Endocrine, *vol. 29, no. 1, 129–134, February 2006* 0969–711X/06/29:129–134/$30.00
ENDO (Online) ISSN 1559-0100

The Estrogenic Effect of *Pueraria mirifica* on Gonadotrophin Levels in Aged Monkeys

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We investigated the effect of *Pueraria mirifica* (PM) on gonadotrophin and estradiol levels in aged animals; nine menopausal cynomolgus monkeys were divided into three groups. Each group (*n* = 3) was fed with 10, 100, and 1000 mg/d of PM for 90 d. PM-10 induced the decrease of follicle stimulating hormone (FSH) levels on d 15–90 in one out of three monkeys. PM-100 and PM-1000 decreased FSH levels of all monkeys throughout the treatment period. After the treatment period, FSH levels continued to decrease for 5 and 10–20 d in PM-100 and PM-1000, respectively, and the levels rebounded in all groups thereafter. PM-10 decreased luteinizing hormone (LH) levels throughout the treatment period in one out of three monkeys and returned to the pretreatment levels immediately after stopping treatment. PM-100 and PM-1000 prominently decreased LH levels between d 10 and 90 during treatment and persisted until d 15–25 and d 20–30 for PM-100 and PM-1000, respectively, during the post-treatment period. Serum LH levels rebounded after returning to pre-treatment levels in a dose-dependent manner. Estradiol levels tended to decrease during the treatment period in all groups. The daily feeding of PM suppressed gonadotrophin levels in aged menopausal monkeys based on dose. Moreover, they can be recovered, and there is a direct correlation between dosage and recovery time. PM may be effective as an alternative medicine in menopausal women because the effects are not permanent.

Key Words: *Pueraria mirifica*; phytestrogen; gonadotrophin; estradiol; menopause monkey.

Introduction

Pueraria mirifica (PM), White Kwao Krua, is a Thai medicinal herb that belongs to the family Leguminoceae. Its tuberous root has been proved to be extremely rich in an isoflavone group of phytoestrogens *(1)*. Also present are small amounts of other phytoestrogens including coumestrol *(2–4)*, miroestrol *(5)*, puerarin *(3)*, deoxymiroestrol, and kwakhurin *(6,7)*. Miroestrol showed a high estrogenic potency when it was assayed by the immature mouse uterine weight and rat vaginal cornification tests *(5,8)*. Miroestrol treatment increased uterine weight in immature female mice *(8)* and produced the cornification of the vaginal epithelium in ovariectomized–adrenalectomized rats *(5)*. PM has recently attracted wide interest in biological research because of its estrogenic properties, as phytoestrogens are increasingly being studied as effective agents in biological systems. Our prior studies showed that a 14-d treatment of 100 and 1000 mg/kg BW/d of PM induced a vaginal cornification and increased uterine weight in ovariectomized rats *(9)*. A single feeding of 10, 100, and 1000 mg/d of PM prolonged the menstrual cycle length in adult cyclic cynomolgus monkeys *(10)*. Moreover, the long-term treatment suppressed serum levels of gonadotropins and ovarian hormones in a dose-dependent manner. The monkeys fed with the highest dose (1000 mg/d) showed symptoms of amenorrhea *(11)*. Clinical trials studying long-term consumption of PM showed reduced postmenopausal symptoms such as hot flush, sleep disorder, and skin dryness *(1)*. From the previous studies, however, there were no data of the effect of PM phytoestrogens on hormones related to reproduction in aged menopausal women that have an ovarian estradiol deficiency.

Several studies have shown that phytoestrogen isoflavones from soy disturb the reproduction and alter the secretion of gonadotrophins [follicle stimulating hormone (FSH) and luteinizing hormone (LH)] in both premenopausal *(12,13)* and postmenopausal women *(14)* who consumed soy daily. Additionally, epidemiological studies showed lower levels of estrone and estradiol in postmenopausal Japanese women who consumed high amounts of soy isoflavones *(15)*.

It is of interest to investigate the long-term effect of PM treatment on serum levels of FSH, LH, and estradiol in aged

Received June 1, 2005; Revised September 11, 2005; Accepted September 20, 2005.

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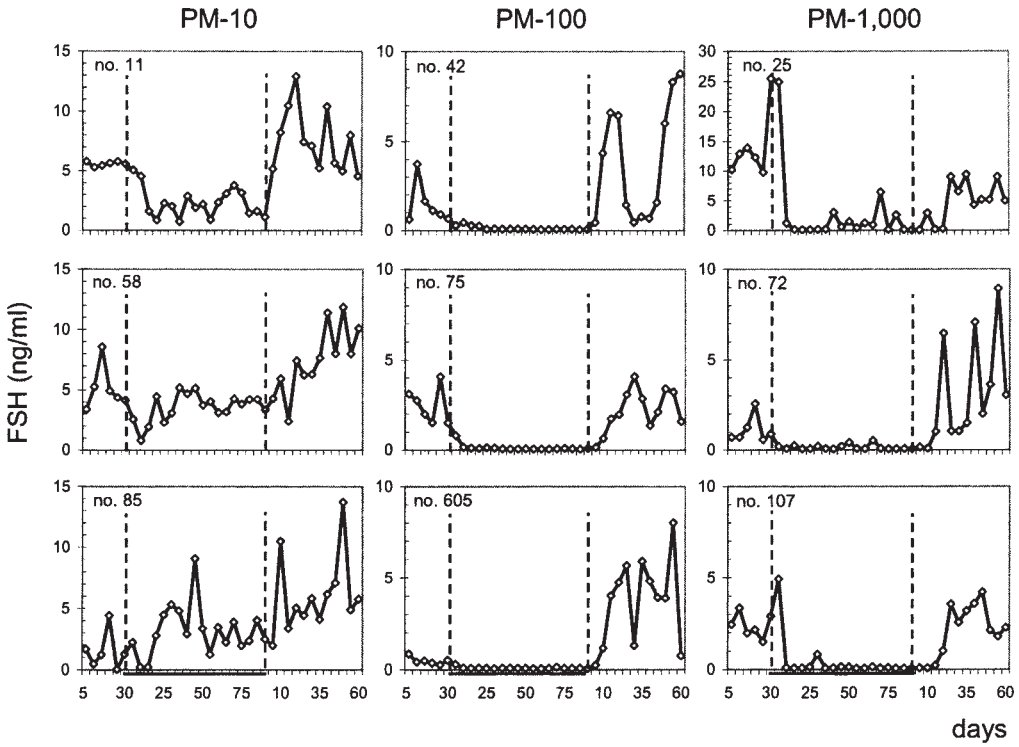

Fig. 1. Serum FSH levels during the pretreatment, treatment, and post-treatment period in aged menopausal monkeys treated with PM-10, PM-100, and PM-1000. The vertical lines separate each period; and a black horizontal line indicates the treatment period.

women. The study on the long-term effect of PM on humans is, however, very difficult to follow up because diet is difficult to control. Female cynomolgus monkeys (*Macaca fascicularis*) were used as a model in this study because of their similarity in the hormonal patterns and reproductive system to those of humans *(16)*. The present study, therefore, aimed to investigate the effect of daily treatment of PM for 90 d on gonadotropin and estradiol levels in aged menopausal monkeys.

Results

Changes in Serum FSH, LH, and Estradiol Levels in Aged Menopausal Monkeys Fed with PM

Changes in serum FSH, LH, and estradiol levels during the treatment and post-treatment periods compared with pretreatment levels are shown in Figs. 1–3. During PM-10 treatment, an obvious decrease in serum FSH levels was observed only in monkey no.11. After cessation of PM-10 treatment, serum FSH levels of all the monkeys (nos. 11, 58, and 85) were increased and higher than pretreatment levels. This is the so-called "rebound" (Fig. 1). In PM-100 and PM-1000 treatments, serum FSH levels of all monkeys were initiated to decrease on d 5–10 and continued decreasing to the end of the treatment period. FSH levels at the post-treatment period were increased gradually from about d 5–10 for PM-100 and d 10–20 for PM-1000 and followed by rebound.

As shown in Fig. 2, serum LH levels showed an obvious decrease throughout PM-10 treatment in monkey no. 11, but did not show this pattern in monkey nos. 58 and 85. All monkeys treated with PM-100 and PM-1000 showed a graded decrease in serum LH levels within 10 d of the start of the treatment period. After cessation of PM-100 and PM-1000 treatment, serum LH levels were maintained at a low level for the first half of the post-treatment period, and slightly increased after that.

As shown in Fig. 3, there were inconsistent changes in serum estradiol levels during PM treatment in all groups. Most of the monkeys treated with PM-10, PM-100, or PM-1000 did not show an obvious decrease in estradiol levels throughout the treatment period.

Efficiency of PM-10, PM-100, and PM-1000 on Hormone Levels

As shown in Figs. 1–3, there were interindividual variations of basal levels and PM-induced suppression of gonadotropin and estradiol levels in serum in addition to a limitation in a small number of monkeys. Serum hormone levels during the treatment and post-treatment periods were adjusted to percentage changes of the pretreatment levels. Percentage changes of hormonal levels of all three monkeys were mean thereafter (Fig. 4).

Compared with pretreatment levels, FSH levels decreased to 37.04, 19.33, and 6.69% on d 10 during PM-10, PM-100, and PM-1000 treatment, respectively. Then, FSH was retained at low levels throughout PM-100 and PM-1000 treatment (range 7.36–19.33% for PM-100 and 3.09–20.15% for PM-1000) and rebounded on d 10 for PM-100 and d 20 for PM-1000 during the post-treatment period. In PM-10

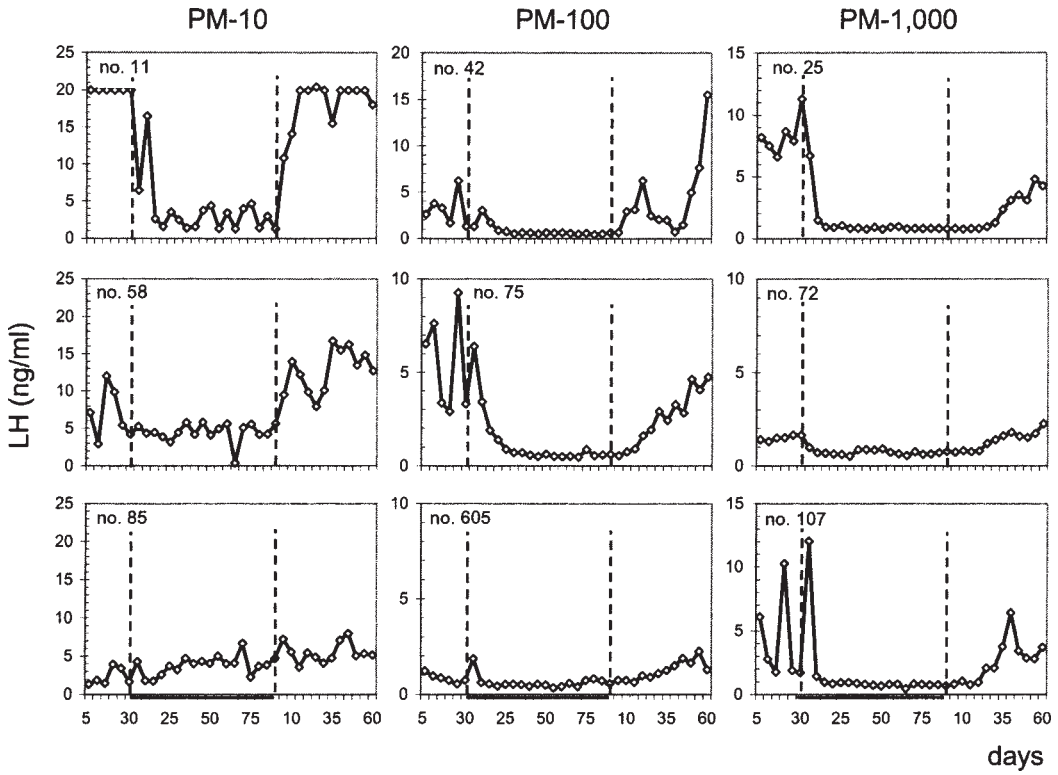

Fig. 2. Serum LH levels during the pretreatment, treatment, and post-treatment period in aged menopausal monkeys treated with PM-10, PM-100, and PM-1000. The meanings of the vertical and black horizontal lines are as indicated in Fig. 1.

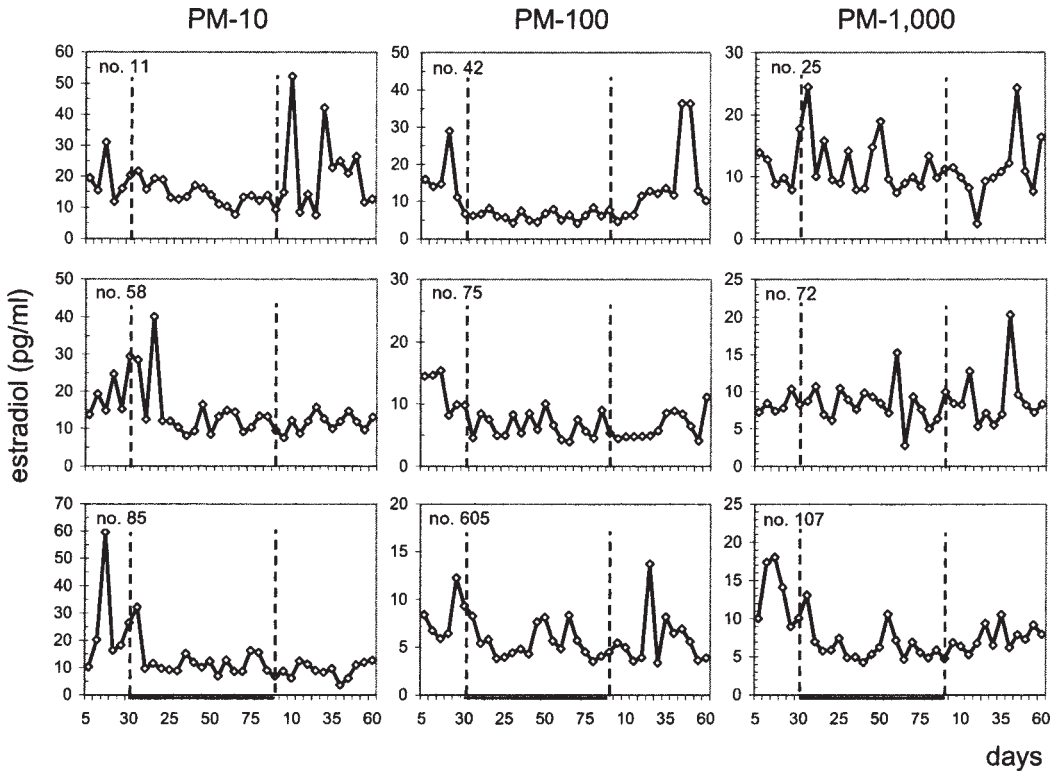

Fig. 3. Serum estradiol levels during the pretreatment, treatment, and post-treatment period in aged menopausal monkeys treated with PM-10, PM-100, and PM-1000. The meanings of the vertical and black horizontal lines are as indicated in Fig. 1.

treatment, FSH levels recovered and rebounded during the treatment period (range 37.04–244.67%). Likewise, LH levels were kept within the range 51.01–128.68% for PM-10, 22.70–77.32% for PM-100, and 18.86–32.98% for PM-1000 between d 10 and 90 during the treatment period. During the post-treatment period, LH levels could recover and rebound

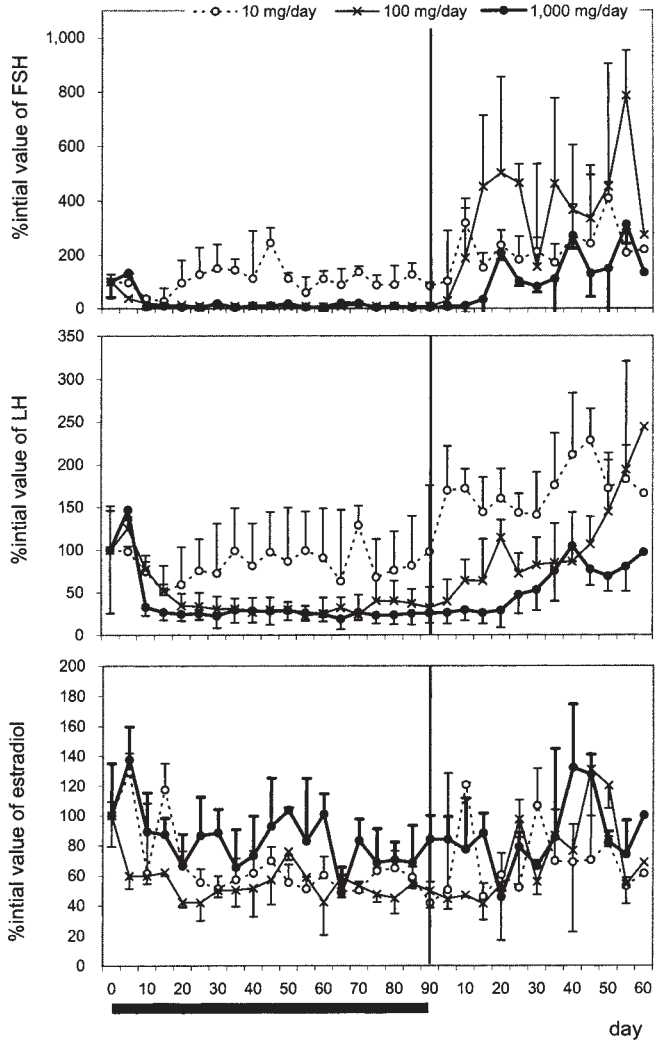

Fig. 4. Changes in serum FSH, LH, and estradiol levels during the treatment and post-treatment periods were adjusted to percentage changes from pretreatment levels, arbitrarily assigned at 100. A black horizontal line indicates the treatment period. Data are expressed as mean ± SEM.

on d 5 for PM-10 and d 50 for PM-100, but could not recover in PM-1000. Estradiol levels did not change throughout the treatment and post-treatment periods in PM-10 and PM-1000, but decreased during the treatment period in PM-100.

Discussion

In this study, we found that the feeding of PM induces a decrease in serum FSH and LH levels in aged menopausal monkeys in a dose-dependent manner. The highest dose (PM-1000) shows high efficiency in serum gonadotropin suppression. It was already known that PM contains many kinds of phytoestrogens *(1–7)*, mainly isoflavones, which present similar nonsteroidal structures and functions to those of estrogens. They can bind to estrogen receptors and exert estrogenic effects in mice *(8)*, rats *(17)*, monkeys *(10,11)*, and humans *(18–20)*. Previous studies demonstrated that genistein administration blocked GnRH-induced rise of LH in ovariectomized rats *(21,22)*. Coumestrol administration led to the decrease in the GnRH pulse generator frequency, and consequently reduced pulsatile secretion of LH and led to suppression of pituitary LH in response to GnRH priming in ovariectomized rats in both in vivo and in vitro studies *(17)*. Concurrently, studies on exogenous estrogen administration showed the direct effect of the negative feedback mechanism. Estradiol benzoate reduced LH release by reducing the sensitivity of pituitary gonadotropes to the GnRH stimulation without altering GnRH secreting pattern in ovariectomized rhesus monkeys, suggesting the target site for estrogen is the pituitary *(23,24)*. In addition, push–pull perfusion of estradiol benzoate decreased pulse amplitude and basal release of GnRH and reduced LH levels in ovariectomized rhesus monkeys, suggesting the target site for estrogen is the hypothalamus *(25,26)*. Although this study did not clearly show the effect of PM treatment on GnRH suppression at the hypothalamus, we could assume that PM has an estrogenic effect on decreasing gonadotropin levels by negative feedback.

Other studies showing estrogenic effect of phytoestrogens from soy diet on serum gonadotropins support our above hypothesis. The daily intake of high amounts of soy containing 165.0 mg isoflavones for 4 wk induced a slight decrease in circulating FSH and LH levels in postmenopausal women *(12)*. A similar observation has been noted in postmenopausal women, who have a reduction of serum FSH levels after high consumption of soy and wheat for 12 mo *(14)*. Isoflavones at lower doses, 7.1–132.0 mg, for 3 mo could not change serum FSH or LH levels in postmenopausal women *(18)*. The same result was obtained in another study; that is, a daily intake of 56.0 and 90.0 mg of isoflavones for 3 or 6 mo could not change serum levels of FSH, LH, or estradiol in postmenopausal women *(27)*. Phytoestrogens from PM seem to show higher potency than those from soy and wheat. Muangman and Cherdshewasart *(1)* analyzed the content of isoflavones in the same PM lot used in this study and reported that PM contains 1.699 mg of total isoflavones/g dried powder. Thus, the isoflavones contained in the PM at doses of 10, 100, and 1000 mg/d should be less than those of soy used in other studies *(12,13,27)*. The lowest dose of PM (10 mg/d) treatment, containing 0.0169 mg isoflavones, clearly suppresses serum FSH and LH levels in aged monkeys; accordingly, it can postulate that PM phytoestrogens have a higher efficiency than isoflavones. From an in vitro study, coumestrol and miroestrol had relative molar binding affinities to estrogen receptors as high as 5% of estradiol; meanwhile, relative molar binding affinities of daidzein and genistien to estrogen receptors are between 0.05% and 1.00% *(28)*. It is assumed that the stronger efficiency of PM is due to the presence of other kinds of phytoestrogens such as coumestrol and miroestrol.

The suppression of serum estradiol was not displayed clearly in our study. Estradiol in menopause mainly comes from the peripheral conversion of androstenedione via estrone

and androstenedione via testosterone. PM phytoestreogens may have an effect on estradiol levels by the conversion of other steroid hormones, and not depend on the decreased gonadotropins through the hypothalamus–pituitary–ovarian axis. There are in vitro studies demonstrating that genistein and coumestrol reduced the conversion *(29,30)*. The additional studies for further understanding, nevertheless, have to be done.

The time course of PM effects has been examined. Pharmacokinetic studies showed that, after ingestion of soy isoflavones, the elimination half-life of genistein and daidzein are 7.0 and 4.0 h in premenopausal women and 4.0 and 3.0 h in men, respectively *(31)*. Other studies also indicated that the elimination half-lives of genistein and daidzein are 5.5 and 7.4 h in premenopausal women *(32)*, and 8.3 and 5.8 h in men, respectively *(33)*. From this evidence, we can assume that at the initial feeding concentrations of phytoestrogens in blood circulation did not reach the threshold for response. After daily treatment of PM, the concentrations of phytoestrogens in the blood circulation was accumulated to reach the threshold concentrations that resulted in the full-physiological response in all monkeys, e.g., the decreases in FSH and LH levels. After cessation of PM treatment, concentrations of phytoestrogens gradually excrete from the blood circulation resulting in recovery of gonadotropin levels in a dose-dependent manner. The lowest dose shows a short duration for the recovery. In addition, there was a rebound of serum gonadotropins after that. The rebound of gonadotropin levels in the study may have been caused by the increase in responsiveness of gonadotroph to GnRH. As found in the other study, a single subcutaneous injection of GnRH antagonist reduced serum FSH and LH levels within 6.0–48.0 h and rebounded serum LH levels at 96 h showing that there is an enhancement of hypothalamic GnRH drive after relief of its antagonist *(34)*.

In summary, the study suggests that the daily treatment of PM containing phytoestrogens exerts an estrogenic effect on the decrease in gonadotropin levels in aged menopausal monkeys in a dose-dependent manner. After cessation of PM treatment, the decreased gonadotropin levels recovered to the pretreatment levels within 60 d and also depended on dose.

Materials and Methods

Animals

Nine aged menopausal monkeys (*Macaca fascicularis*), with complete cessation of menstruation for at least 1 yr before onset of the experiment, aged more than 20 yr, and weighing from 4.0 to 6.5 kg were selected. Menstruation of the monkeys was confirmed and checked daily by the vaginal swabbing method before and during the experiment. The monkeys were housed separately 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 fluctuated slightly depending on the season. The monkeys were fed daily with monkey feed (Pokaphan Animal Feed Co., Ltd., Bangkok, Thailand) in the morning (0900–1000 h) and given 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

The nine monkeys were divided into three groups. The monkeys in each group (n = 3) were fed daily with a suspension of PM at doses of 10, 100, or 1000 mg/5 mL of distilled water/individual (hereafter abbreviated as PM-10, PM-100, or PM-1000) between 0800 and 0830 h. The experiment was separated into the pretreatment, treatment, and post-treatment periods. During the pretreatment and post-treatment periods, the monkeys were fed daily with 5 mL of distilled water for 30 and 60 d, respectively. During the treatment period, the monkeys were fed daily with the suspension of PM for 90 d. Blood samples, 3 mL, were collected from the femoral vein without anesthetization between 0830 and 0930 h every 5 d. The samples were centrifuged 1700*g* at 4°C, for 20 min and stored at –20°C until FSH, LH, and estradiol assays were performed.

Preparation of **P. mirifica** *Suspension*

The tuberous roots of PM used in this study were cultivar-wichai III collected from Chiangmai Province, northern Thailand. To minimize the variation of the phytoestrogen content in PM through seasons and locations, the tuberous roots of PM used in this study were obtained from the same lot. Fresh tuberous roots of PM were sliced, desiccated in a hot air oven at 70°C, and subsequently ground into 100-mesh powder. Then, the powdered stock was kept in the desiccator wrapped with foil until preparation into suspension with distilled water. The PM suspension was kept in a dark bottle at 4°C until feeding time.

Hormonal Analyses

The serum samples were analyzed for FSH and LH levels using a heterologous RIA system described previously *(35, 36)*. The iodinated preparations were rat NIDDK-rat FSH-I-5 and rat NIDDK-rat LH-I-5. The antisera were anti-ovine FSH (NIDDK-H-31) and anti-ovine LH (YM#18). 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%, 7.32% for FSH, 5.71%, 7.02% for LH, respectively. Serum level of estradiol after extraction by fresh diethyl ether was determined by RIA technique using ^{3}H-labeled radioligands as described in the established method of the World Health Organization (WHO) program *(37)*. The intra- and interassay coefficients of variations were 5.07% and 7.02% for estradiol, respectively.

Because the chemical structures of phytoestrogens in PM are similar to that of estradiol, the cross-reactivity of PM phytoestrogens to the estradiol antibody was examined. Phytoestrogens of PM roots were extracted by 5 mL of diethyl ether, dried, and mixed with phosphate buffer solution. The extraction of PM phytoestrogens in the concentration ranging 0.001–1000 μg did not show cross reactivity with the estradiol antibody in the WHO-RIA assay system.

Acknowledgments

We are grateful to Dr.A.F. Parlow and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), CA, USA for providing RIA materials for LH and FSH; and Dr.T.Mori, University of Tokyo, Tokyo, Japan for providing antisera to ovine LH (YM#18). *P. mirifica* was a generous gift from Dr.W. Cherdshewasart, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

This work was supported by The Thailand Research Fund (TRF) contract no. PHD/0014/2544 for Royal Golden Jubilee Program (RGJ network) and no. BGJ/15/2544, Chulalongkorn University, Thailand; and Research Grant and a Grant-in-Aid for Scientific Research (The 21st Century Center-of-Excellence-Program, E-I) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Muangman, V. and Cherdshewasart, W. (2001). *Siriraj Hosp. Gaz.* **53,** 300–309.
2. Ingham, J. L., Markham, K. R., Dziedzic, S. Z., and Pope, G. S. (1986). *Phytochemistry* **25,** 1772–1775.
3. Ingham, J. L., Tahara, S., and Dziedzic, S. Z. (1986). *Z. Naturforsch*. **41c**, 403–408.
4. Ingham, J. L., Tahara, S., and Dziedzic, S. Z. (1989). *Z. Naturforsch*. **44c**, 724–726.
5. Pope, G. S., Grundy, H. M., Jones, H. E. H., and Tait, S. A. S. (1958). *J. Endocrinol*. **17,** 15–16.
6. Chansakaow, S., Ishikawa, T., Seki, H., Sekine, K., Okada, M., and Chaichantipyuth, C. (2000). *J. Nat. Prod*. **63,** 173–175.
7. Chansakaow, S., Ishikawa, T., Sekine, K., et al. (2000). *Planta Medica* **66,** 572–575.
8. Jones, H. E. H. and Pope, G. S. (1960). *J. Endocrinol*. **20,** 229–235.
9. Malaivijitnond, S., Kiatthaipipat, P., Cherdshewasart, W., Watannabe, G., and Taya, K. (2004). *J. Pharmacol. Sci*. **96,** 428–435.
10. Trisomboon, H., Malaivijitnond, S., Watanabe, G., and Kazuyoshi, T. (2004). *J. Pharmacol. Sci*. **94,** 51–59.
11. Trisomboon, H., Malaivijitnond, S., Watanabe, G., and Kazuyoshi, T. (2005). *Endocrine* **26,** 33–40.
12. Baird, D. D., Umbach, D. M., Lansdell, L., et al. (1995). *J. Clin. Endocrinol. Metab*. **80,** 1685–1690.
13. Duncan, A. M., Berz, B. E., Xu, X., Nagel, T., Phipps, W., and Kurzer, M. S. (1999). *J. Clin. Endocrinol. Metab*. **84,** 192–197.
14. Murkies, A. L., Lombard, C., Strauss, B. J. G., Wilcox, G., Burger, H. G., and Morton, M. S. (1995). *Maturitas* **21,** 189–195.
15. Shimizu, H., Ross, R. K., Bernstein, L., Pike, M. C., and Henderson, B. E. (1990). *Br. J. Cancer* **62,** 451–453.
16. Krajewski, S. J., Abel, T. W., Voytko, M. L., and Rance, N. E. (2003). *J. Clin. Endocrinol. Metab*. 8**8,** 665–662.
17. McGarvery, C., Cates, P. S., Brooks, N., et al. (2001). *Endocrinology* **142,** 1202–1208.
18. Cassidy, A., Bingham, S., and Setchell, K. D. (1994). *Am. J. Clin. Nutr*. **60,** 333–330.
19. Cassidy, A., Bingham, S., and Setchell, K. D. R. (1995). *Br. J. Nutr*. **74,** 587–601.
20. Duncan, A. M., Underhilll, K. E. W, Xu, X., Lavalleur, J., Phipps, W. R., and Kurzer, M. S. (1999). *J. Clin. Endocrinol. Metab*. **84,** 3479–3484.
21. Faber, K. A. and Hughes, J. R. (1991). *Biol. Reprod*. **45,** 649–653.
22. Hughes, C. L., Chakinala, M. M., Reece, S. G., Miller, R. N., Schomberg, D. W., and Basham, K. B. (1991). *Reprod. Toxicol*. **5,** 133–137.
23. Nakai, Y., Plant, T. M., Hess, D. L., Keogh, E. J., and Knobil, E. (1978). *Endocrinology* **102,** 1008–1014.
24. Pau, K. Y., Gliessman, P. M., Hess, D. L., Ronnekleiv, O. K., Levine, J. E., and Spies, H. G. (1990). *Brain Res*. **517,** 229–235.
25. Chongthammakun, S. and Terasawa, E. (1993). *Endocrinology* **132,** 735–743.
26. O'Byrne, K. T., Chen, M. D., Nishihara, M., et al. (1993). *Neuroendocrinology* **57,** 588–592.
27. Persky, V. W., Turyk, M. E., Wang, L., et al. (2002). *Am. J. Clin. Nutr.* **75,** 145–153.
28. Shutt, D. A. and Cox, R. I. (1972). *J. Endocrinol*. **52,** 299–310.
29. Makela, S., Poutanen, M., Lehtimaki, J., Kostian, M. L., Santti, R., and Vihko, R. (1995). *Proc. Soc. Exp. Bio. Med*. **208,** 51–59.
30. Whitehead, S. A., Cross, J. E., Burden, C., and Lacey, M. (2002). *Hum. Reprod.* **17,** 589–594.
31. Lu, L. J. W. and Anderson, K. (1998). *Am. J. Clin. Nutr.* **68,** 1500s–1504s.
32. Setchell, K. D. R., Faughnan, M. S., Avades, T., et al. (2003). *Am. J. Clin. Nutr*. **77,** 411–419.
33. Watanabe, S., Yamaguchi, M., Sobue, T., et al. (1998). *J. Nutr*. **128,** 1710–1715.
34. Gianotti, J., Veldhuis, J. D., Destefanis, S., et al. (2003). *Clin. Endocrinol*. **59,** 526–532.
35. Watanabe, G., Nozaki, M., Taya, K., Katakai, Y., and Sasamoto, S. (1990). *Biol. Reprod.* **43,** 196–201.
36. Nozaki, M., Watanabe, G., Taya, K., et al. (1990). *Biol. Reprod*. **43,** 444–449.
37. Sufi, S. B., Donaldson, A., and Jeffcoate, S. L. (1986). *WHO matched reagent program method manual.* WHO Collaborating Center for Immunoassay: London, pp.145–169.