Miroestrol Improves the Expression of Genes Involved in Osteoporosis and Bile Salt Transportation in the β-Naphthoflavone-Treated Mouse Liver

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**Abstract:** Background: Miroestrol, a potent phytoestrogen isolated from the tuberous roots of \textit{Pueraria candollei} var. \textit{mirifica}, exhibits estrogenic activity and has long been used as a Thai traditional medicine for rejuvenation. Objective: To investigate the influence of miroestrol on the regulation of osteoporosis, a worldwide public health problem, and bile salt transportation-related genes was examined as an additional factor in the risk/benefit assessment of the use of miroestrol as an alternative estrogen replacement therapy. Results: Miroestrol at a dose of 0.5 mg/kg/day elevated the expression of OPG mRNA while suppressing the RANKL levels, increasing the OPG/RANKL ratio and consequently inhibiting the progression of osteoporosis at the molecular level. Miroestrol additionally improved the ratio of OPG/RANKL in the livers of β-naphthoflavone-induced mice. In addition to ameliorating osteoclastogenesis, the 0.5 mg/kg/day dose of miroestrol decreased the extent of β-naphthoflavone-induced MRP2 expression without affecting the level of BSEP mRNA in the mouse livers. Conclusion: This is the first report suggesting that miroestrol may be a promising candidate for alternative estrogen replacement therapy due to the beneficial effects on the osteoporosis and bile salt transportation regulatory pathways.

**Keywords:** BSEP, miroestrol, MRP2, OPG, osteoporosis, RANKL.

**INTRODUCTION**

\textit{Pueraria candollei} var. \textit{mirifica} has long been used as a Thai traditional medicine for rejuvenation. The active compounds in the tuberous roots of this plant are isoflavonoids such as puerarin, daidzin, genistin, miroestrol (MR), and deoxymiroestrol. Among these compounds, MR shows high estrogen-like potential [1]. The estrogenic activity of MR was first investigated in rats, with MR showing a mammogenic effect in female rats [2]. MR exhibited estrogenic activity that was approximately 0.25 times as strong as the activity of estradiol in a cornification assay [3]. Our previous study noted that MR possesses various biological activities similar to the activity of estradiol. MR increased the weight and volume of mouse uteri, in addition to altering hepatic cytochrome P450s, which are drug-metabolizing enzymes [4]. MR significantly modified the expression of genes involved in the sex hormone synthesis pathway [5]. Namely, MR suppressed the expression of 3β-HSD, 17β-HSD1, CYP17, and CYP19 mRNA, while 17β-HSD2 mRNA was induced to levels similar to those induced by estradiol. In addition, MR showed antioxidant potential by decreasing the levels of thiobarbituric acid substances produced during the lipid peroxidation process in the mouse brain [4]. The other biological activities of MR, namely its impact on the regulation of osteoporosis and bile salt transportation related genes, are of clear interest for the use of MR as an alternative to estrogen.

Estrogen levels impact many maladies. One of these maladies is osteoporosis, which is currently a global public health problem [6]. The progressive changes in bone structure, quality, and density that occur in osteoporosis lead to pathological fractures and increase the morbidity and mortality of menopausal women. Because some reports have noted the risk of some cancers, such as breast, endometrial, and ovarian cancers, associated with the use of synthetic estrogen replacement therapy, phytoestrogens have become popular in many countries as hormone replacement therapy [7-8]. Women have become interested in phytotherapy and consider this approach an alternative to estrogen replacement therapy for the prevention and treatment of osteoporosis [9].
OGP is an osteoclastogenesis inhibitory factor that is also known as tumor necrosis factor receptor superfamily member 11B (TNFRSF11B) and is a protein encoded by the TNFRSF11B gene in humans [10]. OGP is also a decoy receptor for receptor activator of nuclear factor kappa B ligand (RANKL). The level of OGP influences voltage-dependent calcium channels and reduces the production of osteoclasts by inhibiting the differentiation of osteoclast precursors. Osteoclasts are related to monocytes/macrophages and are derived from granulocyte/macrophage-forming colony units (CFUGM), which in turn regulate the resorption of osteoclasts either in vitro or in vivo. OGP binds to RANKL on osteoblast/stromal cells, blocking the RANKL-RANK ligand interaction between osteoblast/stromal cells and osteoclast precursors. As a result, the differentiation of the osteoclast precursor into a mature osteoclast is inhibited [11]. MR is an interesting phytoestrogen to investigate because of its impact on the expression of OPG and RANKL.

In addition to regulating osteoporosis genes, estrogen has been shown to regulate bile [12]. Bile is a vital secretion that is essential for intestinal digestion and lipid absorption. Moreover, bile is an important route of elimination for environmental toxins, carcinogens, drugs, and their metabolites. In addition, bile represents a major route of excretion for endogenous compounds and metabolic products such as cholesterol, bilirubin, and hormones [13]. Canalicular bile accounts for approximately 75% of the daily bile production in humans and is modified by secretory and absorptive processes throughout the bile ductules and ducts. The canalicular membrane contains a bile salt export pump (BSEP) for monovalent bile salts and a conjugate export pump (MRP2) for divalent bile salts and various other amphipathic conjugates [14]. Both BSEP and MRP2 belong to the ABC superfamily. MRP2 is the major driving force for bile salt-independent bile flow, while BSEP drives bile salt-dependent flow [15]. 17a-ethynylestradiol (EE2) reportedly induced hepatotoxicity by altering bile acid secretion-related genes in the liver [12]. Because MR possesses estrogen-like potential, the impact of MR on the regulation of bile salt transportation-related genes is important.

The present study supports the potential of MR as a phytoestrogen candidate for estrogen replacement therapy, as MR exhibits a lower risk of osteoporosis and hepatotoxicity than estradiol.

MATERIALS AND METHODS

Chemicals

MR was isolated from tuberous roots of P. candollei var. mirifica as described previously [1] and identified in comparison with the authentic standard of MR provided by Dr. Chaiyo Chaichantipyuth, Chulalongkorn University, Bangkok, Thailand. Estradiol benzoate (E2) and β-naphthoflavone (BNF) were purchased from Schering (Kenilworth, NJ, USA) and Sigma-Aldrich® (Saint Louis, MO, USA), respectively. TRIZOL® and dNTP mixture were supplied by Invitrogen® (Carlsbad, CA, USA). RevertAce® and Illustra® Hot Start Master Mix were products of Toyobo®, Osaka, Japan, and GE Healthcare, Bucks, UK, respectively. Random primers and RNase inhibitor were obtained from Takara Bio Inc. (Shiga, Japan). Novel Juice was a product of GeneDirex® (Las Vegas, Nevada, USA). The forward and reverse primers and the product size of the genes were as follows: (1) RANKL, 5'-AGC GTC GCC CTG TTC TTC TAT TT -3' / 5'-ACT TGG GAT TTT GAT TTC TTC TTC TAT TT -3', 441 bp; (2) OPG, 5'-GAA CCC AGA GGG AAA TAC A-3' / 5'-CGC TGT TTT CAC AGA GGT CA-3', 444 bp; (3) BSEP, 5'-CAC ACA AAG CCC GCG AAA TAC A-3' / 5'-CGC TGT TTT CAC AGA GGT CA-3', 441 bp; (4) MRP2, 5'-GCA CTG TAG GCT CTG CTA CCA GT -3'/ 5'-CCA GAG GCA GCT ATC AGG AC-3', 231 bp; (4) MRP2, 5'-GCA CTG TAG GCT CTG GGA AG-3'/ 5'-CAT TTC CAA GAC TGG GAG GA-3', 224 bp; and (5) GAPDH, 5'-TCC ACT CAC GGC AAA TTC AAC G-3' / 5'-TAG ACT CCA CGA CAT ACT CAG C-3', 145 bp. All others chemicals were of the highest available purity from commercial suppliers.

Animals

Six-week-old female ICR mice were supplied by the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand and housed in an animal care unit of the Northeast Laboratory Animal Center (NELAC), Khon Kaen University, Khon Kaen, Thailand. At all times, the mice were housed on wood chip bedding in polycarbonate cages with water and commercial mouse diet supplied ad libitum. The mice were acclimated to a controlled temperature (23 ± 2 °C) for at least 7 days before dosing. The animal handling and the treatment protocols were approved by the Animal Ethics Committee for the care and use of animals at Khon Kaen University, Khon Kaen, Thailand (Approval No. AEKKU18/2555). The protocols were carried out under supervision of a NELAC-certified veterinarian. The mice were subcutaneously administered E2 at a dose of 0.5 mg/kg/day daily for 7 days or MR at doses of 0.5
or 5 mg/kg/day daily for 7 days. A 30 mg/kg/day dose of BNF was injected intraperitoneally once a day for the last 3 days. The control group received a subcutaneous administration of corn oil (0.1 ml) daily for 7 days. The mice were sacrificed 24 h after the last treatment. The liver was immediately excised to isolate total RNA as described elsewhere [16].

Expression of RANKL, OPG, MRP2, and BSEP mRNAs

The mouse RANKL, OPG, MRP2, BSEP, and GAPDH mRNAs were semi-quantified by RT-PCR. Hepatic total RNA was reverse-transcribed using ReverTra Ace® reverse transcriptase, after which the cDNA was amplified under the conditions recommended by the supplier of Illustra® Hot Start Master Mix. The PCR conditions were the same as the conditions described previously [17-18] with some modifications. After the separation of the PCR products by 2% agarose gel electrophoresis, the target cDNAs were detected under ultraviolet illumination in the presence of Novel Juice (GeneDirex®), a non-mutagenic fluorescent reagent, and semi-quantified with the Syngene gel documentation system (Ingenius L, Cambridge, UK) and the GeneTools match program.

Statistical Analysis

The results were analyzed by a one-way analysis of variance (ANOVA) followed by an LSD post hoc test (SPSS ver. 17.0). p values ≤0.05 were considered statistically significant.

Table 1: Body Weight Profiles of the Mice During the Treatment Period

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight (Grams)</th>
<th>% Body Weight</th>
<th>% Increment of Body Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/kg/day)</td>
<td>0  1  3  5  7</td>
<td></td>
</tr>
<tr>
<td>Non-treatment</td>
<td>39.3 ± 1.5</td>
<td>39.8 ± 1.0</td>
<td>40.8 ± 2.1</td>
</tr>
<tr>
<td>β-naphthoflavone (BNF)</td>
<td>30</td>
<td>39.3 ± 3.0</td>
<td>40.0 ± 3.7</td>
</tr>
<tr>
<td>Miroestrol (MR0.5)</td>
<td>0.5</td>
<td>38.8 ± 1.3</td>
<td>40.0 ± 2.0</td>
</tr>
<tr>
<td>Miroestrol (MR5)</td>
<td>5</td>
<td>39.3 ± 1.3</td>
<td>39.8 ± 1.7</td>
</tr>
<tr>
<td>Estradiol benzoate (E2)</td>
<td>0.5</td>
<td>40.8 ± 0.5</td>
<td>41.3 ± 1.3</td>
</tr>
<tr>
<td>BNF + E2</td>
<td></td>
<td>40.8 ± 2.1</td>
<td>41.8 ± 2.2</td>
</tr>
<tr>
<td>BNF + MR0.5</td>
<td></td>
<td>38.8 ± 1.7</td>
<td>39.3 ± 1.7</td>
</tr>
<tr>
<td>BNF + MR5</td>
<td></td>
<td>38.3 ± 1.0</td>
<td>39.3 ± 1.0</td>
</tr>
</tbody>
</table>

Note: * Body weights are present as the mean ± SD (n=5); The % body weight compares the body weights on the day before treatment (day 0) to the body weights on the last day of the treatment (day 7). Total percentage of body weight increment.

RESULTS

Effect of MR on Mouse Body Weight Profiles

The body weight profiles of the mice were analyzed and determined to be unchanged throughout the 7-day treatment period (Table 1). This observation revealed that MR was as safe as corn oil and E2 for short-term treatment.

Effect of MR on the Expression of Hepatic OPG and RANKL mRNA

The mice were treated with MR or E2 to evaluate the influence of MR on osteoclastogenesis through the regulation of hepatic OPG and RANKL expression. In addition, to determine whether MR and E2 modulated osteoclastogenesis factors such as OPG and RANKL in a state of CYP1A1 induction, the mice were co-treated with the typical CYP1A1 inducer BNF and MR or E2. Then, the OPG-to-RANKL ratio, which is a critical factor commonly used for the evaluation of bone formation, was determined. Higher OPG-to-RANKL ratios indicate a higher possibility of bone formation [19]. BNF or E2 alone markedly suppressed the expression of OPG mRNA (Figure 1A). The 5 mg/kg/day dose of MR significantly decreased the level of OPG expression compared to E2, whereas the lower dose (0.5 mg/kg/day) of MR elevated the OPG level. Co-treating the mice with MR and BNF potentiated the increased OPG expression. The expression of RANKL mRNA was significantly suppressed by both the single MR or E2 treatments and the MR or E2 plus BNF treatments (Figure 1B). MR at the dose of 0.5...
mg/kg/day for 7 days significantly increased the OPG-to-RANKL ratio, while the other treatments decreased this ratio (Figure 1C). Interestingly, in the presence of BNF, MR significantly improved the OPG-to-RANKL ratio, whereas this ratio did not increase significantly after co-treatment with E2 and BNF. These observations support the potential of MR in estrogen replacement therapy, as the results show the positive impact of MR on the osteoclastogenesis pathway.

Effect of MR on the Expression of MRP2 and BSEP mRNA

To examine whether MR altered bile salt transportation, we determined the expression of MRP2 and BSEP mRNA in the livers of mice treated with either a single dose of MR or the combination of MR and BNF; these results were then compared with the E2 results. E2 and MR did not significantly change the expression of MRP2 mRNA, but BNF significantly
increased the level of MRP2 mRNA (Figure 2A). Interestingly, the low dose of MR (0.5 mg/kg/day) significantly decreased the BNF-induced MRP2 level, whereas E2 and the high dose of MR (5 mg/kg/day) did not change the expression of this gene. BNF significantly up-regulated the expression of hepatic BSEP mRNA, while E2 and MR did not (Figure 2B). Neither the co-administration of BNF and E2 nor the low dose of MR altered BSEP expression. Co-treatment with the high dose of MR and BNF increased the level of BSEP. These findings indicated an advantage for the optimal dose of MR in lowering the risk of hepatotoxicity associated with bile secretion.

DISCUSSION

Phytoestrogens are plant-derived xenoestrogens that can function as the primary female sex hormone after being consumed [20]. Conflicting studies make it unclear whether the phytoestrogens used in hormone replacement therapy (HRT) have risks or benefits to human health. For example, a Cochrane review of the use of phytoestrogens to relieve the vasomotor symptoms of menopause demonstrated that there was no evidence to suggest any benefit to their use [21]. Epidemiological studies have shown that phytoestrogen has a protective effect against breast cancer [22]. HRT may also be effective at reversing the effects of aging on muscle [23]. *In vitro* studies have concluded that females with current or past breast cancer should be aware of the risk of tumor growth when taking soy products, as these products could stimulate the growth of estrogen receptor-positive cells *in vitro* [24]. Because of these questions, the extensive studies on phytoestrogens have aimed to identify the molecular mechanisms of this therapy that work specifically in selected tissues. *P. candollei* var. *mirifica* is a well-known plant that is traditionally used for estrogen-like effects in Thailand [25]. Many articles have shown that *P. candollei* var. *mirifica* possesses a number of biological activities, such as the prevention of bone loss [26], dose-dependent estrogenic effects [7], and antioxidant activity [27]. MR, a potent phytoestrogen present in the tuberous root of *P. candollei* var. *mirifica*, has demonstrated estrogenic-
like effects and anti-lipid peroxidation effects [4]. Because no studies have investigated the impact of MR on the osteoclastogenesis and bile salt transportation pathways, the present study attempted to evaluate the effects of MR on the expression of genes involved in the osteoclastogenesis and bile salt transportation pathways to unravel the risks and benefits of the use of MR as an alternative to E2.

In this study, the effect of MR on the osteoclastogenesis pathway was investigated by determining the expression of OPG and RANKL mRNA. We found that MR increased the OPG-to-RANKL ratio in a dose-dependent manner. The different effects of the two evaluated doses might be explained by the dose-dependent action of E2; for example, a dose of 0.3 mg E2/day has been recommended for postmenopausal symptoms [28], while 1-2 mg E2/day [28] and 2-3 mg E2/dl [29] doses were required to offset atrophic urethritis and the estrogen-related risk in low-density lipoprotein cholesterol, respectively. On the contrary, BNF, an aryl hydrocarbon receptor (AhR) agonist, affected the expression of OPG and RANKL mRNA differently than MR did. These observations might have been caused by the anti-estrogen effects of BNF. Crosstalk between AhR and the estrogen receptor (ER) has been observed [30,31]. Activated AhR inhibits ER activity through a number of different mechanisms, whereas ERα has been reported to have a positive role in AhR signaling. AhR affects the secretory processes and epithelial proliferation of the mouse uterus by regulating ER expression or activation [32]. Meanwhile, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds were found to have anti-estrogenic activity, the potency of which paralleled their affinities for AhR [33]. These findings support the finding that *P. candollei* var. *mirifica* prevented bone loss in both orchietomized and ovariectomized rats by changing the bone mineral density and bone mineral content [26,34]. A very recent study demonstrated that the extract of *P. candollei* var. *mirifica* and its isoflavone, puerarin, likely enhanced bone formation by up-regulating ALP mRNA expression and increasing the OPG-to-RANKL ratio, thereby promoting osteoblast differentiation [35]. Moreover, there are some article mentions about people who living with chronic liver disease have an additional reason to be concerned about osteoporosis [36,37]. Although the causative reason for this connection remains unclear, improving liver health has been observed to positively affect bone health. For this reason, expression of OPG and RANKL might be factors related with liver injury by BNF. Our results are similar to results found in a clinical trial of conjugated estrogens (0.625 mg/day), which demonstrated a dose-dependent increase in bone density when peri-menopausal women took E2 tablets with doses ranging from 0.3 to 1.2 mg/day [28]. Over 95% of the mouse genome is similar to human [38]. In addition, a close correlation between the mouse strain and incidence of mammary tumor regarding elevation of estradiol 16α-hydroxylation, namely a high incidence of tumor was found in the RIII and C3H strains with a low incidence in the C57BL/6, has been reported [39]. Other forms and routes of E2 administration appeared to give similar results in terms of bone density [40]. These findings suggest that MR probably promotes the prevention of osteoporosis by improving the OPG-to-RANKL ratio of the osteoclastogenesis pathway.

In addition to the impact of MR on bone formation, we also identified an impact on the expression of BSEP and MRP2, two major transporters in the canalicular bile salt export pump. We used E2 rather than the known hepatotoxic compound ethinyl estradiol (EE2) as the reference compound in the present study because E2 has been frequently recommended for HRT [41], while EE2 is commonly used as a contraceptive [42]. The observation that E2 did not change the levels BSEP and MRP2 mRNA might be explained by the short duration of E2 consumption in the present study, which only lasted one week. The effect of E2 on the bile salt export transporter-related genes might not have been missed if the contraceptive estrogen had been used. Because BNF is a CYP1A1 inducer that metabolizes procarcinogens into the active carcinogens in the liver and induces hepatotoxicity [43], we hypothesized that the expression of MRP2 and BSEP might be elevated by BNF treatment. Corresponding to our study, BNF significantly increased the levels of both MRP2 and BSEP. Interestingly, the 0.5 mg/kg/day dose of MR lowered the level of BNF-induced MRP2 expression in the female mouse liver. The MR-mediated suppression of MRP2 expression correlated with the results of a previous study, which found that EE2 suppressed bile acid secretion and ATP-dependent taurocholate transportation in the hepatic canalicular membrane [12]. These observations suggest that MR exerted a hepatoprotective action by suppressing the expression of MRP2 mRNA.

**CONCLUSION**

This is the first study to demonstrate the bimodal actions of 0.5 mg of MR/kg/day, which prevented bone
loss by increasing the OPG-to-RANKL ratio and exhibited hepatoprotective potential by decreasing the expression of MRP2 mRNA, a bile salt transporter, in the female mouse liver. Thus, MR or an MR-containing health product with an optimal titrated dose represents a potential candidate for use in alternative estrogen replacement therapy because this compound exhibits beneficial molecular effects on the osteoclastogenesis and bile salt transportation pathways.

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