รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษากลไกการยับยั้งการตกไข่ของสารไฟโตอีสโตรเจนในกวาวเครือขาวในลิงหางยาวเพศเมีย

The study of blocking ovulation effect of *Pueraria mirifica* phytoestrogens in female cynomolgus monkeys

โดย นางหทัยทิพย์ ไตรสมบูรณ์

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

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ระยะเวลาโครงการ: 2 ปี

ฤทธิ์ของกวาวเครือขาว *Pueraria mirifica* (PM) ต่อระดับโกนาโดโทรฟินส์ และฮอร์โมนเพศในซีรัมและในปัสสาวะของลิงเพศเมียที่อยู่ในวัยเจริญพันธุ์และวัยหมดประจำเดือน ลิงหางยาว *(Macaca fascicularis*) ถูกแบ่งออกเป็น 3 กลุ่ม (n = 3) แต่ละกลุ่มได้รับกวาวเครือขาวขนาด 10, 100 และ 1,000 มิลลิกรัม (PM-10, PM-100 และ PM-1,000 ตามลำดับ) ผลการศึกษาแสดงให้เห็นว่าการได้รับ PM-1,000 จำนวน 1 ครั้ง สามารถยืดความยาวของรอบประจำเดือนแต่ไม่เปลี่ยนแปลงระดับโกนาโดโทรฟินส์ อีสโตรเจนและโปรเจสเตอโรนในซีรัมและปัสสาวะของลิงวัยเจริญพันธุ์ การได้รับกวาวเครือขาวทุกวันเป็นเวลานาน 3 รอบประจำเดือนหรือ 90 วันมีผลยืดความยาวของรอบประจำเดือนและหยุดประจำเดือนในลิงวัยเจริญพันธุ์ การให้กวาวเครือขาวเป็นเวลานานแก่ลิงวัยเจริญพันธุ์และวัยหมดประจำเดือนกดระดับโกนาโดโทรฟินส์ และฮอร์โมนเพศในระหว่างที่ได้รับกวาวเครือขาวและผลต่อฮอร์โมนสามารถกลับสู่ภาวะปกติหลังจากที่หยุดการได้รับกวาวเครือขาว ยกเว้นในกรณีของการให้ PM-1,000 ที่ซึ่งผลของกวาวเครือขาวยังคงมีอยู่จนสิ้นสุดการทดลอง (60 วันหลังจากหยุดการให้) ระดับฟอลลิเคิลสติมูเลทิงฮอร์โมนและอีสตราไดออลในปัสสาวะลดลงในลิงวัยเจริญพันธุ์ที่ได้รับ PM-100 และ PM-1,000 และในลิงที่อยู่ในวัยหมดประจำเดือนที่ได้รับกวาวเครือขาวทุกขนาด ผลการศึกษานี้สรุปได้ว่าแม้ฤทธิ์ของกวาวเครือขาวลดลงหลังจากที่หยุดให้กวาวเครือขาวในปริมาณต่ำ

คำสำคัญ: *Pueraria mirifica*, monkey, gonadotropin, sex steroid hormone, sexual skin color

Abstract

Project code: BT – 06 – 2E – 09 – 3007

Project title: The study of blocking ovulation effect of *Pueraria mirifica* phytoestrogens in female cynomolgus monkeys

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Project period: 2 years

The estrogenic effect of white kwao krua, *Pueraria mirifica* (PM), on serum and urinary gonadotropin and sex steroid hormone levels in adult cyclic and aged menopausal cynomolgus monkeys. The cynomolgus monkeys (*Macaca fascicularis*) were divided into 3 groups. Each group (n = 3) was fed with the 10, 100, and 1,000 mg of PM (PM-10, PM-100, and PM-1,000, respectively). The results showed that a single treatment of PM-1,000 prolonged the length of menstrual cycle, but did not change serum and urinary gonadotropins, estradiol, or progesterone levels. The long-term daily treatment of PM, for there menstrual cycles or 90 days, prolonged the length of menstrual cycle and stopped menstruation of adult cyclic monkeys. Long-term treatment of PM on adult cyclic and aged menopausal monkeys suppressed the serum levels of gonadotropins and sex steroid hormones during the treatment period, and the effect was recovered during the post-treatment period, except the case of PM-1,000 treatment, which the effect continued until the end of experiment (60 days after cessation of treatment). Urinary FSH and estradiol levels were suppressed in adult cyclic monkeys by the treatment of PM-100 and PM-1,000 and in aged menopausal monkeys by all doses of PM. It is concluded that although the effect of PM can be recovered soon after the cessation of treatment in lower dose.

Keywords: *Pueraria mirifica*, female monkey, gonadotropin, sex steroid hormone, sexual skin color

Output จากโครงการวิจัยที่ได้รับทุน สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

- Trisomboon, H., Malaivijitnond, S., Watanabe, G., and Taya, K. 2004. Estrogenic effects of *Pueraria mirifica* on the menstrual cycle and hormone-related ovarian functions in cyclic female cynomolgus monkeys. J Pharmcacol Sci 94: 51-59.

2 การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงพาณิชย์:

- เนื่องจากในปัจจุบันนี้มีกระแสความนิยมของการนำสมุนไพรไทย โดยเฉพาะอย่างยิ่งกวาวเครือขาว ไปใช้ในรูปแบบของอาหารและครีมเสริมความงาม รวมทั้งใช้เป็นยาอายุวัฒนะโดยไม่ทราบถึงผลกระทบที่อาจเกิดขึ้นได้ แต่จากการวิจัยศึกษา พบว่าการบริโภคกวาวเครือขาวเป็นเวลานานจะมีผลไปลดการตกไข่ โดยไปลดระดับฮอร์โมนโกนาโดฟินส์ในหญิงวัยเจริญพันธุ์ และมีผลไปลดอาการต่างๆที่เกิดขึ้นในหญิงวัยหมดประจำเดือน โดยลดการหลั่งของฮอร์โมนโกนาโดฟินส์ รวมทั้งลดการสลายตัวของแคลเซียมออกจากกระดูก โดยลดการหลั่งของพาราไทรอยด์ฮอร์โมน งานวิจัยนี้จะนำไปสู่การศึกษาหาปริมาณที่เหมาะสมของกวาวเครือขาวในคนได้ เชื่อว่าจะสามารถนำไปความรู้ที่ได้นี้ไปใช้ในเชิงพาณิชย์ต่อไปได้

2.2 เชิงวิชาการ:

- งานวิจัยนี้เป็นการศึกษาผลกระทบของการกินพืชสมุนไพรไทย กวาวเครือขาว ต่อการทำงานของระบบสืบพันธุ์และฮอร์โมนที่เกี่ยวข้องในวัยเจริญพันธุ์และวัยหมดประจำเดือน รวมทั้งฮอร์โมนและแคลเซียมในซีรัมซึ่งมีความสัมพันธุ์กับการเปลี่ยนแปลงของกระดูกในวัยหมดประจำเดือน โดยการศึกษานี้ใช้ลิงหางยาวเพศเมียเป็นตัวแทนการศึกษาในคน ผลจากการใช้วิจัยนี้ทำให้เข้าใจกลไกของพืชสมุนไพรชนิดนี้ต่อการเปลี่ยนปลงของระบบสืบพันธุ์และกระดูกในผู้หญิง
- งานวิจัยนี้เป็นการใช้ลิงหางยาวเป็นตัวแทนของการศึกษาในคน เนื่องจากลิงหางยาวมีระบบทางสรีรวิทยา ได้แก่รูปแบบและกลไกการสร้างและการหลั่งของฮอร์โมน การทำงานของระบบสืบพันธุ์ รวมทั้งกลไกและปัจจัยที่เกี่ยวข้องกับการเปลี่ยนแปลงของกระดูกคล้ายคลึงกับคน ซึ่งผลจากการศึกษานี้สามารถนำไปสู่การศึกษาในคนทางคลินิกต่อไปได้

- สมุนไพรกวาวเครือขาว ประกอบด้วยสาร phytoestrogen ชนิดต่างๆ จำนวนมาก ซึ่งสาร phytoestrogen เหล่านี้พบได้ในพืชชนิดต่างๆ โดยเฉพาะพืชตระกูลถั่ว ดังนั้นการศึกษาโดยใช้ลิงหางยาวเป็นสัตว์ทดลอง แทนการศึกษาในคน ทำให้สามารถควบคุมอาหารที่สัตว์ทดลองได้รับในแต่ละวันได้ นอกจากนี้จำนวนครั้งและเวลาของการเก็บตัวอย่างเลือดและปัสสาวะของลิงหางยาว ยังทำได้ง่ายกว่าในคน

- เชื่อว่างานวิจัยนี้จะเป็นการกระตุ้นให้มีการศึกษาคุณสมบัติและผลของสมุนไพรไทยชนิดอื่นๆ ต่อไป

3. การเสนอผลงานในที่ประชุมวิชาการ

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3.4 Trisomboon, H., Malaivijitnond, S., Kazuyoshi, T., Watanabe, G., and Suzuki, J. 2002. Long-term effects of *Pueraria mirifica* on reproductive hormones in aged female cynomolgus monkeys. RGJ Seminar Series: Biodiversity II. pp. 10. Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

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3.9 Trisomboon, H., Malaivijitnond, S., Watanabe, G., and Taya, K. 2003. Biotechnology of phytoestrogen-rich; *pueraria mirifica*: xiv. Ovulation block in adult female cynomolgus monkeys.

Introduction

White kwao krua is one of the indigenous Thai herbs that are classified into family Leguminoceae, subfamily Papilionoideae as soy and other legumes. It was firstly discovered and classified by Vatna in 1939 as red kwao krua (*Butea superba* ROXB) because of their superficial resemblance (Bounds and Pope, 1960). Later, in 1952, white kwao krua has been recognized as a new species and reclassified as *Pueraria mirifica* by Airy Shaw and Kasin Suvatabandhu. Other dialects of *P. mirifica* are tong-krua, tan-jom-tong, po-ta-goo, tan-krua, and jan-krua. This plant is widely found everywhere in Thailand, particularly in the deciduous forests of the northern Thailand especially in Chiang Mai province. It is a liana, which has tuberous roots. Flower color is bluish purple. Leaf shape is closely similar to that of red kwao krua, but thinner and smaller than it is. Its tuberous root with whitish starch granules has a round or ellipse-shape (Kashemsanta et al., 1957; Pisetpakasit, 1976).

The tuberous roots of *P. mirifica* have been analyzed by chromatography technique and found many chemical substances with estrogenic activities such as miroestrol, deoxymiroestrol, daidzein, genistein, coumestrol, puerarin, kwakhurin, and mirificin (Kashemsanta et al., 1957; Pope et al., 1958; Pisetpakasit, 1976; Ingham, Tahara, and Dziedzic, 1986, 1987, 1988, 1989; Chansakaow et al., 2000a, 2000b). These substances are included in phytoestrogens (Pope et al., 1958; Pisetpakasit, 1976; Barnes et al., 1998; Murkies, Wilcox, and Davis, 1998). Phytoestrogens are plant-derived substances with estrogen-like biological activity. Chansakaow (2000) noted that 100 gram of *P. mirifica* dry powder contains 46.1 mg of daidzein and 2-3 mg of miroestrol and deoxymiroestrol. Muangman and Cherdshewasart (2001) also analyzed *P. mirifica* cultivar Wichai III, which is the same lot to our study, with the high performance liquid chromatography (HPLC) technique and found that this cultivar contains the significant amount of isoflavones (169.1 mg total isoflavones/100 gram of the dry powder) whereas small amounts of miroestrol, deoxymiroestrol, and other phytoestrogens present it.

Estrogens, sex steroid hormones, play the main function on reproductive system in women as well as female animals. There are three types of estrogens; estrone, estradiol, and estriol. Estradiol is the major estrogen that is secreted from the ovaries in women. Estrone and estriol are largely products of estradiol metabolism. During the reproductive year, the daily secretion of estrogen varies cyclically throughout the quasi-

monthly menstrual cycle. Estrogen production is governed by two pituitary gonadotropins; follicle stimulating hormone (FSH) and luteinizing hormone (LH). Estrogen cooperated with FSH and LH regulates the growth and development of follicle and stimulates an ovulation. Estrogen and other ovarian hormones, including progesterone and inhibin, regulate FSH and LH secretion from the anterior pituitary gland by both the negative and positive feedback mechanisms (Rhoades and Pflanzer, 1996).

Several studies have been reported that phytoestrogens altered levels of gonadotropins and sex steroid hormones in women (Knight and Eden, 1996; Murkies et al., 1998; Setchell, 1998; Tham, Gardner, and Haskell, 1998). Functions and effects much differ with kind of phytoestrogens. Previous reports demonstrated that premenopausal women who consumed flax seed powder containing lignans, a kind of phytoestrogens, showed a longer luteal phase, but no changes in estradiol and progesterone levels (Phipps et al., 1993). Premenopausal women who consumed 45 mg of isoflavones extracted from soy for the duration of one menstrual cycle have an increase in follicular phase length and a decrease in peak levels of FSH, LH, and progesterone (Cassidy, et al., 1995). Although daily intake of daidzein and genistein for 1 month did not increase significantly the menstrual cycle length, it decreased significantly the serum levels of estradiol and dehydroepi-androstendione sulfate in premenopausal women (Lu et al., 1996). The variation in the effects of phytoestrogens is the subject of research of importance. The phytoestrogens are considered an effective remedy for the various symptoms of estrogen deficiency.

Menopausal state is a state of failure in ovarian function and resulting in low rate of estrogen production. The reduction of estrogen production causes a loss of negative feedback mechanism on the secretion of gonadotropins at the pituitary levels; accordingly, the levels of gonadotropins progressively are increased during this time and kept elevated throughout the menopause (Smith et al., 1983; Gill et al., 2002). The administration of exogenous estrogen reduces, not only the gonadotropin secretion but also the rate of bone loss or bone fracture in menopausal women (Varma et al., 1985; Lindsay et al., 1996; Pinkerton and Santen, 1999). However, the side effects of estrogen administration are concerned, for example nausea, breast tenderness, migraine headaches, hypertension, and carcinomas of endometrium and breast (Pinkerton and Santen, 1999). That is why the phytoestrogens have attracted of researchers, who hope that they would have no such side effects.

Few studies have reported the estrogenic effect of phytoestrogens from soy in menopausal women. Daily consumption of soy decreased serum estrogen (Duncan et al., 1999) and FSH levels in menopausal women (Murkies et al., 1995). Isoflavones also decreased menopausal symptoms (Murkies et al., 1995; Vincent and Fitzpatrick, 2000). It reduced hot flushes and vaginal dryness and slightly increased the vaginal cell maturation (Wilcox et al., 1990; Baird et al., 1995). The epidemiological study in postmenopausal women found that Japanese women who highly consumed a soy diet have the estradiol and estrone levels lower than that of American women (Shimizu et al., 1990). In addition, some reports indicated that daily consumption of soy could reduce bone loss and bone resorption in oophorectomized rats (Draper et al., 1997; Arjmandi et al., 1998) and postmenopausal women (Yamori et al., 2002). These results suggested that consumption of phytoestrogen-rich soy affect reproductive system and bone in estrogen deficiency in menopausal women.

For more than twenty years, tuberous root of *P. mirifica* has been popularly used as a rejuvenating drug in aged persons in Thailand. They believed that *P. mirifica* contains some active substances alike female hormones (Kashemsanta et al., 1957; Pope et al., 1958; Pisetpakasit, 1976). Large quantities of its root were prepared by mixing with honey in a Thai traditional medicine. Native Thai people use it to remedy for various symptoms including cataract in the eyes, exhaustion, and emaciation due to starvation, flatulence, and cough with blood. They also use it to recover the black hair, promote an appetite, and increase the longevity (Wanadorn, 1933). In recent years, many products of *P. mirifica* in the forms of cream, tablet, and solution have been developed and widely used in normal cyclic women as an age rejuvenation drug as well as cosmetics, for example, breast enlargement creams, skin moisturizers, and eye gels.

From these evidences, it is of interest to investigate the exact effect of *P. mirifica* on reproductive system in both adult cyclic women and aged menopausal women. However, studies on the effect of *P. mirifica* in humans may not yield valid results. Most of phytoestrogens can be found in a variety of daily human diet including soy and soy products; it is therefore very difficult to control the diet during experiment in humans. Moreover, the longitudinal determination of hormonal changes in humans is also difficult. Accordingly, the cynomolgus monkey (*Macaca fascicularis*) was chosen and used as an alternative model for the study of changes in reproductive hormones by *P. mirifica* treatment. Their physiological systems e.g. hormonal secretion patterns, menstrual cycle, reproduction, and bone metabolism are similar to those of humans

(Chongthammakun and Terasawa, 1993; Krajewski et al., 2003). This study therefore examined the acute and long-term effects of *P. mirifica* on the menstrual cycle length in adult cyclic monkeys and on changes in serum levels of gonadotropins and sex steroid hormones. To find out whether *P. mirifica* had an estrogenic potency on bone in aged menopausal monkeys, serum calcium and PTH levels were also determined in these monkey groups.

However, the long-term study on changes in serum levels of hormone pose the limitations on frequency of sampling and on the volume of blood to be collected, because losses of high amount of blood and injury from frequent venipuncture may disturb the homeostasis of physiological system in subject monkeys. To avoid these problems, the assays of urinary hormone levels, the non-invasive method, should be the better choice. The changes in urinary levels of reproductive hormones in both adult cyclic and aged menopausal monkeys, which were treated by *P. mirifica* was also investigated in this study.

Title: 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 (PM) 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 PM. 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 PM, but no change in menstrual cycle length in monkeys treated with 10 and 100 mg of PM. 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 PM can disturb ovarian function and menstrual cycle in monkeys.

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

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 (PM) 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 PM 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, PM 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, PM may disturb reproductive function and menstrual cycle in women.

The aim of this study was to examine the endocrine-modulating effect of PM 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 PM 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 4.0 – 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.

Experimental Design

Nine female monkeys were divided into three groups. Each group (n = 3) was force-fed with the suspension of PM 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 PM 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 $^{\circ}$C, 1,700 x g for 20 min. The serum was then separated and stored at –20 $^{\circ}$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 PM (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 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 3H-labeled radioligands as described in the established method of World Health Organization (WHO) (Sufi, Donaldson, and Jeffcoate, 1986).

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.

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 PM 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 PM (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 PM

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

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 1 and Table 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 Fig.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.

Changes in Serum Gonadotropins and Ovarian Hormones in the Monkeys Treated with PM.

As shown in Figs 2-4, 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 PM, respectively. All monkeys exhibited peak levels of serum FSH and LH in the late follicular phase of the first and second menstrual cycles after PM 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 PM), 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 1 Menstrual cycle length of monkeys treated with 10, 100, and 1,000 mg of PM

Treatment groups	Lengths of menstrual cycle after PM treatment (days)	
	The first menstrual cycle	The second menstrual cycle
10 mg	30.67 $\pm$ 2.91 (P = 0.97)	33.33 $\pm$ 3.84 (P = 0.39)
100 mg	30.67 $\pm$ 2.96 (P = 0.97)	35.67 $\pm$ 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 $\pm$ 1.30 days. Asterisks represent a significant difference ($P < 0.05$).

Table 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 ± 0.86	1.82 ± 0.34	59.89 ± 10.66	0.89 + 0.53
- lowest levels	1.60 ± 0.33	0.51 ± 0.03	27.54 ± 4.31	0.27 ± 0.06
Early Luteal phase				
- highest levels	3.93 ± 0.72	0.94 ± 0.11	39.55 ± 14.22	5.09 ± 0.85
- lowest levels	1.29 ± 0.20	0.28 ± 0.05	11.99 ± 4.98	1.19 ± 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 ± S.E.M (n = 13).

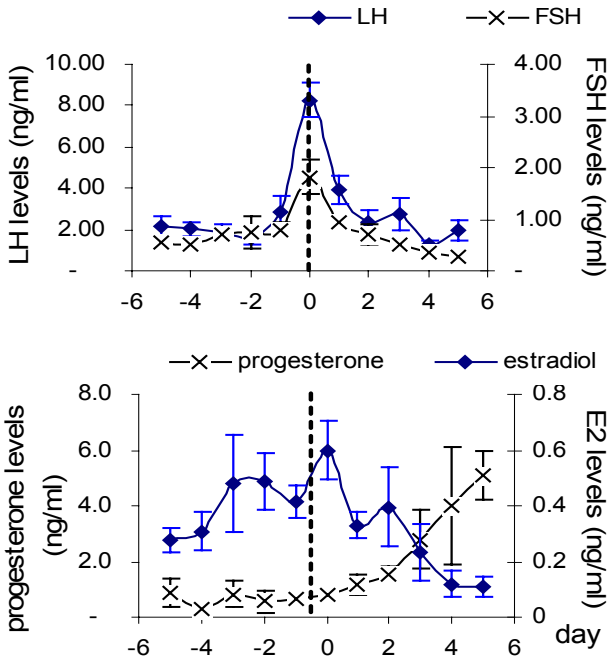

Fig. 1 Serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of normal cyclic monkeys.

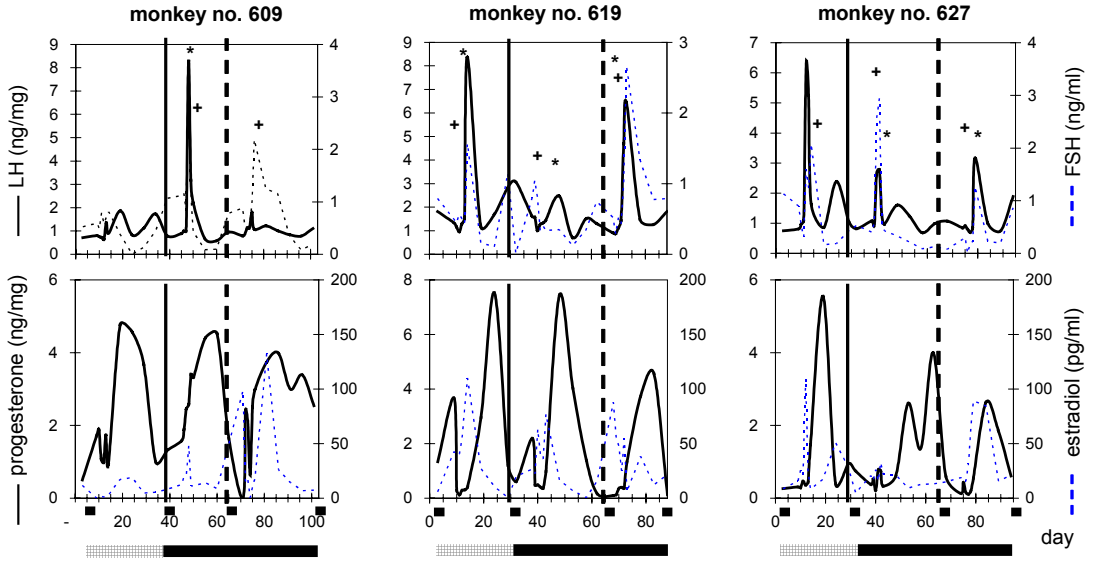

Fig. 2 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of monkeys treated with 10 mg of PM. Horizontal bars represent the day of menses. Day 1 represents the day of menses in the pre-treatment period. The pre-treatment and post-treatment periods 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.

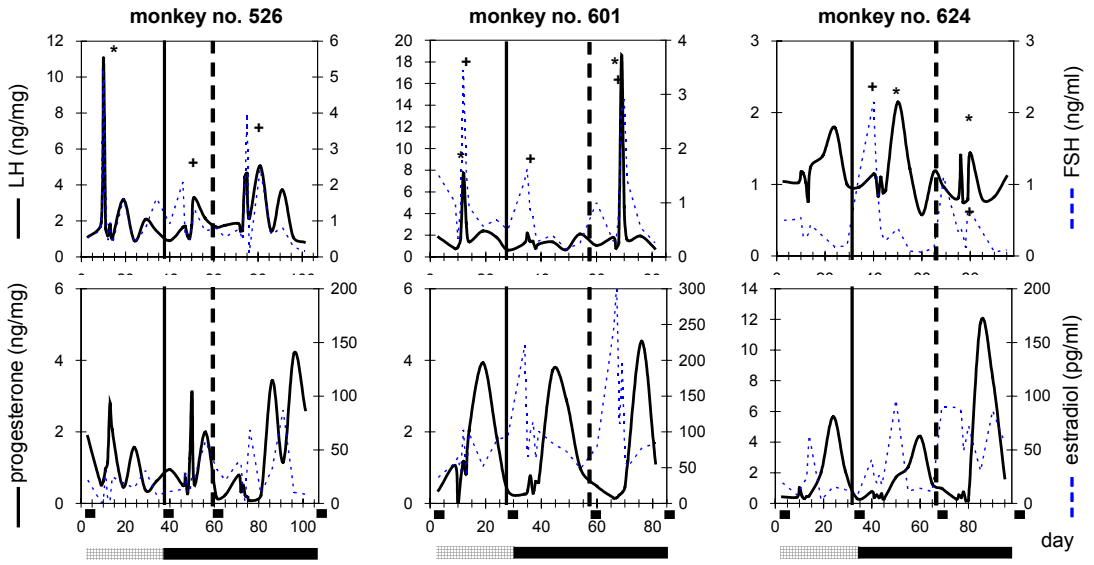

Fig. 3 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of monkey treated with 100 mg of PM. Day 1 represents the day of menses in the pre-treatment period. The meanings of horizontal bars, vertical solid and vertical dotted lines, and symbols are shown in Fig. 2.

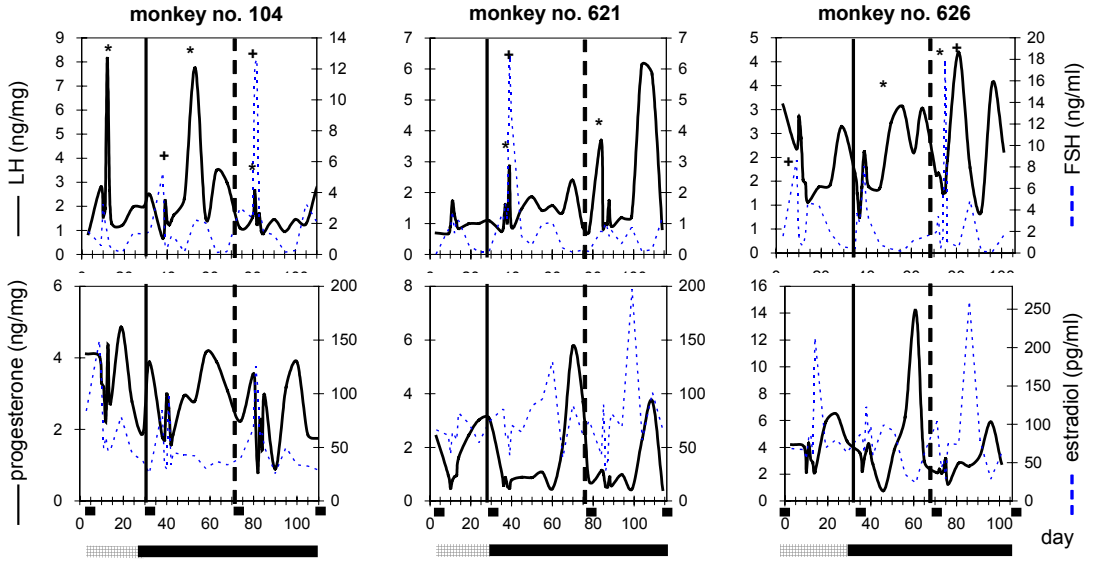

Fig. 4 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of monkey treated with 1,000 mg of PM. Day 1 represents

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.

Discussion

The present study provides the first evidence that PM 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 PM) 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 PM). 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 PM 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 PM 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 PM, 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 PM. The monkeys were fed with a single dose of PM 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 PM. 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 PM disturbs ovarian function and menstrual cycle in normal cyclic monkeys. PM 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.

Title: Ovulation block by *Pueraria mirifica*: a study of its endocrinological effect in female monkeys.

Abstract

Pueraria mirifica (PM), a Thai herb containing phytoestrogens may act as estrogen and disturbs reproduction. To investigate 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 1,000 mg/day 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 -1,000. 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 the menstrual cycles and may suppress the ovulation by lowering serum levels of gonadotropins.

Keywords: 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 (Pope et al., 1958), puerarin (Pope, 1986), deoxymiroestrol, kwakhurin (Chansakaow et al., 2000a, 2000b), and others in the isoflavone and coumestrol groups (Ingham et al., 1986, 1989). In recent years, the use of PM as an alternative medicine has become popular. Many products in the forms of cream, tablet, and solution developed from PM root are widely used in normal cycling women as an age rejuvenation drug and cosmetic products such as breast enlargement cream, skin moisturizer, and eye gel. However, there was no scientific report of its estrogenic effect on reproduction or related hormones in women.

Estrogenic effects of this plant disturbing reproductive function have been found in mice, rats, and monkeys (Jones and Pope, 1960; Trisomboon et al., 2004). Miroestrol, phytoestrogenic substance found only in PM root, increased uterine weight in immature female mice (Jones and Pope, 1960). A single feeding of high dose of PM (1,000 mg) could prolong the menstrual cycle length of female cynomolgus monkeys (Trisomboon et al., 2004). In addition, phytoestrogen isoflavones from other plant was firstly reported as causing sheep infertility (Bennetts et al., 1946). Coumestrol isolated from alfalfa reduced the ovulation rate in mice (Fredricks et al., 1981). Furthermore, previous reports showed that soy isoflavones disturbed hormonal characteristics in premenopausal women, although there have been conflicting data (Cassidy et al., 1994, 1995; Duncan et al., 1999; Lu et al., 1996, 2000). From the epidemiological data, Japanese and Chinese women who consumed high amounts of soy diet lowered circulating level of estradiol during the menstrual cycles (Bernstein et al., 1990; Nagata et al., 1998).

From these evidences, it is important to investigate effects of PM containing phytoestrogens on the menstrual cycle and related hormones. To avoid the limitations of the long-term study effect of PM in human, a nonhuman primate, the adult cyclic cynomolgus monkey (*Macaca fascicularis*) was selected as the model of this study. Female cynomolgus monkey has been proved to have similar reproductive function and hormonal pattern with those in woman (Krajewski et al., 2003), and offer the advantage of allowing control diet and exposure to other environment factor that may confound the

influence. Therefore, our study examined the changes in the menstrual cycle length and related hormones in adult cyclic female cynomolgus monkeys treated with PM.

Material and Methods

Animals

Nine adult female cynomolgus monkeys (*Macaca fascicularis*) with regular menstrual cycles for at least 4 consecutive months, 30 ± 4 days in length and weighing from 4.0 – 6.5 kg before the study, were used. The first day of menstrual bleeding 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. 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 the suspension of PM at doses of 10, 100, and 1,000 mg/5 ml of distilled water/individual/day (abbreviated as PM-10, PM-100, and PM-1,000, respectively.) at 0800 h. The treatment schedule was consisted of 3 periods: the pre-treatment, treatment, and post-treatment period. During the pre-treatment 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 days respectively. Three-ml blood samples were collected from the femoral vein without anesthetization between 0800 - 0900 h on day 3 (the early follicular phase), days 9, 10, 11, 12, 13, 14 (the late follicular phase); days 19, 24, and 29 (the late luteal phase) of the menstrual cycle. However, if monkeys did not show the menstrual bleeding, blood samples were collected every 10 day until 90 and 60 days during the treatment and post-treatment periods, respectively. Blood samples were centrifuged at 4 $^{\circ}$C, 1,700 x g for 20 minutes and stored at –20 $^{\circ}$

C until FSH, LH, estradiol, progesterone, and immunoreactive (ir)-inhibin were assayed. Moreover, the occurrence of menstrual bleeding was checked daily by vaginal swabbing method.

P. Mirifica Suspension Preparation

The fresh tuberous roots of PM were sliced, desiccated in a hot air oven at 70 º C, and subsequently ground into 100 mesh powder. Then, the powdered stock was kept in the dark desiccator until preparation into suspension with distilled water. The PM suspension was kept in a dark bottle at 4 º C until feeding time.

Hormonal Analysis

The serum samples were analyzed for FSH and LH levels using a heterologous RIA system described previously (Watanabe et al. 1990; Nozaki et al. 1990). 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 as in terms of NIDDK rat FSH-RP-2 and NIDDK rat LH-RP-2. The intra- and inter-assay coefficients of variations were 5.82 and 7.32% for FSH and 5.71 and 7.48% for LH, respectively.

Serum level of estradiol after extraction by fresh diethyl ether was determined by double-antibody RIA with 3H-labeled radioligands as described in the established method of World Health Organization (WHO) (Sufi et al.1986). The intra- and inter-assay coefficients of variations were 5.07 and 7.02% for estradiol. Serum concentrations of progesterone were determined by a double-anti body RIA system using 125I-labeled radioligands as described previously (Taya et al., 1985). The intra- and inter-assay 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 (Hamada et al., 1989). 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 variations were 5.47 and 6.78% for ir-inhibin.

Statistical Analysis

The data of the length of menstrual cycle were expressed as mean $\pm$ S.E.M. 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.

Result

Changes In Menstrual Cycle Length of Monkeys Treated with PM

The normal menstrual cycle length during the pre-treatment period in nine monkeys was 28.2 $\pm$ 0.8 days. As shown in table 1, PM-10 extended the menstrual cycle length to 50, 81, and 52 days in monkey no. 601, to 49 and > 90 days in monkey no. 627, and completely stopped the menstruation throughout the treatment period in monkey no. 619. The extended menstrual cycle could recover in all monkeys during the post-treatment period. In monkeys treated with PM-100, the menstrual cycle became shorter during the early treatment period to 15 days in monkey no. 616 and to 21 days 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 show the menstruation throughout the treatment and post-treatment period. All monkeys (nos. 624, 77, and 633) treated with PM-1,000 showed a complete cessation of menstruation throughout the treatment period and did not recover the cycle in the post-treatment period.

Changes in Serum Levels of Gonadotropins and Ovarian Hormones in Monkeys Treated with PM

To evaluate the changes of gonadotropins and ovarian hormones in monkeys treated with PM, their concentration patterns during the treatment and post-treatment periods were compared to those during the pre-treatment period. Although there were the inter-individual 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 agreed. Normal menstrual cycle during the pre-treatment period of the monkeys showed the peak levels of FSH and LH which appeared to coincide with the increase in serum estradiol levels. The peak level of LH was defined as the mid cycle phase or the ovulation day and separated into 2 phases: the follicular and luteal. Serum progesterone and ir-inhibin levels lowered during the follicular phase, and then slightly elevated during the luteal phase.

In monkeys treated with PM-10, as shown in Figs. 1 - 3, there were no changes in patterns of serum gonadotropins or ovarian hormones during the treatment and post-treatment periods compared to those of the pre-treatment period. During PM-10 treatment, all monkeys still showed the peak levels of FSH and LH during the follicular phase and the high levels of estradiol, progesterone, and ir-inhibin afterward.

Comparing to the pre-treatment levels, all three monkeys treated with PM-100 had no surge level of FSH or LH during the treatment period (Figs. 4 - 6). The basal level of FSH decreased significantly and could recover during the post-treatment period; meanwhile, basal level of LH did not differ from the pre-treatment levels. There was no evidence of high level of estradiol, progesterone, or ir-inhibin during PM treatment in all monkeys. The suppression of these hormones could recover during the post-treatment period in some monkeys (monkey nos. 616 and 801).

Serum levels of gonadotropins and ovarian hormones in monkeys treated with PM-1,000, as shown in Figs. 7 - 9, were obviously suppressed. There were no high levels of FSH, LH, estradiol, progesterone, or ir-inhibin throughout the treatment and post-treatment periods in all monkeys. Furthermore, the basal levels of serum FSH and LH during the treatment period were lower than those of the pre-treatment period. After that, their levels recovered to the pre-treatment levels during the post-treatment period in all monkeys. Serum levels of estradiol, progesterone as well as ir-inhibin during the treatment and post-treatment periods were lower than the pre-treatment levels.

Table 1 The menstrual cycle length of monkeys treated with 10, 100, and 1,000 mg/day of PM during the treatment and post-treatment periods.

	Menstrual cycle length (days)	
Treatment group	Treatment period	Post-treatment period
10 mg/day	no. 601: 50, 81, 52	no. 601: 29, 28
	no. 627: 49, > 90 [CC]	no. 627: 27, 36, > 60 [NR]
	no. 619: > 90 [CC]	no. 619: 22, 38, 30
100 mg/day	no. 616: 15, > 90 [CC]	no. 616: > 60 [NR]
	no. 801: 21, > 90 [CC]	no. 801: 39, 38, 32
	no. 108: > 90 [CC]	no. 108: > 60 [NR]
1,000 mg/day	no. 624: > 90 [CC]	no. 624: > 60 [NR]
	no. 77: > 90 [CC]	no. 77: > 60 [NR]
	no. 633: > 90 [CC]	no. 633: > 60 [NR]

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

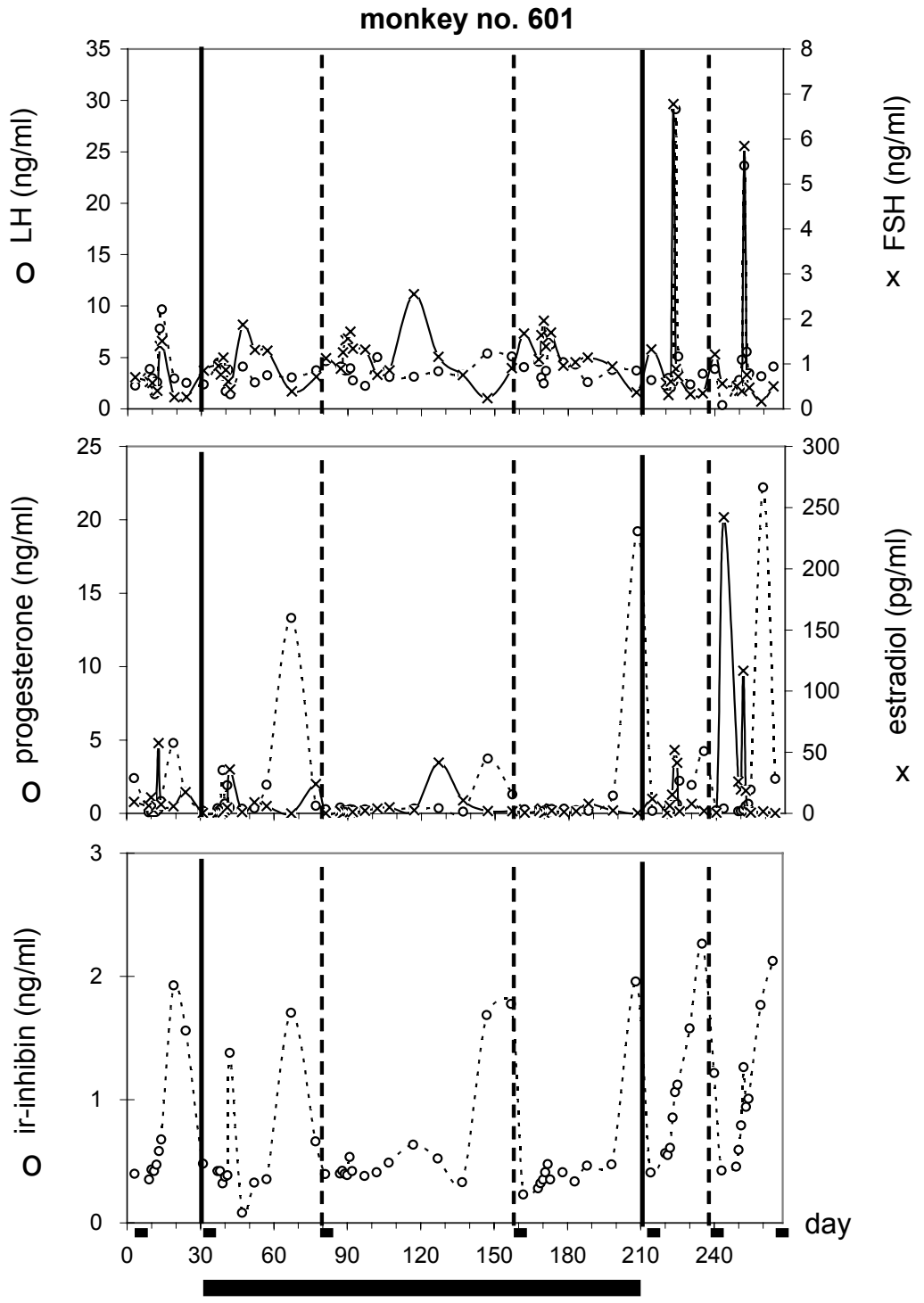

Fig. 1 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey no. 601 treated with PM-10. Day 1 represents the first day of menstruation in the pre-treatment period. The short horizontal bars represent the day of menses. Period between solid vertical lines represents the treatment period.

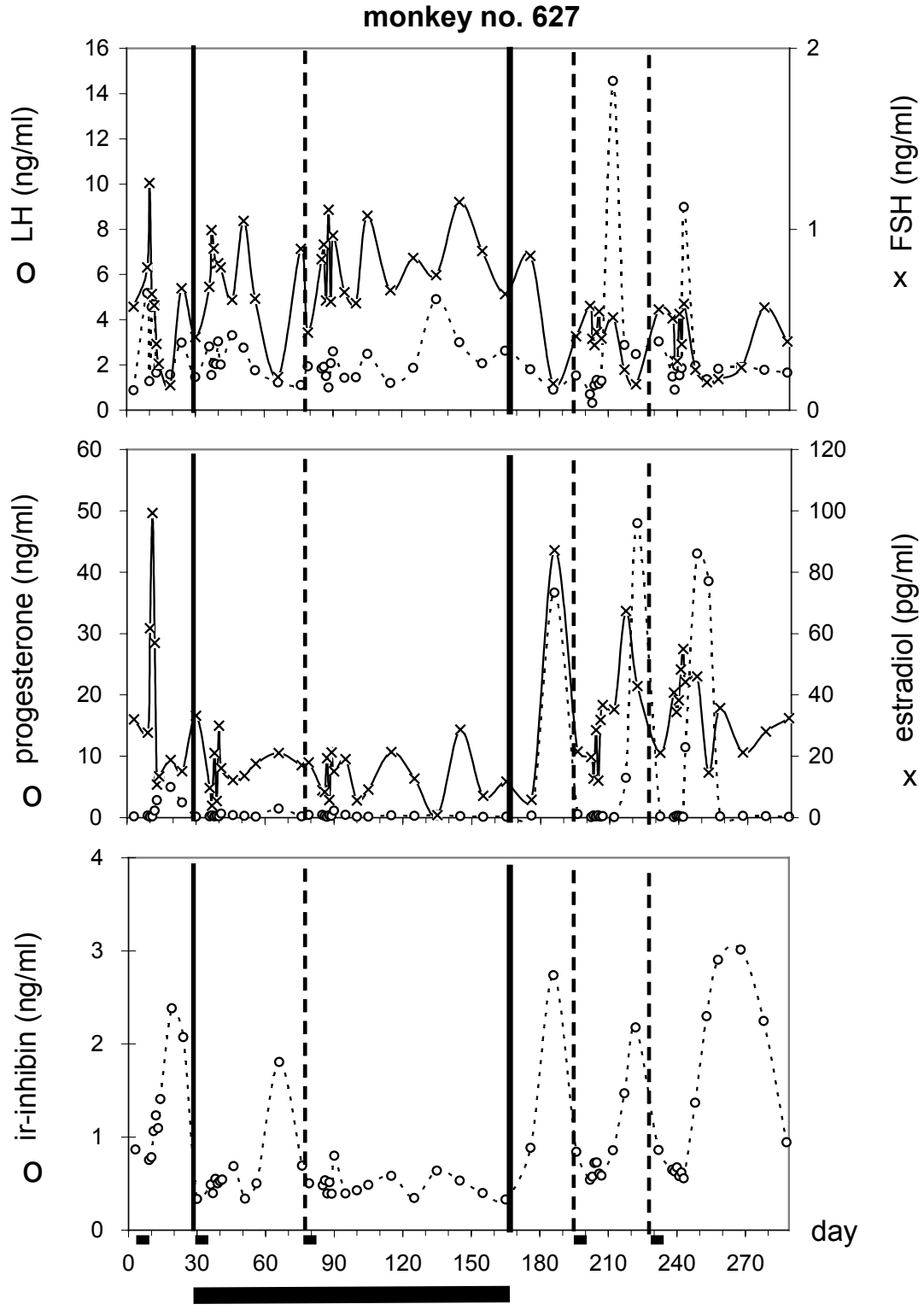

Fig. 2 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey no. 627 treated with PM-10. The meaning of day 1, short horizontal bars, and solid vertical lines are in Fig. 1.

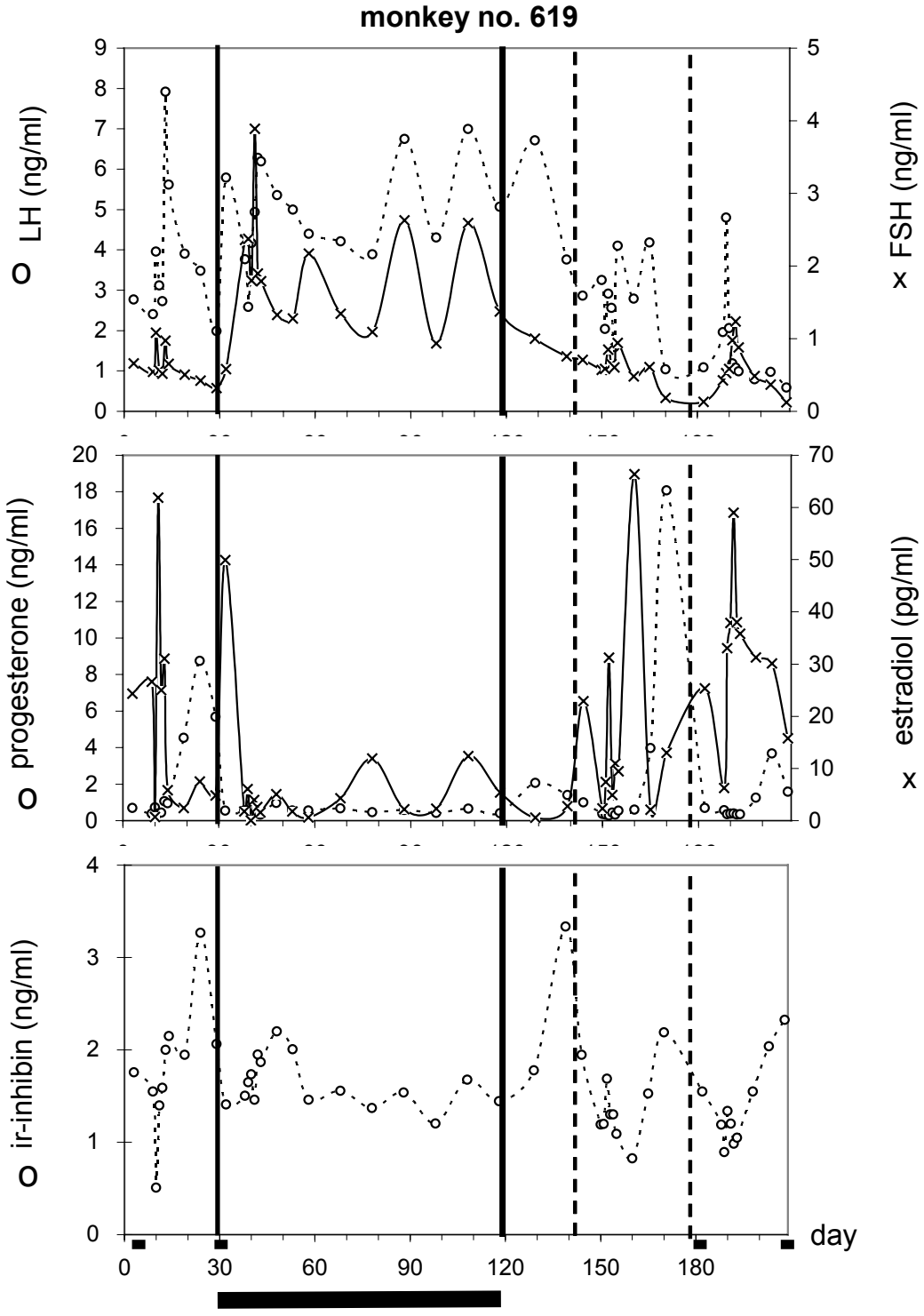

Fig. 3 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey no. 619 treated with PM-10. The meaning of day 1, short horizontal bars, and solid vertical lines were in Fig. 1.

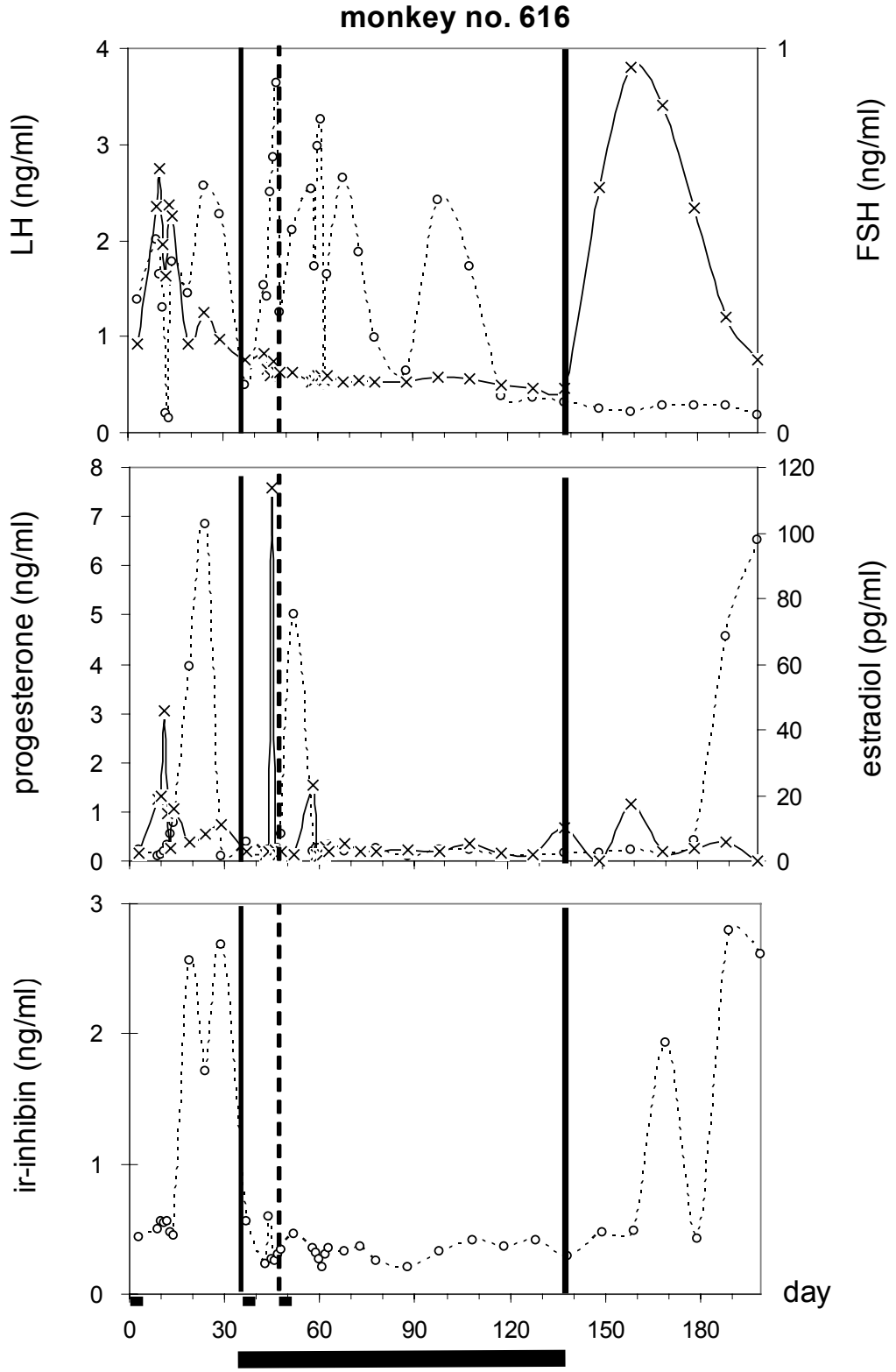

Fig. 4 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey no. 616 treated with PM-100. The meaning of day 1, short horizontal bars, and solid vertical lines are in Fig. 1.

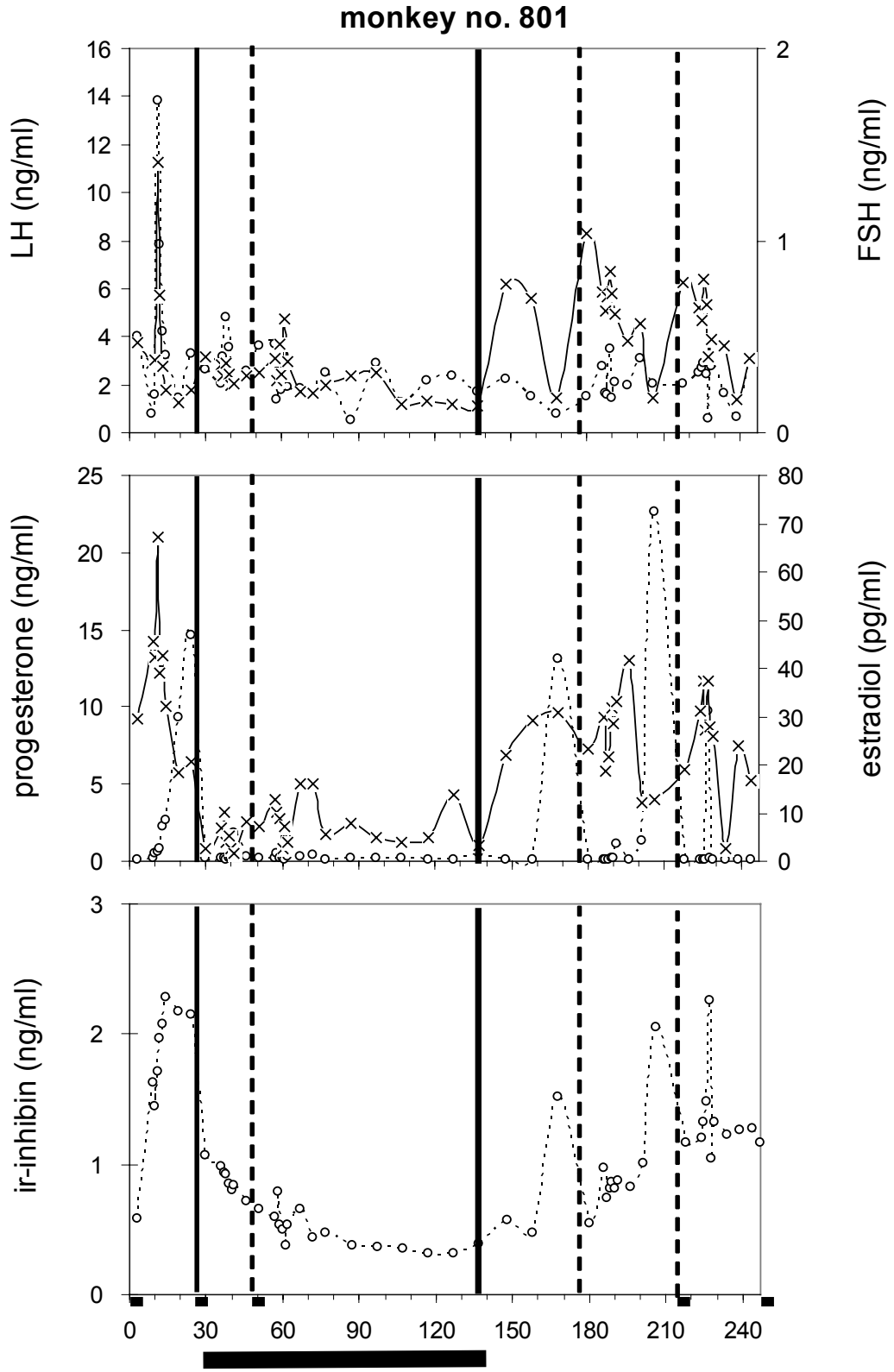
monkey no. 801
LH (ng/ml)
FSH (ng/ml)
progesterone (ng/ml)
estradiol (pg/ml)
ir-inhibin (ng/ml)
0
30
60
90
120
150
180
210
240

Fig. 5 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey no. 801 treated with PM-100. The meaning of day 1, short horizontal bars, and solid vertical lines are in Fig. 1.

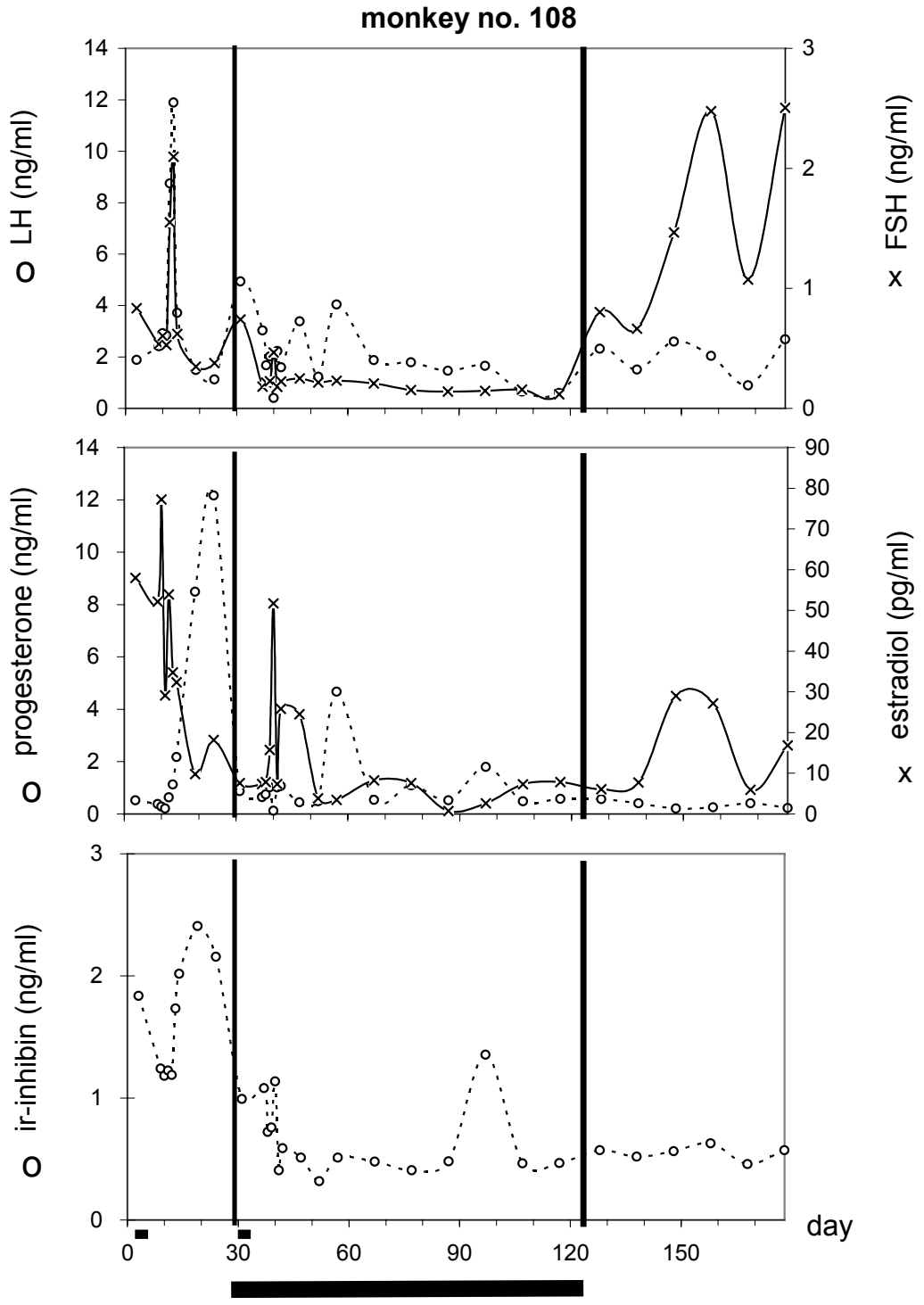

Fig. 6 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey no. 108 treated with PM-100. The meaning of day 1, short horizontal bars, and solid vertical lines are in Fig. 1.

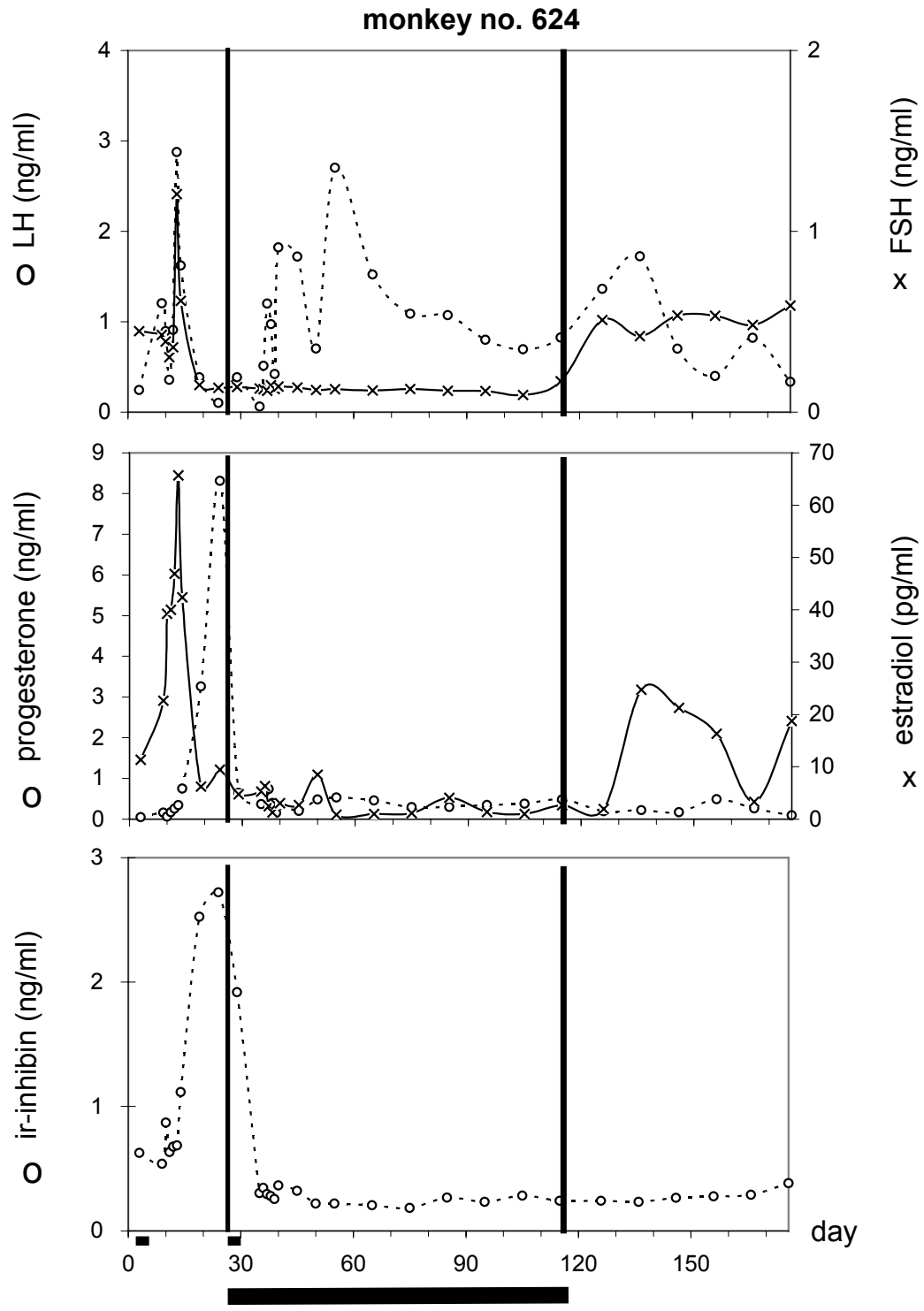

Fig. 7 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey no. 624 treated with PM-1,000. The meaning of day 1, short horizontal bars, and solid vertical lines are in Fig. 1.

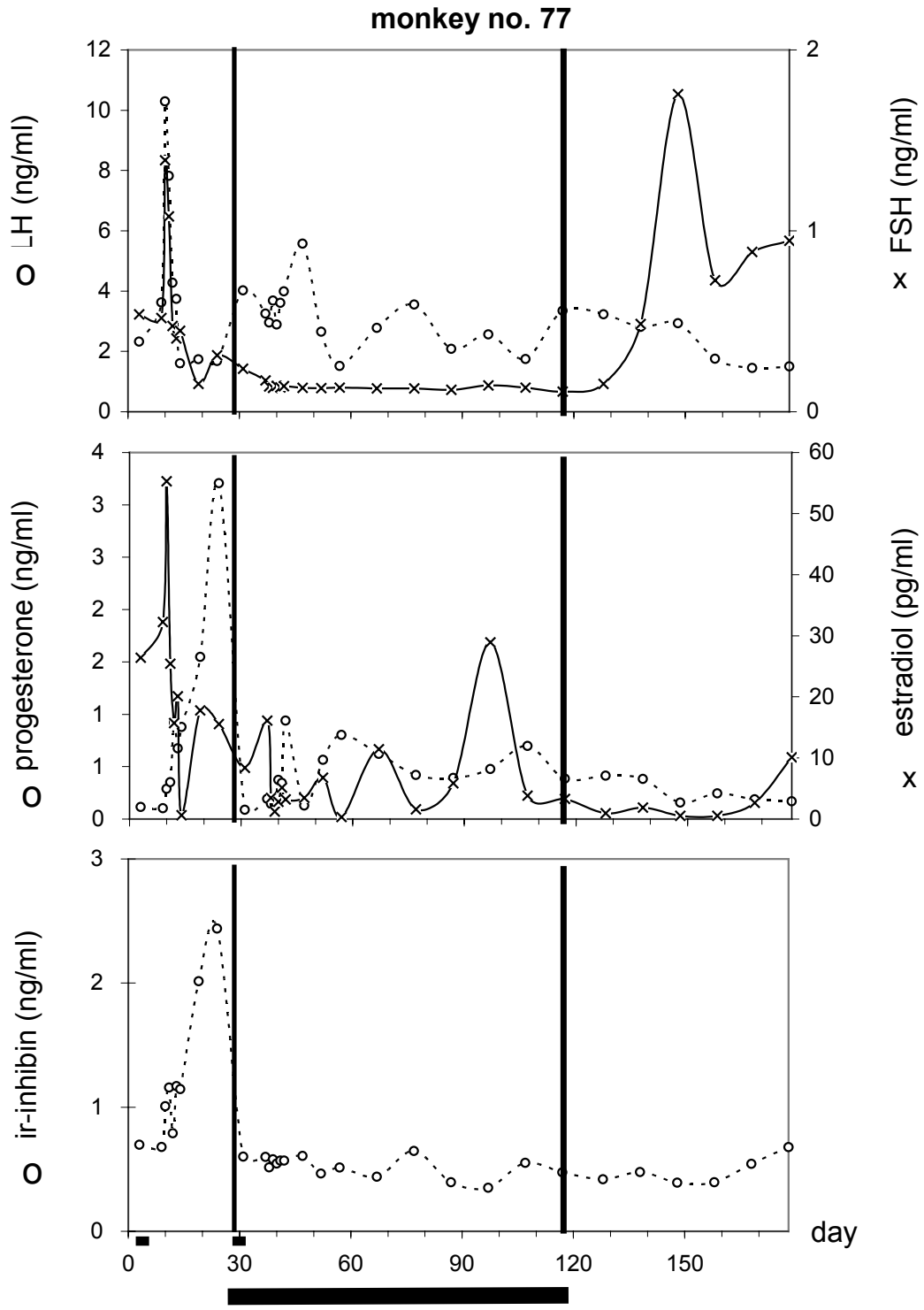

Fig. 8 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey no. 77 treated with PM-1,000. The meaning of day 1, short horizontal bars, and solid vertical lines are in Fig. 1.

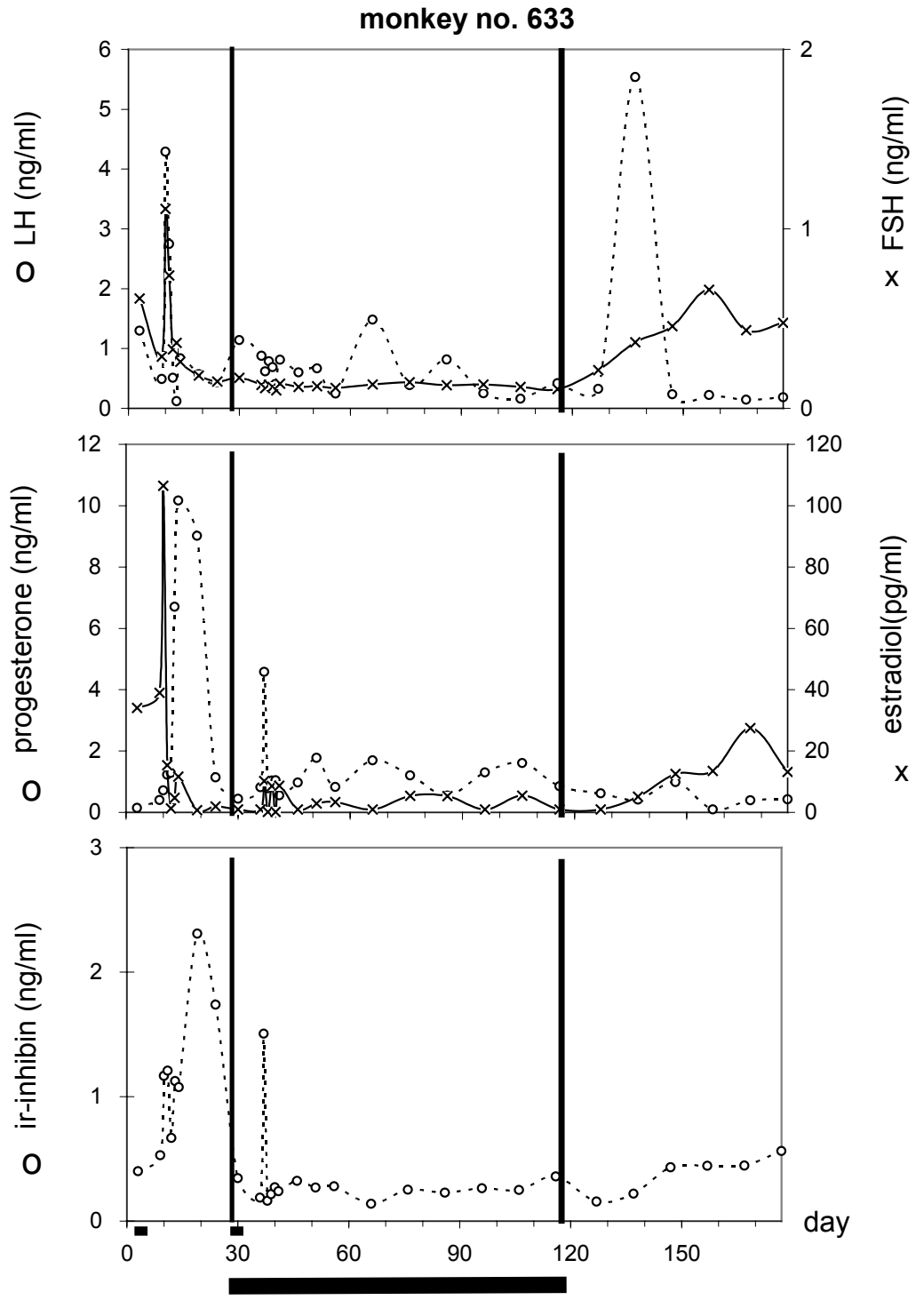

Fig. 9 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol, progesterone, and ir-inhibin) of monkey no. 633 treated with PM-1,000. The meaning of day 1, short horizontal bars, and solid vertical lines are in Fig. 1.

Discussion

Our study is the first report demonstrating the effect of PM, an indigenous Thai herb, on changes of menstrual cycle length and related hormones in adult female monkeys. The present study clearly demonstrated that estrogenic effect of PM disturbed the menstrual cycle in the monkeys. There was an increase in the menstrual cycle length of monkeys treated with PM in a dose dependent manner. The highest dose of PM (PM-1,000) showed a complete cessation of the menstruation throughout the 90-days of PM treatment and 60-days post-treatment. The lower doses of PM (10 and 100 mg/day) 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/day).

The prolongation of the menstrual cycle length was concurrent with the decrease in serum levels of FSH and LH during PM treatment and depended on the dose of treatment. Monkeys treated with the highest dose of PM (PM-1,000) had the complete suppression of serum levels of FSH and LH throughout the 90-days of treatment period and the 60-days of post-treatment period. The suppression of these hormone levels seems to be very strong. 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 levels of serum FSH or LH.

In 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 (Tonetta and Dizerega, 1989). Accordingly, our result can be inferred that PM containing phytoestrogens may impair the follicular growth and development due to decreased FSH and LH levels from the anterior pituitary gland. The impairment of the ovarian function in the monkeys is proved by the decreased in both basal and peak level of estradiol entire menstrual cycles.

Moreover, the high levels of estradiol and progesterone, which were largely secreted from the corpus luteum (Billiar et al., 1991), decreased significantly during the luteal phase. Estradiol and progesterone levels are known to correlated positively with

corpus luteum function. 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 (Goldstein et al., 1982). 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 (Mclachan et al., 1987) and monkeys (Fraser et al., 1989, 1999; Shimizu et al., 2002). The secretion pattern of inhibin during the luteal phase shows a positive correlation with progesterone levels and a negative correlation with FSH levels (Goldstein et al., 1982; Shimizu et al., 2002). The major stimulus of ir-inhibin secretion during the luteal phase is LH. (Goldstein et al., 1982; Shimizu et al., 2002) as shown in the study of LHRH antagonist to inhibit the LH secretion (Fraser et al., 1989). Accordingly, the decrease in serum ir-inhibin and progesterone levels throughout the treatment can support our hypothesis.

Estrogenic effects of PM phytoestrogens are concurrent with the previous reports investigating effect of soy phytoestrogens. Premenopausal women with regular ovulatory cycles who ingested soy protein containing 25 - 60 mg of isoflavones for 1 – 2 months had an extension in the follicular phase length and delayed menstruation (Cassidy et al., 1994, 1995; Lu et al., 1996). In addition, there were the suppression of FSH and LH surge at the follicular phase (Cassidy et al., 1994, 1995; Duncan et al.,1999) and decreased levels of estradiol and progesterone entire menstrual cycle (Lu et al., 1996, 2000).

The potential effect of PM phytoestrogens are mediated by estrogen receptor (ER) at target sites including the pituitary glands, hypothalamus, gonad, and uterus (Shughure et al., 1998). Previous studies of chemical structures of phytoestrogens indicated that phytoestrogens, which are heterocyclic phenol with a close similarity in structure to estradiol, is a prerequisite for binding to ER and then can act as estrogen agonist (Kuiper et al., 1998). 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 was analyzed by the HPLC and found that it contains the high amounts of isoflavones (1.69 mg/g of dried weight) and the lower amounts of miroestrol, deoxymiroestrol, and the other (Muagman and Cherdshewasart, 2001). So, it can deduce that isoflavones are a major phytoestrogens

of PM on gonadotropins and ovarian hormones. However, the present study could not eradicate the potential role of other phytoestrogens.

Summary, our finding strongly indicates that PM containing phytoestrogens can disturbed the menstrual cycle and has an endocrine-modulating effect by acting as a suppressor on the ovulation, resulting from the decrease in serum levels of gonadotropins and ovarian hormones.

Title: Estrogenic effect of *Pueraria mirifica* on Gonadotrophin Levels in Aged Monkeys

Abstract

To investigate the effect of *Pueraria mirifica* (PM) on gonadotrophins and estradiol levels in aged animals, nine menopausal cynomolgus monkeys were divided into 3 groups. Each group (n = 3) was fed with 10, 100, and 1,000 mg/day of PM for 90 days. PM-10 induced the decrease of follicle stimulating hormone (FSH) levels on days 15 – 90 in 1 out of 3 monkeys. PM-100 and PM-1,000 decreased FSH levels throughout the treatment period. After the treatment period, FSH levels continued to decrease for 5 and 10 - 20 days in PM-100 and PM-1,000, respectively, and the levels rebounded in all groups thereafter. PM-10 decreased luteinizing hormone (LH) levels throughout the treatment period in 1 out of 3 of monkeys and returned to the pre-treatment levels immediately after stop treatment. PM-100 and PM-1,000 prominently decreased LH levels between days 10 – 90 during treatment and prolonged to days 15 – 25 and days 20 - 30 for PM-100 and PM-1,000, respectively, during the post-treatment period. Serum LH levels showed the rebound after returning to the 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, it 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.

Keywords: *Pueraria mirifica*, phytestradiol, 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 was proved to be extremely rich in an isoflavone group of phytestradiols (Muangman and Cherdshewasart, 2001). Also present are small amounts of other phytestradiols including coumestrol (Ingham et al., 1986a, 1989), miroestrol (Pope et al., 1958), puerarin (Ingham *et al*., 1986b), deoxymiroestrol, and kwakhurin (Chansakaow et al., 2000a, 2000b). Miroestrol showed a high estrogenic potency when assayed by the immature mouse uterine weight and rat vaginal cornification tests (Pope et al., 1958; Jones and Pope., 1960). Miroestrol treatment increased uterine weight in immature female mice (Jones and Pope et al., 1960) and produced the cornification of the vaginal epithelium in ovariectomized-adrenalectomized rats (Pope et al., 1958). PM has recently gained 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 single feeding of 10, 100, and 1,000 mg/ of PM prolonged the menstrual cycle length in adult cyclic cynomolgus monkeys (Trisomboon et al., 2004). Moreover, the long-term treatment suppressed serum levels of gonadotrophins and ovarian hormones in a dose dependent. The monkeys fed with the highest dose (1,000 mg/day) showed the symptom of amenorrhea (Trisomboon et al., 2002). Clinical trials studying long-term consumption of PM showed reduced postmenopausal symptoms such as hot flush, sleep disorder, and skin dryness (Muangman and Cherdshewasart, 2001). From the previous studies, however, there were no data of the effect of PM phytoestrogens on hormones related reproduction in aged menopausal women that have an ovarian estradiol deficiency. Actually, the number of aged menopausal women who now use natural estrogens, instead of synthetic estrogen, to treat their menopausal symptoms, is increasing.

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 (Baird et al., 1995; Duncan et al., 1999) and postmenopausal women (Murkies et al., 1995) who consumed soy daily. Additionally, epidemiological studies showed lower levels of oestrone and estradiol in postmenopausal Japanese women who consumed high amounts of soy isoflavones (Shimizu et al., 1990).

It is of interest to investigate the long-term effect of PM treatment on serum levels of FSH, LH, and estradiol in aged 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 (Krajewski et al., 2003). The present study, therefore, aimed to investigate the effect of daily treatment of PM for 90 days on gonadotropins and estradiol levels in aged menopausal monkeys.

Materials

Animals

Nine aged menopausal cynomolgus monkeys (*Macaca fascicularis*) with complete cessation of menstruation for at least one year before onset of the experiment and weighing from 4.0 - 6.5 kg were searched from the menstruation record and selected. Menopausal state of the monkeys was confirmed and checked daily by vaginal swabbing method before and during the study period. 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 fed daily with monkey feed (Pokaphan Animal Feed Co., Ltd., Bangkok, Thailand) in the morning (0600-1800 light on) 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 aged menopausal 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, and 1,000 mg/5 ml of distilled water/individual/day (hereafter abbreviated as PM-10, PM-100, or PM-1,000) between 0800 – 0830 h. The treatment schedule was separated into 3 periods: pre-treatment, treatment, and post-treatment. During the pre-treatment and post-treatment periods, monkeys were fed daily with 5 ml of distilled water for 30 and 60 days. During the treatment period, monkeys were fed daily with the suspension of PM for 90 days. Blood samples, 3 ml, were collected from the femoral

vein without anaesthetization between 0830 – 0930 h every 5 days. The samples were centrifuged 1,700 x g at 4 º C, for 20 minutes and stored at –20 º C until FSH, LH, and estradiol assays were performed.

P. Mirifica Suspension Preparation

The tuberous roots of PM used in this study were cultivar-wichai III collected from Chiangmai province, northern Thailand. To minimize the variation of phytoestrogen content in PM with seasons and locations the tuberous roots of PM used in this study were obtained from the same lot. The tuberous roots of PM were sliced, desiccated in a hot air oven at 70 º 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 º C until feeding time.

Hormonal Analyses

The serum samples were analyzed for FSH and LH levels using a heterologous RIA system described previously (Watanabe et al., 1990; Nozaki et al., 1990). 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 inter-assay coefficients of variations were 5.82 and 7.32% for FSH and 5.71 and 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) programme (Sufi et al., 1986). The intra- and inter-assay coefficients of variations were 5.07 and 7.02% for estradiol, respectively.

Since the chemical structures of phytoestrogens 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 1,000 – 0.001 µg did not show a cross reactivity with the estradiol antibody in the WHO-RIA assay system (Fig.1)

Statistical Analysis

Serum hormone levels were expressed as mean ± S.E.M. Analysis of variance (ANOVA) followed by the LSD test was used to determine the significant difference between those three periods and between three groups. Differences were considered significant at a level of $P < 0.05$.

Results

Basal FSH, LH, and Estradiol Levels in Aged Menopausal Monkeys

Menopausal state of nine cynomolgus monkeys in this study was approved by the low level of serum estradiol (14.71 ± 1.18 pg/ml) and the high levels of serum FSH (3.81 ± 0.61 ng/ml) and LH (5.85 ± 0.80 ng/ml) throughout the 30 days of pre-treatment period. When we compared those hormonal levels of aged menopausal monkeys to the normal cyclic monkeys at the early follicular phase of the menstrual cycle in our laboratory, the estradiol levels in cyclic monkeys was higher (27.54 ± 4.31 pg/ml) and the FSH and LH levels were lower (1.60 ± 0.33 ng/ml for FSH, and 0.51 ± 0.03 ng/ml for LH) than that of aged monkeys.

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

Changes in serum levels of FSH, LH, and estradiol during the treatment and post-treatment periods in aged menopausal monkeys treated with PM-10, PM-100, and PM-1,000 are shown in Figs. 2 - 4.

Compared to pre-treatment levels, FSH levels decreased on days 10 - 15 in monkey nos. 58 and 85 and on days 15 - 90 in monkey no. 11 after treatment with PM-10. Serum FSH levels in those three monkeys, however, tended to increase and reached to pre-treatment levels thereafter. Interestingly, the FSH levels were higher than the pre-treatment levels after the PM withdrawal, so-called a rebound effect, during the post-treatment period. In all monkeys treated with PM-100, serum FSH levels decreased on days 5 – 90 during the treatment period. Serum levels of FSH remained low until the first 5 days of the post-treatment period and rebounded thereafter. In monkeys treated with PM-1,000, FSH levels decreased on days 5 – 90 during the treatment period and kept the low levels until days 10 – 20 of the post-treatment period before returning to the pre-treatment levels. FSH levels in monkeys treated with PM-

1,000 showed a rebound slower than the monkeys treated with PM-100 and PM-10, respectively.

The decrease of LH levels in monkeys treated with PM-10 was varied as shown in Fig. 3. Monkey no. 11 showed the high levels of basal LH in serum throughout the 30 days of pre-treatment period and a prominent decrease on days 5 - 90 during PM-10 treatment. In monkey nos. 58 and 85, LH levels did not significantly change from the pre-treatment levels. In monkeys treated with PM-100 and PM-1,000, LH levels showed a graded decrease within 10 days of the treatment period. During the post-treatment period, the levels remained low until the first 15 - 25 days for the PM-100 and the first 20 - 30 days for PM-1,000. LH levels during the post-treatment period were increased to the pre-treatment levels in all monkey groups and rebounded in some monkeys treated with PM-10 (nos. 58 and 85) and PM-100 (nos. 42 and 605).

As shown in Fig. 4, estradiol levels in all nine monkeys tended to be decreased during the treatment period and increased to the pre-treatment levels in monkeys treated with PM-1,000. Furthermore, all monkeys treated with PM-10 and PM-100 did not recover to the pre-treatment levels during the post-treatment period except monkey no. 42.

Comparison the Various Doses of PM on FSH, LH, and Estradiol Levels

As shown in Fig. 5, those nine monkeys showed various basal FSH and LH levels. Thus, the monkeys were divided into 2 groups according to the duration of entering the menopausal stage; less or more than 5 years. Menopausal monkeys that entering the menopausal state less than 5 years showed lower levels of FSH (P = 0.0008) and LH (P = 0.001) than that of more than 5 years. Estradiol levels, however, did not significantly differ between that two groups (P = 0.95), as shown in Fig. 5. For the comparison of changes of FSH and LH levels between 3 groups of monkeys, hormone levels in each monkeys during 30 days of pre-treatment period were arbitrarily assigned to 100. Serum FSH and LH levels during the treatment and post-treatment periods were adjusted to be the percent changes from the pre-treatment levels thereafter and the levels from three monkeys were pooled.

As shown in Fig. 6, comparing to the pre-treatment level, FSH levels decreased to 37.04, 19.33, and 6.69 % on day 10 during the treatment period for PM-10, PM-100, and PM-1,000, respectively. FSH levels were remained low in PM-100 and PM-1,000 groups (7.36 – 19.33 % for PM-100, and 3.09 – 20.15 % for PM-1,000) throughout the

treatment period, but it started to recover to the pre-treatment levels in PM-10 group (37.04 – 244.67 %) before rebounding thereafter. During the post-treatment period, FSH levels increased and rebounded on days 10 and 20 for PM-100 and PM-1,000 groups, respectively. LH levels decreased to 51.07 – 128.68 % for PM-10, 22.70 – 77.32 % for PM-100, and 18.86 – 32.98 % for PM-1,000 between days 10 – 90 during the treatment period. LH levels rebounded on days 5 and 45 of the post-treatment period for PM-10 and PM-100, respectively. The LH levels in PM-1,000 group, however, were kept lower than the pre-treatment levels until 60 days of the post-treatment period.

Estradiol levels decreased within days 10 – 90 during the treatment period and increased to the pre-treatment levels during the post-treatment period in all monkey groups. The suppression of PM phytoestrogens on estradiol levels did depend on dose.

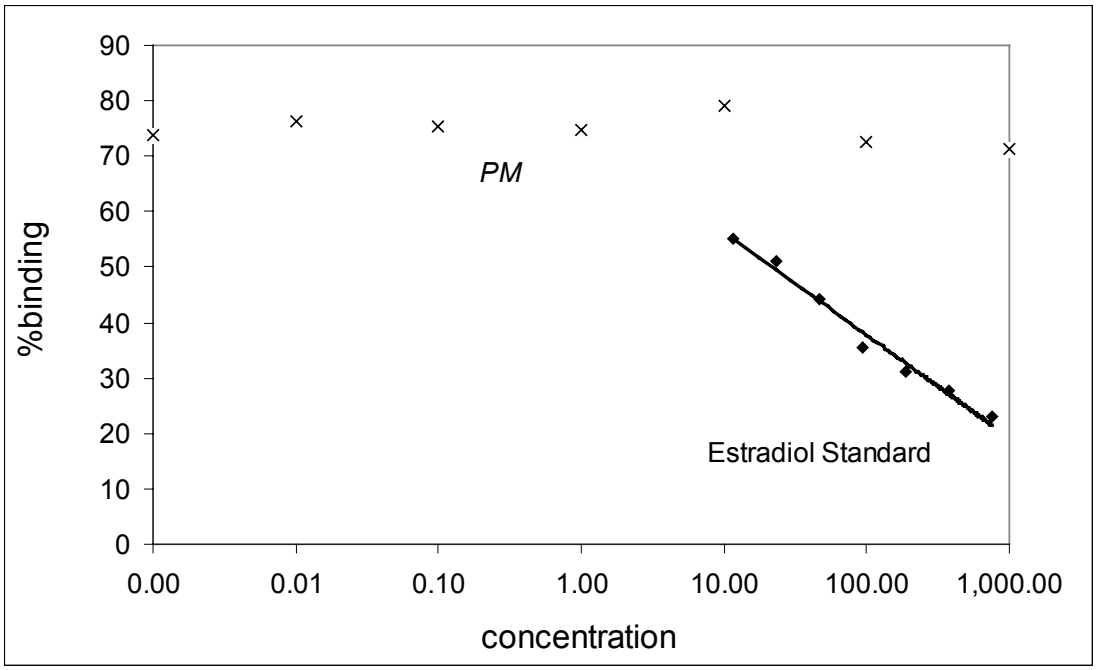

Fig. 1 The percentage of maximum binding to estradiol antibody of PM phytoestrogen against concentration compared to the standard curve of the assay.

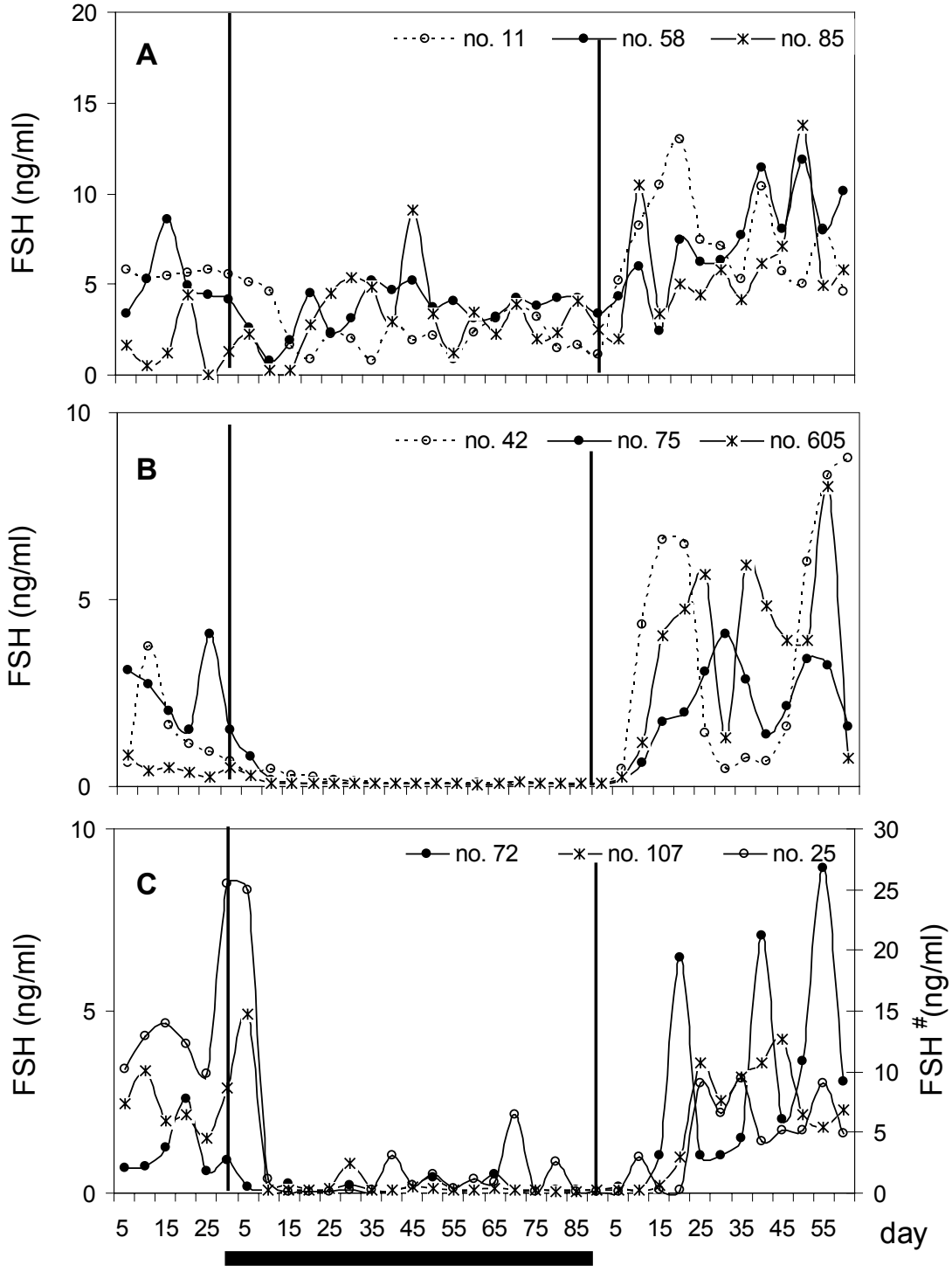

Fig. 2 Changes in FSH levels during the pre-treatment, treatment, and post-treatment periods in aged menopausal monkeys treated with 10 (A), 100 (B), and 1,000 (C) mg/day of *P. mirifica*. The vertical lines separate each period. A black horizontal line indicates treatment period. # indicates serum FSH levels of monkey no. 25.

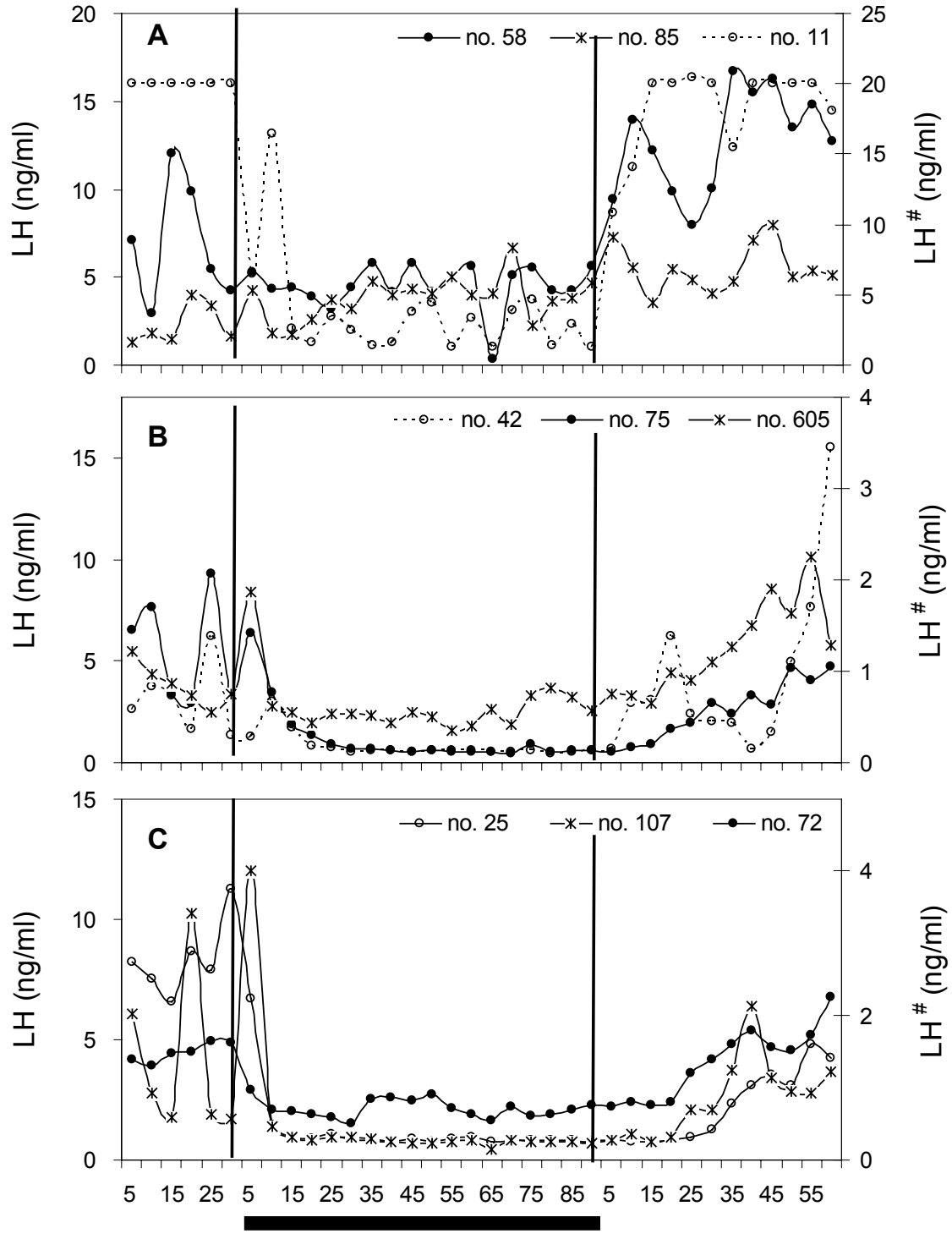

Fig. 3 Changes in LH levels during the pre-treatment, treatment, and post-treatment periods in aged menopausal monkeys treated with 10 (A), 100 (B), and 1,000 (C) mg/day of *P. mirifica*. The meanings of the vertical and black horizontal lines are shown in Fig. 2. # indicates serum LH levels of monkey nos. 11, 605, and 72.

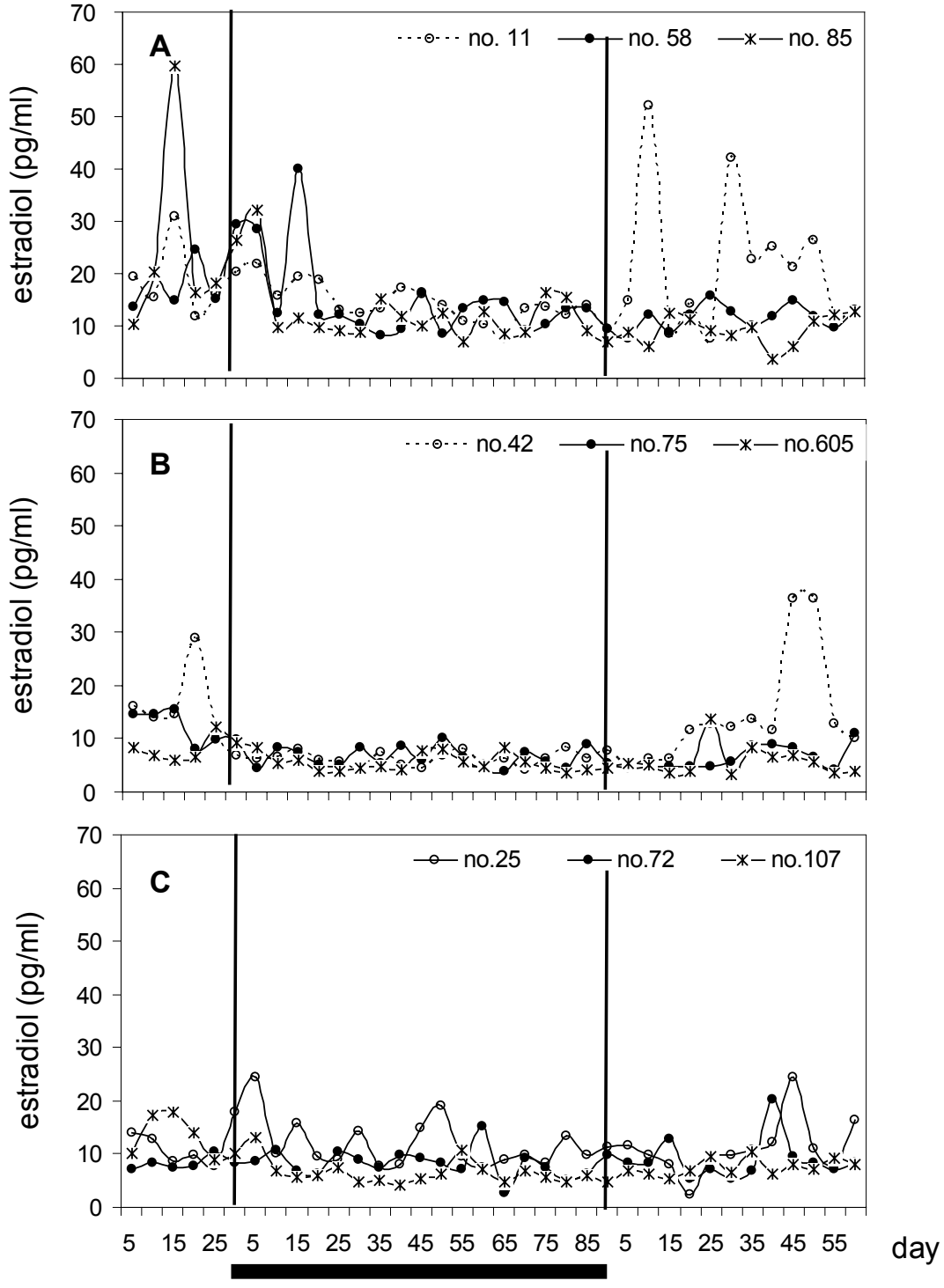

Fig. 4 Changes in estradiol levels during the pre-treatment, treatment, and post-treatment periods in aged menopausal monkeys treated with 10 (A), 100 (B), and 1,000 (C) mg/day of *P. mirifica*. The meanings of the vertical and black horizontal lines are shown in Fig. 2.

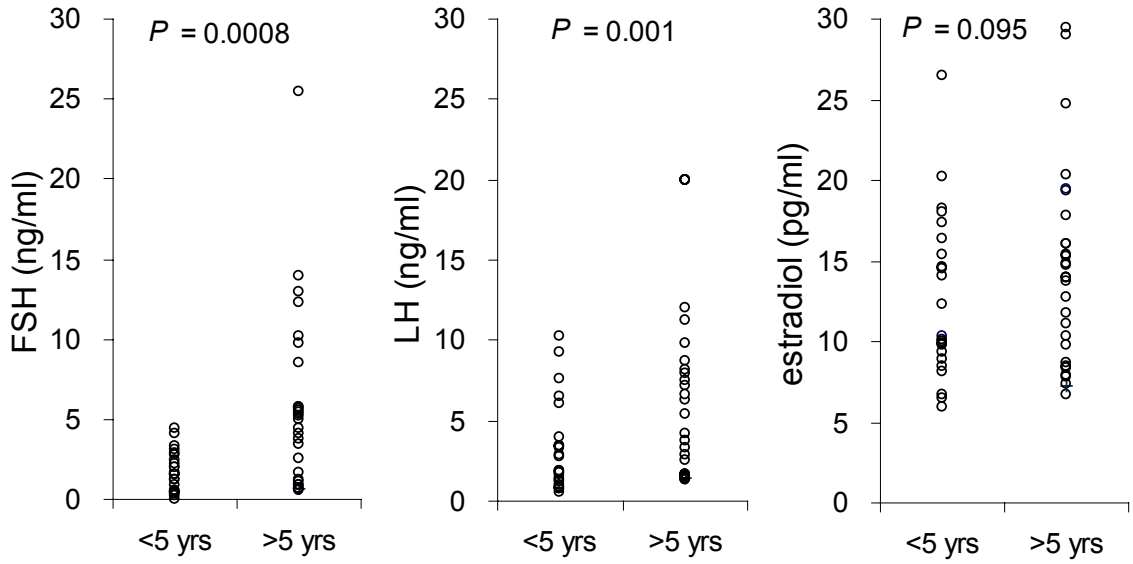

Fig. 5 Basal level of FSH, LH, and estradiol in aged monkeys, which menopause period are longer, and shorter than 5 years. Data are expressed as mean ± S.E.M. *P* value shows the significant difference between longer and shorter menopausal year.

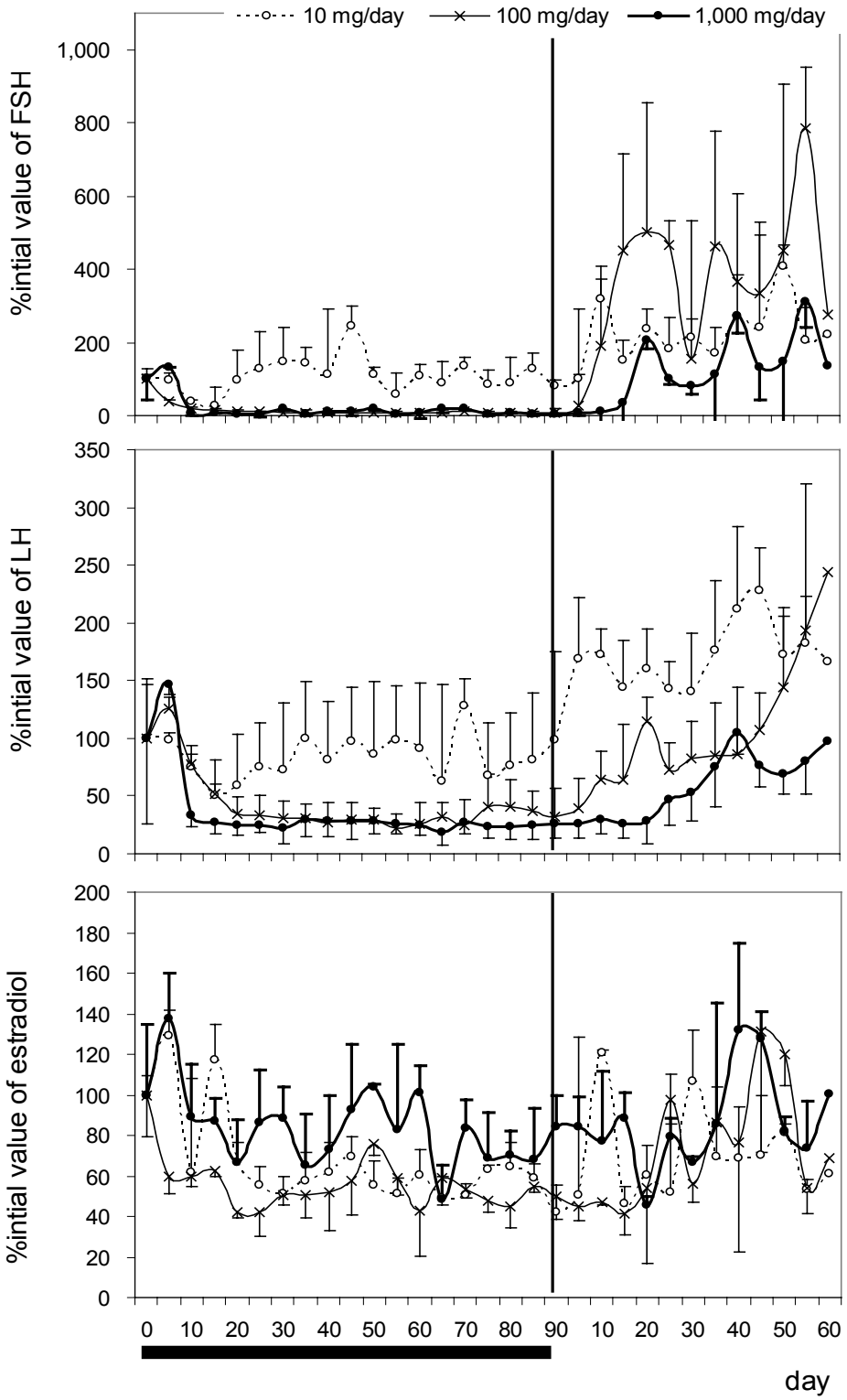

Fig. 6 Serum levels of FSH, LH, and estradiol during the treatment and post-treatment periods were adjusted to percent changes from the pre-treatment levels, arbitrarily assign at 100. A black horizontal line indicates the treatment period. Data are expressed as mean ± S.E.M.

Dicussion

It has long been recognized that estrogen deficiency due to the cessation of ovarian function in aged menopausal monkeys is associated with a significant rise in pituitary gonadotrophin levels, similar to those of postmenopausal women (Park et al., 2002; Woller et al., 2002). Loss of ovarian estrogen with menopause results in the malfunction of the negative feedback mechanism on the hypothalamus and pituitary and, thus, both of FSH and LH levels are increased. The variations of these hormones should be come from the difference in the timing to enter the menopause state. In this study, we found that the oral administration of PM induced a decrease in both of FSH and LH levels in aged menopausal monkeys in a dose-dependent manner. Comparing the response of FSH and LH levels to PM, the higher dose exhibited the higher potency in the decrease of FSH and LH levels.

PM is known to contain many kinds of phytoestrogens (Pope et al., 1958; Ingham et al., 1986a, 1986b, 1989; Chansakaow et al., 2000a, 2000b; Muangman and Cherdshewasart, 2001), mainly isoflavones, which present nonsteroidal structures similar to those of estrogens. It can bind to estrogen receptor and exert estrogenic effect in mice (Jones and Pope, 1960), rats (McGravy et al., 2001) and humans (Cassidy et al., 1994, 1995; Duncan et al., 1999). Previous studies investigated the potential action of phytoestrogens, genistein, on the hypothalamus and pituitary axis in ovariectomized rats and demonstrated that genistein administration blocked the GnRH-induced rise of LH in ovariectomized rats (Faber and Hughes, 1991; Hughes et al., 1991). Coumestrol administration led to the decrease of the GnRH pulse generator frequency, reduced pulsatile secretion of LH in addition to suppression on pituitary LH response to GnRH priming in ovariectomized rats both *in vivo* and *in vitro* studies (Mc Garvery et al., 2001).

Concurrently, studies on exogenous estrogen administration showed the direct effect of the negative feedback mechanism. Estradiol benzoate reduced LH release caused by reducing the sensitivity of pituitary gonadotrope to GnRH stimulation without altering the GnRH secretion pattern in ovariectomized rhesus monkeys, suggesting the target site of estrogen on pituitary level (Nakai et al., 1978; Pau et al., 1990). In addition, administration of estradiol benzoate using push-pull perfusion decreased pulse amplitude and basal release of GnRH and reduced LH levels in ovariectomized rhesus monkeys, suggesting the target site of estrogen on the hypothalamus level (Chongthammakun and Terasawa, 1993; O'Byrne et al., 1993). In our study, although we could not conclude that PM treatment affects at the hypothalamus level, we could assume that PM has an estrogenic effect to decrease in gonadotrophins by the negative feedback mechanism at the hypothalamus and pituitary. We consider that the estrogenic effects

of PM phytoestrogens to suppress gonadotrophin levels support other studies, which reported effect of high consumption of soy phytoestrogens on hormonal levels in both pre- and post-menopausal women (Duncan et al., 1999a, 1999b).

The deficiency of ovarian function in postmenopause results the decrease of the secretion of ovarian estradiol. Aged menopausal monkeys in this study have the lower levels of serum estradiol than those in normal cyclic monkeys at the early follicular phase. During long-term treatment of PM, serum levels of estradiol decreased and returned to the pre-treatment levels after cessation of PM treatment in all monkeys groups because estradiol in menopausal individuals mainly comes from the peripheral conversion of androstenedione via oestrone and androstenedione via testosterone, not from the ovary. These finding seem to imply that PM phytoestrogens may have a direct effect on estradiol production by the conversion of other steroid hormones, not depended on the decreased gonadotrophins through the hypothalamus-pituitary-ovarian axis. There are *in vitro* studies demonstrating that the genistein and coumestrol reduced these conversions (Makella et al., 1995; Whitehead et al., 2002). Nevertheless, the additional studies for the further understanding have to be done.

Estrogenic effect of soy phytoestrogens on serum levels of reproductive hormones was also studied in postmenopausal women. The daily intake of high amounts of soy containing 165 mg isoflavones for 4 weeks induced the slight decrease in circulating FSH, LH, and estradiol levels in postmenopausal women (Baird et al., 1995). At lower doses of isoflavones, 7.1-132.0 mg for 3 months could not change serum FSH or LH levels (Duncan et al., 1999). The same result was obtained in another study that is, the daily intake of 56 and 90 mg of isoflavones for 3 or 6 months, could not change serum levels of FSH, LH, or estradiol in postmenopausal women (Persky et al., 2002). Muangman and Cherdshewasart, (2001) analyzed the content of isoflavones in the same lot of PM used in this study, and reported that it contains 1.691 mg of total isoflavones/g dried powder. Thus, the isoflavones content in the PM at doses of 10, 100, and 1,000 mg/day are less that those contained in soy used in other studies (Baird et al., 1995; Duncan et al., 1999; Persky et al., 2002). However, the lowest dose of PM (10 mg/day) treatment, containing 0.0169 mg isoflavones, can clearly suppress serum FSH and LH levels in aged monkeys. It can postulate that the potency of PM is stronger than soy under the same quantity in isoflavones. From *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 genistein to estrogen receptors are between 1.00 – 0.05 % (Shutt and Cox, 1972). It is possible that the stronger potency of PM than soy is due to the presence of other kind of phytoestrogens in PM, e.g. coumestrol and miroestrol.

The time course of PM function is worthwhile considering. Suppressive effect of PM on serum levels of gonadotrophins was observed on days 10 - 90 during the treatment period. Pharmacokinetics studies showed that after ingestion of soy isoflavones, the elimination half-life of genistein and daidzein are 7 and 4 h in premenopausal women and 4 and 3 h in men, respectively (Lu and Anderson, 1998). Other studies also indicated that the elimination half-lives of genistein and daidzein are 5.5 and 7.4 h in premenopausal women (Setchell et al., 2003) and 8.3 and 5.8 h in men, respectively (Watanabe et al., 1998). From these evidences, we can assume that at the initial feeding, concentrations of phytoestrogens in blood circulation did not reach to the threshold level of response. Although no physiological response could be observed, the biotransformation of phytoestrogens should have occurred and the metabolites were excreted through the urine. After a daily treatment of PM for approximately 10 days, the concentrations of phytoestrogens in the blood circulation were accumulated to reach to the threshold concentrations that resulted in the full-physiological response occurred in all monkeys, e.g. the decrease of FSH and LH levels. This study also found the latency period of the recovery of gonadotrophin levels during the post-treatment period, in a dose-dependent manner. After the cessation of PM treatment, FSH and LH levels remained low for several days before recovery to the pre-treatment levels. FSH and LH levels rebounded abruptly after the cessation of PM-10 on the other hand it took a delaying time for the rebound action in the higher doses. The evidence of rebound effect was also found in the other study (Gianotti et al., 2003). A single subcutaneous injection of GnRH antagonist, a competitive inhibitor of GnRH receptors, reduced serum FSH and LH levels within 6 – 48 h and rebounded serum LH levels at 96 h. From this study, we can postulate that there is the enhancement of hypothalamic GnRH drive after relief of its antagonist (Gianotti et al., 2003). Accordingly, we assume that the rebound of gonadotrophin levels during the post-treatment period maybe caused from the increase in responsiveness of gonadotrope to GnRH.

In summary, the present study suggests that a daily treatment of PM containing phytoestrogens exerts the estrogenic effect on the suppressive gonadotrophin levels in aged menopausal monkeys and depended on doses. After the cessation of PM treatment, the decreased gonadotrophin levels can be recovered to the pre-treatment levels within 60 days and also depended on doses. We can postulate that it suggests that PM has the beneficial effect on the relief of postmenopausal symptoms.

Title: Effect of *Pueraria mirifica* on urinary gonadotropin and sex steroid hormone levels in female monkeys

Abstract

The study was investigate the effect of *Pueraria mirifica* (PM) on urinary hormone levels in female cynomolgus monkeys, both cyclic and aged menopause after a single or long-term feeding. In each experiment, the monkeys were divided into 3 groups. Each group was fed with 10, 100, and 1,000 mg/day of PM, respectively. There were three experiment in this study. Experiments 1 and 2 using adult cyclic monkeys for a single and long-term feeding of PM and experiment 3 using aged menopausal monkeys for a long-term feeding of PM. The experimental schedule was separated into 2 periods, the pre-treatment and post-treatment periods for experiment 1, separated into 3 periods, the pre-treatment, treatment, and post-treatment periods for experiments 2 and 3. Urinary FSH, LH, and estradiol levels were determined in those monkeys, while the adult cyclic monkeys were additionally determined the urinary progesterone levels. The results showed that a single feeding of all doses of PM and long-term feeding of PM-10 did not change pattern of urinary FSH, LH, estradiol, or progesterone in adult cyclic monkeys compared to those in the pre-treatment period. Long-term feeding of PM 100 and PM-1,000 induced the decrease in urinary FSH, LH, and estradiol in adult cyclic monkeys and decrease in urinary FSH and estradiol in aged menopausal monkeys during the treatment period compared to those in the pre-treatment period. However, change of urinary FSH was clearly observed more than that of other hormones. So, urinary FSH is considered as a good indicator of estrogenic effect of PM on hormonal levels in monkeys.

Keywords: *Pueraria mirifica*, phytoestrogen, urine, gonadotropin, estradiol, progesterone, female monkey

Introduction

Pueraria mirifica (PM), a Thai medicinal plant, is classified into the family Leguminoceae. Its tuberous root contains many kinds of phytoestrogens such as genistein, daidzein, coumestrol (Ingham et al., 1986, 1989), miroestrol (Pope et al., 1958), deoxymiroestrol, and kwakhurin (Chansakaow et al., 2000a, 2000b). The previous reports showed conflicting results on the influence of phytoestrogens on hormone-related ovarian function (Cassidy et al., 1995; Duncan et al., 1999; Lu et al., 2000). Daily consumption of soy containing a high amount of isoflavones depressed a midcycle surge of both follicle stimulating hormone (FSH) and luteinizing hormone (LH) in premenopausal women (Cassidy et al., 1995; Duncan et al., 1999). Other report showed, however, that a daily consumption of soy diet in premenopausal women lowered estradiol levels bud did not FSH or LH levels (Lu et al., 2000). The previous studies clearly showed that a long-term treatment of PM could suppress the levels of gonadotropins and sex steroid hormones in both of adult cyclic and aged menopausal monkeys (Trisomboon et al., 2002a, 2002b, 2004). A single feeding of PM on the day of menstruation did not change the levels of FSH, LH, estradiol, or progesterone in adult cyclic monkeys (Trisomboon et al., 2004).

The long-term study on changes in serum levels of hormones is posed the limitations by the frequent sampling. Subject monkeys would suffer from loss of high amount of blood and injury from frequent venipuncture, which may disturb the homeostasis of physiological system. To avoid these problems, the assay of hormonal level in urine, a non-invasive method, should be considered. The present study was investigated the efficacy of urine assay to find out the PM effect on reproductive hormones through three kinds of experiments, a single and the 90-day treatments of PM on either cyclic adult or aged menopausal cynomolgus monkeys. From the previous findings on the effect of PM on serum hormone levels, we considered that the 90-day treatment of PM is long enough to disturb the hormonal patterns that would be reflected in the urinary excretion.

Materials and Methods

Animals

Adult cyclic and aged menopausal cynomolgus monkeys (*Macaca fascicularis*) were used in this study. This study used eighteen adult cyclic female monkeys with regular menstrual cycle for at least 4 consecutive cycles and nine aged menopausal cynomolgus monkeys with a complete cessation of menstrual cycle for at least 1 year before the onset of

the study. They were randomly selected from candidate monkeys. The monkeys were housed in the individual cages at the Primate Research Unit, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The menstruation was monitored daily by vaginal swabbing method. 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 (09:00 – 10:00 h) and supplemented with fresh fruits in the afternoon (14:00 – 15:00 h). The experimental protocol was approved by the ethics committee in accordance with the guide for the care and use of laboratory animals prepared by the Primate Research Unit, Faculty of Science, Chulalongkorn University.

Experimental Designs

Experiment 1: Effect of a Single Feeding of PM on Urinary Gonadotropin and Sex Steroid Hormone Levels in Adult Cyclic Monkeys.

Nine adult cyclic female monkeys were divided into three groups. Subjects of these groups (n = 3) were fed with the suspension of 10, 100, and 1,000 mg/day of *P. mirifica*, abbreviated as PM-10, PM-100, and PM-1,000, at 08:00-08:30 h. The experimental schedule was separated into the pre-treatment and post-treatment periods. The duration for the pre-treatment and post-treatment periods was 1 and 2 menstrual cycles, respectively. The monkeys were fed with PM after a cycle of the pre-treatment period. The first day of the menstrual bleeding was designed as day 1. During these two periods, urinary samples were collected everyday and assayed for FSH, LH, estradiol, and progesterone levels.

Experiment 2: Effect of Long-Term Treatment of PM on Urinary Gonadotropin and Sex Steroid Hormone Levels in Adult Cyclic Monkeys.

Nine adult cyclic female monkeys were divided into three groups. Subjects of these groups (n = 3) were fed daily with PM-10, PM-100, and PM-1,000, respectively, at 08:00 - 08:30 h. The experimental schedule was separated into the pre-treatment, treatment, and post-treatment periods. During the pre-treatment and post-treatment periods, the monkeys were fed daily with 5 ml of distilled water at 08:00-08:30 h for 1 and 2 menstrual cycles, respectively. During the treatment period, the monkeys were fed with the suspension of PM for 3 menstrual cycles. If the monkeys showed an amenorrhea symptom after PM treatment, the duration of experiment was performed 90 and 60 days for the treatment and post-treatment periods, respectively. The first day of the menstrual bleeding was designed as day

1. Urinary samples was collected everyday throughout the study period and assayed for FSH, LH, estradiol, and progesterone levels.

Experiment 3: Effect of Long-Term Treatment of PM on Urinary Gonadotropin And Sex Steroid Hormone Levels in Aged Menopausal Monkeys.

Nine aged menopausal monkeys were divided into three groups. Subjects of these groups (n = 3 in each) were fed daily with PM-10, PM-100, and PM-1,000. The experimental schedule was separated into the pre-treatment, treatment, and post-treatment periods for 30, 90, and 60 days, respectively. The monkeys were fed daily with 5 ml distilled water during the pre-treatment and post-treatment periods and with the suspension of PM during the treatment period at 08:00 - 08:30 h. Urinary samples were collected every 5 days and assayed for FSH, LH, and estradiol levels.

Collection of Urinary Samples.

The tray was inserted and kept under the monkey's cage between 18:00 – 08:00 h. The 14-h urinary samples were collected from the tray with plastic syringes at 08:00 h and then centrifuged at 1,700 xg, 4 º C for 20 minutes. The supernatant was separated and stored at –20 º C until hormonal assays.

Preparation of the Suspension of *Pueraria mirifica*

The fresh tuberous roots of PM were obtained from the same lot. The roots were sliced, dried in hot air oven at 70 º C, and subsequently ground into a powder at size of 100 Mesh. The stock of powder was kept in the dark desiccator before suspended. PM powder was suspended with distilled water and kept in a dark bottle, at 4 $^{\circ}$ C, until the feeding time.

Hormonal Analyses

The urinary samples were analyzed for FSH and LH levels using the heterologous RIA system described previously (Hodgen et al., 1976). Iodinated preparations were rat NIDDK-rat FSH -I-5 and rat LH-I-5. The anitisera were anti-ovine FSH (NIDDK-H-31) and anti-ovine LH (YM#18). Antiserum against ovine LH (YM#18) was kindly provided by Dr.Y.Mori (University of Tokyo, Tokyo, Japan).

Urinary estradiol and progesterone levels were determined by a double-antibody RIA system using ^{125}I-labeled radioligands as described previously (Taya, et al., 1985; Gibori, Anrczak, and Rothchild, 1977). Antisera against estradiol (GDN#244) and progesterone (GDN#377) were kindly provided by Dr.G.D. Niswender (Animal Reproduction and Biotechnology, Colorado State University, Fort Collins, CO, U.S.A.).

Creatinine (Cr) level of each urinary sample was measured by the Jaffe method using an Autoanalyzer to compensate for differences in urine concentration and volume. Urinary hormone levels were calculated as a milligram of creatinine.

Statistical Analyses

Data are expressed as mean ± S.E.M. The student *t*- test was used to determine the difference of hormonal levels in urine between adult cyclic and aged menopausal monkeys. Analysis of variance (ANOVA) followed by the LSD test was applied to determine the significance of differences of urinary levels among each period. The differences of means were considered significant at $P < 0.05$.

Results

Patterns of Urinary Hormones in Normal Menstrual Cycle of Adult Cyclic Monkeys.

The normal length of menstrual cycle of adult cyclic monkeys during the pre-treatment period was 29.29 ± 0.79 days (n = 18). There were an individual variation in urinary hormonal levels depending on the length of each menstrual cycle in subject monkeys. The profiles of hormonal levels in urine were adjusted according to the day of peak level of LH, which was assigned as day 0. It separated into 2 phases: the late follicular and early luteal phases, respectively. As shown in Fig. 1, urinary FSH and LH levels were elevated simultaneously during the late follicular phase and attained the peak on the same day on day 0. The increase in urinary estradiol levels during the late follicular phase coincided with those in urinary FSH and LH levels. All of urinary FSH, LH, and estradiol levels declined during the early luteal phase. Urinary estradiol and progesterone levels showed a high fluctuation throughout the late follicular and early luteal phases. However, urinary progesterone levels trended to be high during the early luteal phase.

To calculate the correlation between hormonal levels in serum and urine of normal adult cyclic monkeys, data of hormonal levels in urine were correlated with levels in serum from the previous report. There was a significant correlation between FSH, LH, and progesterone levels in urine and serum ($r = 0.61$, $P = 0.05$ for FSH, $r = 0.84$, $P = 0.001$ for LH, $r = 0.67$, $P = 0.02$), and no significant correlation between those levels of estradiol ($r = 0.26$, $P < 0.44$).

Experiment 1: Effect of a Single feeding of PM on Urinary FSH, LH, Estradiol, and Progesterone Levels in Adult Cyclic Monkeys.

After a single feeding of PM-10 and PM-100, the menstrual cycle length of adult monkeys are kept in the normal range (for PM-10, 30.97 ± 2.91 and 33.33 ± 3.84 days for the first and second cycle; for PM-100, 33.33 ± 3.84 and 35.67 ± 6.33 days for the first and second cycle, respectively). Adult monkeys treated with PM-1,000 had the prolongation of menstrual cycle length compared to the cycle length during the pre-treatment period (42.00 ± 4.04 days, $P < 0.01$ for the first cycle and 39.67 ± 0.67 days, $P < 0.01$ for the second cycle). Comparing the pattern of urinary hormone levels in all PM treated groups (Figs 2 – 4) to those patterns of normal monkeys in Fig.1, it was shown a similar pattern. Peak levels of urinary FSH and LH are occurred at the mid phase of menstrual cycles or day 0 of Fig.1 in all monkeys treated with PM-10, PM-100, and PM-1,000. After the declining of urinary FSH and LH levels, urinary estradiol and progesterone trended to increase in some monkeys.

Experiment 2: Effect of Long-term Treatment of PM on Urinary FSH, LH, Estradiol, and Progesterone Levels in Adult Cyclic Monkeys.

Changes of urinary FSH, LH, estradiol, and progesterone levels in adult cyclic monkeys treated daily with different dose of PM for 3 menstrual cycles or 90 days are shown in Figs 6 – 8, respectively. As shown in Fig. 5, PM-10 prolonged the menstrual cycle length in all 3 monkeys. The length of 3 consecutives menstrual cycles were prolonged to 50, 81, and 52 days for monkey no. 601, to 40 and > 90 days for monkey no. 627, and to > 90 days for monkey no. 619. However, all adult monkeys treated with PM-10 had the peak levels of FSH and LH when they showed menstrual cycles. There were no changes in pattern of urinary FSH, LH, estradiol, or progesterone levels throughout the treatment and post-treatment periods when compared to the pre-treatment period.

As shown in Fig. 6, adult monkey nos. 616 and 801 showed a shorten menstrual cycle at the beginning of the treatment period (15 and 21 days, respectively) and stopped the menstruation afterward. There was no peak of urinary FSH level in these two monkeys and only monkey no. 801 showed a small peak of urinary LH level. Monkey no. 108 completely stopped menstruation throughout the 90-day of treatment and 60-day of post-treatment periods, and the peak levels of FSH and LH could be found only at the beginning of the treatment period. All adult monkeys in this group showed the low basal level of urinary FSH at the early follicular phase. Urinary estradiol and progesterone levels fluctuated throughout the treatment and post-treatment periods.

As shown in Fig.7, all monkeys treated with PM-1,000 stopped menstruation throughout the 90-day of treatment and 60-day of post-treatment periods. There were no peak levels of

urinary FSH or LH in monkey nos. 624 and 77 throughout the treatment period, while monkey no. 633 showed a small peak of urinary FSH level. Urinary estradiol level in monkey nos. 77 and 624 decreased throughout the treatment and post-treatment periods compared to the pre-treatment levels.

Experiment 3: Effect of Long-term Treatment of PM on Urinary FSH, LH, and Estradiol Levels in Adult Cyclic Monkeys.

The menopausal state of aged monkeys was confirmed by the high levels of urinary FSH (8.00 ± 2.08 ng/mg Cr, P = 0.01) and LH (17.80 ± 4.71 ng/mg Cr, P = 0.23) and by the low levels of urinary estradiol (0.18 ± 0.08 ng/mg Cr, P = 0.01) during the pre-treatment period compared to those levels in the early follicular phase, days 1-3, of adult cyclic monkeys (1.32 ± 0.31 ng/mg Cr for FSH, 10.70 ± 1.68 ng/mg Cr for LH, and 4.44 ± 1.41 ng/mg Cr for estradiol).

Means of urinary levels of FSH, LH, and estradiol in aged menopausal monkeys treated daily with PM-10, PM-100, and PM-1,000 for 90 days are shown in Fig.8. The daily treatment of PM seems to induce, but not significantly, the decrease in urinary FSH levels in a dose dependent manner. In PM-10, urinary FSH levels decreased for the first 15 days during the treatment period and then recovered to the pre-treatment levels afterward. Urinary FSH levels rebounded significantly on day 20 during the post-treatment period. Urinary FSH levels in monkeys treated with PM-100 and PM-1,000 tended to decrease between days 5 – 90 during the treatment period and remained low for the first 10 days of the post-treatment period before the increase and significant rebound on days 20 and 25, respectively.

There were high fluctuations in urinary LH levels throughout the study period in all aged menopausal monkeys. During PM treatment, there was no significant difference in urinary LH levels compared to those during the pre-treatment period in all groups. However, urinary LH levels significantly increased on some days in monkeys treated with PM-100 and PM-1,000. Urinary LH levels tended to increase in general and rebounded after the first 20 days of the cessation of PM treatment in all monkey groups.

Daily treatment of PM seems to insignificantly decrease the urinary estradiol levels in monkeys treated with PM-10 and PM-100. There was no significant difference in urinary estradiol levels during the treatment period compared to those during the pre-treatment period in monkeys treated with PM-1,000.

The Correlation between Hormonal Levels in Serum and Urine in Female Monkeys Treated with PM

The data of hormonal levels in both serum and urine at the same points from adult cyclic and aged menopausal monkeys throughout the study period (n = 27) were selected and pooled together. As shown in Fig.9, there was a significant positive correlation between gonadotropins (FSH and LH) and estradiol levels in serum and urine of the monkeys (r = 0.46, $P < 0.01$ for FSH; r = 0.13, $P < 0.01$ for LH; r = 0.16, $P < 0.01$ for estradiol). There was, however, no correlation between progesterone level in serum and urine (r = 0.05, $P = 0.24$).

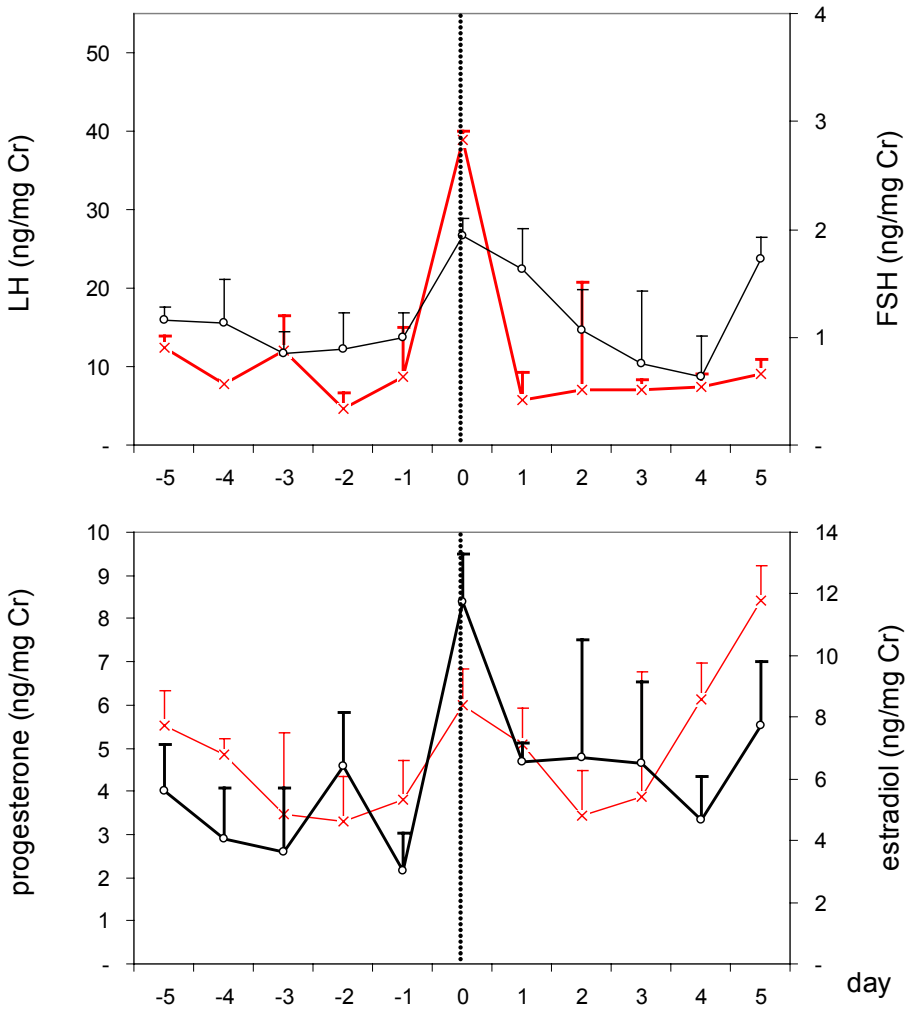

Fig. 1 Pattern of urinary of FSH, LH, estradiol, and progesterone levels in normal menstrual cycle of adult female monkeys.

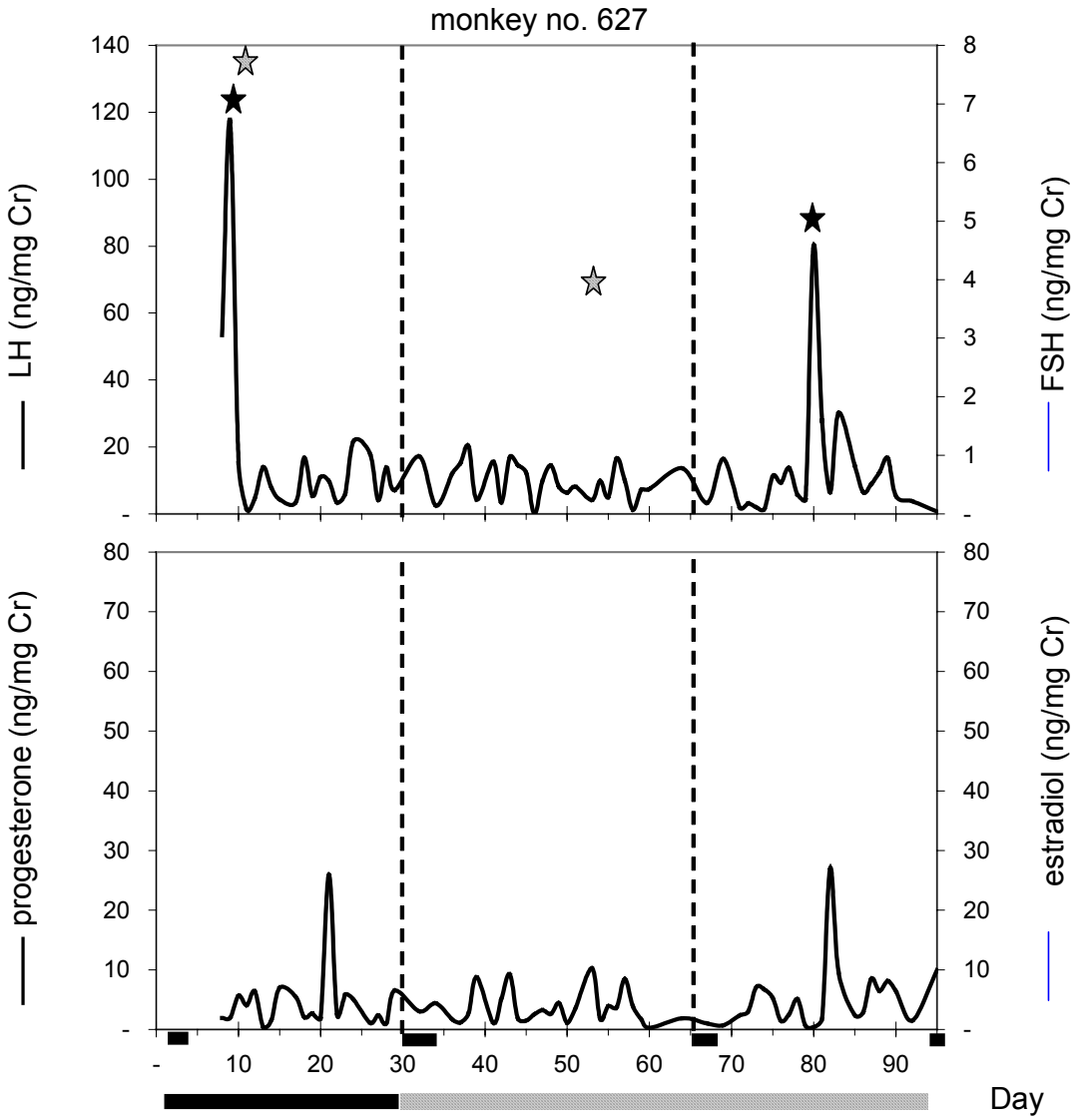

Fig. 2 Changes of urinary FSH, LH, estradiol, and progesterone levels in adult cyclic monkeys on a single feeding of PM-10. The black and stripe lines indicate the pre-treatment and treatment periods, respectively. The dash-vertical lines separated each of cycles. The star indicates the highest peak of urinary FSH and LH levels in each menstrual cycle.

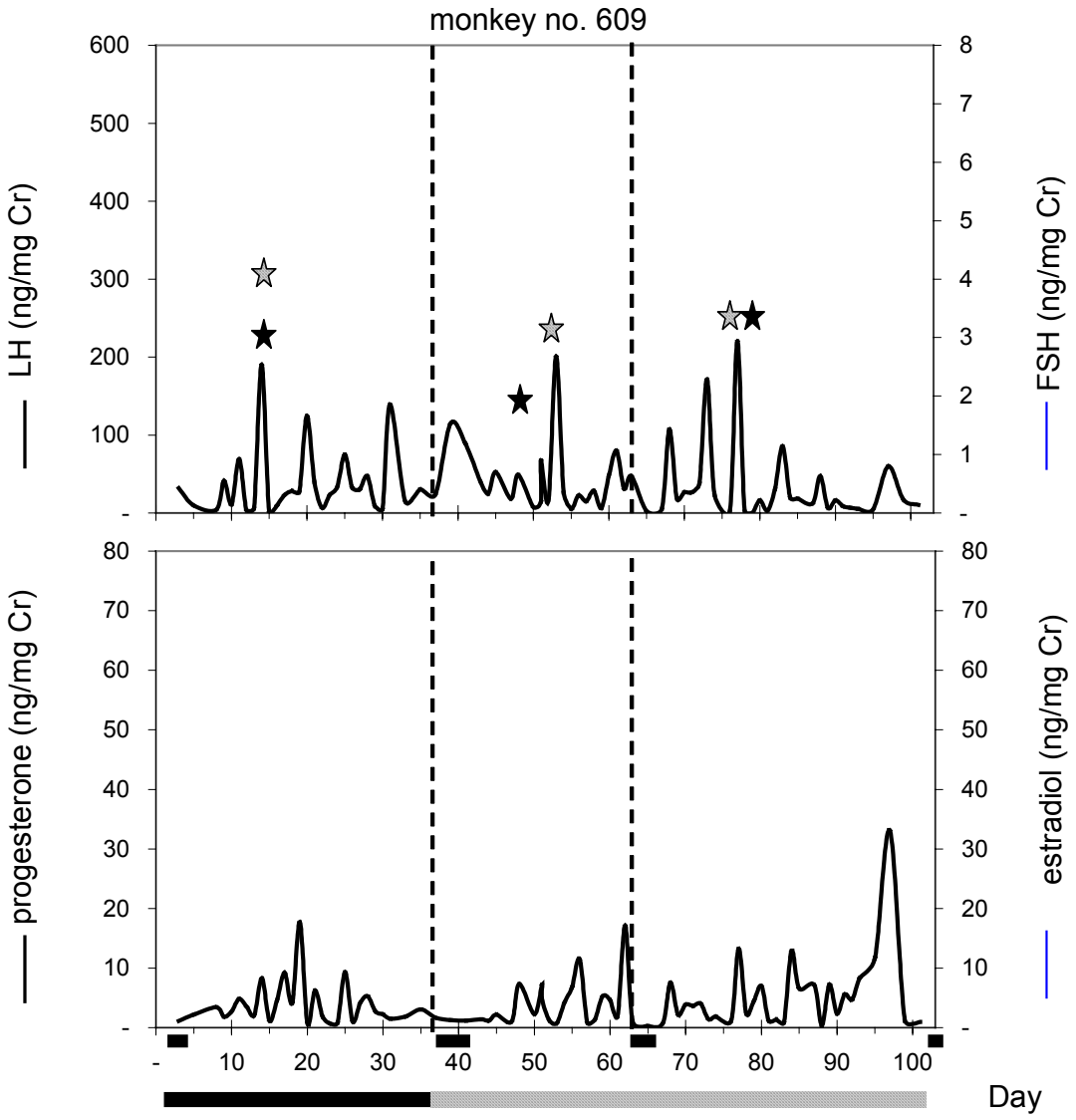
monkey no. 609
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.2 (cont.)

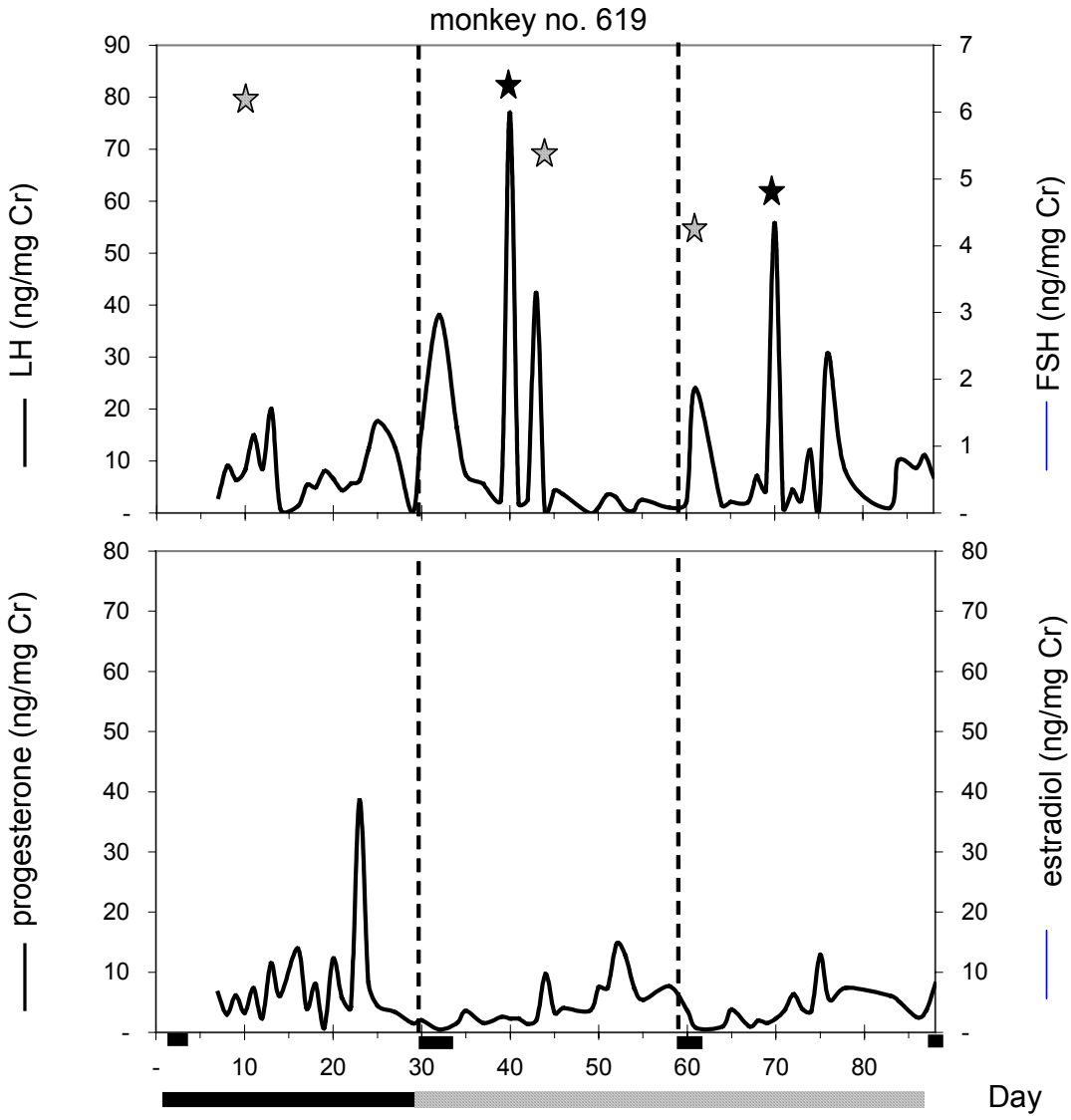
monkey no. 619
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.2 (cont.)

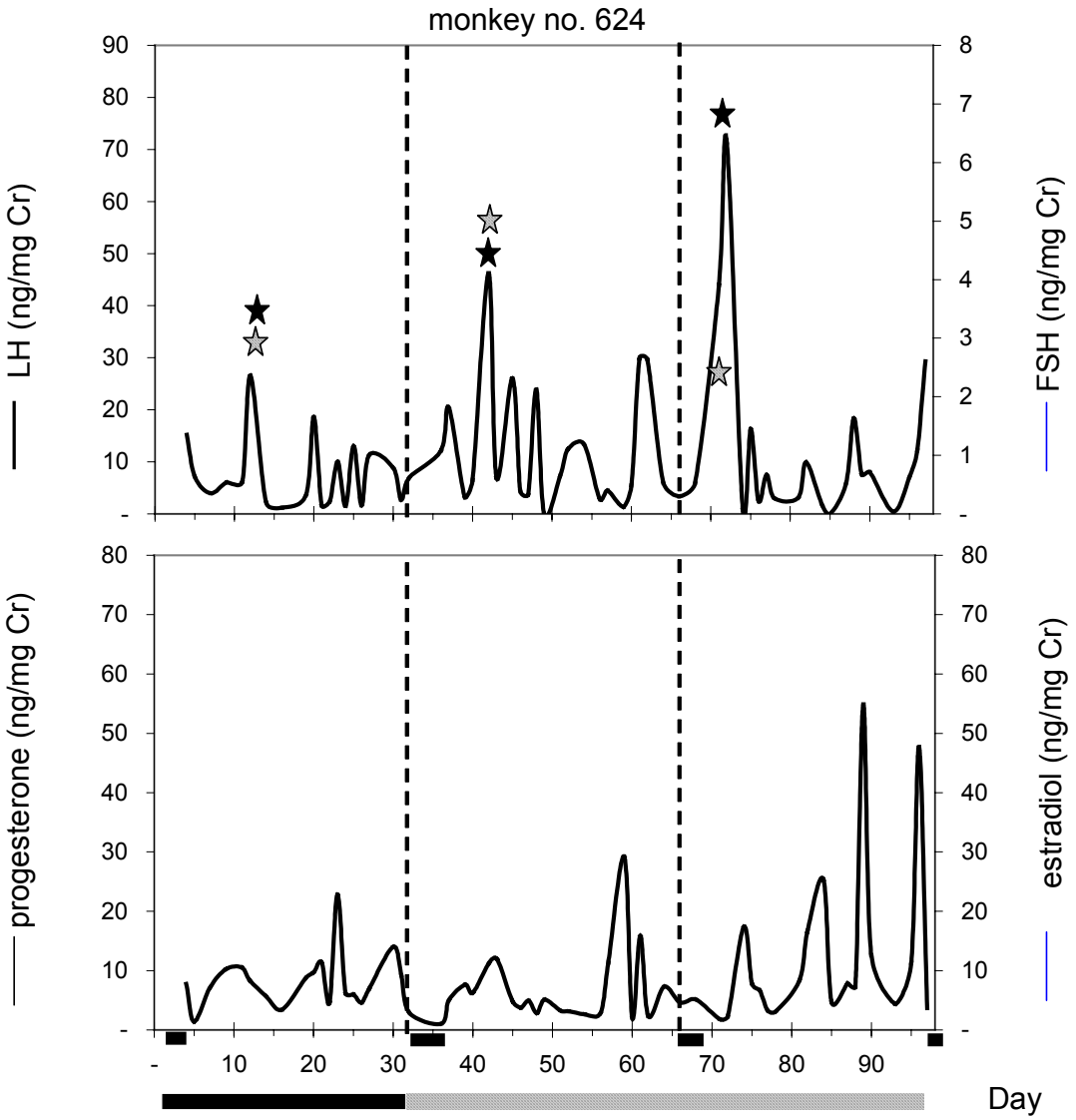

Fig.3 Changes of urinary FSH, LH, estradiol, and progesterone levels in adult cyclic monkeys on a single feeding of PM-100. The meanings of the black and stripe lines and dash-vertical line are the same as explained in Fig.2. The star indicates the highest peak of urinary FSH and LH levels in each menstrual cycle.

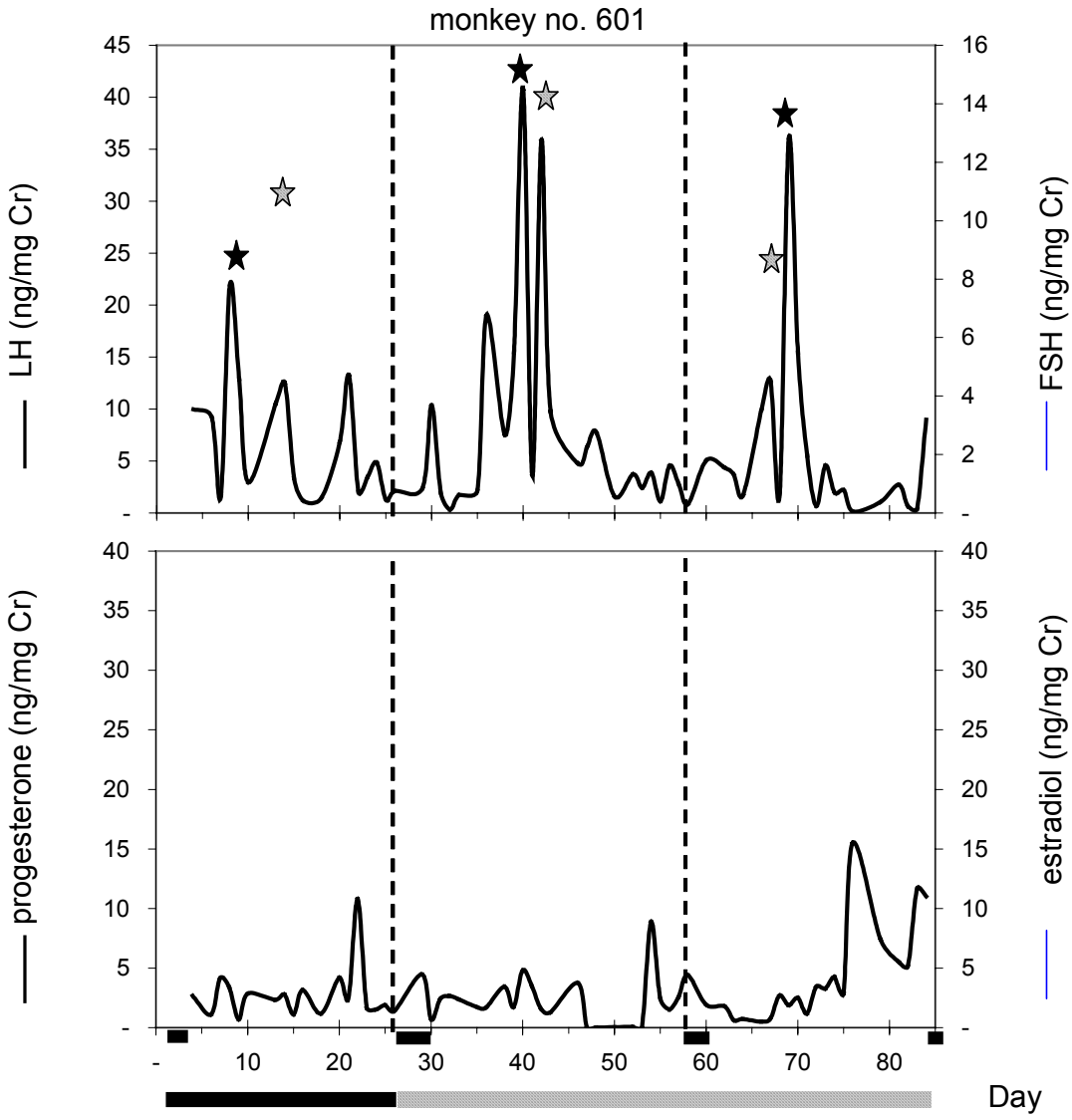
monkey no. 601
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.3 (cont.)

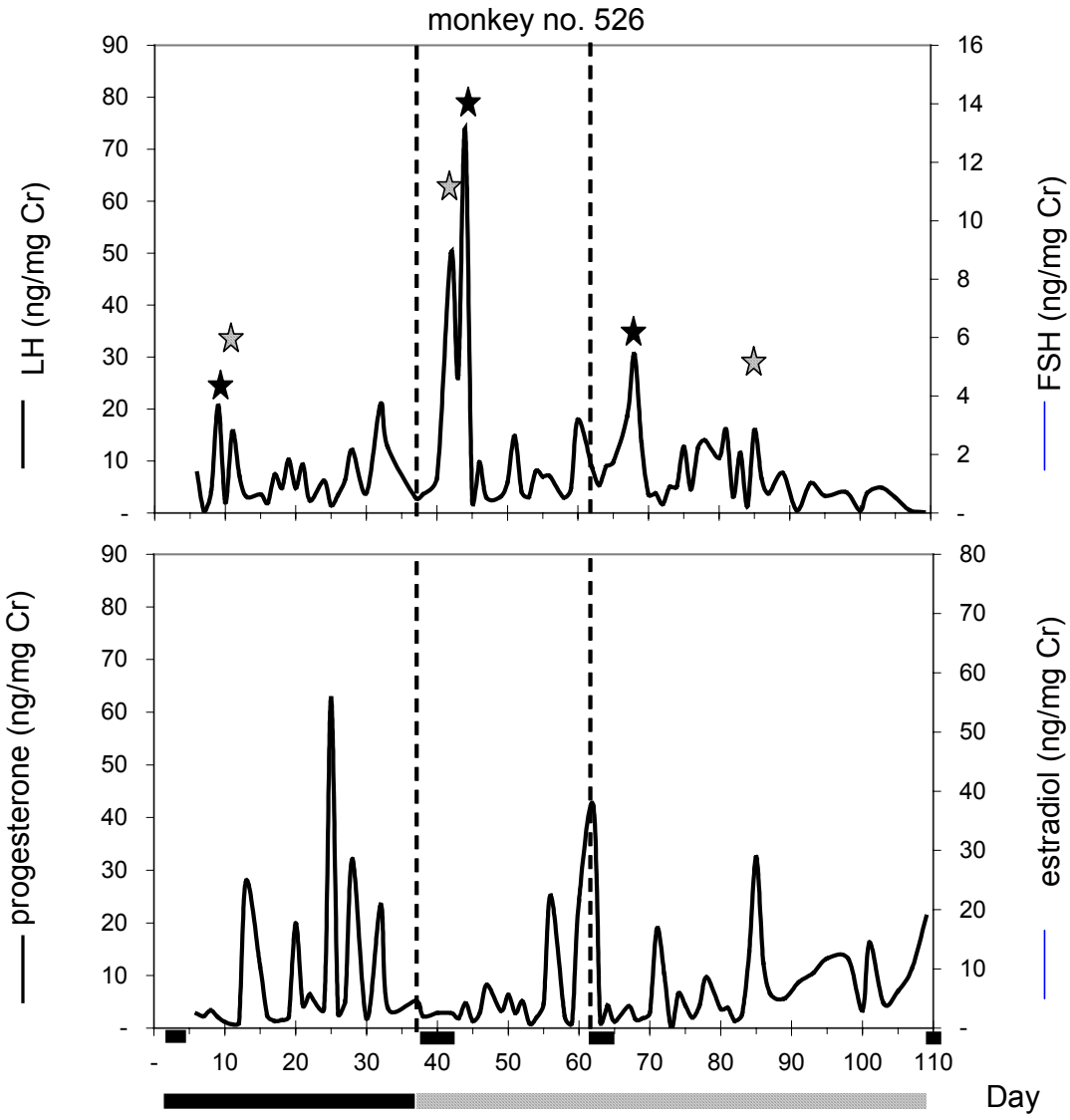
monkey no. 526
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.3 (cont.)

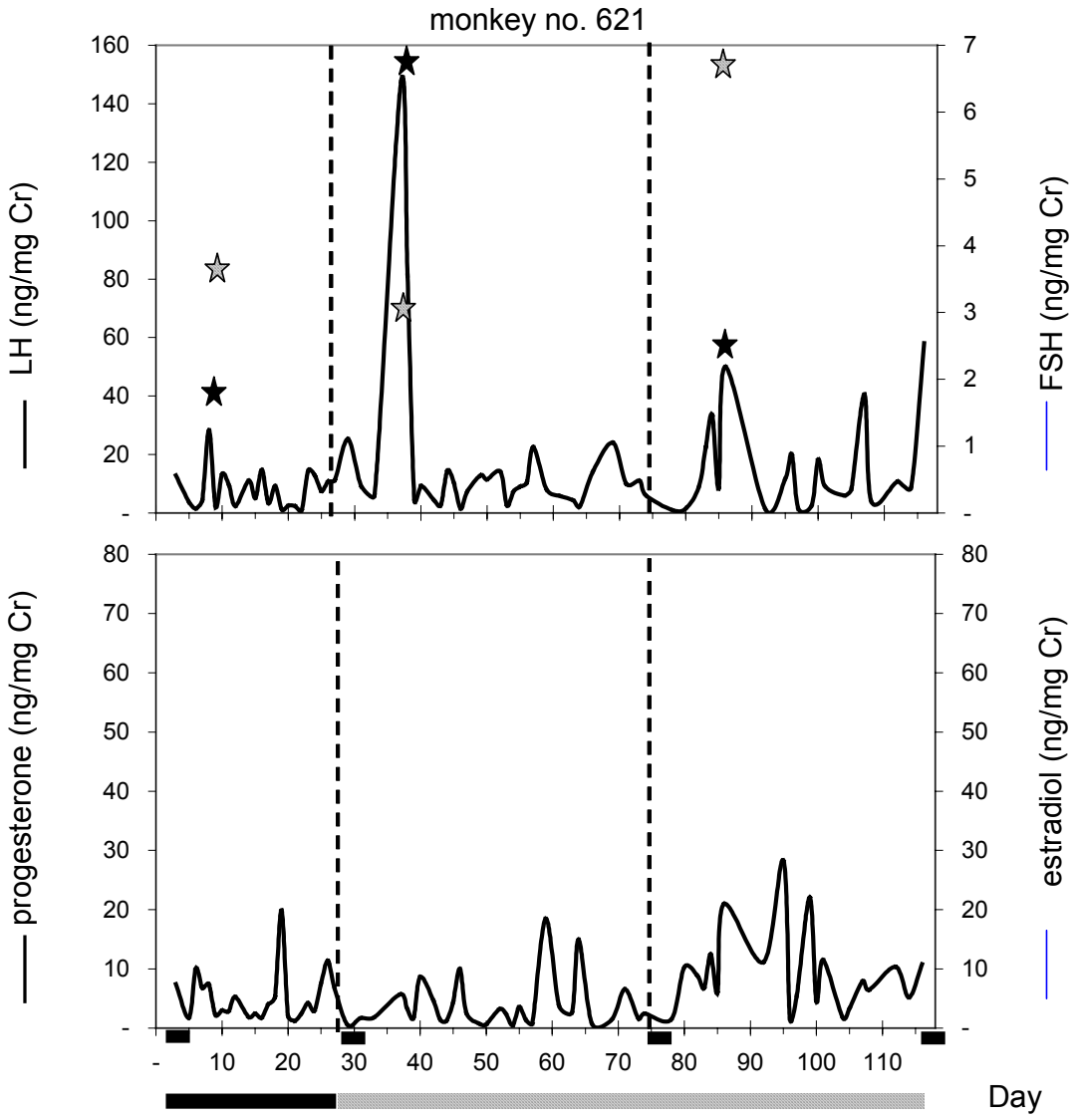

Fig.4 Changes of urinary FSH, LH, estradiol, and progesterone levels in adult cyclic monkeys on a single feeding of PM-1,000. The meanings of the black and stripe lines and dash-vertical line are the same as explained in Fig.2. The star indicates the highest peak of urinary FSH and LH levels in each menstrual cycle.

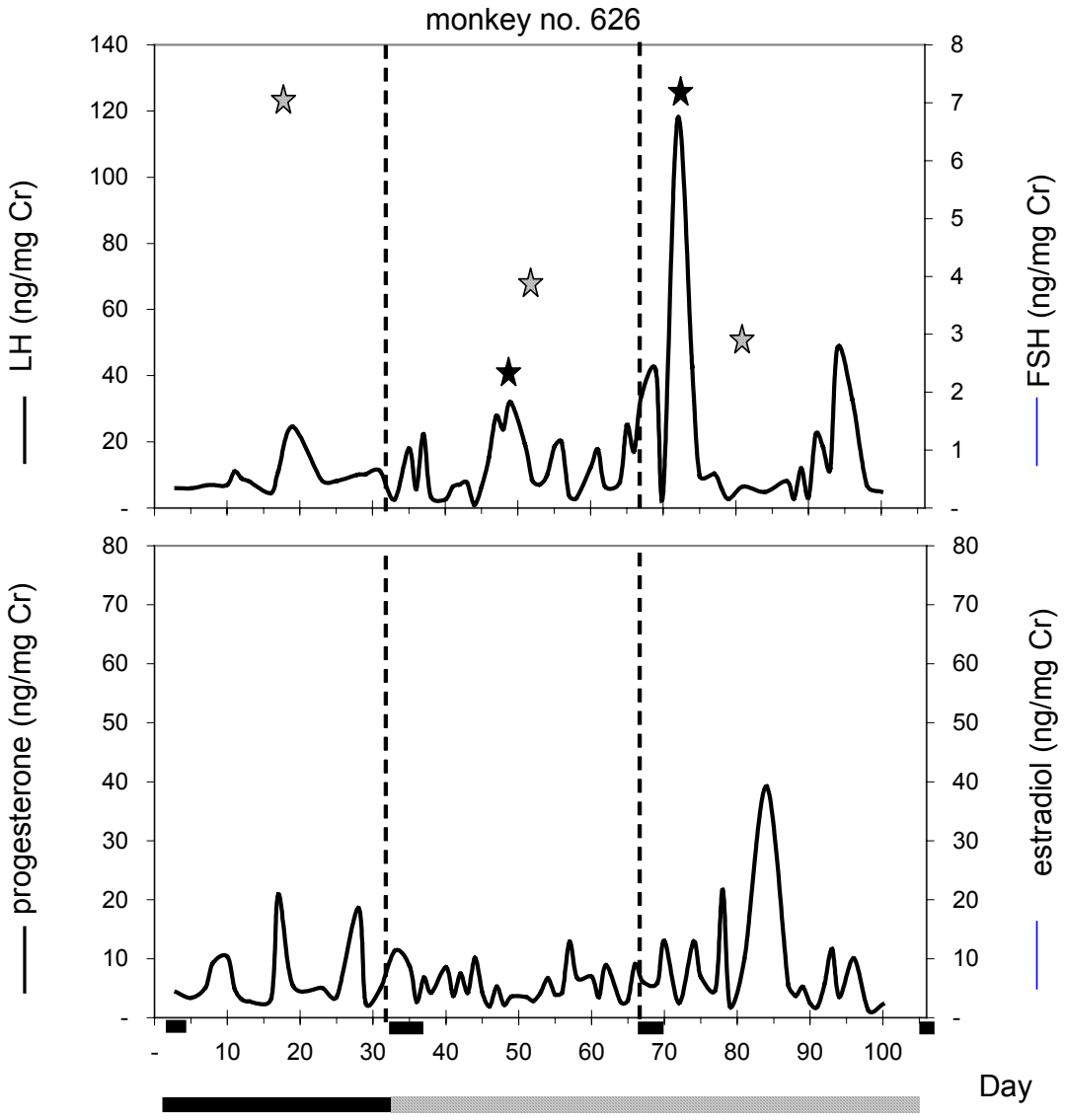
monkey no. 626
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.4 (cont.)

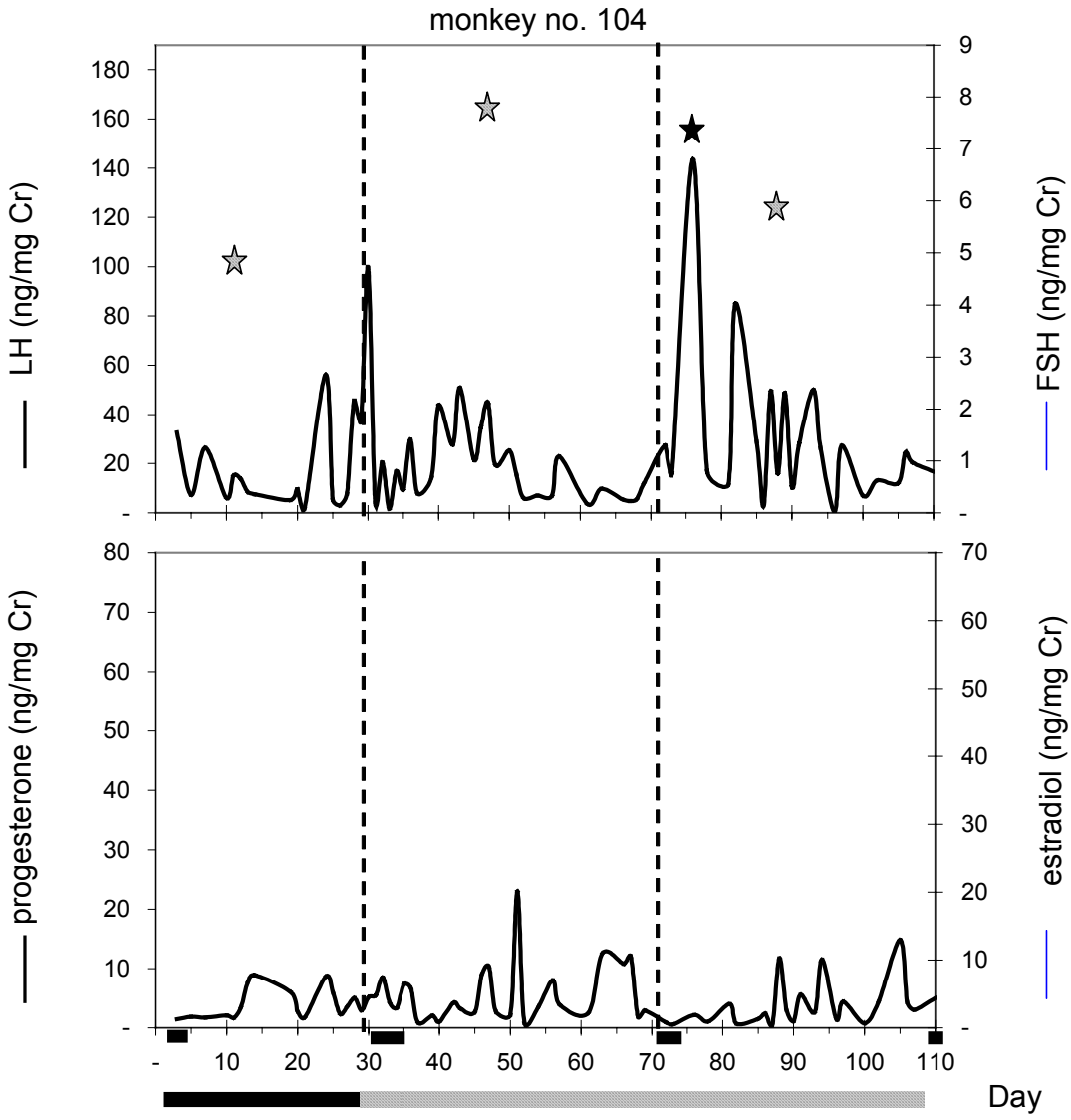
monkey no. 104
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.4 (cont.)

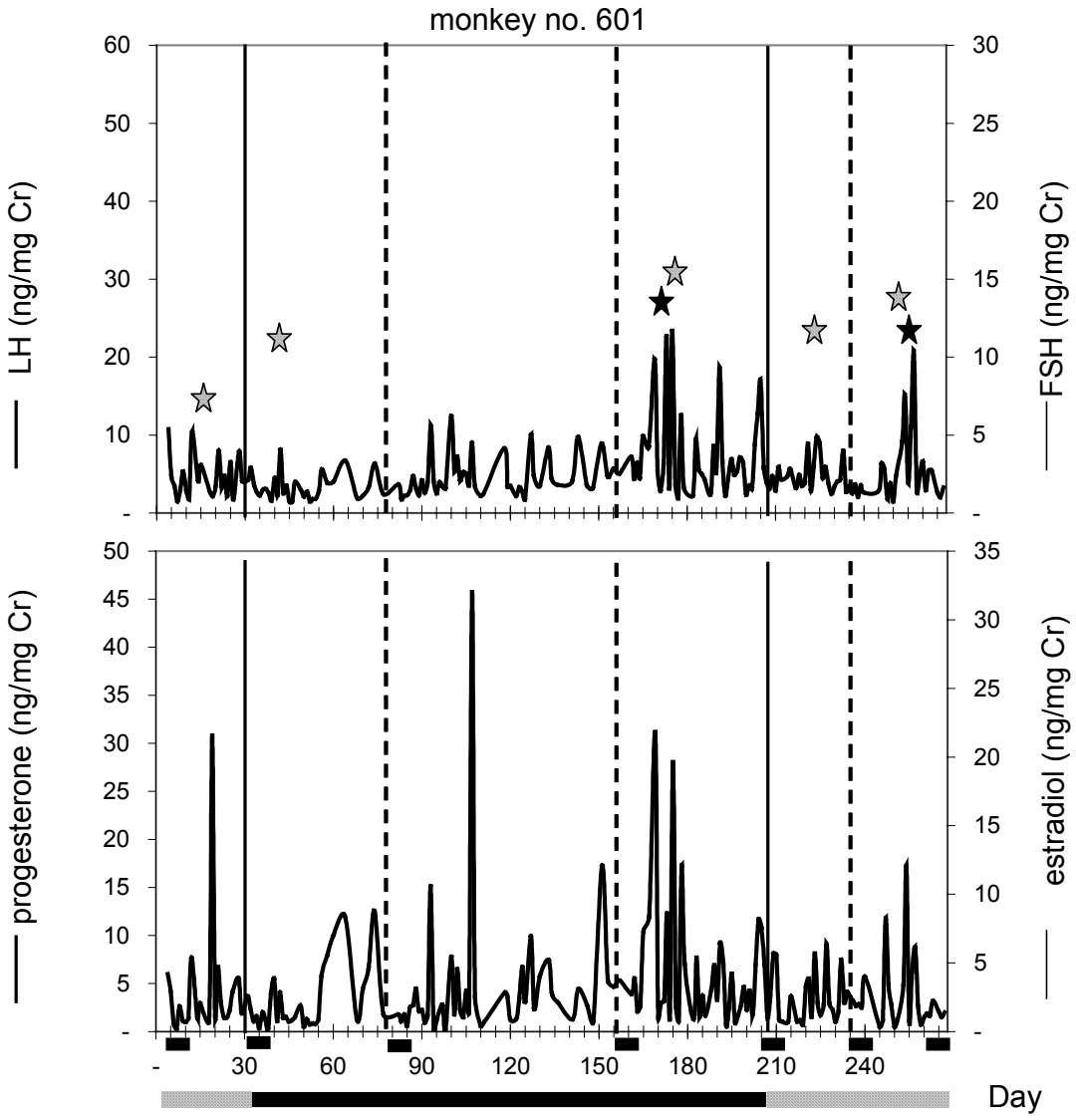

Fig.5 Changes in the levels of urinary FSH, LH, estradiol, and progesterone in adult cyclic monkeys fed daily with PM-10 for 90-day. The black horizontal line indicates the treatment period. The vertical lines separated the menstrual cycles. The short horizontal bars at the abscissa represent the day of menses. The star indicates the highest peak of urinary FSH and LH levels in each menstrual cycle.

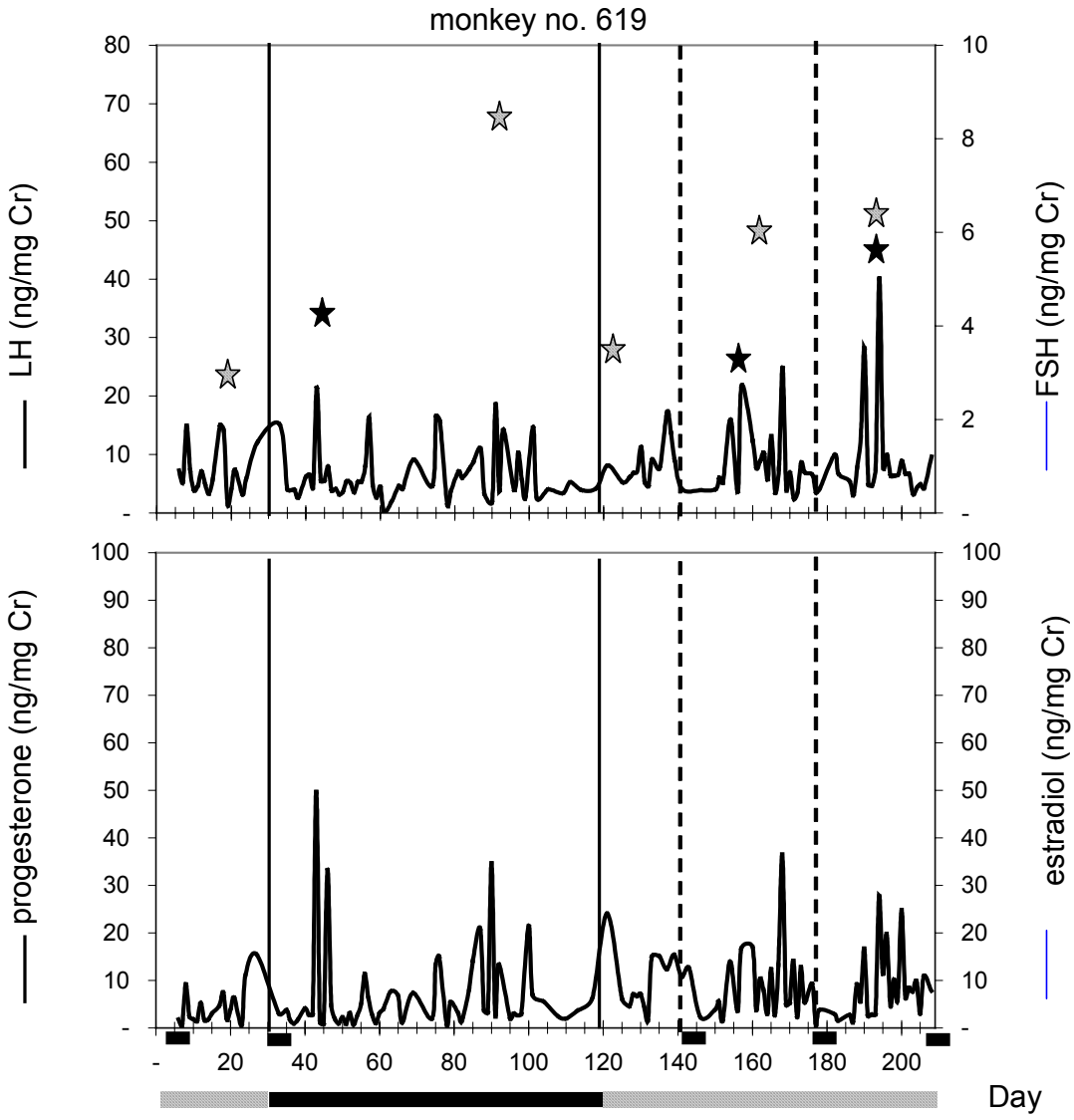
monkey no. 619
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.5 (cont.)

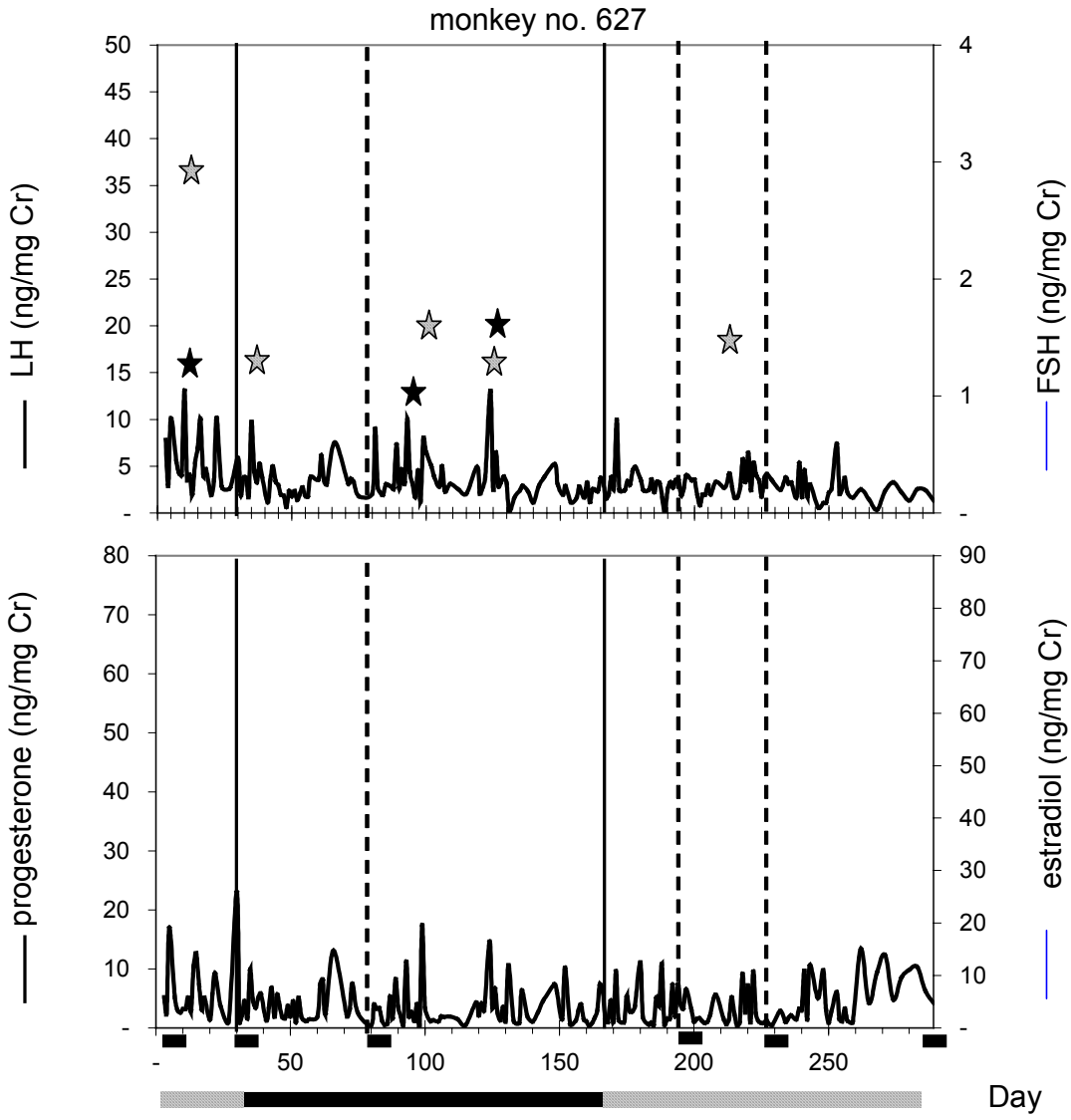
monkey no. 627
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.5 (cont.)

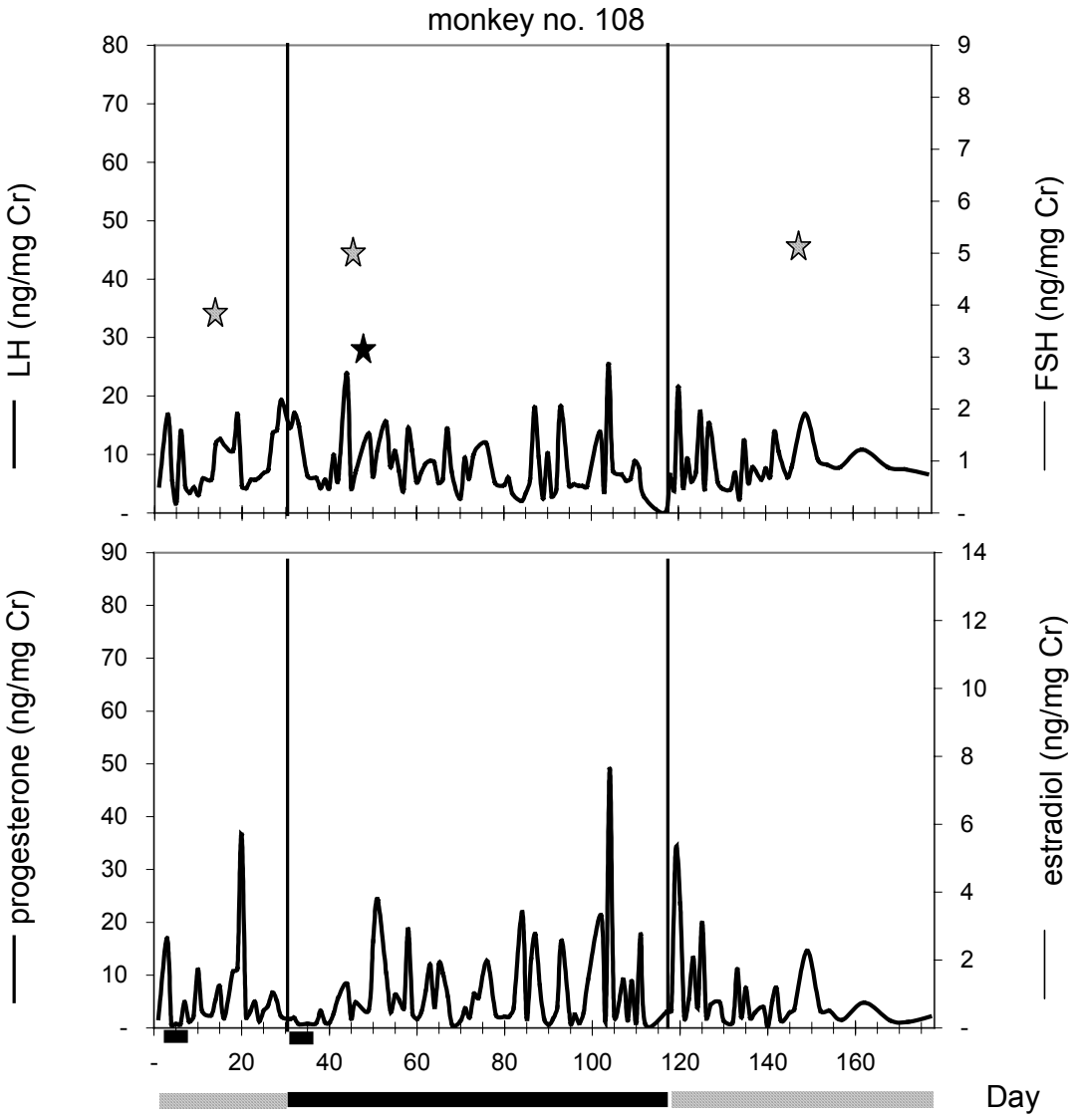

Fig.6 Changes in the levels of urinary FSH, LH, estradiol, and progesterone in adult cyclic monkeys fed daily with PM-100 for 90-day. The meaning of the black horizontal and vertical lines, horizontal bars, and stars are the same as explained in Fig.5.

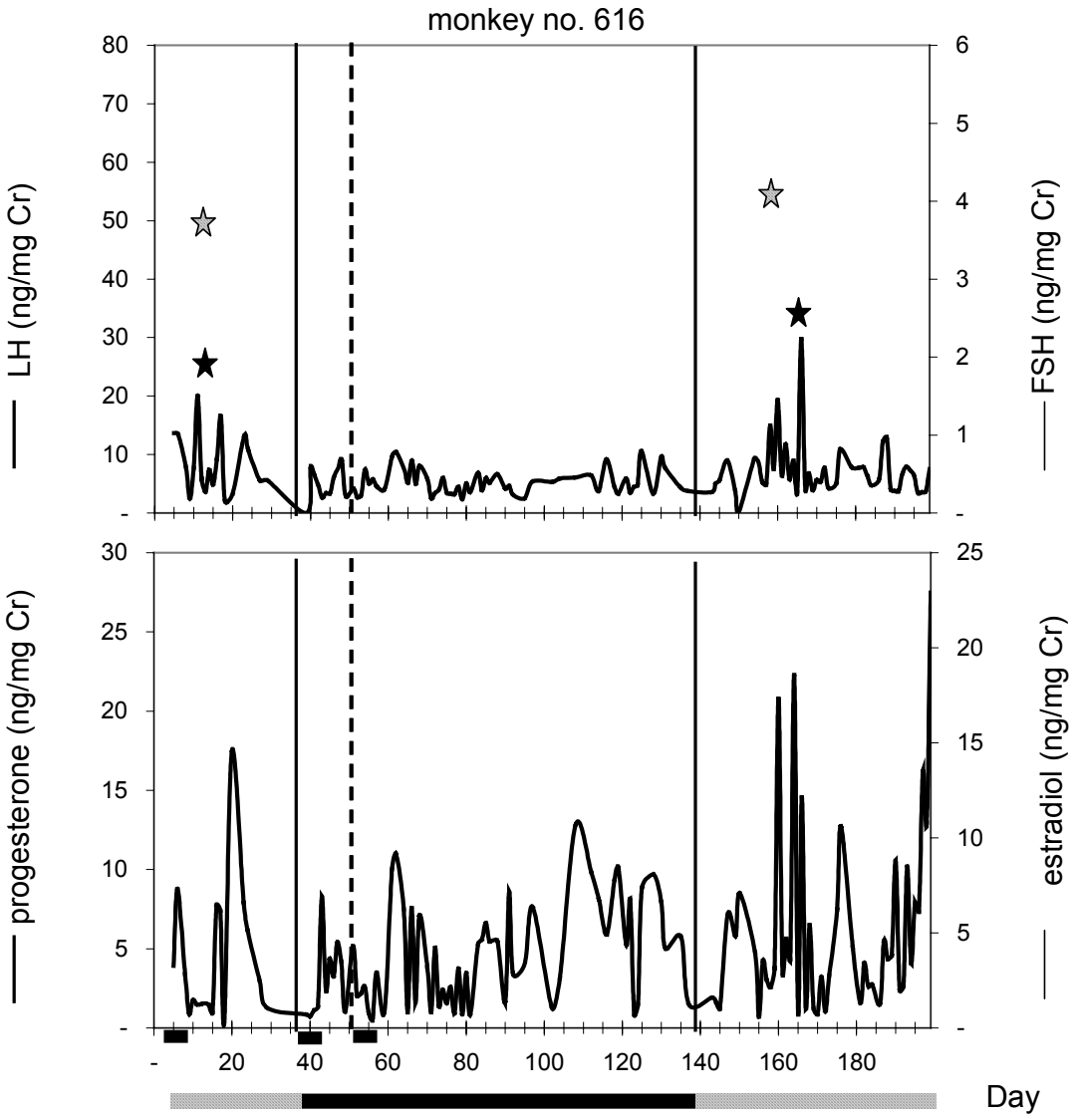
monkey no. 616
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.6 (cont.)

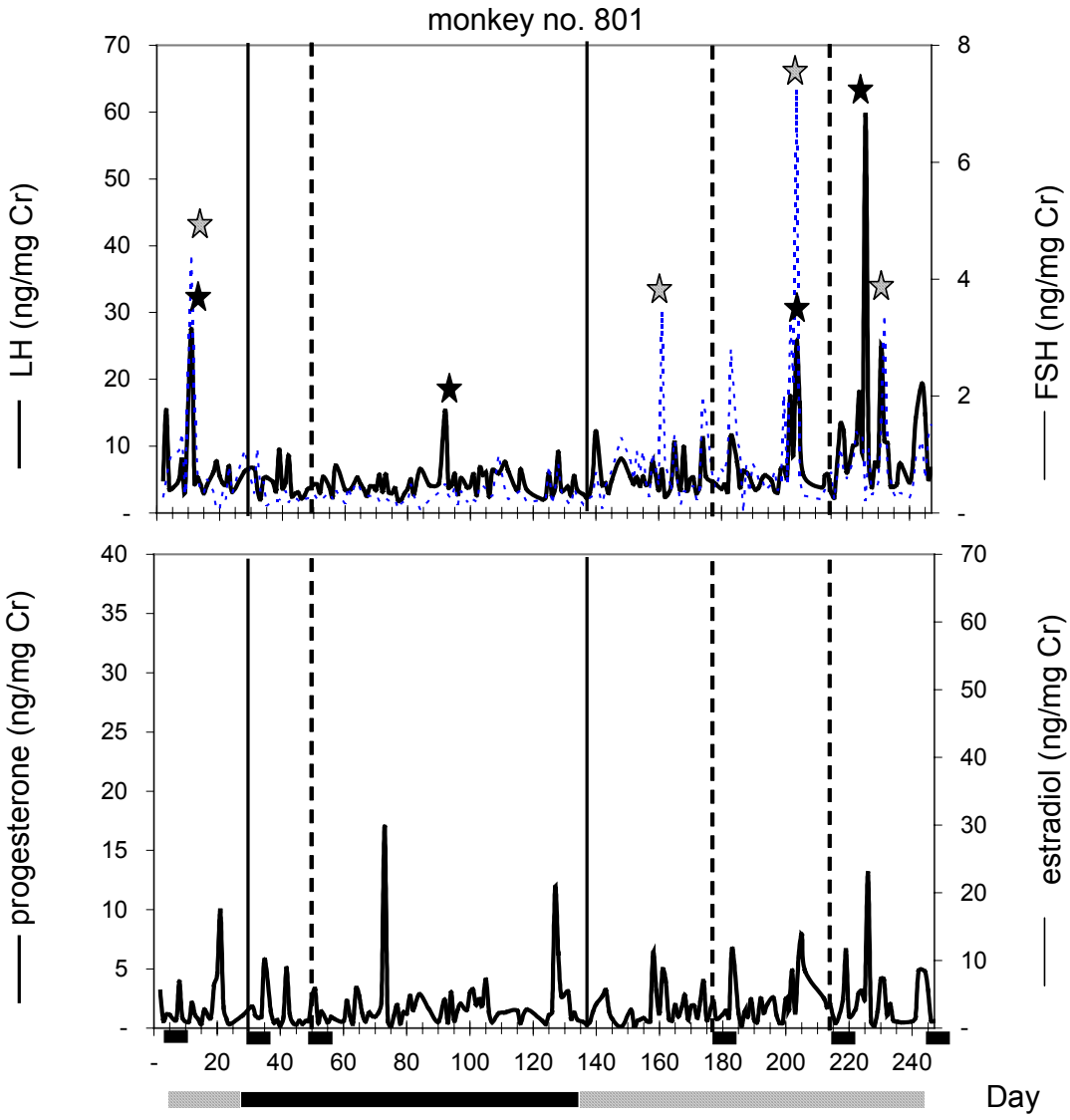
monkey no. 801
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.6 (cont.)

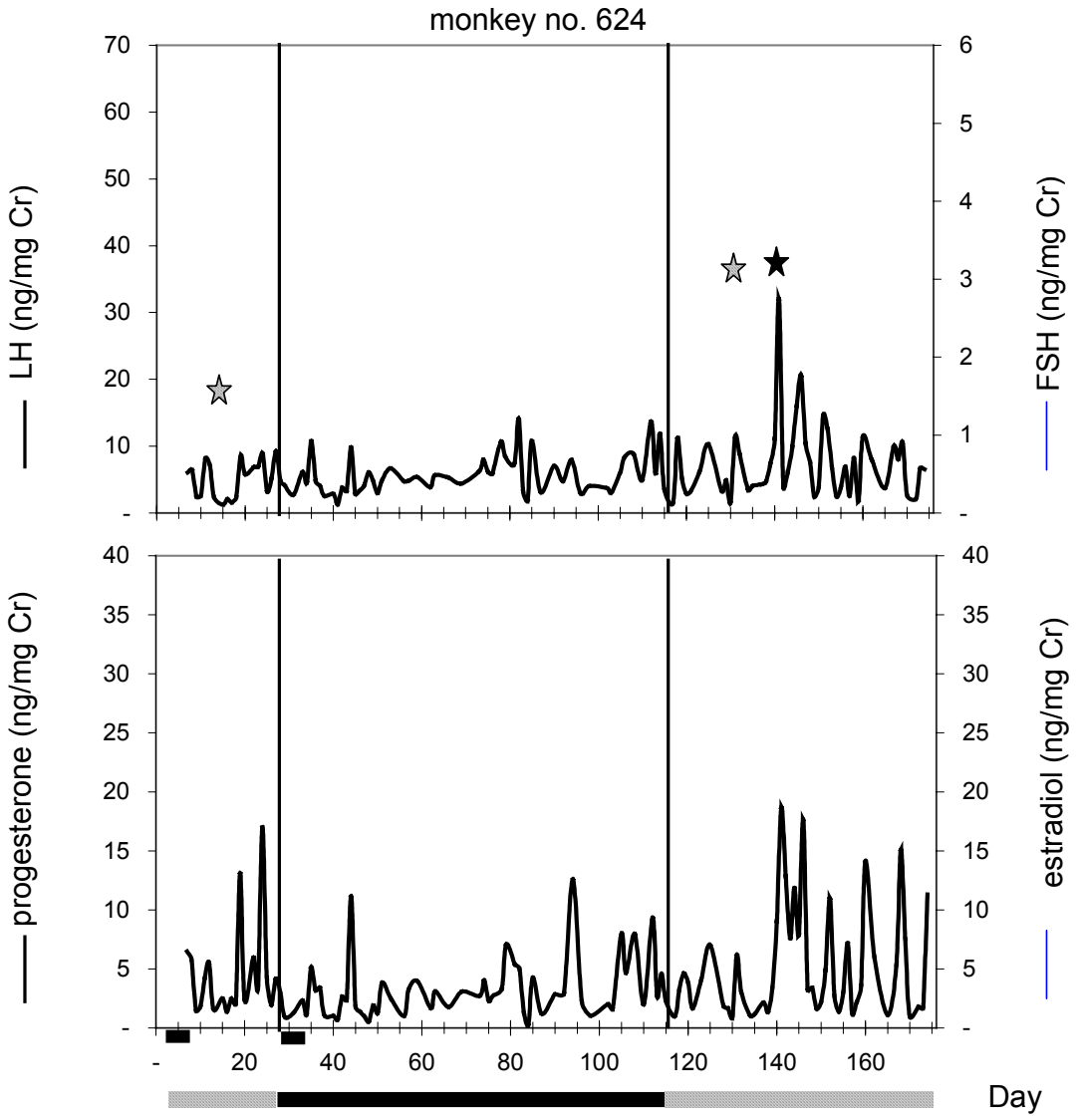

Fig.7 Changes in the levels of urinary FSH, LH, estradiol, and progesterone in adult cyclic monkeys fed daily with PM-1,000 for 90-day. The meaning of the black horizontal and vertical lines, horizontal bars, and stars are the same as explained in Fig.5.

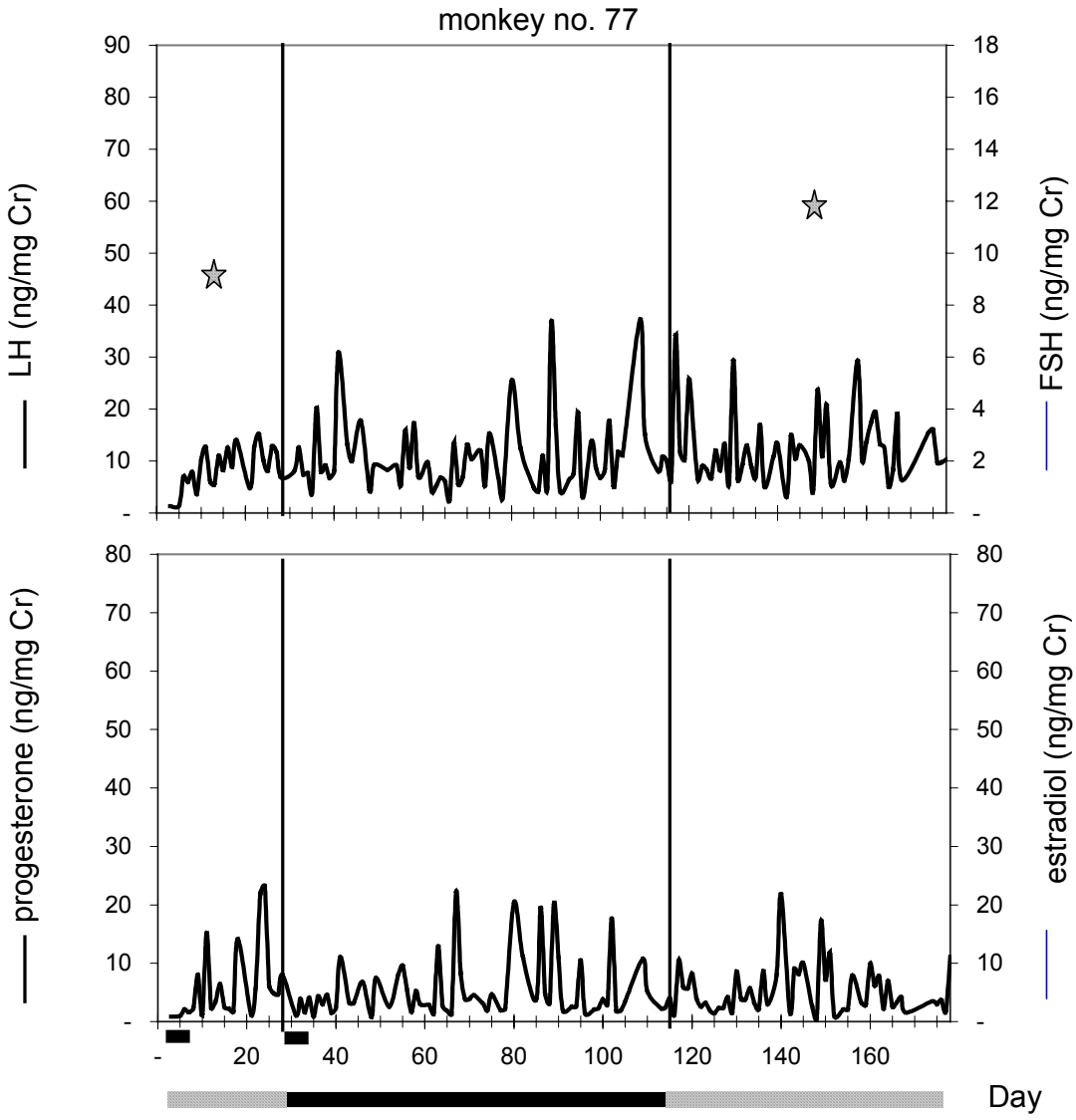
monkey no. 77
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.7 (cont.)

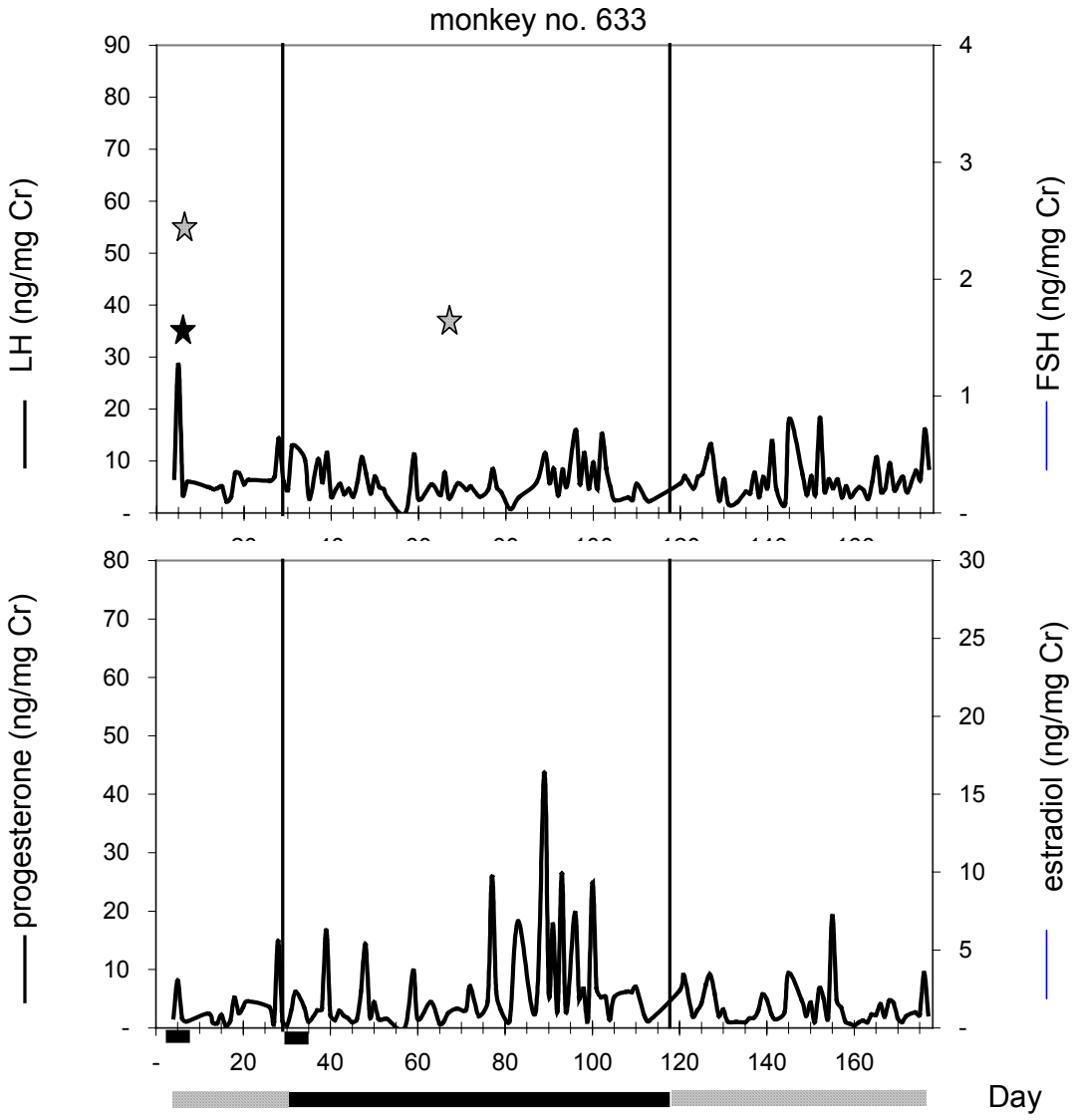
monkey no. 633
LH (ng/mg Cr)
FSH (ng/mg Cr)
progesterone (ng/mg Cr)
estradiol (ng/mg Cr)
Day

Fig.7 (cont.)

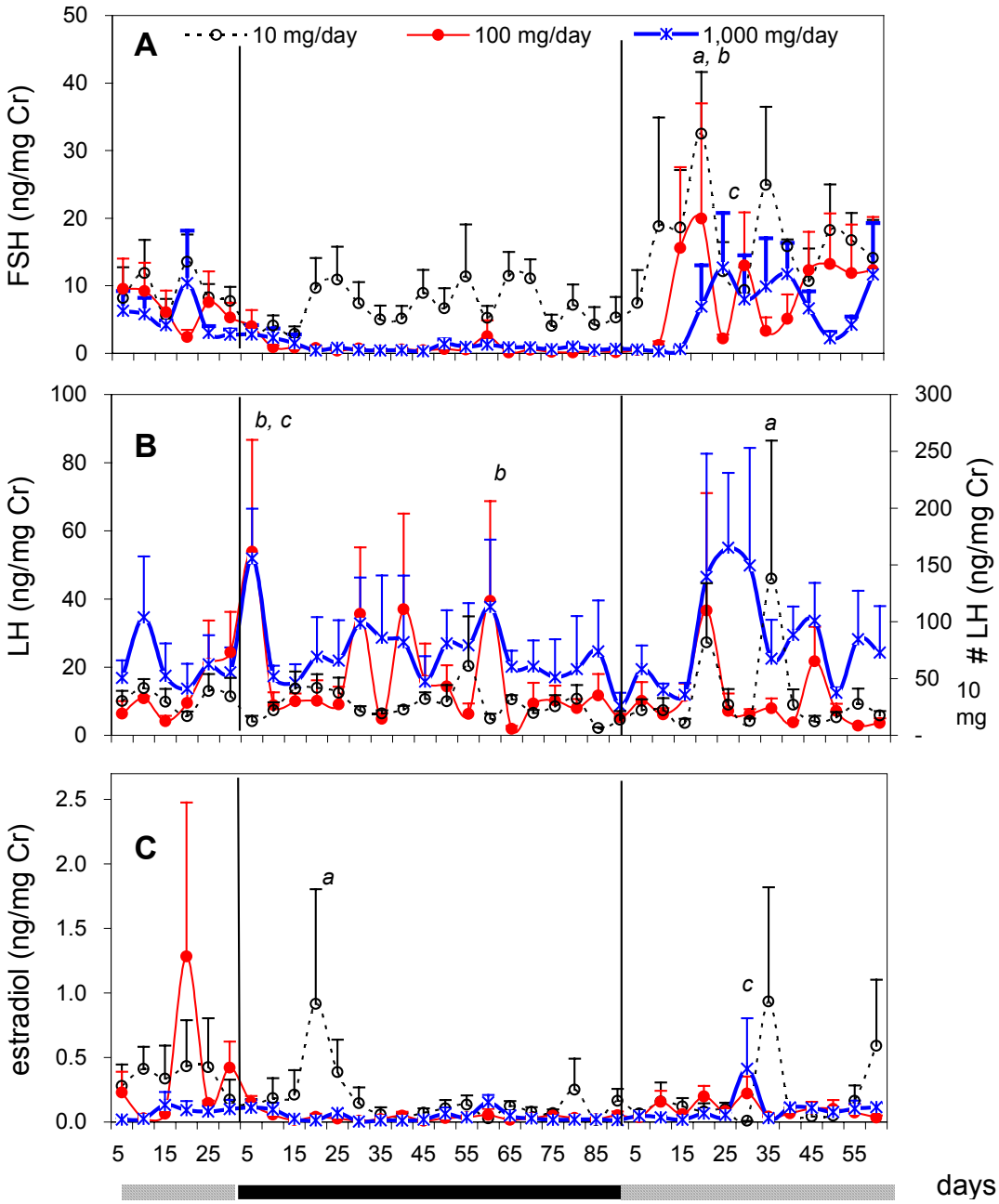

Fig.8 Mean levels of urinary FSH (panel A), LH (panel B), and estradiol (panel C) in aged menopausal monkeys treated with PM-10, PM-100, and PM-1,000. The black horizontal and stripe lines indicates the treatment period. The symbols of *a, b,* and *c* indicated the significant difference with $P < 0.05$.

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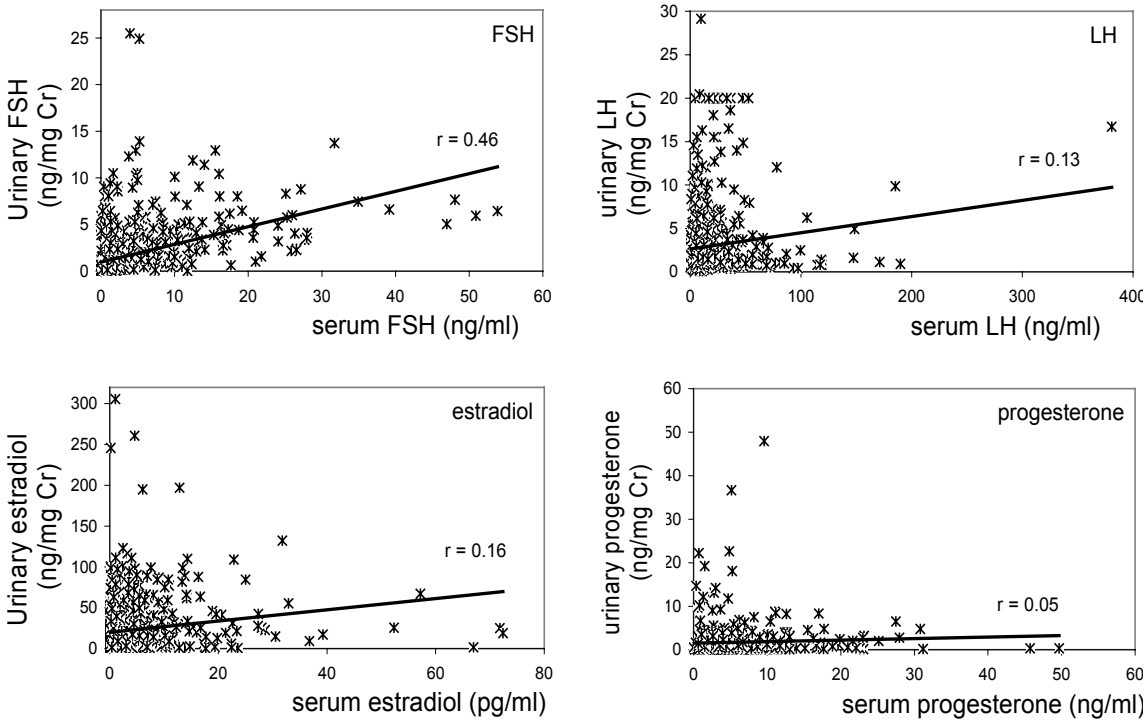

Fig.9 Correlation between FSH, LH, estradiol, and progesterone levels in serum and urine of adult cyclic and aged menopausal monkeys throughout the study period

Discussion

The patterns of urinary excretion of gonadotropins and estradiol during the menstrual cycle in female cynomolgus monkeys in the present study are similar to those secretion patterns in serum, which were previously reported (Trisomboon et al., 2004). Urinary estradiol and progesterone levels showed highly fluctuation throughout the menstrual cycle, although progesterone levels was a trend toward the increase during the early luteal phase. The analysis of correlation coefficient revealed that there was a significant positive correlation in FSH, LH, and progesterone between serum and urine, but no correlation in estradiol levels. The results are in agreement with those form previous studies showing that there are the similar patterns of gonadotropins (Cano and Aliaga, 1995) between in serum and urine of women. Urinary gonadotropin levels can therefore provide a useful index of serum gonadotropin secretion (Frohman, 1995).

The present study also investigated the estrogenic effect of PM on the secretion of urinary gonadotropin and sex steroid hormones in both adult cyclic and aged menopausal monkeys. We hypothesized that the pattern of urinary gonadotropins and sex steroid hormones were parallel with those of the respective hormones in serum, which was revealed

by our previous reports (Trisomboon et al., 2002a, 2002b, 2004). A single treatment of PM in the dosages of PM-10, PM-100, and PM-1,000 did not change the patterns of urinary gonadotropin, estradiol, or progesterone throughout the menstrual cycles in adult cyclic monkeys. The result was coincide with the previous report determining no changes of serum FSH, LH, estradiol, or progesterone levels from the same monkeys (Trisomboon et al., 2004). Furthermore, the previous studies showed that the daily feeding of PM for 90 days significantly suppressed serum gonadotropin and sex steroid hormone levels in both adult cyclic and aged menopausal monkeys in a dose dependent manner (Trisomboon et al., 2002a, 2002b). The similar pattern of changes on those hormones in urine were found, especially for FSH and in aged menopausal monkeys.

Li et al., (2002) found that the peak of urinary FSH level was observed within 1 day of follicular collapse in 96.92% of the menstrual cycle in premenopausal women, indicating that urinary excretion of FSH is a useful biomarker for estimating the day of ovulation. Not only the prominent decrease in basal level but also the absence of peak urinary FSH levels were found in adult monkeys fed daily with PM-100 and PM-1,000 for 90 days. It implies that the daily feeding of PM can disturb the folliculogenesis through the suppression of FSH menstrual cycle. This was partly proved by the fact that the menstrual cycle was either prolonged or stopped in adult monkeys treated with PM-100 and PM-1,000.

Effect of PM on urinary LH level in both adult cyclic and aged menopausal monkeys could not be clearly observed because of the high fluctuation of urinary LH levels throughout the study period. The reason of this fluctuation could not be explained in this study.

Changes in urinary estradiol levels also reflected the daily dose of PM in both adult cyclic monkeys and aged menopausal monkeys. Up to now, no reports showing the effect of phytoestrogen consumption on metabolism of sex steroid hormones. From the previous study, it suggested that PM phytoestrogens act as estrogen and suppressed estradiol through the decrease of gonadotropins in adult cyclic monkeys as well as a direct action on peripheral conversion of androstenedione to estradiol in aged menopausal monkeys (Trisomboon et al., 2002b). It is considered that the decrease in urinary estradiol levels of adult cyclic and aged monkeys determined in the present study are reflects the suppression of estradiol production.

The present study demonstrated that patterns of levels of gonadotropins and estradiol in urine of female cynomolgus monkeys treated with PM are closely related with those of respective hormones in serum in female cynomolgus monkeys. Within these hormones,

urinary FSH is considered to be a good indicator on the study of the estrogenic effect of PM on the disturbances of reproductive system.

Conclusion

White kwao krua or *Pueraria mirifica* (PM), which containing high amounts of phytoestrogens, was proved to be disturb reproductive systems in adult cyclic and aged menopausal cynomolgus monkeys and disturb calcium and related hormone in aged menopausal monkeys.

From this present study, a single treatment of PM prolonged the menstrual cycle length in adult cyclic monkeys in a dose dependent manner. However, there were no changes in hormonal pattern during the menstrual cycle of the adult cyclic monkeys.

The long-term treatment of PM also prolonged the menstrual cycle of adult cyclic monkeys in dose dependence. Adult cyclic monkeys treated with the highest dose (PM-1,000) completely stop menstruation throughout the 90-day of treatment and 60-day of post-treatment period. Serum gonadotropin, estradiol, and progesterone levels decreased significantly in adult cyclic monkeys in a dose-dependent manner. Changes in the menstrual cycle length and serum hormonal levels recovered after the cessation of PM treatment in monkeys treated with PM-10 and PM-100. These finding demonstrated that PM greatly influences the menstrual cycle and suppresses the ovulation by lowering serum levels of gonadotropins.

Aged menopausal monkeys treated daily with PM had a decrease with depend on dose of serum FSH and LH levels. The decrease of serum hormonal levels can be recovered with the durations dependent on doses. The higher dose takes the longer time to recover. From this study, it is suggested that PM can be used as an alternative medicine in aged menopausal women for the estrogen effect, because the effect is reversible after stop using.

In the study of effect of PM on the excretory patterns of urinary hormones in both adult cyclic and aged menopausal monkeys, there were found that the excretory patterns of gonadotropins and estradiol in urine are closely similar to those in serum concentration. In each of gonadotropins and estradiol, there was a significant positive correlation between the levels in serum and urine ($P < 0.01$), but not in progesterone ($P = 0.24$). When urinary pattern of hormone during PM treatment compared to the levels in pre-treatment period, changes of urinary pattern of FSH and estradiol was found in adult cyclic monkeys treated with PM-100 and PM-1,000 and in aged menopausal monkeys treated with all doses of PM. However, changes of urinary FSH level were

clearly observed more than that of urinary estradiol. Accordingly, urinary FSH levels are considered as a good indicator of estrogenic effect of PM on hormonal levels in monkeys.

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