Chapter VII

Long-Term Treatment of *Pueraria mirifica* Phytoestrogens on Parathyroid Hormone and Calcium Levels in Aged Menopausal Cynomolgus Monkeys

Abstract

Prior studies have shown that soy isoflavones have a beneficial effect in preventing bone loss caused by estrogen - deficiency. The aim of the present study is to determine effect of *Pueraria mirifica* (PM) containing phytoestrogens on serum parathyroid hormone (PTH) and calcium levels on estrogen deficiency. Aged menopausal monkeys (*Macaca fascicularis*) were treated with 10, 100, and 1,000 mg/day of PM. Blood samples were collected every 5 days during 30-day, 90-day, and 60-day of the pre-treatment, treatment, and post-treatment periods, respectively, and sera were assayed for PTH, estradiol, and calcium levels. The results showed that PM-1,000 had the strongest effect on the decrease in PTH ($0.001 < P \leq 0.05$) and calcium levels ($0.001 < P \leq 0.03$) during the treatment period. PTH levels remained low for the first 15 days of the post-treatment period ($0.01 \leq P \leq 0.05$). PM-10 induced the significant decrease in PTH level on day 80 ($P = 0.02$) during the treatment period and the significant decrease in calcium level on day 75 ($P < 0.01$). There were no changes in serum PTH and calcium levels throughout the study period in PM-100 group. Estradiol levels decreased significantly during the treatment period in all treatment groups. The result suggests that the long-term treatment of 1,000 mg/day of PM containing phytoestrogens can decrease serum PTH and calcium levels in aged menopausal monkeys, indicating that PM ameliorate bone loss caused by estrogen deficiency.

Keywords: *Pueraria mirifica*, phytoestrogen, PTH, calcium, estrogen, aged menopausal monkey

7.1 Introduction

Menopausal osteoporosis is a disorder of bone characterized by the progressive loss of bone tissue, which begins after estrogen deficiency in both natural and surgical menopause (Pacifici, 1998). The increase in PTH secretion contributes to the increase in bone resorption and osteoporosis, which is related with estrogen deficiency (Silverberg and Bilezikian, 1994; McKane et al., 1997). Although the exact mechanism has not been elucidated yet, parathyroid hormone (PTH) is a major factor involved in the systemic regulation of bone resorption. Overproduction of PTH leads to an increase in bone resorption compared with bone formation and to the general skeletal demineralization. The increased PTH level has been found concomitant with the decreased bone mass in aging (Delmas et al., 1983). The pathogenesis of postmenopausal osteoporosis is complicated, but one pathogenetic mechanism of osteoporosis is a chronic loss of calcium balance on intestinal and renal calcium handling. It is characterized by an increase in PTH concentration, generally thought to be a secondary response to a subtle reduction of serum calcium level.

Several recent reports indicated that soy, a rich source of isoflavone genistein and daidzein, has the beneficial effect on reducing bone loss associated with ovarian hormone deficiency (Ishimi et al., 1999; Mei, Yeng, and Kung, 2001; Yamori et al., 2002). Soy isoflavone treatment induced a great increase in bone mineral density (BMD) in ovariectomized rats (Blum et al., 2003) and mice (Ishimi et al., 1999). Postmenopausal women with habitually high intake of dietary isoflavones had the significantly lower level of serum PTH and higher BMD (Mei et al., 2001). From the

in vitro study, the decrease in bone calcium content induced by bone resorbing factors, PTH, and prostaglandins E2 was inhibited completely by genistein (Gao and Yamaguchi, 1999). Moreover, genistein blocked both the inactivation of acid phosphatase and the activation of alkaline phosphatase due to PTH in bone tissues, resulting in a reduced bone resorption in rats (Yamaguchi and Gao, 1998). These evidences strongly suggested that phytoestrogens play a potential role in preventing bone loss caused by estrogen deficiency in women as well as female animals, possibly through the reduction of PTH levels.

Pueraria mirifica (PM), known as white kwao krua, is a Thai indigenous plant that has long been used as a rejuvenating drug. The chemical contents in its tuberous roots were analyzed by the high performance liquid chromatography technique and many phytoestrogenic substances were found, including miroestrol (Pope et al., 1958; Jones and Pope, 1960), deoxymiroestrol, kwakhurin (Chansakaow et al., 2000), coumestrol, and isoflavones (genistein and daidzein) (Ingham et al., 1988, 1989). Several investigators paid attention to its estrogenic effect on the reproductive organs and functions (Smitasiri et al., 1986, 1989; Trisomboon et al., 2002, 2004). Research with animal model is considered to find and the effect of PM. Treatment of the suspension of PM at various doses influenced serum gonadotropin levels in both of aged menopausal and adult cyclic cynomolgus monkeys (Trisomboon et al., 2002, 2004). The amenorrhea symptom was found in the adult cyclic monkeys as well (Trisomboon et al., 2004). One study found that the administration of the crude extract of PM improved the menopause-related symptoms in women such as hot flushes, frustration, sleep disorder, skin dryness, high blood cholesterol, and amenorrhea with no changes in blood cells and liver and renal functions (Muangman and Cherdshewasart, 2001). There was no report, however, indicating effect of PM on bone, calcium, or PTH levels, especially in menopausal women or female animals that face the problem of bone disorder. The purpose of

this study therefore was to investigate the estrogenic effect of long-term treatment of PM on serum levels of PTH and calcium in aged menopausal monkeys. Aged menopausal cynomolgus monkeys (*Macaca fascicularis*) were used in this study as a representative of menopausal women because the monkeys have physiological systems including hormonal pattern and reproductive function similar to those of humans (Malaivijitnond and Varavudhi, 1998; Krajewski et al., 2003).

7.2 Materials and Methods

7.2.1 Animals

Aged menopausal monkeys (*Macaca fascicularis,* n = 9) with a complete cessation of menstruation for at lest 1 year and weighing 4.0 - 6.5 kg were selected. Menopausal state of the monkeys was confirmed and checked daily by vaginal swabbing method before and during treatment. 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 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, Chulalongkorn University.

7.2.2 Experimental Design

Nine aged menopausal monkeys were divided into three groups. Monkeys in each group (n = 3) were fed daily with the suspension of PM at doses of 10, 100, and 1,000 mg/individual between 08:00 – 08:30 h. The treatment schedule was separated into 3 periods: pre-treatment (30 days), treatment (90 days), and post-treatment (60 days). During the pre-treatment and post-treatment periods, monkeys were fed with 5 ml of distilled water. Three-ml blood samples were collected from the femoral vein without anesthetization between 08:30 – 09:30 h every 5-day, and, then were centrifuged 1,700 x g at 4 ° C, for 20 minutes and stored at –20 ° C until PTH, estradiol, and calcium were assayed.

7.2.3 Preparation of the Suspension of *Pueraria mirifica*

The fresh tuberous roots of PM were sliced, dried in hot air oven at 70 ° C, and subsequently ground into powder at size of 100 Mesh. Then, the stock of its powder was kept in the dark desiccator before the preparation of its suspension. PM powder was suspended into 5 ml of distilled water, and then kept in a dark bottle at 4 °C until the feeding time.

7.2.4 Hormonal Analyses

Serum total calcium levels were measured by the atomic absorptiometry. After extraction of ether, serum level of estradiol was determined by double-antibody RIA with ^{3}H-labeled radioligands as described in the established method of World Health Organization (WHO) (Sufi et al., 1986). Serum PTH levels was assayed by PTH-C radioimmunoassay kit of Eiken Chemical Co.Ltd. (Bunkyo-ku, Tokyo, Japan). The procedure of PTH assay and parallelism check were described in the previous report (Malaivijintnond et al., 2000).

7.2.5 Statistical Analysis

Serum levels of hormones were expressed as mean ± S.E.M. Analysis of variance (ANOVA) followed by the LSD test was applied to determine the significance of difference among three periods of experiment and among three groups. Differences were considered significant at $P < 0.05$.

7.3 Results

7.3.1 Characteristics of Hormonal Pattern in Aged Menopausal Monkeys

The menopausal state of the monkeys was confirmed by the low levels of serum estradiol (14.71 ± 1.18 pg/ml) compared to the levels in normal cyclic monkeys in the late follicular phase of the menstrual cycle in our colony (59.89 ± 10.66 pg/ml). Figure 7.1 shows the relationship between basal levels of estradiol and PTH as well as calcium. There was a slightly positive correlation between the basal levels of calcium and estradiol ($r = 0.46$, $P = 0.001$), and no correlation between the levels of PTH and estradiol ($r = -0.02$, $P = 0.88$).

7.3.2 Changes in Serum PTH, Calcium, and Estradiol Levels in Monkeys Treated with PM

Changes in serum PTH, calcium, and estradiol levels during the pre-treatment, treatment, and post-treatment periods in monkeys treated with PM-10, PM-100, and PM-1,000 are shown in Figures 7.2 – 7.4. As shown in Figure 7.2, monkeys treated with PM-10 showed a trend, but not significant that the level of PTH became lower on days 40 - 90 ($0.06 \leq P \leq 0.51$) in treatment period, than that in the pre-treatment period, except day 80 ($P = 0.02$). After the cessation of PM treatment, PTH level was quickly returned to the pre-treatment levels thereafter. Serum calcium levels

decreased significantly on day 75 (P = 0.01) during treatment period and gradually returned to the basal levels thereafter. Serum estradiol levels significantly sporadically decreased (days 30, 55, 65, 70, and 90 during the treatment period; $0.01 < P < 0.047$, and days 5, 15, and 25 during the post-treatment period; $0.04 < P < 0.05$).

As shown in Figure 7.3, monkeys treated with PM-100, PTH and calcium levels did not significantly change throughout the study period. Estradiol levels decreased significantly in some points during the treatment (days 15, 20, 55, 70, 75, and day 90; $0.03 < P < 0.05$) and post-treatment periods (days 5 and 10; $0.03 < P < 0.05$) compared to the pre-treatment levels.

As shown in Figure 7.4, monkeys treated with PM-1,000, showed the significant decrease in PTH levels during the treatment ($0.001< P < 0.05$) compared to the pre-treatment levels. PTH levels remained low for the first 15 days of the post-treatment period ($0.01< P < 0.05$). Calcium levels decreased significantly only at the latter half of treatment period ($0.001 < P \leq 0.03$) and return to the pre-treatment levels during the early post-treatment period. Estradiol levels were significantly lowered than the pre-treatment levels on day 65 (P = 0.03) of the treatment period and day 20 (P = 0.01) of the post-treatment period.

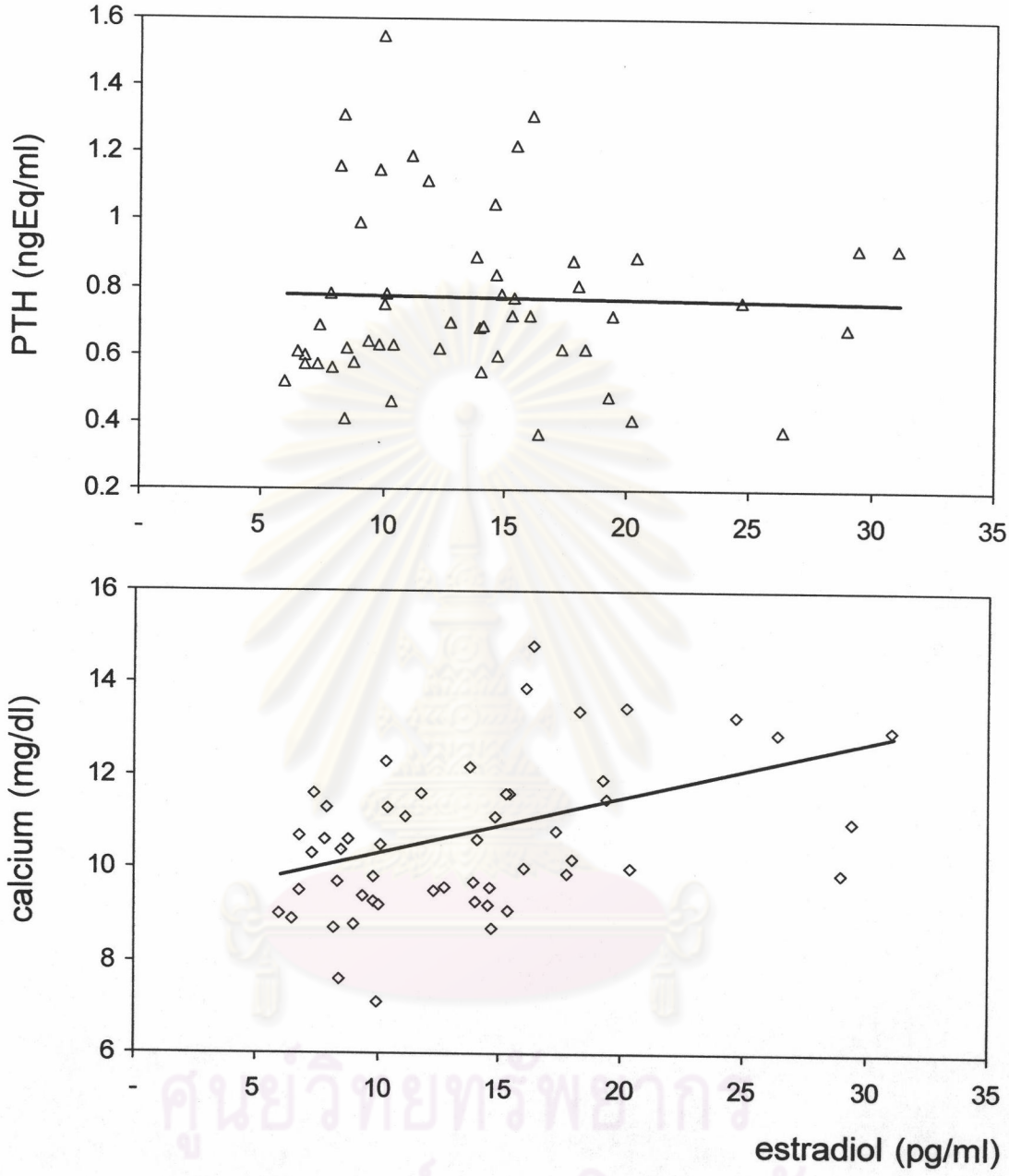

Figure 7.1 Relationship between basal serum levels of PTH and calcium with estradiol in aged menopausal monkeys.

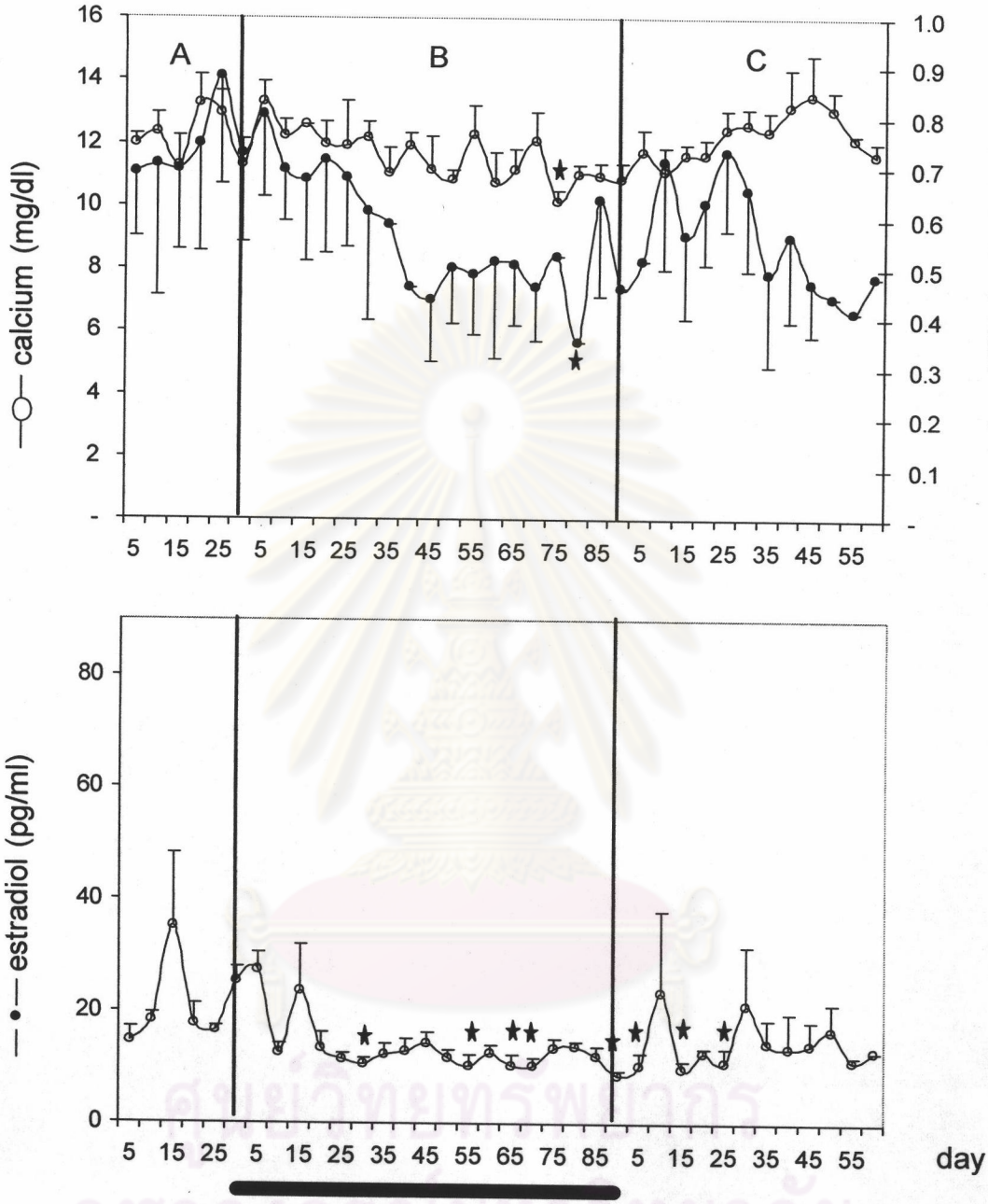

Figure 7.2 Changes in serum PTH, calcium, and estradiol levels during the pre-treatment (A), treatment (B), and post-treatment (C) periods in monkeys treated with PM-10 (n = 3). A horizontal bar indicates the treatment period. The star shows significant difference ($P < 0.05$)

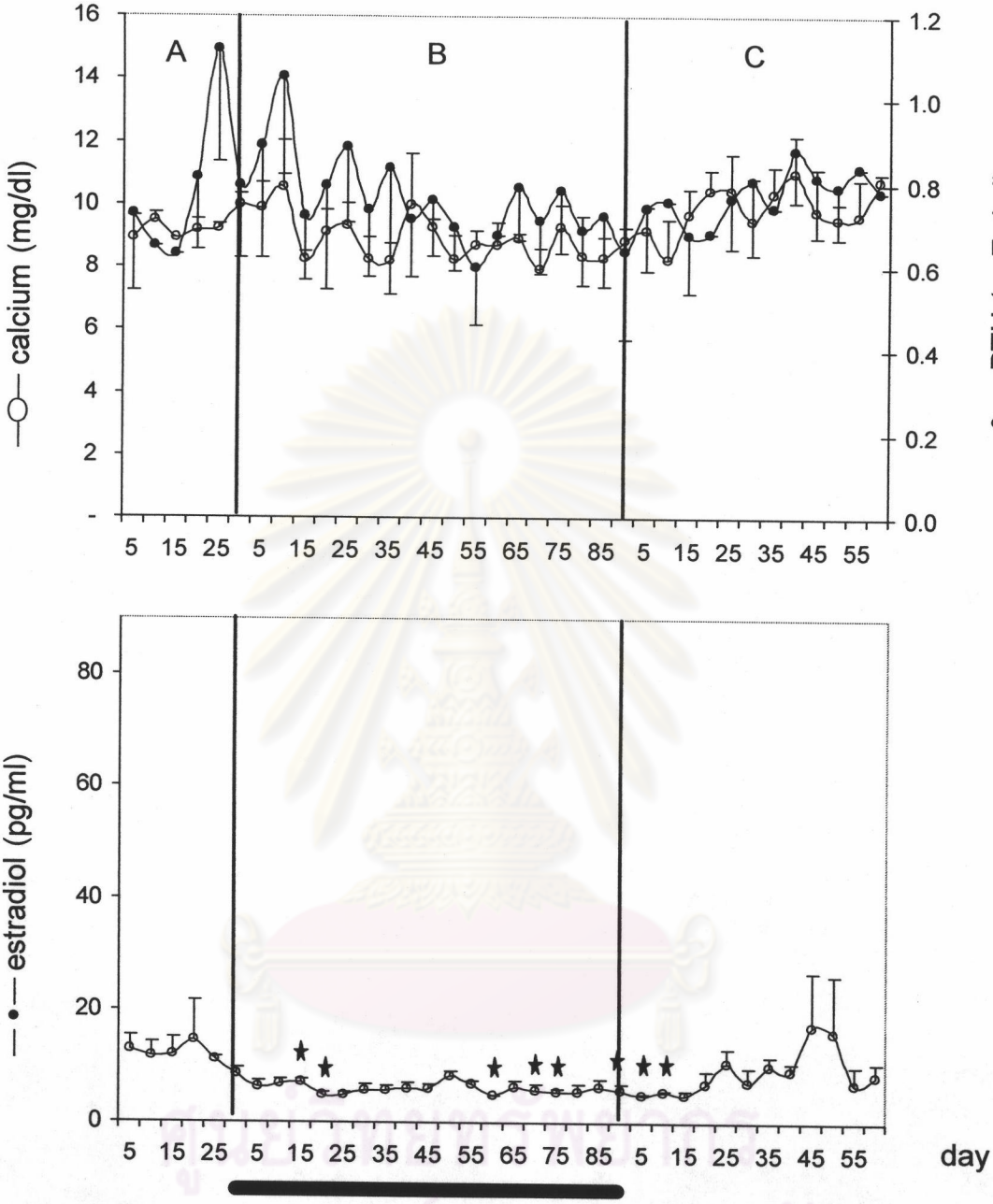

Figure 7.3 Changes in serum PTH, calcium, and estradiol levels during the pre-treatment (A), treatment (B), and post-treatment (C) periods in monkeys treated with PM-100 (n = 3). A horizontal bar indicates the treatment period. The star shows significant difference ($P < 0.05$)

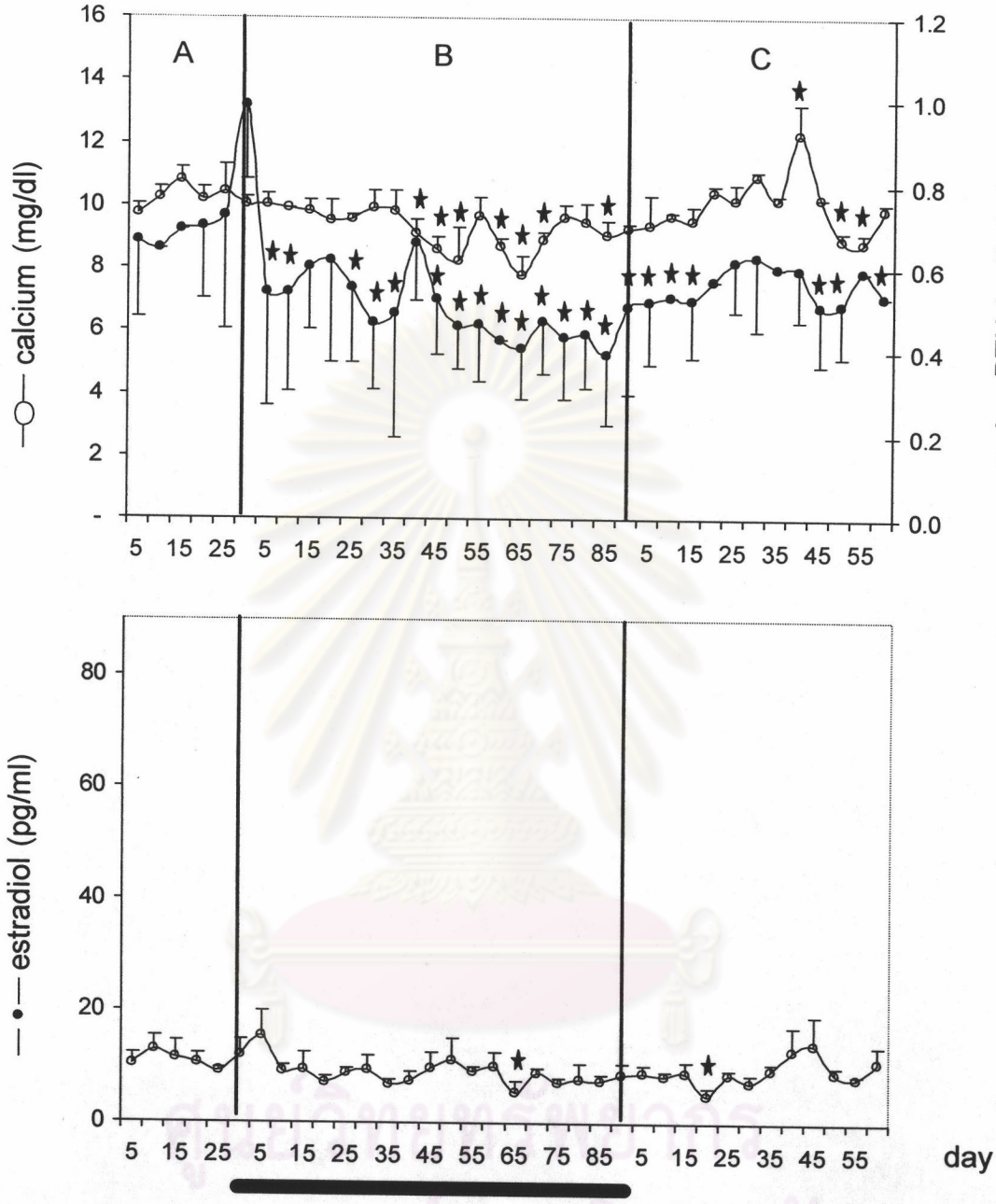

Figure 7.4 Changes in serum PTH, calcium, and estradiol levels during the pre-treatment (A), treatment (B), and post-treatment (C) periods in monkeys treated with PM-1,000 (n = 3). A horizontal bar indicates the treatment period. The star shows significant difference ($P < 0.05$).

7.4 Discussion

This study firstly reports the effect of phytoestrogens from the medicinal plant, PM, on altering serum levels of PTH and calcium in estrogen deficiency animals. The result clearly demonstrated that aged menopausal monkeys treated with PM were induced the decreasing in PTH levels and followed by the decline of serum calcium levels. The highest dose (PM-1,000) seems to be more effective for the decrease of PTH and calcium levels than the lowest dose (PM-10). The effect, however, did not depend on doses, because the significant decrease could not be observed in PM-100 group. The reason why there are no effect of PM-100 on serum PTH and calcium levels could not be explained in this study.

As similar to our result, postmenopausal women consumed high isoflavones from soy diet had the lower level of serum PTH (Mei et al., 2001). The decrease of PTH levels associated with an increased BMD at the lumbar spine and Ward's triangle, suggesting that intake of high phytoestrogens may help to improve the state of hyperparathyroidism in postmenopausal women resulting in both lower rates of bone turnover and bone loss (Mei et al., 2001).

Estrogen administrations have been demonstrated to decrease serum levels of PTH and calcium in postmenopausal women (Stock, Coderre, and Mallette, 1985; Khosla et al., 1997). It was suggested that estrogen directly inhibits bone resorption, leading to decrease the calcium release from bone into blood circulation (Stock, et al., 1985; Khosla et al., 1997). Estrogen may have a direct action on enhancing intestinal and renal tubular calcium absorption and modulating calcium homeostasis (Vincent et al., 2003). In addition, the *in vitro* study showed that estrogen receptor (ER) was found in parathyroid cells, and estradiol treatment caused a decrease in their basal DNA synthesis (Wong et al., 2002), suggesting that estrogen may have a direct effect on PTH secretion.

It is concluded that *P. mirifica* phytoestrogens behave as an estrogen and decrease the PTH levels, since phytoestrogens compete with estradiol for binding to ER, which were found in the renal, gastrointestinal tract, and bone (Onoe et al., 1997; Gustafsson, 1999). Phytoestrogens may have an effect on these organs to improve calcium absorption resulting in a secondary decrease in PTH level. Based on the finding by Wong et al. (2002) described above, this study can hypothesize that PM phytoestrogens have a direct action on decreasing PTH secretion from the parathyroid gland.

It is known that the mechanism of PTH in regulating calcium balance is very complex. Normally, it acts directly on the bone and renal to increase calcium influx into the blood circulation. It also stimulates indirectly the calcium absorption from the intestine. Then, the overall effect of PTH is to increase circulating calcium level (Silverberg and Bilezikian, 1994). Administration of PTH increased the serum calcium level in patients with hypoparathyroidism (Winer et al., 1998). Lower level of serum PTH therefore induced a reduction of serum calcium level in blood circulation. The result of the present study showed that the long-term treatment of PM can suppress PTH level, which is followed by the decrease in calcium level.

Although the long-term treatment of PM led to the decrease in serum calcium, serum calcium levels during the treatment period in all monkey groups (9.91 $\pm$ 0.13 mg/dl) were kept in the narrow range and remained within the normal range (10.57 $\pm$ 0.22 mg/dl) of the aged monkeys. After the cessation of PM treatment, calcium level returned to the pre-treatment level. Calcium is of fundamental importance to all biological activities, and it is also a vital component not only in the mechanism of hormone secretion but also hormone action and involved in neurotransmission and muscle contraction. For this reason, it is vital that calcium concentration is kept within a narrow range. The fact that aged monkeys treated with PM for 90 days, the levels of serum calcium was not greatly lowered than its normal range, means that

the long-term treatment of PM containing phytoestrogens do not have an adverse effect on calcium homeostasis in physiological system.

Although the study can not completely explain the mechanism of PM on the decreasing of PTH and calcium levels, the study, at least, is surely to note that the effect on PTH and calcium levels were not caused by the endogenous ovarian estradiol but by PM phytoestrogens. This was confirmed by low estradiol levels throughout the study period in all monkey groups. During PM treatment, serum estradiol levels were even lowered than that in the pre-treatment period, which is considered to be caused by the reduction in peripheral conversion of estrone and testosterone to estradiol. The previous reports showed that genistein and coumestrol reduced the conversions both of estrone to estradiol (Makella et al., 1995; Whitehead et al., 2002) and of androstenedione and testosterone to estradiol in human granulosa luteal cells (Whitehead et al., 2002).

In summary, the long-term treatment of 1,000 mg/day of PM can decrease the serum levels of PTH and calcium in aged menopausal monkeys. The previous report recommended that 100 mg/day of PM (20mg/kg body weight monkey or 2 mg/kg body weight human) is an appropriate dose for suppression of serum levels of gonadotropins in aged menopausal monkeys (Trisomboon et al., 2002). The present study found, however, that 1,000 mg/day of PM has a beneficial effect on preserve calcium content in bone by reducing PTH secretion. However, to determine the appropriate dose of PM to reduce the bone loss in menopausal women additional studies are necessary.

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