—Full Paper—

Assessment of Fertility and Reproductive Toxicity in Adult Female Mice after Long-Term Exposure to *Pueraria mirifica* Herb

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Abstract. The present study investigated the effects of long-term administration of *Pueraria mirifica* (PM) at non-toxic doses on the ovarian function and fertility of adult female mice based on evaluation of hematological and biochemical parameters [1]. Female mice were divided into 4 groups (36 mice/group). Groups 1–3 were orally treated with a dose of 0 (PM-0), 10 (PM-10) or 100 mg/kg BW/day PM (PM-100), and group 4 was subcutaneously injected with 200 µg/kg BW/day of synthetic estrogen diethylstilbestrol (DES). The treatment schedule was separated into treatment and post-treatment periods. The duration of each period was 8 weeks. The PM-10 mice exhibited regular estrous cycles, while the PM-100 and DES treatments induced prolonged estrous cycles. Although no changes were observed in the uterus and ovary weights of the mice after the PM-100 and DES treatments, hyperplasia of the uterine endothelium and a decrease in the number of growing ovarian follicles were detected. The changes in the ovarian histologies of the PM-100 and DES mice were related to reductions in the levels of LH and FSH, which subsequently caused a decrease in mating efficiency. Once the PM mice were able to copulate, they were capable of successfully becoming pregnant and mothering offspring. No abnormalities were observed in the external morphologies and reproductive organ weights of the 50-day-old offspring. In conclusion, our results suggest that long-term exposure to 100 mg/kg BW of PM has adverse effects on the mating efficiency and reproduction of adult female mice and that administration of 10 mg/kg BW of PM does not induce any changes in the hypothalamic-pituitary-ovarian-uterine axis.

Key words: Diethylstilbestrol (DES), Female mice, Fertility, Phytoestrogens, *Pueraria mirifica*
(J. Reprod. Dev. 53: 995–1005, 2007)

Traditional use of medicinal plants to treat different sorts of diseases, such as reproductive and

fertility-related diseases, is widespread throughout the world, especially in Asian countries. In fact, Dangui in China, Dangui and Kudzu in Korea, Auravedi in India and Jamu in Indonesia all traditionally use medicinal plants for the purposes above. Plants containing phytoestrogens are of

Accepted for publication: May 7, 2007

Published online: June 22, 2007

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long-term interest. *Pueraria mirifica* (PM) is an indigenous herb and known in Thai as White Kwao Krua. It belongs to the family *Leguminosae*, subfamily *Papilionoideae* [2]. Its tuberous root is known to contain at least the following 13 phytoestrogens: daidzin, daidzein, genistin, genistein, deoxymiroestrol, miroestrol, β -sitosterol, stigmasterol, coumestrol, puerarin, mirificoumestan, kwakhurin and mirificin [3–5]. Thus, verification of the estrogenic effects of *P. mirifica* in various species of experimental animals has been conducted, especially in the recent years.

The estrogenic activity of miroestrol isolated from PM was first determined in female rats [3, 6]. Miroestrol increases uterus weight, vaginal growth and the amount of vaginal fluid in intact rats and induces mammogenic effects in ovariectomized rats. Feeding PM powder suspended in water to ovariectomized rats induces proliferation of the vaginal epithelium and uterus endometrium and reduces the levels of serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [7, 8]. Reduction of the levels of FSH and LH has also been observed in adult cyclic and aged menopausal female monkeys [9–12]. PM suppresses folliculogenesis and ovulation in adult cyclic female monkeys after a single or long-term feeding [9, 10]. Intake of crude *P. mirifica* powder can relieve climacteric symptoms, such as hot flashes, frustration, sleep disorders and skin dryness in post-menopausal women [13].

Because of its popular use, the toxicity of PM has also been evaluated for various organs, such as the liver, spleen and kidney. Evaluation of powder and extract from the plant has shown that it has no toxic effects on the eyes and skin of rodents and rabbits [14]; on the other hand, it can stimulate reddening of sex skin in aged menopausal monkeys [15]. The toxicity of PM at doses higher than 100 mg/kg BW has also been tested in rats [1, 16]. It causes anemia symptoms and mutagenicity in subjects. Mutagenicity has also been observed for doses higher than 100 mg/kg BW [16]. In a previous study, acute and subchronic toxicity tests in rats using doses of PM of 10 and 100 mg/kg BW/day did not result in any abnormalities of hematological and biochemical parameters or any changes in the histopathology of the metabolic organs [1]. The authors of that study suggested that doses of 10 and 100 mg/kg BW/day of PM are safe for human consumption on a kg BW basis. Without evaluation

of the toxicity of PM in relation to the reproductive system and fertility, these results should not be considered sufficient to suggest that general use of PM is safe. Thus, the aim of the present study was to investigate this in adult female mice. For this study, we conducted a comparative investigation to determine whether PM can affect the fertility and reproductive system of female mice after long-term treatment at doses of 10 and 100 mg/kg BW/day and to examine the malformations in F1 offspring delivered by the PM-treated mothers.

Diethylstilbestrol (DES) was used as a positive control in order to clarify whether PM has effects similar to those of estrogen. DES is a synthetic estrogen that has a higher binding affinity to estrogen receptor (ER) both the α and β subtypes, and a higher potency of estrogenic biological activity than estradiol [17, 18]. Our previous study found that a dose of 200 μ g/kg BW/day of DES reduced the weights of the testes, epididymides and seminal vesicles of adult male mice after 8 weeks of treatment. Furthermore, the DES-treated mice had no spermatozoa in their seminiferous tubules and epididymides and had reduced levels of LH, FSH and testosterone. None of the DES-treated mice mated with or impregnated any female mice, and therefore a dose of 200 μ g/kg BW/day of DES was chosen for the present study [19].

Materials and Methods

Plant materials

Pueraria mirifica cultivar Wichai-III was collected in Chiang Mai Province, Northern Thailand. The voucher herbarium specimen of the PM (no. BCU 11045) was deposited at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The constituent phytoestrogens of the Wichai-III cultivar, as determined by HPLC technique, have been described previously [7]. The tuberous roots used throughout this study were from the same lot. The roots were sliced and dried at 70–80 °C and pulverized in a mortar. The powder was filtered through a 100-mesh size and stored in desiccators until used. The PM powder was suspended in sterile distilled water before use.

Chemicals

Diethylstilbestrol (DES) and corn oil were

obtained from Sigma-Aldrich (St. Louis, MO, USA). DES was diluted with corn oil and mixed vigorously prior use.

Animals

Adult ICR mice, 50–60 days old and weighing 25–27 g, were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were housed 5 animals/cage in a room with controlled lighting (lights on 0600–2000 h) and temperature (25 ± 1 °C) at the Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. The animals were fed a rat diet (Pokphand Animal Feed, Bangkok, Thailand) and water *ad libitum*. They were habituated by handling regularly for 2 weeks before the experiments. They were checked daily for estrous cyclicity by examining their vaginal cytology. The mice selected had at least 3 consecutive regular estrous cycles of 4–5 days in length before the onset of this study. All experiments were performed between 0800–1100 h. The experimental protocol was approved by the Animal Ethical Committee in accordance with the Guide for Care and Use of Laboratory Animals prepared by Chulalongkorn University, Bangkok, Thailand.

Experimental design

The adult female mice were divided into 4 groups (36 mice/group). Group 1–3 mice were orally treated with 0, 10 or 100 mg/kg BW/day of PM suspended in 0.2 ml distilled water (PM-0, PM-10 and PM-100, respectively). Group 4 mice were subcutaneously injected with 0.2 ml of 200 µg/kg BW/day of synthetic estrogen DES. The body weights of the mice were measured each day and were used to adjust the concentration of PM and DES. The treatment schedule was separated into treatment and post-treatment periods. The duration of each period was 8 weeks. Blood samples were collected from the mice for the LH and FSH assays by cardiac puncture during the proestrous stage if they exhibited a regular estrous cycles or at any stages if they exhibited an irregular estrous cycles; the samples were collected in 4-week intervals for a total of 5 samples (at 0, 4, 8, 12 and 16 weeks). Six female mice were paired with the fertile male mice (3 females per male) after collection of each blood sample, except at 0 weeks. After mating, the female mice were removed from the treatment schedule. Six mice from each group were

selected and decapitated after euthanization at the end of the treatment period or at the end of post-treatment period; 12 mice in total. After necropsy, ovaries and uteri were dissected, weighed and preserved in 10% formalin buffer for histological examination.

The female mice removed after mating were observed for pregnancy and delivery. The copulation rates, pregnancy rates and numbers of offspring delivered and malformations were examined. The number of litters was counted, and the offspring were reared until they reached 50 days old. The offspring were weaned at 21 days of age. External morphologies, such as hair loss and abnormalities in ears, fingers, toes and tails, were observed. The offspring were decapitated after euthanizing at 50 days of age; their gonads and accessory genital glands were then dissected and weighed.

Vaginal smear checks

Vaginal smears were checked daily between 0800–0900 h as described previously [8]. The vaginal epithelium cells were examined under a microscope and classified into the following 3 types: leukocyte cells, nucleated cells and cornified cells. The estrous cycle was categorized into the following 3 stages based on cell type: proestrus (P), estrus (E) and diestrus (D). Cornified cells were used as an indicator of vaginal proliferation.

Mating studies

Six female mice were paired with the fertile male mice (3 females per male) for 1 night from 1800–0600 h every 4 weeks during the experimental period. The criteria for selection of the females were as follows: 1) proestrous mice were chosen if the female mice exhibited regular estrous cycles and 2) mice at any stages of the estrous cycle were chosen if the female mice exhibited irregular estrous cycles. Positive mating was verified either by the presence of copulation or the occurrence of sperm as determined by vaginal smears. The day of positive mating was considered to be the first day of pregnancy. The mated female mice were observed for signs of pregnancy, such as nest building, and delivery after day 20.

Hormonal analysis

The concentrations of serum FSH and LH were measured using NIDDK kits for rat FSH and LH.

The iodination preparations were NIDDK-rat FSH-I-5 and rat LH-I-5. The antisera were anti-rat FSH-S11 and anti-rat LH-S11. The results obtained were expressed in terms of the rat FSH-RP-2 and LH-RP-3 reference standards.

To minimize interassay variation, all samples from each group were run in a single assay for each hormone. The intra-assay coefficients of variation for LH and FSH were 2.24 and 6.34%, respectively.

Histological examination

After the reproductive organs were fixed overnight in 10% formalin buffer, the tissues were dehydrated in a graded series of ethanol and cleared in xylene. They were then embedded and blocked in paraffin, cut into 5- μ m sections and stained with hematoxylin and eosin. Permanent preparations of all collected organs were histologically examined and photographed using a camera (Nikon, Tokyo, Japan) mounted on a microscope (Olympus, Tokyo, Japan).

Statistical analysis

The results were expressed as means $\pm$ SEM. The copulation rate was calculated as the number of copulated females per the total number of females used $\times$ 100. The pregnancy rate was calculated as the number of pregnant females per the number of copulated females $\times$ 100. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) program version 11. The paired-sample *t*-test was used to compare organ weights between the end of the treatment period and the end of the post-treatment period for each group. Comparisons between the control and treatment groups were conducted by one-way analysis of variance (ANOVA) with the factorial or repeated measures designs and post-hoc testing with the least significant difference (LSD) test. *P* values of less than 0.05 were considered to be statistically significant.

Results

General appearance

During this experiment, no deaths or any other clinical signs of toxicity (hair loss, drowsiness or weakness) were observed for the female mice.

Fig. 1. Weights of the uteri (A) and ovaries (B) of female mice treated with 0, 10 or 100 mg/kg BW/day of *Pueraria mirifica* (PM-0, PM-10 and PM-100, respectively) or 200 μ g/kg BW/day of diethylstilbestrol (DES) at the end of treatment period (■) and at the end of post-treatment period (□). Each value represents the mean $\pm$ SEM of six animals. Since the PM-0- and PM-10-treated mice exhibited regular estrous cycles, the mice were decapitated during the proestrous stage. The PM-100- and DES-treated mice exhibited irregular estrous cycles and were decapitated during any stage of the estrous cycle.

Weights of ovaries and uteri

There were no significant differences in the weights of the uteri and ovaries between the PM-0 group and the PM-10, PM-100 and DES groups, respectively (Fig. 1).

Vaginal cytology

Administration of PM-10 did not influence vaginal cornification, and the PM-10 female mice exhibited regular estrous cycles of 4–5 days throughout the experimental period that were the same as those of the PM-0 mice (Fig. 2). Treatment with PM-100 and DES induced cornification of the vaginal epithelium as early as the second day of treatment and maintained the cornified cells (or persistent estrus) until the last day of the treatment period. The mice recovered from treatment and returned to regular estrous cycles 3–4 days after

Fig. 2. Daily monitoring of vaginal cytology for female mice treated with 0, 10 or 100 mg/kg BW/day of *Pueraria mirifica* (PM-0, PM-10 and PM-100, respectively) or 200 μ g/kg BW/day of diethylstilbestrol (DES) during the treatment and post-treatment periods. The first two weeks of data of each period are shown. Each line represents an individual animal. P: proestrus. E: estrus. D: diestrus. The 10 subjects shown for each group are representative of the 36 mice for the treatment period and 12 mice for the post-treatment period; however, the data for the treatment and post-treatment periods (lines at the same horizontal level) came from the same individual mice.

withdrawal of PM-100. In contrast, vaginal cornification lasted for 4–8 days after DES withdrawal, and the mice did not recover or return to a regular estrous cycle until the end of experimental period; only leukocyte cells were found.

Histology of uteri and ovaries

The uterine histology of the PM-10 group did not differ from that of the PM-0 group during the treatment and post-treatment periods (Fig. 3A and B). In contrast, the uteri of the PM-100- and DES-treated mice contained a thicker endometrium and more dilated uterine glands than those of the PM-0 group (Fig. 3C₁ and D₁). The uterine glands of the mice treated with DES also accumulated a lot of secretory material. However, these histological alterations recovered within 8 weeks of withdrawal of PM and DES (Fig. 3C₂ and 3D₂).

There were no morphological differences between the ovaries of the PM-10 group and those of the PM-0 group (Fig. 3F). Edema of ovarian interstitial tissues was observed in the PM-100 and

DES groups. A decrease in primary, secondary and Graafian follicles was also observed in the ovaries of the PM-100- and DES-treated mice (Fig. 3G₁). In addition, the ovaries of the DES-treated mice contained many atretic follicles with some picnotic nuclei (Fig. 3H₁). However, all these morphological changes in the ovary recovered within 8 weeks after withdrawal of PM-100 and DES (Fig. 3G₂ and H₂).

Gonadotropin levels

The serum LH levels of the PM-10 group were significantly higher ($P < 0.05$) than those of the PM-0 group at 8 weeks during the treatment and post-treatment periods, respectively (Fig. 4A). Conversely, the serum LH levels of the PM-100 group were significantly decreased ($P < 0.05$) at 8 weeks during the treatment period. Significant suppression ($P < 0.05$) of the serum LH level was also observed in the DES group throughout the 8 weeks of the treatment period, and recovery was observed within 4 weeks of DES withdrawal.

Fig. 3. Histological comparisons of uteri (A–D) and ovaries (E–H) at the end of the treatment period (1) and at the end of the post-treatment period (2) for mice treated with 0, 10 or 100 mg/kg BW/day of *Pueraria mirifica* (PM-0, PM-10 and PM-100, respectively) or 200 μ g/kg BW/day of diethylstilbestrol (DES). No remarkable lesions were observed in the uteri or ovaries of the PM-10-treated mice. The mice treated with PM-100 and DES exhibited thicker endometria, dilated uterine glands (arrows) and decreased numbers of ovarian growing follicles. The mice treated with DES also exhibited the atretic follicles (arrows) with some picnotic nuclei in the ovary. Hematoxylin and Eosin staining (Scale bars=50 μ m). The day of decapitation was stated in Fig. 1.

The serum FSH levels of the PM-0 group fluctuated throughout the experimental period (Fig. 4B). The fluctuation tended toward increase during the treatment period. Thus, significantly low serum FSH levels were observed at 8 weeks during the

treatment period in the PM-10, PM-100 and DES groups ($P < 0.01$) compared with the PM-0 group.

Fertility

The copulation rate of the female mice treated

Fig. 4. Serum LH (A) and FSH (B) levels of female mice treated with 0 (—●—; PM-0), 10 (---■---; PM-10) or 100 (---▲---; PM-100) mg/kg BW/day of *Pueraria mirifica* or diethylstilbestrol (—×—; DES) during the treatment and post-treatment periods. * $P < 0.05$ vs. the basal level at 0 weeks. # $P < 0.05$ vs. PM-0. Each value represents the mean $\pm$ SEM of six animals. The day of decapitation was stated in Fig. 1.

with PM-10 tended to be similar to that of the PM-0 group (Table 1). Although the copulation rate of the PM-10 mice was higher than that of the PM-0 group at 8 weeks during the treatment period (83.33 vs. 50.00% for PM-10 vs. PM-0), the results for these two groups at this time point could not be compared because the copulation rate of the PM-0 mice at this time point was abnormally lower than at other time points. One explanation for the low pregnancy rate for the PM-0 mice is that all of the tested female mice used in this study were virgin females, which means that none had experienced pregnancy before, and only six individuals were

tested during each period.

In contrast, PM-100 drastically reduced the copulation rate at 4 weeks (33.33%) and 8 weeks (0%) during the treatment period. However, the reductions recovered within 4 weeks (66.67%) of PM-100 withdrawal. Few of the DES-treated mice, only 1 and 2 mice at 4 and 8 weeks during the treatment period, respectively, mated with the untreated male mouse, and absolutely no pregnancies were observed. Although the DES treatment was stopped for 8 weeks, only 1 of the 6 mice (16.67%) recovered and accepted mating from a male mouse and became pregnant at 4 and 8 weeks during the post-treatment period, respectively. All PM-10 and PM-100 mice became pregnant (100%) and delivered offspring once they were able to copulate with a male mouse, except for the PM-100 mice at 8 weeks during the post-treatment period, which had a pregnancy rate of 75%. On the other hand, the pregnancy rate of the PM-0 group varied from 33.33–80.00 % during both the treatment and post-treatment period; the pregnancy rate of the DES group during the treatment period was 0%.

Average number of offspring and relative reproductive organs weights of litters

The number of offspring mothered by the PM- and DES-treated mice corresponded to the observed copulation and pregnancy rate. The number of offspring mothered by the PM-10-treated mice was not different than that of the PM-0 mothers throughout the experimental period, even though the copulation and pregnancy rates of the PM-10 group were higher at 8 weeks during the treatment period (Table 2). The PM-100 group had fewer offspring at 4 weeks and no offspring at 8 weeks during the treatment period. However, the number of offspring was similar to that of the PM-0 group thereafter. No pregnancies were observed in the DES-treated mice, and no offspring were produced by this group. As mentioned previously, 1 of the 6 mice receiving the DES treatment became pregnant during the post-treatment period and produced offspring. However, the number of offspring delivered by the DES-treated mothers was quite low compared with those of the PM-treated mice (ranging between 8 to 12 offspring).

No external morphological abnormalities were found in the offspring mothered by the PM- and DES-treated mice. The relative organ weights of the ovaries and uteri of the female pups and the tes-

Table 1. Numbers of copulations and pregnancy rates for female mice treated with 0, 10 or 100 mg/kg BW/day of *Pueraria mirifica* (PM-0, PM-10 and PM-100, respectively) or 200 µg/kg BW/day of diethylstilbestrol (DES) after mating with a fertile male mouse

Group	At 4 weeks during the treatment period (n=6)		At 8 weeks during the treatment period (n=6)		At 4 weeks during the post-treatment period (n=6)		At 8 weeks during the post-treatment period (n=6)	
	Copulations	Pregnancies	Copulations	Pregnancies	Copulations	Pregnancies	Copulations	Pregnancies
PM-0	5 83.33%*	4 80.00%	3 50.00%	1 33.33%	4 66.67%	3 75.00%	5 83.33%	4 80.00%
PM-10	4 66.67%	4 100%	5 83.33%	5 100%	3 50.00%	3 100%	3 50.00%	4 100%
PM-100	2 33.33%	2 100%	0 0%	0 NA	4 66.67%	3 75.00%	4 66.67%	4 100%
DES	1 16.67%	0 0%	2 33.33%	0 0%	1 16.67%	1 100%	1 16.67%	1 100%

The first row in each group indicates the number of mice and the second row indicates the percent change.

*The copulation rate was calculated as the number of copulated females per the number of total females used × 100. The pregnancy rate was calculated as the number of pregnant females per the number of total females used × 100. NA=not available.

Table 2. Numbers of offspring mothered by female mice treated with 0, 10 or 100 mg/kg BW/day of *Pueraria mirifica* (PM-0, PM-10 and PM-100, respectively) or 200 µg/kg BW/day of diethylstilbestrol (DES)

Group	At 4 weeks during the treatment period	At 8 weeks during the treatment period	At 4 weeks during the post-treatment period	At 8 weeks during the post-treatment period
PM-0	11.0 ± 1.6	10	8.7 ± 3.1	9.4 ± 2.5
PM-10	10.8 ± 2.0	10.0 ± 2.6	11.3 ± 2.7	10.7 ± 2.2
PM-100	8.5 (8, 9)	0	8.8 ± 2.4	8.5 ± 1.3
DES	0	0	3	5

Numbers of offspring are expressed as means ± SEM, if there were more than three treated females that gave birth.

Table 3. Relative organ weights of 50-day-old offspring mothered by female mice treated with 0, 10 or 100 mg/kg BW/day of *Pueraria mirifica* (PM-0, PM-10 and PM-100, respectively) or 200 µg/kg BW/day of diethylstilbestrol (DES)

Relative organ weight	PM-0	PM-10	PM-100	DES
No. of female offspring	35	40	37	3
BW of female offspring	31.89 ± 0.44	32.28 ± 0.41	31.69 ± 0.37	32.92 ± 0.37
Uterus (mg/g)	6.086 ± 0.025	5.422 ± 0.015	5.094 ± 0.021	4.698 ± 0.104
Ovary (mg/g)	0.751 ± 0.009	0.724 ± 0.011	0.753 ± 0.013	0.665 ± 0.006
No. of male offspring	32	40	37	5
BW of male offspring	35.64 ± 1.00	36.27 ± 0.85	36.46 ± 0.72	37.10 ± 0.85
Testis (mg/g)	7.321 ± 0.014	7.153 ± 0.011	7.244 ± 0.012	6.509 ± 0.019
Epididymis (mg/g)	2.353 ± 0.011	2.415 ± 0.009	2.362 ± 0.011	2.571 ± 0.014
Seminal vesicle (mg/g)	6.382 ± 0.008	6.194 ± 0.010	6.515 ± 0.024	5.929 ± 0.019

Data are expressed as means ± SEM.

tes, epididymides and seminal vesicles of the male pups mothered by the PM- and DES-treated mice were not significantly different from those of pups

mothered by PM-0-treated mice (Table 3). The gross morphology of these reproductive organs was also normal.

Discussion

The tuberous root of PM contains many active phytoestrogen compounds, and its estrogenic activity has been shown in animal experiments and clinical trials [7–9, 13, 20]. Previous studies have practically verified pronounced estrogenic activity in rodents using uterotrophic and vaginal cornification assays [3, 7, 8]. Despite the fact no changes were observed in the uterine weights of intact mice after treatment with PM-100 in the present study, vaginal cornification occurred as early as the second day and persistent estrus was maintained throughout the treatment period. In contrast, it has been reported that treatment of ovariectomized rats with PM-100 significantly increases uterine weight [7, 8]. One explanation for this difference is that ovariectomy lowered the level of endogenous estrogens in the rats and increased the sensitivity of their uterine response to phytoestrogen treatment compared with the intact mice in our study. Although no changes in organ weights were found, observation at the cellular level by histological study showed that there was high proliferation of the uterine endometrium in the PM-100 group compared with the PM-0 group. Our results are in agreement with a previous study reporting that the uterine weights of immature female mice administered subcutaneous injections of naringenin and biochanin A do not increase, although uterine epithelial cell height does increase [21]. Ashby *et al.* [22] and Tinwell *et al.* [23] showed that the uterotrophic effect of coumestrol results from both an increase in uterine fluid content and hyperplasia of the endometrium. Rats treated with coumestrol exhibit a rise in uterine DNA content that mimics that of the estradiol effect [24–26]. The present results clearly indicated that the PM-100 has an estrogenic effect and that it acts directly on uterus; however, the changes in the uterus at the cellular levels were not high enough to increase uterine weight. The mechanism of action is probably through estrogen receptors in endometrium cells [27, 28].

Similarly, the ovaries of the PM-treated mice exhibited clear edema of the interstitial tissues, and their ovarian weights were unchanged. Furthermore, histological analyses of ovarian tissues indicated a decrease in ovarian folliculogenesis, that is, a reduction in primary, secondary and Graafian follicles. FSH and LH are key drivers of the

mechanism of follicular development [29]. LH is essential for the final growth of antral follicles, and FSH stimulates small growing follicles. FSH together with insulin-like growth factor-I (IGF-I) and estradiol stimulate proliferation and differentiation of granulosa cells [30, 31]. The results of a previous study showed that a dose of 100 mg/kg BW/day of PM orally administered to ovariectomized rats can reduce the elevation of circulating FSH and LH levels [7]. In the present study, treatment with PM-100 maintained lower levels of FSH and LH than treatment with PM-0. Therefore, arrest of follicular development may have occurred.

The present results clearly demonstrated that long-term exposure to PM-100 induced adverse effects on female mice fertility. That is, the PM-100-treated mice exhibited persistent estrous, and their subsequent mating efficiency after treatment for 4 weeks was partially reduced; it was completely inhibited by treatment for 8 weeks. These results suggest that treatment with PM-100 affects the estrous cycle and mating behavior of female mice and subsequently results in reduced pregnancy rates and litter sizes. Previous studies have shown that treatment with soy supplement and tamoxifen (antiestrogen) suppresses lordosis in estrogen- and progesterone-primed female rats [32–35]. As mentioned above, treatment with PM-100 reduced the serum FSH and LH levels of the mice, and we suspected that there was a subsequent reduction in the estrogen level, although we did not determine the level of this hormone in the current study. Observation of reductions in the numbers of primary, secondary and Graafian follicles in the ovaries of the PM-100 group support this hypothesis. However, reductions in reproductive efficiency recovered as early as 4 weeks after PM-100 withdrawal. On the other hand, reproductive impairments lasted for 8 weeks after DES withdrawal. Although the DES-treated mice exhibited complete recovery of reduced gonadotropin levels and alterations of their uteri and ovaries, only one of the mice accepted mating from a male mouse and produced offspring. Our previous study also demonstrated that recovery of the vaginal cytology of rats after receiving a synthetic estrogen, estradiol valerate, is slower than for those receiving PM containing phytoestrogens. One explanation for this is that phytoestrogens exhibit less binding affinity to both ER α and β , which can be found in large quan-

ties in reproductive organs, whereas synthetic estrogens exhibit high binding affinity [27, 28]. A previous study concluded that this was an advantage of use of a phytoestrogen-rich herb for hormone replacement therapy compared with synthetic estrogens when a short-term effect is needed [8]. However, no abnormalities were observed in the external morphologies and reproductive organ weights of the 50-day-old offspring from the PM- and DES-treated mice, which was in agreement with previous reports concerning other phytoestrogens [36, 37].

In conclusion, long-term exposure to 100 mg/kg BW of PM has adverse effects on mating efficiency and reproduction in adult female mice, whereas administration of 10 mg/kg BW of PM to rats does not induce any changes in the hypothalamic-pituitary-ovarian-uterine axis [8, 9]. However, impairment of mating efficiency and reproduction after treatment with PM recovered faster than after treatment with synthetic estrogen. Thus, the present results suggest that a dosage of 100 mg/kg BW of PM, which has been reported to have no

effect on hematological and biochemical parameters and has been suggested to be safe for human consumption on a kg BW basis, can induce reproductive impairments after long-term treatment. The dosage and duration of PM exposure must be considered depending on the purpose of usage.

Acknowledgments

We are grateful to Dr. A. F. Parlow and the Rat Pituitary Hormone Distribution Program (NIDDK, NIH, Torrance, CA, USA) for providing RIA materials. This study was supported in part by the Inter-department of Physiology, Faculty of Graduate School, Chulalongkorn University, a grant for the Primate Research Unit, Chulalongkorn University, and Grants-in-Aid for Scientific Research (The 21st Century Center of Excellence Program, E-1) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and B-18310044 and the Japan Thailand Joint Research from the Japan Society for the Promotion of Science.

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