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

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Abstract. To determine the effect of *Pueraria mirifica* (PM) on serum parathyroid hormone (PTH) and calcium levels on aged menopausal monkeys (*Macaca fascicularis*), subjects were treated with 10, 100, or 1,000 mg/day of PM. Blood samples were collected every 5 days for 30, 90, and 60 days during pre-treatment, treatment, and post-treatment periods, respectively. Sera were assayed for PTH, estradiol, and calcium levels. PM-1,000 had the strongest effect on the decrease in PTH (0.001<P≤0.05) and calcium levels (0.001<P≤0.03) during the treatment period. PTH levels remained low for the first 15 days of the post-treatment period (0.01≤P≤0.05). PM-10 induced a significant decrease in PTH level on day 80 (P=0.02) during the treatment period and a 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 the PM-100 group. Estradiol levels decreased significantly during the treatment period in all treatment groups. The results suggest that long-term treatment with 1,000 mg/day of PM decreases serum PTH and calcium levels in aged menopausal monkeys, indicating that PM ameliorates bone loss caused by estrogen deficiency.

Key words: *Pueraria mirifica*, Phytoestrogen, PTH, Calcium, Aged menopausal monkey

Menopausal osteoporosis is a disorder of the bone characterized by the progressive loss of bone tissue, and is caused by estrogen deficiency in both natural and surgical menopause [1]. Increased parathyroid hormone (PTH) secretion contributes to an increase in bone resorption and osteoporosis, which is related to estrogen deficiency [2, 3]. Although the exact mechanism has not been elucidated yet, 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 contributes to general skeletal demineralization. Increased PTH levels have been found to be concomitant with decreased bone mass in aging people [4]. One pathogenetic mechanism of osteoporosis involves chronic loss of calcium balance caused by intestinal and renal calcium handling. This mechanism is characterized by an increase in PTH concentration, and is generally though to be a secondary response to a small
reduction of serum calcium levels.

Several recent reports have indicated that soy, a rich source of isoflavone genistein and daidzein, has a beneficial effect, reducing bone loss associated with ovarian hormone deficiency [5–7]. Soy isoflavone treatment induced a large increase in bone mineral density (BMD) in ovariectomized rats [8] and mice [5]. Postmenopausal women with habitually high intakes of dietary isoflavones had significantly lower levels of serum PTH and higher BMD [6]. In an in vitro study, the decrease in bone calcium content induced by bone resorbing factors, PTH, and prostaglandins E2 was inhibited completely by genistein [9]. Moreover, genistein blocked both the inactivation of acid phosphatase and the activation of alkaline phosphatase due to PTH in bone tissue, resulting in reduced bone resorption in rats [10]. The evidence strongly suggests that phytoestrogens play a 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 an indigenous Thai plant that has long been used as a rejuvenating drug. The chemical content of its tuberous roots has been analyzed by high performance liquid chromatography and many phytoestrogenic substances were found, including miroestrol [11, 12], deoxymiroestrol, kwakhurin [13], coumestrol, and isoflavones (genistein and daidzein) [14, 15]. Several investigators have studied PM’s estrogenic effect on the reproductive organs and their functions [16–18]. Prior research found a PM effect using an animal model [16–18]. Treatment with a suspension of PM at various doses influenced serum gonadotropin levels in both aged menopausal and adult cyclic cynomolgus monkeys [18, 19]. One study found that administration of crude extract of PM improved menopause-related symptoms in women such as hot flushes, frustration, sleep disorder, skin dryness, high blood cholesterol, and amenorrhea with no change in blood cells, or liver or renal functions [20].

**Materials and Methods**

**Animals**

Aged menopausal monkeys (*Macaca fascicularis*, n=9) with a complete cessation of menstruation for at least one year, age more than 25 years, and weighing from 4.0–6.5 kg, were selected. The menopausal state of the monkeys was confirmed and checked daily by vaginal swabbing 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 in 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 were given 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.

**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, or 1,000 mg/individual/day between 08:00–08:30 h. (hereafter abbreviated as PM-10, PM-100, and PM-1,000) The treatment schedule was separated into 3 periods: pre-treatment, treatment, and post-treatment for 30, 90, and 60 days, respectively. During the pre-treatment and post-treatment periods, monkeys were fed with 5 ml of distilled water. Blood samples, 3 ml, were collected from the femoral vein without anesthetization between 08:30–09:30 h every 5 days, then centrifuged 1,700 × g at 4 C, for 20 minutes and stored at −20 C until PTH, estradiol, and calcium were assayed.
**Pueraria mirifica suspension preparation**

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

**Hormonal analyses**

Serum total calcium levels were measured by an atomic absorption spectrophotometer (AAS) according to the method of Zettner and Seligson using a Roche/Hitachi system [23]. After extraction with ether, the serum level of estradiol was determined by RIA with tritiated radioligands as described by the established method of the World Health Organization (WHO) [24]. Serum PTH levels were assayed by a PTH-C radioimmunoassay kit (Eiken Chemical Co. Ltd., Bunkyo-ku, Tokyo, Japan). The procedure of the PTH assay and parallel checks were described in a previous report [25].

**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 the three periods of the experiment, and among the three groups. Differences were considered significant at \( P<0.05 \).

**Results**

**Characteristics of hormonal pattern in aged menopausal monkeys**

The menopausal state of the monkeys was confirmed by lower levels of serum estradiol (14.71 ± 1.18 pg/ml) and higher levels of LH (5.85 ± 0.80 ng/ml) compared to normal cyclic monkeys in the early follicular phase of the menstrual cycle in our

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**Fig. 1.** Relationship between basal serum levels of PTH and calcium with estradiol in aged menopausal monkeys.

**Fig. 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). The horizontal bar indicates the treatment period. Stars show significant differences compared to pre-treatment levels (\( P<0.05 \)).
colony (27.54 ± 4.31 pg/ml for estradiol and 0.51 ± 0.03 ng/ml for LH, unpublished data). Figure 1 shows the relationship between basal levels of estradiol and PTH as well as calcium. There was a slight positive correlation between the basal levels of calcium and estradiol (r=0.46, P=0.001), but no correlation between the levels of PTH and estradiol (r=-0.02, P=0.88).

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 Figs. 2–4. As shown in Fig. 2, monkeys treated with PM-10 showed a trend, but not significant, for the PTH level to become lower on days 40–90 (0.06≤P≤0.51) in the treatment period, than that in the pre-treatment period, except day 80 (P=0.02). After the cessation of PM treatment, the PTH level was quickly returned to the pre-treatment levels. Serum calcium levels decreased significantly on day 75 (P=0.01) during the treatment period but gradually returned to the basal levels thereafter. Serum estradiol levels significantly but 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 Fig. 3, monkeys treated with PM-100 did not have significantly changed PTH and calcium levels throughout the study period. Estradiol levels decreased significantly at 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 Fig. 4, monkeys treated with PM-1,000 showed a 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 in the latter half of treatment period \((0.001 < P \leq 0.03)\) and returned to the pre-treatment levels during the early post-treatment period. Estradiol levels were significantly lower than the pre-treatment levels on day 65 \((P=0.03)\) of the treatment period and on day 20 \((P=0.01)\) of the post-treatment period.

**Discussion**

Healthy menopausal monkeys have low levels of serum estradiol and there is a positive correlation between estradiol levels and calcium levels in their serum. It has been proposed that changes in estradiol levels influence intestinal calcium absorption and increase serum calcium levels as found in healthy humans \[26\]. However, we did not find the correlation between estradiol levels and PTH levels because of vary estradiol levels.

Our study is the first to report on the effect of phytoestrogens from the medicinal plant, P. mirifica, on altering serum levels of PTH and calcium in estrogen deficient animals. The results clearly demonstrate that aged menopausal monkeys treated with PM had reduced in PTH levels which were followed by a decline in serum calcium levels. The highest dose (PM-1,000) seems to have been more effective for the decrease of PTH and calcium levels than the lowest dose (PM-10). The effect, however, did not depend on dose, because a significant decrease was not observed in the PM-100 group. The reason why there was no effect of PM-100 on serum PTH and calcium levels in this study remains unknown.

Similar to our result, post-menopausal women consuming high isoflavones from a soy diet had lower levels of serum PTH \[6\]. The decrease of PTH levels was associated with increased BMD in the lumbar spine and Ward’s triangle, suggesting that high intake of phytoestrogens may help to improve the state of hyperparathyroidism in postmenopausal women resulting in both lower rates of bone turnover and bone loss \[6\].

**Estrogen administrations** have been demonstrated to decrease serum levels of PTH and calcium in postmenopausal women \[27, 28\]. It has been suggested that estrogen directly inhibits bone resorption, leading to a decrease in calcium release from the bone into the blood circulation \[27, 28\]. Estrogen may have a direct action on enhancing intestinal and renal tubular calcium absorption and modulating calcium homeostasis \[29\]. In addition, an *in vitro* study showed that estrogen receptor (ER) was found in parathyroid cells, and estradiol, treatment caused a decrease in their basal DNA synthesis \[30\], suggesting that estrogen may have a direct effect on PTH secretion.

We consider that phytoestrogens in *P. mirifica* behave as an estrogen and decrease the PTH levels, since phytoestrogens compete with estradiol to bind to estrogen receptors, which are found in the renal, gastrointestinal tract, and bone \[31, 32\]. Phytoestrogens may effect these organs, improving calcium absorption, resulting in a secondary decrease in the PTH level. Based on the findings of Wong *et al.* \[30\] described above, we 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 kidney to increase calcium influx into the blood circulation. It also stimulates indirectly calcium absorption by the intestine. The overall effect of PTH is to increase the circulating calcium level \[2\]. Administration of PTH increased the serum calcium level in patients with hypoparathyroidism \[33\]. Low levels of serum PTH induced a reduction of serum calcium level in the blood circulation \[2\]. The results of the present study show that long-term treatment with PM can suppress the serum PTH level, followed by a decrease in the serum calcium level.

Although the long-term treatment of PM led to a decrease in serum calcium, serum calcium levels during the treatment period in all monkey groups \((9.91 \pm 0.13 \text{ mg/dl})\) stayed in a narrow range and remained within the normal range \((10.57 \pm 0.22 \text{ mg/dl})\) of aged monkeys. After the cessation of PM treatment, serum calcium levels 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 in hormone action and is involved in neurotransmission and muscle contraction. For this reason, it is vital that the serum calcium concentration is kept within a narrow range. The fact that aged monkeys treated
with PM for 90 days, did not have greatly lowered serum calcium levels outside their normal range, means that the long-term treatment of PM, which contains phytoestrogens, does not have an adverse effect on calcium homeostasis in the physiological system.

Although our study cannot completely explain the mechanism of PM on the decrease of PTH and calcium levels, at the very least it can be said that the effects on PTH and calcium levels were not caused by endogenous ovarian estradiol but by PM phytoestrogens. This was confirmed by the low estradiol levels seen throughout the study period in all monkey groups. During PM treatment, serum estradiol levels were even lower than those in the pre-treatment period, which is considered to have been caused by a reduction in peripheral conversion of estrone and testosterone to estradiol. Previous reports showed that both genistein and coumestrol reduced conversion of estrone to estradiol [34, 35], and of androstenedione and testosterone to estradiol in human granulosa luteal cells [36].

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 present study suggests that 1,000 mg/day of PM has a beneficial effect on preservation of calcium content in the bone by reducing PTH secretion. However, to determine the appropriate dose of PM to reduce bone loss in menopausal women additional studies are necessary.

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**References**


