PHYTOTHERAPY RESEARCH
Phytother. Res. **26**: 1864–1871 (2012)
Published online 15 March 2012 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.4669

Acute Effect of High-dose Isoflavones from *Pueraria lobata* (Willd.) Ohwi on Lipid and Bone Metabolism in Ovariectomized Mice

Hee Joon Cho,[1] Hee-jin Jun,[1] Ji Hae Lee,[1] Yaoyao Jia,[1] Minh Hien Hoang,[1] Jae-Hoon Shim,[2] Kwan-Hwa Park[3] and Sung-Joon Lee[1]*

[1]Division of Food Bioscience and Technology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Korea
[2]Department of Food and Nutrition, Hallym University, College of Natural Sciences, Chuncheon, Gangwon 200-702, Korea
[3]Department of Foodservice Management and Nutrition, Sangmyuung University, Seoul 110-743, Republic of Korea

We investigated the acute metabolic effects of isoflavones from *Pueraria lobata* (Willd.) Ohwi (IPL) in ovariectomized (OVX) mice. After 4 weeks of IPL feeding at 500 mg/day/kg body weight (OVX500), plasma 17β-estradiol concentrations were significantly higher (+25%, $p < 0.05$), whereas plasma triglyceride levels were significantly lower in OVX mice (−15%, $p < 0.05$) compared with controls. Abdominal adipose tissue weight was marginally reduced in IPL-fed groups compared with OVX controls and the plasma levels of liver enzymes were unchanged. In addition, IPL significantly inhibited the reduction of bone mineral density in the femurs of OVX mice (OVX200, +22%; OVX500, +26%; $p < 0.05$) compared with controls after 4 weeks of IPL feeding. In quantitative polymerase chain reaction analysis the expression of aromatase was significantly suppressed and SULT1E1 was increased by IPL feeding, showing that IPL feeding may not alter the risk for breast cancer in mice. Our results suggest that IPL could ameliorate menopausal symptoms in mice. Further studies will confirm the effects of IPL in humans. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: puerarin; *Pueraria lobata* (Willd.) Ohwi; lipid metabolism; bone; ovariectomy.

INTRODUCTION

Menopause is often associated with serious health problems in middle-aged women; thus, appropriate care and management of health after menopause is key to maintaining a woman's quality of life (Pinkerton *et al*., 2010). A reduction in gonadal estrogens is widely believed to cause psychological and mood changes, as well as physiological changes, that result in symptoms such as osteoporosis, breast cancer, hot flashes, obesity, hyperlipidemia, and cardiovascular disease (Greendale and Sowers, 1997). All of these menopausal changes can be at least partly reversed by the local or systemic administration of exogenous estrogens; thus, hormone replacement therapy (HRT) has been used to ameliorate menopausal symptoms (Ishizuka *et al*., 2008). However, HRT increases the risk of several serious diseases, such as breast cancer (Martin *et al*., 2009), thrombosis (Rachon and Teede, 2008), gallbladder disease, heart attack (Stevenson *et al*., 2009), and stroke (Billeci *et al*., 2008); therefore, alternative approaches, including dietary interventions, have been of interest in the control of menopausal symptoms.

Ovariectomy causes a lack of estrogen (Weerachayaphorn *et al*., 2011) and increases body weight and white adipose tissue in rodents (Babaei *et al*., 2010); estrogen reverses these changes. Because similar changes have been found in postmenopausal women after the intake of phytoestrogen isoflavones, there has been considerable interest in their effects on women's health. These biological effects are most likely regulated through changes in gene expression levels.

Phytoestrogens are naturally occurring hormone-like compounds that are structurally similar to 17β-estradiol, sharing a unique nonsteroidal structure with two phenolic ring systems that carry phenolic hydroxyl groups. Due to the structural similarity to the human sex hormone 17β-estradiol, phytoestrogens can bind to estrogen receptors and act as estrogen agonists and antagonists by competing for estradiol at the endoplasmic reticulum (ER) complexes. Isoflavones, which are diphenolic flavonoids, are the most abundant phytoestrogens in human diets and the most studied in both animals and humans. Isoflavones from various plants have various biological activities and can improve metabolic symptoms caused by menopause (Lof and Weiderplass, 2009). Isoflavones, especially those derived from soy, may ameliorate menopausal symptoms with hypolipidemic (Taku *et al*., 2007) and bone-promoting effects (Ma *et al*., 2008).

Meta-analyses of randomized controlled trials have suggested that soy isoflavone intervention significantly attenuates bone loss in the spine, markedly decreases urinary deoxypyridinoline (a bone resorption marker) and increases serum bone-specific alkaline phosphatase (a bone formation marker) in menopausal women. Studies in postmenopausal women have shown similar results. However, no significant effect of isoflavone on bone mass density or biomarkers of bone metabolism has been reported in studies using soy protein

* Correspondence to: Sung-Joon Lee, Division of Food Bioscience and Technology, College of Life Sciences and Biotechnology, Korea University, Seoul 136–713, Korea.
E-mail: junelee@korea.ac.kr

Received 11 October 2011
Revised 13 February 2012
Accepted 15 February 2012

supplementation with isoflavone-poor soy protein as a control. These data indicate that soy protein may interfere with the effect of isoflavone by masking or antagonizing its effect. Although increasing data, especially those from more recent studies, tend to support a positive role of soy intake in the prevention of bone loss, especially on the biomarkers of bone metabolism, in postmenopausal women, more human trials are needed to verify this action. Currently, there is no existing health claim regarding the effect of soy intake on bone health.

Although some studies have suggested that soy isoflavones have potential health benefits in postmenopausal women (Shedd-Wise *et al*., 2011), a significant number of studies have also reported the absence of beneficial effects of soy intake on classic metabolic parameters (Bahr *et al*., 2005), such as body weight, serum lipid profiles, fat mass and blood glucose and insulin profiles. The nutritional committee of the American Heart Association has assessed 22 randomized trials published since 1999. The results showed that the consumption of soy protein containing isoflavones lowered low-density lipoprotein (LDL) cholesterol levels by 3% in comparison with the consumption of milk and other proteins. However, no significant effect on high-density lipoprotein (HDL) cholesterol, triglycerides (TG) or lipoprotein(a) levels was observed. The underlying causes of conflicting results are probably related to variability in experimental designs and study protocols (administration route, composition, dose and duration), the capacity of individuals to produce equol, an active form of daidzein metabolite, and genetic susceptibility. A meta-analysis of 25 trials published between 1966 and 2004 indicated that soy phytoestrogens did not improve hot flashes or other menopausal symptoms (Krebs *et al*., 2004). The intake of soy supplements for the treatment of menopausal symptoms in patients with early-stage breast cancer had no significant effect on menopausal symptom scores or quality of life, compared with the intake of a placebo. Therefore, we currently lack consistent evidence to support any beneficial effect of soy intake on menopausal symptoms. Thus, it is necessary to investigate the effects of phytoestrogens other than soy isoflavones.

Pueraria radix is one of the most popular drugs in Asian countries. Its root, *Pueraria lobata* (Willd.) Ohwi, is generally known as 'kudzu root' and has been used medicinally for more than 5000 years in East Asian countries, including Korea and China. It contains a unique main isoflavone called puerarin (daidzein 8-*C*-glucoside), which shows potent antioxidant and hypolipidemic activities (Chung *et al*., 2008). Many studies have shown various biological activities, such as hypocholesterolemic, antioxidative, hypoglycaemic (Hsu *et al*., 2002; Chung *et al*., 2008) and hypopigmentation effects. *Pueraria lobata* also contains genistein and daidzein, which are common isoflavones in soybeans (Guerra *et al*., 2000).

We tested the effects of isoflavones from *P. lobata* (IPL), which contains the unique isoflavone puerarin, on menopausal symptoms. Puerarin has shown relatively strong estrogenic activities compared with other isoflavones; thus, we hypothesized that puerarin-containing isoflavones may have a significant protective effect against menopausal symptoms, which often include dyslipidemia and osteoporosis. Puerarin may also increase bone formation. Puerarin is commonly consumed as a health supplement in dishes and soups in Asian meals. Therefore, if puerarin can be shown to increase bone in an animal model of bone defect healing, it may be the long-sought-after safe and ideal agent for bone induction and bone defect repair. For this reason, we investigated the effects of IPL on menopausal symptoms. We fed ovariectomized (OVX) mice IPL orally for 4 weeks and then assessed the metabolic parameters related to postmenopausal symptoms.

MATERIALS AND METHODS

Preparation of puerarin. We obtained *P. lobata* (Willd.) Ohwi from the National Academy of Agricultural Science. The IPL was extracted from the dried powder of *P. lobata* with 60% aqueous ethanol by stirring for 24 h at room temperature. The mixture was then centrifuged at 6000 rpm for 30 min at 25 °C. Supernatants were filtered with Whatman No. 1 filter paper (Whatman Inc., Piscataway, NJ, USA), evaporated by a rotary vacuum evaporator (EYELA, Tokyo, Japan), and then freeze-dried into a concentrate that was used for the experiment.

Compositional analysis of IPL by high-performance liquid chromatography. Dried IPL samples were dissolved in 60% aqueous ethanol and filtered using a 0.45 μm syringe filter (Alltech Associates Inc., Deerfield, IL, USA) prior to analysis by high-performance liquid chromatography (HPLC; Waters Associated Inc., Bannockburn Milford, MA, USA). Twenty microlitres of dissolved sample were injected into the HPLC unit. A 4 mm Novakpak C_{18}-column (Waters Associated Inc., Bannockburn Milford, MA, USA) (150 × 3.99 mm ID) protected by a Phenomenex Security Guard C_{18} guard column (4 × 3.0 mm ID) (Phenomenex, Torrance, CA, USA) was used. The mobile phase was 0.1% (v/v) formic acid (A) – 100% acetonitrile (B) filtered through a 0.45 μm filter and degassed prior to use. Separation was achieved with gradient elution using 0.1% formic acid as a solvent. The gradient was reduced by 90% from 0 to 10 min, 75% from 10 to 15 min and 50% from 15 to 20 min, and was increased by 90% from 20 to 28 min to equilibrate the column. The flow rate was set at 1.0 mL/min and samples were detected at 254 nm.

Animals, ovariectomy, and feeding. Twenty female imprinting control region (ICR) mice, aged 30 weeks, were purchased from Samtako Korea (Osan, Korea). Upon arrival, the mice were acclimated for 2 weeks and maintained on regular food (rodent food, 4.5% fat; Purina Laboratories, Framingham, MA, USA) and water *ad libitum* in a specific pathogen-free system at room temperature with a 12-h light/dark cycle. For surgery, mice were placed under anaesthesia with avertin (2,2,2–tribromoethanol). Ovariectomy was performed through two dorsolateral incisions, approximately 1 cm long, located above the ovaries. Using dissection scissors, the skin was cut and the dorsal muscle and peritoneal membrane were accessed. The connection between the fallopian tube and the uterine

horn was cut and the ovary was removed. The incision was then sutured. Three single catgut stitches were placed in the skin.

We created four feeding groups: a sham-operated group and three OVX groups, including OVXc (control), OVX200 (200 mg/kg/day IPL, extracts) and OVX500 (500 mg/kg/day IPL, extracts). Sham and OVXc groups were fed isocaloric cornstarch in distilled water once daily for 4 weeks. The total calories of the samples were adjusted by adding cornstarch. Oral administration was performed at 1100 hours, and mice were observed for clinical symptoms, visible health condition and mortality twice daily before and after administration. The study design was constructed according to the guidelines provided by the Korean Food and Drug Administration. Because we investigated the acute effect of IPL, we used non-toxic but extremely high doses in the experiment. We previously reported that a 250 mg/kg/day dose of puerarin isoflavone was safe, and that a 500 mg/kg/day dose produced no critical sign of toxicity in rats (Chung *et al.*, 2009). All animal experiments were performed according to protocols approved by the Korea University Animal Experiment and Ethics Committee (animal protocol number: KUIACUC-20090421-2).

Blood analysis. Blood samples were collected retro-orbitally and with cardiac puncture at 0 and 4 weeks, respectively, under anaesthesia prior to sacrifice. The liver, uterus and adipose tissue were removed and weighed, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. Blood samples were centrifuged to collect plasma, which was stored at −80 °C until analysis. Plasma 17β-estradiol levels were measured using an estradiol enzyme linked immunosorbent assay (ELISA) kit (Cayman, Ann Arbor, MI, USA), for which the detection limit was 5 pg/nL, according to the manufacturer's protocol. Plasma TG, HDL and LDL cholesterol concentrations, and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, were determined by enzymatic assays using a Cobas C111 automatic analyser (Roche Diagnostics, Basel, Switzerland).

Isolation of total RNA and quantitative real-time polymerase chain reaction. Total RNA was extracted from mouse livers using TRIzol according to the manufacturer's protocol and suspended in RNase-free water. For cDNA synthesis, 2 μg of total RNA was reverse-transcribed by Superscript reverse transcriptase using a combination of oligo$(dT)_{15}$ primers and dNTPs mixture. Then, real-time polymerase chain reaction (RT-PCR) analysis was performed using 9 μL of SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 8 μL of sterile water, 1 μL of cDNA and 1 μL of each primer (10 pmol). The RT-PCR was performed using the aromatase and estrogen-preferring sulphotransferase family 1E (SULT1E1) templates according to the following protocol: one cycle of 3 min at 95 °C, followed by 60 cycles of 20 s at 95 °C, 20 s at 56 °C and 1 min at 72 °C. A final extension was conducted for 1 min at 95 °C and 1 min at 55 °C. Following amplification, a melting curve of amplified DNA was analysed at temperatures of 60–95 °C. All RT-PCRs were performed using an iCycler iQ5 (Bio-Rad). Data were collected and analysed using iCycler iQ5 optical system software (ver. 2.0; Bio-Rad). The following primers were used: for aromatase, F, 5`-CGG AAG AAT GCA CAG GCT CGA G and R, 5`-CGA TGT ACT TCC CAG CAC AGC; for SULT1E1, F, 5`-TGG ACA AAC GGT TCA CCA AA and R, 5`-GCC TTG CCA AGA ACA TTT CAA.

Microcomputed tomography analysis. After sacrifice, mouse femurs were harvested and stored at −80 °C until analysis. To measure the bone mineral density (BMD) of the femurs, a standardized cone-beam microcomputed tomography (μCT) scan of the left limb was performed using a high-resolution (18.1 μm pixel size) *in vivo* X-ray μCT system for small animal imaging (Skyscan 1076; Skyscan, Aartselaar, Belgium). After standardized reconstruction, the data sets for each bone were resampled using Ant: 3D-creator (version 2.2e; Skyscan) so that the medial axis of the bone was oriented centrally. The same thresholds were used for all samples. Thereafter, two- and three-dimensional bone parameter analyses were performed on a 1-cm-long femoral bone segment using CTAn (version 1.9.2.5; Skyscan), and three-dimensional femur images were acquired by CTVol (version 1.11.1.2; Skyscan).

Statistics. All group comparisons were made using Student's *t*-test. Results are represented as means ± standard errors of the mean (SEM), unless otherwise noted. $p < 0.05$ was considered statistically significant.

RESULTS

Compositional analysis of IPL

The HPLC chromatogram of isoflavones is shown in Fig. 1A and the puerarin, daidzin and genistein concentrations in the *P. lobata* extract are listed in Table 1. After purification, puerarin (7.5%) was the major compound in the extract, which also contained daidzin (4.2%) and genistin (1.9%). In addition, small amounts of glucose (1.0%) and maltose (2.5%) were found. The extract of *P. lobata* contained approximately 11% oligosaccharides and approximately 72% of other starches. The IPL for oral administration was calculated based on its isoflavone content.

Effect of puerarin on plasma 17β-estradiol levels

We analysed plasma estradiol levels in sham and OVX mice. Before IPL feeding, plasma estradiol levels were significantly lower (by 23–25%) in OVX groups compared with the sham group ($p < 0.05$; Fig. 1B). Feeding for 4 weeks did not alter the estradiol levels of sham or OVXc mice, but the levels were increased in both OVX200 and OVX500 mice. The estradiol levels in the OVX groups were significantly higher than in the OVXc group ($p < 0.05$) and comparable to that in the sham group (Fig. 1C).

Plasma lipid levels in sham and OVX mice

Body weights of the mice did not differ among groups throughout the study (22.1, 21.8, 22.0 and 22.4 g at 4 weeks in the sham, OVXc, OVX200, and OVX500

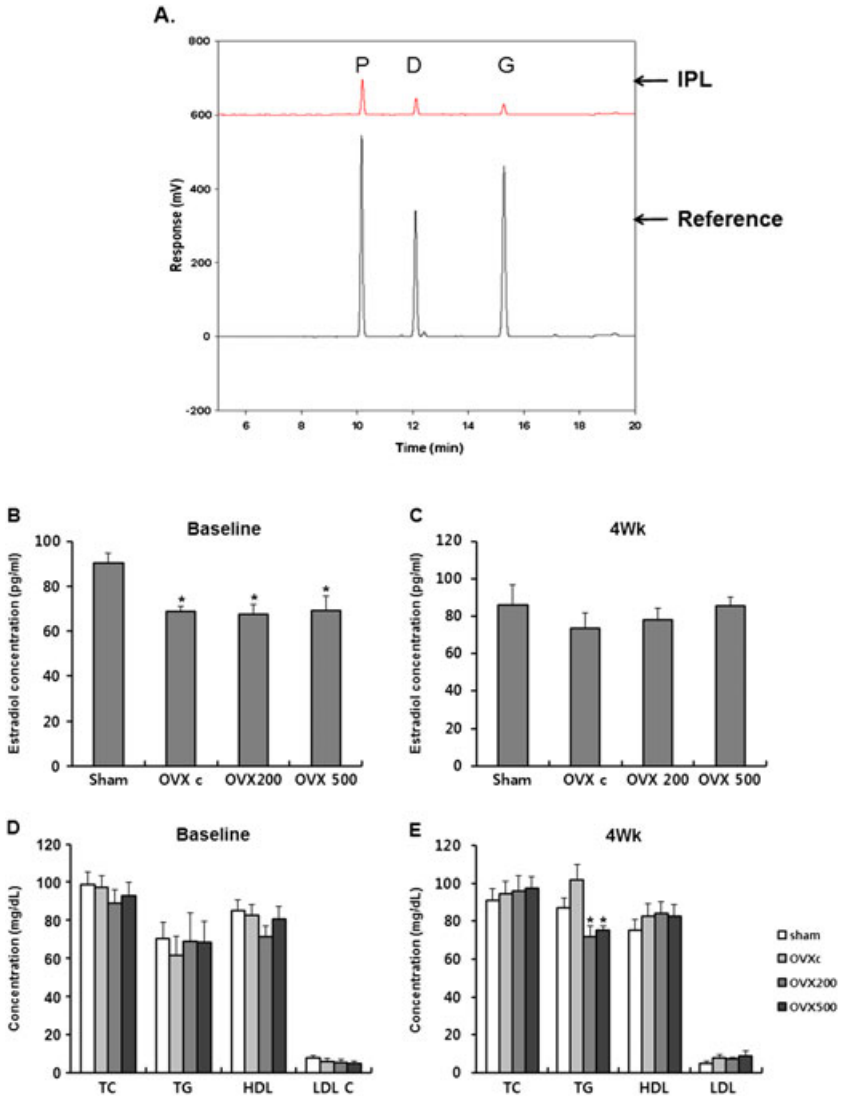

Figure 1. Isoflavones from *Pueraria lobata* (Willd.) Ohwi and their effect on plasma 17β-estradiol and on plasma lipid concentrations in sham-operated and ovariectomized (OVX) mice. (A) Preparative high-performance liquid chromatography of isoflavones from *P. lobata* (Willd.) Ohwi. P, puerarin; D, daidzin; G, genistin; IPL, isoflavones from *P. lobata* (Willd.) Ohwi. (B and C) Effect of isoflavones from *P.lobata* (Willd.) Ohwi on plasma 17β-estradiol concentration in sham-operated and ovariectomized (OVX) mice at (B) baseline and after (C) 4 weeks. (D and E) Effect of isoflavones from *P. lobata* (Willd.) Ohwi on plasma lipid levels in sham-operated and ovariectomized (OVX) mice at (D) baseline and after (E) 4 weeks. TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein. $*p < 0.05$ between OVX and sham groups; $\dagger p < 0.05$ between OVXc and OVX500 groups.

Table 1. Chemical composition of puerarin and other isoflavones from *Pueraria lobata* (Willd.) Ohwi extract

Isoflavone	Concentration (%, w/w)
Puerarin	7.5
Daidzin	4.2
Genistin	1.9
Total isoflavones	13.6

groups, respectively). Total cholesterol (TC), HDL and LDL cholesterol, and TG concentrations were quantified at baseline and after 4 weeks of IPL feeding. Plasma TC, TG, and HDL and LDL cholesterol levels were similar in the sham and OVX groups at baseline (Fig. 1D). The IPL feeding did not change TC, HDL, or LDL cholesterol levels but significantly reduced TG levels in the OVX200 and OVX500 groups compared with the OVXc group (−18% and −15%, respectively; $p < 0.05$; Fig. 1E). The TG level of the sham group was lower than that of the OVXc group.

Organ weights and plasma ALT and AST levels

The weights of the liver, uterus and abdominal adipose tissue were not affected by 4 weeks of IPL feeding, except for a slight increase in liver weight in the OVX200 group (Fig. 2A). The uterus weighed less in the OVXc group than in the sham group; IPL feeding in the OVX groups did not change uterine weight (Fig. 2B). Abdominal fat weights of OVX200 and OVX500 mice did not differ significantly from that of the OVXc group after feeding (OVX200, −8%; OVX500, −11%; $p > 0.1$; Fig. 2C). The plasma concentrations of two liver enzymes, AST and ALT, were quantified. The ALT and AST levels did not differ significantly among groups; thus, IPL did not significantly affect ALT or AST levels (Fig. 2D and E). These

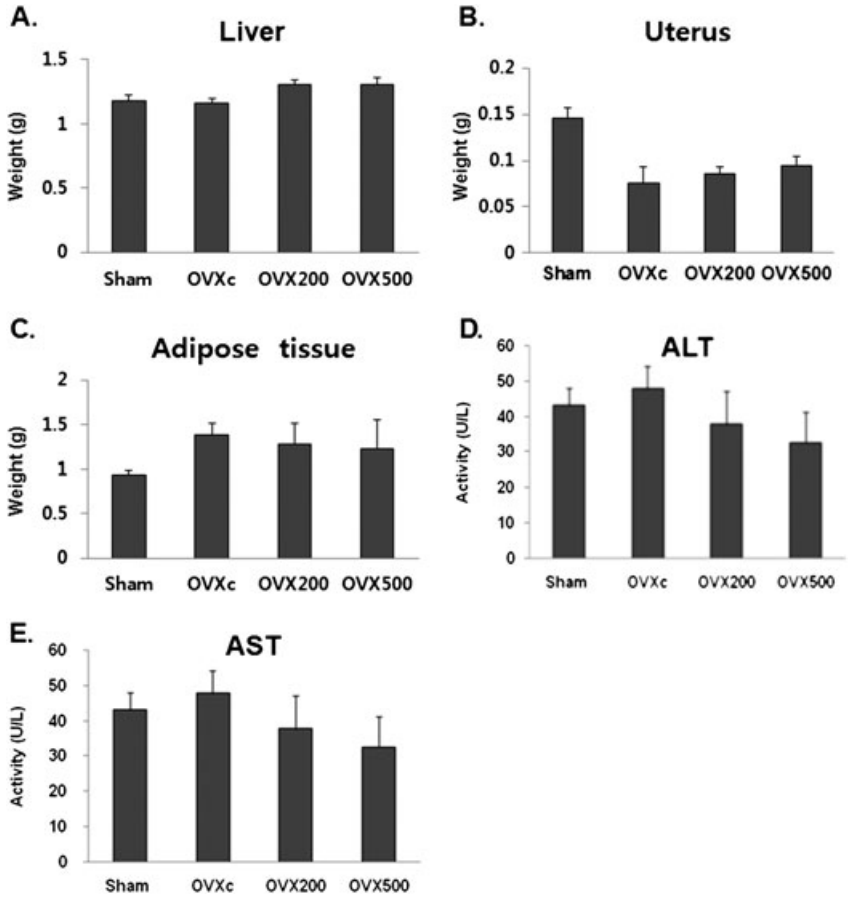

Figure 2. Organ weights and plasma liver enzyme levels after feeding with isoflavones from *Pueraria lobata* (Willd.) Ohwi (IPL) in sham-operated and ovariectomized (OVX) mice. Weights of the (A) liver, (B) uterus and (C) abdominal adipose tissue after oral administration of IPL for 4 weeks. (D) Alanine aminotransferase (ALT) concentrations. (E) Aspartate aminotransferase (AST) concentrations. The plasma samples were collected by cardiac puncture, and liver enzyme levels were analysed using a Cobas C111 automatic analyser according to the enzymatic method.

data suggest that 4 weeks of IPL feeding may not cause significant organ toxicity.

Aromatase and SULT1E1 gene expression

The aromatase enzyme is responsible for a key step in the biosynthesis of estrogens. Because a high level of plasma estrogens is a risk factor for cancer in postmenopausal women, the inhibition of aromatase protein activity or its gene expression is a potential target for breast cancer prevention (Dediu *et al.*, 2009). The results of quantitative RT-PCR indicated that IPL feeding reduced aromatase expression in the mouse liver. The reduction was significant in the OVX500 group compared with the OVXc group (−27%; $p < 0.05$; Fig. 3A). SULT1E1 is an estrogen-preferring sulphotransferase family 1E gene, whose protein desulphonates to produce biologically active estrogen from inactive sulphonated estradiol (Pasqualini, 2009). The quantitative RT-PCR results showed that the expression of SULT1E was markedly reduced after ovariectomy in the OVXc group, but that its concentrations increased in a dose-dependent manner after IPL feeding in the OVX200 and OVX500 groups (Fig. 3B). These findings suggest that IPL could increase active estradiol levels, at least in part, through the induction of SULT1E1 gene expression.

Femur BMD

We obtained three-dimensional images of the femurs using the μCT analyser (Skyscan 1076; Fig. 3C), then calculated BMD using CTAn software. The BMD of OVXc mice was significantly lower than that of the sham group. Interestingly, the BMDs of OVX200 and OVX500 mice were significantly higher than that of the OVXc group (OVX200, +22%; OVX500, +26%; $p < 0.0001$; Fig. 3D) and reached the level of that in sham mice.

DISCUSSION

Kudzu root has been used as a traditional medicine in East Asian countries such as Korea, China and Japan for thousands of years. It contains various isoflavones, including puerarin, genistein and daidzein, which may be responsible for its bioactivity. The major isoflavone in kudzu root, puerarin, has a structure in which a glucose moiety is linked via a *C*-glucosidic bond to the isoflavone core; in contrast, soy isoflavones, including genistein and daidzein, are *O*-linked glycosides. This feature of puerarin provides structural stability against digestive enzymes; puerarin thus remains a water-soluble glycoside, whereas other isoflavones are converted to relatively insoluble aglycons during intestinal uptake. It has been suggested that the high solubility

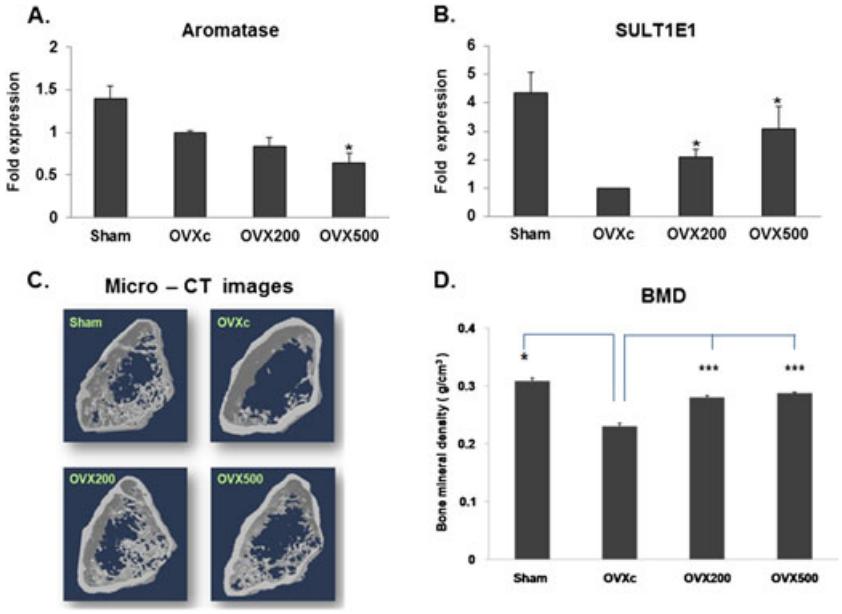

Figure 3. Effect of isoflavones from *Pueraria lobata* (Willd.) Ohwi on the gene expression of aromatase and estrogen-preferring sulphotransferase family 1E (SULT1E1) in the liver and femoral bone mineral density (BMD). (A) Gene expression of aromatase. (B) Gene expression of SULT1E1. Total RNA was isolated from the liver tissue of sham-operated and ovariectomized (OVX) mice. cDNAs were synthesized by reverse transcriptase and analysed by quantitative real-time PCR using the SYBR green method. (C) Three-dimensional images of trabecular femoral bone were obtained by micro-CT (Skyscan 1076; Skyscan, Aartselaar, Belgium). Images were constructed using CTVol (ver. 1.11.1.2; Skyscan). (D) The BMD levels of trabecular femoral bone were analysed by micro-CT. $*p < 0.05$; $***p < 0.0001$. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

of puerarin in the circulation is associated with its potent biological activities *in vivo*, compared with soy isoflavones.

In the present study we found that IPL (14.5%, w/w) induced multiple metabolic changes in a mouse model of postmenopausal symptoms. Four weeks of feeding led to a significant increase in plasma 17β-estradiol levels in the OVX500 group, approaching levels similar to that of the sham group. It also significantly reduced plasma TG concentrations. Notably, IPL feeding did not lead to organ toxicity; no change was observed in liver and uterus weights or plasma AST and ALT levels. Moreover, hepatic aromatase expression decreased while SULT1E1 expression increased, both of which may contribute to appropriate estrogen synthesis and activity in plasma. Finally, IPL feeding significantly improved femur BMD, which could prevent the development of osteoporosis in postmenopausal women.

Menopause is the last physiological menstrual period in a woman's life. It occurs due to the ageing of the ovaries resulting from the exhaustion of ovarian follicles (Wise *et al*., 1996), causing infertility and a progressive loss of hormonal activity (Greendale and Sowers, 1997). The loss of ovarian estrogen synthesis is the critical physiological event responsible for the outcomes of menopause, which include so-called estrogen-deficiency symptoms, such as hot flashes, increased sweating, insomnia, depression, and dyspareunia, as well as long-term health impacts (Yasui *et al*., 2003). These hormonal changes are associated with the development of several chronic metabolic conditions. The prevalence of hyperlipidemia and the risk of cardiovascular disease are increased among postmenopausal women, but isoflavones may reduce plasma lipid levels, including TG concentrations (Taku *et al*., 2007). We found that 4 weeks of IPL feeding reduced plasma TG levels, but had no significant effect on plasma TC, HDL or LDL cholesterol levels. Isoflavones may reduce plasma cholesterol by promoting bile acid excretion from the liver and inducing HMG-CoA reductase expression (Chung *et al*., 2008); however, such effects were not observed in ovariectomized mice in the present study. At present, we do not know the causes for this contradictory finding; however, long-term feeding may be required to confer a hypocholesterolaemic effect in OVX mice. In humans and rodents, the metabolic turnover rate of LDL particles, the primary cholesterol-carrying lipoproteins in plasma, is much slower than that of TG-rich lipoproteins such as very-low-density lipoprotein (VLDL). A human lipoprotein kinetics study has shown that plasma LDL concentrations were much higher in postmenopausal than in premenopausal women due to a reduced fractional catabolic rate of LDL-apoB with no difference in production rate (Matthan *et al*., 2005).

Isoflavones are generally accepted as safe compounds (Reiter *et al*., 2009), although some data suggest potential safety issues. For example, daidzein may induce the formation of micronuclei in cultured Chinese hamster V79 lung fibroblasts (Kulling and Metzler, 1997), and both genistein and daidzein may increase the incidence of DNA strand breaks in human sperm and peripheral lymphocytes (Anderson *et al*., 1997). However, we recently found that IPL shows no mutagenicity in the Ames test with or without metabolic activation, causes no micronucleus formation, and shows no biochemical or pathological evidence of toxicity in rats at a dose of 250 mg/kg/day (Chung *et al*., 2009). In fact, it showed potential antimutagenic effects against nitroquinolin-1-oxide treatment (Chung *et al*., 2009). The levels of daidzin, genistin and the 7-*O*-glycosides of daidzein and genistein are low in IPL; the concentration of puerarin is the highest among isoflavones. Moreover, IPL caused no apparent liver toxicity, based on the slightly increased liver weights and slightly lower plasma AST and ALT levels in treated mice. Several studies,

including a report by El-Demerdash *et al.* (2003), have shown that isoflavone administration reduced ALT and AST activities in *in vivo* models, and IPL might have a similar effect.

Quantitative RT-PCR analysis showed that IPL affected the mRNA expression of aromatase and SULT1E1 in the livers of sham and OVX mice. Aromatase is a cytochrome P450 haemoprotein that biosynthesizes estrogen by converting androgens into estrogens (Pasqualini, 2009). The converted estrogens may promote the proliferation of breast cancer cells. The high expression of aromatase may be associated with breast cancer development; thus, aromatase is a drug target in the treatment of hormone-dependent breast cancer. Our results showing the inhibitory activity of IPL on aromatase gene expression may suggest a protective role of IPL against breast cancer, in line with reports on the activity of soy isoflavone (Dediu *et al.*, 2009).

Two classes of enzymes modulate the activity of estrogen protein in plasma: estrogen sulphatase and sulphotransferase. In plasma, the majority of estrogen is in a relatively inactive sulphated form, and estrogen sulphatase converts inactive estrogen sulphate to active estrogen; estrogen sulphotransferase mediates the reverse reaction. The SULT1E1 enzyme of the estrogen-preferring sulphotransferase family 1E is a key plasma estrogen sulhotransferase that determines the activity of estrogen. Because SULT1E1 reduces active estrogen levels, increasing its expression may help to control plasma estrogen activity, thereby protecting against breast cancer.

In general, the cellular flavonoid uptake mechanism is largely unknown. However, data have suggested the involvement of protein transporters. The role of bilitranslocase in cellular flavonoid transport was shown using common flavonoids such as quercetin; however, isoflavones have not been studied and their transport mechanism remains unknown. It has been shown that genistein and daidzein, isoflavone glycosides, are converted into their aglycon for cellular uptake, although others have suggested that isoflavone glycosides are also taken up by the cells efficiently. There is still a debate regarding this issue. The cellular uptake mechanism of puerarin has not been investigated intensively, but we believe that it may be taken up by the same transporters as genistein and daidzein.

Osteoporosis is a systemic skeletal disease characterized by low bone density and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. The risk for osteoporosis is markedly increased among postmenopausal women (Compston, 2005). Meta-analyses of randomized controlled trials have suggested that soy isoflavone intervention significantly attenuates bone loss in the spine (Ma *et al.*, 2008), markedly decreases deoxypyridinoline (a bone resorption marker), and increases serum bone-specific alkaline phosphatase (a bone formation marker) in menopausal women (Ma *et al.*, 2008). Studies in postmenopausal women have shown similar results (Lydeking-Olsen *et al.*, 2004). In the present study, IPL feeding improved BMD in OVX mice; this effect may have been due to increased bone formation and/or decreased bone resorption. Currently, no data are available regarding the relative effectiveness of IPL and soy isoflavones for bone metabolism. The detailed mechanism of IPL action in bone metabolism needs further study to clarify its role.

In conclusion, our results demonstrated that IPL has multiple metabolic benefits in OVX mice. The IPL feeding led to increased plasma estrogen levels, decreased plasma TG levels and abdominal adipose tissue weight, and significantly improved femur BMD, without elevating the plasma levels of the liver enzymes AST and ALT. Moreover, our results suggest that IPL may help maintain the appropriate levels or activities of estrogen in plasma. Taken together, our findings suggest that IPL may be useful for controlling menopausal symptoms, at least in mice. Further research is necessary to determine whether IPL will have the same effects in humans.

Acknowledgements

We thank Jong-Hyuck Lee for technical assistance at the Research and Development Center of Samlip General Food Cooperation. This work was supported by grant A050376 from the Korean Health 21 Research and Development Project, Ministry of Health and Welfare, Republic of Korea and by the Technology Development Program of the Ministry of Agriculture and Forestry (105054-03-2-HD110), and with support from the "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ008506)" Rural Development Administration, Republic of Korea. Currently, the maximum dose reported by the Korean Food and Drug Administration (KFDA) for the health functionality of isoflavones in humans is 30 mg/day; however, communication with a corresponding officer at the KFDA (Dr Ji Yeon Kim) confirmed that it was acceptable to use extremely high doses of test materials in an acute animal study if the compound was non-toxic at the dosage used.

Conflict of Interest

The authors have declared that there is no conflict of interest

REFERENCES

Anderson D, Dobrzynska MM, Basaran N. 1997. Effect of various genotoxins and reproductive toxins in human lymphocytes and sperm in the comet assay. *Teratog Carcinog Mutagen* **17**: 29–43.

Babaei P, Mehdizadeh R, Ansar MM, Damirchi A. 2010. Effects of ovariectomy and estrogen replacement therapy on visceral adipose tissue and serum adiponectin levels in rats. *Menopause Int* **16**: 100–4.

Bahr JM, Nakai M, Rivera A, *et al*. 2005. Dietary soy protein and isoflavones: minimal beneficial effects on bone and no effect on the reproductive tract of sexually mature ovariectomized Sprague–Dawley rats. *Menopause* **12**: 165–173.

Billeci AMR, Paciaroni M, Caso V, Agnelli G. 2008. Hormone replacement therapy and stroke. *Curr Vasc Pharmacol* **6**: 112–123.

Chung HJ, Chung MJ, Houng SJ, *et al*. 2009. Toxicological evaluation of the isoflavone puerarin and its glycosides. *Eur Food Res and Technol* **230**: 145–153.

Chung MJ, Sung NJ, Park CS, *et al*. 2008. Antioxidative and hypocholesterolemic activities of water-soluble puerarin glycosides in HepG2 cells and in C57BL/6J mice. *Eur J Pharmacol* **578**: 159–170.

Compston J. 2005. How to manage osteoporosis after the menopause. *Best Pract Res Clin Rheumatol* **19**: 1007–1019.

Dediu M, Median D, Alexandru A, Vremes G, Gal C, Gongu M. 2009. Adjuvant therapy with aromatase inhibitors in postmenopausal, estrogen receptor-positive breast cancer patients: upfront or sequential? *J BUON* **14**: 375–379.

El-Demerdash FM, Yousef MI, Al-Salhen KS. 2003. Protective effects of isoflavone on some biochemical parameters

affected by cypermethrin in male rabbits. *J Environ Sci Health B* **38**: 365–378.
Greendale GA, Sowers M. 1997. The menopause transition. *Endocrinol Metab Clin North Am* **26**: 261–77.
Guerra MC, Speroni E, Broccoli M, *et al*. 2000. Comparison between Chinese medical herb Pueraria lobata crude extract and its main isoflavone puerarin – antioxidant properties and effects on rat liver CYP-catalysed drug metabolism. *Life Sci* **67**: 2997–3006.
Hsu HH, Chang CK, Su HC, Liu IM, Cheng JT. 2002. Stimulatory effect of puerarin on alpha(1A)-adrenoceptor to increase glucose uptake into cultured C2C12 cells of mice. *Planta Med* **68**: 999–1003.
Ishizuka B, Kudo Y, Tango T. 2008. Cross-sectional community survey of menopause symptoms among Japanese women. *Maturitas* **61**: 260–267.
Krebs EE, Ensrud KE, Macdonald R, Wilt TJ. 2004. Phytoestrogens for treatment of menopausal symptoms: a systematic review. *Obstet Gynecol* **104**: 824–36.
Kulling SE, Metzler M. 1997. Induction of micronuclei, DNA strand breaks and HPRT mutations in cultured Chinese hamster V79 cells by the phytoestrogen coumoestrol. *Food Chem Toxicol* **35**: 605–13.
Lof M, Weiderplass E. 2009. Impact of diet on breast cancer risk. *Curr Opin Obstet Gynecol* **21**: 80–85.
Lydeking-Olsen E, Beck-Jensen JE, Setchell KDR, Holm-Jensen T. 2004. Soymilk or progesterone for prevention of bone loss - A 2 year randomized, placebo-controlled trial. *Eur J Nutr* **43**: 246–257.
Ma DF, Qin LQ, Wang PY, Katoh R. 2008. Soy isoflavone intake inhibits bone resorption and stimulates bone formation in menopausal women: meta-analysis of randomized controlled trials. *Eur J Clin Nutr* **62**: 155–161.
Martin LJ, Minkin S, Boyd NF. 2009. Hormone therapy, mammographic density, and breast cancer risk. *Maturitas* **64**: 20–26.
Matthan NR, Jalbert SM, Lamon-Fava S, *et al*. 2005. TRL, IDL, and LDL apolipoprotein B-100 and HDL apolipoprotein A-I kinetics as a function of age and menopausal status. *Arterioscler Thromb Vasc Biol* **25**: 1691–1696.
Pasqualini JR. 2009. Estrogen Sulfotransferases in Breast and Endometrial. *Cancers. Ann N Y Acad Sci* **1155**: 88–98.
Pinkerton JV, Guico-Pabia CJ, Taylor HS. 2010. Menstrual cycle-related exacerbation of disease. *Am J Obstet Gynecol* **202**: 221–31.
Rachon D, Teede H. 2008. Postmenopausal hormone therapy and the risk of venous thromboembolism. *Climacteric* **11**: 273–279.
Reiter E, Beck V, Medjakovic S, Jungbauer A. 2009. Isoflavones are safe compounds for therapeutical applications - Evaluation of in vitro data. *Gynecol Endocrinol* **25**: 554–580.
Shedd-Wise KM, Alekel DL, Hofmann H, *et al*. 2011. The soy isoflavones for reducing bone loss study: 3-yr effects on pQCT bone mineral density and strength measures in postmenopausal women. *J Clin Densitom* **14**: 47–57.
Stevenson JC, Hodis HN, Pickar JH, Lobo RA. 2009. Coronary heart disease and menopause management: The swinging pendulum of HRT. *Atherosclerosis* **207**: 336–340.
Taku K, Umegaki K, Sato Y, Taki Y, Endoh K, Watanabe S. 2007. Soy isoflavones lower serum total and LDL cholesterol in humans: a meta-analysis of 11 randomized controlled trials. *Am J Clin Nutr* **85**: 1148–1156.
Weerachayaphorn J, Chuncharunee A, Mahagita C, Lewchalermwongse B, Suksamrarn A, Piyachaturawat P. 2011. A protective effect of *Curcuma comosa* Roxb. on bone loss in estrogen deficient mice. *J Ethnopharmacol* **137**: 956–62.
Wise PM, Krajnak KM, Kashon ML. 1996. Menopause: the aging of multiple pacemakers. *Science* **273**: 67–70.
Yasui T, Uemura H, Takikawa M, Irahara M. 2003. Hormone replacement therapy in postmenopausal women. *J Med Invest* **50**: 136–45.

