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Puerarin exhibits weak estrogenic activity in female rats

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ABSTRACT

A weak estrogenicity of puerarin on reproductive organs was addressed in female rats. In short-term treatment, immature ovariectomized rats were injected with 0.7 mg/kg BW/day of puerarin, for 14 days. Puerarin did not increase uterus weights, endometrium and myometrium areas, and the percent of cornified cells (%Co), but it increased the number of uterine glands. In long-term treatment, mature rats were injected with 7.0 mg/kg BW/day of puerarin for 140 days. Puerarin did not increase uterus weights, endometrium and myometrium areas, and the number of uterine glands, but a significant increase in the %Co was observed from day 98 onwards.

1. Introduction

Pueraria mirifica Airy Shaw et al. Suvatabhandu (Leguminosae), known in Thai as white Kwao Krua, has long been used by Thai people as a traditional medicine for age-rejuvenation. Recently, the estrogenic activity of *P. mirifica* has been evaluated on various animal species and organ systems in both sexes, including the reproductive organs in mice [1,2], rats [3–5], monkeys [6–12], and humans [13], on bones in rats [14,15] and monkeys [7] and on breast cancer in rats [16]. Isoflavone, a class of phytoestrogen chemicals, is the main substance exhibiting estrogenic activity that is found in *P. mirifica*. The isoflavone contents in *P. mirifica* tuberous roots vary widely between locations [17], between seasons [18], and even between roots of the same plant [19]. However, puerarin (daidzein 8-C-glucoside) has always been one of the major isoflavone phytoestrogens isolated from the *P. mirifica* roots [17–19]. In addition to *P. mirifica*, puerarin has also been found at relatively high proportions in the related *P. lobata* [20,21].

The estrogenic activity of puerarin has been widely tested on the bones [22], cardiovascular [23] and nervous systems [24] of mammals. However, the effects of puerarin on the reproductive organs have not been clarified. Rather the results of currently reported studies are controversial; the feeding of puerarin at doses of 150–600 mg/kg BW/day for 5–9 days increased the uterus weight in ovariectomized mature rats and immature female mice, but no obvious effect in mature female mice was observed [25], whilst when the puerarin was mixed with a soybean-free rodent diet at a dose of 600 or 3000 mg/kg of diet (or 10.49 and 48.24 mg/rat/day, respectively) there were no discernable effect on the expression of either the pituitary LH levels or the serum LH levels of ovariectomized mature rats [26].

Although *P. mirifica* contains a relatively high proportion of puerarin, it is not clear whether puerarin is the major substance of *P. mirifica* that exhibits the estrogenic effect. The content of puerarin in *P. mirifica*, as analyzed by HPLC, ranges within 53.20–870.50 μg/ g of *P. mirifica* powder [5,15,17,19], yet the doses of puerarin tested in rodents [25,26] were far above that contained in the effective dose of *P. mirifica* on vaginal cornification and increased uterus weight, that is, 100 mg of *P. mirifica* powder/kg BW of rat or 5.32–87.05 μg of puerarin/kg BW of rat [3–5,17]. It is, therefore, necessary to

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determine if puerarin does have any estrogenic activity on the female reproductive organ and what the effective dose is. The estrogenic activity was evaluated in terms of any change in the uterus weight and histological appearance (uterotropic assay), and in vaginal cell proliferation (vaginal cornification assay). Uterotropic, and vaginal cornification assays in particular, are sensitive, simple and inexpensive methods to predict the estrogenic activity of synthetic estrogens as well as phytoestrogens [4]. The present study aimed to determine the estrogenic activity of short-term and long-term treatment of puerarin at doses of 10 and 100 times higher than those found in *P. mirifica* in ovariectomized and intact female rats, respectively.

2. Materials and methods

2.1. Animals

Immature and mature female Wistar rats aged 21 and 45 days, respectively, were purchased from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were housed in stainless steel cages with sawdust bedding at five animals/cage in a room with controlled lighting (lights on 0600–1800 h) and temperature (25 ± 1 °C) at the Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. The animals were fed with soybean-free rat diet (C.P.082/SBF; S. W. T., Co., Ltd., Samutprakarn, Thailand) and water *ad libitum*. The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University (Protocol Review No. 0823008).

2.2. Experimental procedure

2.2.1. Short-term treatment of puerarin in immature female rats

Twenty immature female rats, within the weight range of 75–80 g and showing a diestrous phase (leukocyte cells), were selected and bilaterally ovariectomized. This day was designated as day-1 of the study period. The rats were then kept for a four day recovery period (approximately 1 estrous cycle) before being submitting to the treatment. The experimental schedule was separated into three periods; pre-treatment, treatment and post-treatment, each for 7, 14 and 14 days, respectively. Rats were divided into four groups (five rats in each group). Three groups of rats were injected daily s.c. with one of 0.2 ml of distilled water (DW), 0.2 ml of 0.7 mg/kg BW/day of estradiol valerate (E_2) or 0.2 ml of 0.7 mg/kg BW/day of puerarin. The fourth group of rats was gavaged daily with 0.7 ml of 100 mg/kg BW/day of *P. mirifica* (PM) suspension during the treatment period. Treatments were performed at 0900–1000 h. At the end of post-treatment period, rats were euthanized under ether, and then the uterus, liver, kidney and spleen were dissected, weighed and then fixed in 10% (w/v) neutral buffered formalin solution and manipulated for histological examination, as described previously [2]. The endometrium and myometrium areas of the uterus were determined. The number of the uterine glands was also counted. The vaginal cytology was checked daily at 0800–0900 h as described previously [4].

2.2.2. Long-term treatment of puerarin in mature female rats

Twenty mature female rats, weighing 120–150 g, with a regular estrous cycle (4–5 days) for at least three consecutive cycles were selected for this study. They were randomly divided into two treatment groups (10 rats in each group), and injected daily s.c. with 0.2 ml of distilled water (DW) or 0.2 ml of 7.0 mg/kg BW/day of puerarin for 140 days. At the end of the treatment period, rats were euthanized under ether, and then submitted to the study following the same protocol as the short-term treatment above.

Throughout the experimental periods all rats in the short-term (2.2.1) and long-term treatments (2.2.2) were weighed once a week.

2.3. The preparation of a P. mirifica suspension and doses of puerarin selected

The tuberous roots of *P. mirifica* Wichai-III used in this study were collected from Chiang Dao District, Chiang Mai Province, Thailand. A voucher specimen of *P. mirifica* (No. BCU 11405) is deposited at the herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University [18]. The *P. mirifica* roots used throughout this study were the same lot, and preparation of the 100-Mesh powder was as described previously [4].

Based upon the four facts that; (i) the puerarin analyzed in *P. mirifica* cultivar Wichai-III is approximately 0.07 mg/ 100 mg of *P. mirifica* powder [19], (ii) *P. mirifica* at the dose of 100 mg/kg BW increased the uterus weight and vaginal cornification in ovariectomized mature rats [4,5], (iii) puerarin could be partially hydrolyzed by cleaving a C-glycosyl bond to daidzein (aglycone form) by rat and human intestinal bacteria [27–29], and (iv) puerarin has a poor solubility, hydrophilicity and liposolubility which cause a limitation of absorption after oral administration [31], the dose of puerarin chosen for this study was 0.7 and 7.0 mg/kg BW/day, equivalent to 10 and 100 times of that contained in 100 mg of *P. mirifica* [15,19], and the route of administration is by injection.

2.4. The preparation of estradiol valerate and puerarin solution

Estradiol valerate (95% purity, Sigma, USA) was weighed and dissolved in a small volume of absolute ethanol. After the powder was completely dissolved, the olive oil was added and the solution was allowed to stand at room temperature to evaporate the ethanol. This stock solution was then diluted with olive oil to give a final dose of 0.7 mg/kg BW/day/0.2 ml olive oil.

Puerarin (96% purity, LKT Laboratories, Inc., USA) was weighed and dissolved in a small volume of absolute ethanol. After the powder was completely dissolved, the peanut oil was added. This stock solution was then diluted with peanut oil to give final doses of 0.7 and 7.0 mg/kg BW/day.

The stock solutions of estradiol valerate and puerarin were kept in dark bottles at room temperature until used.

2.5. Histological analysis

After overnight fixation in formalin, the ovary and uterus were dehydrated in a series of ethanol gradients,