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Ovulation Block by *Pueraria mirifica*

A Study of Its Endocrinological Effect in Female Monkeys

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***Pueraria mirifica* (PM), a Thai herb containing phytoestrogens, may act as estrogen and disturb reproduction. To investigate the effect of PM on the menstrual cycle length and related hormones, nine adult female monkeys (*Macaca fascicularis*) were separated into three groups. Each group (*n* = 3) was fed with 10, 100, and 1000 mg/d of PM for three menstrual cycles. The menstrual cycle length increased significantly in monkeys treated with PM-10 and PM-100 and disappeared completely in monkeys treated with PM-1000. Serum follicle stimulating hormone, luteinizing hormone, estradiol, progesterone, and ir-inhibin were lower during the treatment period in a dose-dependent manner. Changes in menstrual cycle length and the hormonal levels recovered during the post-treatment period only in monkeys treated with PM-10 and PM-100. PM greatly influences menstrual cycles and may suppress ovulation by lowering serum levels of gonadotropins.**

Key Words: Gonadotropins; ovarian hormones; phytoestrogens; *Pueraria mirifica*; cynomolgus monkey.

Introduction

Pueraria mirifica (PM), called white kwao krua in Thai, is an indigenous Thai herb that belongs to the family Leguminoceae. This plant is of interest because its tuberous root contains many phytoestrogens having estrogenic potencies such as miroestrol *(1)*, puerarin *(2)*, deoxymiroestrol, kwakhurin *(3,4)*, and others in the isoflavone and coumestrol groups *(5,6)*. In recent years, the use of PM as an alternative medicine has become popular. Many products in the form of creams, tablets, and solutions developed from PM root are widely used in normal cycling women as age-rejuvenation drugs and cosmetic products such as breast enlargement creams, skin moisturizers, and eye gels. However, there was no scientific report of PMs estrogenic effect on reproduction or related hormones in women.

Estrogenic effects of the ability of this plant to disrupt reproductive function have been observed in mice, rats, and monkeys *(7,8)*. Miroestrol, a phytoestrogenic substance found only in PM root, increased uterine weight in immature female mice *(7)*. A single high dose of PM (1000 mg) could prolong the menstrual cycle length of female cynomolgus monkeys *(8)*. In addition, phytoestrogen isoflavones from another plant were first reported as causing sheep infertility *(9)*. Coumestrol isolated from alfalfa reduced the ovulation rate in mice *(10)*. Furthermore, previous reports showed that soy isoflavones disturbed hormonal characteristics in premenopausal women, although there have been conflicting data *(11–15)*. From the epidemiological data, Japanese and Chinese women who consumed large amounts of soy in their diet lowered circulating levels of estradiol during menstrual cycles *(16,17)*.

From this evidence, it is important to investigate the effects of PM containing phytoestrogens on the menstrual cycle length and related hormones. To avoid the limitations of the long-term study effect of PM in humans, the adult cyclic cynomolgus monkey (*Macaca fascicularis*), a nonhuman primate, was selected as the model of this study. Female cynomolgus monkey has been proven to have similar reproductive function and hormonal patterns with those in woman *(18)*, and offer the advantage of allowing controls over dietary and environmental factors that may influence the results of the study. Therefore, our study examined the changes in the menstrual cycle length and related hormones in adult cyclic female cynomolgus monkeys treated with PM.

Results

Changes in Menstrual Cycle Length of Monkeys Treated with PM

The normal menstrual cycle length during the pretreatment period in nine monkeys was 28.2 ± 0.8 d. As shown

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Table 1
Menstrual Cycle Length of Monkeys Treated with 10, 100, and 1000 mg/d of PM During the Treatment and Post-treatment Periods

	Menstrual cycle length (d)		
Treatment groups	Pretreatment period	Treatment period	Post-treatment period
PM-10	no. 601: 28	no. 601: 50, 81, 52	no. 601: 29, 28
	no. 627: 27	no. 627: 49, > 90 ^{CC}	no. 627: 27, 36, > 60 ^{NR}
	no. 619: 29	no. 619: > 90 ^{CC}	no. 619: 22, 38, 30
PM-100	no. 616: 34	no. 616: 15, > 90 ^{CC}	no. 616: > 60 ^{NR}
	no. 801: 27	no. 801: 21, > 90 ^{CC}	no. 801: 39, 38, 32
	no. 108: 28	no. 108: > 90 ^{CC}	no. 108: > 60 ^{NR}
PM-1000	no. 624: 26	no. 624: > 90 ^{CC}	no. 624: > 60 ^{NR}
	no. 77: 28	no. 77: > 90 ^{CC}	no. 77: > 60 ^{NR}
	no. 633: 27	no. 633: > 90 ^{CC}	no. 633: > 60 ^{NR}

The menstrual cycle length during the pretreatment period in nine monkeys was 28.22 ± 0.78 days. CC represents a complete cessation of menstruation during the treatment period. NR represents the non-recovery of the menstruation during the post-treatment period.

in Table 1, PM-10 extended the menstrual cycle length to 50, 81, and 52 d in monkey no. 601, to 49 and > 90 d in monkey no. 627, and completely stopped the menstruation throughout the treatment period in monkey no. 619. All monkeys recovered an extended menstrual cycle during the post-treatment period. The menstrual cycle decreased in monkeys treated with PM-100, it became shorter during the early treatment period to 15 d in monkey no. 616 and to 21 d in monkey no. 801. Afterward, both monkeys subsequently stopped their menstruations throughout the treatment period, and could recover during the post-treatment period in only monkey no. 801. Monkey no. 108 did not exhibit menstruation throughout the treatment and post-treatment period. All monkeys (nos. 624, 77, and 633) treated with PM-1000 showed a complete cessation of menstruation throughout the treatment period and did not recover the cycle in the post-treatment period.

When the overall of menstrual cycle lengths were averaged and compared with normal length cycles (28.2 ± 0.8 d), a highly significant increase ($p < 0.01$) in menstrual cycle lengths was found during the treatment period in all groups (72.40 ± 9.10, 61.20 ± 20 17.66, and >90.00 ± 0.000 d in PM-10, PM-100, and PM-1000, respectively). Menstrual cycles tended to recover in PM-10 (36.83 ± 4.91 d) and PM-100 groups (47.50 ± 7.32 d).

Changes in Hormonal Pattern of Gonadotropins and Ovarian Hormones Throughout the Menstrual Cycle in Monkeys Treated with PM

To evaluate the changes in gonadotropins and ovarian hormones in monkeys treated with PM, profile hormones during the treatment and post-treatment periods were compared with those during the pre-treatment period. Although there were interindividual variations in serum levels and patterns of FSH, LH, estradiol, progesterone, and ir-inhibin of the monkeys depending on the length of the menstrual cycle, the responding patterns of these hormones to each PM treatment were similar. Normal menstrual cycle during the pre-treatment period of the nine monkeys showed the peaks of FSH and LH, which appeared to coincide with the surge in serum estradiol levels. The peak level of LH was defined as the mid cycle phase or the ovulation day, and was separated into two phases: follicular and luteal. Serum progesterone and ir-inhibin levels decreased during the follicular phase, and then increased slightly and surged during the luteal phase.

As shown in Fig. 1, monkey no. 601 still exhibited peak levels of serum FSH and LH, and estradiol, progesterone, and ir-inhibin levels surged during the treatment and post-treatment periods. Monkey no. 627 showed a prolonged menstruation (49 and >90 d) during the treatment period with no peak of FSH or LH and no surge of estradiol, progesterone, or ir-inhibin levels during the treatment period, particularly during the second cycle; nevertheless, after the recovery of the menstrual cycle, serum FSH and LH levels peaked, and serum estradiol, progesterone, and ir-inhibin levels surged during the post-treatment period. Likewise, monkey no. 619 stopped menstruation during the treatment period, with no surge of progesterone or ir-inhibin levels, and no peak of FSH or LH during the treatment period. After the recovery of the menstrual cycle during the post-treatment period, serum progesterone and ir-inhibin surged after serum estradiol levels increased.

Compared to pre-treatment levels, all three monkeys (nos. 616, 801, and 108) treated with PM-100 had no surge of FSH or LH during the treatment period (Fig. 2). Conspicuously, monkey nos. 616 and 801 had short menstrual cycles during PM-100 treatment, with no peak of FSH or LH. In addition, there was no surge of serum estradiol, progesterone, or ir-inhibin. Basal levels of FSH in all monkeys decreased sig-

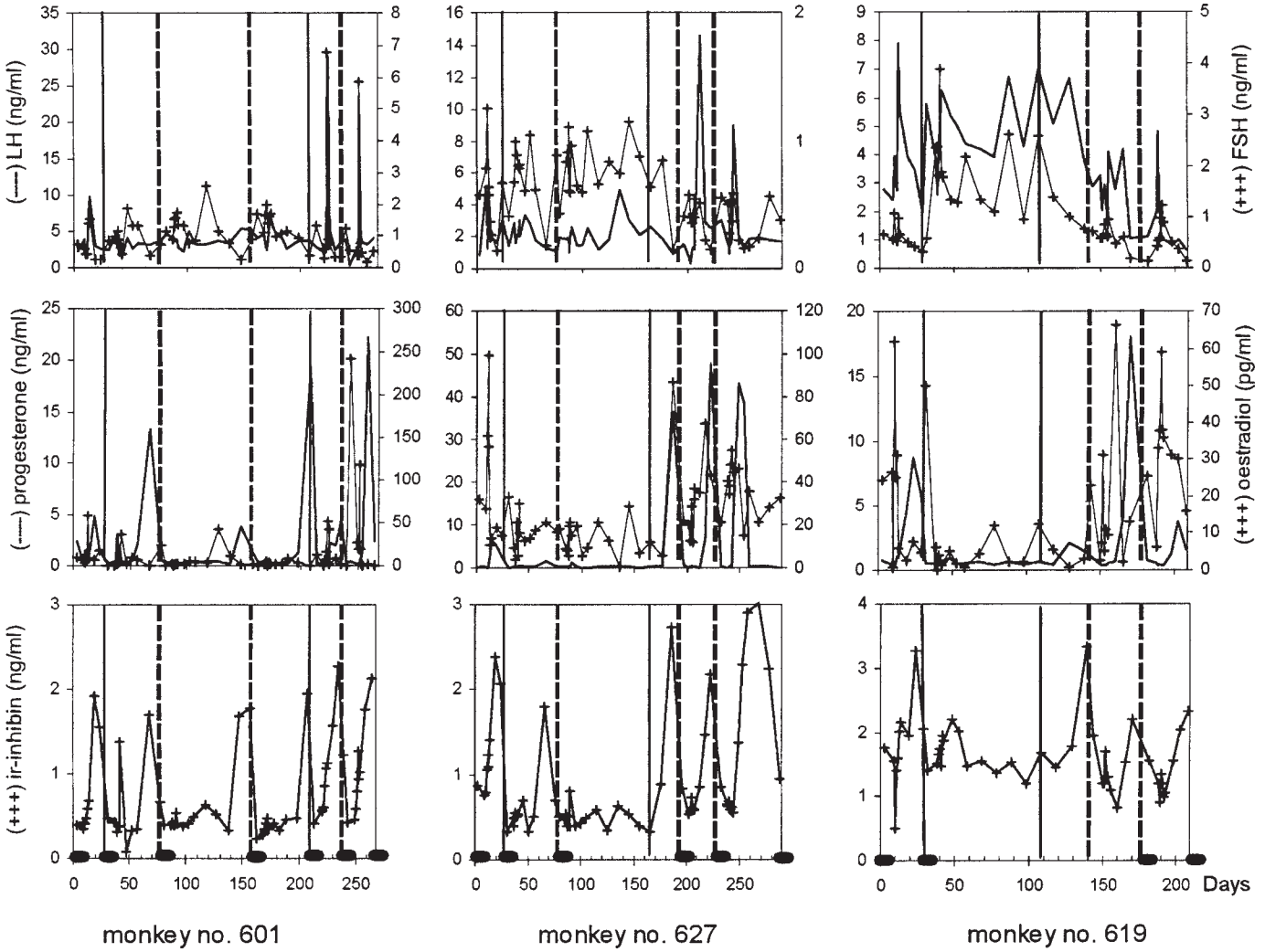

Fig. 1. Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey nos. 601, 627, and 619 treated with PM-10. D 1 represents the first day of menstruation in the pretreatment period. The short horizontal bars represent the day of menses. Period between solid vertical lines represents the treatment period.

nificantly and recovered during the post-treatment period; meanwhile, basal levels of LH did not differ from pre-treatment levels. There was no evidence of estradiol, progesterone, or ir-inhibin surges during PM treatment in any monkeys. Suppression of these hormones recovered during the post-treatment period in some monkeys (monkey nos. 616 and 801).

Profiles of serum gonadotropins and ovarian hormones in monkeys treated with PM-1000, as shown in Fig. 3 were obviously suppressed. There was no surge of serum FSH, LH, estradiol, progesterone, or ir-inhibin throughout the treatment and post-treatment periods in all monkeys (nos. 624, 77, and 633). Furthermore, the basal levels of serum FSH and LH during the treatment period tended to be lower than those of the pretreatment period. Subsequently, their levels tended to recover to pretreatment levels during the post-treatment period in all monkeys. Basal levels of serum estradiol, progesterone and ir-inhibin during the treatment and post-treatment periods were lower than those of the pretreatment levels.

Discussion

Our study demonstrates the effect of PM, an indigenous Thai herb, on changes in menstrual cycle length and related hormones in adult female monkeys. This experiment clearly demonstrated that estrogenic effect of PM disturbs the menstrual cycle of monkeys. The menstrual cycle length of monkeys treated with PM increased in a dose-dependent manner. The highest dose of PM (PM-1000) resulted in complete menstrual cessation throughout the 90 d of PM treatment and 60 d post-treatment. The lower doses of PM (PM-10 and PM-100) showed the prolongation of menstrual cycle length during the treatment period and the recovery during the post-treatment period in some monkeys (2/3 and 1/3 of monkeys treated with the doses of 10 and 100 mg/d, respectively).

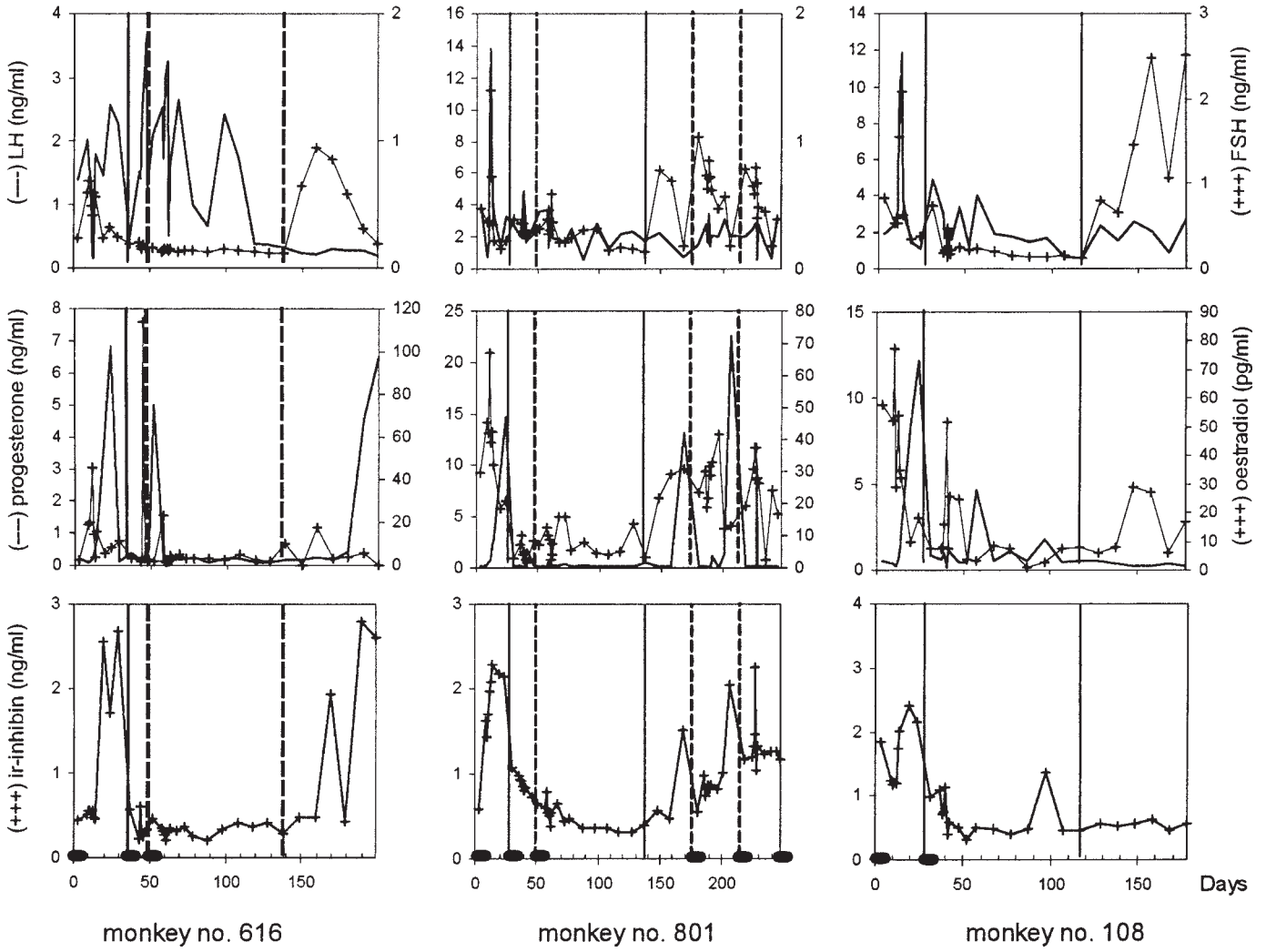

Fig. 2. Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey nos. 616, 801, and 108 treated with PM-100. The meanings of d 1, short horizontal bars, and solid vertical lines are in Fig. 1.

The prolongation of the menstrual cycle length corresponded with the decrease in serum profiles of FSH and LH during PM treatment and depended on dose. Monkeys treated with the highest dose of PM (PM-1000) showed the complete suppression of FSH and LH serum profiles throughout the 90-d treatment period and the 60-d post-treatment period. The suppression of these hormone levels very strongly indicates that use of PM interrupts regular menstrual cycles. The medium dose of PM (PM-100) also suppressed serum FSH and LH levels during the treatment period, but the lowest dose of PM (PM-10) did not change FSH or LH serum levels.

Normally, FSH stimulates granulosa cell aromatization of testosterone to estradiol during the follicular development. In turn, estradiol supports the follicular growth and development by increasing FSH receptor, inducing stimulated estrogen production from the developed granulosa cells. FSH together with estradiol regulates the expression of LH receptor on the granulosa cells of large follicles during the late follicular phase, inducing an increase in serum LH and ovulation *(19)*. Accordingly, our results, indicating FSH and LH suppression during PM treatment, may infer that PM containing phytoestrogens impairs follicular growth and development due to decreased FSH and LH levels from the anterior pituitary gland. The decrease in both basal and peak levels of estradiol during the entire menstrual cycles indicates that ovarian function in the monkeys is impaired.

Moreover, the high levels of estradiol and progesterone, which were largely secreted from the corpus luteum *(20)*, decreased significantly during the luteal phase. Estradiol and progesterone levels are known to correlate positively with ovulation and the corpus luteum function. Thus, the decrease in both serum estradiol and progesterone levels during the luteal phase can be assumed as the indicator of anovulation of the menstrual cycle *(21)*. Furthermore, inhibin is also produced by the corpus luteum and the level is greatest during the luteal phase of the menstrual cycle in both women *(20)* and monkeys *(23–25)*. The secretion pattern of inhibin

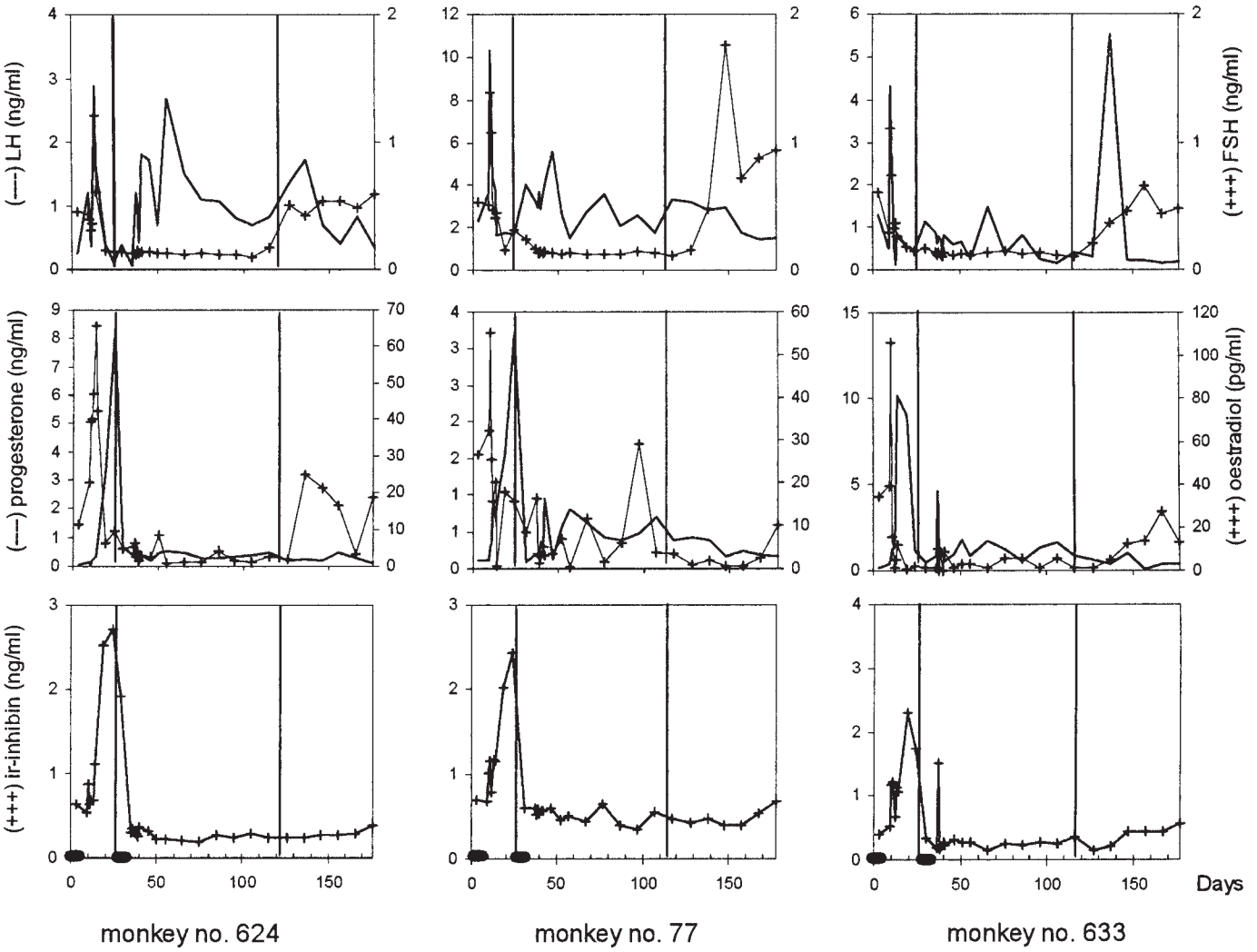

Fig. 3. Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey nos. 624, 77, 633 treated with PM-1000. The meanings of d 1, short horizontal bars, and solid vertical lines are in Fig. 1.

during the luteal phase shows a positive correlation with progesterone levels and a negative correlation with FSH levels *(21,25)*. The major stimulus of ir-inhibin secretion during the luteal phase is LH, *(21,25)*, as shown in the study of LHRH antagonist to inhibit the LH secretion *(23)*; accordingly, the decrease in serum ir-inhibin and progesterone levels throughout the treatment support our hypothesis.

Estrogenic effects of PM phytoestrogens are concurrent with the previous reports investigating the effect of soy phytoestrogens. Premenopausal women with regular ovulatory cycles who ingested soy protein containing 25–60 mg of isoflavones for 1–2 mo had an extension in the follicular phase length and delayed menstruation *(11,14)*. In addition, there were the suppression of FSH and LH surge at the follicular phase *(11–13)* and decreased levels of estradiol and progesterone during the entire menstrual cycle *(14,15)*. However, our previous study could not detect the suppression of serum profile gonadotropins and ovarian hormones in adult cyclic monkeys treated with a single dose of 10, 100, and 1000 mg/d of PM, even though length of menstrual cycle of the monkeys treated with 1000 mg/d of PM prolonged to 42.00 ± 0.04 d ($p = 0.004$) and 39.63 ± 0.67 d ($p = 0.003$) compared with the control (30.56 ± 1.30 d) (*8*). The differences of our results are due to phytoestrogenic concentrations circulating in the body, since phytoestrogens are rapidly removed from circulation in the body. The half-life of genistein, daidzein, and equol were 7, 4, and 9 h in women and 4, 3, and 5 h in men, respectively *(26)*. Concentration of genistein and daidzein in serum are highest at 5.5 and 7.4 h in premenopausal women and cleared from the body within 48 h *(27)*. So, our previous study could not detect changes of serum hormones after 72 h of a single feeding of PM. Daily feeding of PM containing phytoestrogens resulted in the accumulation of phytoestrogens in the blood as it reached to threshold for disturbing hormonal levels in this study.

The potential effect of PM phytoestrogens is mediated by estrogen receptor (ER) at target sites including the pituitary gland, hypothalamus, gonads, and uterus *(28)*. Previ-

ous studies of chemical structures of phytoestrogens indicated that phytoestrogens, which are heterocyclic phenols with a close similarity in structure to estradiol, are necessary for binding to ER, which can then act as estrogen agonist *(29)*. Therefore, phytoestrogens contained in PM may have an estrogenic effect by direct interaction with the ER, at least at the levels of pituitary and/or ovary and result in decreased serum levels of FSH and LH secreted from the pituitary by a negative feedback mechanism.

In recent years, the tuberous roots of PM were analyzed with high performance liquid chromatography (HPLC) and found that they contain the high amounts of isoflavones (1.69 mg/g of dried weight) and the lower amounts of miroestrol, deoxymiroestrol, and other compounds *(30)*. Therefore, we can deduce that isoflavones are major phytoestrogens of PMs effect on gonadotropins and ovarian hormones. However, the present study could not eliminate the potential role of other phytoestrogens such as miroestrol. It is of interest to investigate the half-life and pharmacokinetics of the individual phytoestrogens contained in PM in further study, and also to examine the effect of each component on reproductive function and related hormones.

In summary, our findings strongly indicate that PM containing phytoestrogens can disturb the menstrual cycle and have an endocrine-modulating effect by acting as a suppressor on ovulation, resulting from a decrease in serum levels of gonadotropins and ovarian hormones.

Material and Methods

Animals

Nine adult female cynomolgus monkeys (*Macaca fascicularis*) with regular menstrual cycles (30 ± 4 d in length) for at least four consecutive months and weighing from 4.0 to 6.5 kg before the study were used. The first day of menstrual bleeding was designated as d 1 of the menstrual cycle. The monkeys were housed in individual cages at the Primate Research Unit, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Lighting conditions of the animal room were controlled (12:12 h light to dark cycle). Temperature and humidity fluctuated slightly depending on the season. The monkeys were fed daily with monkey chow (Pokaphan Animal Feed Co., Ltd., Bangkok, Thailand) in the morning (0900–1000 h) and supplemented with fresh fruits in the afternoon (1400–1500 h). The experimental protocol was approved in accordance with a guide for the care and use of laboratory animals prepared by Chulalongkorn University.

Experimental Design

Nine female monkeys were divided into three groups. Each monkey group was fed with a suspension of PM at doses of 10, 100, and 1000 mg/5 mL of distilled water/individual/d (abbreviated as PM-10, PM-100, and PM-1000, respectively) at 0800 h. The treatment schedule consisted of three periods: the pretreatment, treatment, and post-treatment. During the pretreatment and post-treatment periods, all monkey groups were fed daily with 5 mL of distilled water at 0800 h for one and two menstrual cycles, respectively. During the treatment period, all monkey groups were fed with the suspension of PM for three menstrual cycles. However, if monkeys had no menstrual bleeding during the treatment and post-treatment periods, the treatment time was proceeded to 90 and 60 d, respectively. Three milliliter blood samples were collected from the femoral vein without anesthetization between 0800 and 0900 h on d 3 (the early follicular phase); d 9, 10, 11, 12, 13, 14 (the late follicular phase); d 19, 24, and d 29 (the late luteal phase) of the menstrual cycle. However, if monkeys did not show menstrual bleeding, blood samples were collected every 10 d until 90 and 60 d during the treatment and post-treatment periods, respectively. Blood samples were centrifuged at 4°C, 1,700*g* for 20 min and stored at –20°C until FSH, LH, estradiol, progesterone, and immunoreactive (ir)-inhibin were assayed. The occurrence of menstrual bleeding was checked daily by vaginal swabbing method.

P. mirifica *Suspension Preparation*

Fresh tuberous roots of PM were sliced, desiccated in a hot air oven at 70°C, and subsequently ground into 100-mesh powder. The powdered stock used for the entire study was kept in the dark desiccator until preparation into suspension with distilled water. The PM suspension was prepared every 7 d and kept in a dark bottle at 4°C until feeding time.

Hormonal Analysis

Serum FSH and LH levels were analyzed by a heterologous radioimmunoassay (RIA) described previously *(31,32)*. Iodinated preparations were rat NIDDK rat FSH -I-5 and rat NIDDK rat LH-I-5, and antisera were anti-ovine FSH (NIDDK-H-31) and anti-ovine LH (YM#18), respectively. These assays specify for determination of FSH and LH levels in monkeys *(31,32)*. The results are expressed in terms of NIDDK rat FSH-RP-2 and NIDDK rat LH-RP-2. The intra- and interassay coefficients of variations were 5.82% and 7.32% for FSH and 5.71% and 7.48% for LH, respectively.

After extraction by fresh diethyl ether, serum estradiol levels was determined by RIA with ^{3}H-labeled radioligands as described in the established method of the World Health Organization *(33)*. The intra- and interassay coefficients of variations were 5.07% and 7.02% for estradiol. Serum concentrations of progesterone were determined by a double-antibody RIA system using ^{125}I-labeled radioligands as described previously *(34)*. The intra- and interassay coefficients of variations were 7.45% and 7.72% for progesterone.

Serum concentrations of ir-inhibin were measured by a double-antibody RIA, as described previously *(35)*. 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 interassay coefficients of variations were 5.47% and 6.78%.

Statistical Analysis

The data of menstrual cycle length were expressed as mean ± SEM. Analysis of variance (ANOVA) evaluated the significance of the differences between the mean. The observed significance was then confirmed using the least significant difference (LSD) test. $p < 0.05$ was considered statistically significant.

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