Chapter III

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

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 (FSH), luteinizing hormone (LH), estradiol, or progesterone did not change during the first and second menstrual cycles of the post-treatment period for all monkey groups. Our findings demonstrate that although a clear 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.

Key words: *Pueraria mirifica*, phytoestrogen, monkey, reproductive hormone, menstrual cycle

3.1 Introduction

Phytoestrogens, which are structurally and functionally similar to17β-estradiol, are naturally occurring phytochemicals found in plants and plant products (Murkies et al., 1998). 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 (Wakai et al., 1999; Horn-Ross et al., 2000; Liggins et al., 2000; Boker et al., 2002). Estrogenic effects of phytoestrogens were reported on hormonal and reproductive disturbances in both animals (Whitten et al., 1993, 1995; Medlock et al., 1995; Burton and Wells, 2002) and humans (Wilcox et al., 1990; Phipps et al., 1993; Whitehead et al., 2002) to include causing infertility in sheep (Bennetts, Underwood and Shier, 1946), the reduction of ovulation rate in mice (Fredcricks et al., 1981), and disruption of reproductive hormones and ovarian function in cyclic women (Cassidy et al., 1994, 1995; Duncan et al., 1999; Kurzer, 2000). In addition, epidemiological studies have shown that premenopausal and postmenopausal Japanese and Chinese women who consumed high amounts of isoflavones from soy had a decrease in serum levels of estrone and estradiol (Bernstein et al., 1990; Shimizu et al., 1990; Nagata et al., 1998).

Pueararia mirifica (*P. mirifica*) is an indigenous Thai herb of the family Leguminoceae. Its tuberous roots also contain phytoestrogenic substances including miroestrol (Pope et al., 1958), puerarin (Ingham et al., 1986a), deoxymiroestrol, kwakhurin (Chansakaow et al., 2000a, 2000b), and other phytoestrogens that belong to the isoflavone and coumestrol class (Ingham et al., 1986b, 1988, 1989). Miroestrol compound isolated from the roots of *P. mirifica* prevented the implantation of blastocysts, promoted uterine weight and vaginal growth, and increased vaginal fluid in normal female rats (Pope et al., 1958), and produced cornification of the

vaginal epithelium in ovariectomized-adrenalectomized rats (Jones and Pope, 1960; Cantero et al., 1996), but did not stimulate the secretion of endogenous estrogen by the ovaries or the adrenal gland (Jones and Pope, 1960). Miroestrol also exhibited mammogenic potency in both ovariectomized rats and mice by restoring the mammary duct growth as estradiol did (Pope et al., 1958). The potency of subcutaneously injected miroestrol is about 0.7 times that of estradiol and twice as potent as estrone (Benson et al., 1961).

In recent years, *P. mirifica* has been widely used in premenopausal 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 (Hotchkiss et al., 1982; Harrison et al., 1999). 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.

3.2 Materials and Methods

3.2.1 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 4.0 – 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 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 the Primate Research Unit, Faculty of Science, Chulalongkorn University.

3.2.2 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 1000 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 and 29 (the late luteal phase); and

every 10 days until the next menstruation. Blood samples were immediately centrifuged at 4 °C, 1,700 x g for 20 min. The serum was then separated and stored at −20 °C until follicle stimulating hormone (FSH), luteinzing hormone (LH), estradiol, and progesterone 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.

3.2.3 Hormonal Analyses

Concentrations of serum FSH and LH were measured by heterologous 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) and anti-ovine LH (YM#18) (Hodgen et al., 1976). Antiserum against ovine LH (YM#18) was kindly provided by Dr.Y.Mori (University of Tokyo, Tokyo, Japan). After extraction of ether, serum levels of estradiol and progesterone were determined by double-antibody RIA with ^{3}H-labeled radioligands as described in the established method of World Health Organization (WHO) (Sufi, Donaldson, and Jeffcoate, 1986).

3.2.4 Statistical Analysis

All data including changes of menstrual cycle length and serum levels of hormones were expressed as the mean $\pm$ 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.

3.2.5 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. 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.

3.3 Results

3.3.1 Changes in Menstrual Cycle Length of Monkeys Treated with *P. mirifica*

Nine monkeys had normal menstrual cycle length during the pre-treatment period for 30.56 ± 1.30 days, as shown in Table 3.1. 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*.

3.3.2 Serum Levels of Gonadotropins and Ovarian Hormones in Normal Cycling Monkeys

Serum profiles of gonadotropins (FSH and LH), and ovarian hormones (estradiol, and progesterone) in 13 normal cyclic monkeys during the menstrual cycle

are shown in Figure 3.1 and Table 3.2. Changes in these hormonal profiles during 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 Figure 3.1, peak levels of serum FSH and LH were 1.82 $\pm$ 0.34 and 8.27 $\pm$ 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 levels remain low during the late follicular phase, and then become slightly elevated during the early luteal phase, indicating that cyclic ovulation occurred in the menstrual cycle.

3.3.3 Changes in Serum Gonadotropins and Ovarian Hormones in the Monkeys Treated with *P. mirifica*.

As shown in Figures 3.2, 3.3, and 3.4, there were no apparent changes in serum levels of FSH, LH, estradiol, or progesterone throughout the first and second menstrual cycles as compared to controls 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 levels were low throughout the early follicular phase and gradually increased in the early luteal phase of the 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 (Figure 3.4).

Table 3.1 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	$42.00 \pm 4.04^*$ ($P = 0.004$)	$39.67 \pm 0.67^*$ ($P = 0.003$)

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 ($P <
0.05$).

Table 3.2 Mean concentrations of serum gonadotropins and ovarian hormones during the late follicular phase and the early luteal phase in normal cyclic monkeys

	LH (ng/ml)	FSH (ng/ml)	estradiol (pg/ml)	progesterone (ng/ml)
Day of peak levels	0	0	0	5
Late follicular phase				
- highest levels	8.27 $\pm$ 0.86	1.82 $\pm$ 0.34	59.89 $\pm$ 10.66	0.89 + 0.53
- lowest levels	1.60 $\pm$ 0.33	0.51 $\pm$ 0.03	27.54 $\pm$ 4.31	0.27 $\pm$ 0.06
Early Luteal phase				
- highest levels	3.93 $\pm$ 0.72	0.94 $\pm$ 0.11	39.55 $\pm$ 14.22	5.09 $\pm$ 0.85
- lowest levels	1.29 $\pm$ 0.20	0.28 $\pm$ 0.05	11.99 $\pm$ 4.98	1.19 $\pm$ 0.3

The day of LH surge (Day 0) was used as a reference point for the separation between the follicular phase and the luteal phase. Results are expressed as mean $\pm$ S.E.M (n = 13).

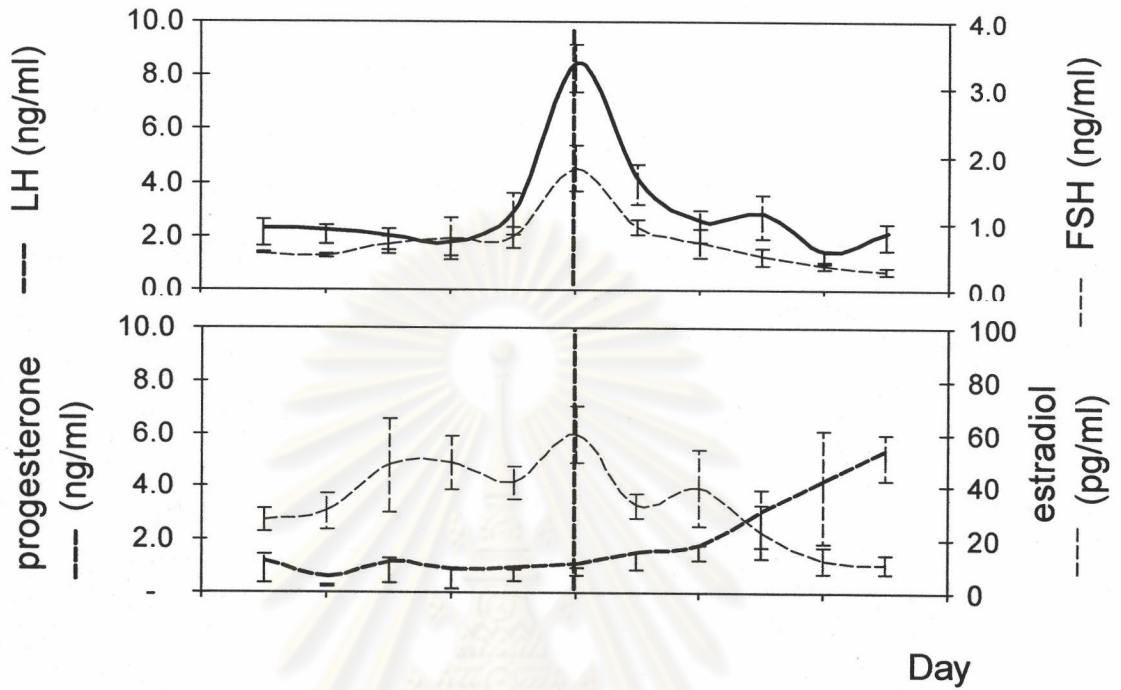**Figure 3.1** Serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of normal cyclic monkeys.
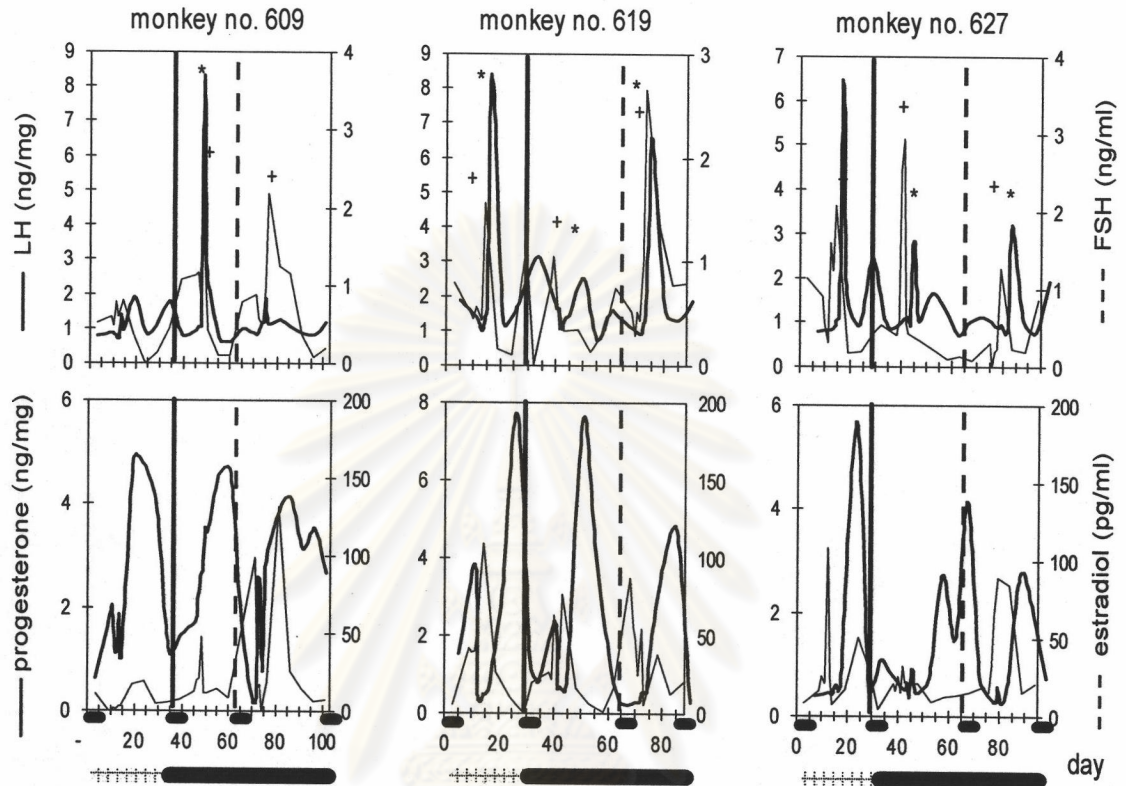
Figure 3.2 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of monkeys treated with 10 mg of *P. mirifica*. Horizontal bars represent the day of menses. Day 1 represents the day of menses in the pre-treatment period. The pre-treatment and post-treatment 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.
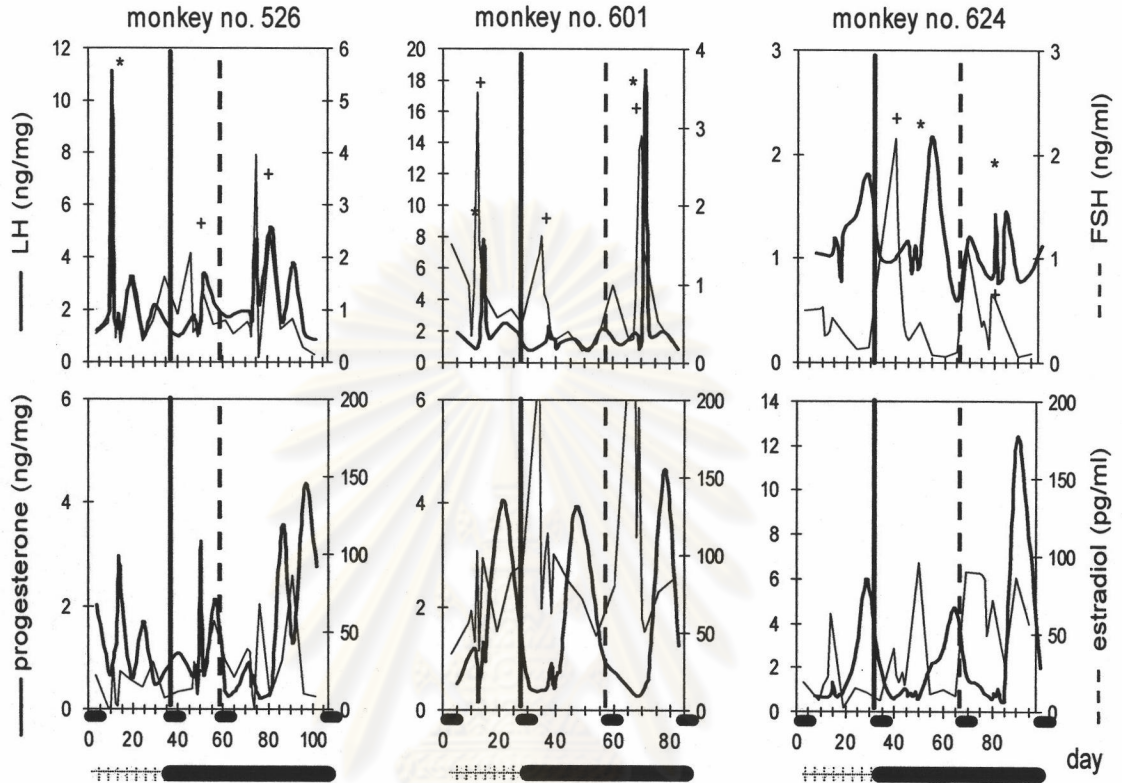
Figure 3.3 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of monkey treated with 100 mg of *P. mirifica*. Day 1 represents the day of menses in the pre-treatment period. The meanings of horizontal bars, vertical solid and vertical dotted lines, and symbols are shown in Figure 3.2.
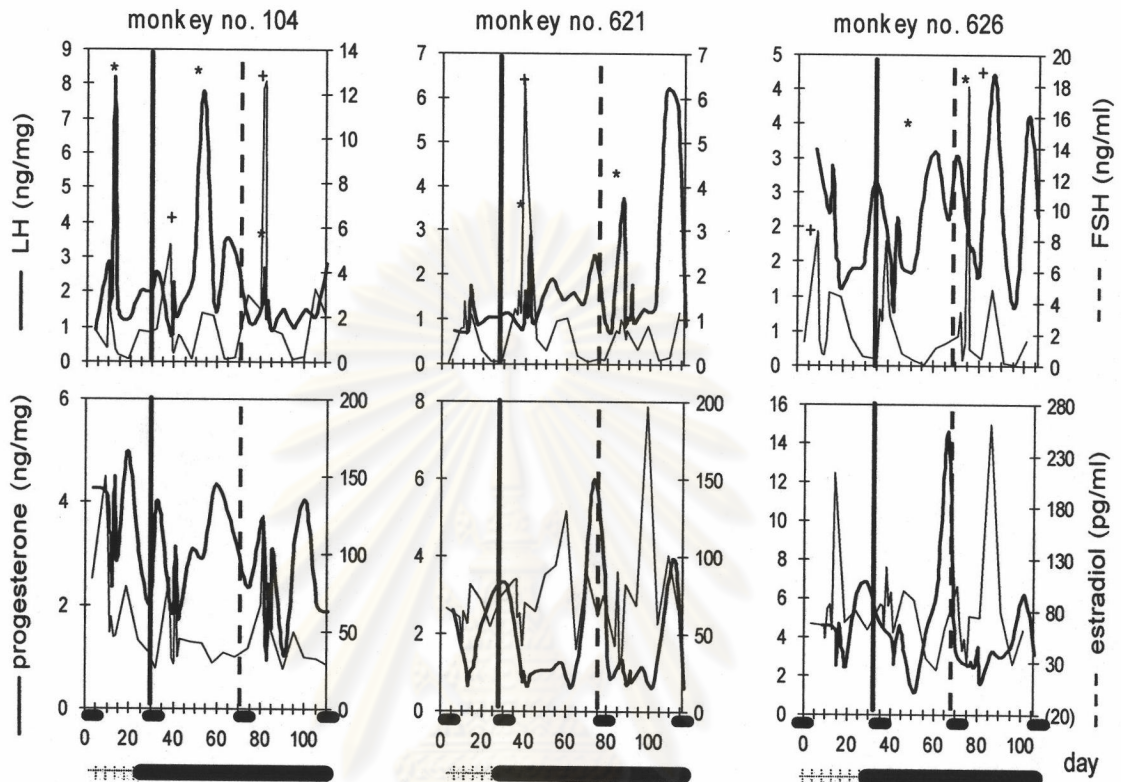
Figure 3.4 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of monkey treated with 1,000 mg of *P. mirifica.* Day 1 represents the day of menses in the pre-treatment period. The meanings of horizontal bars, vertical solid and vertical dotted lines, and symbols are shown in Figure 3.2.

3.4 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 medium doses (10 and 100 mg of *P. mirifica*). Additionally, the present study did not observe an apparent changes in profile levels of serum FSH, LH, estradiol, or progesterone 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) (Muangman and Cherdshewasart, 2001) compared to soybean using the HPLC technique (Murkies et al., 1998). There are many published reports showed that isoflavones from soy is the major component that has an effect on the reproductive system (Cassidy et al., 1994, 1995; Duncan et al., 1999). 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 (Duncan et al., 1999; Lu et al., 1996, 2000), 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 (Cassidy et al 1994), and luteal phase length increased in premenopausal women who ingested lignans from flax seed daily (Phipps et al., 1993).

Effect of phytoestrogens from soy on reproductive hormones in premenopausal women has also been demonstrated. There was no change (Lu et al., 2000) or

significant decrease in serum levels of FSH and LH in premenopausal women who consumed daily dietary phytoestrogens throughout their menstrual cycle (Cassidy et al., 1995; Duncan et al., 1999; Nicholls et al., 2002). Serum levels of estradiol and progesterone showed no change (Duncan et al., 1999) or decreased (Lu et al., 1996, 2000).

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 (McGarvey et al., 2001). Estradiol administration also has an effect on reduction in both pulsatile GnRH and LH secretion (McGarvey et al., 2001; Wuttke et al., 2003), and caused decreases in serum LH and FSH levels (Wildt et al., 1981). The inhibitory effect of coumestrol on LH pulse frequency was greater than that of estradiol (McGarvey et al., 2001). This evidence 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 (Berga et al., 1989; Scheweiger et al., 1989) and luteal phase deficiency (Soules et al., 1989). There is a high correlation between pulse amplitude and frequency of LH and mean LH levels (Bennet et al., 1991).

However, our study could not detect clear 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 (Murkies et al., 1998). 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 (King and Bursill, 1988). 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 (Lu and Anderson, 1998). 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 (Busby et al., 2002). The elimination rates of isoflavones from the circulation are different and affected by sex. Lu and Anderson (1998) shows 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 (Setchell et al., 2003). 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 menstrual 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, the present 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, and estradiol decrease during the menstrual cycle. These changes are consistent with an increase in length of the follicular phase and total menstrual cycle (Mais et al., 1986; Kettle et al., 1991). 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.

Acknowledgments

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