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

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Abstract

Epimedii 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 Epimedii 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), Epimedii 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 Epimedii 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 Epimedii Herba displayed relatively weak estrogenic activity, its repeated administration could affect pubertal development in female rats.

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

INTRODUCTION

Epimedii 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, icariin (ICI), epimedin A, B, C, and hyperin are the main components in Epimedii Herba (Chen et al., 2008; Islam et al., 2008). ICI, which is the principal Epimedium prenyllavonoid, 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, in submission).

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., in submission). 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 (Patiasaul 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 prenylflavonoid 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., in submission).

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 (Kangwondo, Korea) and authenticated by Prof. Je-Hyun Lee at the College of Oriental Medicine at Dongguk University (Gyeonggi, 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.

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 treatment 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).

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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER alpha</td>
<td>(F) TCA CAC CAA AGC TCT GGG AA (R) GGC CAA AGG TTG GCA GCT CT</td>
<td>879</td>
</tr>
<tr>
<td>ER beta</td>
<td>(F) GGC ACC CAT TGC CAA TCA TC (R) GAA AAT GAG CTT GCC GGG GT</td>
<td>805</td>
</tr>
<tr>
<td>C3</td>
<td>(F) TGG TGC GCA ATG AAC AGG TG (R) AGC CAT TTG ACA GCC CCA CA</td>
<td>839</td>
</tr>
<tr>
<td>CaBP9K</td>
<td>(F) AGG TGG GCA CAG TGG CAA AA (R) CAC ATG CAG GCA AAA TGC AA</td>
<td>862</td>
</tr>
<tr>
<td>PR</td>
<td>(F) TTC AGC TGC CCA TTC TGG CT (R) TTA TGC TGC CCT TGC ATC GC</td>
<td>802</td>
</tr>
<tr>
<td>IGFBP1</td>
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<td>802</td>
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