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Evaluation of *in vivo* estrogenic potency of natural estrogen-active chemical, puerarin, on pituitary function in gonadectomized female rats

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ABSTRACT

Aims: Previous research has revealed that puerarin, the major phytoestrogen in tuberous roots of *Pueraria lobata* and *Pueraria mirifica*, acts as a selective estrogen receptor modulator that displays predominantly estrogenic potential for health benefit. However, little is known about the estrogenic potency of puerarin in pituitary, especially in the rat model of postmenopausal females.
Main methods: Plasma prolactin and growth hormone levels as well as mRNA expression levels of pituitary estrogen-regulated genes, such as estrogen receptor (ER) subtypes alpha (ERα) and beta (ERβ), truncated ER product-1 (TERP-1) and -2 (TERP-2) and gonadotropin alpha subunit, were examined using radioimmunoassay and TaqMan® real-time PCR, respectively. The effects were compared with the potent ER agonist, 17β-estradiol-3-benzoate (E2B), and both substances were supplemented at low and high doses, *i.e.*, 0.6 or 3 g puerarin and 0.0043 or 0.0173 g E2B per kilogram of phytoestrogens-free rat chow, and applied to ovariectomized rats (five groups; 11–12 rats per group) for 12 weeks.
Key findings: Puerarin possessed weak E2B-like activities on pituitary function by acting as ERβ and TERP-1/-2 agonists, which resulted in the downregulation and upregulation of ERβ and TERP-1/-2 mRNA expressions, respectively, and elevation of growth hormone levels. There were trends of decreased levels of alpha subunit mRNA transcripts and increased levels of prolactin in puerarin-treated rats as observed in E2B-treated animals.
Significance: This is the first report in ovariectomized rats the effects of puerarin on somatotropes and pituitary estrogen-responsive mRNA expressions, which are very weakly estrogenic by acting through ERβ- and TERP-1/-2 mediated pathways.

1. Introduction

Isoflavone puerarin is a major plant-derived phytoestrogen found at a relatively high level in the tuberous roots of *Pueraria lobata* and *Pueraria mirifica*, which are indigenous medicinal herbs that have been used in traditional oriental medicine for several decades [1–4]. There is a large body of evidence that puerarin may act as a putative natural estrogen-active compound in both *in vitro* and *in vivo* models [1,5–9]. Compared to most other estrogen-active compounds found in the roots of *P. lobota* and *P. mirifica*, puerarin binds to estrogen receptors (ERs), but with a higher relative binding affinity for the estrogen receptor subtype beta (ERβ) compared to subtype alpha (ERα) in the *in vitro* ERs competitive binding assay. In intracellular ERs-induced transcriptional activation assay puerarin possesses the highest ability to induce the growth and proliferation of the ERs-dependent human mammary adenocarcinoma cancer cell line (MCF-7) and show the highest levels of ERs agonistic activity through ERα- and ERβ-dependent modes of action, and these properties may make it more effective than many isoflavones found in the roots of *P. lobota* and *P. mirifica* to disrupt estrogen-dependent physiological functions [1,5,10].

Much of the previous *in vivo* research has revealed that puerarin exerted weak estrogenic activities on the stimulation of uterine growth and estrogen-sensitive gene expression [8,9] and vaginal proliferation [6,9], and may act as a selective estrogen receptor modulator (SERM) that displays predominantly the desired estrogenic potential for human health and therapeutic benefits [6–10]. However, most of the previous studies investigating the potential impacts and desired SERM-like properties of puerarin have focused intensively on the reproductive toxicology and physiology [6–9]. In contrast, very little is known about the estrogenic potency of puerarin in the pituitary, which has been implicated in the reproductive neuroendocrine axis function [11], especially in the rat model of postmenopausal females. It has also been documented that both ER subtypes and truncated products of ERs are highly expressed in the rat pituitary [11–15], and have been shown to mediate the estrogenic activity of reproductive steroid

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hormones and, potentially, SERM on the pituitary function [11,12,15–17]. Although in a previous study puerarin is shown to have no estrogenic effects on the pituitary luteinizing hormone (LH) gene expression and serum LH levels [8], the analysis of the mRNA expression of the other estrogen-regulated genes and ERs in the pituitary of this rat model has not yet been clearly elucidated for the estrogenic or antiestrogenic potential of puerarin. Therefore, the main aim of the current project was to shed new light on the possible effects of puerarin on the pituitary estrogen-responsive gene expressions in the mature ovariectomized rat, which has been validated and widely accepted as a clinical animal model of ovarian-derived estrogen-deficiency or postmenopause in the adult woman, and to determine the biological action of the isoflavones [6–9]. To test our hypothesis, in the present experiment, 17β-estradiol benzoate (E2B) was used as the positive control and the animals were long-term administered estrogen-active compounds *via* phytoestrogen-free dietary supplementation. Uterine weight and plasma levels of prolactin (PRL) and growth hormone (GH) were also included to get a better understanding of the potential estrogenic effects of puerarin.

2. Materials and methods

2.1. Test chemicals

Puerarin (PubChem CID: 5281807: IUPAC Name: 7-hydroxy-3-(4-hydroxyphenyl)-8-[(2*S*,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one; Molecular formula: $C_{21}H_{20}O_9$, purity 98%) was obtained from Changzhou Dahua Import and Export Group, PR China. 17beta-estradiol-3-benzoate (PubChem CID: 222757: IUPAC Name: [(8*R*,9*S*,13*S*,14*S*,17*S*)-17-hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthren-3-yl]benzoate: Molecular formula: $C_{25}H_{28}O_3$, purity 98.5%) was purchased from Sigma-Aldrich Chemicals GmbH, St. Louis, USA.

2.2. Animal maintenance and treatments

Animal maintenance and treatments complied with the ARRIVE guidelines and were carried out in accordance with the EU directive 2010/63/EU for animal experiments. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Mahasarakham University (IACUC-MSU) and University Medical Center Göttingen according to the German animal welfare regulations with the permit issued in Braunschweig (No. 509.42502/01-36.03).

Two-month-old female virgin Sprague-Dawley rats (Faculty of Science, University Medical Center, Georg-August University of Göttingen, Germany) were housed (5–6 animals per Makrolon® cage type IV) in a humidity and temperature controlled room with a 12-h light, 12-h dark cycle (lights on from 06:00 a.m. to 18:00 p.m.) at 22–24 °C, 50–55% average relative humidity and air change per hour of 16 h^{-1} at the Animal Research Unit, Faculty of Medicine, University Medical Center, Georg-August University of Göttingen, Germany. As the original laboratory rat diet was soy-based, and thus contained significant amounts of major soy-derived phytoestrogens, daidzein and genistein [18], all of the animals were fed with a special phytoestrogen-free formulated rat chow (Ssniff Spezialdiäten GmbH, Soest, Germany) throughout the maintenance and treatment periods and had water *ad libitum*.

At three months of age, normal intact cycling rats with at least two consecutive regular estrus cycles were bilaterally ovariectomized under Xylazin (Rompan® 2%, Bayer, Germany) and Ketamin (Ketavet®, Pfizer, Germany) anesthesia with a dose of 0.00225 g Xylazin and 0.01875 g Ketamin per rat. Immediately after ovariectomy, 58 rats were weighed (mean body weights 243.2 ± 1.337 g) and randomly divided into one negative control group, *i.e.*, vehicle-treated control (n = 12 rats per group); two positive estrogenic reference control groups, *i.e.*, E2B low and E2B high (n = 12 rats per group); and two treatment groups, *i.e.*, puerarin low and puerarin high (n = 11 rats per group). In the vehicle control group, the rats received a phytoestrogen-free diet without test chemicals. In the E2B low and high groups, animals were fed with the special phytoestrogen-free diet containing 0.0043 g and 0.0173 g E2B per kilogram diet, respectively. In the puerarin low and high groups, rats received the special phytoestrogen-free rat chow supplemented with 0.6 and 3 g puerarin per kilogram diet, respectively. The treatment period was 12 weeks. Animal and food weights were determined twice weekly, and pathological symptoms were recorded daily. The low dose of E2B was chosen because our previous studies demonstrated that estrogenic effects on the expression pattern of pituitary and uterine estrogen-sensitive genes were observed in mature gonadectomized female rats, and circulating E2 levels were within the physiological range observed during a regular estrus cycle [8,19–21]. The high dose of E2B was selected based on previous data to evaluate the estrogenic potencies of E2B at a pharmacologically relevant dose [19,20]. The doses of puerarin were the levels at which the apparent estrogen-like actions could be observed at the uterus and vagina levels in adult ovariectomized rats [6–9].

2.3. Necropsy, collection of blood and target organs, and measurement of organ weights

At the end of the 12 week treatment period, animals were rapidly exposed to CO_2 asphyxiation and then decapitated. To minimize the effects of inter-individual and time variations, necropsy and euthanasia were carried out across five groups of rats, and trunk blood was collected between 08:00 a.m. and 12:00 a.m. and kept in a normal fridge for a few hours. Serum was prepared using a refrigerated microcentrifuge at the controlled temperature of 4 °C and the centrifugal speed of 2500 rounds per minute for 30 min. Serum samples were then stored at −20 °C until hormonal analysis. The anterior pituitaries were immediately collected, then snap frozen in liquid nitrogen and stored at −80 °C for further mRNA expression analysis. The uteri were also dissected, and the associated tissue and fat were removed. After weighing and recording, the uteri were immediately frozen in liquid nitrogen and stored at −80 °C.

2.4. Hormone analysis

Circulating concentrations of PRL and GH in the rat sera were measured using the specific radioimmunoassay supplied by the National Hormone and Pituitary Program of the National Institutes of Health and the National Institute of Diabetes and Digestive and Kidney Diseases (Baltimore, Maryland, USA) as described previously [17,22].

2.5. Total RNA extraction for the homogenized pituitary tissue

The dissected pituitary tissue from each animal in the five groups was cut into small pieces and placed in a 2-ml microcentrifuge tube containing 500 μl lysis buffer of the RNeasy Mini Kit (Qiagen, Hilden, Germany) using a new and sharped hypodermic needle. Tissue homogenization was performed on ice for 10 s using a single-cell ultrasonic Sonifier® Cell disruptor B-12 (Branson Sonic Power Company, Danbury, CT, USA) as described previously [17,22].

The extraction of the total RNA was conducted using the RNeasy Mini Kit according to the manufacturer's handbook. RNase-Free DNase I Set (Qiagen, Hilden, Germany) was used following the instructions of the manufacture. The total RNA concentration was measured using an Eppendorf BioPhotometer (Hamburg, Germany) at the wavelengths of 260 and 280 nm. After determination of the total RNA concentration, the samples were adjusted to a 20 ng/μl final concentration utilizing RNase-free water and kept at −80 °C for gene expression analysis.