Estrogenic Effects of *Pueraria mirifica* on the Menstrual Cycle and Hormone-Related Ovarian Functions in Cyclic Female Cynomolgus Monkeys

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**Abstract.** This study investigated the estrogenic effect of *Pueraria mirifica* (*P. mirifica*) on menstrual cycle length and hormone-related ovarian function. Nine normal cyclic monkeys (*Macaca fascicularis*) were separated into 3 groups; each group was force fed with a single dose of 10, 100, and 1,000 mg of *P. mirifica*. The experimental schedule was separated into the pre-treatment and post-treatment periods. Blood samples were collected on days 3, 9 – 14, 19, 24, 29, and every 10 days until the next menstruation for one and two menstrual cycles during two consecutive periods and assayed for serum levels of gonadotropins and ovarian hormones. The result showed a significant increase in lengths of the follicular phase and total menstrual cycle in monkeys treated with 1,000 mg of *P. mirifica*, but no change in menstrual cycle length in monkeys treated with 10 and 100 mg of *P. mirifica*. Serum levels of follicle stimulating hormone, luteinizing hormone, estradiol, progesterone, or immunoreactive-inhibin did not change during the first and second menstrual cycles of the post-treatment period for all monkey groups. Our findings demonstrate that although changes in hormonal levels could not be observed in this study, a single dose of 1,000 mg of *P. mirifica* can disturb ovarian function and menstrual cycle in monkeys.

**Keywords:** *Pueraria mirifica*, phytoestrogen, monkey, reproductive hormone, menstrual cycle

**Introduction**

Phytoestrogens, which are structurally and functionally similar to 17β-estradiol, are naturally occurring phytochemicals found in plants and plant products (1). The principle classes of phytoestrogens are isoflavones (including daidzein and genistein), coumestans (including coumestrol), and lignans (including esterodiol and enterolactone) that are mainly found in soy and soy food (2 – 5). Estrogenic effects of phytoestrogens were reported on hormonal and reproductive disturbances in both animals (6 – 9) and humans (10 – 12) to include causing infertility in sheep (13), the reduction of ovulation rate in mice (14), and disruption of reproductive hormones and ovarian function in cyclic women (15 – 18). In addition, epidemiological studies have shown that pre- and postmenopausal Japanese and Chinese women who consumed high amounts of isoflavones from soy had a decrease in serum levels of estrone and estradiol (19 – 21).

*Pueraria mirifica* (*P. mirifica*) is an indigenous Thai herb of the family Leguminosae. Its tuberous roots also contain phytoestrogenic substances including miroestrol (22), puerarin (23), deoxymiroestrol, kwakhurin (24, 25), and other phytoestrogens that belong to the isoflavone and coumestrol class (26 – 28). Miroestrol compounds isolated from the roots of *P. mirifica* prevented the implantation of blastocysts, promoted uterine weight, vaginal growth, increased
vaginal fluid in normal female rats (22), and produced cornification of the vaginal epithelium in ovariectomized-adrenalectomized rats (29, 30), but did not stimulate the secretion of endogenous estrogen by the ovaries or the adrenal gland (29). Miroestrol also exhibited mammogenic potency in both ovariectomized rats and mice by restoring the mammary duct growth as estradiol did (22). The potency of subcutaneously injected miroestrol is about 0.7 times that of estradiol and twice as potent as estrone (31).

In recent years, *P. mirifica* has been widely used in pre- and postmenopausal women. They believe that phytoestrogens contained in the plant, especially isoflavones, support female characteristics including breast and skin appearances as well as improve bone structure and the cardiovascular system. However, *P. mirifica* may disturb reproductive function and menstrual cycle in women.

The aim of this study was to examine the endocrine-modulating effect of *P. mirifica* on menstrual cycle length and hormones related ovarian function in normal cyclic monkeys. Female cynomolgus monkeys (*Macaca fascicularis*) were used as the alternative model to study reproductive hormones and function because the monkeys have physiological systems including hormonal pattern, ovarian cycle, and reproductive function that are similar to those of humans (32, 33). Moreover, to study the effects of *P. mirifica* containing phytoestrogens on changes of serum levels of hormones in humans is very difficult due to uncontrolled diet and follow-up factors.

**Materials and Methods**

**Animals**

Seventeen adult female cynomolgus monkeys (*Macaca fascicularis*) with regular menstrual cycles for at least 4 consecutive months, 26 – 37 days in length and weighing 4 – 6.5 kg prior to the study, were used. The first day of menstrual bleeding was considered as day 1 of the menstrual cycle. The monkeys were housed in individual cages at the Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Lighting conditions of the animal room were controlled (12:12 h light to dark cycle). Temperature (25 – 30°C) and humidity (37 – 41%) fluctuated slightly depending on the season. The monkeys were fed daily with monkey chow (Pokaphan Animal Feed Co., Ltd., Bangkok, Thailand) in the morning (09:00 – 10:00 h) and supplemented with fresh fruits in the afternoon (14:00 – 15:00 h). The experimental protocol was approved in accordance with the guide for the care and use of laboratory animals prepared by Faculty of Science, Chulalongkorn University.

**Experimental design**

Nine female monkeys were divided into three groups. Each group (*n* = 3) was force-fed with the suspension of *P. mirifica* at doses of 10, 100, and 1,000 mg/5 ml of distilled water/individual, at 08:00 – 08:30 h. The schedule was separated into the pre-treatment and post-treatment periods. The pre-treatment was performed on one menstrual cycle and the post-treatment was performed after a single forced-feeding of *P. mirifica* within 2 menstrual cycles. Day 1 of menstrual bleeding was used as a reference for the first day of a period. During these periods, 3-ml blood samples were collected from the femoral vein without anesthetization between 08:00-09:00 h on day 3 (the early follicular phase); days 9, 10, 11, 12, 13 (the late follicular phase); days 14, 19 (the early luteal phase); days 24, 29 (the late luteal phase); and every 10 days until the next menstruation. Blood samples were immediately centrifuged at 4°C, 1,700 ×g for 20 min. The serum was then separated and stored at −20°C until follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, progesterone, and immunoreactive (ir)-inhibin were assayed. Occurrence of menstrual bleeding was daily checked by vaginal swabbing. The suspension of *P. mirifica* (10, 100, and 1,000 mg) was prepared from powder of the tuberous roots and suspended into 5-ml distilled water and then kept in a dark bottle at 4°C until feeding time.

**Hormonal analysis**

Concentrations of serum FSH and LH were measured by a heterologous radioimmunoassay (RIA) system. Iodination preparations were rat NIDDK-rat FSH-I-5 and rat LH-I-5. The antisera were anti-ovine FSH (NIDDK-H-31) (34) and anti-ovine LH (YM#18) (35). Antiserum against ovine LH (YM#18) was kindly provided by Dr. Y. Mori (University of Tokyo, Tokyo). The results are expressed in terms of NIDDK rat FSH-RP-2 and rat LH-RP-2. The intra- and inter-assay coefficients of variations were 7.8% and 8.5% for FSH and 7.8% and 10.3% for LH, respectively.

Concentrations of estradiol and progesterone were measured by using the established method of the World Health Organization (WHO) (36). The intra- and inter-assay coefficients of variation were 6.15% and 10.96% for estradiol and 5.98% and 11.01% for progesterone, respectively.

Serum concentrations of ir-inhibin were measured by a double-antibody RIA, as described previously (37). The antiserum used was raised in rabbits against bovine inhibin (TNDH-1). Purified bovine 32-kDA inhibin...
was used as the standard. The intra- and inter-assay coefficients of variation were 8.8% and 14.4%, respectively.

**Statistical analyses**

All data including changes of menstrual cycle length and serum levels of hormones were expressed as the mean ± S.E.M. The significance of the differences between the mean was evaluated by the paired t-test. *P*<0.05 was considered to be statistically significant.

**Control levels of hormones in normal menstrual cycles**

According to the interval of blood collection schedule, the prominent peaks of LH and FSH levels during the pre-treatment period could not be caught in 4 out of 9 monkeys (nos. 609, 624, 621, and 626). However, the ovulation was confirmed by the increase of progesterone and ir-inhibin. To evaluate changes of serum levels of gonadotropins and ovarian hormones in the nine monkeys after *P. mirifica* treatment, serum levels of those hormones were compared with those of eight monkeys showing normal menstrual cycle, hormonal pattern, and prominent peak LH level from the same colony. To increase the animal numbers in this group, the hormonal levels during the pre-treatment period of 5 monkeys treated with *P. mirifica* (nos. 619, 627, 526, 604, and 104) showing prominent LH level were combined to this control group. Thus, the total number of monkeys in this group is thirteen.

**Results**

**Changes in menstrual cycle length of monkeys treated with 10, 100, and 1,000 mg of *P. mirifica***

Nine monkeys had normal menstrual cycle length during the pre-treatment period for 30.56 ± 1.30 days, as shown in Table 1. After *P. mirifica*-treatment, lengths of the first or second menstrual cycles of monkeys treated with 10 and 100 mg of *P. mirifica* were not different from the menstrual cycle length at the pre-treatment period. Lengths of the first and second menstrual cycles were 30.67 ± 2.91 and 33.33 ± 3.84 days for monkeys treated with 10 mg of *P. mirifica* and 30.67 ± 2.96 and 35.67 ± 6.33 days for monkeys treated with 100 mg of *P. mirifica*, respectively. Lengths of the first and second menstrual cycles were significantly extended to 42.00 ± 4.04 (P = 0.004) and 39.67 ± 0.67 (P = 0.003) days, respectively, in monkeys treated with 1,000 mg of *P. mirifica*.

**Serum levels of gonadotropins and ovarian hormones in normal cycling monkeys**

Serum profiles of gonadotropins (FSH and LH), and ovarian hormones (estradiol, progesterone, and ir-inhibin) in 13 normal cyclic monkeys during the menstrual cycle are shown in Fig. 1 and Table 2. Changes in these hormonal profiles during the menstrual cycle were adjusted according to the day of peak levels of serum LH, defined as an ovulation day (day 0), and separated into 2 phases: the late follicular phase and the early luteal phase. As shown in the Fig. 1, peak levels of serum FSH and LH were 1.82 ± 0.34 and 8.27 ± 0.86 ng/ml on the same day (day 0). The increase of peak serum estradiol levels appears to coincide with the mid-cycle peak levels of FSH and LH. Serum progesterone and ir-inhibin levels remain low during the early follicular phase and then become slightly elevated during the early luteal phase, indicating that cyclic ovulation occurred in this menstrual cycle.

**Changes in serum gonadotropins and ovarian hormones in the monkeys treated with 10, 100, and 1,000 mg of *P. mirifica***

As shown in Figs. 2, 3, and 4, there were no changes in serum levels of FSH, LH, estradiol, progesterone, or ir-inhibin throughout the first and second menstrual cycles in monkeys treated with 10, 100, and 1,000 mg of *P. mirifica*, respectively. All monkeys exhibited peak levels of serum FSH and LH in the late follicular phase of the first and second menstrual cycles after *P. mirifica* treatment, concurrent with high levels of serum estradiol. Serum progesterone and ir-inhibin levels were low throughout the early follicular phase and gradually increased in the early luteal phase of the

| Table 1. | Menstrual cycle length of monkeys treated with 10, 100, and 1,000 mg of *P. mirifica* |
| --- | --- | --- |
| **Treatment groups** | **Lengths of menstrual cycle after *P. mirifica* treatment (days)** |  |
|  | **The first menstrual cycle** | **The second menstrual cycle** |
| 10 mg | 30.67 ± 2.91 (P = 0.97) | 33.33 ± 3.84 (P = 0.39) |
| 100 mg | 30.67 ± 2.96 (P = 0.97) | 35.67 ± 6.33 (P = 0.23) |
| 1,000 mg | 42.00 ± 4.04* (P = 0.004) | 39.67 ± 0.67* (P = 0.003) |

Mean length of menstrual cycle during the pre-treatment period of nine cyclic female monkeys was 30.56 ± 1.30 days. Asterisks represent a significant difference (*P*<0.05).
first and the second menstrual cycles. However, at the highest dose (1,000 mg of P. mirifica), peak levels of serum LH were delayed from day 10 during the pre-treatment period to days 34 and 29 of the first and second menstrual cycles for monkey no. 621, from day 12 during the pre-treatment period to days 24 and 19 of the first and second menstrual cycles for monkey no. 104, and from day 11 during the pre-treatment period to days 24 and 19 of the first and second menstrual cycles for monkey no. 626 (Fig. 4).

**Discussion**

The present study provides the first evidence that P. mirifica containing phytoestrogens has a profound, dose-dependent effect on the menstrual cycle length. The lengths of the follicular phase and the entire first and second menstrual cycles in monkeys treated with the highest dose (1,000 mg of P. mirifica) increased significantly; meanwhile, there were no changes in the lengths of the follicular phase, luteal phase, or total menstrual cycle in monkeys treated with the lowest and
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Medium doses (10 and 100 mg of *P. mirifica*). Additionally, our study did not observe changes in profile levels of serum FSH, LH, estradiol, progesterone, or ir-inhibin during the menstrual cycle.

It has been reported that the tuberous roots of *P. mirifica* contained a higher amount of isoflavones (169.1 mg/100 gram of dry powder) (38) compared to soyabean using the HPLC technique (1). There are many published reports showing that isoflavones from soy are the major component that has an effect on the reproductive system (15 – 17). Thus, it seems to be that isoflavones in *P. mirifica* are the major component to influence the menstruation in this study. Although some reports showed no effect from daily consumption of isoflavones in length of the follicular phase, the luteal phase, or total menstrual cycle in premenopausal women (17, 39, 40), other reports support our finding of a phytoestrogenic effect on menstrual cycle length. Follicular phase length increased in premenopausal women who consumed isoflavones from soy daily (15), and luteal phase length increased in premenopausal women who ingested lignans from flax seed daily (11).

Effect of phytoestrogens from soy on reproductive hormones in premenopausal women has also been demonstrated. There was no change (40) or significant decrease in serum levels of FSH and LH in premenopausal women who consumed daily dietary phytoestrogens throughout their menstrual cycle (16, 17, 41). Serum levels of estradiol and progesterone showed no change (17) or decreased (39, 40).

Phytoestrogens have also been shown to inhibit GnRH-induced LH release. Intravenous administration of coumestrol to ovariectomized rats resulted in reduction in GnRH pulse frequency, as well as reduction in LH pulse frequency and amplitude (42). Estradiol administration also has an effect on reduction in both pulsatile GnRH and LH secretion (42, 43) and caused decreases in serum LH and FSH levels (44). The inhibitory effect of coumestrol on LH pulse frequency was greater than that of estradiol (42). This evidence

![Fig. 2. Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey nos. 609 (left panel), 619 (middle panel), and 627 (right panel) treated with 10 mg of *P. mirifica*. Horizontal bars represent the day of menses. Day 1 represents the day of menses in the pre-treatment period. The pre-treatment and post-treatment periods are separated by a vertical solid line. Two menstrual cycles during the post-treatment period are separated by a vertical dotted line. The symbols of asterisk and plus show peak levels of LH and FSH, respectively.](image-url)
indicates that both phytoestrogens and estradiol have profound effect on decreasing GnRH-induced LH secretion from the pituitary gland. An alteration of pulsatile secretion of serum gonadotropins, leading to decreased of gonadotropin levels, results in disorder of the follicular growth and ovulation found in women with functional hypothalamic amenorrhea (45, 46) and luteal phase deficiency (47). There is a high correlation between pulse amplitude and frequency of LH and LH levels (48).

However, our study could not detect changes in serum levels of either gonadotropins or ovarian hormones in monkeys because after intake of *P. mirifica*, phytoestrogenic substances, including genistein and daidzein, may convert to a phytoestrogen metabolite and excreted in the urine (1). The slow increase in plasma concentrations of the glycosidic forms of the isoflavones is consistent with the facilitation of absorption by hydrolysis in the small and large intestines (49). There have been reports showing that after a single dose of soy in the diet, both genistein and daidzein were excreted in the urine as conjugated metabolite by 15% and 47% in men, respectively, and by 24% and 66% in women, respectively (50). Pharmacokinetic studies show that after a single dose of genistein and daidzein intake, measurable quantities of free genistein and free daidzein are present in the circulation with half-life ($t_{1/2}$) of 3.2 and 4.2 h for free genistein and free daidzein in men, respectively. The elimination half-life values for total genistein and total daidzein in men were 9.2 and 8.2 h, respectively (51). The elimination rates of isoflavones from the circulation are different and affected by sex. Lu and Anderson (50) showed that the elimination half-life values for genistein, daidzein, and equol were 7, 4, and 9 h in women and 4, 3, and 5 h in men, respectively. Another study showed that serum concentration of genistein and daidzein were highest at 5.5 and 7.4 h in premenopausal women (52). These investigations suggest that phytoestrogens are rapidly cleared from the circulation in both males and females.

In this study, it is very difficult to ascribe the reproductive hormonal changes to the acute effect of phytoestrogens in *P. mirifica*. The monkeys were fed with a single dose of *P. mirifica* on day 1 of the men-

![Fig. 3. Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey nos. 526 (left panel), 601 (middle panel), and 624 (right panel) treated with 100 mg of *P. mirifica*. Day 1 represents the day of menses in the pre-treatment period. The meanings of horizontal bars, vertical solid and vertical dotted lines, and symbols are shown in Fig. 2.](image-url)
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Struthional cycle and blood sample collection began on day 3. It is possible that phytoestrogens are completely removed from the blood circulation within 24 h. Accordingly, at 48 h after feeding time, we did not observe any phytoestrogenic effects on changes in hormonal levels in serum. Moreover, in this study, changes in serum levels of hormones were detected on day 3; days 9 – 14, 19, 24, 29; and every 10 days until menstruation. So, we could not detect whether there are changes in pulsatile secretion of these hormones. However, our result showed the prolongation of menstrual cycle length in the monkeys treated with 1,000 mg of *P. mirifica*. It can be assumed that at the highest dose, phytoestrogens may reduce pulse amplitude and frequency of gonadotropins, especially LH, to support follicular growth and ovulation, resulting in increased length of the follicular phase and total menstrual cycle of the monkeys.

Normal cycling women who were administrated with GnRH antagonist reduced the pulse amplitude and frequency of LH. Serum levels of FSH, LH, estradiol, and ir-inhibin decrease during the menstrual cycle. These changes are consistent with an increase in length of the follicular phase and total menstrual cycle (53, 54). The results of this report support our hypothesis described in the previous paragraph.

In conclusion, the result of the study suggests that a single dose of 1,000 mg of *P. mirifica* disturbs ovarian function and menstrual cycle in normal cyclic monkeys. *P. mirifica* may have positive effects on female characteristics; however, use of this plant or its products in normal cyclic women may have an effect on ovarian function and may induce menstrual cycle disruption. Its lowest dose should be recommended for cyclic women.

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