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APPENDICES

LIST OF PUBLICATIONS

Research papers

1. **Urasopon, N.,** Hamada, Y., Cherdshewasart, W., Asaoka, K., and Malaivijitnond, S. 2007. *Pueraria mirifica*, a phytoestrogen-rich herb, prevents bone loss in orchidectomized rats. Maturitas 56: 322-331. (IF 2006 = 1.947)

2. Malaivijitnond, S., Chansri, K., Kijkuokul, P., **Urasopon, N.,** and Cherdshewasart, W. 2006. Using vaginal cytology to assess the estrogenic activity of phytoestrogen-rich herb. J Ethnopharmacol 107: 354-360. (IF 2006 = 1.625)

Proceedings

1. **Urasopon, N.,** Hamada, Y., and Malaivijitnond S. 2007. *Pueraria mirifica*, a phytoestrogen-rich herb, prevents bone loss in ovariectomized rats. In proceedings of RGJ-Ph.D. Congress VIII in Jomtien Palm Beach Resort Pattaya, Chonburi, April 20 – 22, 2007, Chonburi, Thailand. 158. (oral presentation)

2. **Urasopon, N.,** Hamada, Y., and Malaivijitnond, S. 2004. Morphometric study on the cross-section of distal radius: Preparatory work for bone turnover study. In proceedings of the fifth congress of AOSCE in conjunction with the annual meeting of JSCE, March 26-30, 2004, Nara, Japan. 567-569. (poster presentation)

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Pueraria mirifica, a phytoestrogen-rich herb, prevents bone loss in orchidectomized rats

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Abstract

Objective: Estrogens and estrogen-like substances have been reported to play an important role in male bone homeostasis and to prevent bone loss. *Pueraria mirifica* (Leguminosae), a Thai herbal plant, containing a high amount of phytoestrogens was a choice of interest for this study. We examined the effects of crude *P. mirifica* on bone loss and influences on reproductive organs in male rats.

Methods: Using fully mature and orchidectomized (ORX) rats, the effects of 0, 10, 100 and 1000 mg/kg B.W./day of *P. mirifica* and 0.1 mg/kg B.W./day of 17 alpha-ethinylestradiol (a positive control) were evaluated on bone mineral density (BMD) and bone mineral content (BMC) measured with a peripheral Quantitative Computerized Tomography (pQCT) densitometry.

Results: Bone loss in trabecular and cortical bones of the various sites of axial bone (fourth lumbar vertebral body) and long bones (tibia and femur) after ORX was dose-dependently prevented by *P. mirifica*. The effects were specific on bone types and sites. The weights of the accessory sex organs, seminal vesicle and ventral prostrate gland, which significantly decreased after 3-month of ORX, were not altered by *P. mirifica*.

Conclusion: The results suggest that *P. mirifica* treatment may be useful to prevent an osteoporosis in elderly hypogonadism subjects without influences on reproductive organs.

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Keywords: Bone loss; Male rats; Phytoestrogens; *Pueraria mirifica*

1. Introduction

Hypogonadism is considered to be one of the most important risk factors of osteoporosis in men [1,2]. It is established that androgen withdrawal induced by orchidectomy (ORX) results in the significant loss of

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bone mass in experimental animals [3,4]. However, during recent years, it was shown that estrogens also play an important role in male bone homeostasis. The men with a mutation in the estrogen-receptor gene [5] or those with a defective aromatase enzyme [6,7] exhibited a low bone mass. In the latter, testosterone and other androgen levels were all greatly raised, while estradiol and estrone levels were undetectable, and the subsequent treatment with estradiol increased bone mass at all bone sites [7,8]. Additionally, the bone mass in elderly men was more significantly related with the serum level of estradiol than testosterone [9,10]. Therefore, estrogen replacement therapy is proposed to prevent bone loss in males as well as in females [8,11]. Estrogen, however, has been considered as one of the hormonal risk factors of benign prostatic hyperplasia and prostate cancer, the most frequent tumor in males [12,13]. Thus, an alternative estrogenic substance for bone loss therapy has been sought.

Phytoestrogens are considered to be an effective alternative estrogenic substance in preventing bone loss caused by the deficiency of either estrogen and/or androgen. Administration of phytoestrogens or plant containing phytoestrogens, *Pueraria lobata*, showed a preventive effect on bone loss in ovariectomized (OVX) rats and mice [14–18], and in ORX mice [19,20]. Thus, in this study, we investigated other herb containing high amount of phytoestrogens, *Pueraria mirifica*, for bone therapy.

P. mirifica, a Thai herb, belongs to the same family of soybean and *P. lobata*. Its tuberous root was found to contain at least 13 known phytoestrogens [21–23]. The estrogenic effects of *P. mirifica* were exhibited in various reproduction organs, that is, induction of vaginal cornification and increased uterine weight in OVX rats [23,24], prolongation of the menstrual cycle in mature female monkeys [25], suppression of serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels in male rats [23] and female monkeys [26,27], and alleviation of menopausal symptoms in women [28]. However, no published reports on the influence of *P. mirifica* in bones have been found. Thus, the effects of *P. mirifica* on bone loss in male rats are evaluated in the present study. The determination has been performed both in the axial bone and the long bone, in trabecular and cortical compartments, and in metaphyseal and diaphyseal sites.

Table 1
Composition of the soybean-free diet

Ingredients	Percentage of diet
Moisture	5.41
Protein	26.3
Fat (ether extraction)	2.92
Fat (acid hydrolysis)	7.98
Fiber	2.14
Ash	4.71
Calcium	0.94
Phosphorus	0.83
Sodium chloride	0.44
Vitamin D	4000 i.u.

2. Materials and methods

2.1. Animals

Two-month old male Sprague–Dawley rats were purchased from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were housed at the Primate Research Unit, Chulalongkorn University, Bangkok, Thailand in a room at 23 ± 2 °C with 12-h light:12-h dark cycles. The rats were fed with a standard rat diet (C.P. 082, Lot No. 2, S.W.T. Co., Ltd., Thailand) for 4.5 months or until they were 6.5 months old. Two weeks in advance and during experimental period, rats were kept in three animals/cage and fed a soybean-free rat diet (C.P. 082/SBF, Lot No. 050119, S.W.T. Co., Ltd., Thailand) to minimize the phytoestrogen content in the diet. The soybean-free diet contained yellow corn, rice, rice by-product, fish meal, corn gluten meal, vegetable oil, salt, vitamins and minerals. The composition of the soybean-free diet is shown in Table 1. The rat diet and water were supplied *ad libitum*. The experimental protocol was approved by the Animal Ethical Committee in accordance with guide for the care and use of laboratory animals prepared by Chulalongkorn University.

2.2. Experimental design

At 7 months of age, rats were grouped by means of body weight (B.W.) and assigned for experiments. The duration of treatment period was 3 months. Two series of experiments were performed. In the first, rats were kept their testes intact. They were separated into

two groups; the initial control and the Sham control groups. The initial control group (IC), nine rats were sacrificed at the first day of the experimental period and their data were kept as a base line control. The Sham control group (SH), nine rats were cut the scrotal skin and sutured to induce a stress similar to that obtained with bilateral ORX, and gavaged daily with 1 ml of distilled water during the experimental period. In the second, rats were bilaterally ORX and separated into five groups, nine rats per group; EE, P0, P10, P100 and P1000. The EE group, rats were gavaged daily with 0.1 mg/kg B.W./day of 17 alpha-ethinylestradiol, and the P0, P10, P100 and P1000 groups, respectively, rats were gavaged daily with 0, 10, 100 and 1000 mg/kg B.W./day of *P. mirifica* during experimental period. The feeding of all treatments was performed in the afternoon at 01:00–03:00 p.m.

Body weights of rats were measured weekly and used to adjust the quantity of *P. mirifica* or 17 alpha-ethinylestradiol treatments, thereafter. At the end of 3 months of experimental period, all rats were sacrificed, and their seminal vesicles and ventral prostate glands were weighed. The tibia, femur and lumbar vertebra were defleshed from adjacent tissues, wrapped in saline-soaked gauze bandages to prevent dehydration, and stored frozen at −20 °C in small Ziploc freezer bags until bone mineral density (BMD) and bone mineral content (BMC) were measured [29].

2.2.1. Preparation of *P. mirifica* suspension

The same lot of fresh tuberous roots of *P. mirifica* cultivar Wichai-III was used throughout this study, which were collected from the field in Chiang Mai Province, Thailand. The voucher specimen of the *P. mirifica* (No. BCU 11045) was deposited at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. *P. mirifica* suspension was prepared as described previously [24,30].

2.2.2. Preparation of 17 alpha-ethinylestradiol solution

The 17 alpha-ethinylestradiol powder (Sigma, St. Louis, MO) was dissolved by minimum volume of absolute ethanol and then diluted by distilled water. The ethanol was left to evaporate overnight and the solution was kept in a dark bottle at 4 °C until the feeding time.

2.3. Bone measurement

Bone mineral density and content were measured using a peripheral Quantitative Computed Tomography (pQCT) in the research M mode (XCT Research SA+, Stratec Medizintechnik GmbH., Germany). This system has a 50 kV X-ray tube with a current of 0.307 mA as the source of a 0.5 mm-thick beam. The determination has been performed both in the axial bone and the long bone, in cortical and trabecular parts, and in metaphyseal and diaphyseal sites as follows:

Tibia: Proximal tibial metaphysis (TM) was cross-sectionally scanned at 2, 2.5 and 3 mm below the growth plate of the tibia. Tibial diaphysis (TD) was scanned at the midpoint (50% of the length of the tibia) and at both sides of the midpoint, 1 mm apart.
Femur: Distal femoral metaphysis (FM) was cross-sectionally scanned at 2, 2.5 and 3 mm above the growth plate and femoral diaphysis (FD) was scanned at the midpoint (50% of the length of the femur) and at both sides of the midpoint, 1 mm apart.
The fourth lumbar vertebra: Fourth lumbar vertebra (L4) was cross-sectionally scanned at the midpoint of cranio-caudal axis and at both sides of the midpoint, 1 mm apart from the midpoint along the longitudinal axis of the vertebra.

The voxel size of each bone slice was 0.09 mm × 0.09 mm × 0.09 mm and a slice thickness was 0.46 mm. The trabecular bone is determined by the contour mode 2 and the peel mode 2 with the threshold value of 720 mg/cm^3, and the cortical bone is determined by the separation mode 2 with the threshold value of 900 mg/cm^3. Upon the completion of scanning, the following parameters were analyzed for each bone slice using XCT-5.50E software (Stratec Medizintechnik GmbH., Germany): the trabecular bone mineral density (TbBMD), trabecular bone mineral content (TbBMC), cortical bone mineral density (CtBMD) and cortical bone mineral content (CtBMC) for the TM, FM and L4; and the CtBMD and CtBMC for the TD and FD. The average of three scans made in all bone sites described above was analyzed.

2.4. Statistical analysis

Results were reported as mean ± S.E.M. for each group. The significance of differences between groups

were examined by one-way analysis of variance followed by Fisher' s protected least significant difference. Differences were considered significant at the level of $p < 0.05$. The bone parameters, BMD and BMC, in treatment groups, which were compared with those in P0 group were also expressed by 'the percent prevention' calculated using the following formula:

$$\text{percent prevention} = \left[\frac{\text{mean score of treatment group} - \text{mean score of P0 group}}{\text{mean score of SH group} - \text{mean score of P0 group}}\right] \times 100.$$

In case of non-significant difference between SH and P0 group, changes of BMD and BMC in treatment groups were expressed as 'the percent change' of P0 group which was calculated using the following formula:

$$\text{percent change} = \frac{\text{mean score of treatment group} - \text{mean score of P0 group}}{\text{mean score of P0 group}} \times 100$$

3. Results

3.1. Seminal vesicle and ventral prostate gland weights

After ORX for 3 months, the weights of seminal vesicles and ventral prostate glands of P0 rats were highly significantly lower than those in Sham control (SH) rats (seminal vesicle = 2047 ± 119 mg for SH rats versus 194 ± 5 mg for P0 rats and prostate gland = 549 ± 36 mg for SH rats versus 92 ± 12 mg for P0 rats). Feeding of 17 alpha-ethinylestradiol (EE) and all three doses of *P. mirifica* did not affect the weights of these organs and non-significant difference from those of P0 group.

3.2. Bone mineral density

The trabecular bone mineral densities (TbBMDs) in the proximal tibial metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebral body (L4) for all treatment groups are shown in Fig. 1. The difference of bone measurements between IC and SH groups was regarded as the bone change by means of age increase during the experimental period, from 7 to 10 months old. The TbBMDs in SH group were not significantly lower than those in IC group in TM and L4, but it was in FM by 8.48% ($p < 0.01$). The influence of ORX was shown by the differences between TbBMDs in P0 and SH groups. The TbBMDs in TM, FM and L4 of P0 group were significantly lower, by 28.32%, 34.38% and 27.90%, respectively, than those in the SH group ($p < 0.001$). Treatment of P10 slightly prevented the decrease of TbBMDs in TM and FM ($p > 0.05$). On the other hand, *P. mirifica* at the same dose significantly prevented the decrease of TbBMD in L4 by 50.81% ($p < 0.001$). Treatment of P100 absolutely prevented the decrease of TbBMDs in TM and L4 by 100.12% and 93.91%, respectively ($p < 0.001$). However, it prevented only 72.52% in FM ($p < 0.001$). Treatment of P1000 highly prevented the decrease of TbBMDs in TM, FM and L4 by 134.46%, 124.17% and 137.37%, respectively ($p < 0.001$). As expected, EE administration absolutely prevented the decrease in the TbBMDs in TM, FM and L4 by 92.45%, 86.91% and 94.28%, respectively ($p < 0.001$).

The cortical bone mineral densities (CtBMDs) measured for all treatment groups in TM, FM, L4, TD and FD are shown in Fig. 2. As the 3 months of age change, the CtBMDs in TM, FM, TD and FD significantly increased in SH group from those in IC group, by 4.00%, 3.07%, 1.82% and 2.03%, respectively ($p < 0.001$). The increase of CtBMD in L4 was only 0.79% in SH group, and non-significant difference from that of the IC group. The influence of ORX in P0 group did not affect the CtBMDs in TM and FM compared with those in the SH group. On the other hand, the CtBMDs in L4, TD and FD of P0 group were significantly lower than those of SH group by 2.80% ($p < 0.001$), 0.82% ($p < 0.05$) and 0.90% ($p < 0.001$), respectively. The treatment of P10 did not affect the CtBMDs in TM, FM, L4, TD and FD compared with those in P0 group. Treatment of P100, however, significantly increased the CtBMDs in TM and FM compared with those in P0 group by 1.87% and 1.60%, respectively ($p < 0.01$). Treatment of P1000 also increased the CtBMDs in TM and FM by 2.31% and 1.78% from those in P0 group ($p < 0.001$). The treatment of P100 prevented the decrease of CtBMDs in L4, TD and FD by 63.09% ($p < 0.001$), 24.37% ($p = 0.79$) and 67.56% ($p < 0.01$), respectively. The treatment of

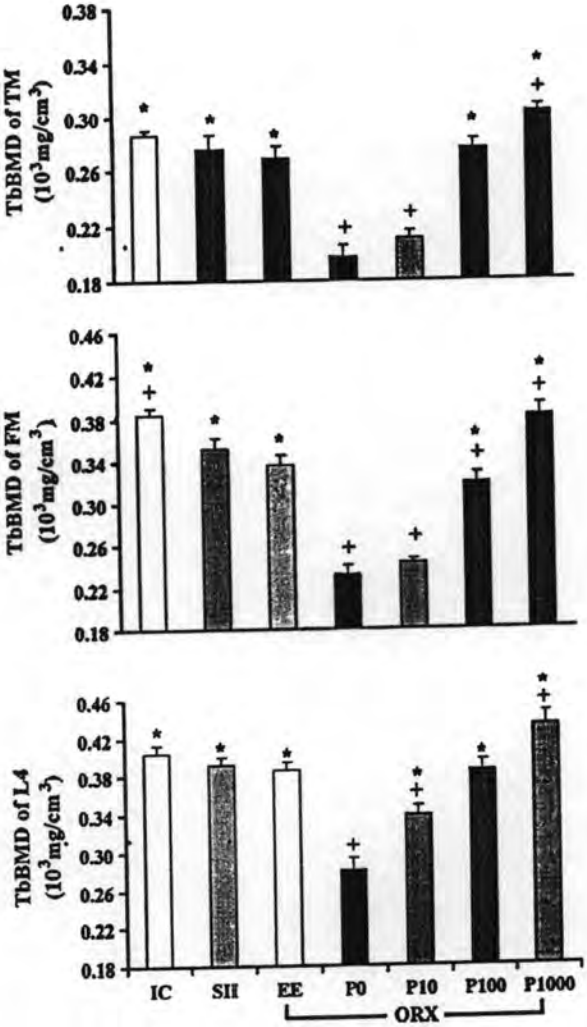

Fig. 1. Trabecular bone mineral densities (TbBMDs) at the proximal tibial metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebral body (L4) in initial control (IC) rats, Sham control (SH) rats and orchidectomized (ORX) rats treated with 0.1 mg/kg B.W./day of 17 alpha-ethinylestradiol (EE), 0, 10, 100 and 1000 mg/kg B.W./day of *Pueraria mirifica* (P0, P10, P100 and P1000, respectively), were determined at day 0 in IC group and at the end of 3-month experimental period in other remaining six groups. Data are mean ± S.E.M. $^{+}p<0.05$ vs. SH; $^{*}p<0.05$ vs. P0.

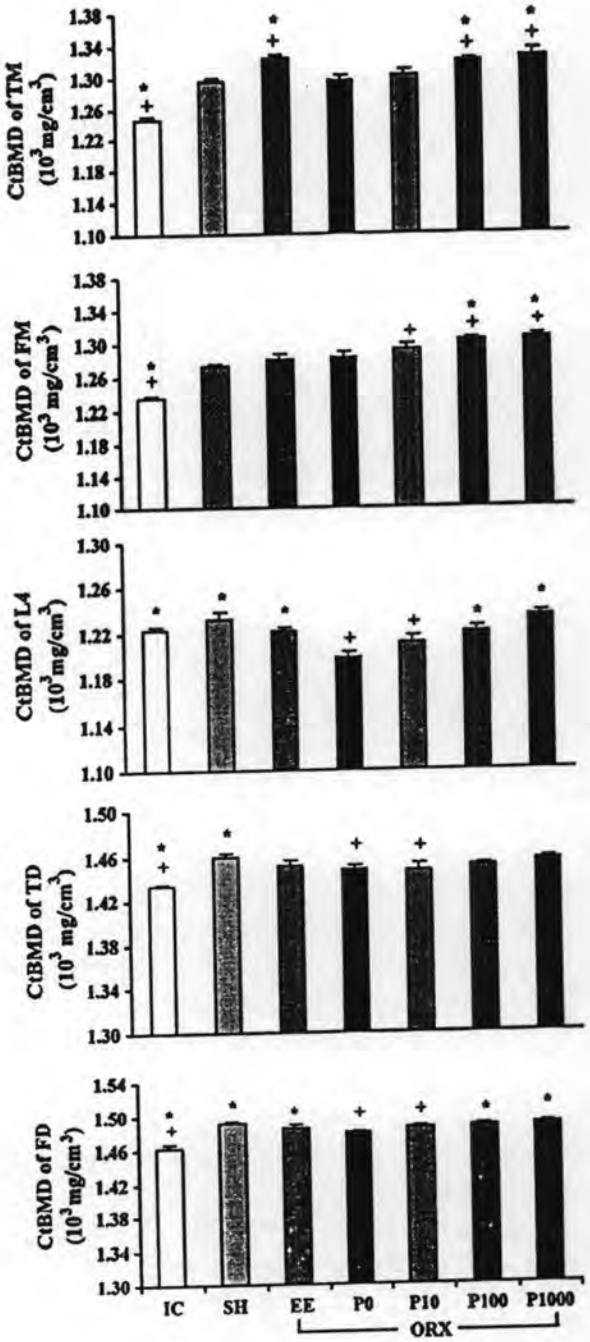

Fig. 2. Cortical bone mineral densities (CtBMDs) at the proximal tibial metaphysis (TM), distal femoral metaphysis (FM), the fourth lumbar vertebral body (L4), tibial diaphysis (TD) and femoral diaphysis (FD) in IC, SH, EE, P0, P10, P100 and P1000 groups (abbreviations for groups were the same as those written in Fig. 1), were determined at day 0 in IC group and at the end of 3-month experimental period in other remaining six groups. Data are mean ± S.E.M. $^{+}p<0.05$ vs. SH; $^{*}p<0.05$ vs. P0.

P1000 prevented the decrease of CtBMDs in L4, TD and FD by 103.24% ($p<0.001$), 64.36% ($p=0.18$) and 80.81% ($p<0.001$), respectively. The EE administration significantly increased the CtBMD in TM by 2.20% ($p<0.001$), but not that in FM from those in P0 group. The EE treatment also prevented the decrease of CtBMDs in L4, TD and FD by 66.36% ($p<0.01$), 35.48% ($p=0.58$) and 55.33% ($p<0.05$), respectively.

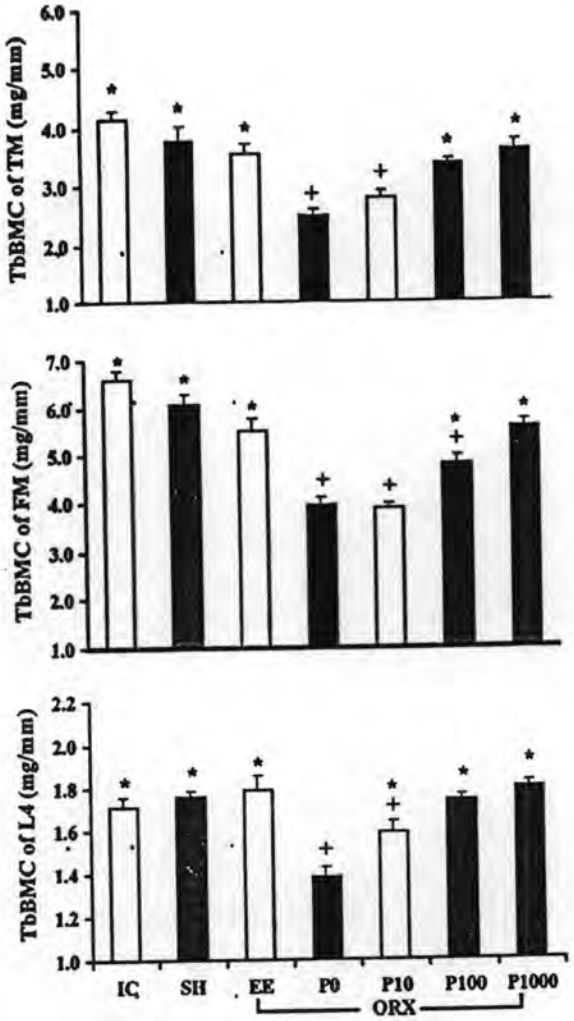

Fig. 3. Trabecular bone mineral contents (TbBMCs) at the proximal tibial metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebral body (L4) in IC, SH, EE, P0, P10, P100 and P1000 groups (abbreviations for groups were the same as those written in Fig. 1), were determined at day 0 in IC group and at the end of 3-month experimental period in other remaining six groups. Data are mean ± S.E.M. $^{+}p<0.05$ vs. SH; $^{*}p<0.05$ vs. P0.

3.3. Bone mineral content

The effects of *P. mirifica* on bone mass in ORX rats were further examined by changes on BMC. The results for trabecular bone mineral contents (TbBMCs) in TM, FM and L4 are shown in Fig. 3. At the end of experimental period, the TbBMCs in TM, FM and L4 slightly changed in SH group from those in IC group ($p>0.05$). The ORX for 3 months in P0 group obviously decreased TbBMCs in TM, FM and L4 by 34.13%, 35.31% and 21.59%, respectively, from those of the SH group ($p<0.001$). The treatment of P10 significantly prevented the decrease of TbBMC in L4 by 55.26% ($p<0.01$), but did not significantly prevent those in the TM and FM. The treatment of P100 significantly prevented the decrease of TbBMCs in TM, FM and L4 by 67.44% ($p<0.001$), 41.12% ($p<0.01$) and 94.74% ($p<0.001$), respectively. The treatment of P1000 highly prevented the decrease of TbBMCs in TM, FM and L4 by 85.27%, 76.17% and 110.53%, respectively ($p<0.001$). Administration of EE highly prevented the decrease of TbBMCs in TM, FM and L4 by 82.17%, 74.30% and 107.89%, respectively ($p<0.001$).

The cortical bone mineral contents (CtBMCs) in all treatment groups are shown in Fig. 4. At the end of 3-month experiment, the CtBMCs in SH group significantly increased in TM, FM, TD and FD, by 11.77%, 17.84%, 11.72% and 11.91%, respectively ($p<0.01$), while that of L4 did neither increase nor decrease from those in IC group. The ORX for 3 months in P0 group significantly decreased CtBMCs in L4, TD and FD in comparison with those in SH group by 25.90%, 11.20% and 11.53%, respectively ($p<0.001$), however, the CtBMCs in TM and FM were not significantly different from those of SH group. The treatment of P10 slightly increased the CtBMCs in all bone sites from those in P0 group ($p>0.05$). The treatment of P100 significantly increased the CtBMCs in TM and FM by 8.09% ($p<0.05$) and 13.32% ($p<0.001$), respectively, from those in P0 group. However, P100 did not significantly prevent the decrease of CtBMDs in TD (28.72%; $p=0.39$) and FD (36.13%; $p=0.09$), respectively. Interestingly, the P100 highly significantly prevented the decrease of CtBMC in L4 by 59.72% ($p<0.01$). The P1000 increased the CtBMCs in TM and FM by 6.95% ($p=0.09$) and 11.55% ($p<0.01$), respectively, from those of P0 group. The P1000 also highly prevented the decrease of CtBMCs in L4, TD and FD by 83.33% ($p<0.001$), 69.09% ($p<0.01$) and 70.97% ($p<0.01$), respectively. Administration of EE significantly increased the CtBMCs in TM and FM by 13.4% and 12.56%, respectively, compared with those of P0 group ($p<0.001$). The EE also significantly prevented the decrease of CtBMCs in L4, TD and FD by 55.56% ($p<0.01$), 79.79% ($p<0.001$) and 45.81% ($p<0.05$), respectively.

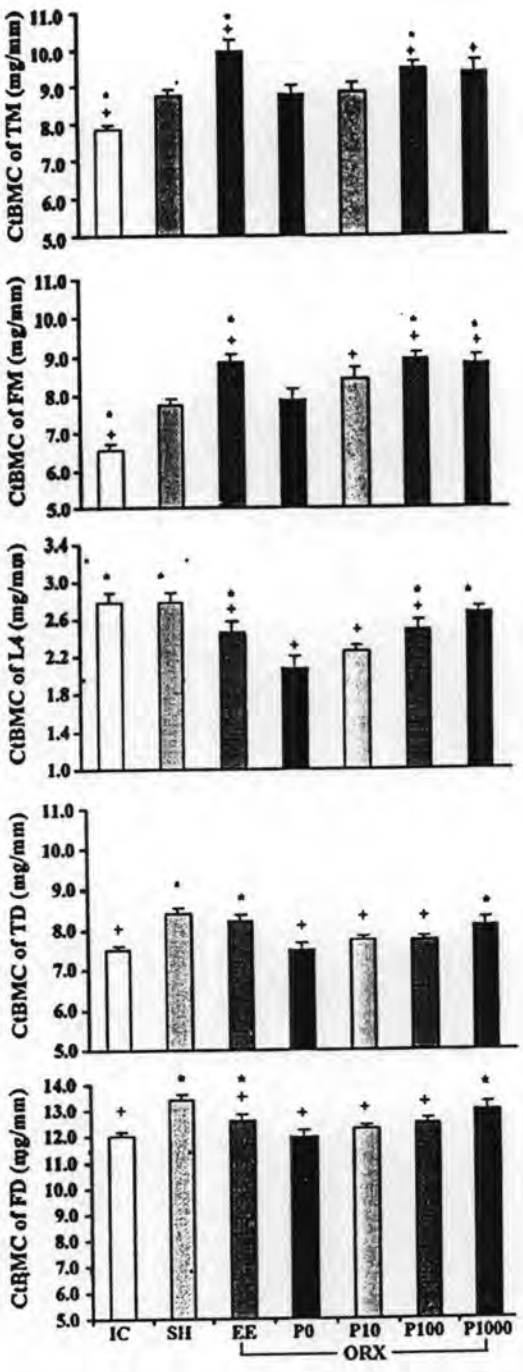

Fig. 4. Cortical bone mineral contents (CtBMCs) at the proximal tibial metaphysis (TM), distal femoral metaphysis (FM), the fourth lumbar vertebral body (L4), tibial diaphysis (TD) and femoral diaphysis (FD) in IC, SH, EE, P0, P10, P1 00 and P1000 groups (abbreviations for groups were the same as those written in Fig. 1), were determined at day 0 in IC group and at the end of 3-month experimental period in other remaining six groups. Data are mean ± S.E.M. $^{+}p<0.05$ vs. SH; $^{*}p<0.05$ vs. P0.

4. Discussion

Elderly men, especially those with hypogonadism, suffered from osteoporosis and increased in considerable number. Estrogen, one of the sources of treatment, has been reported to have side effects on benign prostatic hyperplasia and prostate cancer [12,13]. Therefore, herbs containing phytoestrogens are sought as the alternative medicines. We here report the preventive effects of *P. mirifica*, a herb containing phytoestrogens, on bone loss in ORX rats. Changes in bone with an advancing age and by ORX in male rats were also considered.

The age-related change of bone mineral density and content were examined in the subject rats between the ages of 7–10 months, or in turn, between IC and SH groups. In this study, age change patterns were observed to be specific to bone types (axial bone (fourth lumbar vertebra) and long bone (tibia and femur)), bone sites (metaphysis and diaphysis) and bone compartments (trabecular and cortical). In the cortical compartment of long bone, both at metaphyseal and diaphyseal sites and both of BMD and BMC, were kept increase with an advancing age. On the other hand, in the trabecular compartment of long bone, BMD significantly decreased at the femoral metaphyseal site and slightly decreased in tibial metaphyseal site. Similar age-related bone change was also reported for long bones in rats [31,32]. As expected and agreed with the previous report, there were no changes in BMD and BMC and in trabecular and cortical compartments of the fourth lumbar vertebra in 10-month old rats [31].

After ORX, changes of bone were examined by comparison of the BMD and BMC between SH and P0 groups. Changes of bone after ORX are also considered to be specific to bone types, bone sites as well as bone compartments. The effects of ORX on BMD and BMC in the cortical compartment were much smaller than those in the trabecular compartment. The decreases of trabecular BMD measured at metaphyseal sites in tibia and femur, and lumbar vertebral body ranged 27–34% in the present study. On the other hand, those changes of cortical BMD ranged as small as 0–2.8%. The severe decreases of trabecular BMD in the metaphyseal sites after ORX were similar to the previous reports [33,34]. Venken et al. [34] also reported a diminutive decrease in cortical BMD at the metaphyseal site of femur in ORX rats.

The preventive effect of bone loss by *P. mirifica* in ORX rats was significant and comparable to that by 17 alpha-ethinylestradiol, both in BMD and BMC. The effects appeared to be similar to that of *P. lobata* administered on ORX mice [20]. Although the ORX procedure makes the rats to the sex hormone-deficient stage [3], the direct substance controlling the bone metabolism is seemingly being an estrogen. The preventive effects of bone loss by specific phytoestrogens on cortical part were previously reported in cortical bone culture of female rats, that is, daidzein and genistein-induced increase of calcium content and alkaline phosphatase activity in bone tissues [35].

The effect of *P. mirifica* (10–1000 mg/kg B.W./day) observed in the present study was depended on doses. Its effects ranged from the prevention of decrease of BMD and BMC caused by ORX, the maintenance till the increase, respectively, from the lower to the higher doses. The highest dose of 1000 mg/kg B.W./day did not only completely prevent bone loss, but it also increased the BMD, the increase was higher than those of the Sham control group. The effect of *P. mirifica* is somewhat similar to those of the *P. lobata* effects reported previously [18,20]. The dose of *P. mirifica* for the prevention of bone loss, which is comparable to 0.1 mg/kg B.W./day of 17 alpha-ethinylestradiol, is considered to be 100 mg/kg B.W./day. The effect of *P. mirifica* and the response to the ORX appear to be related to the states of local bone tissues which are reflected by bone changes in an advancing age observed in control rats as mentioned previously. At the local bone tissues, which show either increase or decrease of BMD and BMC during the experimental period, *P. mirifica* feeding enhances the increase or prevents the decrease, respectively. Thus, the lowest effective dose for osteoporotic therapy should be carefully adjusted according to the states of local bone target tissues.

The effect of *P. mirifica* in the prevention of bone loss should depend on quantities and types of phytoestrogens. Base on the high performance liquid chromatography technique analyzed by Malaivijitnond et al. [23] and our team (data not shown), the phytoestrogens found in *P. mirifica* cultivar Wichai-III are puerarin (daidzein-8-c-glucoside), daidzin (daidzein glycoside form), daidzein, genistin (genistein glycoside form) and genistein. Puerarin [36], a major isoflavone found in *P. mirifica* as well as in *P. lobata* [37], daidzin [15], daidzein [17,35], genistin [15] and genistein [19,35] exhibited an anabolic effect on bone metabolism. However, it is probable that other phytoestrogens, which we do not mention them here and found in *P. mirifica* may be attributable in the preventive effects on bone loss. Upon the latest information, at least 13 phytoestrogen substances were analyzed in *P. mirifica* [21,22]. Thus, further study of the effects of other phytoestrogens on bone loss is necessary.

Feeding of *P. mirifica* did not influence weights of male reproductive organs, seminal vesicle and ventral prostate gland, which were greatly decreased by ORX [3,34]. Similar results were reported in ORX mice for *P. lobata* [20] and genistein [19]. These results suggest that use of *P. mirifica* in curing an osteoporosis caused by the hypogonadism in men may have no risk of prostate cancer, whereas the androgen/estrogen administration could stimulate male cancers [12,13]. Additionally, the previous research found that phytoestrogens have an anti-prostate cancer effect [38].

In conclusion, this report clearly showed that the *P. mirifica*, an edible Thai herb, completely inhibits the bone loss, both in long bones and axial bones, in sex hormone-deficient male rats. It also significantly increases the bone density at the higher dose treatment without affecting accessory sex organs. These results suggest that *P. mirifica* may be useful for treatment or prevention of osteoporosis in elderly men with hypogonadism.

Acknowledgments

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Using vaginal cytology to assess the estrogenic activity of phytoestrogen-rich herb

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Abstract

To assess the estrogenic activities of synthetic estrogen, synthetic phytoestrogen, *Pueraria lobata* and three distinct cultivars of *Pueraria mirifica* a phytoestrogen-rich herb, a vaginal cytology assay in ovariectomized rats were used. Rats were ovariectomized and treated with DW, estradiol valerate (1 mg/kg BW), genistein (0.25–2.5 mg/kg BW), *Pueraria lobata* and *Pueraria mirifica* (10–1000 mg/kg BW) for 14 days. The vaginal cytology was checked daily and the uteri were dissected and weighed at the end of treatment or post-treatment periods. The treatments of DW, genistein and *Pueraria lobata* did not influence the vaginal epithelium, but the injection of estradiol valerate induced a vaginal cornification from day-3 of treatment to day-14 of post-treatment period. The occurrence of vaginal cornification after treatment and the recovery after the cessation was dependent on dosages and cultivars of *Pueraria mirifica*. The increments of uterus weight in all rats agreed with the cornification of vaginal epithelium. Although both uterotropic and vaginal cytology assays can be used to assess the estrogenic activity of phytoestrogen-rich herb, however using vaginal cytology assay has two advantages: (1) we do not need to kill the animals and (2) we can follow up the recovery after the cessation of treatment.
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Keywords: *Pueraria mirifica*; *Pueraria lobata*; Phytoestrogens; Vaginal cytology assay; Ovariectomized rats

1. Introduction

Nowadays, it has become popular to use phytoestrogens as an alternative choice for estrogen replacement therapy. The biological potencies of phytoestrogens vary greatly and have a stronger binding affinity to ER-β than ER-α. The majority of the compounds are nonsteroidal structures and vastly less potent than synthetic estrogens (10^{-2} to 10^{-5}) (Kuiper et al., 1997, 1998). Phytoestrogens can be found in various species of plants especially from soy and leguminous herb. The *Pueraria* spp. is one among other genera of an indigenous herb that contained phytoestrogens. The *Pueraria lobata* (Leguminosae), also known as Kadzu, is a leguminous plant and possesses a high content of isoflavonoids such as daidzein and genistein (Wang et al., 2003). As regards to the isoflavonoids contents, *Pueraria lobata* slightly increased the uterus weight in ovariectomized (OVX) rats (Wang et al., 2003, 2004) and remarkably increased the bone mineral density in OVX mice (Wang et al., 2003). However, it had neither proliferation nor anti-proliferation effect on growth of MCF-7 in vitro (Cherdshewasart et al., 2004). It is noteworthy that no reports of its estrogenic activity on the vaginal cytology have been found. Therefore, the estrogenic activity of *Pueraria lobata* was assessed using a vaginal cytology assay in comparison to that of synthetic estrogen and synthetic phytoestrogen in the present study.

The *Pueraria mirifica* (Airy Shaw et. Suvatabandhu (Leguminosae)) is a Thai vine herbal plant, known in Thai as "white kwao krua". It was firstly discovered and identified by Vatn in 1939 as "red kwao krua" (*Butea superba* Roxb.) because of their superficial resemblance. Later, in 1952, it was recognized as a new species and reidentified as *Pueraria mirifica* (Kasemsanta et al., 1952). Its tuberous root and the chemica

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constituents were analyzed by HPLC technique and at least 13 known phytoestrogens were characterized (Chansakaow et al., 2000a,b). As regards to Thai traditional medicine, the consumption of *Pueraria mirifica* has been believed to improve the human physical appearances such as re-growing hair, promoting black hair, improving flexibility of the body and sexual performance, enlarging breast, recovering smooth skin and prolonging life (Kasemsanta et al., 1952). By this view, *Pueraria mirifica* has recently become popular in Thailand, Korea and Japan as age-rejuvenation and anti-menopausal symptom drugs and a breast enlargement cream. Many researches studied the estrogenic activity of *Pueraria mirifica* on reproductive functions and cell growths were conducted afterwards. The *Pueraria mirifica* stimulated the proliferation of vaginal and uterus epithelium in female rats and women (Sukavattana, 1940; Pope et al., 1958; Malaivijitnond et al., 2004). Feeding a suspension of *Pueraria mirifica* suppressed serum gonadotropin levels in gonadectomized rats (Malaivijitnond et al., 2004) and adult female monkeys (Trisomboon et al., 2004a, 2005). The clinical trial of *Pueraria mirifica* consumption in Thai menopausal women showed a decrease in menopausal symptoms such as hot flushes, frustration, sleep disorder and skin dryness (Muangman and Cherdshewasart, 2001). The plant also decreased serum parathyroid hormone and calcium levels in aged menopausal monkeys (Trisomboon et al., 2004b). *Pueraria mirifica* had a dual effect on the growth of MCF-7 human mammary adenocarcinoma cells, stimulated by low doses and suppressed by high doses (Cherdshewasart et al., 2004).

Pueraria mirifica is commonly found in the forests throughout Thailand and 28 cultivars are found at present. But the plants collected in different seasons induced different responses in vaginal cornification (Sukavattana, 1940). In addition, the analysis of phytoestrogen contents of *Pueraria mirifica* by thin layer chromatography (TLC) techniques showed that *Pueraria mirifica* collected in the different locations in Thailand contained the different amounts of phytoestrogens (Panriansaen, 2000). Based on these evidences, the estrogenic efficacy of drugs or cosmetics containing *Pueraria mirifica* can vary lot by lot even though it contains the same amount of plant. This has been considered as one of the problems for manufacturing *Pueraria mirifica* drugs or cosmetics in the large scale to serve the market demand. Thus, a sensitive, simple and inexpensive method to predict the estrogenic activity of *Pueraria mirifica* is needed. The present study aimed to compare the estrogenic activity of three distinct cultivars of *Pueraria mirifica* collected from different locations in Thailand using a vaginal cytology assay.

2. Materials and methods

2.1. Animals

Adult female Wistar rats, 60 and 100 days of age, with regular estrous cycles (4–5 days) for three consecutive cycles before the study period were used in this study. They were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. Five animals per cage were housed in a room with controlled lighting (lights on at 06:00–20:00 h) in which the temperature was maintained at 25 ± 1 °C at the Primate Research Unit, Chulalongkorn University, Bangkok, Thailand. The animals were fed with the rat chow diet (Pokaphan Animal Feed Co. Ltd., Thailand) and water ad libitum. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University.

2.2. Experimental design

2.2.1. Experiment I: comparison of the estrogenic activity of synthetic estrogen, synthetic phytoestrogens and a herb containing phytoestrogens

Fifty adult female rats, 60 days of age and 185–230 g of body weight, were used. When the rats showed the diestrous phase (leukocyte cells) on the fourth estrous cycle, they were ovariectomized under ether anesthesia. The treatment schedule was separated into three periods: pre-treatment, treatment and post-treatment. The duration for each period was 14 days. Five series of experiments were performed. In the first, rats were gavaged daily with 0.7 ml distilled water and kept as a negative control. In the second, rats were subcutaneously injected with 1 mg/kg BW of synthetic estrogen (estradiol valerate; Sigma Chemical Company, Merck, USA) in 200 μl of olive oil, three times a week, and kept as a positive control. In the third, rats were subcutaneously injected with 0.25 and 2.5 mg/kg BWof synthetic phytoestrogen (genistein; Sigma Chemical Company, Merck) in 200 μl of 2% dimethyl sulfoxide (DMSO; Sigma Chemical Company, Merck), three times a week. In the fourth, rats were gavaged daily with *Pueraria lobata* at doses of 10, 100 and 1000 mg/kg BW in 0.7 ml distilled water, respectively. At the end of post-treatment period in these four series, rats were euthanized, the uteri were dissected and weighed, thereafter. In the fifth, rats were treated with distilled water, 1 mg/kg BW of estradiol valerate and 1000 mg/kg BW of *Pueraria lobata* as in the first, second and fourth series, but they were euthanized at the end of treatment period, the uteri were dissected and weighed, thereafter. Vaginal smears were checked daily between 09:00 and 10:00 h in all rats throughout the experimental period. Generally, five rats were used in each group and treatments were performed at 10:00–11:00 h.

2.2.2. Experiment II: comparison of the estrogenic activity of different cultivars of the Pueraria mirifica herb

Fifty adult female rats, 100 days of age and 230–245 g of body weight, were used. When the rats showed the diestrous phase, which was determined by the presence of leukocyte cells, on the fourth estrous cycle, they were ovariectomized under ether anesthesia. The treatment schedule, duration in each period and the number of rats used in Experiment II were the same as in Experiment I. The rats were divided into three main groups and gavaged daily with *Pueraria mirifica* cultivars WichaiIII, Saraburi and Prachuab at doses of 10, 100 and 1000 mg/kg BW in 0.7 ml of distilled water, respectively. The additional negative control group of 0.7 ml of distilled water was also performed. At the end of post-treatment period, all rats were euthanized, the uteri were dissected and weighed, thereafter. Vaginal smears were

checked daily between 09:00 and 10:00 h in all rats throughout the experimental period.

2.3. Vaginal smear checks

Vaginal smears were checked daily between 09:00 and 10:00 h. The vaginal epithelium cells observed under the microscope were classified into three types: leukocyte cells (L), nucleated cells (O) and cornified cells (Co). The representative cell type was determined by choosing the majority of cells. The results of examined vaginal smear cells from five rats in each treatment group were expressed as a mode value (the most frequently occurring cell type in five rats). The appearance of cornified cells (or the majority of Co-cell type) was used as an indicator of estrogenic activity.

2.4. The preparation of Pueraria suspension and feeding

To minimize the seasonal variation of phytoestrogens content in *Pueraria* spp., the tuberous roots of *Pueraria mirifica* (voucher specimen no. BCU 11045; Cherdshewasart et al., 2004) and *Pueraria lobata* (the specimen was identified by Zhang Yam; Cherdshewasart et al., 2004) used in this study were collected at once. The roots of *Pueraria mirifica* cultivar WichaiIII were collected from Chiang Mai province, northernmost Thailand. The constituents of phytoestrogens of WichaiIII cultivar investigated by the HPLC technique were described previously (Malaivijitnond et al., 2004). The roots of *Pueraria mirifica* cultivar Saraburi were collected from Saraburi province, central Thailand. The roots of *Pueraria mirifica* cultivar Prachuab were collected from Prachuabkhirikhan province, upper southern Thailand. The roots of *Pueraria lobata* were collected from Guangzhou province, China (Cherdshewasart et al., 2004). The roots were sliced, dried in a hot air oven at 70 °C and subsequently ground into powder at a size of 100 mesh. The powder was kept in the desiccators until used. The suspensions of *Pueraria* spp. were freshly prepared from the powder and suspended into distilled water. The rats were fed daily with the suspension of *Pueraria* spp. between 10:00 and 11:00 h by gavage.

2.5. Statistical analysis

Results of vaginal smear were expressed as a mode value. Analysis of variance (ANOVA) was used to determine the differences of means of uterus weights. The observed significance was then confirmed using the least significant difference (LSD) test. Significance level was set at $P < 0.05$. The SPSS, a statistical analysis program, Version 10, was used.

3. Results

3.1. Experiment I: comparison of the estrogenic activity of synthetic estrogen, synthetic phytoestrogen and a herb containing phytoestrogens

3.1.1. Vaginal cytology

All rats had only L-type cells throughout the pre-treatment period after ovariectomy. It was confirmed by the fact that the ovaries were completely removed and no endogenous ovarian estrogens were produced. The administration of distilled water, synthetic phytoestrogen (genistein, 0.25 and 2.5 mg/kg BW) and a herb containing phytoestrogens (*Pueraria lobata*, 10, 100 and 1000 mg/kg BW) did not influence the vaginal epithelium, and only L-type cells were found. In contrast, subcutaneous injection of 1 mg/kg BW of synthetic estrogen (estradiol valerate) induced a cornification of the vaginal epithelium as early as the third day of treatment, and kept the Co-type cells until the last day of post-treatment period.

Table 1
Uterus weights at the end of treatment and post-treatment periods in 60-day-old ovariectomized rats treated with distilled water, estradiol valerate, genistein and *Pueraria lobata*

Treatment	Uterus weight (mg)	
	Treatment period	Post-treatment period
Distilled water (negative control)	94.00 ± 3.22	93.00 ± 3.34
Estradiol valerate (positive control)	438.80 ± 2.56***	299.00 ± 3.81***
Genistein		
0.25 mg/kg BW	–	102.20 ± 10.90
2.5 mg/kg BW	–	90.00 ± 1.00
Pueraria lobata		
10 mg/kg BW	–	94.00 ± 4.02
100 mg/kg BW	–	97.80 ± 4.17
1000 mg/kg BW	98.00 ± 2.98	98.00 ± 1.37

*** $P < 0.001$ compared to the mean of negative control group.

3.1.2. Uterus weight

The increment of uterus weights at the end of treatment period in rats treated with distilled water, estradiol valerate and 1000 mg/kg BW of *Pueraria lobata* in the fifth series agreed with changes of vaginal cytology (Table 1). The uterus weight of rats treated with estradiol valerate was highly significantly heavier than those of distilled water and *Pueraria lobata*-treated rats ($P < 0.001$), and the rats kept the high uterus weights until the end of post-treatment period. Even though it was less heavy than the weight at the end of treatment period ($P < 0.022$), it is significantly heavier than those of rats treated with distilled water and 1000 mg/kg BW of *Pueraria lobata* ($P < 0.001$). However, no differences between the uterus weights of rats treated with distilled water and *Pueraria lobata* were found in both the end of treatment and the end of post-treatment periods. Based on these results, the uterus weights of rats in Experiment II were decided to determine only at the end of post-treatment period after follow up of the recovery of vaginal cytology changes in rats was ended.

The uterus weights at the end of post-treatment period of rats treated with genistein and *Pueraria lobata* also agreed with non-changes of vaginal cytology since the uterus weights did not significantly differ from that of negative control (distilled water) group (Table 1).

Table 2
The appearance of cornified cells (Co) and leukocyte cells (L) during treatment and post-treatment periods in ovariectomized rats treated with 10, 100 and 1000 mg/kg BW of three distinct cultivars of *Pueraria mirifica* and distilled water

Dose (mg/kg BW)	Appearance of Co (day of treatment)			Appearance of L (day of post-treatment)		
	Saraburi	WichaiIII	Prachuab	Saraburi	WichaiIII	Prachuab
10	N	N	N	N	N	N
100	N	5	3	N	2	3
1000	4	3	2	3	3	4
Distilled water	N			N		

N: no appearance of Co-cell type with consistent L-cell type.

3.2. *Experiment II: comparison of the estrogenic activity of three cultivars and three doses of Pueraria mirifica*

3.2.1. *Vaginal cytology*

All rats had only L-type cells throughout the pre-treatment period after ovariectomy. The occurrence of vaginal cornification in rats was dependent on doses of *Pueraria mirifica* treatment. The higher dose of *Pueraria mirifica* showed an earlier response and slower recovery on vaginal cornification (Table 2). At dosage of 10 mg/kg BW of all three cultivars, rats did not show any changes on vaginal epithelium, only L-type cells were found. Treatment of 100 and 1000 mg/kg BW, except 100 mg/kg BW of cultivar Saraburi, induced a cornification of the vaginal smear, and a 1000 mg/kg BW produced a greater effect. When the estrogenic activity of *Pueraria mirifica* was compared between cultivars at the same dosage of 100 mg/kg BW, it was found that cultivar Prachub had the estrogenic activity higher than cultivars WichaiIII and Saraburi, respectively, by showing the Co-type cells the earliest and recovering to L-type cells the latest (Fig. 1).

3.2.2. *Uterus weight*

The increment of uterus weight at the end of post-treatment period in all *Pueraria mirifica* groups of rats agreed with changes of vaginal epithelium, that is, the higher doses of *Pueraria mirifica* showed the heavier of uterus weight (Table 3). For instance, the uterus weights of rats treated with 1000 mg/kg BW of *Pueraria mirifica* cultivar Prachuab were heavier than those treated with 100 and 10 mg/kg BW, respectively. The increment of uterus weight also depended on the cultivars of *Pueraria mirifica*. The uterus weights of rats treated with 1000 mg/kg BW of *Pueraria mirifica* cultivar Prachuab were also heavier than those treated with 1000 mg/kg BW of cultivars WichaiIII and Saraburi, respectively.

4. Discussion

Vaginal cytology assay is particularly used to determine the estrogenic activity of the synthetic estrogens (Ashby et al., 2000; Diel et al., 2000; Stroheker et al., 2003; Wuttke et al., 2003), xenoestrogens (Stroheker et al., 2003) and phytoestrogens (Shukla et al., 1987; Diel et al., 2000; Balk et al., 2002; Okazaki et al., 2002; Chiechi et al., 2003; Stroheker et al., 2003; Wang et al., 2003). It has been firstly used by Cook et al. in 1933. It is a sensitive, simple and inexpensive method to predict the estrogenic activity. The assay can be performed in either immature or ovariectomized rodents (Ashby et al., 2000; Stroheker et al., 2003).

The vagina is covered by a mucosa with a Malpighian pluristratified epithelium with marked sensitivity to sex steroids, par-

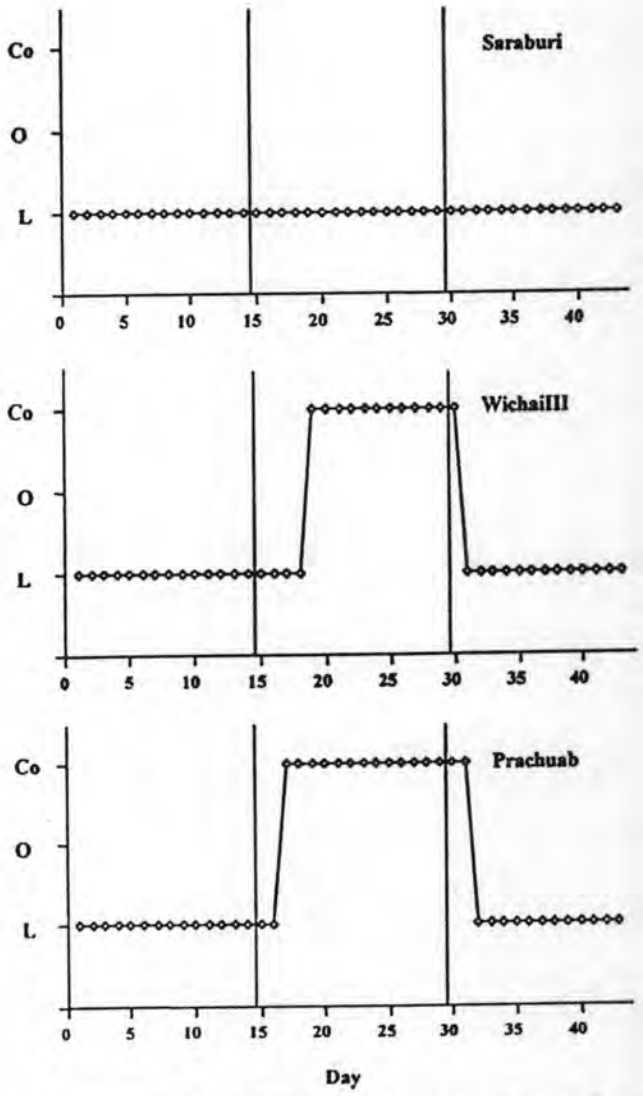

Fig. 1. Comparison of the estrogenic activity of *Pueraria mirifica* at dosag of 100 mg/kg BW between three distinct cultivars: Saraburi, WichaiIII an Prachuab, by vaginal cytology assay in ovariectomized rats. L, O and Co indicat leukocyte cells, nucleated cells and cornified cells, respectively.

Table 3
Uterus weights at the end of post-treatment period in 100-day old ovariectomized rats treated with distilled water and three distinct cultivars of *Pueraria mirifica*

Treatment	Uterus weight (mg)
Distilled water	158.00 ± 3.70
Pueraria mirifica cultivar Saraburi	
10 mg/kg BW	152.00 ± 2.00
100 mg/kg BW	168.00 ± 3.70
1000 mg/kg BW	172.00 ± 2.00*
Pueraria mirifica cultivar Wichai111	
10 mg/kg BW	160.00 ± 5.40
100 mg/kg BW	154.00 ± 4.00
1000 mg/kg BW	178.00 ± 4.80**
Pueraria mirifica cultivar Prachuab	
10 mg/kg BW	168.00 ± 8.60
100 mg/kg BW	188.00 ± 4.80***
1000 mg/kg BW	200.00 ± 5.40***

* $P<0.05$ compared to the mean of distilled water group.
** $P<0.01$ compared to the mean of distilled water group.
*** $P<0.001$ compared to the mean of distilled water group.

ticularly to estrogens. This specific action of estrogens is due to the presence of specific receptors, ER-α and ER-β (Johnson and Everitt, 1995; Kuiper et al., 1997, 1998). In OVX rats, treated for 3 months with estradiol (0.5 mg/day/animal, via food) vaginal cornification was regularly observed, whereas the sham-treated animals have an unestrous vaginal smear (Wuttke et al., 2003). There are many studies about the effects of phytoestrogens on genital tract of women and female animals. Miroestrol produced cornification of the vaginal epithelium in the immature female mice (Jones and Pope, 1960). Dietary supplementation with phytoestrogens led to increased vaginal cytological maturation in women (Wilcox et al., 1990). Six-month treatment of soy-rich diet to the asymptomatic post-menopausal women increased vaginal cornification of epithelium, karyopycnotic index (KI) and maturation value (MV), identical to those found in the hormonal replacement women (Chiechi et al., 2003).

To compare our result with the previous reports, the 60-day-old rats were used for vaginal cornification assay of estradiol valerate, genistein and *Pueraria lobata* (Ashby et al., 2000) and the 100-day-old rats were used for the *Pueraria mirifica* (Malaivijitnond et al., 2004). Additionally, from our previous study (Kijkuokool et al., in press), 2% DMSO did not show the vaginal cornification effect in female rats; thus, we did not perform this vehicle control group in the present study. The dosage of 1 mg/kg BW of estradiol valerate used in our study produced a vaginal cornification in OVX rats with the similar degree as observed in the previous studies injecting 0.022 or 0.2 mg/kg BW/day of estradiol (Ashby et al., 2000; Stroheker et al., 2003). However, the subcutaneous injection of genistein, as high as 2.5 mg/kg BW, in this study did not stimulate the proliferation of vaginal epithelium and uterus in OVX rats. Our results are in agreement with the studies of Stroheker et al. (2003) who treated 100 mg/kg BW/day of genistein by gavage and found that it did not induce an increase in vaginal cornification and uterine weight in OVX rats. The doses of 0.25–2.5 mg/kg BW of genistein might be too low to exhibit the estrogenic effects on reproductive organs. However, the doses of 0.25 and 1.0 mg/kg BW of genistein were reported to be the effective doses for treatment of vasodilation disorder and for stimulation of breast cancer in OVX rats, respectively (Khemapech et al., 2003; Kijkuokool et al., in press). The previous reports also confirmed our study, that is, genistein in the dosage that significantly prevented bone loss in OVX mice did not exhibit the estrogenic action in the uterus (Ishimi et al., 1999, 2000; Wu et al., 2001). Thus, it may be concluded that the low dose of genistein could affect the hypertension and breast cancer without the adverse effect on the endometrium cancer.

We found that the cornification in vaginal smear was dependent on doses and cultivars of *Pueraria mirifica*. Three cultivars of *Pueraria mirifica* showed the different estrogenic activities which are seemingly related to their phytoestrogen contents. In addition, the results of estrogenic activity determined by vaginal cytology assay are in accordance with the increase of uterus weight or the uterotropic assay. From the results, it can be concluded that vaginal cytology assay can be a practical method to assess the estrogenic activity of other cultivars of *Pueraria mirifica* found in Thailand. It is also known that the phytoestrogen content in soy varied considerably (Price and Fenwick, 1985; Reinli and Block, 1996). In two batches of one rat diet (Altromin A and B) produced mainly from soy, an approximately two-fold difference in daidzein and genistein content was found (Degen et al., 2002). Thus, the applicable dosage of *Pueraria mirifica* to human should be adjusted according to the cultivars. In addition to the stimulation of vaginal cornification, returning of Co-type cells to L-type cells after cessation of *Pueraria mirifica* treatment was also dependent on doses; the higher dose prolonged the time for recovery. The recovery of rats on vaginal cytology after receiving the synthetic estrogen, estradiol valerate, is also slower than the feeding of *Pueraria mirifica* containing phytoestrogens. This might be taken as an advantage of using phytoestrogen-rich herb for hormone replacement therapy compared to the synthetic estrogens when the short-term effect is needed.

We found that *Pueraria mirifica* showed the estrogenic activity greater than *Pueraria lobata*, both with uterotropic and vaginal cornification assays. In agreement with our study, OVX mice fed a diet containing *Pueraria lobata* (20% of diet or equivalent to 7.2 mg isoflavone) for 4 weeks did not show changes in uterine weight (Wang et al., 2003), but it could increase the trabecular bone volume. However, although slightly, the consumption of 100 mg/kg BW of *Pueraria lobata* for 5 weeks has been proved to significantly ($P<0.05$) increase the uterus weight in OVX rats (Wang et al., 2004).

5. Conclusion

The present study showed that both the uterotropic and vaginal cytology assays gave the similar results in comparison to estrogenic effects of the estradiol valerate, genistein, *Pueraria lobata* and *Pueraria mirifica* in OVX rats. However, using a vaginal cytology to assess the estrogenic effects has at least two advantages: (1) we do not need to kill the animals, and

therefore, (2) we can follow up the recovery after the cessation of treatment. Thus, vaginal cytology assay could be useful in the assessment of the estrogenic activity of phytoestrogen-rich herb.

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MORPHOMETRIC STUDY ON THE CROSS-SECTION OF DISTAL RADIUS: PREPARATORY WORK FOR BONE TURNOVER STUDY

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Summary

Animal models, especially macaques, have been widely used to correlate in *vivo* bone turnover with age and physiological states. Although X-ray microdensitometry method (MD) is considered to be inferior to the peripheral quantitative computed tomography (pQCT), it is still an advantageous, especially from the viewpoint of practical use. In this study, using male Japanese macaques (age range: 0.3 – 24.4 years) and dry bone specimens, we assessed the morphometric properties distal radius, to establish the reliable parameters of bone turnover by MD with the simultaneous application of pQCT. We found MD sizes, width and cortical thickness, can give such bone turnover parameters of diameter of radius, density, and cortical and trabecular cross-sectional areas.

Introduction

The application and advantages of the micro-density method (MD), using ordinary x-ray film with the penetrometer (Al calibration wedge), have been described by several researches. Presently, pQCT and dual energy x-ray absorptiometry (DEXA), however, are the most widespread methods to determine bone mineral density (BMD). Their high accuracy, low radiation exposure, and the filmless way to obtain densitometry data make them valuable diagnostic and research tools. It is especially true for the measurement of trabecular part of bone, which is the most susceptible part in bone turnover. On the other hand, plain x-ray radiography is still an advantageous tool in both routine clinical practice and research, since its high resolution, general availability, relatively low cost and shorter time exposure[1]. The reliability and validity of radiographic absorptiometry compared to DEXA readings were significant[2]. The MD has been applied to metacarpal bone because its mid-shaft consists almost only of cortical bone. However, taking the morphological parameters into consideration, the density profile obtained from MD is considered to afford useful parameters for bone turnover. In the present study, we analyzed morphometrical properties of cross-section of radius at 4% from distal end,

and elucidate such parameters related with the bone turnover, such as density, diameter, cortical thickness, and trabecular and cortical cross-sectional areas.

Materials and Methods

Subjects and dry bone specimens: We used 23 living male Japanese macaques (Macaca fuscata) with various ages (range: 0.33 – 24.38 years), which are reared at the PRI, Kyoto University. We also used 20 dry bone specimens of Japanese macaques obtained from PRI.

X-rays and pQCT: X-ray photos at the distal part of forearm with the aluminum penetrometer (1-20 mm thickness with 1 mm step height increments). They were taken using an ordinary X-ray apparatus (Hitachi, Co. ltd.), with the exposure condition of 60 KV, 20mA, and 0.1 to 0.3 seconds, the tube-film distance of 110 cm, and ordinary medical film (RX, Fuji Film, Co. ltd.). The film was digitized at 200 dpi using scanner. The densities at the target sites on the radius, 4% and 30% from distal end, and on penetrometer with software (Scion Image, Scion corp., Frederick, MA). The bone density at every pixel was converted to the Al thickness. From the density profile, we obtained average density, diameter and cortical thickness at left and right margins. We applied pQCT (Norland Stratec Co. ltd., XCT-Research) to the same subjects and bone specimens, with software version 5.40, and density was calculated by the threshold protocol of half-maximum height[3]. We also measured from the cross-sectional image, the diameter, height, thickness at left (ulnar side), right, upper (dorsal), and lower parts. Fundamental statistical analyses were applied to extract valuable parameters obtained from MD.

Results

At first we examined graphically the age change of average density by MD and found two phases, one is increasing phase from infantile to about 7 years of age, and another is level after 7 years. The simple Pearson correlation was calculated between densities obtained by the two methods. The average MD density at 4% from distal radius bone specimens of male macaque monkey were significantly correlated with pQCT total BMD ($r=0.44$, $p<0.05$) and pQCT trabecular BMD ($r=0.57$, $p<0.01$) but it was not correlated with pQCT cortical.

We statistically examined morphological parameters obtained from pQCT. The height/diameter proportion increases from infant to about 5 years of age, and then is kept level (average=75.27%). The cortical thickness at four sites are highly correlated with each other, but the thickness at the right side (radial) increased in individuals older than 10

years. The dimensions (diameter and cortical thickness) obtained from MD and pQCT are almost identical with each other, and the trabecular and cortical cross-sectional areas can be estimated from small number of MD morphological parameters. Similar morphometric analysis was made on the site at 30% of radius, where there is substantially no trabecular bone.

Discussion

The accurate measurement of quantity and quality of bone are the most important necessity for the bone turnover research. The MD (micro-density) method, although it is convenient method as it does not need such special apparatuses as pQCT or DXA but ordinary X-ray apparatus and scanner; it is considered to be short of accuracy to follow the course of bone turnover. It is especially true in the quantity of trabecular bones, and it has been applied to the mid-haft of metacarpal because the shaft is almost exclusively composed of cortical bone. Invariant shape of cross-section of bone is, if there were, considered to improve the bone turnover parameters obtained from MD. MD affords the density profile, which is in turn translated into average density, width, and cortical thickness at radial and ulnar sides of radial cross-section. By the application of pQCT, the cross-sectional morphometric parameters were obtained in the present study. They were, then, used to calculate the coefficients with which cross-sectional areas of cortical and trabecular parts can be obtained separately from width and cortical thickness.

By the application of these morphometric coefficients, and with the combination of measurements and parameters obtained at the sites of 4% and 30% of distal radius, minute bone turnover will be detected by MD in aged macaques.

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