Assessment of Urinary Gonadotropin and Steroid Hormone Profiles of Female Cynomolgus Monkeys after Treatment with *Pueraria mirifica*

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Abstract. This study investigated the changes in the urinary hormone levels of female monkeys (*Macaca fascicularis*) after single-dose and long-term treatments with *Pueraria mirifica* (PM). The monkeys were separated into 3 groups (n=3) and orally treated with 10, 100, or 1,000 mg of PM in each group. Two series of experiments were performed. In the first series of experiments, the monkeys were orally treated with a single dose of PM. The experimental schedule was divided into a one menstrual cycle pretreatment period and a two menstrual cycle post-treatment period. In the second series of experiments, the monkeys were orally treated daily with PM for 90 days. The experiment schedule was divided into a one menstrual cycle pretreatment period, a three menstrual cycle treatment period, and a two menstrual cycle post-treatment period. Urinary samples were collected daily and assayed for the FSH, LH, estradiol, and progesterone levels. The results showed that there were no changes in the FSH, LH, estradiol, and progesterone levels after treatment with a single dose of 10, 100, or 1,000 mg of PM or after daily treatment with 10 mg of PM for 90 days compared with the levels observed during the pretreatment period. Daily treatment with 100 mg and 1,000 mg of PM for 90 days only produced a clear reduction in the urinary FSH levels. This suggests that changes of urinary FSH levels can be considered an indicator for study of estrogenic effects on hormonal levels in female monkeys.

Key words: Gonadotropins, *Macaca fascicularis*, Monkey, Phytoestrogen, *Pueraria mirifica*, Sex steroid hormones, Urine

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Investigators wishing to examine hormonal changes in blood samples should consider the ethics of animal use and the health problems of the subject animals. Blood loss from sampling can induce stress and increase plasma adrenocorticotropic hormone (ACTH) and insulin levels [1]. In addition, immobilization and anesthetization during blood sampling induces animal stress and produces changes in hormones related to reproduction, such as luteinizing hormone (LH) [2–4].

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PM, a Thai herbal plant, belongs to family Leguminosae [5–7]. The PM root has been analyzed and found to contain many phytoestrogens, including isoflavone genistein, daidzein, and coumestrol [5]. PM influences the reproductive system by reducing serum gonadotropin levels and increasing uterine weight and vaginal cornification in rats [8]. Our previous studies have found that daily treatment with PM induces suppression of serum follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and progesterone in adult female cynomolgus monkeys (Macaca fascicularis) in a dose-dependent manner [6], but also that treatment with a single dose of PM has no effect on these hormones [7]. Reduction of serum gonadotropin and sex steroid hormone levels from long-term PM treatment implies disturbance of folliculogenesis and ovulation in monkeys. However, the examination of serum hormonal levels by daily blood sampling can induce stress in animals. To avoid this stress, a non-invasive method of specimen collection should be considered as an alternative choice for the study of changes in hormone levels.

In this study, we investigated the effects of PM treatment on the changes in gonadotropins, estradiol, and progesterone levels in the urine of adult cyclic monkeys. We hypothesized that the changes in these hormone levels in urine would be congruent with the levels in serum as shown in our previous reports [6, 7]. If the gonadotropin and ovarian hormone levels are the same in serum and urine, urinary gonadotropin and ovarian hormone levels would be a good indicator of the effect of PM for use in further study in order to avoid stress on subject animals from experimental procedures.

Materials and Methods

Animals

Adult female cynomolgus monkeys with at least 4 consecutive regular menstrual cycles before the start of this study were chosen for the study. Menstruation of the monkeys was monitored daily by visual observation and the vaginal swabbing method. The first day of menstruation was designated as day 1 of the menstrual cycle. The monkeys were housed in individual cages at the Primate Research Unit, Faculty of Science, Chulalongkorn University (Bangkok, Thailand). The lighting conditions of the animal house were controlled and included a 12 h light/12 h dark cycle. Temperature and humidity fluctuated slightly depending on the season. The monkeys were fed a monkey chow (Pokphand Animal Feed, Bangkok, Thailand) each morning (0900–1000 h), and this was supplemented with fresh fruit in the afternoon (1400–1500 h). The experimental protocol was approved by the ethics committee in accordance with the guide for care and use of laboratory animals prepared by Chulalongkorn University.

Study design

Experiment I: Effect of a single treatment of PM on hormone levels in urine

Nine adult cyclic monkeys were divided into three groups. Each group was treated with a suspension of 10, 100 and 1,000 mg of PM (abbreviated as PM-10, PM-100 and PM-1,000, respectively). The experimental schedule was separated into a one menstrual cycle pretreatment period and a two menstrual cycle post-treatment period. After the pretreatment period, the monkeys were fed a single dose of PM and observed for the two menstrual cycles during the post-treatment period. Urinary samples were collected daily and assayed for the hormone levels of FSH, LH, estradiol and progesterone.

Experiment II: Effect of long-term treatment with PM on hormone levels in urine

Nine adult cyclic monkeys were divided into three groups, PM-10, PM-100 and PM-1,000, as in Experiment I. The experimental schedule in Experiment II was divided into a one menstrual cycle pretreatment period, a three menstrual cycle treatment period, and a two menstrual cycle post-treatment period. All three monkey groups were fed 5 ml of distilled water daily during the pre- and post-treatment periods and 5 ml of PM suspension during the treatment period. Experiments were conducted during the 90-day treatment and 60-day post-treatment periods if no menstrual bleeding was detected.

Preparation of PM suspensions

PM Cultivar-Wichai III roots were collected in Chiang Mai Province, Thailand. The voucher herbarium specimen for the PM (no. BCU 11045) was deposited at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn
University (Bangkok, Thailand). In brief, fresh tuberous PM cultivar-Wichai III roots were sliced and desiccated in a hot air oven at 70°C and subsequently ground into powder as described in previous reports [5, 6]. Each dose of 100 mesh PM powder, 10, 100 and 1,000 mg, was suspended in 5 ml of distilled water and kept in a sunlight-protected bottle at 4°C until feeding time.

Urine collection
To avoid contamination by water from monkey food, the pans for urine collection were placed under monkey cages at 1800 h daily and were removed the next day at 0800 h. The 14-h urine samples were filtrated through cotton cloth, poured into 15-ml glass tubes, and centrifuged at 4°C, 1700 × g, for 20 min. The supernatant was collected, and aliquots were stored at –20°C for the FSH, LH, estradiol, and progesterone assays.

Urinary hormone measurement
The urine samples were analyzed radioimmunoassay for their FSH and LH levels using a heterologous radio immuno assay (RIA) system as described previously [9–11]. The iodinated preparations used were rat NIDDK-rat FSH-I-5 and rat LH-I-5. The antisera used were anti-ovine FSH (NIDDK-H-31) [10] and anti-ovine LH (YM#18) [9]. The antiserum against ovine LH (YM#18) was kindly provided by Dr. Y. Mori (University of Tokyo, Tokyo, Japan). The results are expressed in terms of NIDDK rat FSH-RP-2 and rat LH-RP-2. The intra- and inter-assay coefficients of variation were 7.8 and 8.5% for FSH and 7.8 and 10.3% for LH, respectively.

The urinary estradiol and progesterone levels were determined using a double-antibody RIA system and 125I-labeled radioligands as described previously [12–14]. Antisera against estradiol (GDN#244) and progesterone (GDN#377) were kindly provided by Dr. G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, USA). The intra- and interassay coefficients of variation were 2.7 and 7.9% for estradiol and 2.8 and 5.7% for progesterone.

The creatinine (Cr) level of each urinary sample was measured by the Jaffe method using an Autoanalyzer [15]. The creatinine levels were used to compensate for differences in urine concentration and volume. Urinary hormone levels were expressed as milligrams of creatinine.

Results
Urinary hormone profiles in the regular menstrual cycles of the adult monkeys
The mean length of the menstrual cycle during the pretreatment period of the 18 adult monkeys used in Experiments I and II was 29.3 ± 0.8 days. The urinary hormonal profiles of FSH, LH, estradiol and progesterone varied for each monkey and depended on the menstrual cycle length. Thus, the profiles were adjusted according to the day of urinary LH peak, which was designated as day 0 of the menstrual cycle for this determination. The menstrual cycle was separated into the follicular phase (before day 0) and luteal phase (after day 0). The hormonal profiles are only shown for 5 days in each phase.

As shown in Fig. 1, the urinary FSH and LH levels elevated simultaneously and reached a peak on day 0. The increase in urinary estradiol level coincided with the increase in the urinary FSH and
LH levels. The urinary FSH, LH and estradiol levels all declined simultaneously during the early luteal phase. The urinary progesterone levels fluctuated greatly throughout the late follicular and early luteal phases. No peak of urinary progesterone level was observed during the 10 days around day 0; however, the level tended to increase on day 5 of the luteal phase.

Experiment I: Effect of a single treatment of PM on hormonal profiles in urine

As shown in Figs. 2–4, the urinary hormone profiles of FSH, LH estradiol and progesterone after all PM treatments were similar to those during the pretreatment period. Comparison of the urinary hormone profiles of each monkey showed that there were no differences between the pre- and post-treatment periods. The peak levels of urinary FSH and LH for all monkeys treated with PM-10, PM-100 and PM-1,000 were observed in the middle of menstrual cycles and were comparable to the levels observed on day 0 of the regular menstrual cycles.

Experiment II: Effect of long-term treatment with PM on hormonal profiles in urine

Figs. 5–7 show comparisons of the hormonal levels of urinary FSH, LH, estradiol and progesterone for the monkeys during the pretreatment period with those during the PM-10, PM-100 and PM-1,000 treatment and post-treatment periods.

As shown in Fig. 5, no changes were observed in the urinary gonadotropin, estradiol, or progesterone profiles of the 3 monkeys (nos. 601, 619 and 627) treated with PM-10 throughout the treatment and post-treatment periods compared with the pretreatment period.

As shown in Fig. 6, monkey nos. 616 and 801 had shorter menstrual cycles at the beginning of the treatment period (15 and 21 days, respectively) and ceased menstruation afterward. No urinary FSH surges were found during PM-100 treatment. Although monkey no.108 had complete cessation of menstruation throughout the 90-day treatment and 60-day post-treatment periods, the peak level of FSH was only observed at the beginning of the
Fig. 3. Urinary FSH, LH, estradiol and progesterone profiles during the pretreatment and post-treatment periods in monkeys treated with a single PM-100 dose. The meanings of the horizontal and vertical lines are explained in Fig. 2.

Fig. 4. Urinary FSH, LH, estradiol and progesterone profiles during the pretreatment and post-treatment periods in monkeys treated with a single PM-1,000 dose. The meanings of the horizontal and vertical lines are explained in Fig. 2.
Fig. 5. Urinary FSH, LH, estradiol and progesterone profiles during the pretreatment, treatment and post-treatment periods in monkeys treated with PM-10 daily for 90 days. The meanings of the horizontal and vertical lines are explained in Fig. 2.

Fig. 6. Urinary FSH, LH, estradiol and progesterone profiles during the pretreatment, treatment and post-treatment periods in monkeys treated with PM-100 daily for 90 days. The meanings of the horizontal and vertical lines are explained in Fig. 2.
treatment period. After cessation of PM-100 treatment, urinary FSH levels tended to increase in all monkeys. The urinary estradiol levels of monkey no. 616 were decreased during PM-100 treatment; however, no changes were observed in the urinary estradiol profile of monkey nos. 801 and 108. We did not detect any clear changes in the hormonal profiles of urinary LH or progesterone throughout the PM treatment and post-treatment periods. It is noteworthy that high fluctuations in urinary LH and progesterone levels were observed throughout the experimental period.

As shown in Fig. 7, the 3 monkeys (nos. 77, 624 and 633) treated with PM-1,000 ceased menstruation throughout the 90-day treatment and 60-day post-treatment periods. Monkey nos. 624 and 633 did not exhibit peak levels of urinary FSH throughout the treatment period, whereas monkey no. 77 showed a transient increase in urinary FSH levels during the early PM treatment period. After cessation of PM-1,000 treatment, urinary FSH levels tended to increase in all monkeys, especially in monkey no. 77. Urinary estradiol levels were suppressed throughout the treatment period in all monkeys and tended to increase during the latter half of the post-treatment period. The urinary LH and progesterone levels fluctuated throughout the treatment and post-treatment periods.

**Discussion**

The hormonal profiles of urinary gonadotropins, estrogen, and progesterone in the regular menstrual cycles of the female cynomolgus monkeys in the present study were similar to the profiles of these hormones in serum in our previous report [6]. Our results are in agreement with a previous study that reported similar patterns of gonadotropins in serum and urine in women [7]. Therefore, urinary gonadotropin should provide a useful index of serum gonadotropin secretion [16]. The urinary estradiol and progesterone levels fluctuated greatly throughout the entire menstrual cycle, although the progesterone levels tended to increase during the early luteal phase.

The present study examined the estrogenic effects of single-dose and long-term treatment with
PM on the profiles of urinary gonadotropin and sex steroid hormones in 18 cyclic female monkeys. As mentioned in the introduction, the gonadotropin and sex steroid hormone levels in the serum of 18 monkeys have been determined previously by our team and reported elsewhere [6, 7]. Thus, we hypothesized that the patterns of the gonadotropin and sex steroid hormone levels in urine after PM treatment could be congruent with those of the respective hormones in serum. A single treatment of PM at a dose of 10, 100, or 1,000 mg did not disturb the patterns of the urinary gonadotropin, estradiol, or progesterone levels throughout the two menstrual cycles of post-treatment period, which coincides with the patterns for these hormones in serum [7]. Daily treatment with PM for 3 menstrual cycles or 90 days, however, induced suppression of FSH and estradiol in urine in a dose-dependent manner. Li et al. [16] found that the peak urinary FSH levels were observed within 1 day of follicular collapse in 96.92% of menstrual cycles in premenopausal women, indicating that urinary excretion of FSH is a useful biomarker for estimating the day of ovulation. In addition to a prominent decrease in the basal levels of urinary FSH, peak levels of urinary FSH were absent from the monkeys treated daily with PM-100 and PM-1,000. This implies that daily treatment with PM can disturb folliculogenesis through suppression of serum and urinary FSH levels. This was partly proved by the fact that the menstrual cycle was either prolonged or disappeared. However, we could unable to detect prominent changes in LH levels in urine. It is possible that augmentation of LH secretion has been found at night [9]. In addition, the half-life of LH is short, approximately 50 min [17]. We believe that collection of the urine samples between 1800 and 0800 h caused the high fluctuation in the LH levels and resulted in inability to detect a difference in the LH profiles between the pre- and post-PM treatment periods.

Changes in the estradiol levels in urine were also found in the present study after long-term treatment with PM. Based on the latest information we have obtained, there are currently no reports concerning the effects of phytoestrogens consumption on the metabolism sex steroid hormones. Previous studies have suggested that PM phytoestrogens may act as estrogen and reduce estradiol levels through a decrease in gonadotropin secretion in serum [6] and may also have a direct effect on peripheral conversion of androstenedione to estradiol [18]. Previous reports have also shown that consumption of phytoestrogens from soy decreases the serum estradiol, estriol, and estrone-sulfate levels of post-menopausal women [19] and decreases the excretion of urinary estradiol and estrogen metabolites of premenopausal women [20]. Taken together, the decrease in urinary estradiol level is probably the result of suppression of estradiol production. Although the serum progesterone levels were suppressed during PM treatment in a dose-dependent manner in a previous study [6], we were unable to detect changes in urinary progesterone levels during the PM treatment period in the present study, and the urinary progesterone levels fluctuated greatly throughout the study period. The reason for this disagreements is unclear and could not be explained in the present study.

The present study clearly demonstrates that the profiles of FSH and estradiol in urine after single or long-term treatments with PM are closely related to the profiles of these hormones in serum. Nevertheless, of the four hormones examined in this study, urinary FSH levels are considered to be a candidate indicator for avoiding the stress on animals from experimental procedures in study of the estrogenic effects of PM on disturbance of the reproductive system.

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