides (Liu et al., 2003). Xu et al. (2007) reported that ICI is rapidly metabolized to icariside II by bacteria in the rat intestine, where icariside II is absorbed faster than ICI, and relatively high concentrations of icariside II are detected in plasma after intragastric administration of ICA to rats. Phase II metabolism such as glucuronidation in young rats would be limited, because the capacity for phase II metabolism is underdeveloped relative to adults (Coughtrie et al., 1988; Jefferson et al., 2009). Therefore, intestinal bacteria might be underdeveloped in young rats compared to adult rats; thus, the glycoside form of flavonoids in Ext or ICI could not be readily converted to their aglycone form and the rate of absorption might have been lower compared to ICT in prepubertal rats.

The weights of the uterus and pituitary gland increased significantly following the E2 and ICT treatments but not after the Ext and ICI treatments. However, the profile of ovary weight changes was different. Ovary weight decreased significantly following E2 treatment (p<0.05) but increased following the Ext, ICT, and ICT treatments (Fig. 4). The blood estrogen profile among these four groups was similar to that of ovarian weight (Fig. 6). E2 treatment significantly lowered serum estradiol levels, whereas *Epimedium* Herba treatment elevated serum estradiol levels in the following order: ICT, ICI, and Ext (Fig. 8). Estrogens are synthesized in the ovary. Thus, exogenous estrogen might stimulate feedback inhibition of estrogen synthesis via the hypothalamus-pituitary-ovary axis resulting in ovarian atrophy. The changes in gene expression were very similar between E2 and ICT in the uterus, ovary and liver, except for aromatase. Therefore, atrophy of the ovary might be related to aromatase activity, i.e., induction of aromatase expression in ovaries or stimulation of aromatase activity. Although further study is needed, aromatase mRNA expression in the ovary of the E2 and ICI groups was correlated with the
level of blood estrogen (Fig. 5). Thus, the estrogenic effect of ICT might be due not only to estrogenic activity but also to elevated blood levels of E2.

Relatively few phytoestrogens or natural plant extracts have been studied in the context of pubertal timing compared to environmental chemicals, and most of these studies evaluated the effects of soy-related phytoestrogens or formula (Kouki et al., 2003; Jefferson et al., 2009). Although the estrogenic activities of Ext and ICI were weak, weaning rats (21 days of age) were exposed to relatively low doses of the compounds. For example, we used ICI and ICT at doses of 10 mg/kg, and Ext at 200 mg, which can be obtained from 1 g of dried Epimedi Herba (about 22% yield). Whitten and Naftolin (1992) reported that prepubertal dietary exposure to coumestrol (0.01% concentration, d22-60) in female rats accelerated VO by 4 days and produced irregular cycles at 116-131 days. They suggested that chronic coumestrol treatment may have induced some permanent changes in reproductive function. The pituitary/hypothalamic axis in peripubertal female Sprague-Dawley rats is less sensitive than that of adult female Sprague-Dawley rats (Ashby et al., 2002). Therefore, the strength of the estrogenic activity of phytoestrogens or plant extracts could have a greater affect on pubertal timing and plant estrogens at natural dietary levels and produce significant agonistic actions in several estrogen-dependent tissues and processes.

The estrogenic effect of phytoestrogens has been widely evaluated in terms of human health. However, phytoestrogens can also interfere with endogenous estrogen action either by acting as agonists at times of low endogenous estrogen or by acting as antagonists at times of higher endogenous estrogen levels. Thus, estrogenic substances that are not only synthetic chemicals but also plant extracts may act as endocrine disruptors and may alter sex maturation in male and female children who are sensitive to sex hormones.

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