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Effects of *Pueraria mirifica*, an Herb Containing Phytoestrogens, on Reproductive Organs and Fertility of Adult Male Mice

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The effects of *Pueraria mirifica* (PM) on reproductive organs and fertility of adult male mice were investigated. Male mice were divided into four groups (10 mice/group). Groups 1–3 were orally treated with PM at doses of 0 (PM-0), 10 (PM-10), and 100 (PM-100) mg/kg BW/d in 0.2 mL distilled water, and group 4 was subcutaneously injected with 200 µg/kg BW/d of synthetic estrogen diesthylstilbestol (DES). The treatment schedule was separated into two periods: treatment and posttreatment (8 wk for each period). The PM-10 and PM-100 treatments had no effect on testicular weight, sperm number, and serum LH, FSH, and testosterone levels. Only the PM-100 treatment reduced weights of epididymes and seminal vesicle and the sperm motility and viability. Histopathological examination demonstrated that testis, epididymis, and seminal vesicle were normal in all doses of PM treatment. PM-treated males showed no alterations in mating efficiency and on causing pregnancy of their female partners. DES injection impaired all those parameters. Offspring fathered by the PM- and DES-treated males exhibited neither malformations nor change of body weight gains, and the reproductive organ weights of 50-d old pups were in the normal range. The present data clearly demonstrate that a long-term treatment of PM at doses 10 and 100 mg/kg BW/d, via oral route, does not alter a male fertility and a hypothalamus–pituitary–testis axis. Although PM-100 can cause some moderate impairment, no persistent effects were observed. Most of PM-treated mice increased the mating efficiency after stop treatment.

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Key Words: Diethylstilbestrol; male mice; *Pueraria mirifica*; gonadotropins; sperm quality; testis.

Introduction

Many current studies have been conducted to evaluate the actual efficacy and adverse effects of plants on reproductive organs and functions *(1–3)*. *Pueraria mirifica* (PM) is one indigenous herb among those plants. PM, known in Thai as White Kwao Krua, belongs to the family *Leguminosae*, subfamily *Papilionoideae* or soybean and pea subfamily *(4)*. Its tuberous root accumulates at least 13 known phytoestrogens: daidzin, daidzein, genistin, genistein, deoxymiroestrol, miroestrol, β-sitosterol, stigmasterol, coumestrol, puerarin, mirificoumestan, kwakhurin, and mirificin *(5–7)*. Thus, the effects of PM on the reproductive system have been throughly examined in female Japanese quails *(8)*, ovariectomized mice *(9)*, gonadectomized rats *(10,11)*, adult and aged female monkeys *(12–14)*, and women *(15)*. Mostly, the effects of PM on reproductive organs have been conducted in female animals or in women. Probably, it is because PM has been widely used in women as age rejuvenation drugs and cosmetics. However, in the recent years, its use has become popular in men as well.

We previously evaluated the effects of PM at doses of 10–1000 mg/kg BW/d on changes of reproductive organs and gonadotropin levels in ochidectomized male rats after 14 d of feeding and found that only LH levels were decreased after 1000 mg/kg BW/d of the PM treatment *(11)*. However, the effects of long-term treatment of PM on hormone-related reproductive function and fertility in adult males have not been studied thoroughly. Considering the growing popular use of PM in men and the presence of estrogen-like substances in its constituents, we aim to study the effects of PM plant on reproductive organs, testosterone and gonadotropin levels, and fertility of adult male mice after an 8-wk treatment.

Results

Clinical Signs

No evidence of overt toxicity and abnormal clinical signs were observed in any of the groups during the experimental period. The mortalities of animals were not appeared as well.

Body Weight and Weights of Testes, Epididymes, and Seminal Vesicles

There were no significant differences in body weight between the PM-0 group and the PM-10, PM-100, and DES groups, respectively (Fig. 1). The testes weights were not significantly different between the control and PM-treated mice (Fig. 1), except for the decrease in PM-100 treated mice during the post-treatment period. Significant increase in the seminal vesicle weights ($p < 0.05$) was observed in PM-10 at post-treatment period, while no changes in epididymes weights were found. Significant reduction of the weights of epididymes ($p < 0.05$) and seminal vesicles ($p < 0.05$) was observed in the PM-100 group at treatment period compared to PM-0 group. Likewise, weights of testes, epididymes, and seminal vesicle in DES group at treatment period were lower than those of the PM-0 group ($p < 0.01$), whereas at the post-treatment period only testes weights were significantly lower ($p < 0.05$).

Histology of Testes, Epididymes, and Seminal Vesicles

Considering the testes of the PM-0 group, the compartmentalization of germ cell within the seminiferous tubules was observed. Spermatozoa were found in normal sized lumens of testes during treatment and post-treatment periods (Fig. 2, A1, A2). PM-10 and PM-100 groups showed no gross morphological changes (Fig. 2, B1, B2, C1, C2). The normal cytoarchitecture and compartmentalization of germ cells within the seminiferous tubules during treatment and post-treatment periods were maintained as in the control. Spermatozoa were found in both PM-10 and PM-100 groups. However, abundant spermatozoa in the seminiferous tubules were found at post-treatment period in PM-10 group, and more condensed than those of the PM-0 group (Fig. 2, B2). DES-treated mice showed a thin layer of spermatogenic lineage, an absence of spermatid and spermatozoa, a few spermatogenic cells, and an evidence of Leydig cells hypertrophy (Fig. 2, D1). However, all these histological alterations could be partially recovered within 8 wk after DES withdrawal (Fig. 2, D2).

Judging the epididymes of the PM-0 group, the tubules are lined with pseudostratified columnar epithelium (Fig. 2, E1, E2). Long cytoplasmic processes or the stereocilia projected into the lumen. The lumen was filled with spermatozoa. The PM-10 and PM-100 groups showed no gross morphological changes of epididymis (Fig. 2, F1, F2, G1, G2). The normal cytoarchitectures and a number of spermatozoa within the tubules were maintained as in the PM-0 group. DES-treated mice showed an absence of spermatozoa in the lumens (Fig. 2, H1); however, the histological alteration was recovered within 8 wk after DES withdrawal (Fig. 2, H2).

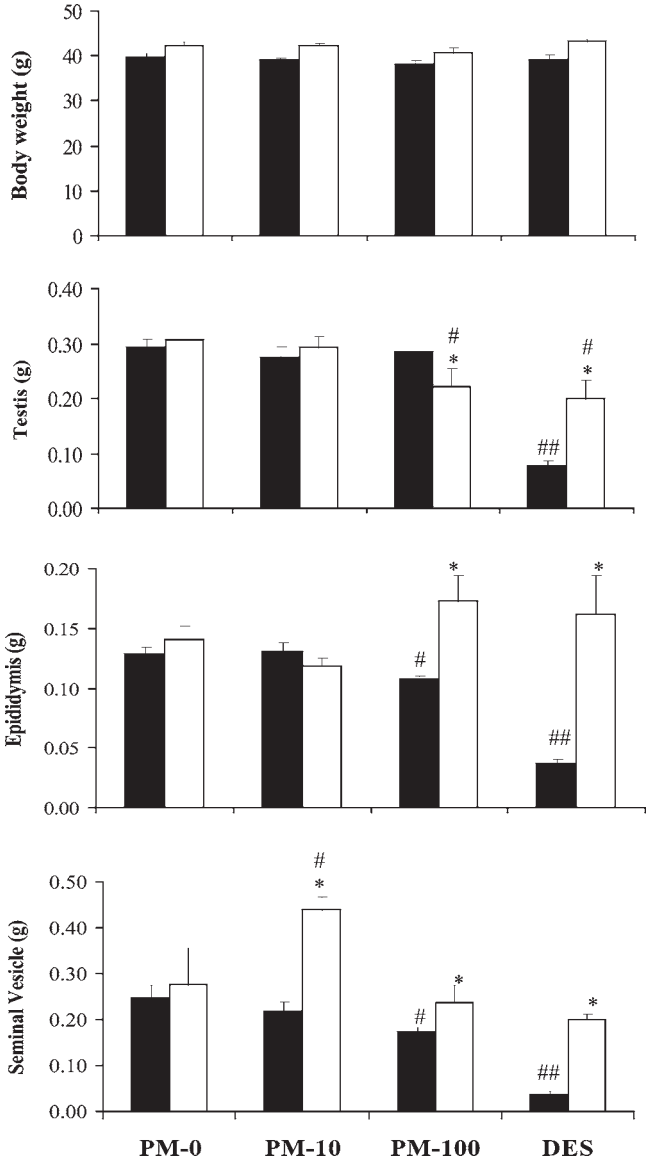

Fig. 1. Body weight and weights of testes, epididymes, and seminal vesicle of male mice treated with *Pueraria mirifica* (PM) at doses 0, 10, 100 mg/kg BW/d (PM-0, PM-10, PM-100) and diethylstilbestrol (DES) 200 μg/kg BW/d at the end of treatment (black column) and post-treatment periods (clear column). Each value is expressed as the mean ± SEM of five animals. $^{*}p < 0.05$ vs treatment period, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs PM-0.

Considering the seminal vesicle of the PM-0 group, the lumen was lined by a tall columnar secretory epithelium and filled with the secretion material (Fig. 2, I1, I2). PM-10 and PM-100 groups showed normal for both gross morphology and cytoarchitecture of seminal vesicles (Fig. 2, J1, K1). In contrast, mice treated with DES showed a thin layer of columnar secretory epithelium, a reduced lumen diameter, and an absence of secretion material in lumen (Fig. 2, L1). However, all these histological alterations were recovered within 8 wk after DES withdrawal (Fig. 2, L2).

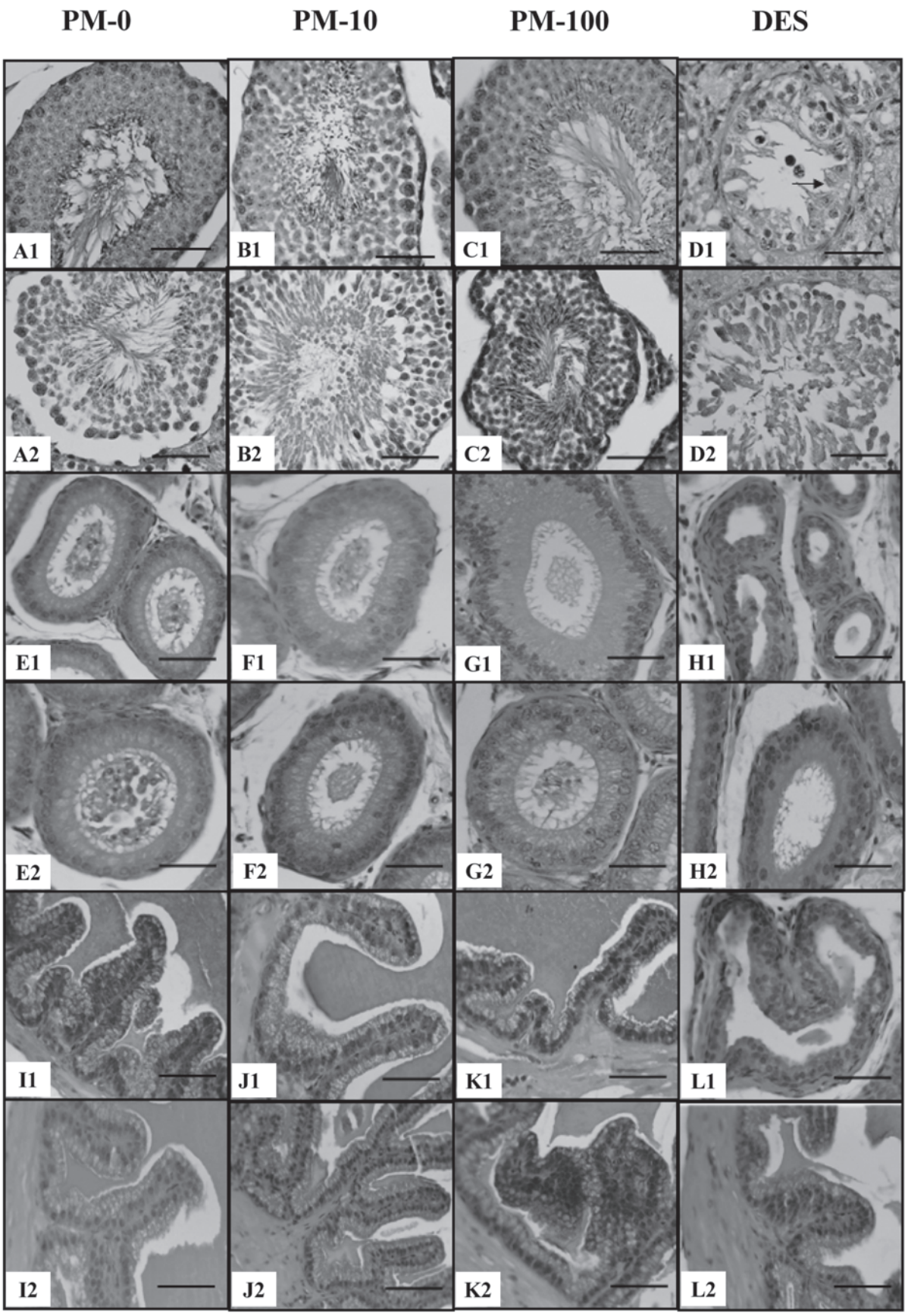

Fig. 2. Histological comparison of testis (**A–D**), epididymis (**E–H**) and seminal vesicle (**I–L**) at the end of treatment period (1) and the end of post-treatment period (2) of mice treated with *Pueraria mirifica* (PM) at doses 0, 10, 100 mg/kg BW/d (PM-0, PM-10, PM-100) and diethylstilbestrol (DES) at 200 μg/kg BW/d. Hematoxylin and eosin stain. (Scale bars = 50 μm.)

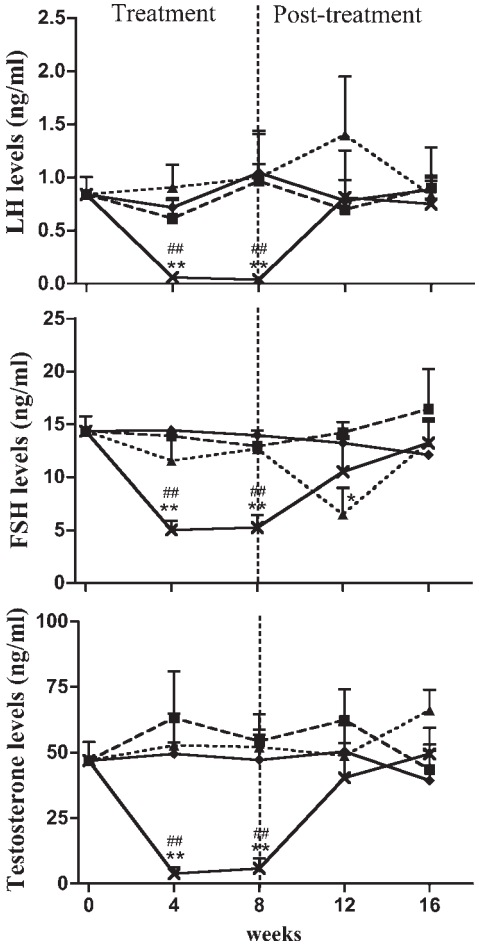

Fig. 3. Serum LH, FSH, and testosterone levels in male mice treated with *Pueraria mirifica* (PM) at doses 0 (–◆–; PM-0), 10 (–■–; PM-10), 100 (--▲--; PM-100) mg/kg BW/d and diethylstilbestrol at 200 μg/kg BW/d (—×—; DES). * $p < 0.05$ vs basal levels, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs PM-0. Each value represents the mean ± SEM of five animals.

Gonadotropin and Testosterone Levels

Serum LH, FSH, and testosterone levels in mice treated with PM-10 and PM-100 were not different from those of the PM-0 group, throughout the study period (Fig. 3). However, a significant reduction of serum FSH levels ($p < 0.05$) was observed at 4 wk of post-treatment period in PM-100 group as compared to the 0-wk level. As expected, serum LH, FSH, and testosterone levels in mice treated with DES were significantly lower at 4–8 wk of treatment period and recovered within 4 wk after the DES withdrawal.

The Number, Viability, and Motility of Sperm from the Cauda Epididymis

The sperm concentration, viability, and motility in mice treated with PM-10 is not significantly different from those of the PM-0 treatment for both treatment and post-treatment periods, excepting that the sperm concentration at the post-treatment period was significantly lower (Table 1). The sperm viability and motility at the treatment period and the sperm concentration at the post-treatment period in mice treated with PM-100 were lower than those of the PM-0 group. Sperm concentration, viability, and motility in mice injected with DES were significantly lower than those of PM-0 group for both treatment and post-treatment periods.

Fertility

The numbers of untreated (or virgin) females observing sperm plug and pregnancy after mating with the PM-10 and PM-100 treated males were similar to those number of females mating with the PM-0 treated males, throughout the experimental period (Table 2). Unexpectedly, the number of females with sperm plug and pregnancy were increased by 4 wk of PM-100 withdrawal. All of DES-treated males could not produce sperm plug and pregnant females, throughout the treatment period; however, they could recover and mate with untreated females within 4 wk of DES withdrawal. Although after mating the male mice could produce 53.33% of the sperm plugs as those of the PM-0 group, no pregnant females were found. The complete recovery of mating (indicated by the number of sperm plugs) and pregnancy by DES-treated male mice were found after 8 wk of DES withdrawal.

The Number of Litters Delivery, Body Weight and Reproductive Organ Weights of 50-d Old Pups

The average number of litters fathered by PM-10– and PM-100–treated male mice were not different from that of PM-0 fathers (Table 3). All of DES-treated male mice did not produce a litter throughout the treatment and the first 4 wk of post-treatment periods. However, they were completely recovered from the DES treatment and could produce litters at the 8th wk of post-treatment period.

The body weights of litters and the weekly monitoring body weight gains of the pups fathered by the PM- and DES-treated males did not differ from those delivered by the PM-0 fathers (data not shown). No malformations of the offspring, from the neonatal age to 50-d-old, were found. The relative organ weights of ovary and uterus in female pups, and testis, epididymis, and seminal vesicles in male pups fathered by PM- and DES-treated male mice were not different from those delivered by PM-0 fathers (Table 4). Gross morphology of the reproductive organs in all 50-d-old pups were also normal.

Discussion

The present study was designed to investigate the effects of PM on reproductive organs and fertility in male mice. Because changes in number and mobility of sperm were used as one of the parameters of fertility, the duration of PM treatment was performed in accord with the mouse's spermatogenic cycle, that is, 56 d or 8 wk. The doses of PM used in the present study were assigned from the previously published report, that is, 10 and 100 mg/kg BW/d of PM were safe to apply for human use *(16)*. As the PM-supple-

Table 1
Sperm Concentration, Viability, and Motility of Treated-Male Mice[a]

	Sperm count ($\times 10^6$)		Sperm viability (%)		Sperm motility (%)	
Group	Treatment	Post-treatment	Treatment	Post-treatment	Treatment	Post-treatment
PM-0	12.04 ± 1.58	24.02 ± 3.30*	60.40 ± 3.26	73.80 ± 5.29	58.67 ± 2.65	59.91 ± 1.74
PM-10	8.56 ± 1.64	19.20 ± 3.58*#	62.60 ± 1.81	71.80 ± 2.61	56.52 ± 3.88	64.39 ± 3.78
PM-100	9.04 ± 1.99	13.44 ± 5.75*#	42.20 ± 3.45#	55.60 ± 15.73	40.69 ± 3.25#	50.91 ± 15.97
DES	N/A	8.64 ± 2.77*#	N/A	43.20 ± 11.63*#	N/A	32.75 ± 2.91*#

Each value is expressed as the mean ± SEM of 5 animals.
*$p < 0.05$ vs treatment period, #$p < 0.05$ vs PM-0.
N/A: Not available.
[a]Male mice were treated with *Pueraria mirifica* (PM) at doses 0, 10, 100 mg/kg BW/d (PM-0, PM-10, PM-100) and diethylstilbestrol (DES) at 200 μg/kg BW/d.

Table 2
Sperm Plug and Pregnancy Observed in Untreated Female Mice after Mating with Treated Male Mouse[a]

	At 4 wk of treatment ($n = 30$)		At 8 wk of treatment ($n = 30$)		At 4 wk of post-treatment ($n = 15$)**		At 8 wk of post-treatment ($n = 15$)**	
Group	Sperm plug	Pregnancy	Sperm plug	Pregnancy	Sperm plug	Pregnancy	Sperm plug	Pregnancy
PM-0	16	13	18	14	8	7	8	8
	53.33%*	43.33%	60.00%	46.67%	53.33%	46.67%	53.33%	53.33%
PM-10	17	15	16	15	8	8	7	7
	56.67%	50.00%	53.33%	50.00%	53.33%	53.33%	46.67%	46.67%
PM-100	13	11	14	14	12	12	10	10
	43.33%	36.67%	46.67%	46.67%	80.00%	80.00%	66.67%	66.67%
DES	0	0	0	0	8	0	11	6
	0%	0%	0%	0%	53.33%	0%	73.33%	40.00%

*The percentage of sperm plug and pregnancy were calculated as the number of females found sperm plug and pregnancy, respectively, per the number of total females used ×100.
**"n" = number of female mice. Ten male mice/group were used during treatment period and mated with untreated female mice at ratio of 1: 3; $n = 30$. Five of 10 male mice were autopsied at the end of treatment period, and only five males were remained and mated with untreated-female mice in this period; $n = 15$.
[a]Male mice were treated with *Pueraria mirifica* (PM) at doses 0, 10, 100 mg/kg BW/d (PM-0, PM-10, PM-100) and diethylstilbestrol (DES) at 200 μg/kg BW/d.

Table 3
Average Number of Litters Fathered by Treated Male Mice[a]

Group	At 4 wk of treatment	At 8 wk of treatment	At 4 wk of post-treatment	At 8 wk of post-treatment
PM-0	12.80 ± 0.60	11.00 ± 0.70	10.10 ± 1.00	10.10 ± 0.90
PM-10	12.30 ± 0.80	10.90 ± 0.90	11.30 ± 0.40	8.70 ± 1.70
PM-100	10.70 ± 0.90	10.60 ± 0.90	13.00 ± 0.60	12.10 ± 0.70
DES	0	0	0	11.50 ± 1.10

[a]Male mice were treated with *Pueraria mirifica* (PM) at doses 0, 10, 100 mg/kg BW/d (PM-0, PM-10, PM-100) and diethylstilbestrol (DES) at 200 μg/kg BW/d.

mented dietary products for hair-loss protection, new hair growing stimulation, and rejuvenation have been widely used in mature men, especially in Asian men, the investigation of the effects of PM on reproductive organs and fertility in male mice should shed some light on the potential health risk for men as well.

Estrogen, an intrinsic hormone in vertebrates, is present in the blood circulation and its receptor is expressed in various germ cell types during spermatogenesis *(17)*. Bidirectional effects of estrogens, stimulation and suppression, on the male reproductive organs and spermatogenesis were reported *(17–21)*. A long period exposure to estrogens leads

Table 4
Body Weight and Relative Organ Weights
(Organ Weight/Body Weight) of 50-d-old Pups Fathered by Treated Male Mice[a]

	PM-0	PM-10	PM-100	DES
Body weight (g)	32.77 ± 0.47	33.21 ± 0.45	32.81 ± 0.44	32.46 ± 0.44
Relative organ weights (mg/g)				
Uterus	5.81 ± 0.02	5.82 ± 0.01	5.77 ± 0.03	6.22 ± 0.04
Ovary	0.63 ± 0.01	0.63 ± 0.01	0.64 ± 0.01	0.62 ± 0.01
Testes	7.77 ± 0.01	7.61 ± 0.01	7.60 ± 0.03	7.85 ± 0.04
Epididymes	2.52 ± 0.02	2.69 ± 0.02	2.87 ± 0.04	2.61 ± 0.01
Seminal vesicle	6.42 ± 0.02	6.19 ± 0.01	6.26 ± 0.03	6.19 ± 0.02

[a]Male mice were treated with *Pueraria mirifica* (PM) at doses 0, 10, 100 mg/kg BW/d (PM-0, PM-10, PM-100) and diethylstilbestrol (DES) at 200 μg/kg BW/d.

to reduction in gonadal size, feminization of the genetic male, and low sperm count and sperm motility *(18)*. Robaire et al. *(20)* reported that the percentage of motile sperm was significantly reduced in rats treated with 10 mg/kg of ethinyl estradiol for 1 wk or at dose of 1 mg/kg for 2 wk. Goyal et al. *(21)* also reported that male rats treated with synthetic estrogen DES of 8 μg/rat/d, for 12 d, significantly reduced the epididymal sperm number and sperm motility, but no changes in testis weights, daily sperm production, or spermatogenesis. Conversely, low dose of estradiol treatment promoted the spermatogonial stem cell renewal *(17)* and acted as a germ cell survival factor *(19)*. It implies that a low level of estrogens may be necessary for the normal spermatogenesis and could inhibit a male germ cell apoptosis *(19)*. These distinctive estrogenic responses are also found in our study when the different doses of PM were used. That is, treatment of PM at a dose of 10 mg/kg BW/d, but not 100 mg/kg BW/d, for 8 wk resulted in a slight proliferation of seminiferous epithelium. The increase of seminal vesicle weights and the sperm number in the seminiferous tubules were also found at the post-treatment period. Treatment of PM at a dose of 100 mg/kg BW/d, but not 10 mg/kg BW/d, reduced weights of epididymes and seminal vesicles and suppressed both sperm viability and motility in the cauda epididymis. These findings indicate that phytoestrogens may have controversial effects on spermatogenesis depended on the dosage and the exposure duration.

In the present study, we found that PM at both 10 and 100 mg/kg BW/d had no effects on serum LH, FSH, and testosterone levels in intact male mice. These results agreed with our previous finding that feeding of 10 and 100 mg/kg BW/d of PM to ochidectomized rats could not affect the serum LH and FSH levels, and only the suppression of LH levels were found after the PM dose was increased up to 1000 mg/kg BW/d *(11)*. As mentioned above, male mice exposed to 100 mg/kg BW/d of PM significantly reduced weights of the epididymes and seminal vesicles with no changes at the cellular levels. Generally, the weights of the accessory genital glands are remarkably low during an absence of androgens *(22)* and a full development and maintenance of their structures and functions also depend on testosterone hormone. However, the mechanisms of actions of phytoestrogen on accessory genital glands have not been clarified, only few reports were found *(23–25)*. The isoflavone phytoestrogen, genistein, was reported to act as an estrogen agonist in the stimulation of prostate gland of adult rodents *(26)*. Genistein and daidzein probably exerted their antiestrogenic effects indirectly via a decrease of the androgen receptor expression *(23)*. Thus, it is possible that the reduction in androgen receptor expression can lower the sensitivity of circulating testosterone, even though the testosterone levels were normal *(24,25)*. As in the reports cited above, the biological roles played by phytoestrogens in the androgenic response are still unclear; further studies in this field are necessary, such as the analyses on the expression and binding of estrogen and androgen receptors in the accessory genital glands after phytoestrogen treatment.

Following the reduction of epididymis and seminal vesicle weights, feeding of 100 mg/kg BW/d of PM caused a significant reduction in both sperm viability and motility in the cauda epididymis of male mice without reducing sperm number. Such a reduction could be attributed to a direct effect of PM on epididymis function and caused alterations in spermiogenesis. It is well known that the normal function of epididymis critically depends on testicular androgens as well as other factors including estrogen *(22,27–30)*. These functional disorders in the epididymis may have resulted from an imbalance between estrogen and testosterone, because spermiogenesis and sperm maturation depend on the delicate balance of the hypothalamus–pituitary–testis axis. These findings indicate that PM could affect the function of spermatogenic organs and then the production of spermatozoa. However, the reductions in sperm motility and viability caused by PM had no further effects in mating efficiency, pregnancy rate, and average number of offspring delivery.

Male mice treated with PM could father normal offspring. No malformation of litters and no alterations of reproductive organs of 50-d-old pups were found. Their body weights are in the normal range *(31)*. Similarly, female rats exposed

to 0.4 and 4 mg/kg BW/d of genistein during gestation and lactation did not have the effect on body weight of their offspring during development *(32)*. Our results can conclude that the exposure of male mice to PM at doses of 10 and 100 mg/kg BW/d has no adverse effects on the reproductive organs, growth rate, and prenatal development in offspring, at least in the F1 generation. Moreover, the PM at 10 and 100 mg/kg BW/d can promote the fertility after stop feeding.

Taken together, our results clearly suggest that treatment of 10 mg/kg BW/d of PM for 8 wk, via the oral route, has no impairment effects on the reproductive organs and functions of adult male mice; however, a 10-times higher dose, 100 mg/kg BW/d, could cause some moderate changes. Additionally, PM treatment at doses of 10 and 100 mg/kg BW/d which is suggested to be safe to apply for human consumption in kg BW basis, does not alter the male fertility and the hypothalamus–pituitary–testis axis when taken orally *(16,33)*. Although most of the PM commercial products are formulated as a topical cream or solution, not for consumption, which is convenient for the customer to use, we could not emphasize that an administration of PM by oral route is higher potency than the tropical route, or vice versa. Based on the latest information in our hands, no scientific reports on those aspects could be found. However, most of the PM commercial products manufactured for women, such as a breast enlargement cream or skin recovery solution, have no side effects on reproductive organs and functions, and the response to PM in females is greater and more sensitive than in males *(11)*. Thus, the results of our study should somewhat facilitate our understanding of the PM side effects, when either the oral or tropical routes are used, on the reproductive functions in men as well.

Materials and Methods

Chemicals

Pueraria mirifica cultivar Wichai-III was collected from Chiangmai province, northern Thailand, voucher specimen No. BCU11045. The tuberous powder was prepared and stored as previously described *(34)*, and the same lot was used throughout this study. The constituents of phytoestrogens of Wichai-III cultivar investigated by HPLC technique were described previously *(11)*.

Diethylstilbestrol (DES) and corn oil were obtained from Sigma Chemical Company (St. Louis, MO, USA). DES was diluted with corn oil and mixed vigorously prior to use.

Animals

Adult ICR mice, age 50–60 d, weighing 30–35 g, from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand, were used in this study. 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, Chulalongkorn University, Bangkok, Thailand. They were fed with the rat diet (Pokphand, Animal Feed Co., Ltd., Bangkok, Thailand) and water *ad libitum*. The animals were habituated by handling regularly for 2 wk before the experiments were conducted. All experiments were performed between 0800 and 1100 h. The experimental protocol was approved by the Animal Ethical Committee in accordance with guide for the care and use of laboratory animals prepared by Chulalongkorn University.

Experimental Design

Forty adult male mice were divided into four groups (10 mice/group). Groups 1–3 rats were orally treated with 0, 10, and 100 mg/kg BW/d of PM suspended in 0.2 mL distilled water, abbreviated as PM-0, PM-10, and PM-100, respectively. Group 4, rats were subcutaneously injected with a synthetic estrogen DES at 200 μg/kg BW/d, and kept as a positive control group. To ensure that the estrogenic effect of DES on reproductive functions and fertility, such as accessory sex organ weights, sperm quality, and gonadotropin and testosterone levels *(21)*, could be observed, a high dose of 200 μg/kg BW/d of DES was chosen for this study. The treatment schedule was separated into two periods; treatment and post-treatment. The duration in each period was 8 wk.

Blood was collected by cardiac puncture every 4 wk for LH, FSH, and testosterone assays. After each time of blood collection, a male mouse was paired with three virgin female mice during the night (approximately 12 h/day) for 4 d. Male mouse was returned to the treatment schedule after mating. Five of 10 mice in each group were randomly decapitated at the end of treatment or post-treatment periods. The testes, epididymes, and seminal vesicles were dissected and weighed. The cauda epididymis parts were collected for the examination of sperm concentration, motility, and viability. All collected organs were preserved in 10% formalin buffer for histological examination afterward. After mating, all females were quantified as to the presence of sperm plug and pregnancy rates. Consequently, the number of delivered offspring and their malformations were determined in case the female mice were pregnant. The weights of offspring were recorded at birth, 21 d of age (or weaning age), and every week thereafter. The pups were decapitated at 50 d of age; gonad and accessory sex organs were dissected, weighed, and examined.

Hormonal Analysis

Concentrations of serum FSH and LH were measured using NIDDK kits for rat FSH and LH. Iodination preparations were rat 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 are expressed in terms of the rat FSH-RP-2 and LH-RP-3 reference standards.

Serum concentrations of testosterone were measured using the established RIA methods of World Health Organization (WHO) after the samples were extracted with ether *(35)*.

To minimize the interassay variation, all samples were assayed in a single run for each hormone. The intraassay

coefficients of variation for LH, FSH, and testosterone were 2.24%, 6.34%, and 12.64% respectively.

Semen Quality Analysis

Mice were sacrificed and the cauda epididymis was removed and placed into 1 mL of phosphate buffer • saline (PBS). Cauda epididymis was cut into three pieces and incubated at 37°C in incubator maintaining at 5% CO_2 in air for 10 min. The determination of semen quality were done thereafter.

Semen quality was determined by three parameters: sperm concentration, viability, and motility. "Sperm concentration" was analyzed by the hemocytometer method *(36)*. Semen collected from the cauda epididymis was diluted 1:20 with diluents (50 g $NaHCO_3$, 10 mL of 35% formalin, and distilled water for a final volume of 1000 mL). From this homogenate, a sample was taken and the number of sperm was counted using a hemocytometer with improved double Neubauer ruling. Counts for two chambers were averaged. "Sperm viability" was analyzed by the eosin–nigrosin staining method *(36)*. The viable sperm, which were not stained with eosin y, and the dead sperm, which were stained with eosin y, were counted under a light microscope. The percentage of viable sperm was calculated as the number of viable sperm per total number of sperm counted × 100. "Sperm motility" was analyzed by the multiexposure photography method *(37)*. The percentage of sperm motility was calculated as the number of motile sperm per the total number of sperm counted.

Histological Examination

After the overnight fixation of reproductive organs in 10% formalin buffer, tissues were dehydrated in a series of ethanol gradient and clearing in xylene. Tissues were then embedded and blocked in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin *(38)*. Permanent preparations of all tissues were histologically examined and photographed using a camera (Nikon) mounted on the microscope (Olympus).

Statistical Analysis

The results were expressed as means ± SEM. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) program version 11.

Paired-sample *t* test was submitted for the comparison of organ weights between the end of treatment and the end of post-treatment periods in each group. Comparisons between treatment groups were done by one-way analyses of variance (ANOVA) for factorial or repeated measure design with post–hoc testing by least significant difference (LSD) test. *p* values of less than 0.05 were considered to be statistically significant.

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